Nerve Growth Factor

New Research

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NERVE GROWTH FACTOR: NEW RESEARCH

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Contents

Preface

Nerve growth factor (NGF) is a small secreted protein which induces the differentiation and survival of particular target neurons (nerve cells). It is perhaps the prototypical growth factor, in that it is one of the first to be described - that work by Rita Levi-Montalcini and Stanley Cohen was rewarded with a Nobel Prize. NGF is critical for the survival and maintenance of sympathetic and sensory neurons. NGF is released from the target cells, binds to and activates its high affinity receptor (TrkA), and is internalized into the responsive neuron. There are some data that show that NGF can be transported from the axon tip to soma, but it is unclear if this is necessary for effective cell signalling; in fact there are data showing that it is not. What is clear is that NGF binding and activation of TrkA is required for NGF-mediated neuronal survival and differentiation. This new volume presents important recent research in this rapidly-expanding field.

Chapter 1 – Clinical patients with peripheral nerve injury often suffer from persistent neuropathic pain, which are refractory to conventional analgesic therapy. The mechanisms underlying post-traumatic neuropathic pain remain unclear. Over the past decade evidence has been accumulated that neurotrophic factors play critical roles in persistent pain states and emerged as an exciting new class of potential targets for development of new drugs to control neuropathic pain on the basis of their profound roles as mediators and modulator of pain. In this review, the authors present an overview of pathophysiological changes of neurotrophic factors in pain pathway in different neuropathic pain animal models, and discuss roles of neurotrophic factors in particular nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in peripheral and central pathomechanisms of nerve injury-induced neuropathic pain. Moreover, current progress on therapeutic intervention targeting neurotrophic factors in neuropathic pain and inflammatory pain is summarized.

Chapter 2 – Itching is a characteristic symptom of various dermatoses, especially atopic dermatitis. The most effective strategy for treatment of atopic dermatitis would be to prevent aggravation of skin lesions and improve quality of life by reducing itching and scratching. Nerve growth factor (NGF) is an important substance in the skin, where it plays roles in nerve maintenance and repair. However, the nature of involvement of NGF in pruritic diseases such as atopic dermatitis is not yet fully understood. The authors used the NC/Nga mouse, an animal model of atopic dermatitis, to test the hypothesis that NGF plays important roles in the pathogenesis, development, and maintenance of the skin lesions of this condition.

In these mice, nerve fibers were significantly increased in number in the epidermis of lesional skin, and NGF contents in serum and skin were significantly elevated. Furthermore, repeated administration of anti-NGF antibody or high-affinity NGF receptor inhibitors significantly improved established dermatitis and scratching behavior and decreased innervation of the epidermis. The authors' findings suggest that NGF plays important roles in the pathogenesis of atopic dermatitis-like skin lesions, particularly via the high-affinity NGF receptor, and that inhibition of the physiological effects of NGF or suppression of increase in NGF production may prevent or moderate the symptoms of several pruritic skin diseases.

Chapter 3 – Chemokines and their receptors are essential for the development and organization of the hematopoietic/lymphopoietic system. A member of the CXC chemokine subfamily, stromal cell-derived factor-1 (SDF-1) and its unique receptor CXCR4 have been shown to be expressed in embryonic and mature central nervous system (CNS). The present data show that SDF-1 causes the morphological and molecular differentiation of PC12 cells in a manner similar to nerve growth factor (NGF). PC12 cells are shown to express CXCR4. Neurite outgrowth stimulated by SDF-1 was attenuated by small interfering RNA-mediated knockdown of CXCR4 and pertussis toxin (PTX), which uncouples Gi protein. Comparison of extracellular signal-regulated kinase signaling pathways between SDF-1and NGF shows that these pathways are crucial for SDF-1 action as well as NGF. CXCR4 mRNA is up-regulated by neuron in the rat facial nucleus following axotomy. Recently, several studies have demonstrated that SDF-1 and its receptor CXCR4 play an important role in the CNS development and adulthood by mediating cell migration, enhancing precursor cell proliferation. In addition to these functions, SDF-1/CXCR4 signaling may be required for neurite outgrowth during nerve regeneration.

Chapter 4 – Neurotrophic molecules have a deep influence on developmental events such as naturally occurring cell death, differentiation and process outgrowth, and could be used for treating degenerative neurological conditions and promoting neural regeneration. The neurotrophin family members (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), neurotrophins 3 (NT-3) and 4 (NT-4)) are proteins which cannot cross the blood-brain barrier and therefore need to be administered directly to their target in the brain.

Drug delivery to the nervous system remains a challenge despite advances in understanding the mechanisms involved in the development of neurodegenerative disorders and the actions of neuroactive agents. The systemic administration of neuroactive biomolecules to support neuronal regeneration has several intrinsic problems, including the toxicity and poor stability associated with many bioactive factors and access limitation by the blood-brain barrier.

The best characterized trophic molecule, NGF, has a spectrum of effects on peripheral and central neurons. It has received particular attention as a potential treatment for Alzheimer's disease because of its protective action on basal forebrain cholinergic neurons. Intraventricular infusion of NGF can prevent cholinergic cell death following fimbrial transsection in adult rodents and monkeys. Based on these data, the entrapment of NGF was performed in monolithic implantable devices.

A variety of techniques to deliver therapeutics to the CNS has been established, including osmotic pumps and silicone reservoirs. However, pumps frequently become

clogged, thus limiting their ability to sustain effective concentrations; moreover these methods are often associated to highly invasive drawbacks, including device failure and higher potentials for inflammation and infection due to their non degradable components.

This chapter will focus on innovative strategies for delivering NGF both for *in vivo* and *in vitro* applications. Development of polymeric films, hydrogels and microparticles will be considered. Polymeric delivery systems, in fact, have the potential to maintain therapeutic levels of a drug, to reduce side effects and to facilitate the delivery of drugs with short *in vivo* half-lives. Synthetic and naturally derived polymers are widely used in controlled release devices for protein delivery. These devices are designed such that bioactive factors are released in a spatially and temporally controlled manner; for example, release can occur as the polymer degrades or by diffusion through pores in the polymer matrix. In neural applications, two types of delivery devices have been primarily used: polymer matrices and microspheres.

Particular attention will be devoted to the recent advancements in the field of neuronal tissue engineering and of neuronal regeneration. Finally, possibility to integrate NGF delivery techniques to regeneration-type (sieve) neuronal interfaces will be analysed and discussed.

Chapter 5 – Nerve growth factor (NGF) is widely recognized as a target-derived factor responsible for the survival and maintenance of the phenotype of specific subsets of peripheral neurons and basal forebrain cholinergic nuclei during development and maturation. Considerable evidence has accumulated over the last twenty years to indicate that the actions of NGF extend far beyond “classical” effects on cells of the nervous system, to encompass a role for this molecule in the interplay between the nervous, immune, and endocrine systems. NGF as well as brain-derived neurotrophic factor, another member of the protein family of neurotrophins, have been implicated in the pathophysiological mechanisms of many diseases of the nervous and the immune systems, such as multiple sclerosis, neuropathy, pain, allergic bronchial asthma and neurotrophic keratitis. The concentration of NGF is elevated in a number of inflammatory and autoimmune states in conjunction with increased accumulation of mast cells. Like NGF, brain-derived neurotrophic factor serum levels are also increased in asthmatics. Mast cells and NGF appear to be involved in neuroimmune interactions and tissue inflammation. Mast cells themselves are capable of producing and responding to NGF, suggesting that alterations in mast cell behavior may trigger maladaptive neuroimmune tissue responses, including those of an autoimmune nature. Moreover, NGF exerts a modulatory role on sensory nociceptive nerve physiology in the adult, and appears to correlate with hyperalgesic phenomena occurring in tissue inflammation. NGF can thus be viewed as a multifactorial modulator in the reciprocal crosstalk between neurons, immune cells and endocrine cells.

Chpater 6 – A major consequence of severe chronic infection is the often massive loss of body weight (=cachexia), which is directly linked to increased morbidity and reduced survival, similarly large increases in body weight leading to obesity are also associated with reduced survival. Animal models must be used to determine the mechanisms involved and this may led to the development of appropriate therapy. The authors’ use of chronic murine infection models and brain inflammatory models suggests that *in vivo* the site of initiation of the inflammatory process be it peripheral or central is of prime importance to the subsequent pathology. Neuroendocrine interactions with the immune system have recently been

suggested to be of importance in determining the degree of pathology due to dysregulation of the immune system. In particular peripheral hormones involved in energy balance, such as leptin and ghrelin have been shown to be important in regulating immune function and susceptibility to infection. The authors observed that following permanent middle cerebral artery occlusion (pMCAO) or in acute/chronic infection models all of which induce changes in circulating ghrelin. These changes in ghrelin are associated with altering nerve growth factor (NGF) and redox regulatory factors such as monoamine oxidase (MAO), uncoupling protein 2 (UCP2) and glutathione (GSH). The authors' studies suggest that NGF can play a role in modifying neuroendocrine systems involving peripheral/central MAO activity, UCP2 and GSH. The authors also have evidence that NGF may play a specific role in food intake regulation following inflammation. In this review the authors will focus on their *in vivo* studies demonstrating regulatory circuits involving NGF modulation by ghrelin and leptin. Furthermore they will show a role for NGF in modifying central MAO activity, UCP2, cytokine production and glutathione levels. Finally the authors will discuss the role of NGF on food intake and energy expenditure based on their studies. The role of NGF as a integrator of neuroendocrine system, immune function and physiological systems will be evaluated.

Chapter 7 – Nerve growth factor (NGF), the founder member of the neurotrophin family, plays an essential role in the development and functioning of the vertebrate nervous system. It regulates survival and function of different neuron populations of the central and peripheral nervous system through two different membrane-associated receptors: the p75 common neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor superfamily, and the tyrosine kinase receptor TrkA. NGF is not only active in the nervous system, but also in other different cell types including tumor cells. Changes in the neurotrophin signaling system are significant for the pathogenesis of malignancies at the initiation stage as well as during subsequent tumor progression steps. NGF and its receptors can affect malignant cells and tissues in different ways, acting on cell proliferation, cell maintenance and survival, apoptosis, and metastasis regulation. Differences in mechanisms and outcomes of NGF action depend on the cell and tissue type in which this neurotrophin works, as well as the ratio $p75^{NTR}$/TrkA present in the cell. In this chapter, the authors summarize the current information on the NGF signaling network in various neural tumors and demonstrate its contribution to the disease course. Furthermore, they show that nuclear translocation of the intracellular domain of $p75^{NTR}$ ($p75^{ICD}$) induced by NGF in RN22 schwannoma cells, reduces the proliferative capacity of these cells. This provides a novel mechanism by which $p75^{NTR}$ can act as a tumor suppressor gene.

Chapter 8 – The neurotrophins - nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4 - represent a family of proteins essential for neuronal survival and plasticity. Recent reports have suggested that these molecules, particularly BDNF, play an important role in neuropsychiatric disorders such as major depression (MDD), suicide, bipolar disorder (BD) and eating disorders (ED). A comprehensive study of the biology of BDNF is an important step in developing its clinical relevance.

Following the neuroplastic hypothesis of affective disorders the authors have led an investigation of BDNF's biology and related molecules. Blood and brain BDNF and NT-3 levels were measured in rodents and in psychiatric subjects. These studies aimed to associate the dysregulation of neurotrophic factors and psychiatric pathologies. The authors also

investigated the regulation of BDNF mRNA expression in drug-treated cells through cAMP transduction pathways. Results showed that, in major depression disorder (MDD), subjects are characterized by decreased serum BDNF levels, and that the decrease was correlated with the severity of illness. Moreover, the decrease was reversed after an antidepressant treatment. The authors also observed that, during the postnatal maturation period in the rat, changes in serum and brain BDNF protein levels followed the same development scheme and were positively correlated. Neurotrophin protein levels (BDNF and NT-3) have also been measured in postmortem human brains. The data indicated a significant decrease in BDNF and NT-3 levels in both the hippocampus and prefrontal cortex of drug-free depressed suicide subjects compared to non-depressed controls. The decrease was observed in different suicide groups whatever the diagnosis, indicating either a suicide-specific trait or a trait common to other psychiatric diagnoses. Subjects who were under drug treatment at the time of death did not differ from non-suicide controls. By using lymphoblasts (from BD patients), the authors assessed the drug-manipulated BDNF expression as downstream target of the cAMP-Protein kinase A (PKA) and the transcription factor CREB signaling axis. It was observed that, in BD-derived cells, BDNF mRNA expression was decreased, but that the decrease was masked by an upregulated PKA-CREB signaling pathway, resulting in normalization of BDNF mRNA levels. In conclusion, these studies on neurotrophins have identified BDNF and NT-3 as potential biological markers of neuroplasticity in the affective disorders. Given the reported morphological deficits associated with affective disorders, the study of neurotrophins could be a promising track for drug development.

Chapter 9 – The neurotrophins (NT) including nerve growth factor (NGF) are a family of related growth factors and their respective receptor tyrosine kinases that are of major importance in the regulation of neuronal survival and differentiation. Since Rita Levi Montalcini and Stanley Cohen received Nobel Prize for their pioneering work on NGF, its role in female reproductive system has been reinforced in last two decades. While role of NGF in mast cell-mediated egg implantation and inhibition of rejection were primary concern at their time, in the ovary they can help in the differentiation process by which ovarian follicles become responsive to gonadotrophins. They help in follicular maturation, steroid secretion and ovulation in the ovary, by inducing the FSH receptor (FSHR). Due to the pleiotropism, NGF is mandatory for the success of pregnancy, while progesterone helping to maintain local levels of NGF in utero. Perimenopausal ovarian surface epithelium (OSE) can also express FSHR. NGF deregulates expression of FSHR in OSE and secretion of FSH from the pituitary and also has the ability to increase VEGF expression. This phenomenon strongly suggests an autocrine role of NGF in epithelial ovarian cancer (EOC). From endometriosis to EOC, NGF is implicated in a variety of female reproductive disorder. Thus its study will infuse new insight in health and disease of female reproductive system.

Chapter 10 – The nerve growth factor (NGF) receptor-signaling system is composed of the neurotrophin receptor p75 and the tyrosine kinase A (TrkA) receptor. Recent studies suggest that NGF plays important roles in both the normal development and function of the human ovary as well as with the promotion of ovarian cancer.

NGF and its receptors were investigated in human ovaries from fetuses, girls and women and in granulosa cells (GCs) and oocytes from in vitro fertilization (IVF) cycles. The NGF protein was identified in all GCs, and in oogonia and oocytes from preantral follicles. The

TrkA protein was expressed mainly in oocytes, but also in GCs and in theca cells of antral follicles. The mRNA transcripts of NGF and TrkA were also identified. The p75 protein and mRNA transcripts were predominantly localized in stroma cells surrounding the oogonia/oocytes of fetuses aged up to 22 gestational weeks, but also in neuronal-like cells or fibers among blood vessels, in neuronal bodies, and in theca cells from fetuses and babies. The proteins and mRNA transcripts for NGF and TrkA were identified in cultured GCs and unfertilized mature oocytes from IVF cycles. NGF treatment of these GCs resulted in an increase in 17-β estradiol (E2) production and a decrease in progesterone levels; the addition of NGF together with FSH, induced higher E2 secretion. NGF also promoted an increase in FSH receptor mRNA; this action was blocked by a Trk inhibitor (K252a).

Studies indicate that p75 might be involved in follicular assembly during normal fetal ovarian development, as it is expressed mainly around this period. NGF, by promoting E2 production and reducing progesterone levels, prevents early luteinization and induces follicular acquisition of the FSH receptors required for their growth.

NGF and its two receptors have also been identified in various ovarian cancers. The NGF and TrkA proteins as well as TrkA mRNA were overexpressed in epithelial ovarian cancer, and the expression of TrkA was found to be a prediction of better outcome in the advanced-stage serous epithelial ovarian cancer. NGF probably promotes postmenopausal epithelial ovarian cancer by reducing FSH levels and by stimulating the expression of FSH receptors on the ovarian surface epithelium. NGF also stimulates angiogenesis in surface epithelium cancer directly or by promoting the production of vascular endothelial growth factor. Studies have shown that this increase is inhibited by the addition of an anti-NGF antibody and by K252a. Therefore, blocking NGF action could be a therapeutic target in ovarian cancer.

Chapter 11 – Nerve growth factor (NGF) and other members of the neurotrophin family are critical for the survival and differentiation of neurons in the central nervous system (CNS) and have been implicated in the pathophysiology of numerous disease states. Several studies have sought to demonstrate that neurodegeneration during disease and in old age is associated with reduced support of neurotrophins. Because NGF maintains the magnocellular cholinergic neurons that are damaged in Alzheimer's disease (AD), over the past decade, this neurotrophin has gained attention as a candidate therapeutic agent for the disease. However, because NGF does not cross the blood-brain barrier, inconvenient pharmacokinetics and adverse side-effect profiles have limited its clinical usefulness and therapeutic potential. Nevertheless, in recent years, alternative strategies have been developed with particular emphasis on small molecules able to modulate NGF function in AD. Compounds that mimic NGF signaling and overcome the pharmacokinetic and side-effect barriers may have therapeutic potential.

Here, the authors review the past and recent preclinical studies and advances into clinical development on the therapeutic effect of NGF in AD and illustrate additional strategies to target (modulate) NGF production and/or its signal transduction pathways. Moreover, since inflammation has been proposed as a possible pathogenic mechanism of AD and NGF is a key mediator of neuronal and immune cross talk, the potential involvement of immune system on NGF-based therapeutic approaches are also discussed. The development of studies on the role of immune-mediated mechanisms in AD and in the regulation of NGF expression can open the door to new frontiers for therapeutic intervention in AD.

Chapter 12 – Objective: The neurotrophic factor S100B has been promoted as a clinical marker of brain damage and high serum levels are considered to correlate with the severity of injury and a poor prognosis. However, experimental research demonstrated increased cerebral levels of S100B to enhance hippocampal neurogenesis and to improve cognitive recovery following traumatic brain injury (TBI). The purpose of the present study was to elucidate the cellular effects of S100B and thus to differentiate beneficial and detrimental ones.

Methods: Following lateral fluid percussion injury in male rats (n=32), the authors infused S100B (50ng/hr) or vehicle into the lateral ventricle for 7 days using an osmotic micro-pump. The animals were sacrificed on day 5 or 5 weeks post-injury, and 5μm sections, 100μm apart (bregma -3.3 to -5.6mm) were analysed histologically. Cell death was assessed using TUNEL and hematoxylin-eosin staining, axonal damage and microglial activation by APP and ED1 immunostaining, and gliosis applying the glial markers GFAP and S100B.

Results: TUNEL-positive cells were present directly beneath the lesion site in vehicle and S100B-treated animals on day 5 post-injury (238±6 and 234±24 cells/mm, respectively, n.s.), but not after 5 weeks. The intraventricular S100B infusion did not significantly affect the early (TBI $p=0.004$, TBI+S100B $p=0.036$) or late (TBI $p=0.039$, TBI+S100B $p=0.002$) axonal injury, but resulted in an unspecific microglial activation opposite to the injury site as documented by an increased ED1 expression (TBI+S100B $p=0.001$). After 5 weeks, both injury and S100B treatment resulted in an increased number of GFAP expressing cells in the corpus callosum (TBI $p=0.005$, TBI+S100B $p=0.003$, sham+S100B $p=0.005$), while in the hippocampal granular cell layer (GCL) this effect was only present in non-injured control animals ($p=0.048$). The S100B expression in the GCL was increased by a S100B treatment after 5 weeks, both in injured and non-injured animals ($p=0.017$ and $p<0.001$).

Conclusion: In the authors' model, the exogenous application of S100B did not exert an effect on early cell death or late axonal injury. The significance of some delayed APP and ED1 accumulation and stimulation of reactive astrocytosis following injury and S100B treatment has to be clarified by long-term experiments in order to exclude any participation in neurodegenerative processes.

Chapter 13 – Cell biology faces a sharp rise in the amount of experimental data and thus, it's difficult to interpret those data into an integral functional plan of a living cell. There is a demand for adequate tools for the analysis of highly interconnected signaling networks at different levels of complexity. This would allow an understanding of how to do signaling network process multiple receptor inputs to produce adoptive physiological responses both at the single cell level and in the a multicellular organism. Here the authors discuss the use of Boolean networks for the interpretation of experimental results on converging signaling pathways induced by nerve growth factor (NGF) and extracellular matrix (ECM) in neurons. The authors consider Boolean logics as a convenient tool for the systematization of causal connections between signaling events. As an illustration, a Boolean analysis of NGF-Src signaling is performed that triggers axonal growth. High-throughput quantitative studies of signaling events allows a more detailed understanding of signaling network connectivity based on the modular response analysis and other mathematical methods. A possible structural mechanism of NGF- and ECM-induced signal integration is then discussed at the level of the Src-FAK molecular complex. They hypothesize that FAK mediates alternative signaling pathways for axonal growth in the presence versus the absence of NGF. Finally, the

importance of quantification of subcellular targeting of signaling events is emphasized for the analysis of signaling networks.

Chapter 14 – Nerve growth factor (NGF) is not restricted to cells of neuronal origin but also extends to cells of the immune system, and to cells that neurons innervate such as muscles, glands and adipose tissue. NGF plays a role in inflammatory responses and in tissue repair via its two classes of receptors, p75 and tyrosin kinase (TrkA).

Graves' orbitopathy represents a special link between thyroid and orbital autoimmunity with a complexity of neuronal activity. The clinical symptoms of hyperthyroidism could associate - depending on catecholamine amount - with an increase of myocyte hypertrophy or apoptosis. Production of NGF could be induced by T helper -2 activation. An aberrant apoptosis has been demonstrated in nodular goitre due to increased proteasome activity contrary to the death receptor mediated form in thyroid autoimmunity. The proper balance between NGF mediated proliferative processes and enhanced apoptosis could be demonstrated during the manifestation of Graves' orbitopathy.

The expression of NGF receptors could locally modulate the inflammatory and reparative responses. Data about the role of NGF in conjunctival and corneal epithelium integrity suggest its importance in orbitopathy. NGF displays stimulatory effects on fibroblasts and does not influence collagen production but promotes their phenotype into myofibroblasts.

NGF via its dual effect on survival and apoptosis, which could manifest in hypertrophy and degenerative damages represents a special neuronal regulatory system in Graves' orbitopathy. NGF mediated events in the development of orbitopathy associated with Graves' disease are independent of direct effects of thyroid hormones. Recombinant human NGF may be useful therapy in orbitopathy, which has been applied for corneal ulcers successfully.

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Chapter 1

Roles of Neurotrophic Factors in Neuropathic Pain: From Pathogensis to Therapy

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Abstract

Clinical patients with peripheral nerve injury often suffer from persistent neuropathic pain, which are refractory to conventional analgesic therapy. The mechanisms underlying post-traumatic neuropathic pain remain unclear. Over the past decade evidence has been accumulated that neurotrophic factors play critical roles in persistent pain states and emerged as an exciting new class of potential targets for development of new drugs to control neuropathic pain on the basis of their profound roles as mediators and modulator of pain. In this review, we present an overview of pathophysiological changes of neurotrophic factors in pain pathway in different neuropathic pain animal models, and discuss roles of neurotrophic factors in particular nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in peripheral and central pathomechanisms of nerve injury-induced neuropathic pain. Moreover, current progress on therapeutic intervention targeting neurotrophic factors in neuropathic pain and inflammatory pain is summarized.

1. Introduction

Major advances in the field of pain research are occurring at every level of analysis, from development to neural plasticity in the adult and from the transduction of a noxious stimulus

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in primary afferent neurons to the impact of this stimulus on cortical circuitry (Dubner and Gold, 1999). Cellular- and molecular-biological, pharmacological and neurobiological techniques have generated tremendous information towards the understanding of the pathogenesis of pain in the recent years. This is especially true in respect of neuropathic pain which is a major clinical problem and difficult to treat. Thus, it is critical to understand the mechanisms of neuropathic pain in order to develop a more effective treatment. In this review, an emphasis is placed on primary sensory neurons, the first relay of neurons in pain transduction, we concentrate on analysis of the pathophysiological changes of primary sensory neurons in dorsal root ganglion (DRG) in neuropathic pain models. We will review (1) the physiological properties of primary sensory neurons in DRG; (2) pathophysiological changes in these neurons in response to peripheral nerve injury; (3) diverse biological actions of neurotrophic factors in the physiology and pathophysiology of primary sensory neurons; and (4) how these nerve injury-induced plastic changes contribute to the understanding of peripheral and central mechanisms in the generation of peripheral neuropathic pain on the level of primary sensory neurons and the dorsal horn in the spinal cord.

2. Gross Organization of DRG

Dorsal root ganglion lies between the pedicles of the adjacent vertebrae. Each spinal segment is associated with a pair of DRGs that contain the cell bodies of sensory neurons, a dorsal root and a ventral root (Devor, 1999a). The dorsal root contains the axons of those neurons, which bring sensory information into the spinal cord, while the ventral root contains the axons of motor neurons that extend into the periphery to control somatic and visceral effectors. Distal to each DRG, the spinal nerve is formed from the fusion of the dorsal and ventral roots. All spinal nerves are mixed nerves because they contain both afferent (sensory) and efferent (motor) fibers. Each spinal nerve is divided into dorsal and ventral rami, with dorsal rami serving the skin and musculature of the posterior body trunk at their approximate level of emergence, and the ventral rami of adjacent spinal nerve forming a complex network of nerves called plexuses. The plexuses supply the pelvic region of the trunk and the lower limbs called the lumbosacral plexus, including lumbar plexus and sacral plexus. In rats, lumbar 4 to 6 spinal nerves converge to form lumbosacral trunk. The major peripheral nerve of sacral plexus is the sciatic nerve, which leaves the pelvis through the greater sciatic notch and travels down the posterior thigh, serving its flexor muscles and skin in the popliteal region. The sciatic nerve divides into the common peroneal nerve and tibial nerve, which together supply the balance of the leg muscles and skin.

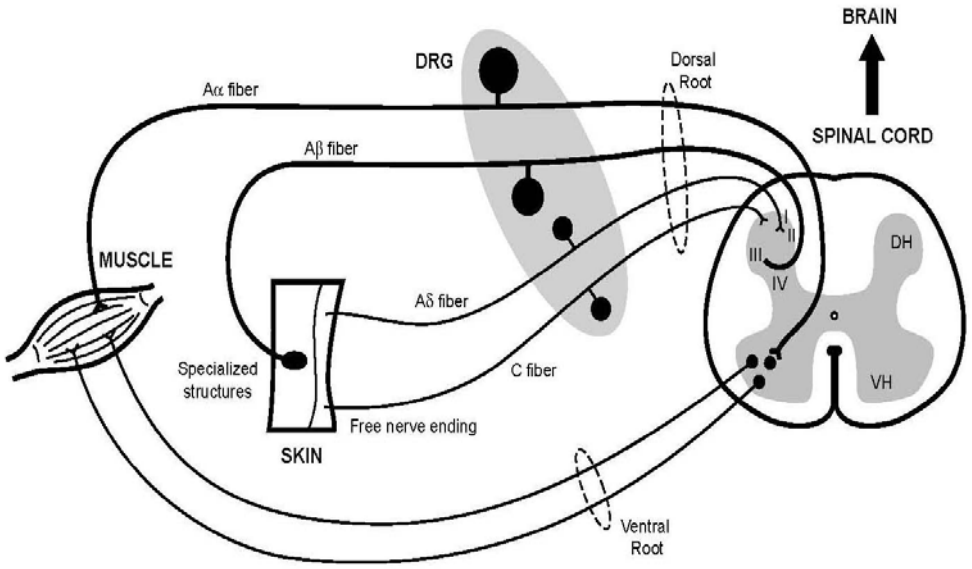
3. Heterogeneity of Primary Sensory Neurons in DRG

Dorsal root ganglia contain most of body’s primary sensory neurons, which are responsible for transducing stimuli from peripheral tissues and passing the sensory signals on to the central nervous system (CNS). DRG neurons have their peculiar pseudounipolar

morphology, and give rise to two branches. One branch enters the spinal cord or brainstem through the dorsal root. The other branch projects into the spinal nerve and forms a sensory ending in skin, muscle and viscera in the periphery (Devor, 1999). The terminal of the peripheral branches is sensitive to stimuli and the properties of the nerve terminal determine the sensory function of each DRG neuron. The remainder of the peripheral branch, together with the central branch, is called the primary afferent fiber. It transmits the encoded stimulus information to the spinal cord or brain stem from the peripheral nerve terminals.

DRG neurons are wrapped in a layer of ensheathing satellite cells and separated from each other without synaptic interaction (Devor, 1999a). However, electrophysiological studies have indicated that nearly all neurons are linked to each other by cross-excitation (Utzschneider et al., 1992; Amir and Devor, 2000).

The prominent feature of mature primary sensory neurons is their heterogeneity, which is reflected by their morphological, biochemical and functional diversity. Heterogeneous populations in DRG subserve diverse sensory modalities including painful stimuli (nociception), innocuous stimuli such as light touch (mechanoreception), the sense of static position and movement of the limbs and body (proprioception) and temperature sense. The neurons serving these distinct modalities can also be classified based on their histochemical and functional properties (Figure 1).



Cell size	Myelination	Axons	Diameter (μm)	Conduction velocity (m/sec)	Action potential	Sensory modality
Large neurons	Myelinated	Aα fibre	13 - 20	30 - 120	Fast	Proprioception
Large neurons	Myelinated	Aβ fibre	6 - 12	35 - 75	Fast	Mechanoreception
Small neurons	Unmyelinated	C fibres	0.2 -1.5	0.5 - 2	Slow	Nociception
Small neurons	Thin myelinated	Aδ fibres	1.5	5 - 30	Slow	Nociception

Figure 1. Schematic drawing showing heterogeneity of primary sensory neurons in DRG.

The rodent adult lumbar DRG neurons are divided into three minimally overlapping subgroups. One group represents about 40% of DRG neurons. These neurons are large in diameter and lightly stained histologically with myelinated axons and have fast action potential (AP). They have rich neurofilament and can be identified immunocytochemically with neurofilament antibodies such as RT97 or N52. These neurons include muscle afferent sensory neurons and cutaneous afferent sensory neurons. The former mediates proprioception, and has the largest myelinated axons (Aα fibers, 13-20 μm in diameter, 30-120 m/sec in conduction velocity) (Figure 1) projecting to motor neurons in the ventral horn of the spinal cord centrally; and to muscle spindles and Golgi tendons peripherally. The latter mediates mechanoception, and has myelinated axons (Aβ fibers, 6-12 μm in diameter, 35-75m/sec in conduction velocity) (Figure 1) projecting to specialized structures such as Pacinian corpuscles, Ruffini's corpuscles and Meissner's corpuscles in the skin, and projects to deeper layers III and IV of the spinal cord (Ralston et al., 1984; Shortland et al., 1989).

The remaining 60% of rat DRG neurons are the small-dark neurons with the finest unmyelinated axons (C-fibers, 0.2-1.5μm in diameter, 0.5-2m/sec in conduction velocity) or thinly myelinated axons (Aδ fibers, 1-5μm in diameter, 5-30m/sec in conduction velocity) (Figure 1). They also have slow APs (Lawson, 1979) and project as free nerve endings to skin. The majority of small fibers, called nociceptors, are specialized to detect noxious stimuli (Meyer, 1994). Alternatively, these nociceptive neurons are subdivided into two classes on the basis of anatomical localization, neurotrophic dependence, histological markers and target innervation (Snider and McMahon, 1998). One major group represents about 40% of DRG neurons. They mainly express nerve growth factor (NGF) high affinity receptor TrkA and neuropeptides calcitonin gene-related protein (CGRP) and substance P (SP) (Verge et al., 1992; McMahon et al., 1994; Averill et al., 1995; Kashiba et al., 1995). These neurons are NGF-dependent during development. They mainly project to lamina I and the outer layer of lamina II of dorsal horn in the spinal cord, and innervate peripherally in the superficial layers of skin (Snider, 1994). The other group, comprising about 30% of DRG neurons, does not express TrkA and neuropeptides (Snider, 1994; Averill et al., 1995). They are nonpeptidergic, and can be identified by their binding of lectin, *Griffonia simplicifolia* isolectin B4 (IB4) (Silverman and Kruger, 1990) or expressing enzyme activities of fluoride resistant acid phosphatase or thiamine monophosphatase. The lectin-binding neurons also coexpress glial cell line-derived neurotrophic factor (GDNF) receptor Ret and GDNF receptor α1 (GFRα1) or GFRα2 and adenosine triphosphate (ATP) receptor P2X3 (Vulchanova et al., 1997; Bradbury et al., 1998). They mainly project to the deeper inner layer of lamina II, and are GDNF-dependent in the postnatal and adult life. Electrophysiological study has confirmed that IB4-positive and negative neurons are functionally distinct from each other on the magnitudes of voltage-gated sodium currents and heat-evoked currents (Stucky and Lewin, 1999b).

These histological distinctions of the two classes of nociceptive neurons and their distinct central projections may contribute differentially to the initiation and maintenance of pain. Peptidergic neurons are involved in chronic pain (Woolf et al., 1998; Cao et al., 1998). Selective ablation of neurons expressing the SP receptor neurokinin 1 (NK1) in lamina I by intrathecal delivery of neuropeptide ribosome-inactivating protein saporin (Mantyh et al., 1997), has no effect on baseline behavioral responsiveness to mild noxious stimuli, but

markedly attenuates the hypersensitivity induced by capsaicin. Thus, the functional roles of SP/TrkA-expressing sensory neurons projecting to lamina I are critical to hyperalgesic responses induced by inflammation. In contrast, nonpeptidergic neurons involve chronic pain resulting from nerve injury. It has been shown that protein kinase C γ (PKCγ) is specifically expressed in the inner layer of lamina II in the target field of Ret-expressing neurons. Elimination of PKCγ by gene targeting leads to remarkable alleviation of hyperalgesic responses induced by nerve injury (Malmberg et al., 1997). Evidence from the analysis of IB4-saporin-treated rats suggests that nonpeptidergic IB4-binding neurons are involved in thermal and mechanical nociception and are required for the development of capsaicin-mediated hyperalgesia (Vulchanova and Olson, 2000). Using the same methodology, Tarpley et al have demonstrated that acute NGF-induced hyperalgesia was also attenuated after IB4-saporin treatment (Tarpley et al., 2004). Moreover, in the lumbar 5 (L5) spinal nerve injury model, the mechanical allodynia fail to develop after 2 weeks postsurgery, the expression of PKCγ in the spinal cord and P2X3 in uninjured L4 DRG are all reduced in the treated animals indicating that a reduction of IB4-positive neurons in L4 are required for the expression of NGF-induced hyperalgesia and nerve injury-induced behavioral and neurochemical changes.

Recently, several newly-identified ion channels such as transient receptor potential (TRP) cation channels and acid-sensing ion channel provide further contribution to functional heterogeneity of primary sensory neurons, and their correlation to pain-related behavorial responses are being investigated (Kobayashi et al., 2005; Hjerling-Leffler et al., 2007; Lin et al., 2008).

4. Roles of Neurotrophic Factors in Primary Sensory Neurons during Development and in Adulthood

Before sensory neurons have the capacity of detecting noxious stimuli, the crucial step for sensory neurons is to make the appropriate connections in their peripheral and central targets. Although transcription factors have been identified to play important roles in pathfinding of peripheral and central targets of sensory neurons (Saito et al., 1995; Arber et al., 2000; Chen et al., 2001), trophic factors from target tissues are essential for the establishment of target innervation by regulating the magnitude and specificity of neuronal survival (Davies, 1996; Lewin and Barde, 1996). The heterogeneous populations of DRG neurons have provided a model system for investigating the actions of different neurotrophic factors. Here we overview the dependence of primary sensory neurons on neurotrophin family during development and adulthood.

4.1. The Neurotrophin Family

The basic concept of neurotrophic factor is classically defined by the hypothesis that trophic proteins are synthesized in a limited amount in the target tissues and delivered, via retrograde transport, to the neuronal soma where they exert a trophic and survival effect

(Oppenheim, 1991; Lindsay, 1996). The neurotrophins (the general term coined for members of the NGF gene family) are the most intensively studied neurotrophic factors. They are structurally related secretory proteins including NGF (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Maisonpierre et al., 1990; Kaisho et al., 1994), neurotrophin-4/5 (NT-4/5) (Ip et al., 1992), neurotrophin-6 (NT-6) (Gotz et al., 1994) and neurotrophin-7 (NT-7) (Lai et al., 1998; Nilsson et al., 1998). They are all derived from a common ancestral gene (Hallböök, 1999). The members of the neurotrophin family share around 50% homology to one another and each of them functions as a homodimer (Butte, 2001). They elicit a variety of biological responses via their preferred receptors that interact with diverse types of intracellular secondary messenger systems. Two classes of cell surface receptors have been identified. One is the 75 Kilo Dalton neurotrophin receptor (p75NTR), which is the first identified as a NGF receptor and has fast dissociation with a low affinity. It binds with approximately equal affinity to each of the neurotrophins (Hallböök et al., 1991). P75NTR also acts as a receptor for the immature pro-neurotrophin (Barker, 2004), moreover, the binding of pro-neurotropin to p75NTR is implicated to be mediated by interaction with a coreceptor sortilin, leading to promote apoptosis in cultured neurons. The other class is the tyrosine receptor kinase (Trk) which includes TrkA, TrkB and TrkC. Members of the Trk family show different specificities for the different neurotrophins. TrkA preferably binds to NGF (Klein et al., 1991) and is also the receptor for NT-3, NT-6 and NT-7 (Nilsson et al., 1998). TrkB is the receptor for both BDNF (Squinto et al., 1991) and NT-4/5 (Klein et al., 1992; Rydén et al., 1995). Although both NT-4 and BDNF have a rapid excitatory action via TrkB (Kafitz et al., 2000), no overlapping roles for NT-4 and BDNF have been observed both *in vivo* and *in vitro* (Ardelt et al., 1994; Fischer et al., 1994; Hyman et al., 1994). TrkC is the high affinity receptor for NT-3 (Lamballe et al., 1991b). NT-3 can also activate TrkA but TrkB receptors less efficiently (Farinas et al., 1998).

4.2. Neurotrophin Receptor-Mediated Signaling Pathways

4.2.1. Trk Receptor Signal Transduction Via Multiple Signaling Pathways

Trk is a single-chain member of the receptor tyrosine kinase superfamily, in which TrkA, TrkB and TrkC share 66% amino acid sequence identity (Lamballe et al., 1991a). Trk receptors have a complex structure, including an extracelllular portion involved in neurotrophin binding, and an intracellular portion with protein-tyrosine kinase activity. Each of the Trk members has multiple transcripts (Middlemas et al., 1991; Barker et al., 1993; Lamballe et al., 1993), which lead to complexity in neurotrophin signaling (Barbacid, 1995). Neurotrophins bind to two Trk monomers, causing the formation of a homodimer, which in turn leads to Trk trans- and auto-phosphorylation. The Trk phosphorylation of specific tyrosine residues creates binding sites for the second messengers like Shc, phosphatidylinositol 3 (PI3) and phospholipase C-γ1 (PLC-γ1), which are involved in protein trafficking. Recruitment of these proteins into a ligand-receptor complex initiates multiple signaling cascades for different functions.

Trk signaling pathways include ras-mitogen-activated protein kinase (Ras-MAPK) pathway and Ras-MAPK independent pathways. Ras/MAPK pathway is the major

downstream cascade pathway mediating neuritogenesis and cell survival in response to neurotrophins (Kaplan and Miller, 2000; Arevalo and Wu, 2006). Although the Ras-MAPK pathway plays a predominant role in many NGF-mediated differentiation events (Kaplan and Stephens, 1994), several Ras-independent pathways have been indicated to be associated with some differentiation responses in peripheral and central nervous systems (Kuo et al., 1996; Klinz et al., 1996). These pathways include PI3 kinase/Akt kinase signaling pathway (Dudek et al., 1997), the PLC-γ1 pathway (Stephens et al., 1994), and suc-associated neurotrophic factor-induced tyrosine-phosphorylated target (SNT) pathway (Rabin et al., 1993). Null mutations of the tyrosine kinase domains of each Trk receptor gene have demonstrated that these receptors are necessary components in determining the specificity of neurotrophic effects.

4.2.2. p75NTR-mediated Signaling Pathways and their Biological Activities

p75NTR has three domains. The extracellular domain contains four cysteine repeats, which contribute to formation of the ligand binding sites (Baldwin and Shooter, 1995). The juxtamembrane region is the most highly conserved and represents a region of protein-protein interaction. The Chopper domain, a flexible linker site at the juxtamembrane region has been implicated in cell death signaling (Coulson et al., 2004). The poorly conserved cytoplasmic domain of p75NTR lacks a tyrosine kinase domain, but contains the death domains (Casaccia-Bonnefil et al., 1999).

Great attention has been focused on roles of p75NTR in regulating NGF functions through the modulation of TrkA. P75NTR acts synergistically with Taka receptor to increase affinity of NGF binding (Barker and Shooter, 1994; Verdi et al., 1994; Ridden and Ibanez, 1997) either by concentrating NGF within the Taka local environment or by forming a molecular complex with Taka to generate a high-affinity site (Hempstead et al., 1991). In addition, p75NTR may also contribute to the ability of Trk receptors to discriminate a preferred ligand from other neurotrophins (Ryden et al., 1995).

p75NTR not only modulates and modifies TrkA signaling but also acts as a signaling receptor itself to exert biological activities. Dobrowsky et al first reported that NGF binding to p75NTR activated sphingomyelin hydrolysis and induced the production of lipid secondary messenger ceramide in a TrkA-independent way (Dobrowsky et al., 1994). p75NTR has been demonstrated to function as an apoptosis-inducing receptor in a neurotrophin-independent (Barrett and Bartlett, 1994; Lu et al., 2005) or -dependent way in a variety of cell types in the peripheral nervous system (PNS) (Bamji et al., 1998) and CNS (Von Bartheld et al., 1994; Frade and Barde, 1999) *in vivo* and *in vitro* (Friedman and Greene, 1999; Gu et al., 1999; Soilu-Hanninen et al., 1999). It should be noted that in some cases expression of p75NTR alone is not sufficient to elicit apoptosis (Ladiwala et al., 1998). The cellular ratio of p75NTR to Trk is a fundamental determinant of the signaling by exposure to a given neurotrophin (Lee et al., 1994; Barrett and Georgiou, 1996). p75NTR can antagonize TrkA-mediated cell survival response, and conversely, TrkA-mediated rescue involves not only activation of survival signals but also simultaneous suppression of p75NTR-mediated apoptosis. Nevertheless, there is no doubt that p75NTR death signaling participates in the pathological changes in human neurodegenerative diseases, such as motor neuron disease, Alzheimer's disease and ischemia (Dechant and Barde 2002; Coulson, 2006).

In addition to of the regulation of apoptosis, p75NTR is involved in a wider range of biological actions such as cell survival (Hamanoue et al., 1999), proliferation of precursor cells (Young et al., 2007), migration of Schwann cells (Anton et al., 1994), and retrograde transport of neurotrophins (Curtis et al., 1998), target innervation (Bentley and Lee,; Lee et al., 1992; Lee et al., 1994; Yamashita et al., 1999; Walsh et al., 1999a; Walsh et al., 1999c), myelination (Song et al., 2006). Increasing evidence has also shown that p75NTR can interact with other coreceptor such as the Nogo receptor (NgR) and LINGO-1 to inhibit axonal growth (Wang et al., 2002; Mi et al., 2004). Nevertheless, the controversial role of p75NTR in nerve regeneration has been recently raised (Song et al 2004; Zhou and Li, 2007).

p75NTR mediates signaling transduction through interactions between the intracellular domain of p75NTR and a number of secondary messenger systems such as TRAF6 (Carter et al., 1996), Rho (Montaner et al., 1998), NFκB (Carter et al., 1996), JNK (Verheij et al., 1996; Yoon et al., 1998), ceramide (Bredesen and Rabizadeh, 1997; Chao et al., 1998), a zinc finger-containing molecule called neurotrophin receptor interacting factor (NRIF) (Casademunt et al., 1999). In addition to NGF, BDNF and other trophic factors like leukemia inhibitory factor (LIF) (Savitz and Kessler, 2000) induces apoptosis via p75NTR as well (Bamji et al., 1998).

4.3. Neurotrophic Factor Dependence of Primary Sensory Neurons during Development and in Adulthood

4.3.1. Development of Primary Sensory Neurons in DRG

Despite the heterogeneity of anatomical and functional properties, primary sensory neurons originally are derived from a common sensory precursor, the neural crest and placode (Le Douarin and Smith, 1988). Various genetic approaches lead to the identification of regulators of cell fate during sensory neurogenesis (Abson et al., 2001; Marmigere and Ernfors 2007). Two different sublineages of sensory precursor populations (A and B) have been shown to use neurogenin (*ngn)1* and *ngn2* genes to control the timing of sensory neurogenesis and generate different subtypes of primary sensory neurons. *ngn2* is required in the early differentiating sensory sublineage that gives rise to large TrkB and TrkC neurons whereas *ngn1* is needed by a later differentiating lineage that generates small TrkA neurons, as well as a subset of large neurons (Ma et al., 1998; 1999). It has been shown that a distinct subpopulation of sensory neurons requires different regulators to promote their axonal growth. The Ets-domain transcription factors *ER81* and *PEA3* are expressed by muscle afferent sensory neurons and their central targets, the motor neurons. *ER81* is required for the establishment of the monosynaptic circuit between *ER81*-expressing sensory and motor neurons that innervate the same muscle groups (Arber et al., 2000). Whereas *DRG11*, a homeodomain transcription factor, is uniquely expressed by cutaneous afferent sensory neurons and required for the formation of connection between cutaneous afferent sensory neurons and the central termination in dorsal horn (Saito et al., 1995; Chen et al., 2001), *NGF/TrkA* signaling is critical for the proper peripheral process of cutaneous afferent sensory neurons (Patel et al., 2000). Further studies demonstrated that NGF promotes differentiation and maturation of nonpeptidegic neurons through both Ret-dependent and Ret-independent

signaling mechanisms (Luo et al., 2007). Moverover, the Runt domain transcription factor RunX1 is critical for Ret-expressing nonpeptidergic nociceptors and axonal projections of DRG neurons (Chen et al., 2006, Yoshikawa et al., 2007). Generally, primary sensory neurons in rats are generated over a period of 4 days, with the peak production at embryonic day 12 (E12) and E13 (Lawson et al., 1974). Axons appear in the distal parts of limbs by E14 (Altman and Bayer, 1984). In addition to the regulation by transcription factors, external environmental influences and other diffusible signaling molecules like leukemia inhibitory factor, fibroblast growth factor, heparin growth factor and tumor growth factorβ also have effects on the specification of cell fate (Abson et al., 2001). The following section highlights the dependency of neurotrophic factors in DRG neurons during development and adulthood.

4.3.2. Effects of Neurotrophic Factors on Developing Sensory Neurons before Target Innervation

Before target innervation, early DRG neurons survive initially independent of neurotrophic factors at a brief phase (Wright et al., 1992). However, NT-3 from the tissue en route to their peripheral targets and BDNF autocrine loop play a major role in promoting early maturational changes in these neurons during the earliest stage (Wright et al., 1992). A later study shows that the BDNF autocrine loop is important in sustaining the survival of a subset of adult DRG neurons (Acheson et al., 1995). During the early stages of gangliogenesis, NT-3 has pleiotrophic effects on proliferation, migration, survival, and differentiation of neuronal precursors (Elshamy and Ernfors, 1996; Ockel et al., 1996; Sieber-Blum, 1998). NT-3 mRNA and TrkC mRNA are expressed earlier than NGF mRNA and TrkA mRNA in the neural tube and neural crest cells (Henion et al., 1995). TrkC mRNA (Kahane and Kalcheim, 1994; Lamballe et al., 1994) and protein (Lefcort et al., 1996) are expressed by the majority of DRG neurons in which NT-3 was synthesized (Elshamy and Ernfors, 1996). The analysis of NT-3 or TrkC knock out mice (Farinas et al., 1994; Ernfors et al., 1994a), and rats with NT-3 or TrkC antibody deprivation (Gaese et al., 1994; Lefcort et al., 1996) reveal a substantial loss of TrkC-expressing DRG neurons as early as E11.5, before any of the sensory neurons innervate their peripheral targets (Kucera et al., 1995). These findings suggest that most DRG neuronal precursors and postmitotic neurons express TrkC and locally synthesized NT-3 acts on these neurons by an autocrine/paracrine mechanism well before the period of target innervation (around E13-14) (Wright et al., 1992; Lewin and Barde, 1996).

4.3.3. Neurotrophic Factor Dependence during and after Target Innervation

During the phase of target field innervation, the developing neurons compete for the limited amount of neurotrophic factors provided by the target fields, and those that do not obtain enough neurotrophic factors will undergo programmed cell death shortly after their axons reach their target fields (Oppenheim, 1981). The survival of sensory neurons sequentially depends on BDNF or NT-3 or NGF (Davies, 1996) by the time when neurotrophic factors are not only required for the survival but also related to their sensory modality. In later development, particular neurotrophin modulates sensory neurons to differentiate into functional identified subtypes (Lewin and Barde, 1996), and continues to exert multiple biological actions during adulthood.

4.3.4. NGF is a Survival Factor for Nociceptive Neurons at the Earlier Development

While the majority of DRG neurons express TrkC before E 11.5, by E13.5 in mouse, most of the neurons have downregulated TrkC that becomes restricted to a subset of large neurons called proprioceptive neurons. In contrast, 80% of DRG neurons with small-diameters express TrkA until birth (Farinas, 1999), while only a subset synthesizes TrkB and p75NTR (Ernfors et al., 1988). The major action of NGF has been found to be involved in promoting the survival of a subpopulation of embryonic sensory neurons (Levi-Montalcini, 1987). This finding firstly came from a study which found that in utero deprivation of NGF by antibody treatment on fetal rodents resulted in a substantial loss of DRG neurons (Johnson and Gorin, 1980; Goedert et al., 1984). Analysis of mice defective in TrkA receptor or NGF has further confirmed the survival role of NGF in TrkA-expressing neurons. In these mice, histological analysis of DRG shows profound neuronal loss in neuropeptidergic neurons, which express TrkA. Furthermore, peripheral and central processes from these neurons were completely absent. These mice are hypoalgesic, which is characterised by a complete loss of response to noxious mechanical and thermal stimuli (Crowley et al., 1994; Smeyne et al., 1994). Similarly, genetic study has shown a mutation in the TrkA gene leads to congenital insensitivity to pain in human patients (Indo et al., 1996). The observations demonstrate that most TrkA-expressing neurons convey nociceptive information and NGF is required for the survival of nociceptive neurons and the regulation of nociception.

4.3.5. Sensitivity Changes of a Subset of Nociceptive Neurons to Neurotrophic Factors at Later Developmental Stage

As discussed above, about 80% of all DRG neurons express TrkA and are dependent on NGF for survival until birth. However, between birth and postnatal weeks 3, a subset of these neurons changes NGF sensitivity and becomes responsive to GDNF (Molliver et al., 1997). Nearly half of small neurons that express TrkA lose NGF sensitivity (Bennett et al., 1998b; Molliver et al., 1997)**.** Thus, 40%-45% of adult DRG neurons express TrkA and are small-sized, however, about 20% of the TrkA-expressing neurons are large in cell size and are likely to be high-threshold mechanoreceptors. At the late embryogenesis, the subpopulation of small neurons that have a downregulated TrkA expression turns to bind IB4, and do not express neurotrophin receptors during adulthood. The IB4-binding neurons account for about 30% of DRG neurons (McMahon et al., 1994; Averill et al., 1995; Wright and Snider, 1995). They begin to express Ret or GFRα (Molliver et al., 1997; Fundin et al., 1999; Huang and Reichardt, 2001) that the expression persists to adult (Pachnis et al., 1993). These neurons become sensitive to GDNF (Molliver et al., 1997). Moreover, a high level of GDNF synthesis is mainly found in the periphery during development (Wright and Snider, 1996). These findings indicate that GDNF may act as a trophic factor for sensory neurons during target innervation. It has been demonstrated that in NGF knockout mice, the IB4-binding population of DRG cells fails to develop (Silos-Santiago et al., 1995). Moreover, recent evidence showed that NGF regulates expression of Ret, instructing a subset of TrkA-expressing sensory neurons to adopt the nonpeptidergic sensory neuron fate (Luo et al., 2007). Taken together, IB4-binding neurons are sensitive to NGF during embryogenesis, whereas during the postnatal period IB4-binding neurons switch from NGF to GDNF

dependence. These findings provide evidence that a single population of sensory neurons at different stages requires differential neurotrophic factors for their survival.

4.3.6. Roles of NGF in the Postnatal Period and Adulthood

Both NGF and its receptors continue to be expressed throughout adult life (Averill et al., 1995; Wright and Snider, 1995), suggesting its importance continues to the postnatal period and adulthood. However, unlike NGF functions as a survival factor during embryogenesis (Kirstein and Farinas 2002), nociceptive neurons do not depend on NGF for survival beyond postnatal day 2 (Lewin et al., 1992), and mature sensory neurons do not die after NGF withdrawal (Johnson and Deckwerth, 1993). Once sensory neurons reach maturity, NGF switches its supportive roles from neuronal survival to the regulation of neuronal phenotypes and to mainly mediate neuroinflammatory/neuroimmune response and initiate hypersensitivity (Nicol and Vasko 2007). For example, withdrawal of NGF by the treatment of NGF antiserum during the early postnatal period results in a phenotypic switch. The Aδ fibers that respond to high-threshold mechanical stimulation fail to develop, become down hair (D hair) fibers which respond to light touch, but D hair fibers still conduct via Aδ fibers (Ritter et al., 1991). It has been shown that NGF is required for the development of Aδ cutaneous nociceptors. Moreover, NGF also regulates morphology of sensory neurons and gene expressions such as TrkA and p75NTR, neuropeptides, non-neuropeptides and ion channels (Sofroniew et al., 2001, Xu and Hall, 2007, Luo et al., 2007). In addition to the regulation of phenotype properties, NGF plays important roles in the functional properties of nociceptive neurons. Application of exogenous NGF or overexpression of NGF in skin using the keratin-14 promoter results in increased survival of both the C and Aδ class of nociceptive neurons and an increase in the responsiveness to nociceptive and heat stimuli or capsaicin (Stucky et al., 1999a; Shu and Mendell, 2001). Transgenic animals overexpressing NGF in the epidermis from mid-developmental stages display hyperalgesia when tested in adults (Davis et al., 1993). Conversely, in NGF knockout mice, there is a loss of adrenergic sympathetic function, and the levels of SP and CGRP, the capsaicin sensitivity and axon reflex vasodilatation are reduced (Anand et al., 1991). Subcutaneous infusion of TrkA-IgG fusion protein by selectively antagonising NGF into the hindpaw markedly decreases responsiveness to heat and chemical stimuli (McMahon et al., 1995). Similarly, treatment of neonatal NGF antiserum induces reduction of mechanical sensitivity of the Aδ high-threshold mechanoreceptors and the noxious heat sensitivity of C-fibers (Lewin et al., 1992). On the other hand, mice with a deletion of p75NTR exhibit a loss of heat injury-induced hyperalgesia (Bergmann et al., 1997), indicating that p75NTR may play a role in pain, but the mechanism of p75NTR in the mediation of nociceptive action of NGF needs to be further investigated. These findings suggest that the action of NGF in maintenance of sensitivity of nociception.

4.3.7. NT-3/TrkC Signaling in the Development of Survival and Modality of Proprioceptive Neurons

NT-3/TrkC is critically required for the survival of proprioceptive neurons in DRG both *in vitro* (Hohn et al., 1990) and *in vivo* (Farinas et al., 1994; Gaese et al., 1994). By E13.5, 70% of DRG neurons downregulate TrkC expression, with only 10% of neurons expressing

TrkC. The TrkC-positive neurons have medium to large soma areas, and many of their axons express calcium-binding proteins parvalbumin and calretinin (Copray et al., 1994; Klein et al., 1994; McMahon et al., 1994). In the absence of one of RUNX transciption factors Runx3, TrkC neurons do not survive long enough to extend their axons towards target cells, suggesting that Runx3 is a neurogenic TrkC neurons-specific transcription factor (Levanon et al., 2002). TrkC-expressing neurons give rise to the largest myelinated axons (Aα fibers) in the dorsal roots, and convey proprioceptive information of joint movement and position from stretch and tension receptors (muscle spindles and Golgi tendon organs in skeletal muscle) (Klein et al., 1994). Evidence that proprioceptive neurons are dependent on NT-3/TrkC signaling comes from the analysis of NT-3 (Ernfors et al., 1994b; Farinas et al., 1994; Tessarollo et al., 1994; Tessarollo et al., 1997) and TrkC knockout mice (Klein et al., 1994; Minichiello et al., 1995; Liebl et al., 1997, Stephens et al., 2005) and the deprivation of NT-3 by anti-NT-3 treatment (Oakley et al., 1995). The administration of NT-3 antiserum to chicken embryos results in the elimination of proprioceptive neurons. Analysis of NT-3 or TrkC deficient mice reveals different severities of loss of large parvalbumin-positive neurons, and absence of Ia muscle afferents derived from the proprioceptive neurons, which connect primary endings of muscle spindles to motor neurons in the ventral horn. Moreover, muscle spindles do not develop in mice lacking NT-3 or TrkC (Klein et al., 1994; Silos-Santiago et al., 1995). Neutralization of NT-3 by immunoadhesive TrkC-IgG results in a reduction of axonal conduction velocity of intact group I muscle afferents, whereas administration of NT-3 elevates excitatory postsynaptic potential amplitude in skeletal muscle in neonatal rats (Seebach et al., 1999). Conversely, introduction of the NT-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous NT-3 (Wright et al., 1997)**.** Moreover, overexpression of NT3 in muscle rescues spindles and monosynaptic inputs from Ia afferents to motoneurons in the spinal cord in the absence of ER81, a transcription factor is also essential for the formation of muscle spindles and the function of afferent-motoneuron synapses, suggesting that muscle-derived NT3 can modulate spindle density and afferent-motoneuron connectivity independently of ER81 (Li et al., 2006a).

In the adult, proprioceptive neurons remain responsive to NT-3. NT-3 is diffusively expressed in muscle during development, however, its expression becomes restricted to muscle spindles postnatally (Copray and Brouwer, 1994; Griesbeck et al., 1995) and TrkC is also expressed in the spindle fibers (Oakley et al., 1995). Target-derived NT-3 is transported retrogradely to the largest neurons in the lumbar DRG via the sciatic nerve (DiStefano et al., 1992). Correspondingly, about 30-35% of adult DRG neurons strongly express TrkC mRNA, whereas another 10% express TrkC at low levels (Karchewski et al., 1999). In contrast to TrkA, TrkC expression is primarily detected in medium- to large-diameter neurons and also in some small-diameter neurons (McMahon et al., 1994; Wetmore and Olson, 1995; Wright and Snider, 1995). TrkC and TrkA expressions have been reported to overlap in small-diameter DRG neurons (Wright and Snider, 1995). Administration of NT-3 attenuates the loss of conduction velocity in both sensory and motor nerves in the adult rat (Munson et al., 1997a) and cat (Mendell et al., 1999), and successfully prevented behavioral, electrophysiological and morphological changes of large sensory neurons caused by neurotoxic agents (Helgren et al., 1997). Taken together, these findings demonstrate that the

proprioceptive neurons require NT-3/TrkC signaling for the survival and proprioceptive modality during development and adulthood.

4.3.8. Cutaneous Mechanoreceptive Neurons and Neurotrophin Dependence

As stated above, cutaneous mechanoreceptive neurons have large- and medium-sized somata with Aβ fibers and terminate centrally in the deep dorsal horn. In the periphery, they supply a variety of distinct endings to various components of the skin and adjacent subcutaneous connective tissues. Corresponding to this heterogeneity is a complex trophic dependence. Innervation of some endings is dependent on more than one neurotrophin (Fundin et al., 1997). NT-3/TrkC signaling is required for early postnatal survival of cutaneous afferents innervating D hair receptors, low-threshold slowly adapting mechanoreceptors and Merkel cells (Airaksinen et al., 1996a; Fundin et al., 1997). NT-4, but not BDNF is also important for the survival of D-hair receptors (Stucky et al., 1998). However, it acts sequentially after NT3 (Stucky et al., 2002). Thus, the survival D-hair fibers are age-dependent and require NT-3 or NT-4 at different developmental stages.

The role of BNDF/TrkB signaling in the development of cutaneous mechanoreceptors is less well-defined. It has been shown that BDNF is synthesized in developing dermis and TrkB is expressed in small and medium-sized sensory neurons (Mu et al., 1993; Wright and Snider, 1995; McMahon et al., 1994). One would expect that BDNF supports these neurons, however, some conflicting results have been reported. Some studies showed limited cell death in newborn mice with BDNF or TrkB knockout (Minichiello et al., 1995; Liebl et al., 1997) whereas other studies observed about 30% reduction in the number of DRG neurons in 2-week-old BDNF null mutant mice (Ernfors et al., 1994b). Nevertheless, BDNF has functional roles in regulating the mechanosensitivity of slow adapting mechanoreceptor (Carroll et al., 1998) and rapidly adapting cutaneous mechanoreceptors (Meissner corpuscles) (Gonzalez-Martinez et al., 2005). A recent study showed that NT4 overexperssion also enhanced the innervations of Meissner corpuscles and the projection of myelinated axons in the skin (Krimm et al., 2006). Thus, in postnatal life, BDNF, NT-3 and NT4 synergistically control the survival and mechanotransduction properties of mechanoreceptors.

In summary, neurotrophins play distinct functional roles at different stages of development and adulthood. In embryonic life, following a brief phrase of independence of neurotrophins, discrete populations of sensory neurons are dependent on specific neurotrophic factors for survival. This comes from studies of the different Trk receptors expressed by different kinds of sensory neurons and has been further confirmed by studies on the types of sensory neurons being eliminated in neurotrophins or their receptors knockout mice. In later embryonic life and early postnatal life, neurotrophins play critical roles in regulating innervation density of peripheral targets and influence the physiological phenotype of peripheral sensory neurons. Discrete subpopulations of DRG neurons conveying specific sensory modalities exhibit differential neurotrophic factor dependencies. Moreover, a subpopulation of nociceptive neurons switches sensitivity from NGF to GDNF. During adulthood, although neurotrophins are no longer essential for neuronal survival, they continue to exert profound effects on the regulation of gene expression and phenotypic and functional properties of neurons.

5. Plastic Changes of Neurotrophins and their Receptors after Peripheral Nerve Injury

Peripheral nerve injury is associated with profound and extensive debilitating consequences, with the most severe one being the emergence of chronic neuropathic pain. The etiology and mechanisms of pain are poorly understood and existing clinical treatments are largely ineffective. Over the last decade, efforts have been taken to characterise plastic changes in somatosensory system, particularly in primary sensory neurons in a variety of nerve injury models. Neuronal plasticity is confined exclusively to activity-dependent, prolonged functional changes, accompanied by corresponding biochemical and possibly morphological alterations, which lead to functional and behavioral disorders. The understanding of plasticity also provides insights into pain mechanisms. Pain hypersensitivity is an expression of neuronal plasticity, which has diverse reactions to changes in neuronal activity or environment.

Besides their well-established actions in regulating the survival, and differentiation of distinct populations of primary sensory neurons as discussed in previous sections, neurotrophins are actively involved in the processes of neuronal plasticity. Various studies have shown that levels of neurotrophins and their receptors are subject to marked changes in response to peripheral nerve injury. Moreover, neurotrophins remain crucial for regulation and maintenance of the morphological and functional phenotypes of adult sensory neurons. Alterations in the supply of neurotrophic factors are likely to cause changes of neurotransmitter phenotypes and ion channels, which are closely associated with an abnormal transduction, transmission and modulation of nociceptive information in the painful neuropathy. These pathophysiological changes have a broad range of effects on primary sensory neurons such as neuroprotection and pain transmission. Here a review is presented on changes in the expression of neurotrophins and their receptors along the sensory pathway in response to peripheral nerve injury and on the functional roles of neurotrophins in the maintenance of primary sensory neurons after a nerve injury.

5.1. Expression of Neurotrophins in DRG after Peripheral Nerve Injury

In normal adult rodent DRG cells, NGF protein but not NGF mRNA is detectable (Lee et al., 1998). This is in line with a target-derived mechanism of NGF trophic support for nociceptive sensory neurons. NGF is synthesized by peripheral targets of sensory neurons such as keratinocytes (Tron et al., 1990; Mearow et al., 1993) and fibroblasts (Anand et al., 1995) in skin, then retrogradely transported to sensory neurons after binding to their receptors (Reynolds et al., 1999). In contrast to NGF, NT-3 serves as a target-derived neurotrophic factor for proprioceptive neurons. It is synthesized in muscle spindle (Copray and Brouwer, 1994). The retrograde transportation of NT-3 is similar to NGF in a receptor-mediated manner (Nitta et al., 1999). NT-3 protein and very low level of NT-3 mRNA can be detected mostly in large neurons and some small neurons (Zhou and Rush, 1995; Chen et al., 1996; Zhou et al., 1999b). Thus, NGF and NT-3 are target-derived neurotrophins for a distinct population of sensory neurons. Although NGF and NT-3 have been demonstrated to have

capability of anterograde transport in CNS (Von Bartheld et al., 2000), neither of them is anterogradely transported by DRG neurons.

BDNF falls in the same target-derived model of neurotrophic support on responsive neurons as NGF. BDNF is produced in target tissues of peripheral neurons (Koliatsos et al., 1993)**,** and retrogradely transported by responsive neurons (Altar and DiStefano, 1998). Both p75NTR and TrkB seem to be able to mediate retrograde transport of BDNF (Curtis et al., 1995; Bhattacharyya et al., 1997; Watson et al., 1999). On the other hand, unlike NGF, BDNF is also a neuronal-derived neurotrophin. DRG neurons themselves are capable of synthesizing BDNF in TrkA-positive neurons (Kashiba et al., 1997, Apfel et al., 1996; Cho et al., 1997; Michael et al., 1999a; Zhou et al., 1999a) in a NGF-dependent manner. However, since DRG neurons do not receive synaptic contacts on their cell bodies, BDNF produced by these cells is unlikely to play a role as a target-derived trophic support for an innervating neuronal population. The production of BDNF by sensory neurons leads to investigation on the possibilities of anterograde transport of endogenous BDNF towards the targets of their axonal processes. Later studies have demonstrated that endogenous BDNF is transported anterogradely to peripheral and central nerve terminals in primary sensory neurons (Zhou and Rush, 1996; Michael et al., 1997). Ultrastructural studies show that BDNF is packaged in dense- and/or clear-cored vesicles (Michael et al., 1997; Luo et al., 2001), and is released locally within DRG (Acheson et al., 1995) and from central terminals in the dorsal horn (Michael et al., 1999a; Lever et al., 2001), where it controls spinal cord excitability (Thompson et al., 1999). The anterogradely transported BDNF might serve as a neurotransmitter or neuromodulator to act on TrkB expressing neurons in DRG or on postsynaptic neurons in dorsal horns, modifying the properties of second order neurons. Thus, BDNF serves as both a classical target-derived neurotrophin and an anterograde source of neurotrophin to neuronal targets (Tonra, 1999).

It is worth noting that different models of nerve injury have different effects on NGF expression in DRG neurons. Following spinal nerve injury, the amount of NGF protein in DRG decreases (Raivich et al., 1991; Lee et al., 1998), while NGF mRNA increases after CCI (Sebert and Shooter, 1993; Wells et al., 1994; Herzberg et al., 1997). In situ hybridization studies show that upregulated NGF and NT-3 mRNA are localized in the pericellular satellite cells after sciatic nerve injury (Lee et al., 1998; Zhou et al., 1999a). While L5 VRT without sensory axotomy induces upreguation of NGFmRNA and protein in the small diameter sensory neurons in uninjured DRG (Li et al., 2003).

Sensory axotomy increases BDNF mRNA and protein in injured DRG (Ernfors et al., 1993; Sebert and Shooter, 1993; Tonra et al., 1998; Zhou et al., 1999b, Michael et al.,1999; Obata et al., 2006a) and uninjured DRG (Fuokuka et al., 2001), and also enhances BDNF anterograde transport (Tonra et al., 1998). Moreover, DRG neurons switch BDNF phenotype in response to axotomy. Small TrkA-expressing neurons which normally express BDNF switch off their BDNF expression, while medium and/or large TrkB- and TrkC-expressing neurons which are normally negative for BDNF switch on BDNF production (Michael et al., 1999a, Kashiba, 1999, Zhou, 1999b). The pathophysiology of this BDNF phenotypic switch in response to a nerve injury remains unclear. However, the down-regulation of BDNF in small neurons may be related to the blockade of axonal transport of NGF, and a reduced supply of NT3 to injured sensory neurons may contribute to the elevated expression observed

in the large neurons (Michael et al., 1999a; Zhou, 1999b). Further study confirmed that differential modulation of neuronal BDNF expression in injured sensory neurons by NGF and NT3 (Karchewski et al., 2002). NGF can regulate BDNF expression in TrkA-expressing neurons regardless of the axonal state and that elevated levels of BDNF may contribute to the down-regulation in TrkB expression associated with these states. By contrast, NT-3 acts in an antagonistic fashion to NGF by mitigating expression BDNF in injured neurons. Interestingly, L5 VRT also induced BDNF upregulation in intact DRG neurons and pericellular satellite cells (Obata et al., 2004; Li et al., 2006b).

The rat BDNF gene has four major transcript forms, each containing a distinct 5' exon and a shared 3' exon encoding BDNF protein (Timmusk et al., 1993; Bishop et al., 1994). Thus, multiple differentially regulated BDNF transcripts contribute to the different anatomical and functional patterns of BDNF expression in response to external and internal stimuli. It is likely that different BDNF gene promoters (Timmusk et al., 1993) are involved in the regulation of BDNF expression in small and large sensory neurons after nerve injury (Timmusk et al., 1995) or even different types of nerve injury (Kim et al., 2001). It is known that NGF regulates BDNF expression. An elevated NGF level in the periphery induced upregulation of BDNF expression in TrkA-positive DRG neurons and their projections in the dorsal horn (Apfel et al., 1996; Cho et al., 1997; Michael et al., 1997). Blocking NGF action has been shown to prevent an inflammation-induced rise in BDNF level (Cho et al., 1997). Moreover, NGF alters the electrical activity of nociceptors (Djouhri et al., 2001; Shu and Mendell, 2001) which stimulates BDNF synthesis in the DRG via calcium influx and/or activation of extracellular signal-regulated protein kinase (ERK) (Dai et al., 2002; Obata et al., 2004) and transcription factors such as CREB (Shieh et al., 1998; Tao et al., 1998). A recent study further investigated the effect of NGF on the multiple BDNF promoters and the signaling pathway by which NGF activates these promoters in PC12 cells, and have demonstrated that the NGF-actitvated ERK1/2 pathways promote BDNF promoter IV to the greatest extent in response to NGF but independently of NGF-activated signaling pathways involving protein kinase A (PKA) and protein kinase C (PKC) (Park et al., 2006). Recently, among all of BDNF splice variants, exon 1-9 has been identified to have the greatest increase in response to stimulation of NGF and peripheral inflammation (Matsuoka et al., 2007).

5.2. Expression of Neurotrophin Receptors in DRG after Peripheral Nerve Injury

The expression pattern of Trk receptors in normal developing and adult DRG has been described as above. As for p75NTR, it is expressed by many DRG cells both during development and in the adult (Kashiba et al., 1995; Yamamoto et al., 1996; Zhou et al., 1996; Bennett et al., 1996b). It is generally coexpressed with most of the Trk receptors, except that about 50% of TrkC-expressing neurons do not express p75NTR, suggesting that p75NTR is important in mediating the actions of NGF and BDNF on DRG neurons but it is negligible for a subpopulation of neurons that require NT-3.

Peripheral nerve injury induces differential alternation in the expression of neurotrophin receptors in DRG. Sciatic nerve lesion leads to a down-regulation of TrkA mRNA (Krekoski

et al., 1996; Shen et al., 1999) and protein expression (Li et al**.,** 1999; Shen et al., 1999), while the number of TrkA-expressing cells slowly decline reducing by 25% at 1 week and 35% at 3 weeks after spinal nerve ligation (Shen et al., 1999), indicating the decreased TrkA is due to deprivation of target-derived NGF. By contrast, sciatic nerve lesion induces upregulation of TrkB mRNA and TrkC mRNA (Ernfors et al., 1993; Foster et al., 1994; Verge et al., 1996; Mannion 1999; Narita 2000), unlike Trk expression, nerve injury induces up-regulation of p75NTR expression in the pericellular satellite cells but down-regulation in sensory neurons (Zhou et al., 1996; Li et al., 2002).

5.3. Expression of Neurotrophins in Sciatic Nerve after Peripheral Nerve Injury

In the normal sciatic nerve of adult rats, NT-3 mRNA level is the highest, followed by NT-4 mRNA and BDNF mRNA (Funakoshi et al., 1993). NGF is produced by immature Schwann cells in peripheral nerve during development (Mirsky and Jessen, 1999), but it is not expressed to a significant extent in Schwann cells in adult (Heumann et al., 1987b). After a peripheral nerve injury, although the retrograde transport of NGF from target tissues to sensory neurons is reduced in sciatic nerve (Heumann et al., 1987; Raivich et al., 1991), there is a compensatory supply of NGF by non-neuronal cells expressing NGF mRNA. NGF mRNA displays a biphasic increase, including rapid production by Schwann cells (Lindholm et al., 1987; Heumann et al., 1987; Matsuoka et al., 1991; Frostick et al., 1998), and a long-lasting increase from invading macrophage (Safieh-Garabedian et al., 1995). In contrast, BDNF displays a monophasic increase in Schwann cells and fibroblasts at a slower rate in the distal part of the injured sciatic nerve (Otten et al., 1980; Meyer et al., 1992; Funakoshi et al., 1993 Nitta et al., 1999, Omura et al., 2005), but the increase of BDNF level is much higher than that of NGF. The NT-3 protein level transiently increases in the distal transected nerve but returns to normal by 21 days (Cai et al., 1998). NT-4 mRNA level decreases shortly after injury but increases thereafter (Funakoshi et al., 1993). Thus, in contrast to the intact nerve, BDNF mRNA level is the highest while NT-3 mRNA level is the lowest in the injured nerve.

5.4. Expression of Neurotrophin Receptors in Sciatic Nerve after Peripheral Nerve Injury

In the normal adult rat sciatic nerve, TrkA mRNA is undetectable while TrkB and TrkC are present depending on their isoforms and maturation (Funakoshi et al., 1993)**.** A significant level of expression of truncated TrkB is found in adult nerve (Frisén et al., 1993). High levels of full-length and truncated TrkC mRNA and protein are found in the sciatic nerve during early development, whereas in the adult sciatic nerve full-length TrkC is downregulated and truncated TrkC is maintained in Schwann cells (Offenhauser et al., 1995). p75NTR is expressed by cells in peripheral nerve during development, but the expression reduces significantly in adult nerve (Heumann et al., 1987). After peripheral nerve injury, TrkA mRNA is still undetectable in the injured sciatic nerve (Sebert and Shooter, 1993), but

levels of truncated TrkB and TrkC mRNAs in Schwann cells increase in the nerve proximal to the transection (Funakoshi et al., 1993). Majority of Schwann cells express a high level of p75NTR mRNA and protein in the denervated distal sciatic nerve segment (Taniuchi et al., 1986).

5.5. Roles of Neurotrophins in the Maintenance of Phenotypic and Functional Properties of Primary Sensory Neurons after Peripheral Nerve Injury

Nerve injury leads to profound changes within sensory neurons, ranging from changes in neurochemistry to functional properties. Expression of neuropeptides, neurotransmitters, ion channels, receptors, and morphology are altered with the formation of sprouting in DRG and sprouting of Aβ fibers in the dorsal horn. Some of the effects of nerve injury are thought to reflect changes in the availability of target-derived growth factors. It has been extensively demonstrated that neurotrophins are involved in the regulation of these changes.

Substantial evidence suggests that NGF signaling subserves neuroprotection and facilitates regrowth and repair during the injury response. Peripheral nerve injury results in neuronal atrophy, death and loss in DRG (Ekstrom, 1995; Groves et al., 1997) and transganglionic degeneration characterized by the withdrawal of central axons from synaptic sites in the superficial layer of dorsal horn (Aldskogius et al., 1985; Kitchener et al., 1994). The degree of cell loss may vary considerably in their vulnerability to axotomy due to different kinds of injuries, types of neurons and maturation (Coggeshall et al., 1997; Vestergaard et al., 1997; Groves et al., 1997). Administration of exogenous NGF prevents the loss of the responsive populations of DRG neurons (Cheema et al., 1996; Matheson et al., 1997).

In addition to the selective protection by NGF, differential regulation of peptide expression in small-diameter sensory neurons has been demonstrated. Exogenous NGF prevents the down-regulation of neuropeptides such as SP, CGRP and PACAP (Fitzgerald et al., 1985; Verge et al., 1995; Jongsma et al., 2001), its own receptors (Verge et al., 1992) and ion channels such as sensory neuron specific sodium (SNS) channel (Dib-Hajj et al., 1998; Fjell et al., 1999), transient receptor potential channels (TRPA1), Nav1.8 and Nav 1.9 (Gould et al., 2000). NGF also partially reverses a reduction in neurofilament expression, axon diameter and conduction velocity particularly in small sensory neurons (Verge et al., 1990; Gold et al., 1991). On the other hand, NGF prevents the up-regulation of transcription factors such as c-jun (Gold et al., 1993) and ATF3 (Averill et al., 2004), and it also partially prevents the upregulation of galanin, vasoactive intestinal polypepide, neuropeptide Y (NPY) and cholecystokinin (Verge et al., 1995). Thus, the nerve injury-induced increase of NGF from injured nerve plays important roles in modifying the expression of neuropeptides in primary sensory neurons. Furthermore, NGF subserves neural regeneration. A marked upregulation of both NGF and p75NTR in proliferative cells following axotomy is indicated to enhance axon elongation (Madison and Archibald, 1994) by promoting cell migration (Anton et al., 1994). Further studies have shown that NGF also prevents sema 3A-mediated growth cone collapse in the adult rat DRG neurons (Wanigasekara et al., 2006).

In contrast to NGF, NT-3 plays crucial roles in the maintenance of proprioceptive neurons following axotomy. Exogenous NT-3 prevents nerve injury-induced loss of proprioceptive neurons (Groves et al., 1999), and attenuates the down-regulation of TrkC, p75NTR and neurofilament (Verge et al., 1996) and the upregulation of NPY (Ohara et al., 1995; Sterne et al., 1998) and PACAP (Jongsma et al., 2001) in proprioceptive neurons. Moreover, electrophysiological studies demonstrate that NT-3 restores sensory conduction velocity in a dose-dependent manner in injured DRG (Verge et al., 1996; Munson et al., 1997a).

Taken together, these studies support roles of NGF and NT-3 in the selective maintenance of distinct phenotypes of DRG neurons after peripheral nerve injury. In addition to these protective roles of NGF and NT-3 in the nerve injury-induced neuropathy, they also play roles in the neuroprotection in other kinds of experimental peripheral neuropathy, such as diabetic neuropathy (White et al., 1996; Apfel et al., 1998) and toxic sensory neuropathy (Apfel et al., 1991; Apfel et al., 1992; Gao et al., 1995; Helgren et al., 1997).

BDNF has neurotrophic effects on axotomized sensory neurons. In neonates, BDNF delays sensory neuron death after sciatic nerve section (Eriksson et al., 1994), and reduces naturally occurring degeneration of sensory neurons (Hofer and Barde, 1988). In addition to neurotrophic effects, BDNF could affect the responses of Schwann cells to nerve injury, acting through an increased expression of p75NTR (Funakoshi, 1993) to promote cell death or cell migration (Casaccia-Bonnefil et al., 1999). Furthermore, BDNF has been implicated in axonal regeneration of adult sensory neurons *in vitro* (Lindsay, 1988) and *in vivo* (Lewin et al., 1997; Zhang et al., 2000; Song et al., 2008). Peripheral nerve injury induced DRG-derived BDNF has been indicated for a source of nociceptive stimuli for neuropathic pain, which is demonstrated by a reduction in allodynia after antibodies to BDNF delivered to injured DRG in the L5 SNT model (Zhou et al., 2000).

6. Neuropathic Pain

6.1. Types of Pain

Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Millan, 1999). It is a constellation of different sensitivities in normal and diseased states (Woolf and Decosterd, 1999). Two distinct kinds of pain exist, namely physiological pain and pathological pain. Physiological pain is a key component of the body's normal defence mechanisms, i.e. it is an essential early warning device protecting the body from a potentially hostile external environment by initiating behavioral and reflex avoidance strategies. This pain is adaptive or has biological function, and is characterised by the activation of high-threshold C/Aδ fibers and limited duration. It is only elicited when noxious stimuli threaten to damage normal tissues, thus, it can be also termed nociceptive pain. Whereas pathological pain with persistent pain syndromes has no biological advantages and causes suffering and distress.

Neuropathic pain is a kind of pathological pain due to a dysfunction of or damage to the nervous system. Peripheral neuropathic pain means pain due to a dysfunction of or damage to

a nerve or group of nerves, primarily peripheral nociceptor afferents (Millan, 1999). Etiologically, it has different types, such as traumatic, metabolic, infectious, toxic, ischaemic, hereditary, compression by tumor and immune-mediated neuropathies (Figure 2). Syndromes of neuropathic pain comprise a complex combination of positive and negative symptoms. Negative symptoms include hypothesia with loss of sensation. Positive symptoms manifest as spontaneous pain (stimulus-independent pain) or pain hypersensitivity elicited by a stimulus after damage to sensory neurons (stimulus-evoked pain) including hyperalgesia and allodynia. Hyperalgesia is an increase in the sensitivity to noxious stimuli (Woolf and Mannion, 1999). It is classified into subgroups on the basis of modality-mechanical, thermal or chemical. Allodynia is characterized as pain due to an innocuous stimulus, typically gentle tactile stimulation. It can be produced in two ways: by the actions of low-threshold myelinated Aβ fibers on an altered CNS, and by a reduction in the threshold of nociceptor terminals in the periphery.

Neuropathic pain is a persistent pain. Patients not only have physical complications but also have emotional suffering. Such a chronic pain cycle leads to a series of extensive and severe problems such as social withdrawal, financial pressure and psychological problems (Figure 2). The main problem is that there is no effective treatment to prevent or control neuropathic pain suffered by clinical patients.

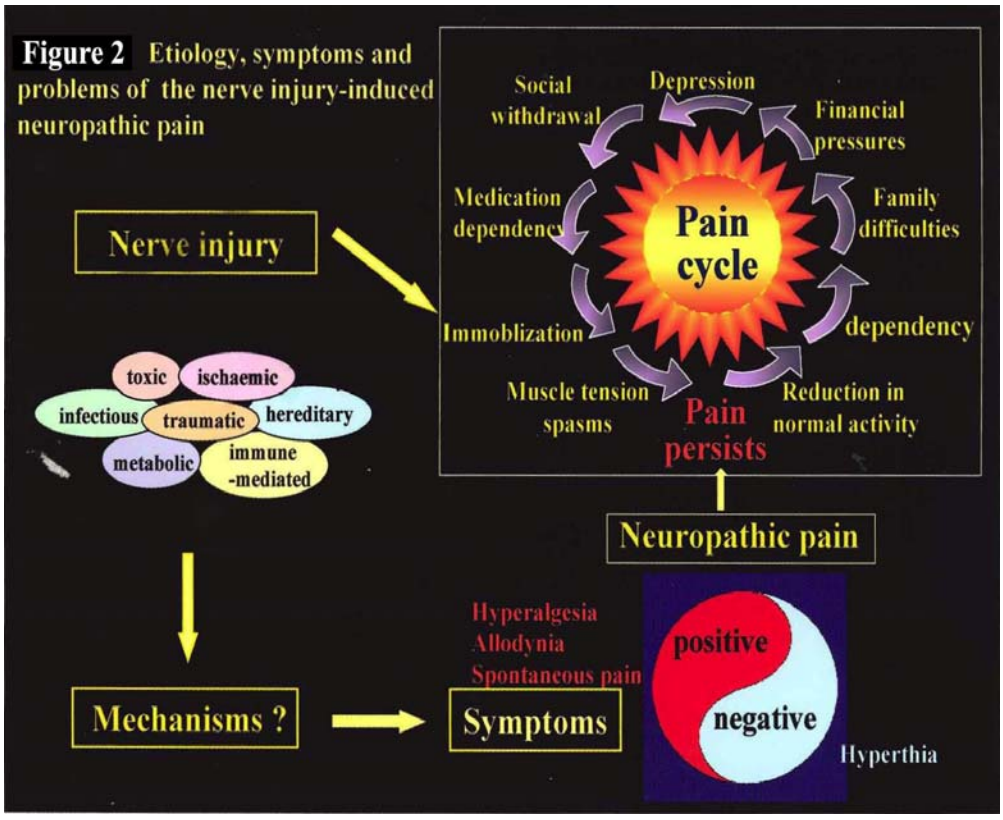


Figure 2. Schematic diagram showing etiology, symptoms and problems of the nerve injury-induced neuropathic pain, which contributes to the pain cycle, leading to a series of extensive problems such as social withdrawal, financial pressure, physical and psychological problems.

The clinical importance of these neuropathic pain syndromes provides a strong incentive for understanding the underlying pathophysiological mechanisms. Prior to 1960, neuropathic pain lacked evidence-based theories, until the first specific hypothesis of pain mechanism came out of the Melzack-Wall Gate Control theory (Melzack and Wall, 1965)**.** Up to now, enormous progress has been made by the exploration of modern neurobiological techniques in the elucidation of mechanisms that contribute to the pathogenesis of pain (Woolf and Salter, 2000). Clinical investigations have shown that distinct symptoms can be related to distinct neurobiological changes and this has led to the currently evolving concept of a mechanism-based classification of neuropathic pain which supplements the traditional etiology-based classification, furthermore, clinically, Identifying neurobiological mechanisms together with pharmacological agents that specifically target these mechanisms, seems to be the most logical and rational way of improving neuropathic pain treatment (Finnerup and Jensen , 2006). There is a general consensus today that both the peripheral and central nervous system contribute to different aspects of neuropathic pain. This part of the review will highlight the peripheral and central mechanisms of neuropathic pain on the level of nociceptors, primary sensory neurons and dorsal horn of spinal cord based on peripheral neuropathic pain animal models.

6.2 Experimental Models of Peripheral Neuropathic Pain

The imperative in developing laboratory animal models of neuropathic pain has been trying to reproduce or mimic pain in human. The development of several peripheral nerve injury-induced neuropathic pain models has provided us useful tools to gain insight into the pain mechanisms, through investigating of the correlation between the cellular and molecular changes occurring in painful neuropathies and pain behaviors (Sah et al., 2003). Different types of peripheral neuropathic pain have been developed typically by physical trauma, including transection, crush, loose or tight ligation and crynoneurolysis. In addition, based on variations in nerve injury, partial and complete peripheral nerve injury animal models have been employed. Complete peripheral nerve injury by cutting or crushing the sciatic or spinal nerve will eliminate all inflow from the periphery. However, this model produces motor weakness and precipitates autotomy, and thus it is not practical for behavioral studies. The following neuropathic pain models are based on partial denervation. (1) Only the lumbar 5 or both the lumbar 5 and 6 spinal nerves on one side are tightly ligated or cut (Kim and Chung, 1992; Kinnman and Levine, 1995, Li et al., 2000), removing a major part of the sensory inflow for the sciatic plexus**.** (2) The chronic constriction injury (CCI) model involves four loose chromic suture ligatures tied around the sciatic nerve, producing a variable amount of axotomy as a consequence of the swelling and inflammation generated by the sutures (Bennett and Xie, 1988). (3) Partial sciatic nerve injury model in which about a third to a half of the sciatic nerve is tightly ligated (Seltzer et al., 1990) or transected (Lindenlaub and Sommer, 2000) proximal to its division into its terminal branches. (4) A spared nerve injury model, which involves a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact (Decosterd and Woolf, 2000). The common features of these models are varied extent of sensory neuron

injury and sensory inflow in a partially denervated system. (5) A recently developed L5 ventral root transection (L5 VRT) model, which selectly injure the motor nerve in the L5 ventral root but leave sensory neurons uninjured. The rats with L5 VRT produce neuropathic pain similar to those with L5 spinal nerve injury (Li et al., 2002). This indicates that sensory axotomy is not necessary to induce the neuropathic pain. In addition to physical trauma to the nerves, inflammation-inducing agents have been directly applied to induce sciatic inflammatory neuritis via inflammation and immune activation. These agents include proinflammatory cytokines (high mobility group-1) and zymosan (yeast cell walls) (Eliav et al., 1999; Chacur et al., 2001; Twining et al., 2004). Each model relies on detecting a change in the flexion withdrawal reflex threshold or response to an applied stimulus such as heat, cold or mechanical stimuli), but very few reliable measurements of spontaneous pain are currently available.

6.3. Peripheral and central Mechanisms Underlying Neuropathic Pain

6.3.1. Peripheral Mechanisms in the Generation of Neuropathic Pain

6.3.1.1 Peripheral Sensitization

Ectopic hyperexcitability of primary nociceptive afferents following nerve injury is the main mechanism for peripheral sensitization (Devor, 1999a), which manifests as an increased excitability of the nociceptor terminal membrane, leading to a decreased amount of depolarization for initiation of an action potential discharge in response to noxious stimuli. It is believed that inflammatory mediators (prostaglandins, protons, bradykinin, serotonin, adenosine), cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and/or growth factors (NGF) released from activated inflammatory or immune cells after nerve injury sensitize these primary afferent nociceptors directly or indirectly (Bolin et al., 1995; Fu and Gordon, 1995; Wagner and Myers, 1996; Sorkin et al., 1997; Liu et al., 2000)**.** The sensitization involves the activation of intracellular kinases by G protein-coupled and tyrosine kinase membrane-binding receptors activating protein kinase A (PKA) (Aley, KO, 1999) and/or protein kinase Cε (PKCε) (Khasar et al., 1999; Cesare et al., 1999)**,** or phosphorylating tetrodotoxin-resistant SNS ion channels (Novakovic et al., 1998; Fitzgerald et al., 1999). Nerve injury induces abnormal accumulation of sodium channels and quickly reprimes sodium current in the axonal membrane, particularly in the area of demyelination, at the neuroma and/or at the site of collateral sprouting (Koltzenburg, 1998; Waxman, 1999). Moreover, accumulation of sodium channel renders “ectopic pacemaker” sites of hyperexcitability (Devor, 1999a). Thus, phosphorylation of sodium channel alters the functional properties of nociceptors, as a consequence of increased excitabilities in response to noxious stimuli. In addition, overactive N type Ca^{2+}-channels (Bowersox et al., 1996; Wright and Angus, 1996) and a decrease in K^+ conductance also contribute to spontaneous excitability after nerve injury (Kajander et al., 1992). Ectopic hyperexcitability acts as a primary source of spontaneous pain and exaggerated hyperalgesia in response to noxious stimulation, and also as a factor that triggers and maintains central sensitization (Devor, 2001).

6.3.1.2 Sympathetic and Sensory Neurons Coupling in DRG after Peripheral Nerve Injury

Normally, the activity of primary afferent nociceptors is not affected by sympathetic outflow. However, after peripheral nerve injury, the communication of sympathetic and sensory neurons can be established (Baron et al., 1999; Janig and Habler, 2000). The cutaneous afferents develop noradrenergic sensitivity by increased response to the stimulation of sympathetic efferents in neuroma (Sato and Perl, 1991; Matzner and Devor, 1987) and DRG (Michael M 1996). The increased sensitivity is paralleled by morphological changes in DRG. After peripheral nerve injury, sympathetic postganglionic vasoconstrictor axons that normally innervate blood vessels in DRG sprout to form pericellular baskets around the somata of large-diameter neurons (McLachlan et al., 1993), which are also surround by sprouting fibers from peptidergic sensory neurons containing CGRP, SP, galanin and BDNF (McLachlan and Hu, 1998, Hu and McLachlan, 2001). Ultrastructural observation revealed that these sprouts did not make direct contacts with the soma membrane but actually grew on the surface of proliferated glial cells around large-diameter neurons (Chen et al., 1996; Shinder et al., 1999). Moreover, sympathetic sprouting preferentially occurs on spontaneously active cells and is reduced by nerve block, indicating that a highly localized correlation of neuronal activity is essential for the formation of the sprouting (Xie et al., 2007). Different types and sites of nerve injury appear to trigger distinct patterns of sympathetic sprouting with different mechanisms involved (Ramer et al., 1999). In addition, nonpeptidergic sensory sprouting fibers, which bind to IB4, also occurs in the injured DRG (Li et al., 2000). Multiple sources of neurotrophins and cytokines such as interleukin-6 (IL-6) and LIF contribute to induce the formation of sympathetic and sensory sprouting (Ramer et al., 1999; Zhou et al.,1999).

Although sympathetic efferents and sensory neurons do not have direct morphological connection, upregulation of $\alpha 2$ adrenoreceptors has been found on the membrane of DRG neurons and neuromas after nerve injury (Chen et al., 1996; Shinder et al., 1999). The interaction of sympathetic efferents and sensory neurons is mainly mediated by noradrenaline released from postganglionic sympathetic endings, binding to adrenoreceptors on injured hyperexcitable sensory neurons. Therefore, sympathetic terminals constitute a source of pronociceptive mediators in the periphery.

Sympathetic-sensory coupling is believed to contribute to some degree of sympathetically maintained pain (Ramer et al., 1999). Chemical or surgical sympathectomy attenuates increased sympathetic innervation to DRG and also simultaneously alleviates symptoms of neuropathic pain in some peripheral neuropathy and in patients (Baron et al., 1999a). On the other hand, pain can be rekindled by local administration of noradenergic drug into the injured tissue (Torebjork et al., 1995; Xie et al., 1995).

6.3.1.3. Cross-Excitation of Sensory-Sensory Neurons and their Afferents

Cross-excitation among sensory neurons and fibers is normally very limited. However, after nerve damage, they may exaggerate ectopic activity by setting up reverberating circuits and recruiting silent neurons through ephaptic contacts (Amir and Devor, 2000a). This processing may be mediated by the diffusible factors due to a loss of normal insulation from neurons and neuromas (Utzschneider et al., 1992; Amir and Devor, 1996; Liu et al., 1999).

Cross-excitation might occur between fibers of different classes of DRG neurons. For example, Aβ fibers may evoke allodynia by indirectly recruiting C-fibers in the dorsal horn (Sluka and Westlund, 1993; Cervero and Laird, 1996). On the other hand, cell bodies of sensory neurons also display crossed afterdischarge, which is involved in the depolarization of large-diameter DRG neurons on the repetitive firing of their neighbours (Devor and Wall, 1990). In the normal DRG, the membrane potential of most of small neurons can be affected by the spike activity in large neurons via cross-excitation (Utzschneider et al., 1992; Amir and Devor, 1996). It would be reasonable to predicate that the abnormal structure correlation between sprouting of nerve terminals of small sensory neurons and somata of large neurons (Li et al., 2000) would provide an anatomical basis for the occurrence of neuropathic pain via cross excitation.

6.3.2. Central Mechanisms in the Generation of Neuropathic Pain

6.3.2.1. Central Sensitization

Central sensitization is a C-fiber-mediated increase in membrane excitability of dorsal horn neurons that generate hypersensitivity by recruiting previously subthreshold mechanoreceptor inputs to threshold and initiate action potential discharge (Torebjork et al., 1992). Central sensitization is characterised by a reduction in the stimulation threshold, allodynia, Aβ fiber-mediated hyperalgesia, an expansion of cutaneous receptive fields in the periphery and an increase in background activity of spinal neurons. Central sensitization is transient, fading in minutes or hours, but it may be maintained if nociceptor input persists, and can be produced by injury of peripheral tissues, but in some cases, also after wind-up-inducing repetitive electrical stimulation (Herrero et al., 2000).

The spontaneous firing in C-fibers triggers central sensitization. During peripheral nerve injury, low frequency C-fibers generate cumulative depolarization by summation in the neuroma and DRG, which causes the release of excitatory amino acid (glutamate) and/or neuropeptide transmitters (tachykinins). These released transmitters act on dorsal horn neurons postsynaptically by the activation of N-methyl-D-aspartate (NMDA) receptors and/or tachykinins receptors (NK1 and NK2), producing calcium inward currents as well as phosphorylation of ion channels. The increased excitability will lead to the activation of signal transduction cascades (Woolf and Salter, 2000). It has been found that several different intracellular signal transduction cascades converge on MAPK, activation of which appears to be a key switch or gate for the regulation of central sensitization (Ji and Woolf, 2001) and facilitate excitatory synaptic response in the spinal dorsal horn.

6.3.2.2. Involvement of Non-Nociceptive Low-Threshold Aβ Fibers in Neuropathic Pain

Activation of Aβ low-threshold mechanoreceptors normally never produces pain. Evidence has shown that in the presence of central sensitization peripheral input entering along non-nociceptive, myelinated, Aβ touch afferents may evoke pain after nerve injury (Torebjork et al., 1992). The transduction sensitivity of high-threshold mechanosensitive, nociceptive C-fibers is not remarkably modified in the condition of mechanical allodynia, but this can be eliminated by blocking conduction in Aβ fibers (Koltzenburg et al., 1994). The

mechanisms of mechanical allodynia mediated via Aβ fibers include nerve injury-induced redistribution of Aβ fibers in all laminae of the dorsal horn and Aβ fiber phenotypic switch.

Redistribution of Aβ afferent fibers in the dorsal horn. Neuroanatomical studies have demonstrated that peripheral nerve injury induces reorganization of central connections of Aβ fibers, which sprout from their normal termination site in the deep dorsal horn into lamina II (substantia gelatinosa), the site which receives most of their monosynaptic input from C and A δ fibers but not Aβ fibers (Koerber et al., 1994; Woolf et al., 1995). It is believed that aberrant connection of Aβ fibers in novel territories results from the combination of vacant synaptic sites due to transganglionic degeneration of C-fibers and the reexpression of growth associated molecules such as growth associated protein-43 (Shortland and Woolf, 1993). This new pattern of synaptic input may provide an anatomical substrate for tactile pain hypersensitivity. A recent electrophysiological study showed that following reorganization of Aβ fibers, most of substantia gelatinosa neurons started to receive Aβ afferent input (Nakatsuka et al., 1999).

Aβ fiber phenotypic switch. Pronociceptive molecules such as SP and BDNF are normally expressed only in nociceptive sensory neurons. After peripheral nerve injury or inflammation, large myelinated Aβ fibers begin to express and release these molecules (Noguchi et al., 1995; Neumann et al., 1996; Miki et al., 1998; Li et al., 1999). This switch of expression of the pronociceptive molecules is coupled with their receptors which are also increased in the dorsal horn (Woolf and Doubell, 1994; Abbadie et al., 1996; Narita et al., 2000). Therefore, these molecules may contribute to the generation of central hyperexcitability via activation of signal cascades as mentioned in section 6.3.2.1.

6.3.2.3. Disinhibition

Central sensitization also involves the disinhibition (Woolf and Wall, 1982; Ren and Dubner, 1996) by depression of spinal inhibitory mechanisms to facilitate pain transmission. A decrease in inhibition occurs in three ways (Woolf and Mannion, 1999): (1) by reduction in inhibitory transmitter γ-aminobutyric acid (GABA) and glycine receptors on the central terminals of axotomized neurons, thereby reducing presynaptic inhibition; (2) by a reduction in GABA and glycine in interneurons; and (3) by a reduction of numbers in inhibitory interneurons due to a transynaptic degeneration of the inhibitory interneurons after peripheral nerve injury.

7. Neurotrophins and Pain

The role of trophic factors in the development and maintenance of pain in response to various forms of tissue injury is an area of intensive research in the last several years. In addition to the critical roles during development, neurotrophins also play important roles in persistent pain states. NGF was the first neurotrophin factor to be linked with pain. A recent discovered mutation in the gene that encodes human NGF is associated with diminished pain perception (Einarsdottir et al, 2004). In addition to its role in the regulation of nociceptive pain, under pathological conditions, it acts as an important inflammatory mediator, modulating the sensitivity of the sensory nervous system to noxious stimuli via peripheral

and central sensitization. Emerging evidence implies that BDNF functions as a central neuromodulator in pain transmission. In particular, neurotrophins closely involves nerve injury-induced neuropathic pain. However, the exact roles of neurotrophins in the induction, development and maintenance of neuropathic pain are still to be defined.

7.1. NGF and Pain

7.1.1. NGF is an Inflammatory Mediator

NGF itself is sufficient to elicit hyperalgesia. Local or systemic administration of NGF induces both acute thermal hyperalgesia and delays mechanical hyperalgesia in rodents (Lewin et al., 1993, Ma and Woolf, 1997; Bennett et al., 1998a, Sivilia et al., 2008). In humans, intravenous or intramuscular injection of very low doses of NGF (1μg/kg) produces widespread pains in deep tissues (myalgia-like symptom) and hyperalgesia at the injection sites (Dyck et al., 1997; McArthur, 2000; Svensson et al., 2003). Endoneurial administration of NGF into the sciatic nerve also produces hyperalgesia (Ruiz et al, 2004). Blocking NGF bioactivity systemically or locally largely blocks the effects of inflammation on sensitivity of sensory neurons. Application of NGF antibody (Lewin et al., 1994; Ishikawa et al., 1999) prevents the development of thermal hyperalgesia induced by inflammatory reagents. Thus, endogeneous NGF is necessary to mediate inflammatory hyperalgesia (Mendell et al., 1999).

NGF expression at both the mRNA and protein levels is elvated in skin in a wide range of animal models of inflammation, including those induced by complete Freund's adjuvant (CFA) (Donnerer et al., 1992; Woolf et al., 1994), subcutaneous carageenan (Aloe et al., 1992b), interleukin-1 beta (IL-1β) (Safieh-Garabedian et al., 1995), ultraviolet light (Gillardon et al., 1995) or TNFα (Woolf et al., 1997) and in a rat model of cystitis (Oddiah et al., 1998; Dupont et al., 2001). Elevated NGF levels have been also found in a variety of inflammatory states in human including rheumatoid arthritis (Aloe et al., 1992a; Halliday et al., 2004), cystitis (Lowe et al., 1997), and inflammatory autoimmune disorders such as multiple sclerosis (Aloe et al., 1994; Falcini et al., 1996). These pools of NGF are derived from various cellular sources including immune cells (mast cells) (Horigome et al., 1994), lymphocytes (Santambrogio et al., 1994), macrophages (Brown et al., 1991), fibroblasts (Woolf et al., 1997), Schwann cells (Campana, 2007) and keratinocytes (English et al., 1994; Pincelli and Yaar, 1997). In addition, many proinflammatory cytokines are also released from these cells during inflammation. In particular, IL-1β and TNFα are important in the synthesis and release of NGF during inflammation (Friedman et al., 1990; Hattori et al., 1993). Thus, it is no doubt that peripheral NGF substantially contributes to the development of inflammatory hyperalgesia. However, it is essential to consider action of NGF on sensitizaton of sensory neurons is direct or indirect due to effects of NGF on multiple cell types (Nicol and Vasko, 2007).

7.1.2. NGF and Neuropathic Pain

Unlike the definitive roles of NGF in inflammatory pain, the action of NGF in neuropathic pain following peripheral nerve injury is so far controversial. The confusion over exactly what happens to NGF levels following various models of nerve injury is reflected in

the conflicting results from the effects of NGF supplementation or antagonism on pain-related behaviors (Hefti et al., 2007). Ren et al showed that the direct infusion of NGF to a ligated sciatic nerve abolishes thermal hyperalgesia and mechanical sensitivity following CCI (Ren et al., 1995). This effect has been confirmed in other studies (Theodosiou et al., 1999; Ramer et al., 1999). Furthermore, a newly developed synthesized NL1L4 peptide mimics the activity of NGF and can reduce thermal and mechanical hyperalgesia induced by CCI (Colangelo et al., 2008), suggesting the potential therapeutic application for this NGF-mimetic peptide to peripheral neuropathy. However, in the same nerve injury model, Ro et al reported that delivery of either NGF or antibody to NGF results in a reduction in hyperalgesia at different onset time (Ro et al., 1999). A later study demonstrated that local and systemic administration of antibody to NGF decreases L5 spinal nerve transection-induced mechanical allodynia (Deng et al., 2000a). In CCI model, neutralizing anti-NGF antibodies reverses tactile allodynia and thermal hyperalgesia even when administered 2 weeks after surgery, suggesting that NGF play a critical role in a models of established neuropathic pain with no development of tolerance to antagonism (Wild et al., 2007). It is indicated that antagonists of NGF may have therapeutic utility in analogous human pain conditions (Hefti et al., 2006; Schulte-Herbruggen et al., 2007). The different effects of NGF on neuropathic pain in the different peripheral nerve injury models suggest that neuropathic pain is a multifactorial process. Thus, in clinical setting, a pro- or anti-NGF strategy would be appropriate depending on the distinct mechanisms underlying neuropathic pain.

7.1.3. Roles of NGF in Peripheral Sensitization

7.1.3.1. Direct Action of NGF via TrkA Receptor

Evidence has shown that NGF directly sensitizes the nociceptive fibers. Systemic administration of NGF induces thermal hypersalgesia for a short period (30 minutes) (Lewin et al., 1994; Woolf et al., 1994). Because NGF does not cross the blood brain barriers and the rapid onset of hyperalgesia after NGF treatment, the local effect of NGF on the peripheral terminals of nociceptors is suggested. Evidence has shown that visceral afferents innervating the urinary bladder is sensitized to mechanical stimuli after NGF administration (Dmitrieva and McMahon, 1996). Acute administration of NGF to cutaneous afferents of skin-nerve preparation *in vitro* sensitizes nociceptive afferents to thermal and chemical stimuli (Dmitrieva and McMahon, 1996; Rueff and Mendell, 1996). In the same preparation, neutralization of NGF by TrkA-IgG in carageenan-induced infammation decreased thermal and chemical sensitivity (Bennett et al., 1998a; Koltzenburg, 1999). Injection of synthetic NGF sequestering protein (tyrosine receptor kinase A Ig2 (TrkAIg2), a smaller protein than TrkA-IgG (an NGF-specific immunoadhesion molecule) (McMahon et al., 1995b) prevents a decrease in somatic action potential and an increase in firing rate and spontaneous activity (Djouhri et al., 2001). Moreover, neuralizing antibodies directed against the TrkA produces a significant anti-allodynic effect on both neuropathic pain and formalin-evoked inflammatory pain in mice, suggesting that the antibody display potent analgesic effects in pain state (Ugolini et al., 2007). Taken together, these results strongly support the idea that the action of NGF on the sensitivity of the peripheral terminals can be directly mediated via TrkA signaling pathways. It is known that NGF binds to TrkA on the peripheral nerve terminals

and activates intracellular tyrosine kinase, which leads to phosphorylation of transduction-related molecules. The activation could alter sensitivity by increasing excitability of the terminal membrane, which contributes to hypersensitivity (Nicol and Vasko 2007).

7.1.3.2. Action of NGF on Peripheral Sensitization Via p75NTR

The role of p75NTR in neuropathic pain has not been widely explored. In particular, role of p75NTR in mediating NGF-induced pain is not well established. One study showed that NGF-induced hyperalgesia occurs in mice that lack p75NTR indicated the TrkA receptor is sufficient to mediate the acute noxious action of NGF in the absence of p75NTR signalling (Bergmann et al., 1998). However, a few other studies indicate direct or indrect correlation of p75NTR with pain-related behavioral. NGF regulates the binding sites of bradykinin, a molecule related to pain transmission on sensory neurons, through an interaction with the p75NTR receptor (Petersen et al., 1998), and blockade of the BK1 receptors reduces the NGF-induced hyperalgesia (Rueff et al., 1996). More direct evidence is provided by pharmacological blockade of p75NTR which functionally inhibits p75NTR in primary sensory neurons and suppresses L5 spinal nerve injury-induced neuropathic pain. The blockade decreases the phosphorylation of TrkA and p38 mitogen-activated protein kinase and the induction of TRP channels in L4 DRG neurons, indicating that p75NTR induced in undamaged DRG neurons facilitates TrkA signaling and contributes to heat and cold hyperalgesia (Obata et al., 2006b). In addition, in vitro experiments also demonstrated that antibody-mediated blockade of p75NTR reduces NGF-induced excitability in capsaicin-sensitive small-diameter sensory neurons (Zhang and Nicol, 2004). Based on the evidence that p75NTR is coexpressed with TrkA in a subset of sensory neurons, it is suggested that NGF action on sensitivity of sensory neurons is mediated via activation p75NTR intrinsic signaling pathways (Nicol and Vasko 2007).

7.1.3.3. Actions of NGF Via Post-Translational Modification and Regulation of Gene Expression

Electrophysiological study has shown that NGF can directly sensitize the adult sensory neurons by increasing the amplitude of membrane current stimulated by capsaicin (Shu and Mendell, 2001). NGF can change sensitivity of sensory neurons acutely by initiating rapid post-translational modification of ion channels such as TRPV1 (Nicol and Vasko 2007). TRPV1 is a cation channel which is activated by pungent vanilloid compounds (such as capsaicin) and is also sensitive to noxious heat and extracellular protons (Caterina and Julius, 1999). TRPV1-deficient mice do not develop thermal hyperalgesia after subcutaneous injection of NGF (Chuang et al., 2001). The peripheral sensitization of NGF on sensory neurons is mediated via a number of intracellular signaling cascades to activate TRP channels, which play critical roles in inflammation and injury-induced thermal hyperalgesia (Walker et al., 2003, Xing et al., 2007, Kasama et al., 2007). It has been shown that the upregulation of NGF after peripheral inflammation leads to the activation of mitogen-activated protein kinase (MAPK) p38, consequently, stimulates the upreglation of TRPV1 (Widmann et al., 1999; Ji and Woolf 2001; Ji et al., 2002). A recent study has shown that phosphoinositide-3-kinase (PI3K) also mediate acute NGF sensitization of TRPV1 (Zhu and Oxford, 2007). Moreover, NGF rapidly increases membrane expression of TRPV1 heat-gated

ion channels (Zhang et al., 2005) and TRPV1-positive sensory neurons coexpress TrkA (Michael and Priestley, 1999b). These findings strongly support that NGF is linked to TRPV1-mediate thermal hyperalgesia and positively regulates sensitization via TRPV1 (Xue et al., 2007).

TRPA1, another member of TRP, has been also shown to mediate cold hyperalgesia (Babes et al., 2004; Obata et al., 2005; Katsura et al., 2006). Intrathecal injection of NGF increases TRPA1 expression in DRG neurons. Conversely, intrathecal administration of antibody against NGF decreased the induction of TRPA1 and suppressed inflammation- and nerve injury-induced cold hyperalgesia, suggesting that NGF-induced TRPA1 increase in sensory neurons is necessary for cold hyperalgesia (Obata et al., 2005).

In addition to its acute action of NGF via regulating ion channels, which occurs rapidly by posttranslational regulation via phosphorylation, in a chronic pain state, however, NGF can change sensitivity of sensory neurons chronically by modulating gene expression (i.e. by transcriptional regulation) such as neuropeptides and receptors conveying nociceptive signals (Nicol and Vasko 2007). These include: (1) P2X3, which is *de novo* expressed in NGF-sensitive nociceptors after intrathecal delivery of NGF (Ramer et al., 2001). (2) SP (Lindsay et al., 1990; Yang et al., 2007), which can be released from central terminal of sensory neurons, increases the activity of nociceptive spinal neurons. (3) Voltage-gated sodium channels: $Na_v1.8$ (Dib-Hajj et al., Kerr et al., 2001) and $Na_v1.9$ (Ostman et al., 2008), which are specifically expressed by nociceptor. $Na_v1.8$ is implicated in NGF-induced hyperalgesia. Moreover, enhanced nociceptor excitability and inflammatory hyperalgesia also require $Na_v1.9$ as demonstated by the decreased pain responses observed in NaV1.9 knockout mice during inflammation. (4) NGF has been implicated in regulating inflammation-induced electrophysiological membrane properties in nociceptive neurons by regulating potassium conductance (Everill and Kocsis, 2000). (5) BDNF, which is regulated by NGF and its roles in nociception are described in section 7.2.

7.1.3.4. Indirect Action of NGF on Peripheral Sensitization

In addition to direct effects, sensitization of nocieptors by NGF may arise indirectly via postganglionic sympathetic nerve terminals (Dixon and McKinnon, 1994; Smeyne et al., 1994; Fagan et al., 1996). It has been shown that surgical or chemical sympathectomy can transiently reduce the NGF-evoked thermal and mechanical hyperalgesia (Andreev et al., 1995a; Woolf et al., 1996). Changes in NGF levels are implicated in the induction of sympathetic sprouting in DRG (McLachlan et al., 1993), one of plasticity changes occurs in the neuropathic pain models. These abnormal sympathetic sprouting fibers in DRG have been also found in transgenic mice with overexpression of NGF in the skin (Davis et al., 1994). These mice are also hyperalgesic (Davis et al., 1993). Delivery of exogenous NGF to adult animals promotes sympathetic sprouting formation (Jones et al., 1999), conversely the administration of NGF antiserum prevents sympathetic sprouting into the DRG in the CCI model (Ramer and Bisby, 1999). In addition, abnormal Aβ fiber sprouting also occurs within the dorsal horn of the spinal cord in several neuropathic pain models, including sciatic nerve injury (Woolf et al., 1992), CCI (Shortland and Woolf, 1993) and spinal nerve ligation (Lekan et al., 1996). However, it is believed that the Aβ sprouting may be overestimated because the method of using cholera toxin B (CTB) labeling also traces the C-fibers which

switch to express the CTB receptor after axotomy (Tong et al., 1999). Exogenous NGF but not NT-3 or BDNF prevents CTB labeling in the dorsal horn (Bennett et al., 1996a), probably as an indirect effect following specific protection of C-fibers from expressing the CTB receptor by NGF (Bennett et al., 1996a; Eriksson et al., 1997).

In addition to sympathetic sprouting, mast cells also plays important role in mediating the indirect action of NGF on peripheral sensitization. Mast cells can express TrkA but not p75NTR (Horigome et al., 1993). Mast cells release inflammatory mediators such as histamine and serotonin known to excite and sensitize primary afferent nociceptors. NGF stimulates degranulation of mast cells (Horigome et al., 1993; Lewin et al., 1994); (Griesbeck et al., 1999), which also synthesize and release NGF (Leon et al., 1994). Mast cell degranulators and serotonin antagonists reduce the elevated NGF and partially prevent thermal but not the mechanical hyperalgesia induced by NGF (Lewin et al., 1994; Woolf et al., 1996). Thus, mast cell activation could be the principal mechanism involved in the long-term thermal hyperalgesia provoked by NGF in rats (Rueff and Mendell, 1996). Furthermore, NGF can activate immune cells such as T and B lymphocytes from which consequently cytokines are released to alter the sensitivity of sensory neurons (MaMchon et al. 1995; Simon et al., 1999)

Evidence has also shown that NGF may produce peripheral sensitization via activation of the 5-lipoxygenase production of arachidonic acid and leukotriene B4 (LTB4), which are demonstrated to sensitize nociceptive afferents to thermal and mechanical stimuli (Martin et al.,1987). NGF stimulates the production of LTB4 in skin and inhibitors of lipoxygenase prevent the development of NGF-induced thermal hyperalgesia (Amann et al., 1996). The actions of LTB4 are mediated via its chemotactic action on neutrophils. Intraplantar injection of NGF results in local neurtophil accumulation and animals, which are neutrophil depleted, do not develop thermal hyperalgesia in response to NGF (Bennett et al., 1998a).

7.1.4. Effects of NGF on Central Sensitization

NGF has been implicated in the spinal processing of nociceptive information. The peripheral action of NGF on the activation and sensitization of primary afferent nociceptors may trigger central changes in the spinal cord. Systemic NGF treatment (Thompson et al., 1995) to peripheral targets has been shown to increase spinal neuron excitability to inputs from unmyelinated afferents. Peripheral NGF administration to some visceral tissues results in the induction of *c-fos* in dorsal horn (Dmitriva, 1996). NGF may exert long-term effects on nociceptive neurons via modulating gene expression within neurons (Park and Deboni, 1996; Koltzenburg, 1999; Nicol and Vasko, 2007) and then lead to secondary upregulation of neuropeptides and BDNF (Leslie et al., 1995; Michael et al., 1997; Petersen et al., 1998)**.** In addition, NGF regulates central release of excitatory neurotransmitters such as glutamate (Ishikawa et al., 1999) and SP (Malcangio et al., 1997). Released neurotransmitters, coupled with an upregulation of SP receptor NK1 in the dorsal horn neurons, become triggers for the induction of central sensitization (McMahon et al., 1993). Although the direct effect of NGF administration in the spinal cord is less well defined, evidence has shown that overexpression of NGF by injection of adenovirus encoding NGF into adult dorsal spinal cord induces thermal hyperalgesia (Romero et al., 2001). The NGF-induced hyperalgesia is believed to be mediated by regulating central synaptic connectivity (Ramer et al., 2000; Romero et al.,

2001). NGF participates in central plasticity by increasing TrkA expression in central nociceptive spinoreticular neurons in the deeper layers (laminae V and VI) of dorsal horn in a rat model of adjuvant-induced chronic arthritis (Pezet et al., 2001). Furthermore, arachinoid injection of NGF induced a later phase of thermal hyperalgesia, which can be prevented by NMDA or NK receptor antagonist (Woolf et al., 1998), indicating that effects of NGF likely involve central NMDA receptors (Lewin et al., 1994). A recent electrophysiological study has further demonstrated that intramuscular injection of NGF enhanced sensitization of dorsal horn neurons (Hoheisel et al., 2007).

A considerable body of evidence implicates endogenous NGF in conditions in which pain is a prominent feature, including neuropathic pain. However, previous studies of NGF antagonism in animal models of neuropathic pain have examined only the prevention of hyperalgesia and allodynia after injury, whereas the more relevant issue is whether treatment can provide relief of established pain, particularly without tolerance. In the complete Freund's adjuvant-induced hind-paw inflammation, spinal nerve ligation and streptozotocin-induced neuropathic pain models, a single intraperitoneal injection of a polyclonal anti-NGF antibody reversed established tactile allodynia from approximately day 3 to day 7 after treatment. Effects on thermal hyperalgesia were variable with a significant effect observed only in the spinal nerve ligation model. In the mouse chronic constriction injury (CCI) model, a mouse monoclonal anti-NGF antibody reversed tactile allodynia when administered 2 weeks after surgery. Repeated administration of this antibody to CCI mice for 3 weeks produced a sustained reversal (days 4 to 21) of tactile allodynia that returned 5 days after the end of dosing. In conclusion, NGF seems to play a critical role in models of established neuropathic and inflammatory pain in both rats and mice,

7.2. BDNF and Pain

7.2.1 BDNF Acts as a Central Modulator

BDNF has important implications in nociceptive signaling. During development, BDNF regulates the mechanosensitivity of slowly adapting mechanoreceptors (Carroll et al., 1998; Sedy et al., 2004). In adulthood, BDNF continues to play a role in the physiological regulation of tactile sense in rats (Watanabe et al., 2000b)**.** BDNF is mainly present in nociceptive TrkA-expressing neurons and their central terminals in the superficial dorsal horn, and released in an activity-dependent manner (Griesbeck et al., 1999; Lever et al., 2001; Walker et al., 2001; Balkowiec et al., 2000). The anatomical and neurochemical features of BDNF indicate that it might modify nociceptive neurotransmission at the level of central connections in the dorsal horn of the spinal cord.

BDNF has been recognized as a regulatory molecule involved in pain processing under pathological conditions. The local application of BDNF to the rat hindpaw induces thermal hyperalgesia (Shu et al., 1999a). Systemic or local delivery of BDNF antibody or sequestering fusion molecule TrkB-IgG prevents the development of nerve injury-induced neuropathic pain (Theodosiou et al., 1999; Zhou et al., 1999b; Fukuoka et al., 2001; Li et al., 2006) or inflammation (Kerr et al., 1999). BDNF knockout mice display a reduced sensitivity to inflammation-mediated pain (MacQueen et al., 2001; Zhao et al., 2006). Baseline thermal

thresholds are reduced in a conditional null mouse in which BDNF in most sensory neurons are deleted (Zhao et al., 2006). Carrageenan-induced thermal hyperalgesia is also inhibited. Formalin-induced pain behavior was attenuated in a later phase. This effect is correlated with abolition of NMDA receptor NR1 Ser896/897 phosphorylation and activation of ERK1 and ERK2 in the dorsal horn, which has been shown to be associated with pain states (Ji et al., 1999; Zhuang et al., 2005). By contrast, neuropathic pain behavior developed normally. Thus, nociceptor-derived BDNF plays an important role in regulating inflammatory pain thresholds and secondary hyperalgesia, which may be mediated via central sensitization.

A large body of evidence has shown that endogenous BDNF plays important roles in central sensitization in the persistent pain states (Pezet and McMahon 2006). Application of exogenous BDNF facilitates the flexor reflex (Mannion et al., 1999) and rapid induction of c-fos in superficial dorsal horn neurons (Dassab et al., 1998; Dupont et al., 2001). Intraspinal administration of BDNF antagonist TrkB-IgG fusion protein (an antagonist of BDNF) largely attenuates an enhanced excitability of C-fiber reflexes and the progressive mechanical hypersensitivity elicited by CFA-induced inflammation (Dassab et al., 1998). Moreover, the expression of BDNF and its full-length TrkB increases in the dorsal horn (Frisen et al., 1992) in the nerve injury-induced pain models (Cho et al., 1998; Li et al., 1999) and inflammation (Cho et al., 1997; Lee et al., 1999). The anterograde transport and activity-dependent release of DRG-derived BDNF in dorsal horn may act presynaptically and/or postsynaptically via TrkB to sensitize the sensory pathway (Griesbeck et al., 1999; Balkowiec and Katz, 2000; Lever et al., 2001; Walker et al., 2001). Activation of TrkB leads to phosphorylation of the NMDA receptor (Lin et al., 1998; Levine and Kolb, 2000), which is involved in central hyperexcitable states (McMahon et al., 1993; Kerr et al., 1999; Slack et al., 2004). BDNF-induced hyperalgesia can be blocked by NMDA receptor antagonist (Groth et al., 2002). In addition, it has been recently shown that nerve injury-induced TrkB-dependent reduction in potassium chloride co-transporter 2 (KCC2) in spinal dorsal horn may also contribute to spinal neuronal excitability via a loss of KCC2-mediated GABA inhibition (Miletic and Miletic 2007). Therefore, BDNF might contribute to transferring the information of noxious stimuli from primary afferents to second order neurons in the dorsal horn. These actions potentiate the efficacy of synaptic inputs from nociceptors, and represent one of the mechanisms underlying the stimulus-evoked central sensitization, which contributes to the abnormal pain sensitivity.

7.2.2. Peripheral Action of BDNF

In addition to the role in central sensitization, BDNF has a peripheral action. Electrophysiological recordings from C-fibers in an *in vitro* skin-saphenous nerve preparation reveal increased sensitization to noxious heat stimuli after direct application of BDNF to the receptive field (Le Douarin and Smith, 1988; Shu et al., 1999a). Moreover, BDNF enhances the sensitivity of primary afferent fibers of rat vagal ganglion neurons to capsaicin in vitro (Winter, 1998). Furthemore, a subpopulation of small sensory neurons coexpresses TrkA and TrkB receptors (McMahon et al., 1994), suggesting the possibility for a direct sensitization of some peripheral nociceptors to both NGF and BDNF via Trk signaling (Shu et al., 1999). Thus, it is possible that elevated BDNF from L5 DRG and the non-neuronal cells in sciatic nerve after peripheral nerve injury directly sensitizes the nociceptive afferents. Moreover,

DRG-derived BDNF could be locally released in an activity-dependent manner as observed in cultured cranial ganglion cells (Balkowiec and Katz, 2000), acting on nociceptive neurons in an autocrine or a paracrine manner (Acheson et al., 1995; Lee et al., 1999).

On the other hand, BDNF may activate Aβ fiber mechanoreceptors which normally do not mediate pain. It has been shown that nerve injury induces a phenotypic switch with novel expression of BDNF by large neurons which project Aβ fibers, and sympathetic sprouting preferentially surrounds these large neurons and upregulation of p75NTR is also observed in pericellualr satellite cells (Zhou, 1999b), indicating that BDNF and p75NTR could form chemoattractive gradient to attract axonal sprouting which could provide anatomic basis for the nociceptive action of BDNF. Administration of exogenous BDNF to the normal DRG induces a persistent mechanical allodynia (Zhou et al., 2000) and triggers sympathetic sprouting (Deng et al., 2000) whereas antibody against BDNF prevents the sprouting (Deng et al., 2000). Moreover, administration of BDNF antibody to the injured DRG reduces mechanical allodynia and thermal hyperalgesia after spinal nerve injury (Zhou et al., 2000; Yajima et al., 2002, 2005). These findings indicate pronociceptive role of BDNF in the periphery.

7.2.3. Anti-Nociceptive Action of BDNF

The actions of exogenous BDNF are complex and dose- and site-dependent within the nonciceptive pathway (Miki et al., 2000). Chronic spinal delivery of BDNF alleviates hyperalgesia in rats (Gartner et al., 2000). Evidence has shown that exogenous BDNF infusion near the spinal cord produces potent antinociception (Frank et al., 1997) by increasing the synthesis and release of serotonin in descending raphe serotonergic/peptidergic neurons (Martin-Iverson et al., 1994; Croll et al., 1994; Nawa et al., 1994). BDNF regulates expression of neuropeptides such as somatostatin and NPY, which are located in the spinal dorsal horn (Munglani et al., 1996; Mark et al., 1997), contribute to anti-nociceptive efffect (Hua et al., 1991; Mollenholt et al., 1994; Helmchen et al., 1995). The antinociceptive effect of BDNF is not only limited to the spinal level (Eaton et al., 1997; Cejas et al., 2000) but also extend to brainstem in both normal and inflammatory condition (Siuciak et al., 1994; Siuciak et al., 1995; Dyck et al., 1997). It is believed that BDNF strongly inhibits AMPA-mediated currents via TrkB receptor activation (Balkowiec et al., 2000; Cejas, et al., 2000).

7.3. NT-3 and Pain

It is known that the development of nociceptive afferents is largely independent of NT-3. Nevertheless, subcutaneous administration of recombinant NT-3 in healthy human induces mild injection-site pain (Chaudhry et al., 2000). In rodent, intrathecal administration of NT-3 antisense oligonucleotides via an osmotic pump for 14 days, attenuates nerve injury-induced sprouting of Aβ fibers and allodynia (White, 2000). Intraperitoneal injection of NT-3 antiserum reduces the mechanical allodynia and sympathetic sprouting induced by L5 spinal nerve injury (Deng et al., 2000a), indicating that NT-3-induced sympathetic sprouting contributes to the mechanical hyperalgesia. It is believed that the activation of TrkC by NT-3 may be also relevant to hyperalgsia during the neuropathic or inflammatory pain. It has been

shown that a number of changes occurred in large sensory neurons, which express TrkC after tissue injury. These changes include altered expression of neuropeptides, neuromodulators, ion channels, as well as anatomical rearrangement of myelinated Aβ fibers. Moreover, treatment with NT-3 mitigates some of these changes (Ohara et al., 1995, Munson et al., 1997a). On the other hand, intrathecal administration of NT-3 potentiates SP release (Malcangio 1997), further supporting the role of NT-3 in nociception.

Several lines of evidence have shown that NT-3 also has anti-nociceptive action in both inflammatory pain model and neuropathic pain model. Local injection of recombinant NT-3 reverses transiently but specifically the inflammatory hyperalgesia induced by intraplantar injection of complete Freund's adjuvant to the rat hind paw. Moreover, the expression of NT3 is decreased in the plantar skin (Watanabe et al., 2000a). These results suggest an inhibitory role of NT-3 in the regulation of inflammatory pain sensitivity. Similarly, transgenic mice overexpressing NT3 in muscle (myo/NT3 mice) show a robust reduced mechanical hyperalgesia in response to intramuscular injection of acidic saline (Gandhi et al., 2004). This anti-nociceptive effect could be mimicked by intramuscular administration of exogenous NT3 but not by other neurotrophins, indicating that the anti-nociceptive action of NT-3 is selective and not caused by general trophic effects. In addition, intrathecal infusion of NT-3 to rats receiving CCI suppresses thermal but not mechanical hyperalgesia. NT-3 infusion mitigates CCI-induced elevated TRPV1 and activated p38 MAPK (Wilson-Gerwing et al., 2005) and galanin (Wilson-Gerwing et al., 2006), which has been shown to be causally linked to pain-related behavior. Since the major role of NGF in thermal hyperalgesia is mediated via regulation of these nociceptor phenotypes, it is believed that NT-3 acts antagonistically to NGF and identified as a potent negative modulator of this state. The findings indicate a beneficical role of NT-3 in the treatment of neuropathic pain mediated by NGF.

Taken together, these studies identify circumstances in which NT-3 plays a role in modulating nociceptive signaling. Nevertheless, it is also reported that intradermal injection of NT-3 has no effect on the response to noxious heat stimuli (Shu et al., 1999). Thus, effects of NT-3 on pain could be differential depending on administration routes or types of tissue injury or the interplay among different neurotrophin via the cognate Trk receptors.

7.4. NT-4 and Pain

The role of NT-4 in the pain transmission is not extensively investigated as that of BDNF. NT4 is expressed by a subpopulation of DRG neurons (Heppenstall et al 2001). One study has shown that NT-4 can sensitize individual sensory afferents to noxious thermal stimulation by facilitating inward currents *in vitro* in a skin-nerve preparation (Rueff and Mendell, 1996). Moreover, NT-4 has a more potent effect than BDNF (Shu et al., 1999a). However, intrathecal delivery of antibody against NT4 does not prevent the nerve injury-induced thermal hyperalgesia (Yajima et al., 2002).

8. Summary

In summary, primary sensory neurons have morphological, neurochemical and functional diversity. Neurotrophic factors have a broad scope of actions on these primary sensory neurons. These factors have a developmental role in regulating the survival and specification of primary sensory neurons, and continue to influence the properties of primary sensory neurons through the postnatal period and into adult life. It is now known that peripheral nerve injury results in widespread morphological, neurochemical and functional changes in the sensory nervous system both at the level of the primary sensory neurons and the dorsal horn of spinal cord. Neurotrophic factors orchestrate many of these dynamic plasticity changes, and significantly contribute to the pathophysiology of peripheral neuropathic pain by peripheral and central sensitization.

Given the high degree of redundancy and pleiotropy known to exist in biological systems, the determination of which genes participate in which physiological mechanisms remains a daunting task. Thus, it is essential to redirect efforts towards the development of experimental strategies for testing which of these are essential parts of the pain process and which are tangential. Genetically, the recent high-throughput genomic technologies (e.g. gene chips) may permit one to assay for changes in nearly all genes coded by the genome simultaneously. These studies will accelerate the rate of discovery of pain-related gene and pave the way to study the function of pain gene-encoded proteins in the pain transmission and down-stream of signal transduction.

However, consideration must remain open for the nature of pain, which has considerable complexity, subjectivity and heredity (Mogil, 1999). Moreover, the symptoms and the mechanisms of pain are dynamic during the course of the disease. It seems to be advisable to shift from etiology-based treatment to mechanism-based approach. Although pharmacological intervention plays a predominant role in the treatment of pain, the new strategies, such as “molecular killer” and cellular minipumps for delivery of biological antinociceptive molecules and synthesis of small molecules are being actively explored (Hentall and Sagen 2000; Golangelo et al., 2008). In particular, gene therapy is engendering considerable interest due to locally restricated and highly concentreated application of neurotrophins (Pohl and Braz, 2001). Direct somatic gene transfer for neuropathic pain will eventually overcome the problems associated with transplantation of non-autologous and xenogenic cells (Eaton, 2000; Siniscalco et al., 2005). Antibody against neurotrophins can be a promising treatment and it is likely to see the outcome of this therapy in the near future as drug companies are focusing on the development of this novel therapy. Understanding the mechanisms of pain will certainly contribute to these new endeavors.

References

Abbadie C, Brown JL, Mantyh PW, Basbaum AI (1996) Spinal cord substance p receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. *Neuroscience* 70: 201-209.

Abson N, Sukumaran M, Wood JN (2001) Genetic appraches to nociceptor development. Molecular basis of pain induction, Wood JN (ed). John Wileyand Sons, Inc, 2001. p1-22.

Acheson A, Conover JC, Fandl JP, DeChlara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* 374: 450-453.

Airaksinen MS, Koltzenburg M, Lewin GR, Masu Y, Helbig C, Wolf E, Brem G, Toyka KV, Thoenen H, Meyer M (1996a) Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* 16: 287-295.

Aldskogius H, Arvidsson J, Grant G (1985) The reaction of primary sensory neurons to peripheral nerve injury with particular emphasis on transganglionic changes. *Brain Res* 357: 27-46.

Aley KO, Levine JD. (1999) Role of protein kinase A in the maintenance of inflammatory pain. *J. Neurosci.*19:2181-6.

Aloe L, Tuveri MA, Carcassi U, Levi-Montalcini R (1992a) Nerve growth factor in the synovial fluid of patients with chronic arthritis. *Arthritis Rheum.* 35: 351-355.

Aloe L, Tuveri MA, Levi-Montalcini R (1992b) Studies on carrageenan-induced arthritis in adult rats: presence of nerve growth factor and role of sympathetic innervation. *Rheumatol Int.* 12: 213-216.

Aloe L, Skaper SD, Leon A, Levi-Montalcini R (1994) Nerve growth factor and autoimmune diseases [review]. *Autoimmunity* 19: 141-150.

Altar CA and DiStefano PS (1998) Neurotrophin trafficking by anterograde transport. *Trends Neurosci.* 21: 433-437.

Altman J and Bayer SA (1984) The development of the rat spinal cord. *Adv. Anat. Embryol. Cell Biol.* 85:1-164.

Amann R, Schuligoi R, Lanz I, Peskar BA (1996) Effect of a 5-lipoxygenase inhibitor on nerve growth factor- induced thermal hyperalgesia in the rat. *Eur. J. Pharmacol.* 306: 89-91.

Amir R and Devor M (1996) Chemically mediated cross-excitation in rat dorsal root ganglia. *J. Neurosci.* 16: 4733-4741.

Amir R and Devor M (2000) Functional cross-excitation between afferent A- and C-neurons in dorsal root ganglia. *Neuroscience* 95: 189-195.

Anand P, Chen FE, Reid CD, Cotes PM, Rudge P, Coates M (1991) Correction by erythropoietin of anaemia in autonomic failure. *Lancet* 338: 759-760.

Anand P, Foley P, Navsaria HA, Sinicropi D, Williams-Chestnut RE, Leigh IM (1995) Nerve growth factor levels in cultured human skin cells: effect of gestation and viral transformation. *Neurosci. Lett.* 184: 157-160.

Andreev NY, Dimitrieva N, Koltzenburg M, McMahon SB (1995) Peripheral administration of nerve growth factor in the adult rat produces a thermal hyperalgesia that requires the presence of sympathetic post-ganglionic neurones. *Pain* 63: 109-115.

Anton ES, Weskamp G, Reichardt LF, Matthew WD (1994) Nerve growth factor and its low-affinity receptor promote Schwann cell migration. *Proc. Natl. Acad. Sci. USA* 91: 2795-2799.

Apfel SC, Lipton RB, Arezzo JC, Kessler JA (1991) Nerve growth factor prevents toxic neuropathy in mice. *Ann. Neurol.* 29: 87-90.

Apfel SC, Arezzo JC, Lipson L, Kessler JA (1992) Nerve growth factor prevents experimental cisplatin neuropathy. *Ann. Neurol.* 31: 76-80.

Apfel SC, Wright DE, Wiideman AM, Dormia C, Snider WD, Kessler JA (1996) Nerve growth factor regulates the expression of brain-derived neurotrophic factor mRNA in the peripheral nervous system. *Mol. Cell Neurosci.* 7: 134-142.

Apfel SC, Kessler JA, Adornato BT, Litchy WJ, Sanders C, Rask CA (1998) Recombinant human nerve growth factor in the treatment of diabetic polyneuropathy. NGF Study Group. *Neurology* 51: 695-702.

Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2000) ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell* 101: 485-498.

Ardelt AA, Flaris NA, Roth KA (1994) Neurotrophin-4 selectively promotes survival of striatal neurons in organotypic slice culture. *Brain Res.* 647: 340-344.

Arévalo JC, Wu SH (2006) Neurotrophin signaling: many exciting surprises! *Cell Mol. Life Sci.* 2006 Jul;63(13):1523-37.

Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur. J. Neurosci*. 7: 1484-1494.

Averill S, Michael GJ, Shortland PJ, Leavesley RC, King VR, Bradbury EJ, McMahon SB, Priestley JV (2004) NGF and GDNF ameliorate the increase in ATF3 expression which occurs in dorsal root ganglion cells in response to peripheral nerve injury. *Eur. J. Neurosci.* 19:1437-45.

Babes A, Zorzon D, Reid G. (2004) Two populations of cold-sensitive neurons in rat dorsal root ganglia and their modulation by nerve growth factor. *Eur. J. Neurosci.* 20:2276-82.

Baldwin AN and Shooter EM (1995) Zone mapping of the binding domain of the rat low affinity nerve growth factor receptor by the introduction of novel N- glycosylation sites. *J. Biol. Chem.* 270: 4594-4602.

Balkowiec A and Katz DM (2000a) Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. *J. Neurosci.* 20: 7417-7423.

Balkowiec A, Kunze DL, Katz DM (2000b) Brain-derived neurotrophic factor acutely inhibits AMPA-mediated currents in developing sensory relay neurons. *J. Neurosci.* 20: 1904-1911.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J. Cell Biol.* 140: 911-923.

Barbacid M (1995) Neurotrophic factors and their receptors. *Curr Opin Cell Biol*. 7: 148-155.

Barde YA, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* 1: 549-553.

Barker PA, Lomen-Hoerth C, Gensch EM, Meakin SO, Glass DJ, Shooter EM (1993) Tissue-specific alternative splicing generates two isoforms of the *trkA* receptor. *J. Biol. Chem.* 268: 15150-15157.

Barker PA and Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* 13: 203-215.

Barker PA (2004) p75NTR is positively promiscuous: novel partners and new insights. *Neuron* 42(4):529-33.

Baron R, Levine JD, Fields HL (1999) Causalgia and reflex sympathetic dystrophy: does the sympathetic nervous system contribute to the generation of pain? *Muscle Nerve* 22: 678-695.

Barrett GB and Bartlett PF (1994) The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc. Natl. Acad. Sci. USA* 91: 6501-6505.

Barrett GL and Georgiou A (1996) The low-affinity nerve growth factor receptor $p75^{NGFR}$ mediates death of PC12 cells after nerve growth factor withdrawal. *J. Neurosci. Res.* 45: 117-128.

Bennett DL, Averill S, Clary DO, Priestley JV, McMahon SB (1996a) Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. *Eur. J. Neurosci.* 8: 2204-2208.

Bennett DL, French J, Priestley JV, McMahon SB (1996b) NGF but not NT-3 or BDNF prevents the A fiber sprouting into lamina II of the spinal cord that occurs following axotomy. *Mol. Cell Neurosci.* 8: 211-220.

Bennett DL, Koltzenburg M, Priestley JV, Shelton DL, McMahon SB (1998a) Endogenous nerve growth factor regulates the sensitivity of nociceptors in the adult rat. *Eur. J. Neurosci*. 10: 1282-1291.

Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998b) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J. Neurosci.* 18: 3059-3072.

Bennett GJ and Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33: 87-107.

Bentley CA and Lee KF (2000) p75 is important for axon growth and schwann cell migration during development. *J. Neurosci*. 20: 7706-7715.

Bergmann I, Priestley JV, McMahon SB, Brocker EB, Toyka KV, Koltzenburg M (1997) Analysis of cutaneous sensory neurons in transgenic mice lacking the low affinity neurotrophin receptor p75. *Eur. J. Neurosci.* 9:18-28.

Bergmann I, Reiter R, Toyka KV, Koltzenburg M (1998) Nerve growth factor evokes hyperalgesia in mice lacking the low-affinity neurotrophin receptor p75. *Neurosci. Lett*. 255:87-90.

Bhattacharyya A, Watson FL, Bradlee TA, Pomeroy SL, Stiles CD, Segal RA (1997) Trk receptors function as rapid retrograde signal carriers in the adult nervous system. *J. Neurosci.* 17: 7007-7016.

Bishop JF, Mueller GP, Mouradian MM (1994) Alternate 5' exons in the rat brain-derived neurotrophic factor gene: differential patterns of expression across brain regions. *Brain Res. Mol. Brain Res*. 26: 225-232.

Bolin LM, Verity AN, Silver JE, Shooter EM, Abrams JS (1995) Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. *J. Neurochem.* 64: 850-858.

Bowersox SS, Gadbois T, Singh T, Pettus M, Wang YX, Luther RR (1996) Selective N-type neuronal voltage-sensitive calcium channel blocker, SNX-111, produces spinal antinociception in rat models of acute, persistent and neuropathic pain. *J. Pharmacol. Exp. Ther.* 279: 1243-1249.

Bradbury EJ, Burnstock G, McMahon SB (1998) The Expression of P2X3 Purinoreceptors in Sensory Neurons: Effects of Axotomy and Glial-Derived Neurotrophic Factor. *Mol. Cell Neurosci.* 12: 256-268.

Bredesen DE and Rabizadeh S (1997) p75NTR and apoptosis: Trk-dependent and Trk-independent effects. *Trends Neurosci*. 20: 287-290.

Brown MC, Perry VH, Lunn ER, Gordon S, Heumann R (1991) Macrophage dependence of peripheral sensory nerve regeneration: Possible involvement of nerve growth factor. *Neuron.* 6: 359-370.

Butte MJ (2001) Neurotrophic factor structures reveal clues to evolution, binding, specificity, and receptor activation. *Cell Mol. Life Sci*. 58: 1003-1013.

Cai F, Tomlinson DR, Fernyhough P (1998) Effect of sciatic nerve crush on local and target tissue production of neurotrophin-3 transcripts in rats. *Neurosci. Lett*. 252: 45-48.

Campana WM (2007) Schwann cells: activated peripheral glia and their role in neuropathic pain. *Brain Behav. Immun*. 21:522-7.

Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI (1998) Primary afferent tachykinins are required to experience moderate to intense pain. *Nature* 392: 390-394.

Carroll P, Lewin GR, Koltzenburg M, Toyka V, Thoenen H (1998) A role for BDNF in mechanosensation. *Nature Neuroscience* 1: 42-46.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohmmatthaei R, Baeuerle PA, Barde YA (1996) Selective activation of NF-kappa-B by nerve growth factor through the neurotrophin receptor p75. *Science* 272: 542-545.

Casaccia-Bonnefil P, Gu C, Khursigara G, Chao MV (1999) p75 neurotrophin receptor as a modulator of survival and death decisions. *Microsc. Res. Tech.* 45: 217-224.

Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, Barde YA (1999) The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. *EMBO J.* 18: 6050-6061.

Caterina MJ and Julius D (1999) Sense and specificity: a molecular identity for nociceptors. *Curr. Opin. Neurobiol.* 9: 525-530

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 14;288:306-13.

Cejas PJ, Martinez M, Karmally S, McKillop M, McKillop J, Plunkett JA, Oudega M, Eaton MJ (2000) Lumbar transplant of neurons genetically modified to secrete brain-derived neurotrophic factor attenuates allodynia and hyperalgesia after sciatic nerve constriction. *Pain* 86: 195-210.

Cervero F and Laird JM (1996) Mechanisms of touch-evoked pain (allodynia): a new model. *Pain* 68: 13-23.

Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999) Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. *Neuron* 23: 617-624.

Chao M, Casaccia-Bonnefil P, Carter B, Chittka A, Kong H, Yoon SO (1998) Neurotrophin receptors: mediators of life and death. *Brain Res. Brain Res. Rev.* 26: 295-301.

Chacur M, Milligan ED, Gazda LS, Armstrong C, Wang H, Tracey KJ, Maier SF, Watkins LR (2001) A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. *Pain* 94: 231-244.

Chaudhry V, Giuliani M, Petty BG, Lee D, Seyedsadr M, Hilt D, Cornblath DR (2000) Tolerability of recombinant-methionyl human neurotrophin-3 (r-metHuNT3) in healthy subjects. *Muscle Nerve* 23:189-92.

Cheema SS, Barrett GL, Bartlett PF (1996) Reducing p75 nerve growth factor receptor levels using antisense oligonucleotides prevents the loss of axotomized sensory neurons in the dorsal root ganglia of newborn rats. *J. Neurosci. Res*. 46: 239-245.

Chen Y, Michaelis M, Janig W, Devor M (1996) Adrenoreceptor subtype mediating sympathetic-sensory coupling in injured sensory neurons. *J. Neurophysiol*. 76: 3721-3730.

Chen Z, Rebelo S, White F, Malmberg AB, Baba H, Lima D, Woolf CJ, Basbaum AI, Anderson DJ (2001) The paired homeodomain protein drg11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. *Neuron* 31: 59-73

Chen CL, Broom DC, Liu Y, de Nooij JC, Li Z, Cen C, Samad OA, Jessell TM, Woolf CJ, Ma Q (206) Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. *Neuron* 49:365-77.

Cho HJ, Kim JK, Zhou XF, Rush RA (1997) Increased brain-derived neurotrophic factor immunoreactivity in rat dorsal root ganglia and spinal cord following peripheral inflammation. *Brain Res.* 764: 269-272.

Cho HJ, Kim JK, Park HC, Kim DS, Ha SO, Hong HS (1998) Changes in brain-derived neurotrophic factor immunoreactivity in rat dorsal root ganglia, spinal cord, and gracile nuclei following cut or crush injuries. *Exp. Neurol*. 154: 224-230.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411: 957-962.

Colangelo AM, Bianco MR, Vitagliano L, Cavaliere C, Cirillo G, De Gioia L, Diana D, Colombo D, Redaelli C, Zaccaro L, Morelli G, Papa M, Sarmientos P, Alberghina L, Martegani E (2008) A new nerve growth factor-mimetic peptide active on neuropathic pain in rats. *J. Neurosci*. 28:2698-709.

Coggeshall RE, Lekan HA, Doubell TP, Allchorne A, Woolf CJ (1997) Central changes in primary afferent fibers following peripheral nerve lesions. Neuroscience 77: 1115-1122.

Copray JCVM and Brouwer N (1994) Selective expression of neurotrophin-3 messenger rna in muscle spindles of the rat. *Neuroscience* 63: 1125-1135.

Coulson EJ, Reid K, Shipham KM, Morley S, Kilpatrick TJ, Bartlett PF (2004) The role of neurotransmission and the Chopper domain in p75 neurotrophin receptor death signaling. *Prog. Brain Res*.146:41-62.

Coulson EJ (2006) Does the p75 neurotrophin receptor mediate Abeta-induced toxicity in Alzheimer's disease? *J. Neurochem*. 98:654-60.

Croll SD, Wiegand SJ, Anderson KD, Lindsay RM, Nawa H (1994) Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF. *Eur. J. Neurosci*. 6: 1343-1353.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD, Phillips HS (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* 76: 1001-1011.

Curtis R, Adryan KM, Stark JL, Park JS, Compton DL, Weskamp G, Huber LJ, Chao MV, Jaenisch R, Lee KF (1995) Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. *Neuron* 14: 1201-1211.

Curtis R, Tonra JR, Stark JL, Adryan KM, Park JS, Cliffer KD, Lindsay RM, DiStefano PS (1998) Neuronal injury increases retrograde axonal transport of the neurotrophins to spinal sensory neurons and motor neurons via multiple receptor mechanisms. *Mol. Cell Neurosci.* 12: 105-118.

Dassab P, Trevedi P, McMahon SB, Shelton II D, Jones M, Swanson G, Thompson SWN (1998) BDNF induces prolonged increases in spinal reflex activity *in vitro*. *Soc. Neurosci. Abstr*. 24 (154.13): 394.

Dai Y, Iwata K, Fukuoka T, Kondo E, Tokunaga A, Yamanaka H, Tachibana T, Liu Y, Noguchi K. 2002. Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. *J. Neurosci.* 22: 7737-7745.

Davis BM, Lewin GR, Mendell LM, Jones ME, Albers KM (1993) Altered expression of nerve growth factor in the skin of transgenic mice leads to changes in response to mechanical stimuli. *Neuroscience* 56: 789-792

Davis BM, Albers KM, Seroogy KB, Katz DM (1994) Overexpression of nerve growth factor in transgenic mice induces novel sympathetic projections to primary sensory neurons. *J. Comp. Neurol.* 349: 464-474.

Davies AM (1996) The neurotrophic hypothesis - where does it stand. Philosophical Transactions of the Royal Society of London - Series B: Biological 351: 389-394.

Dechant G, Barde YA (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat. Neurosci.* 5:1131-6.

Decosterd I and Woolf CJ (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87: 149-158.

Deng YS, Zhong JH, Zhou XF (2000a) Effects of endogenous neurotrophins on sympathetic sprouting in the dorsal root ganglia and allodynia following spinal nerve injury. *Exp. Neurol*. 164: 344-350.

Deng YS, Zhong JH, Zhou XF (2000b) BDNF is involved in sympathetic sprouting in the dorsal root ganglia following peripheral nerve injury in rats. *Neurotoxicity Research* 1:311-322.

Devor M and Wall PD (1990) Cross-excitation in dorsal root ganglia of nerve-injured and intact rats. *J. Neurophysiol.* 64: 1733-1746.

Devor M (1999a) The pathophysiology of damaged peripheral nerves in relation to chronic pain. In: Wall PD, Mezack R, eds Textbook of pain, 4 th edn London: Churchill Livingstone, 1999. 129-164.

Devor M (1999b) Unexplained peculiarities of the dorsal root ganglion. *Pain Suppl.* 6: S27-S35.

Devor M (2001) Neuropathic pain: what do we do with all these theories? *Acta Anaesthesiol. Scand*. 45: 1121-1127.

Dib-Hajj SD, Black JA, Cummins TR, Kenney AM, Kocsis JD, Waxman SG (1998) Rescue of alpha-SNS sodium channel expression in small dorsal root ganglion neurons after axotomy by nerve growth factor in vivo. *J. Neurophysiol.* 79: 2668-2676.

DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ (1992) The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron* 8: 983-993.

Dixon JE and McKinnon D (1994) Expression of the *trk* gene family of neurotrophin receptors in prevertebral sympathetic ganglia. *Dev. Brain Res.* 77: 177-182.

Djouhri L, Dawbarn D, Robertson A, Newton R, Lawson SN (2001) Time course and nerve growth factor dependence of inflammation-induced alterations in electrophysiological membrane properties in nociceptive primary afferent neurons. *J. Neurosci*. 21: 8722-8733.

Dmitrieva N and McMahon SB (1996) Sensitisation of visceral afferents by nerve growth factor in the adult rat. *Pain* 66: 87-97.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* 265: 1596-1599.

Donnerer J, Schuligoi R, Stein C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: Evidence for a regulatory function of nerve growth factor *in vivo*. *Neuroscience* 49: 693-698.

Dubner R and Gold M (1999) The neurobiology of pain. *Proc. Natl. Acad. Sci. USA* 96: 7627-7630.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 275: 661-665.

Dupont MC, Spitsbergen JM, Kim KB, Tuttle JB, Steers WD (2001) Histological and neurotrophic changes triggered by varying models of bladder inflammation. *J. Urol.* 166: 1111-1118.

Dyck PJ, Peroutka S, Rask C, Burton E, Baker MK, Lehman KA, Gillen DA, Hokanson JL, O'Brien PC (1997) Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain threshold in humans. *Neurology* 48: 501-505.

Eaton MJ, Santiago DI, Dancausse HA, Whittemore SR (1997) Lumbar transplants of immortalized serotonergic neurons alleviate chronic neuropathic pain. *Pain* 72: 59-69.

Eaton MJ (2000) Emerging cell and molecular strategies for the study and treatment of painful peripheral neuropathies. *J. Peripher. Nerv. Syst*. 5: 59-74.

Ekstrom PAR (1995) Neurones and glial cells of the mouse sciatic nerve undergo apoptosis after injury in vivo and in vitro. *Neuroreport* 6: 1029-1032

Einarsdottir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, Holmgren G, Holmberg D, Holmberg M (2004) A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Hum. Mol. Genet.* 13:799-805.

Eliav E, Herzberg U, Ruda MA, Bennett GJ (1999) Neuropathic pain from an experimental neuritis of the rat sciatic nerve. *Pain* 83: 169-182.

Elshamy WM and Ernfors P (1996) A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. *Neuron* 16: 963-972.

English KB, Harper S, Stayner N, Wang ZM, Davies AM (1994) Localization of nerve growth factor (NGF) and low-affinity NGF receptors in touch domes and quantification of NGF mRNA in keratinocytes of adult rats. *J. Comp. Neurol*. 344: 470-480.

Eriksson NP, Aldskogius H, Grant G, Lindsay RM, Rivero-Melian C (1997) Effects of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 on the laminar distribution of transganglionically fransported choleragenoid in the spinal cord dorsal horn following transection of the sciatic nerve in the adult rat. *Neuroscience* 78: 863-872.

Eriksson NP, Lindsay RM, Aldskogius H (1994) BDNF and NT-3 rescue sensory but not motoneurones following axotomy in the neonate. *Neuroreport* 5: 1445-1448.

Ernfors P, Hallböök F, Ebendal T, Shooter EM, Radeke MJ, Misko TP, Persson H (1988) Developmental and regional expression of b-nerve growth factor receptor mRNA in the chick and rat. *Neuron* 1: 983-996.

Ernfors P, Rosario CM, Merlio JP, Grant G, Aldskogius H, Persson H (1993) Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. *Brain Res. Mol. Brain Res.* 17: 217-226.

Ernfors P, Lee K-F, Kucera J, Jaenisch R (1994a) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* 77: 503-512.

Ernfors P, Lee K-F, Jaenisch R (1994b) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* 368: 147-150.

Everill B and Kocsis JD (2000) Nerve growth factor maintains potassium conductance after nerve injury in adult cutaneous afferent dorsal root ganglion neurons. *Neuroscience* 100: 417-422.

Fagan AM, Zhang H, Landis S, Smeyne RJ, Silossantiago I, Barbacid M (1996) TrkA, but not trkC, receptors are essential for survival of sympathetic neurons *in vivo. J. Neurosci*. 16: 6208-6218.

Falcini F, Matucci CM, Lombardi A, Generini S, Pignone A, Tirassa P, Ermini M, Lepore L, Partsch G, Aloe L (1996) Increased circulating nerve growth factor is directly correlated with disease activity in juvenile chronic arthritis. *Ann. Rheum. Dis.* 55: 745-748.

Farinas I, Jones KR, Bachus C, Wang XY, Reichardt LF (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* 369: 658-661.

Farinas I (1999) Neurotrophin actions during the development of the peripheral nervous system. *Microsc. Res. Tech.* 45: 233-242.

Farinas I, Wilkinson GA, Backus C, Reichardt LF, Patapoutian A (1998) Characterization of neurotrophin and Trk receptor functions in developing sensory ganglia: direct NT-3 activation of TrkB neurons in vivo. *Neuron* 21: 325-334.

Finnerup NB, Jensen TS (2006) Mechanisms of disease: mechanism-based classification of neuropathic pain-a critical analysis. *Nat. Clin. Pract. Neurol*. 2:107-15.

Fischer W, Sirevaag A, Wiegand SJ, Lindsay RM, Bjorklund A (1994) Reversal of spatial memory impairments in aged rats by nerve growth factor and neurotrophins 3 and 4/5 but not by brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* 91: 8607-8611.

Fitzgerald M (1982) Alterations in the ipsi- and contralateral afferent inputs of dorsal horn cells produced by capsaicin treatment of one sciatic nerve in the rat. *Brain Res.*248: 97-107.

Fitzgerald M, Wall PD, Goedert M, Emson PC (1985) Nerve growth factor counteracts the neurophysiological and neurochemical effects of chronic sciatic nerve section. *Brain Res.*332: 131-141.

Fitzgerald EM, Okuse K, Wood JN, Dolphin AC, Moss SJ (1999) cAMP-dependent phosphorylation of the tetrodotoxin-resistant voltage-dependent sodium channel SNS. *J. Physiol*. 516: 433-446.

Fjell J, Cummins TR, Dib-Hajj SD, Fried K, Black JA, Waxman SG (1999) Differential role of GDNF and NGF in the maintenance of two TTX- resistant sodium channels in adult DRG neurons. *Brain Res. Mol. Brain Res*. 67: 267-282.

Foster E, Robertson B, Fried K (1994) trkB-like immunoreactivity in rat dorsal root ganglia following sciatic nerve injury. *Brain Res.* 659: 267-271.

Frade JM, Rodrigueztebar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* 383: 166-168.

Frade JM and Barde YA (1999) Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. *Development* 126: 683-690.

Frank L, Wiegand SJ, Siuciak JA, Lindsay RM, Rudge JS (1997) Effects of BDNF infusion on the regulation of TrkB protein and message in adult rat brain. Exp Neurol 145: 62-70.

Friedman WJ, Lärkfors L, Ayer-LeLievre C, Ebendal T, Olson L, Persson H (1990) Regulation of b-nerve growth factor expression by inflammatory mediators in hippocampal cultures. *J. Neurosci. Res.* 27: 374-382.

Friedman WJ and Greene LA (1999) Neurotrophin signaling via Trks and p75. *Exp. Cell Res.* 253: 131-142.

Frisen J, Verge VM, Cullheim S, Persson H, Fried K, Middlemas DS, Hunter T, Hokfelt T, Risling M (1992) Increased levels of trkB mRNA and trkB protein-like immunoreactivity in the injured rat and cat spinal cord. *Proc. Natl. Acad. Sci. USA* 89: 11282-11286.

Frisén J, Verge VMK, Fried K, Risling M, Persson H, Trotter J, Hökfelt T, Lindholm D (1993) Characterization of glial trkB receptors: Differential response to injury in the central and peripheral nervous systems. *Proc. Natl. Acad. Sci. USA* 90: 4971-4975.

Frostick SP, Yin Q, Kemp GJ (1998) Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery* 18: 397-405.

Fu SY and Gordon T (1995) Contributing factors to poor functional recovery after delayed nerve repair - prolonged denervation. *J. Neurosci*. 15:3886-3895.

Fukuoka T, Kondo E, Dai Y, Hashimoto N, Noguchi K (2001) Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. *J. Neurosci*. 21: 4891-4900.

Funakoshi H, Frisén J, Barbany G, Timmusk T, Zachrisson O, Verge VMK, Persson H (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J. Cell Biol*. 123: 455-465.

Fundin BT, Mikaels A, Westphal H, Ernfors P (1999) A rapid and dynamic regulation of GDNF-family ligands and receptors correlate with the developmental dependency of cutaneous sensory innervation. *Development* 126: 2597-2610.

Fundin BT, Silos-Santiago I, Ernfors P, Fagan AM, Aldskogius H, DeChiara TM, Phillips HS, Barbacid M, Yancopoulos GD, Rice FL (1997) Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and P75 LNGFR. *Dev. Biol*. 190: 94-116.

Gaese F, Kolbeck F, Barde YA (1994) Sensory ganglia require neurotrophin-3 early in development. *Development* 120: 1613-1619.

Galoyan SM, Petruska JC, Mendell LM. (2003) Mechanisms of sensitization of the response of single dorsal root ganglion cells from adult rat to noxious heat. *Eur. J. Neurosci.* 18:535-41.

Gandhi R, Ryals JM, Wright DE (2004) Neurotrophin-3 reverses chronic mechanical hyperalgesia induced by intramuscular acid injection. *J. Neurosci*. 24: 9405-13.

Gao WQ, Dybdal N, Shinsky N, Murnane A, Schmelzer C, Siegel M, Keller G, Hefti F, Phillips HS, Winslow JW (1995) Neurotrophin-3 reverses experimental cisplatin-induced peripheral sensory neuropathy. *Annals of Neurology* 38: 30-37.

Gartner A, Shostak Y, Hackel N, Ethell IM, Thoenen H (2000) Ultrastructural identification of storage compartments and localization of activity-dependent secretion of neurotrophin 6 in hippocampal neurons. *Mol. Cell Neurosci*. 15: 215-234.

Gillardon F, Eschenfelder C, Rush RA, Zimmerman M (1995) Increase in neuronal jun immunoreactivity and epidermal ngf levels following uv exposure of rat skin. *Neuroreport* 6: 1322-1324.

Goedert M, Otten U, Hunt SP, Bond A, Chapman D, Schlumpf M, Lichtensteiger W (1984) Biochemical and anatomical effects of antibodies against nerve growth factor on developing rat sensory ganglia. *Proc. Natl. Acad. Sci. USA* 81: 1580-1584.

Gold BG, Mobley WC, Matheson SF (1991) Regulation of axonal caliber, neurofilament content, and nuclear localization in mature sensory neurons by nerve growth factor. *J. Neurosci*. 11: 943-955.

Gold BG, Storm-Dickerson T, Austin DR (1993) Regulation of the transcription factor c-JUN by nerve growth factor in adult sensory neurons. *Neurosci. Lett*. 154: 129-133.

Gonzalez-Martinez T, Farinas I, Del Valle ME, Feito J, Germana G, Cobo J, Vega JA (2005) BDNf, but not NT-4, is necessary for normal development of Meissner corpuscles. *Neurosci. Lett.* 377: 12-5.

Gotz R, Koster R, Winkler C, Raulf F, Lottspeich F, Schartl M, Thoenen H (1994) Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* 372: 266-269.

Gandhi R, Ryals JM, Wright DE (2004) Neurotrophin-3 reverses chronic mechanical hyperalgesia induced by intramuscular acid injection. *J. Neurosci*. 2004 Oct 20; 24 (42): 9405-13.

Griesbeck O, Parsadanian AS, Sendtner M, Thoenen H (1995) Expression of neurotrophins in skeletal muscle: Quantitative comparison and significance for motoneuron survival and maintenance of function. *J. Neurosci. Res.* 42: 21-33.

Griesbeck O, Canossa M, Campana G, Gartner A, Hoener MC, Nawa H, Kolbeck R, Thoenen H (1999) Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. *Microsc. Res. Tech*. 45: 262-275. (check where cite it).

Gould HJ 3rd, Gould TN, England JD, Paul D, Liu ZP, Levinson SR (2000) A possible role for nerve growth factor in the augmentation of sodium channels in models of chronic pain. *Brain Res*. 854:19-29.

Groth R and Aanonsen L (2002) spinal brain-derivd neruotrophic factor (BDNF) produces hyperalgesia in normal while antisense directed against wither BDNF or TrkB, prevents inflammation-induced hyperalgesia. *Pain* 100:171-81.

Groves MJ, An SF, Giometto B, Scaravilli F (1999) Inhibition of sensory neuron apoptosis and prevention of loss by NT-3 administration following axotomy. *Exp. Neurol.* 155: 284-294.

Groves MJ, Christopherson T, Giometto B, Scaravilli F (1997) Axotomy-induced apoptosis in adult rat primary sensory neurons. *J. Neurocytol*. 26: 615-624.

Gu C, Casaccia-Bonnefil P, Srinivasan A, Chao MV (1999) Oligodendrocyte apoptosis mediated by caspase activation. *J. Neurosci*. 19: 3043-3049.

Hallböök F, Ibáñez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron* 6: 845-858.

Hallböök F (1999) Evolution of the vertebrate neurotrophin and Trk receptor gene families. *Curr. Opin. Neurobiol.* 9: 616-621.

Halliday DA, Zettler C, Rush RA, Scicchitano R and McNeil JD (2004). Elevated nerve rowth factor levels in the synovial fluid of patients with inflammatory joint disease *Neurochem. Res.* 23. 919-922.

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. *Mol. Cell Neurosci.* 14: 28-40.

Hattori A, Tanaka E, Murase K, Ishida N, Chatani Y, Tsujimoto M, Hayashi K, Kohno M (1993) Tumor necrosis factor stimulates the synthesis and secretion of biologically active nerve growth factor in non-neuronal cells. *J. Biol. Chem.* 268: 2577-2582.

Hefti FF, Rosenthal A, Walicke PA, Wyatt S, Vergara G, Shelton DL, Davies AM. (2006) Novel class of pain drugs based on antagonism of NGF. *Trends Pharmacol. Sci.* 27:85-91.

Helgren ME, Cliffer KD, Torrento K, Cavnor C, Curtis R, DiStefano PS, Wiegand SJ, Lindsay RM (1997) Neurotrophin-3 administration attenuates deficits of pyridoxine-induced large-fiber sensory neuropathy. *J. Neurosci.* 17: 372-382.

Helmchen C, Fu QG, Sandkuhler J (1995) Inhibition of spinal nociceptive neurons by microinjections of somatostatin into the nucleus raphe magnus and the midbrain periaqueductal gray of the anesthetized cat. *Neurosci. Lett*. 187: 137-141.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. *Nature* 350: 678-683.

Henion PD, Garner AS, Large TH, Weston JA (1995) trkC-mediated NT-3 signaling is required for the early development of a subpopulation of neurogenic neural crest cells. *Dev. Biol*. 172: 602-613.

Hentall ID and Sagen J (2000) The alleviation of pain by cell transplantation. *Prog. Brain Res*. 127:535-50.

Heppenstall PA, Lewin GR. (2001) BDNF but not NT-4 is required for normal flexion reflex plasticity and function. *Proc. Natl. Acad. Sci. USA* 98:8107-12.

Herrero JF, Laird JM, Lopez-Garcia JA (2000) Wind-up of spinal cord neurones and pain sensation: much ado about something? *Prog. Neurobiol.* 61: 169-203.

Herzberg U, Eliav E, Dorsey JM, Gracely RH, Kopin IJ (1997) NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve. *Neuroreport* 8: 1613-1618.

Heumann R, Korsching S, Bandtlow C, Thoenen H (1987) Changes of nerve growth factor synthesis in non-neuronal cells in response to sciatic nerve transection. *J. Cell Biol.* 104: 1623-1631.

Hjerling-Leffler J, Alqatari M, Ernfors P, Koltzenburg M (2007) Emergence of functional sensory subtypes as defined by transient receptor potential channel expression. *J. Neurosci.* 27: 2435-43.

Hofer MM and Barde YA (1988) Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature* 331: 261-262.

Hoheisel U, Unger T, Mense S (2007) Sensitization ofrat dordsal horn neurons by NGF-induced subthreshold potentials and low-frequency activation. A study employing intracellular recordings in vivo. *Brain Res.* 1169:34-43.

Hohn A, Leibrock J, Bailey K, Barde YA (1990) Identification and characterization of a novel member of the nerve growth factor/brain derived neurotrophic factor family. *Nature* 344: 339-341.

Horigome K, Pryor JC, Bullock ED, Johnson EMJ (1993) Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. *J. Biol. Chem.* 268: 14881-14887.

Horigome K, Bullock ED, Johnson EM, Jr. (1994) Effects of nerve growth factor on rat peritoneal mast cells. Survival promotion and immediate-early gene induction. *J. Biol. Chem.* 269: 2695-2702.

Hu P and McLachlan EM (2001) Long-term changes in the distribution of galanin in dorsal root ganglia after sciatic or spinal nerve transection in rats. *Neuroscience* 103: 1059-1071.

Hua XY, Boublik JH, Spicer MA, Rivier JE, Brown MR, Yaksh TL (1991) The antinociceptive effects of spinally administered neuropeptide Y in the rat: systematic studies on structure-activity relationship. *J. Pharmacol. Exp. Ther.* 258: 243-248.

Huang EJ and Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* 24: 677-736.

Hyman C, Juhasz M, Jackson C, Wright P, Ip NY, Lindsay RM (1994) Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. *J. Neurosci.* 14: 335-347.

Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, Mitsubuchi H, Tonoki H, Awaya Y, Matsuda I (1996) Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat. Genet.* 13: 485-488.

Ip NY, Ibanez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa R3, Squinto SP, et al (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* 89: 3060-3064.

Ishikawa T, Nakanishi O, Funatsu N, Kameyama H (1999) Nerve growth factor inducer, 4-methyl catechol, potentiates central sensitization associated with acceleration of spinal glutamate release after mustard oil paw injection in rats. *Cell Mol. Neurobiol.* 19: 587-596.

Janig W and Habler HJ (2000) Sympathetic nervous system: contribution to chronic pain. *Prog. Brain Res.* 129:451-68: 451-468.

Janig W, Levine JD, Michaelis M (1996) Interactions of sympathetic and primary afferent neurons following nerve injury and tissue trauma. *Prog. Brain Res*. 113: 161-184.

Ji RR, Baba H, Brenner GJ, Woolf CJ (1999) Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat. Neurosci*. 2(12):1114-9.

Ji RR and Woolf CJ (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol. Dis.* 8: 1-10.

Johnson JrEM and Gorin PD (1980) Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science* 210: 916-918.

Johnson EM, Jr. and Deckwerth TL (1993) Molecular mechanisms of developmental neuronal death. *Annu. Rev. Neurosci.* 16: 31-46.

Jones MG, Munson JB, Thompson SW (1999) A role for nerve growth factor in sympathetic sprouting in rat dorsal root ganglia. *Pain* 79: 21-29.

Jongsma WH, Danielsen N, Johnston JM, Gratto KA, Karchewski LA, Verge VM (2001) Exogenous NT-3 and NGF differentially modulate PACAP expression in adult sensory neurons, suggesting distinct roles in injury and inflammation. *Eur. J. Neurosci.* 14: 267-282.

Kafitz KW, Rose CR, Konnerth (2000) A Neurotrophin-evoked rapid excitation of central neurons. *Prog. Brain Res.* 128: 243-249.

Kahane N and Kalcheim C (1994) Expression of trkC receptor mRNA during development of the avian nervous system. *J. Neurobiol.* 25: 571-584.

Kaisho Y, Shintani A, Nishida M, Fukumoto H, Igarashi K (1994) Developmental changes of neurotrophin-3 level in the mouse brain detected by a highly sensitive enzyme immunoassay. *Brain Res.* 666: 143-146.

Kajander KC, Wakisaka S, Bennett GJ (1992) Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat. *Neurosci. Lett.* 138: 225-228.

Kaplan DR and Stephens RM (1994) Neurotrophin signal transduction by the Trk receptor. *J. Neurobiol.* 25: 1404-1417.

Kaplan DR and Miller FD (2000) Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* 10: 381-391.

Karchewski LA, Kim FA, Johnston J, McKnight RM, Verge VM (1999) Anatomical evidence supporting the potential for modulation by multiple neurotrophins in the majority of adult lumbar sensory neurons. *J. Comp. Neurol.* 413: 327-341.

Karchewski LA, Gratto KA, Wetmore C, Verge VM. 2002 Dynamic patterns of BDNF expression in injured sensory neurons: differential modulation by NGF and NT-3. *Eur. J. Neurosci.* 16:1449-62.

Kasama S, Kawakubo M, Suzuki T, Nishizawa T, Ishida A, Nakayama J. (2007) RNA interference-mediated knock-down of transient receptor potential vanilloid 1 prevents forepaw inflammatory hyperalgesia in rat. *Eur. J. Neurosci.* 25:2956-63.

Kashiba H, Noguchi K, Ueda Y, Senba E (1995) Coexpression of trk family members and low-affinity neurotrophin receptors in rat dorsal root ganglion neurons. *Mol. Brain Res.* 30: 158-164.

Kashiba H and Senba E (1999) Up- and down-regulation of BDNF mRNA in distinct subgroups of rat sensory neurons after axotomy. *Neuroreport* 10: 3561-3565.

Kashiba H, Ueda Y, Ueyama T, Nemoto K, Senba E (1997) Relationship between BDNF- and trk-expressing neurones in rat dorsal root ganglion: an analysis by in situ hybridization. *Neuroreport* 8: 1229-1234.

Katsura H, Obata K, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Sakagami M, Noguchi K. (2006) Antisense knock down of TRPA1, but not TRPM8, alleviates cold hyperalgesia after spinal nerve ligation in rats. *Exp. Neurol*. 200:112-23.

Kerr BJ, Bradbury EJ, Bennett DL, Trivedi PM, Dassan P, French J, Shelton DB, McMahon SB, Thompson SW (1999) Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. *J. Neurosci.* 19: 5138-5148.

Kerr BJ, Souslova V, McMahon SB, Wood JN. (2001) A role for the TTX-resistant sodium channel Nav 1.8 in NGF-induced hyperalgesia, but not neuropathic pain. *Neuroreport*. 12(14):3077-80.

Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO (1999) A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron* 24: 253-260.

Kim D, Lee S, Cho H (2001) Differential usage of multiple brain-derived neurotrophic factor promoter in rat dorsal root ganglia following peripheral nerve injuries and inflammation. *Brain Res. Mol. Brain Res.* 92: 167-171.

Kim SH and Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50: 355-363.

Kinnman E and Levine JD (1995) Sensory and sympathetic contributions to nerve injury-induced sensory abnormalities in the rat. *Neuroscience* 64: 751-767.

Kirstein M, Fariñas I (2002) Sensing life: regulation of sensory neuron survival by neurotrophins. *Cell Mol. Life Sci.* 59:1787-802.

Kitchener PD, Wilson P, Snow PJ (1994) Sciatic axotomy compromises axonal transport of transganglionic tracer BSI-B4 from the soma to the central terminals of C-fiber afferents. *Neurosci. Lett.* 166: 121-125.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991) The *trk* proto-oncogene encodes a receptor for nerve growth factor. *Cell* 65: 189-197.

Klein R, Lamballe F, Bryant S, Barbacid M (1992) The *trk*B tyrosine protein kinase is a receptor for neurotrophin- 4. *Neuron* 8: 947-956.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M (1994) Disruption of the neurotrophin-3 receptor gene *trk*C eliminates Ia muscle afferents and results in abnormal movements. *Nature* 368: 249-251.

Klinz FJ, Wolff P, Heumann R (1996) Nerve growth factor-stimulated mitogen-activated protein kinase activity is not necessary for neurite outgrowth of chick dorsal root ganglion sensory and sympathetic neurons. *J. Neurosci. Res.* 46: 720-726.

Krimm RF, Davis BM, Noel T, Albers KM (2006) Overexpression of neurotrophin 4 in skin enhances myelinated sensory endings but does not influence sensory neuron number. *J. Comp. Neurol*. 498(4): 455-65

Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, Noguchi K. (2005) Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. *J. Comp. Neurol*. 493: 596-606.

Koerber HR, Mirnics K, Brown PB, Mendell LM (1994) Central sprouting and functional plasticity of regenerated primary afferents. *J. Neurosci.* 14: 3655-3671.

Koliatsos VE, Clatterbuck RE, Winslow JW, Cayouette MH, Price DL (1993) Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron* 10: 359-367.

Koltzenburg M (1999) The changing sensitivity in the life of the nociceptor. *Pain Suppl.* 6: S93-102.

Koltzenburg M, Torebjork HE, Wahren LK (1994) Nociceptor modulated central sensitization causes mechanical hyperalgesia in acute chemogenic and chronic neuropathic pain. *Brain* 117: 579-591.

Krekoski CA, Parhad IM, Clark AW (1996) Attenuation and recovery of nerve growth factor receptor mRNA in dorsal root ganglion neurons following axotomy. *J. Neurosci. Res.* 43: 1-11.

Kucera J, Fan GP, Jaenisch R, Linnarsson S, Ernfors P (1995) Dependence of developing group Ia afferents on neurotrophin-3. *J. Comp. Neurol.* 363: 307-320.

Kuo WL, Abe M, Rhee J, Eves EM, McCarthy SA, Yan M, Templeton DJ, McMahon M, Rosner MR (1996) Raf, but not MEK or ERK, is sufficient for differentiation of hippocampal neuronal cells. *Mol. Cell Biol.* 16: 1458-1470.

Ladiwala U, Lachance C, Simoneau SJ, Bhakar A, Barker PA, Antel JP (1998) p75 neurotrophin receptor expression on adult human oligodendrocytes: signaling without cell death in response to NGF. *J. Neurosci.* 18: 1297-1304.

Lai KO, Fu WY, Ip FCF, Ip NY (1998) Cloning and Expression of a Novel Neurotrophin, NT-7, from *Carp. Mol. Cell Neurosci*. 11: 64-76.

Lamballe F, Klein R, Barbacid M (1991a) The trk family of oncogenes and neurotrophin receptors. *Princess Takamatsu Symp*. 22: 153-170.

Lamballe F, Klein R, Barbacid M (1991b) trkC, a New member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* 66: 967-979.

Lamballe F, Tapley P, Barbacid M (1993) *trk*C encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities. *EMBO J*. 12: 3083-3094.

Lamballe F, Smeyne RJ, Barbacid M (1994) Developmental expression of *trkC*, the neurotrophin-3 receptor, in the mammalian nervous system. *J. Neurosci.* 14: 14-28.

Lawson SN (1979) The postnatal development of large light and dark neurons in mouse dorsal root ganglia. *J. Neurocytol.* 8: 275-294.

Lawson SN, Caddy KW, Biscoe TJ (1974) Development of rat dorsal root ganglion neurones. Studies of cell birthdays and changes in mean cell diameter. *Cell Tissue Res.* 153: 399-413.

Le Douarin NM and Smith J (1988) Development of the peripheral nervous system from the neural crest. *Annu. Rev. Cell Biol.* 4:375-404.

Lebl DJ, Tessarollo L Palko ME, Parada LF (1997) Absence of sensory neurons before target innervation in brain-derived neurotrophic facto-, neurotrophin 3-, and TrkC-deficient embryonic mice. *J. Neurosci*. 17: 9113-21.

Lee KF, Davies AM, Jaenisch R (1994) p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* 120: 1027-1033.

Lee KF, Li E, Huber LJ, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* 69: 737-749.

Lee KF, Davies AM, Jaenisch R (1994) p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* 120: 1027-1033.

Lee SE, Shen H, Taglialatela G, Chung JM, Chung K (1998) Expression of nerve growth factor in the dorsal root ganglion after peripheral nerve injury. *Brain Res.* 796: 99-106.

Lee SL, Kim JK, Kim DS, Cho HJ (1999) Expression of mRNAs encoding full-length and truncated TrkB receptors in rat dorsal root ganglia and spinal cord following peripheral inflammation. *Neuroreport* 10: 2847-2851.

Lefcort F, Clary DO, Rusoff AC, Reichardt LF (1996) Inhibition of the NT-3 receptor trkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. *J. Neurosci.* 16: 3704-3713.

Lekan HA, Carlton SM, Coggeshall RE (1996) Sprouting of A beta fibers into lamina II of the rat dorsal horn in peripheral neuropathy. *Neurosci. Lett.* 208: 147-150

Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, Levi-Montalcini R (1994) Mast cells synthesize, store, and release nerve growth factor. *Proc. Natl. Acad. Sci. USA* 91: 3739-3743.

Leslie TA, Emson PC, Dowd PM, Woolf CJ (1995) Nerve growth factor contributes to the up-regulation of growth- associated protein 43 and preprotachykinin a messenger rnas in primary sensory neurons following peripheral inflammation. *Neuroscience* 67: 753-761.

Levanon D, Bettoun D, Harris-Cerruti C, Woolf E, Negreanu V, Eilam R, Bernstein Y, Goldenberg D, Xiao C, Fliegauf M, Kremer E, Otto F, Brenner O, Lev-Tov A, Groner Y (2002) The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. *EMBO J.* 21:3454-63.

Lever IJ, Bradbury EJ, Cunningham JR, Adelson DW, Jones MG, McMahon SB, Marvizon JC, Malcangio M (2001) Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. *J. Neurosci.* 21: 4469-4477.

Levi-Montalcini R (1987) The nerve growth factor: 35 years later. Science 237: 1154-1162.

Levine ES and Kolb JE (2000) Brain-derived neurotrophic factor increases activity of NR2B-containing N-methyl-D-aspartate receptors in excised patches from hippocampal neurons. *J. Neurosci. Res.* 62: 357-362.

Lewin GR, Ritter AM, Mendell LM (1992) On the role of nerve growth factor in the development of myelinated nociceptors. *J. Neurosci.* 12: 1896-1905.

Lewin GR, Ritter AM, Mendell LM (1993) Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. *J. Neurosci.* 13: 2136-2148.

Lewin GR, Rueff A, Mendell LM (1994) Peripheral and central mechanisms of ngf-induced hyperalgesia. *Eur. J. Neurosci.* 6: 1903-1912.

Lewin GR (1996) Neurotrophins and the specification of neuronal phenotype. Philosophical Transactions of the Royal Society of London - Series B: Biological 351: 405-411.

Lewin SL, Utley DS, Cheng ET, Verity AN, Terris DJ (1997) Simultaneous treatment with BDNF and CNTF after peripheral nerve transection and repair enhances rate of functional recovery compared with BDNF treatment alone. *Laryngoscope* 107: 992-999.

Li L, Deng YS, Zhou XF (2000) Downregulation of TrkA expression in primary sensory neurons after unilateral lumbar spinal nerve transection and some rescuing effects of nerve growth factor infusion. *Neurosci. Res.* 38:183-91. Erratum in: *Neurosci. Res.* 2001 Feb;39(2):259. Yan-Shen Deng, LL [corrected to Deng, YS].

Li L, Zhou XF (2001) Pericellular Griffonia simplicifolia I isolectin B4-binding ring structures in the dorsal root ganglia following peripheral nerve injury in rats. *J. Comp. Neurol.* 2001. 439:259-74.

Li L, Xian CJ, Zhong JH, Zhou XF (2002) Effect of lumbar 5 ventral root transection on pain behaviors: a novel rat model for neuropathic pain without axotomy of primary sensory neurons. Exp Neurol. 175:23-34. Erratum in: *Exp. Neurol.* 176:407.

Li L, Xian CJ, Zhong JH, Zhou XF (2003) Lumbar 5 ventral root transection-induced upregulation of nerve growth factor in sensory neurons and their target tissues: a mechanism in neuropathic pain. *Mol. Cell Neurosci.* 23:232-50.

Li LY, Wang Z, Sedý J, Quazi R, Walro JM, Frank E, Kucera J (2006a) Neurotrophin-3 ameliorates sensory-motor deficits in Er81-deficient mice. *Dev. Dyn.* 235:3039-50

Li L, Xian CJ, Zhong JH, Zhou XF (2006b) Upregulation of brain-derived neurotrophic factor in the sensory pathway by selective motor nerve injury in adult rats. *Neurotox Res.* 9: 269-83.

Li WP, Xian C, Rush RA, Zhou XF (1999) Upregulation of brain-derived neurotrophic factor and neuropeptide Y in the dorsal ascending sensory pathway following sciatic nerve injury in rat. *Neurosci. Lett.* 260: 49-52.

Liebl DJ, Tessarollo L, Palko ME, Parada LF (1997) Absence of sensory neurons before target innervation in brain-derived neurotrophic factor-, neurotrophin 3-, and TrkC-deficient embryonic mice. *J. Neurosci.* 17: 9113-9121.

Lin SY, Wu K, Levine ES, Mount HT, Suen PC, Black IB (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. *Brain Res. Mol. Brain Res.* 55: 20-27.

Lin YW, Min MY, Lin CC, Chen WN, Wu WL, Yu HM, Chen CC. (2008) Identification and characterization of a subset of mouse sensory neurons that express acid-sensing ion channel 3. *Neuroscience* 151:544-57.

Lindenlaub T and Sommer C (2000) Partial sciatic nerve transection as a model of neuropathic pain: a qualitative and quantitative neuropathological study. *Pain* 89: 97-106.

Lindholm D, Heumann R, Meyer M, Thoenen H (1987) Interleukin-1 regulates synthesis of nerve growth factor in non- neuronal cells of rat sciatic nerve. *Nature* 330: 658-659.

Lindsay RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J. Neurosci.* 8:2394-405.

Lindsay RM, Shooter EM, Radeke MJ, Misko TP, Dechant G, Thoenen H, Lindholm D (1990) Nerve Growth Factor Regulates Expression of the Nerve Growth Factor Receptor Gene in Adult Sensory Neurons. *Eur. J. Neurosci*. 2:389-396.

Lindsay RM (1996) Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons - an overview. Philosophical Transactions of the Royal Society of London - Series B: Biological 351: 365-373.

Liu CN, Amir R, Devor M (1999) Effect of age and nerve injury on cross-excitation among sensory neurons in rat dorsal root ganglia. *Neurosci. Lett.* 259: 95-98.

Liu X, Eschenfelder S, Blenk KH, Janig W, Habler H (2000) Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats. *Pain* 84: 309-318.

Lowe EM, Anand P, Terenghi G, Williams-Chestnut RE, Sinicropi DV, Osborne JL (1997) Increased nerve growth factor levels in the urinary bladder of women with idiopathic sensory urgency and interstitial cystitis. *Br. J. Urol.* 79: 572-577.

Lu B, Pang PT, Woo NH (2005) The yin and yang of neurotrophin action. *Nat. Rev. Neurosci.* 6:603-14.

Luo W, Wickramasinghe SR, Savitt JM, Griffin JW, Dawson TM, Ginty DD (2007) A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. *Neuron* 54:673-5.

Luo X, Rush RA, Zhou X (2001) Ultrastructural localization of brain-derived neurotrophic factor in rat primary sensory neurons. *Neurosci. Res.* 39: 377-384.

Ma Q, Chen Z, del Barco B, de la Pompa JL, Anderson DJ (1998) neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* 20: 469-482.

Ma Q, Fode C, Guillemot F, Anderson DJ (1999) Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes Dev.* 13: 1717-1728.

Ma QP and Woolf CJ (1997) The progressive tactile hyperalgesia induced by peripheral inflammation is nerve growth factor dependent. *Neuroreport* 8: 807-810.

MacQueen GM, Ramakrishnan K, Croll SD, Siuciak JA, Yu G, Young LT, Fahnestock M (2001) Performance of heterozygous brain-derived neurotrophic factor knockout mice on behavioral analogues of anxiety, nociception, and depression. *Behav. Neurosci.* 115: 1145-1153.

Madison RD and Archibald SJ (1994) Point sources of Schwann cells result in growth into a nerve entubulation repair site in the absence of axons: effects of freeze-thawing. *Exp. Neurol*. 128: 266-275.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM, Yancopoulos GD (1990) NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. *Neuron* 5: 501-509.

Malcangio M, Garrett NE, Cruwys S, Tomlinson DR (1997a) Nerve growth factor- and neurotrophin-3-induced changes in nociceptive threshold and the release of substance P from the rat isolated spinal cord. *J. Neurosci.* 17: 8459-8467.

Malmberg AB, Chen C, Tonegawa S, Basbaum AI (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma *Science* 278: 279-283.

Marmigere F. and Ernfors P (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. *Nat. Rev. Neurosci.* 8, 114–127.

Mannion RJ, Costigan M, Decosterd I, Amaya F, Ma QP, Holstege JC, Ji RR, Acheson A, Lindsay RM, Wilkinson GA, Woolf CJ (1999) Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc. Natl. Acad. Sci. USA* 96: 9385-9390.

Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA (1997) Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* 278: 275-279.

Mark MA, Jarrott B, Colvin LA, MacMillan SJ, Duggan AW (1997) The release of immunoreactive neuropeptide Y in the spinal cord of the anaesthetized rat and cat. *Brain Res.* 754: 195-203.

Martin-Iverson MT, Todd KG, Altar CA (1994) Brain-derived neurotrophic factor and neurotrophin-3 activate striatal dopamine and serotonin metabolism and related behaviors: interactions with amphetamine. *J. Neurosci.* 14: 1262-1270.

Martin HA, Basbaum AI, Kwiat GC, Goetzl EJ, Levine JD (1987) Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs. *Neuroscience* 22: 651-659.

Matheson CR, Carnahan J, Urich JL, Bocangel D, Zhang TJ, Yan Q (1997a) Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor for sensory neurons: Comparison with the effects of the neurotrophins. *J. Neurobiol.* 32: 22-32.

Matsuoka I, Meyer M, Thoenen H (1991) Cell-type-specific regulation of nerve growth factor (NGF) synthesis in non-neuronal cells: Comparison of Schwann cells with other cell types. *J. Neurosci*. 11: 3165-3177.

Matsuoka Y, Yokoyama M, Kobayashi H, Omori M, Itano Y, Morita K, Mori H, Nakanishi T (2007) Expression profiles of BDNF splice variants in cultured DRG neurons stimulated with NGF. *Biochem. Biophys. Res. Commun*. 363:682-8.

Matzner O and Devor M (1987) Contrasting thermal sensitivity of spontaneously active A- and C-fibers in experimental nerve-end neuromas. *Pain* 30: 373-384.

McAllister AK, Katz LC, Lo DC (1999) Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci*. 22:295-318:.

McArthur JC, Yiannoutsos C, Simpson DM, Adornato BT, Singer EJ, Hollander H, Marra C, Rubin M, Cohen BA, Tucker T, Navia BA, Schifitto G, Katzenstein D, Rask C, Zaborski L, Smith ME, Shriver S, Millar L, Clifford DB, Karalnik IJ (2000) A phase II trial of nerve growth factor for sensory neuropathy associated with HIV infection. AIDS Clinical Trials Group Team 291.Neurology. Mar 14;54(5):1080-8. Erratum in: *Neurology* 13; 55: 162

McLachlan EM, Janig W, Devor M, Michaelis M (1993) Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. *Nature* 363: 543-545.

McLachlan EM and Hu P (1998) Axonal sprouts containing calcitonin gene-related peptide and substance P form pericellular baskets around large diameter neurons after sciatic nerve transection in the rat. *Neuroscience* 84: 961-965.

McMahon SB, Armanini MP, Ling LH, Phillips HS (1994) Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* 12: 1161-1171.

McMahon SB, Bennett DL, Priestley JV, Shelton DL (1995) The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nat. Med.* 1: 774-780.

McMahon SB, Lewin GR, Wall PD (1993) Central hyperexcitability triggered by noxious inputs. *Curr. Opin. Neurobiol.* 3: 602-610.

Mearow KM, Kril Y, Diamond J (1993) Increased NGF mRNA expression in denervated rat skin. *Neuroreport* 4: 351-354.

Melzack R and Wall PD (1965) Pain mechanisms: a new theory. Science 150: 971-979.

Mendell LM, Albers KM, Davis BM (1999) Neurotrophins, nociceptors, and pain. *Microsc. Res. Tech.* 45: 252-261.

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *J. Cell Biol.* 119: 45-54.

Meyer RA CJRS (1994) Peripheral neural mechanisms of nociception. In: Textbook of Pain, Wall PD and Melzack R (eds). Edinburgh: Churchill Livingstone. 1994. p13-14.

Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat. Neurosci.* 7:221-228.

Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DL, Yan Q, Priestley JV (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in

TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *J. Neurosci.* 17: 8476-8490.

Michael GJ, Averill S, Shortland PJ, Yan Q, Priestley JV (1999a) Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. *Eur. J. Neurosci.* 11: 3539-3551.

Michael GJ and Priestley JV (1999b) Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its down-regulation by axotomy. *J. Neurosci.* 19: 1844-1854.

Middlemas DS, Lindberg RA, Hunter T (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol. Cell Biol.* 11: 143-153.

Miletic G, Miletic V (2007) Loose ligation of the sciatic nerve is associated with TrkB receptor-dependent decreases in KCC2 protein levels in the ipsilateral spinal dorsal horn. *Pain* Nov 30; [Epub ahead of print].

Miki K, Fukuoka T, Tokunaga A, Kondo E, Dai Y, Noguchi K (2000) Differential effect of brain-derived neurotrophic factor on high-threshold mechanosensitivity in a rat neuropathic pain model. *Neurosci. Lett*. 278: 85-88.

Miki K, Fukuoka T, Tokunaga A, Noguchi K (1998) Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: up-regulation in a subpopulation of primary afferent sensory neurons. *Neuroscience* 82: 1243-1252.

Millan MJ (1999) The induction of pain: an integrative review. Prog Neurobiol 57: 1-164.

Minichiello L, Piehl F, Vazquez E, Schimmang T, Hökfelt T, Represa J, Klein R (1995) Differential effects of combined *trk* receptor mutations on dorsal root ganglion and inner ear sensory neurons. Development 121: 4067-4075.

Mirsky R and Jessen KR (1999) The neurobiology of Schwann cells. *Brain Pathol.* 9: 293-311.

Mogil JS (1999) The genetic mediation of individual differences in sensitivity to pain and its inhibition. *Proc. Natl. Acad. Sci. USA* 96: 7744-7751.

Mollenholt P, Rawal N, Gordh TJ, Olsson Y (1994) Intrathecal and epidural somatostatin for patients with cancer. Analgesic effects and postmortem neuropathologic investigations of spinal cord and nerve roots. *Anesthesiology* 81: 534-542.

Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 19: 849-861.

Montaner S, Perona R, Saniger L, Lacal JC (1998) Multiple signaling pathways lead to the activation of the nuclear factor kappaB by the Rho family of GTPases. *J. Biol. Chem.* 273: 12779-12785.

Mu X, Silos-Santiago I, Carroll SL, Snider WD (1993) Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. *J. Neurosci.* 13: 4029-4041.

Munglani R, Harrison SM, Smith GD, Bountra C, Birch PJ, Elliot PJ, Hunt SP (1996) Neuropeptide changes persist in spinal cord despite resolving hyperalgesia in a rat model of mononeuropathy. *Brain Res.* 743: 102-108.

Munson JB, Shelton DL, McMahon SB (1997a) Adult mammalian sensory and motor neurons: roles of endogenous neurotrophins and rescue by exogenous neurotrophins after axotomy. *J. Neurosci.* 17: 470-476.

Munson JB, Johnson RD, Mendell LM (1997a) NT-3 increases amplitude of EPSPs produced by axotomized group Ia afferents. *J. Neurophysiol*. 77: 2209-2212.

Munson JB, Shelton DL, McMahon SB (1997b) Adult mammalian sensory and motor neurons: roles of endogenous neurotrophins and rescue by exogenous neurotrophins after axotomy. *J. Neurosci*. 17: 470-476.

Nakatsuka T, Park JS, Kumamoto E, Tamaki T, Yoshimura M (1999) Plastic changes in sensory inputs to rat substantia gelatinosa neurons following peripheral inflammation. *Pain* 82: 39-47.

Narita M, Yajima Y, Aoki T, Ozaki S, Mizoguchi H, Tseng LF, Suzuki T (2000) Up-regulation of the TrkB receptor in mice injured by the partial ligation of the sciatic nerve. *Eur. J. Pharmacol.* 401: 187-190.

Nawa H, Pelleymounter MA, Carnahan J (1994) Intraventricular administration of BDNF increases neuropeptide expression in newborn rat brain. *J. Neurosci*. 14: 3751-3765.

Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature* 384: 360-364.

Nicol, GD and Vasko MR 2007 unraveling the story of NGF-mediated sensitization of nociceptive sensory neurons: on or off the Trks? *Molecular interventions* 7:26-41.

Nilsson AS, Fainzilber M, Falck P, Ibanez CF (1998) Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. *FEBS Lett*. 424: 285-290.

Nitta A, Ohmiya M, Jin-nouchi T, Sometani A, Asami T, Kinukawa H, Fukumitsu H, Nomoto H, Furukawa S (1999) Endogenous neurotrophin-3 is retrogradely transported in the rat sciatic nerve. *Neuroscience* 88: 679-685.

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K (1995) Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. *J. Neurosci.* 15: 7633-7643.

Novakovic SD, Tzoumaka E, McGivern JG, Haraguchi M, Sangameswaran L, Gogas KR, Eglen RM, Hunter JC (1998) Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J. Neurosci.* 18: 2174-2187.

Oakley RA, Garner AS, Large TH, Frank E (1995) Muscle sensory neurons require neurotrophin-3 from peripheral tissues during the period of normal cell death. *Development* 121: 1341-1350.

Obata K, Yamanaka H, Kobayashi K, Dai Y, Mizushima T, Katsura H, Fukuoka T, Tokunaga A, Noguchi K (2004) Role of mitogen-activated protein kinase activation in injured and intact primary afferent neurons for mechanical and heat hypersensitivity after spinal nerve ligation. *J. Neurosci.* 24:10211-22.

Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Tominaga M, Noguchi K. (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J. Clin. Invest* 115:2393-401.

Obata K, Yamanaka H, Kobayashi K, Dai Y, Mizushima T, Katsura H, Fukuoka T, Tokunaga A, Noguchi K (2006a) The effect of site and type of nerve injury on the expression of brain-derived neurotrophic factor in the dorsal root ganglion and on neuropathic pain behavior. *Neuroscience* 137:961-70.

Obata K, Katsura H, Sakurai J, Kobayashi K, Yamanaka H, Dai Y, Fukuoka T, Noguchi K. (2006b) Suppression of the p75 neurotrophin receptor in uninjured sensory neurons reduces neuropathic pain after nerve injury. *J. Neurosci*. 26:11974-86.

Ockel M, Vonschack D, Schropel A, Dechant G, Lewin GR, Barde YA (1996) Roles of neurotrophin-3 during early development of the peripheral nervous system. Philosophical Transactions of the Royal Society of London - Series B: Biological 351: 383-387.

Oddiah D, Anand P, McMahon SB, Rattray M (1998) Rapid increase of NGF, BDNF and NT-3 mRNAs in inflamed bladder. *Neuroreport* 9: 1455-1458.

Offenhauser N, Bohmmatthaei R, Tsoulfas P, Parada L, Meyer M (1995) Developmental regulation of full-length trkC in the rat sciatic nerve. *Eur. J. Neurosci.* 7: 917-925.

Ohara S, Tantuwaya V, DiStefano PS, Schmidt RE (1995) Exogenous NT-3 mitigates the transganglionic neuropeptide Y response to sciatic nerve injury. Brain Res 699: 143-148.

Omura T, Sano M, Omura K, Hasegawa T, Doi M, Sawada T,Nagano A (2005) Different expressions of BDNF, NT3, and NT4 in muscle and nerve after various types of peripheral nerve injuries. *J. Peripher. Nerv. Syst.* 10:293-300.

Oppenheim RW (1991) Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453-501.

Ostman JA, Nassar MA, Wood JN, Baker MD. (2008) GTP up-regulated persistent Na+ current and enhanced nociceptor excitability require NaV1.9. *J. Physiol*. 586:1077-87.

Otten U, Goedert M, Mayer N, Lembeck F (1980) Requirement of nerve growth factor for development of substance P-containing sensory neurones. *Nature* 287: 138-159.

Pachnis V, Mankoo B, Costantini F (1993) Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* 119: 1005-1017.

Park PC and Deboni U (1996) Transposition of dnase hypersensitive chromatin to the nuclear periphery coincides temporally with nerve growth factor-induced up-regulation of gene expression in pc12 cells. *Proc. Natl. Acad. Sci. USA* 93: 11646-11651.

Park SY, Lee JY, Choi JY, Park MJ, Kim DS (2006) Nerve growth factor activates brain-derived neurotrophic factor promoter IV via extracellular signal-regulated protein kinase 1/2 in PC12 cells. *Mol. Cells* 21:237-43.

Patel TD, Jackman A, Rice FL, Kucera J, Snider WD (2000) Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. *Neuron* 25: 345-357.

Petersen M, Segond VB, Heppelmann B, Koltzenburg M (1998) Nerve growth factor regulates the expression of bradykinin binding sites on adult sensory neurons via the neurotrophin receptor p75. *Neuroscience* 83: 161-168.

Pezet S, Onteniente B, Jullien J, Junier MP, Grannec G, Rudkin BB, Calvino B (2001) Differential regulation of NGF receptors in primary sensory neurons by adjuvant-induced arthritis in the rat. *Pain* 90: 113-125.

Pezet S, McMahon SB. (2006) Neurotrophins: mediators and modulators of pain. *Annu. Rev. Neurosci.* 29:507-38.

Pincelli C and Marconi A (2000) Autocrine nerve growth factor in human keratinocytes. *J. Dermatol. Sci*. 22: 71-79.

Pohl M and Braz J (2001) Gene therapy of pain: emerging strategies and future directions. *Eur. J. Pharmacol*. 429: 39-48.

Rabin SJ, Cleghon V, Kaplan DR (1993) SNT, a differentiation-specific target of neurotrophic factor- induced tyrosine kinase activity in neurons and PC12 cells. *Mol. Cell Biol.* 13: 2203-2213.

Raivich G, Hellweg R, Kreutzberg GW (1991) NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. *Neuron* 7: 151-164.

Ralston HJ, Light AR, Ralston DD, Perl ER (1984) Morphology and synaptic relationships of physiologically identified low-threshold dorsal root axons stained with intra-axonal horseradish peroxidase in the cat and monkey. *J. Neurophysiol*. 51: 777-792.

Ramer MS and Bisby MA (1999) Adrenergic innervation of rat sensory ganglia following proximal or distal painful sciatic neuropathy: distinct mechanisms revealed by anti- NGF treatment. *Eur. J. Neurosci*. 11: 837-846.

Ramer MS, Bradbury EJ, McMahon SB (2001) Nerve growth factor induces P2X(3) expression in sensory neurons. *J. Neurochem*. 77: 864-875.

Ramer MS, Priestley JV, McMahon SB (2000) Functional regeneration of sensory axons into the adult spinal cord. *Nature* 403: 312-316.

Ramer MS, Thompson SW, McMahon SB (1999) Causes and consequences of sympathetic basket formation in dorsal root ganglia. *Pain Suppl.* 6: S111-S120.

Ren K and Dubner R (1996) Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation. *J. Neurophysiol*. 76: 3025-3037.

Ren K, Thomas DA, Dubner R (1995) Nerve growth factor alleviates a painful peripheral neuropathy in rats. *Brain Res.* 699: 286-292.

Reynolds AJ, Heydon K, Bartlett SE, Hendry IA (1999) Evidence for phosphatidylinositol 4-kinase and actin involvement in the regulation of 125I-beta-nerve growth factor retrograde axonal transport. *J. Neurochem.* 73: 87-95.

Ritter AM, Lewin GR, Kremer NE, Mendell LM (1991) Requirement for nerve growth factor in the development of myelinated nociceptors in vivo. *Nature* 350: 500-502.

Ro LS, Chen ST, Tang LM, Jacobs JM (1999) Effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve. *Pain* 79: 265-274.

Robinson RC, Radziejewski C, Spraggon G, Greenwald J, Kostura MR, Burtnick LD, Stuart DI, Choe S, Jones EY (1999) The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site. *Protein Sci*. 8: 2589-2597.

Romero MI, Rangappa N, Garry MG, Smith GM (2001) Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy. *J. Neurosci.* 21: 8408-8416.

Rueff A and Mendell LM (1996) Nerve growth factor and NT-5 induce increased thermal sensitivity of cutaneous nociceptors in vitro. *J. Neurophysiol*. 76: 3593-3596.

Rueff A, Dawson AJLR, Mendell LM (1996) Characteristics of nerve growth factor induced hyperalgesia in adult rats: Dependence on enhanced bradykinin-1 receptor activity but not neurokinin-1 receptor activation. *Pain* 66: 359-372.

Ruiz G, Ceballos D, Baños JE (2004) Behavioral and histological effects of endoneurial administration of nerve growth factor: possible implications in neuropathic pain. *Brain Res.* 1011:1-6.

Ryden M and Ibanez CF (1997) A second determinant of binding to the p75 neurotrophin receptor revealed by alanine-scanning mutagenesis of a conserved loop in nerve growth factor. *J. Biol. Chem.* 272: 33085-33091.

Rydén M, Murray-Rust J, Glass D, Ilag LL, Trupp M, Yancopoulos GD, McDonald NQ, Ibáñez CF (1995) Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for $p75^{LNGFR}$ in NT- 4 signaling. *EMBO J.* 14: 1979-1990.

Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995) Contribution of interleukin-1b to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Br. J. Pharmacol.* 115: 1265-1275.

Sah DW, Ossipo MH, Porreca F (2003) Neurotrophic factors as novel therapeutics for neuropathic pain. *Nat. Rev. Drug Discov*. 2:460-72.

Saito T, Greenwood A, Sun Q, Anderson DJ (1995) Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. *Mol. Cell Neurosci.* 6: 280-292.

Santambrogio L, Benedetti M, Chao MV, Muzaffar R, Kulig K, Gabellini N, Hochwald G (1994) Nerve growth factor production by lymphocytes. *J. Immunol.* 153: 4488-4495.

Sato J and Perl ER (1991) Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury. *Science* 251: 1608-1610.

Savitz SI and Kessler JA (2000) Leukemia inhibitory factor requires concurrent p75LNTR signaling to induce apoptosis of cultured sympathetic neurons. *J. Neurosci.* 20: 4198-4205.

Schulte-Herbrüggen O, Braun A, Rochlitzer S, Jockers-Scherübl MC, Hellweg R. (2007) Neurotrophic factors--a tool for therapeutic strategies in neurological, neuropsychiatric and neuroimmunological diseases? *Curr. Med. Chem.* 14:2318-29.

Sebert ME and Shooter EM (1993) Expression of mRNA for neurotrophic factors and their receptors in the rat dorsal root ganglion and sciatic nerve following nerve injury. *J. Neurosci. Res.* 36: 357-367.

Seebach BS, Arvanov V, Mendell LM (1999) Effects of BDNF and NT-3 on development of Ia/motoneuron functional connectivity in neonatal rats. *J. Neurophysiol.* 81: 2398-2405.

Sedý J, Szeder V, Walro JM, Ren ZG, Nanka O, Tessarollo L, Sieber-Blum M, Grim M, Kucera J. (2004) Pacinian corpuscle development involves multiple Trk signaling pathways. *Dev. Dyn*. 231(3):551-63.

Seltzer Z, Dubner R, Shir Y (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 43: 205-218.

Shen H, Chung JM, Coggeshall RE, Chung K (1999) Changes in TrkA expression in the dorsal root ganglion after peripheral nerve injury. *Exp. Brain Res*. 127: 141-6.

Shinder V, Govrin-Lippmann R, Cohen S, Belenky M, Ilin P, Fried K, Wilkinson HA, Devor M (1999) Structural basis of sympathetic-sensory coupling in rat and human dorsal root ganglia following peripheral nerve injury. *J. Neurocytol.* 28: 743-761.

Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20: 727-740.

Siniscalco D, de Novellis V, Rossi F, Maione S (2005) Neuropathic pain: is the end of suffering starting in the gene therapy? *Curr. Drug Targets* 6:75-80.

Shortland P and Woolf CJ (1993) Chronic peripheral nerve section results in a rearrangement of the central axonal arborizations of axotomized A beta primary afferent neurons in the rat spinal cord. *J. Comp. Neurol* 330: 65-82.

Shu XQ, Llinas A, Mendell LM (1999) Effects of trkB and trkC neurotrophin receptor agonists on thermal nociception: a behavioral and electrophysiological study. *Pain* 80: 463-470.

Shu X and Mendell LM (2001) Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. *J. Neurophysiol*. 86: 2931-2938.

Sieber-Blum M (1998) Growth factor synergism and antagonism in early neural crest development. *Biochem. Cell Biol*. 76: 1039-1050.

Silos-Santiago I, Molliver DC, Ozaki S, Smeyne RJ, Fagan AM, Barbacid M, Snider WD (1995) Non-TrkA-expressing small DRG neurons are lost in *TrkA* deficient mice. *J. Neurosci.* 15: 5929-5942.

Simon MD, DeSantis S, Vigneti E, Papa G, Amador S, Aloe L (1999) Nerve growth factor: A survery of activity on immune and hematopoietic cells. *Hematol. Oncol*. 17, 1-10.

Siuciak JA, Altar CA, Wiegand SJ, Lindsay RM (1994) Antinociceptive effect of brain-derived neurotrophic factor and neurotrophin-3. *Brain Res.* 633: 326-330.

Silverman JD and Kruger L (1990) Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *J. Neurocytol*. 19: 789-801.

Siuciak JA, Wong V, Pearsall D, Wiegand SJ, Lindsay RM (1995) Bdnf produces analgesia in the formalin test and modifies neuropeptide levels in rat brain and spinal cord areas associated with nociception. *Eur. J. Neurosci.* 7: 663-670.

Sivilia S, Paradisi M, D'Intino G, Fernandez M, Pirondi S, Lorenzini L, Calzà L. (2008) Skin homeostasis during inflammation: a role for nerve growth factor. *Histol Histopathol*. Jan;23(1):1-10.

Slack SE, Pezet S, McMahon SB, Thompson SW, Malcangio M (2004) Brain-derived neurotrophic factor induces NMDA receptor subunit one phosphorylation via ERK and PKC in the rat spinal cord. *Eur. J. Neurosci*. 20:1769-78.

Sluka KA and Westlund KN (1993) Behavioral and immunohistochemical changes in an experimental arthritis model in rats. *Pain* 55: 367-377.

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature* 368: 246-249.

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* 77: 627-638.

Snider WD and McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron* 20: 629-632.

Soilu-Hanninen M, Ekert P, Bucci T, Syroid D, Bartlett PF, Kilpatrick TJ (1999) Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway. *J. Neurosci*. 19: 4828-4838.

Song XY, Zhong JH, Wang X, Zhou XF (2004) Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. *J. Neurosci*. 24:542-6.

Song XY, Zhou FH, Zhong JH, Wu LL, Zhou XF (2006) Knockout of p75(NTR) impairs re-myelination of injured sciatic nerve in mice. *J. Neurochem.* 96:833-42.

Song XY, Li F, Zhang FH, Zhong JH, Zhou XF (2008) Peripherally-derived BDNF promotes regeneration of ascending sensory neurons after spinal cord injury. *PLoS ONE* 3:e1707.

Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. *Annu. Rev. Neurosci*. 24: 1217-1281.

Sorkin LS, Xiao WH, Wagner R, Myers RR (1997) Tumour necrosis factor-alpha induces ectopic activity in nociceptive primary afferent fibers. *Neuroscience* 81: 255-262.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, DiStefano PS, Yancopoulos GD (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* 65: 885-893.

Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma1 to mediate NGF responses. *Neuron* 12: 691-705.

Stephens HE, Belliveau AC, Gupta JS, Mirkovic S, Kablar B (2005). The role of neurotrophins in the maintenance of the spinal cord motor neurons and the dorsal root ganglia proprioceptive sensory neurons. *Int. J. Dev. Neurosci*. 23:613-20.

Sterne GD, Brown RA, Green CJ, Terenghi G (1998) NT-3 modulates NPY expression in primary sensory neurons following peripheral nerve injury. *J. Anat*. 193: 273-281.

Stucky CL, Koltzenburg M (1997) The low-affinity neurotrophin receptor p75 regulates the function but not the selective survival of specific subpopulations of sensory neurons. *J. Neurosci.* 17: 4398-4405.

Stucky CL, Dechiara T, Lindsay RM, Yancopoulos GD, Koltzenburg M (1998) Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. *J. Neurosci.* 18: 7040-7046.

Stucky CL, Koltzenburg M, Schneider M, Engle MG, Albers KM, Davis BM (1999a) Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. *J. Neurosci*. 19: 8509-8516.

Stucky CL and Lewin GR (1999b) Isolectin B4-positive and negative nociceptors are functionally distinct. *J. Neurosci.* 19: 6497-6505.

Stucky CL, Shin JB, Lewin GR (2002) Neurotrophin-4: a survival factor for adult sensory neurons. *Curr. Biol.* 12(16:1401-4.

Svensson P, Cairns BE, Wang K, Arendt-Nielsen L (2003) Injection of nerve growth factor into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia. *Pain* 104:241-7.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* 20: 709-726.

Taniuchi M, Clark HB, Johnson EM (1986) Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proc. Natl. Acad. Sci. USA* 83: 4094-4098

Tarpley JW, Kohler MG, Martin WJ. (2004) The behavioral and neuroanatomical effects of IB4-saporin treatment in rat models of nociceptive and neuropathic pain. *Brain Res.* 1029: 65-76.

Tessarollo L, Vogel KS, Palko ME, Reid SW, Parada LF (1994) Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. *Proc. Natl. Acad. Sci. USA* 91: 11844-11848.

Tessarollo L, Tsoulfas P, Donovan MJ, Palko ME, Blair-Flynn J, Hempstead BL, Parada LF (1997) Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis. *Proc. Natl. Acad. Sci. USA* 94: 14776-14781.

Theodosiou M, Rush RA, Zhou XF, Hu D, Walker JS, Tracey DJ (1999) Hyperalgesia due to nerve damage: role of nerve growth factor. *Pain* 81: 245-255.

Thompson SW, Bennett DL, Kerr BJ, Bradbury EJ, McMahon SB (1999) Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. *Proc. Natl. Acad. Sci. USA* 96: 7714-7718.

Thompson SWN, Dray A, McCarson KE, Krause JE, Urban L (1995) Nerve growth factor induces mechanical allodynia associated with novel A fiber-evoked spinal reflex activity and enhanced neurokinin-1 receptor activation in the rat. *Pain* 62: 219-231.

Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* 10: 475-489.

Timmusk T, Lendahl U, Funakoshi H, Arenas E, Persson H, Metsis M (1995) Identification of brain-derived neurotrophic factor promoter regions mediating tissue-specific, axotomy-, and neuronal activity-induced expression in transgenic mice. *J. Cell Biol*. 128: 185-199.

Tong YG, Wang HF, Ju G, Grant G, Hokfelt T, Zhang X (1999) Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: possible implications for sensory sprouting. *J. Comp. Neurol* 404: 143-158.

Tonra JR (1999) Classical and novel directions in neurotrophin transport and research: anterograde transport of brain-derived neurotrophic factor by sensory neurons. *Microsc. Res. Tech.* 45: 225-232.

Tonra JR, Curtis R, Wong V, Cliffer KD, Park JS, Timmes A, Nguyen T, LindsayRM, Acheson A, DiStefano PS (1998) Axotomy upregulates the anterograde transport and expression of brain- derived neurotrophic factor by sensory neurons. *J. Neurosci.* 18: 4374-4383.

Torebjork HE, Lundberg LE, Lamotte RH (1992) Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J. Physiol.* 448: 765-780.

Torebjork E, Wahren L, Wallin G, Hallin R, Koltzenburg M (1995) Noradrenaline-evoked pain in neuralgia. *Pain* 63: 11-20.

Tron VA, Coughlin MD, Jang DE, Stanisz J, Sauder DN (1990) Expression and modulation of nerve growth factor in murine keratinocytes (PAM 212). *J. Clin. Invest* 85: 1085-1089.

Twining CM, Sloane EM, Milligan ED, Chacur M, Martin D, Poole S, Marsh H, Maier SF, Watkins LR (2004) Peri-sciatic proinflammatory cytokines, reactive oxygen species, and complement induce mirror-image neuropathic pain in rats. *Pain* 110:299-309.

Ugolini G, Marinelli S, Covaceuszach S, Cattaneo A, Pavone F (2007) The function neutralizing anti-TrkA antibody MNAC13 reduces inflammatory and neuropathic pain. *Proc. Natl. Acad. Sci. USA* 104: 2985-90.

Utzschneider D, Kocsis J, Devor M (1992) Mutual excitation among dorsal root ganglion neurons in the rat. *Neurosci. Lett.* 146: 53-56.

Van Beers RJ, Sittig AC, Van der Gon JJD (1996) How humans combine simultaneous proprioceptive and visual position information. *Exp. Brain Res*. 111: 253-261.

Verdi JM, Birren SJ, Ibáñez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994) $p75^{LNGFR}$ Regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* 12: 733-745.

Verge VM, Merlio JP, Grondin J, Ernfors P, Persson H, Riopelle RJ, Hokfelt T, Richardson PM (1992) Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. *J. Neurosci.* 12: 4011-4022.

Verge VM, Richardson PM, Wiesenfeld Hallin Z, Hokfelt T (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. *J. Neurosci.* 15: 2081-2096.

Verge VM, Tetzlaff W, Bisby MA, Richardson PM (1990) Influence of nerve growth factor on neurofilament gene expression in mature primary sensory neurons. *J. Neurosci.* 10: 2018-2025.

Verge VM, Gratto KA, Karchewski LA, Richardson PM (1996) Neurotrophins and nerve injury in the adult. Philosophical Transactions of the Royal Society of London - Series B: Biological 351: 423-430.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN (1996) Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. *Nature* 380: 75-79.

Vestergaard S, Tandrup T, Jakobsen J (1997) Effect of permanent axotomy on number and volume of dorsal root ganglion cell bodies. *J. Comp. Neurol* 388: 307-312.

Von Bartheld CS, Kinoshita Y, Prevette D, Yin QW, Oppenheim RW, Bothwell M (1994) Positive and negative effects of neurotrophins on the isthmo- optic nucleus in chick embryos. *Neuron* 12: 639-654.

Von Bartheld CS, Butowt R (2000) Expression of neurotrophin-3 (NT-3) and anterograde axonal transport of endogenous NT-3 by retinal ganglion cells in chick embryos. *J. Neurosci.* 2: 736-748.

Vulchanova L, Riedl MS, Shuster SJ, Buell G, Surprenant A, North RA, Elde R (1997) Immunohistochemical study of the P2X2 and P2X3 receptor subunits in rat and monkey sensory neurons and their central terminals. *Neuropharmacology* 36: 1229-1242.

Vulchanova L and Olson RE (2000) Roles of IB4-bindng sensory neurons in the effects of intradermal capsaicin injection. *Soc. Neurosci. Abstr*. 26: 561.

Wagner R and Myers RR (1996) Endoneurial injection of TNF-alpha produces neuropathic pain behaviors. *Neuroreport* 7: 2897-2901.

Walker SM, Mitchell VA, White DM, Rush RA, Duggan AW (2001) Release of immunoreactive brain-derived neurotrophic factor in the spinal cord of the rat following sciatic nerve transection. *Brain Res*. 899: 240-247.

Walker KM, Urban L, Medhurst SJ, Patel S, Panesar M, Fox AJ, McIntyre P (2003) The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J. Pharmacol. Exp. Ther.* 2003 Jan;304(1):56-62.

Walsh GS, Krol KM, Crutcher KA, Kawaja MD (1999a) Enhanced neurotrophin-induced axon growth in myelinated portions of the CNS in mice lacking the p75 neurotrophin receptor. *J. Neurosci.* 19: 4155-4168.

Walsh GS, Krol KM, Kawaja MD (1999b) Absence of the p75 neurotrophin receptor alters the pattern of sympathosensory sprouting in the trigeminal ganglia of mice overexpressing nerve growth factor. *J. Neurosci.* 19: 258-273.

Wang XX, Chun SJ, Treloar H, Vartanian T, Greer CA, Strittmatter SM (2002) Localization of Nogo-A and Nogo-66 Receptor Proteins at Sites of Axon-Myelin and Synaptic Contact. *J. Neurosci.* 22:5505-5515

Wanigasekara Y, Keast JR (2006) Nerve growth factor, glial cell line-derived neurotrophic factor and neurturin prevent semaphorin 3A-mediated growth cone collapse in adult sensory neurons. *Neuroscience* 142:369-79.

Watanabe M, Endo Y, Kimoto K, Katoh-Semba R, Arakawa Y (2000a) Inhibition of adjuvant-induced inflammatory hyperalgesia in rats by local injection of neurotrophin-3. *Neurosci. Lett.* 282(1-2): 61-4.

Watanabe M, Endo Y, Kimoto K, Katoh-Semba R, Arakawa Y (2000b) Functional regulation of tactile sense by brain-derived neurotrophic factor in adult rats during acute inflammation. *Neuroscience* 97: 171-175.

Watson FL, Heerssen HM, Moheban DB, Lin MZ, Sauvageot CM, Bhattacharyya A, Pomeroy SL, Segal RA (1999) Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complex. *J. Neurosci.* 19: 7889-7900.

Wells MR, Vaidya U, Schwartz JP (1994) Bilateral phasic increases in dorsal root ganglia nerve growth factor synthesis after unilateral sciatic nerve crush. *Exp. Brain Res*. 101: 53-58.

Wetmore C and Olson L (1995) Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions [review]. *J. Comp. Neurol* 353: 143-159.

White FA, Silos-Santiago I, Molliver DC, Nishimura M, Phillips H, Barbacid M, Snider WD (1996) Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. *J. Neurosci.* 16: 4662-4672.

White DM (2000) Neurotrophin-3 antisense oligonucleotide attenuates nerve injury-induced Abeta-fiber sprouting. *Brain Res.* 885: 79-86.

Wilson-Gerwing TD, Dmyterko MV, Zochodne DW, Johnston JM, Verge VM (2005) Neurotrophin-3 suppresses thermal hyperalgesia associated with neuropathic pain and

attenuates transient receptor potential vanilloid receptor-1 expression in adult sensory neurons. *J. Neurosci*. 25:758-67.

Wilson-Gerwing TD, Verge VM (2006) Neurotrophin-3 attenuates galanin expression in the chronic constriction injury model of neuropathic pain. *Neuroscience* 141:2075-85.

Wood JN, Boorman JP, Okuse K, Baker MD. (2004) Voltage-gated sodium channels and pain pathways. *J. Neurobiol*. 61:55-71.

Woolf CJ and Doubell TP (1994) The pathophysiology of chronic pain--increased sensitivity to low-threshold A beta-fiber inputs. *Curr. Opin. Neurobiol*. 4: 525-534.

Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S (1997) Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. *Br. J. Pharmacol*. 121: 417-424.

Woolf CJ, Mannion RJ, Neumann S (1998) Null mutations lacking substance: elucidating pain mechanisms by genetic pharmacology [published erratum appears in Neuron 1998 21:453]. *Neuron* 20: 1063-1066.

Woolf CJ and Decosterd I (1999a) Implications of recent advances in the understanding of pain pathophysiology for the assessment of pain in patients. *Pain Suppl*. 6: S141-S147.

Woolf CJ and Mannion RJ (1999b) Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* 353: 1959-1964.

Woolf CJ and Salter MW (2000) Neuronal plasticity: increasing the gain in pain. *Science* 288: 1765-1769.

Woolf CJ, Shortland P, Coggeshall RE (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355: 75-78.

Wright DE and Snider WD (1995) Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. *J. Comp. Neurol.* 351: 329-338.

Wright CE and Angus JA (1996) Effects of N-, P- and Q-type neuronal calcium channel antagonists on mammalian peripheral neurotransmission. Br J Pharmacol 119: 49-56.

Wright DE and Snider WD (1996) Focal expression of glial cell line-derived neurotrophic factor in developing mouse limb bud. *Cell Tissue Res.* 286: 209-217.

Wright DE, Zhou L, Kucera J, Snider WD (1997) Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3. *Neuron* 19: 503-517.

Wright EM, Vogel KS, Davies AM (1992) Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron* 9: 139-150.

Xie W, Strong JA, Li H, Zhang JM (2007) Sympathetic sprouting near sensory neurons after nerve injury occurs preferentially on spontaneously active cells and is reduced by early nerve block. *J. Neurophysiol.* 97:492-502.

Xie Y, Zhang J, Petersen M, Lamotte RH (1995) Functional changes in dorsal root ganglion cells after chronic nerve constriction in the rat. *J. Neurophysiol.* 73: 1811-1820.

Xing H, Chen M, Ling J, Tan W, Gu JG (2007) TRPM8 mechanism of cold allodynia after chronic nerve injury. *J. Neurosci*. 27:13680-90.

Xu P, Hall AK (2007) Activin acts with nerve growth factor to regulate calcitonin gene-related peptide mRNA in sensory neurons. *Neuroscience* 150:665-74.

Xue Q, Jong B, Chen T, Schumacher MA. Transcription of rat TRPV1 utilizes a dual promoter system that is positively regulated by nerve growth factor. *J. Neurochem.* 2007 Apr;101(1):212-22.

Yajima Y, Narita M, Narita M, Matsumoto N, Suzuki T (2002) Involvement of a spinal brain-derived neurotrophic factor/full-length TrkB pathway in the development of nerve injury-induced thermal hyperalgesia in mice. *Brain Res.* 958:338-46.

Yajima Y, Narita M, Usui A, Kaneko C, Miyatake M, Narita M, Yamaguchi T, Tamaki H, Wachi H, Seyama Y, Suzuki T (2005) Direct evidence for the involvement of brain-derived neurotrophic factor in the development of a neuropathic pain-like state in mice. *J. Neurochem.* 93:584-94.

Yang XD, Liu Z, Liu HX, Wang LH, Ma CH, Li ZZ. (2007) Regulatory effect of nerve growth factor on release of substance P in cultured dorsal root ganglion neurons of rat. *Neurosci. Bull*. 23:215-20.

Yamamoto M, Sobue G, Yamamoto K, Terao S, Mitsuma T (1996) Expression of mRNAs for neurotrophic factors (NGF, BDNF, NT-3, and GDNF) and their receptors (p75NGFR, trkA, trkB, and trkC) in the adult human peripheral nervous system and nonneural tissues. *Neurochem. Res.* 21: 929-938.

Yamashita T, Tucker KL, Barde YA (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* 24: 585-593.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV (1998) Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J. Neurosci.* 18: 3273-3281.

Yoshikawa M, Senzaki K, Yokomizo T, Takahashi S, Ozaki S, Shiga T (2007) Runx1 selectively regulates cell fate specification and axonal projections of dorsal root ganglion neurons. *Dev. Biol.* 303:663-74.

Young KM, Merson TD, Sotthibundhu A, Coulson EJ, Bartlett PF (2007) p75 neurotrophin receptor expression defines a population of BDNF-responsive neurogenic precursor cells. J Neurosci. 27:5146-55.

Zhang JY, Luo XG, Xian CJ, Liu ZH, Zhou XF (2000) Endogenous BDNF is required for myelination and regeneration of injured sciatic nerve in rodents. *Eur. J. Neurosci.* 12: 4171-4180.

Zhang X, Huang J, McNaughton PA. 2005 NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J*. 2005 Dec 21;24(24):4211-23.

Zhang YH, Nicol GD (2004) NGF-mediated sensitization of the excitability of rat sensory neurons is prevented by a blocking antibody to the p75 neurotrophin receptor. *Neurosci. Lett.* 2004 Aug 12;366(2):187-92.

Zhao J, Seereeram A, Nassar MA, Levato A, Pezet S, Hathaway G, Morenilla-Palao C, Stirling C, Fitzgerald M, McMahon SB, Rios M, Wood JN; (2006) London Pain Consortium. Nociceptor-derived brain-derived neurotrophic factor regulates acute and inflammatory but not neuropathic pain. *Mol. Cell Neurosci.* 31:539-48.

Zhou XF and Rush RA (1995) Peripheral projections of rat primary sensory neurons immunoreactive for neurotrophin 3. *J. Comp. Neurol*. 363: 69-77.

Zhou XF and Rush RA (1996) Endogenous brain-derived neurotrophic factor is anterogradely transported in primary sensory neurons. *Neuroscience* 74: 945-951.

Zhou XF, Rush RA, McLachlan EM (1996) Differential expression of the p75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection. *J. Neurosci.* 16: 2901-2911.

Zhou XF, Deng YS, Chie E, Xue Q, Zhong JH, McLachlan EM, Rush RA, Xian CJ (1999a) Satellite-cell-derived nerve growth factor and neurotrophin-3 are involved in noradrenergic sprouting in the dorsal root ganglia following peripheral nerve injury in the rat. *Eur. J. Neurosci.* 11: 1711-1722.

Zhou XF, Chie ET, Deng YS, Zhong JH, Xue Q, Rush RA, Xian CJ (1999b) Injured primary sensory neurons switch phenotype for brain derived neurotrophic factor in the rat. *Neuroscience* 92: 841-853.

Zhou XF, Deng YS, Xian CJ, Zhong JH (2000) Neurotrophins from dorsal root ganglia trigger allodynia after spinal nerve injury in rats. *Eur. J. Neurosci.* 12: 100-105.

Zhou XF, Deng YS, Xian CJ, Zhong JH (2000) Neurotrophins from dorsal root ganglia trigger allodynia after spinal nerve injury in rats. *Eur. J. Neurosci.* 12: 100-105.

Zhou XF, Li HY (2007) Roles of glial p75NTR in axonal regeneration. *Journal of Neuroscience Research* 85:1601-1605.

Zhu W, Oxford GS (2007) Phosphoinositide-3-kinase and mitogen activated protein kinase signaling pathways mediate acute NGF sensitization of TRPV1. *Mol. Cell Neurosci.* 34: 689-700.

Zhuang ZY, Gerner P, Woolf CJ, Ji RR (2005) ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 114:149-59.

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Chapter 2

Nerve Growth Factor in Animal Models of Atopic Dermatitis

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Abstract

Itching is a characteristic symptom of various dermatoses, especially atopic dermatitis. The most effective strategy for treatment of atopic dermatitis would be to prevent aggravation of skin lesions and improve quality of life by reducing itching and scratching. Nerve growth factor (NGF) is an important substance in the skin, where it plays roles in nerve maintenance and repair. However, the nature of involvement of NGF in pruritic diseases such as atopic dermatitis is not yet fully understood. We used the NC/Nga mouse, an animal model of atopic dermatitis, to test the hypothesis that NGF plays important roles in the pathogenesis, development, and maintenance of the skin lesions of this condition. In these mice, nerve fibers were significantly increased in number in the epidermis of lesional skin, and NGF contents in serum and skin were significantly elevated. Furthermore, repeated administration of anti-NGF antibody or high-affinity NGF receptor inhibitors significantly improved established dermatitis and scratching behavior and decreased innervation of the epidermis. Our findings suggest that NGF plays important roles in the pathogenesis of atopic dermatitis-like skin lesions, particularly via the high-affinity NGF receptor, and that inhibition of the physiological effects of NGF or suppression of increase in NGF production may prevent or moderate the symptoms of several pruritic skin diseases.

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Introduction

Itching is a characteristic symptom of various dermatoses, especially atopic dermatitis, and is a major diagnostic criterion [1, 2]. The itch-scratch cycle – in which strong scratching increases susceptibility to itching and aggravation of skin lesions in patients with atopic dermatitis – is a well-known phenomenon [3, 4]. The most effective strategy for the treatment of atopic dermatitis would be to prevent this aggravation of skin lesions and thereby improve the quality of life for patients [5].

Cutaneous nerve fibers are present at higher densities in the epidermis of skin affected by atopic dermatitis [6, 7]. This increase in nerve fibers is partly responsible for the intense itching sensation in the lesional skin of patients with atopic dermatitis. It is now generally accepted that nerve growth factor (NGF), which is released from keratinocytes in the skin, is one of the major determinants of skin innervation density [8-10]. NGF is an important substance in the skin environment, where it plays roles in nerve maintenance and repair [11, 12]. However, the nature of the relationship between NGF and pruritic conditions such as atopic dermatitis is still not fully understood. A recent study reported that NGF is overexpressed in prurigo nodularis, suggesting the possibility that NGF and its receptors contribute to the neurohyperplasia seen in this disease [13]. NGF protein levels are higher in psoriatic skin than in non-lesional and normal skin [14], and psoriatic keratinocytes express larger amounts of NGF than normal keratinocytes [15]. Patients with atopic dermatitis also exhibited significantly higher NGF plasma levels than controls, and a strong correlation was noted between plasma NGF and severity of symptoms [16]. These findings suggest that NGF may play an important role in the pathogenesis of atopic dermatitis, possibly via regulation of the development of atopic dermatitis lesions. Few studies have addressed the roles of NGF in the pathogenesis, development, and maintenance of atopic dermatitis in vivo. We used an animal model to test the hypothesis that NGF plays important roles in the pathogenesis, development, and maintenance of the skin lesions associated with atopic dermatitis [17, 18].

The Nc/Nga Mouse,a Model of Atopic Dermatitis

NC/Nga mice were originally established as an inbred strain of Japanese fancy mice (Figure 1). Under conventional conditions, NC/Nga mice develop spontaneous skin lesions with characteristics including high concentrations of total immunoglobulin E in plasma and invasion of inflammatory cells into these skin lesions [19, 20]. NC/Nga mice with severe skin lesions frequently scratch their face, ears, and rostral portion of the back with their hind paws [21]. Since these traits are similar to those observed in patients with atopic dermatitis, NC/Nga mice are considered a suitable model of human atopic dermatitis.

Nerve fiber density in skin in NC/Nga mice was compared in regions with severe dermatitis (lesional skin) and regions without dermatitis (non-lesional skin). The rostral skin of the back of mice was extracted and fixed in formalin solution, and then cut perpendicular to the dermal-epidermal surface. The skin sections were incubated with polyclonal rabbit antibodies to PGP9.5, and were then incubated with fluorescent antibodies. Sections were observed by conventional fluorescence microscopy.

Figure 1. A photograph of NC/Nga mice.

The area of immunoreactive nerve fibers in the epidermis (fluorescently stained area) was quantified into ten randomly selected fields per mouse using imaging software. In non-lesional skin, nerve fibers were observed in the dermis, but relatively few were observed in the epidermis (Figure 2A). In lesional skin, nerve fibers were observed at many sites in the epidermis, and distinct acanthosis and hyperkeratosis were noted (Figure 2B). Nerve fiber counts in the epidermis of lesional skin were increased significantly compared to those in non-lesional skin. In addition, serum and skin NGF contents were measured by enzyme-linked immunosorbent assays. Mouse blood was centrifuged to acquire serum for the NGF assay. The rostral back skin was homogenized and centrifuged, and the extracted supernatant was assayed for NGF. Compared to NC/Nga mice without lesions, serum NGF content was significantly higher in NC/Nga mice with lesions [17]. The NGF content of rostral back skin was also higher in NC/Nga mice with lesions [17]. Following immunohistochemical staining, lesional skin exhibited distinct acanthosis and increased levels of NGF in keratinocytes compared to non-lesional skin [17].

NC/Nga mice with skin lesions frequently scratch affected regions using their hind paws [21], a behavior believed to be elicited by cutaneous itching sensation [22]. In our study, PGP9.5 positive nerve fibers were significantly increased in the epidermis of lesional compared with non-lesional skin. These findings appear similar to those observed in humans with atopic dermatitis. Moreover, serum NGF content significantly higher in NC/Nga mice with lesions in NC/Nga mice without lesions, as reported for humans as well [16]. Enzyme-linked immunosorbent assay and immunohistochemical studies have revealed higher NGF levels in lesional than in normal skin. In other studies, comparable results were obtained using NC/Nga mice [23-25]. NGF was initially identified as the main neurotrophic factor controlling the survival, development, differentiation, and function of sympathetic and sensory neurons [26].

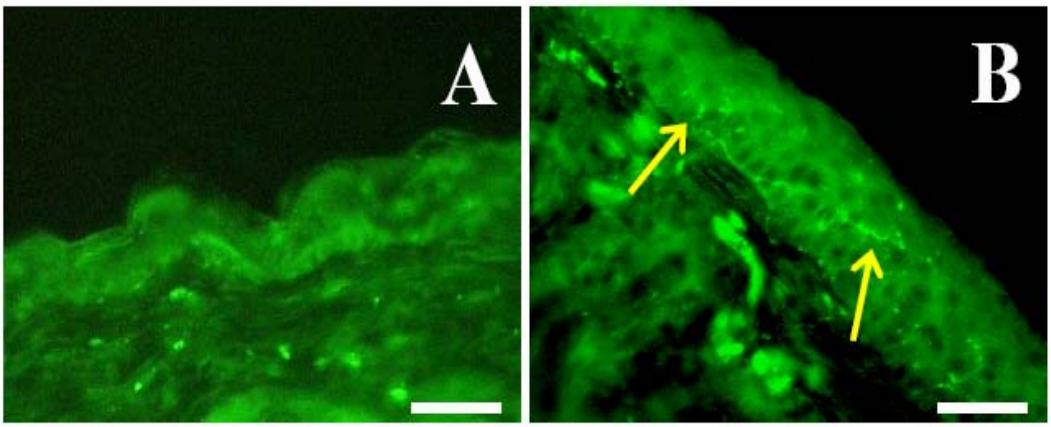

Figure 2. Immunofluorescence of PGP9.5 in skin of NC/Nga mice. A: Non-lesional skin of NC/Nga mice, B: Lesional skin of NC/Nga mice. Arrows indicate immunoreactive nerve fibers. Scale bar: 50 μm [17].

These findings suggest that overproduction of NGF increases innervation of the epidermis, intensifies itching sensation, and increases scratching behavior, thereby contributing to the maintenance of skin lesions.

Effects of Anti-Ngf Antibody

To determine whether NGF plays an important role in the pathogenesis, development, and maintenance of atopic dermatitis-like skin lesions, we investigated the effects of anti-NGF antibodies on signs of dermatitis in vivo. Anti-NGF or control (rabbit polyclonal IgG) antibodies were administered to NC/Nga mice with lesional skin intraperitoneally twice a week for 4 weeks. Dermatitis scores for three parts of the body (face, ear, and rostral back) were assessed once a week for 4 weeks using the following criteria: no lesion, 0; minor hair loss or wound without bleeding, 1; bleeding wounds in parts, 2; broad regions of serious wounds, 3. In mice administered the control antibody, dermatitis scores remained relatively constant during the study. In mice administered the anti-NGF antibody, dermatitis scores improved significantly from week 2, compared to pre-administration, and at week 4 were comparable to those in a group administered control antibody (Figure 3A). The scratching behavior of NC/Nga mice was measured using the MicroAct device (Neuroscience, Tokyo, Japan), which automatically detects and evaluates the scratching behavior of mice [27, 28]. Repeated administration of anti-NGF antibody also decreased scratching behavior, though not to a statistically significant extent (statistical comparisons were made with Student's unpaired *t*-test, P=0.054 vs. control, Figure 3B). Fluorescence staining of the rostral skin of the back was performed as described above. In the case of control antibody, nerve fibers were observed in many sites of the epidermis, and distinct acanthosis and hyperkeratosis were noted (Figure 3C). On the other hand, few nerve fibers were observed in the epidermis and acanthosis and hyperkeratosis were markedly improved in the mice administered anti-NGF antibody (Figure 3D).

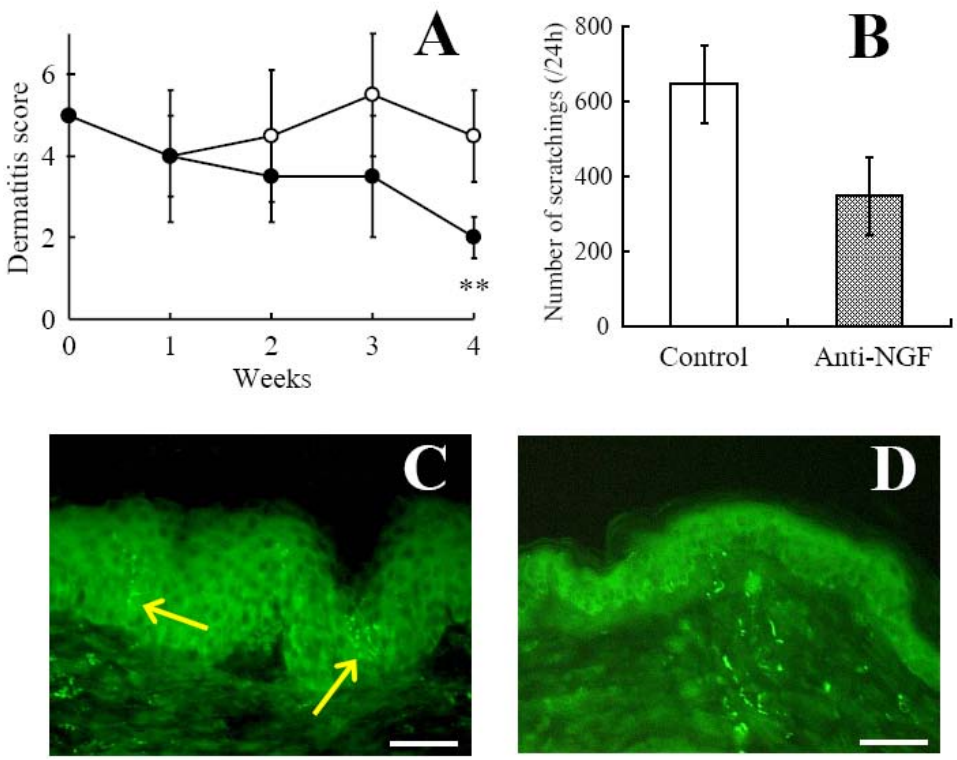

Figure 3. Effects of anti-NGF antibody on signs of established dermatitis in NC/Nga mice. A: Dermatitis scores. ○: control antibody, ●: anti-NGF antibody. Values are the median and quartile deviation for eight mice. **P<0.01. B: Number of scratching behaviors. Values are the mean and S.E.M. for eight mice. C, D: PGP9.5 immunoreactivity of skin of NC/Nga mice treated with control (C) or anti-NGF antibody (D). Arrows indicate immunoreactive nerve fibers. Scale bar: 50 μm [17].

The number of nerve fibers in the epidermis declined significantly with repeated administration of anti-NGF antibody. Furthermore, repeated administration of anti-NGF antibody also significantly inhibited the development of skin lesions, epidermal innervation, and scratching behavior [17]. On the other hand, in neither experiment did administration of anti-NGF antibody altered either serum NGF concentrations or NGF content in the rostral skin of the back.

Antibody to 2.5 S NGF can block the effects of NGF in vitro [29] and in vivo [30]. In the skin, NGF synthesized and released by keratinocytes [31-33] plays important roles in the survival and development of the peripheral nervous system [12]. Our findings suggest that anti-NGF antibody blocks the effects of NGF on the peripheral nervous system and suppresses epidermal innervation, dermatitis, and scratching behavior. There is also growing evidence that NGF has biological effects on immune cells, such as mast cells [34], B cells [35], T cells [36], neutrophils [37], eosinophils [38], and basophils [39]. Anti-NGF antibody may suppress signs of dermatitis in NC/Nga mice by blocking responses of these immune cells.

Our findings indicate that repeated administration of anti-NGF antibody may help heal skin lesions and decrease innervation of the epidermis and scratching behavior in the NC/Nga

mice, an animal model atopic dermatitis, and in turn suggest that NGF plays important roles in the pathogenesis, development, and maintenance of atopic dermatitis-like skin lesions.

Effects of Ngf Receptor Inhibitors

NGF exhibits effects by binding to two classes of transmembrane receptors, a high affinity receptor (tropomyosin-related kinase A, TrkA), for which NGF is the primary ligand, and the non-specific neurotrophin receptor p75, which binds all known neurotrophins, including NGF, brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 4/5 with low affinity [40-42]. The binding of NGF to the TrkA receptor is responsible for the transmission of NGF signaling related to mitogenesis and differentiation [43, 44]. Although the roles of p75 in these processes are less clear, it may function as an accessory protein that modifies ligand-binding affinity [45], and recent findings have suggested that it plays a role in the apoptotic cell death cascade [46-48]. The roles played by the two types of NGF receptors in the pathogenesis of atopic dermatitis remain unclear. To determine the roles played by NGF receptors in the pathogenesis of atopic dermatitis, we evaluated the efficts of an alkaloid-like material isolated from the culture broth of *Nacardiopsis sp.* (K252a) and 3,5-Di-tert-butyl-4-hydroxybenzaldehyde (AG879), which are high affinity NGF receptor inhibitor [49-52], in NC/Nga mice.

K252a or AG879 was applied to the rostral part of the back of NC/Nga mice with skin skin lesions five times a week for 4 weeks. Dermatitis score for the rostral back, based on the severity of erythema/hemorrhage and psilosis (0, no lesions; 1, light; 2, moderate; 3, severe; minimum 0, maximum 6) was assessed once a week. The number of scratching behavior movements directed at the rostral back was measured for 30 min as an index of itching sensation. In the K252a group, dermatitis scores improved significantly (Figure 4A), and scratching behavior decreased (Figure 4B). Furthermore, immunofluorescence examination for nerve fibers, NGF, and TrkA receptors was performed in the rostral skin of the back. In the mice treated with vehicle, nerve fibers were observed at many sites in the epidermis, and epidermal thickening was noted (Figure 4C). On the other hand, few nerve fibers were observed in the epidermis of mice treated with K252a (Figure 4D). NGF immunoreactivity was strongly observed in keratinocytes and mast cells of mice treated with vehicle, and was observed in mast cells but only weakly in keratinocytes of mice treated with K252a. High expression of TrkA was observed in the stratum germinativum of the epidermis of mice treated with vehicle (Figure 4E) compared with mice treated with K252a (Figure 4F). Each finding in the K252a group was also observed in the group treated with AG879 [18].

As noted above, NGF mediates its effects by binding to two classes of transmembrane receptors, TrkA and p75, which are expressed not only in neurons but also in keratinocytes [32, 53]. AG879 and K252a are protein kinase inhibitors. In this study, repeated application of AG879 or K252a significantly improved established dermatitis, scratching behavior, and innervation of the epidermis in NC/Nga mice compared to those in the group treated with vehicle. These results were similar to those obtained with NGF-neutralizing antibody. These findings strongly suggest that TrkA mediates the effects of NGF in the pathogenesis of the atopic dermatitis-like symptoms in NC/Nga mice.

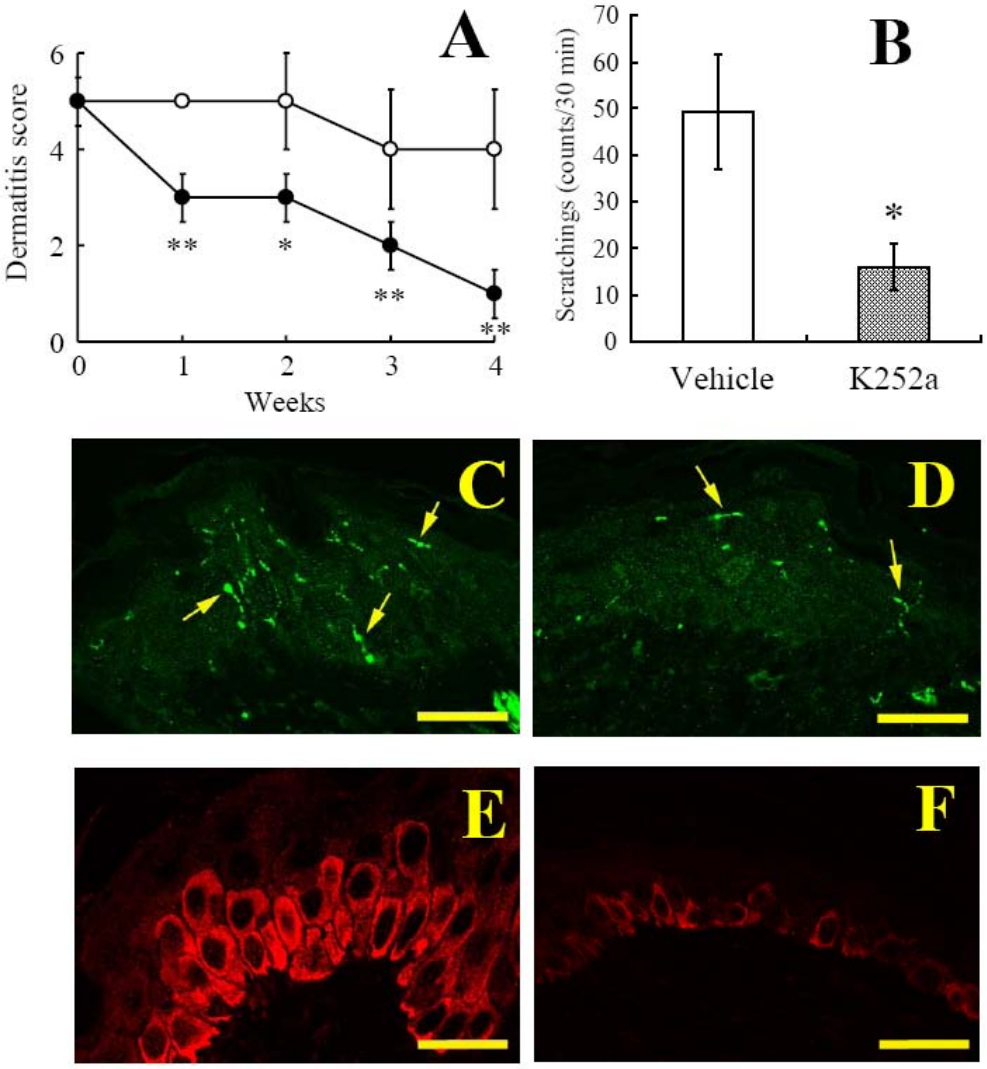

Figure 4. Effects of K252a on signs of established dermatitis in NC/Nga mice. A: Dermatitis scores. ○: vehicle, ●: K252a. Values are the median and quartile deviation for eight mice. B: Number of scratching behaviors. Values are the mean and S.E.M. for eight mice. *P<0.05, **P<0.01. C, D: PGP9.5 immunoreactivity of skin of NC/Nga mice treated with vehicle (C) or K252a (D). Arrows indicate immunoreactive nerve fibers. Scale bar: 25 μm. E, F: Immunofluorescence for tropomyosin-related kinase A (TrkA) in the skin of NC/Nga mice treated with vehicle (E) or K252a (F) [18].

NGF stimulates TrkA phosphorylation in human keratinocytes, and functions as a survival factor for keratinocytes through TrkA [32, 54, 55]. Although levels of p75 mRNA and protein in human keratinocytes increase during the exponential growth phase [53], K252a, but not anti-p75, inhibits NGF-induced keratinocyte proliferation [32], strongly suggesting that TrkA is the functional NGF receptor in neurons and keratinocytes. NGF is released from keratinocytes in the skin. Thus far, no studies have determined NGF receptor expression in the skin in atopic dermatitis. In NC/Nga mice, expression of TrkA was higher in the epidermis of lesional than in non-lesional skin. Expression of TrkA in lesional skin was

declined to the same level as that in non-lesional skin with repeated application of AG879 or K252a, as also observed for NGF levels. As noted above, NGF is released from keratinocytes in the skin, and TrkA is expressed in keratinocytes. In addition, TrkA inhibitor can induce apoptosis in keratinocytes [56]. There is some concern that the apoptosis induced by TrkA inhibitors may result in lower NGF levels and TrkA expression in the epidermis. However, no significant difference was found in expression of p75 between lesional and non-lesional skin, and no change in expression of p75 was observed following administration of AG879 or K252a in our study (data not shown). Studies of the effects of p75 have indicated that this it is a TrkA coreceptor able to enhance or suppress neurotrophin-mediated TrkA activity, and that it autonomously activates signaling cascades that result in the induction of apoptosis or in the promotion of survival [57-60]. However, there is a report that p75 plays an important role in the induction of innervation in inflamed skin [61]. Further study is needed to determine how the effects of NGF are mediated via p75 in the pathogenesis of atopic dermatitis.

Our findings demonstrated that repeated application of TrkA inhibitors can suppress skin lesions, scratching behavior, and innervation of the epidermis in the NC/Nga mouse, an animal model of atopic dermatitis, and suggest that NGF plays important roles in the pathogenesis of atopic dermatitis-like skin lesions via the high affinity NGF receptor.

Conclusion

Our findings suggest that NGF plays important roles in the pathogenesis of atopic dermatitis-like skin lesions, particularly via the high affinity NGF receptor, and that inhibition of the physiological effects of NGF or suppression of increased NGF production may prevent or moderate the symptoms of atopic dermatitis. These findings suggest a potential approach to amelioration of the symptoms of several pruritic skin diseases, including not only atopic dermatitis but also dry skin and psoriasis, which also features increase in innervation of the epidermis.

References

[1] Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm. Venereo*l., 1980, 92, 44-47.

[2] Williams HC, Burney PGJ, Perbroke AC, Hay RJ. The U. K. working party's diagnostic criteria for atopic dermatitis, III. Independent hospital validation. *Br. J. Dermatol.*, 1994, 131, 406-416.

[3] Kimura T, Miyazawa H. The 'butterfly' sign in patients with atopic dermatitis: evidence for the role of scratching in the development of skin manifestations. *J. Am. Acad. Dermatol.*, 1989, 21, 579-580.

[4] Wahlgren CF. Itch and atopic dermatitis: an overview. *J. Dermatol.*, 1999, 26, 770-779.

[5] Koblenzer CS. Itching and the atopic skin. *J. Allergy Clin. Immunol.*, 1999, 104, S109-113.

[6] Tobin D, Nabarro G, de la Faille HB, van Vloten WA, van der Putte SC, Schuurman HJ. Increased number of immunoreactive nerve fibers in atopic dermatitis. *J. Allergy Clin. Immunol.*, 1992, 90, 613-622.

[7] Urashima R, Mihara M. Cutaneous nerves in atopic dermatitis. *Virchows Arch.*, 1998, 432, 363-370.

[8] Albers KM, Wright DE, Davis BM. Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. *J. Neurosci.*, 1994, 14, 1422-1432.

[9] Leslie TA, Emson PC, Dowd PM, Woolf CJ. Nerve growth factor contributes to the up-regulation of growth-associated protein 43 and preprotachykinin A messenger RNAs in primary sensory neurons following peripheral inflammation. *Neuroscience*, 1995, 67, 753-761.

[10] Bennett DL, Koltzenburg M, Priestley JV, Shelton DL, McMahon SB. Endogenous nerve growth factor regulates the sensitivity of nociceptors in the adult rat. *Eur. J. Neurosci.*, 1998, 10, 1282-1291.

[11] Ansel JC, Kaynard AH, Armstrong CA, Olerud J, Bunnett N, Payan D. Skin-nervous system interactions. *J. Invest Dermatol*., 1996, 106, 198-204.

[12] Pincelli C, Yaar M. Nerve growth factor: its significance in cutaneous biology. *J. Invest. Dermatol .Symp. Proc.*, 1997, 2, 31-36.

[13] Johansson O, Liang Y, Emtestam L. Increased nerve growth factor- and tyrosine kinase A-like immunoreactivities in prurigo nodularis skin - an exploration of the cause of neurohyperplasia. *Arch. Dermatol .Res.*, 2002, 293, 614-619.

[14] Fantini F, Magnoni C, Bracci-Laudiero L, Pincelli C. Nerve growth factor is increased in psoriatic skin. *J. Invest. Dermatol*., 1995, 105, 854-855.

[15] Raychaudhuri SP, Jiang WY, Farber EM. Psoriatic keratinocytes express high levels of nerve growth factor. *Acta Derm .Venereol.*, 1998, 78, 84-86.

[16] Toyoda M, Nakamura M, Makino T et al. Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br. J. Dermatol.*, 2002, 147, 71-79.

[17] Takano N, Sakurai T, Kurachi M. Effects of anti-nerve growth factor antibody on symptoms in the NC/Nga mouse, an atopic dermatitis model. *J. Pharmacol. Sci.*, 2005, 99, 277-286.

[18] Takano N, Sakurai T, Ohashi Y, Kurachi M. Effects of high-affinity nerve growth factor receptor inhibitors on symptoms in the NC/Nga mouse atopic dermatitis model. *Br. J. Dermatol.*, 2007, 156, 241-246.

[19] Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase PW, Ra C. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol*, 1997, 9, 461-466.

[20] Suto H, Matsuda H, Mitsuishi K, Hira K, Uchida T, Unno T, Ogawa H, Ra C. NC/Nga mice: a mouse model for atopic dermatitis. *Int. Arch. Allergy Immunol.*, 1999, 120, 70-75.

[21] Tohda C, Yamaguchi T, Kuraishi Y. Increased expression of mRNA for myocyte-specific enhancer binding factor (MEF) 2C in the cerebral cortex of the itching mouse. *Neurosci .Res.* 1997, 29, 209-215.

[22] Kuraishi Y, Nagasawa T, Hayashi K, Satoh M. Scratching behavior induced by pruritogenic but not algesiogenic agents in mice. *Eur. J. Pharmacol.*, 1995, 275, 229-233.

[23] Horiuchi Y, Bae S, Katayama I. Nerve growth factor (NGF) and epidermal nerve fibers in atopic dermatitis model NC/Nga mice. *J. Dermatol. Sci.*, 2005, 39, 56-58.

[24] Tanaka A, Matsuda H. Expression of nerve growth factor in itchy skins of atopic NC/NgaTnd mice. *J. Vet. Med .Sci.*, 2005, 67, 915-919.

[25] Tominaga M, Ozawa S, Ogawa H, Takamori K. A hypothetical mechanism of intraepidermal neurite formation in NC/Nga mice with atopic dermatitis. *J. Dermatol. Sci*., 2007, 46, 199-210.

[26] Levi-Montalcini R. The nerve growth factor: thirty-five years later. *Eur. Mol. Biol. Org. J.*, 1987, 6, 1145-1154.

[27] Takano N, Arai I, Kurachi M. Analysis of the spontaneous scratching behavior by NC/Nga mice: a possible approach to evaluate antipruritics for subjects with atopic dermatitis. *Eur. J. Pharmacol.*, 2003, 471, 223-228.

[28] Takano N, Arai I, Hashimoto Y, Kurachi M. Evaluation of antipruritic effects of several agants on scratching behavior by NC/Nga mice. *Eur .J. Pharmacol.*, 2004, 495, 159-165.

[29] Varilek GW, Neil GA, Bishop WP, Lin J, Pantazis NJ. Nerve growth factor synthesis by intestinal epithelial cells. *Am. J. Physiol.*, 1995, 269, G445-452.

[30] De Vries A, Engels F, Henricks AJ, Leusink-Muis T, Fischer A, Nijkamp FP. Antibodies directed against nerve growth factor inhibit the acute bronchoconstriction due to allergen challenge in guinea-pigs. *Clin. Exp. Allergy*, 2002, 32, 325-328.

[31] Di Marco E, Marchisio PC, Bondanza S, Franzi AT, Cancedda R, De Luca M. Growth-regulated synthesis and secretion of biologically active nerve growth factor by human keratinocytes. *J. Biol. Chem.*, 1991, 266, 21718-21722.

[32] Pincelli C, Sevignani C, Manfredini R, Grande A, Fantini F, Bracci-Laudiero L, Aloe L, Ferrari S, Cossarizza A, Giannetti A. Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes. *J. Invest. Dermatol.*, 1994, 103, 13-18.

[33] Burbach GJ, Kim KH, Zivony AS, Kim A, Aranda J, Wright S, Naik SM, Caughman SW, Ansel JC, Armstrong CA. The neurosensory tachykinins substance P and neurokinin A directly induce keratinocyte nerve growth factor. *J. Invest. Dermatol.*, 2001, 117, 1075-1082.

[34] Aloe L, Levi-Montalcini R. Mast cells increase in tissues of neonatal rats injected with the nerve growth factor. *Brain Res.*, 1977, 133, 358-366.

[35] Torcia M, Bracci-Laudiero L, Lucibello M, Nencioni L, Labardi D, Rubartelli A, Cozzolino F, Aloe L, Garaci E. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell*, 1996, 85, 345-356.

[36] Brodie C, Oshiba A, Renz H, Bradley K, Gelfand EW. Nerve growth factor and anti-CD40 provide opposite signals for the production of IgE in interleukin-4-treated lymphocytes. *Eur. J. Immunol.*, 1996, 26, 171-178.

[37] Kannan Y, Ushio H, Koyama H, Okada M, Oikawa M, Yoshihara T, Kaneko M, Matsuda H. 2.5S nerve growth factor enhances survival, phagocytosis, and superoxide production of murine neutrophils. *Blood*, 1991, 77, 1320-1325.

[38] Hamada A, Watanabe N, Ohtomo H, Matsuda H. Nerve growth factor enhances survival and cytotoxic activity of human eosinophils. *Br. J. Haematol.*, 1996, 93, 299-302.

[39] Bischoff SC, Dahinden CA. Effect of nerve growth factor on the release of inflammatory mediators by mature human basophils. *Blood*, 1992, 79, 2662-2669.

[40] Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M. Expression and structure of the human NGF receptor. *Cell*, 1986, 47, 545-554.

[41] Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science*, 1991, 252. 554-558.

[42] Hempstead BL. The many faces of p75NTR. *Curr. Opin. Neurobiol.*, 2002, 12, 260-267.

[43] Loeb DM, Maragos J, Martin-Zanca D et al. The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. *Cell* 1991; **66**: 961-6.

[44] Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, Kaplan DR. Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron*, 1992, 9, 883-886.

[45] Schechter AL, Bothwell MA. Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. *Cell*, 1981, 24, 867-874.

[46] Barrett GL. The p75 neurotrophin receptor and neuronal apoptosis. Prog Neurobiol 2000, 61, 205-229.

[47] Hirata H, Hibasami H, Yoshida T, Ogawa M, Matsumoto M, Morita A, Uchida A. Nerve growth factor signaling of p75 induces differentiation and ceramide-mediated apoptosis in Schwann cells cultured from degenerating nerves. *Glia*, 2001, 36, 245-258.

[48] Miller FD, Kaplan DR. Neurotrophin signaling pathways regulating neuronal apoptosis. *Cell. Mol. Life Sci.*, 2001, 58, 1045-1053.

[49] Koizumi S, Contreras ML, Matsuda Y, Hama T, Lazarovici P, Guroff G. K252a: A specific inhibitor of the action of nerve growth factor on PC 12 cells. *J. Neurosci.*, 1988, 8, 715-721.

[50] Lazarovici P, Levi BZ, Lelkes PI, Koizumi S, Fujita K, Matsuda Y, Ozato K, Guroff G. K-252a inhibits the increase in c-fos transcription and the increase in intracellular calcium produced by nerve growth factor in PC12 cells. *J. Neurosci Res.*, 1989, 23, 1-8.

[51] Berg MM, Sternberg DW, Parada LF, Chao MV. K252a inhibits nerve growth factor induced trk proto-oncogene tyrosine phosphorylation and kinase activity. *J. Biol. Chem.*, 1992, 267, 13-16.

[52] Ohmichi M, Pang L, Ribon V, Gazit A, Levitzki A, Saltiel AR. The tyrosine kinase inhibitor tyrphostin blocks the cellular actions of nerve growth factor. *Biochem*, 1993, 32, 4650-4658.

[53] Di Marco E, Mathor M, Bondanza S, Cutuli N, Marchisio PC, Cancedda R, De Luca M. Nerve growth factor binds to normal human keratinocytes through high and low

affinity receptors and stimulates their growth by a novel autocrine loop. *J. Biol. Chem.*, 1993, 268, 22838-22846.

[54] Kaplan DR, Miller FD. Signal transduction by the neurotrophin receptors. *Curr. Opin. Cell Biol.*, 1997, 9, 213-221.

[55] Pincelli C, Haake AR, Benassi L, Grassilli E, Magnoni C, Ottani D, Polakowska R, Franceschi C, Giannetti A. Autocrine nerve growth factor protects human keratinocytes from apoptosis through its high affinity receptor (TRK): a role for BCL-2. *J. Invest. Dermatol.*, 1997, 109, 757-764.

[56] Pincelli C, Marconi A. Autocrine nerve growth factor in human keratinocytes. *J. Dermatol. Sci*., 2000, 22: 71-79.

[57] Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature*, 1991, 350, 678-683.

[58] Ross AH, Daou MC, McKinnon CA, Condon PJ, Lachyankar MB, Stephens RM, Kaplan DR, Wolf DE. The neurotrophin receptor, gp75, forms a complex with the receptor tyrosine kinase TrkA. *J. Cell Biol.*, 1996, 132, 945-953.

[59] Maliartchouk S, Saragovi HU. Optimal nerve growth factor trophic signals mediated by synergy of TrkA and p75 receptor-specific ligands. *J. Neurosci.*, 1997, 17, 6031-6037.

[60] Majdan M, Walsh GS, Aloyz R, Miller FD. TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal. *J. Cell Biol.*, 2001, 155, 1275-1285.

[61] Taniguchi M, Matsuzaki S, Tohyama M. P75 plays a key role in the induction of the sprouting of sensory nerve fibers in inflamed skin. *J. Invest. Dermatol.*, 2007, 127, 2062-2065.

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Chapter 3

The Chemokine Stromal Cell-Derived Factor-1 (SDF-1) Promotes PC12 Cell Differentiation

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Abstract

Chemokines and their receptors are essential for the development and organization of the hematopoietic/lymphopoietic system. A member of the CXC chemokine subfamily, stromal cell-derived factor-1 (SDF-1) and its unique receptor CXCR4 have been shown to be expressed in embryonic and mature central nervous system (CNS). The present data show that SDF-1 causes the morphological and molecular differentiation of PC12 cells in a manner similar to nerve growth factor (NGF). PC12 cells are shown to express CXCR4. Neurite outgrowth stimulated by SDF-1 was attenuated by small interfering RNA-mediated knockdown of CXCR4 and pertussis toxin (PTX), which uncouples Gi protein. Comparison of extracellular signal-regulated kinase signaling pathways between SDF-1and NGF shows that these pathways are crucial for SDF-1 action as well as NGF. CXCR4 mRNA is up-regulated by neuron in the rat facial nucleus following axotomy. Recently, several studies have demonstrated that SDF-1 and its receptor CXCR4 play an important role in the CNS development and adulthood by mediating cell migration, enhancing precursor cell proliferation. In addition to these functions, SDF-1/CXCR4 signaling may be required for neurite outgrowth during nerve regeneration.

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Introduction

Neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) are critical for the development, differentiation and maintenance of distinct populations of neurons [1]. In the human brain, there may be about 85 billion neurons that form sophisticated neuronal networks [1]. Compared with a large number of neurons and their networks, it is thought that a small number of neurotrophic factors are known until now.

The PC12 cell line was derived from a pheochromocytoma tumor of the rat adrenal medulla [2], and it has been used as a model system for neuronal differentiation and death. PC12 cells respond to NGF by extension of neurites and the acquisition of sympathetic neuronal phenotype [2]. PC12 cells undergo apoptosis when cultured in serum free medium without NGF. However, supplementation of serum-free medium with NGF promotes the survival of PC12 cells [3]. In order to obtain novel neurotrohic factors like NGF, the effect of over 200 small secreted proteins, such as cytokines, chemokines, and bioactive peptides on PC12 cells was investigated. The study revealed that one of the CXC chemokines, stromal cell-derived factor-1 (SDF-1) promotes the neuronal differentiation of PC12 cells. SDF-1 is thought to act exclusively through its receptor CXCR4 [4]. The present data show that CXCR4 is expressed on PC12 cells, and up-regulated by neuron in the rat facial nucleus following axotomy. SDF-1/CXCR4 signaling might be participating in neurite outgrowth during nerve regeneration.

Materials and Methods

Materials - PC12 cell line was purchased from ATCC. Antibodies for phospho-ERK1/2, ERK1/2, were from Santa Cruz Biotechnology. Anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase were purchased from Amersham, all human chemokines (SDF-1, IP-10), PD98059 from Sigma, and mouse NGF (2.5S) from Promega.

Western Blot Analysis - Cells were lysed in lysis buffer [25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$ mM EDTA, 5 mM NaF, 1 mM vanadate, 1% Nonidet P-40, EDTA-free CompleteTM protease inhibitor mixture (Roche Molecular Biochemicals)]. Protein extracts transferred to nitrocellulose membranes were incubated with primary antibodies followed by horseradish peroxidase-coupled secondary antibodies. Signals were detected by the ECL PLUS system (Amersham) using Hyperfilm (Amersham) for exposure.

Determination of Neurite Outgrowth - PC12 cells were maintained at 37^{o}C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 5% heat-inactivated horse serum (Invitrogen). To measure neurite outgrowth, 2×10^4 cells were seeded in each well of a 24-well plate that had been coated with mouse collagen type IV (BD Biosciences) in DMEM containing 1% heat-inactivated fetal bovine serum and 0.5% heat-inactivated horse serum with or without ligand (SDF-1, NGF) as indicated. After incubation for 24 h, for quantification of neurite outgrowth, random photographs were taken (3 or 4 per well) and

process-bearing neurites were counted, scoring as possible processes those of length greater than the cell's diameter. Three independent experiments were performed in duplicate for each data point presented. Neurites were identified and counted from approximately 100 cells per photograph.

Flow cytometry – Cells were stained with specific monoclonal antibody against CXCR4 (rabbit anti-CXCR4, Abcam), followed by secondary anti-rabbit Alexa 488-labeled secondary antibody (Molecular Probes). The samples were analyzed by flow cytometry (Epics XL, Beckman Courter).

Animals and surgical procedures- Forty adult male Sprague–Dawley rats (six- to eight-weeks-old) were anaesthetized with sodium pentobarbital, and the left facial nerve was transected at the level of the stylomastoid foramen. On day 7 after axotomy, rats were killed under pentobarbital or ether anaesthesia, and brains were removed immediately. Using a 15-gauge stainless-steel bore tubing, the left and right facial nuclei were punched out of the brainstem.

Quantitative RT-PCR- cDNA synthesis was carried out using first strand cDNA synthesis kit (Invitrogen) according to the recommendations of the manufacture. In a typical experiment, each cDNA sample was submitted to 2 PCR amplifications: one for the normalizing glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene and the other for rat CXCR4, each in triplicate. Real-Time PCR analysis was performed on the 7500 Fast Real Time PCR System with TaqMan Universal PCR Master Mix (Applied Biosystems).

Results

SDF-1 Increases Neurite Extension in PC12 Cells

SDF-1 -treatment of PC12 cells induced their differentiation, as shown clearly by the appearance of neurite outgrowth within 24 h (Figure 1A). In contrast, interferon-inducible protein 10 (IP-10) that belongs to the CXC chemokine (non-ELR motif) subfamily did not stimulate neurite extension in PC12 cells (Figure 1A). SDF-1 promoted the extension in PC12 cells in a dose-dependent manner according to quantitative analysis of neurite extension (Figure 1B). Substantial neurite outgrowth was observed at 50 ng/ml SDF-1 or higher concentrations. With 100 ng/ml SDF-1, neurite outgrowth was about the same level as that by 100 ng/ml NGF.

Neurite Outgrowth Action of SDF-1 is Mediated by ERK Signal Transduction

NGF-induced sustained activation of extracellular signal-regulated kinase (ERK) pathway has been suggested to be crucial to the neuronal differentiation of PC12 cells (5). This led us to investigate whether SDF-1 induces neurite outgrowth via sustained activation of the ERK pathway. ERK kinase inhibitor PD98059 markedly inhibited neurite outgrowth activity of NGF in a dose-dependent manner, supporting previous findings (Figure 2A).

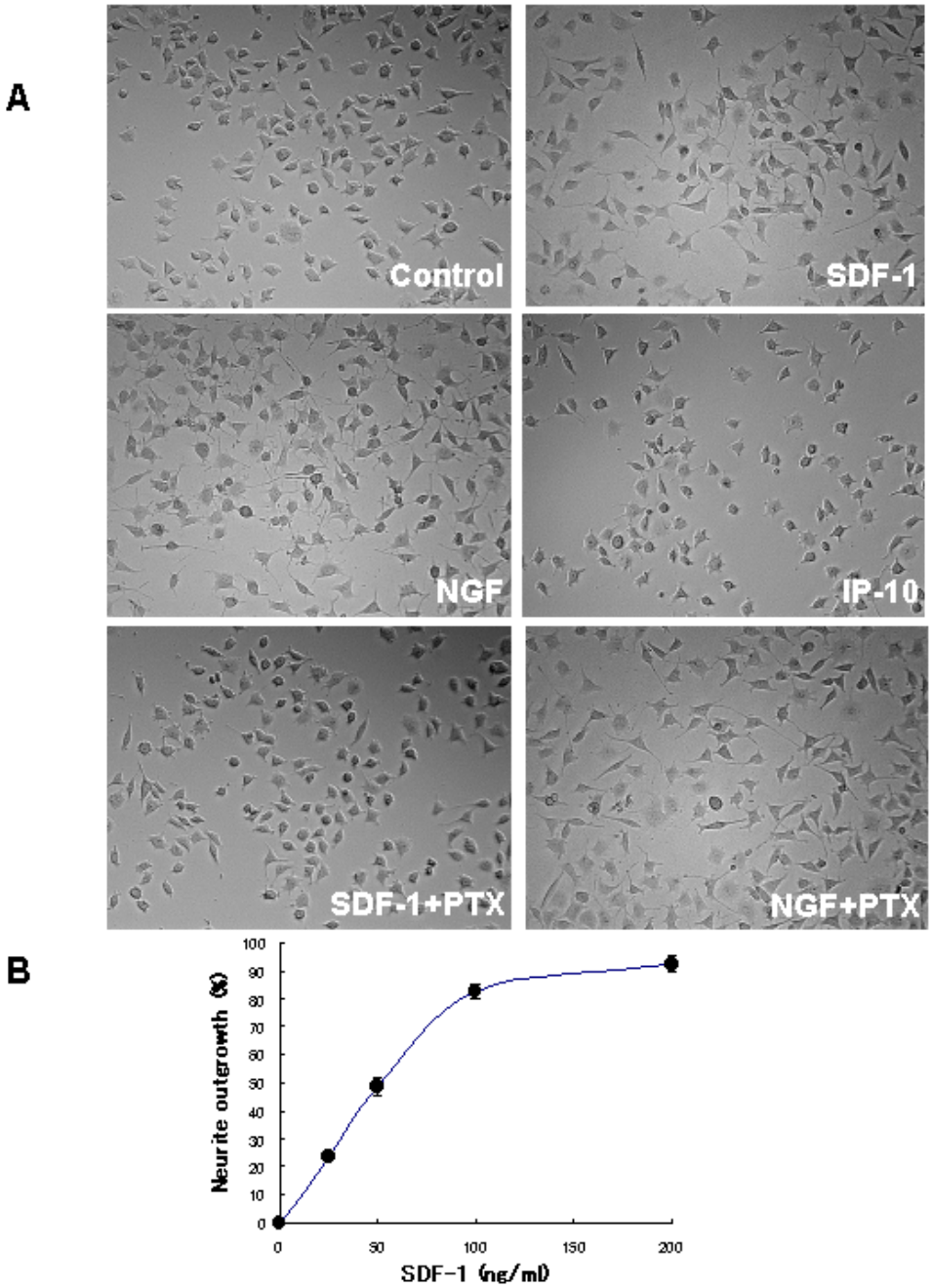

Figure 1. Induction of neurite outgrowth in PC12 cells by SDF-1. A, Morphological examination of the effect of SDF-1 on PC12 cells. PC12 cells were treated with 100 ng/ml SDF-1, 100 ng/ml NGF, 100 ng/ml IP-10, 100 ng/ml SDF-1 plus 1 g/ml PTX, or 100 ng/ml NGF plus 1 g/ml PTX, for 24 h. The experiment shown was repeated three more times, with similar results. For experiments using PTX, PC12 cells were preincubated for 60 min with 1 g/ml PTX and 100 ng/ml SDF-1 or NGF was added, followed by further incubation for 24 h. B, Dose-dependent curve showing neurite outgrowth activity of SDF-1. PC12 cells were treated with varying concentrations of SDF-1 for 24 h. The percentage of cells with neurites is plotted against the concentration of SDF-1. Each value is the mean ±S.E. for approximately 100 cells sampled from three independent experiments.

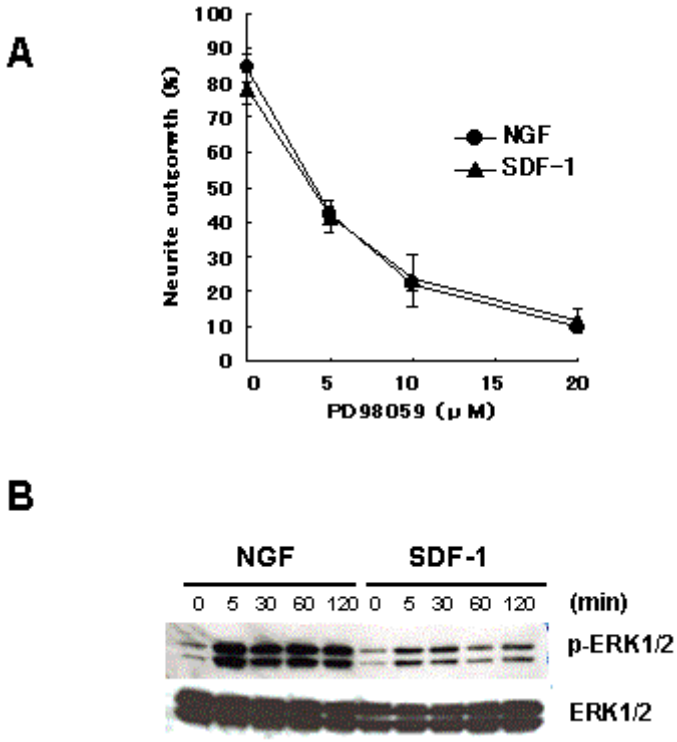

Figure 2. Activation of ERK by SDF-1. A, Effect of PD98059 on neurite outgrowth induced by SDF-1. PC12 cells were plated and preincubated in DMEM containing the indicated concentration of PD98059 for 15 min, and then 100 ng/ml SDF-1 or 100 ng/ml NGF was added. After further incubation for 24 h, the percentage of cells with neurites was determined. Each value is the mean ± S.E. for about 100 cells sampled from three independent experiments. B, Time course of ERK phosphorylation in PC12 cells stimulated by SDF-1 or NGF. PC12 cells (2×104 cells) were treated with DMEM containing 100 ng/ml SDF-1 or 100 ng/ml NGF. At the indicated times, the cells were lysed, and subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with an anti-phospho-ERK antibody (p-ERK). The blots were stripped and reprobed with anti-ERK antibody (ERK) to verify that the protein levels were uniform. The experiments were repeated three times with similar results.

SDF-1-induced neurite outgrowth was also inhibited by PD98059 in a similar dose-dependent manner (Figure 2A), suggesting that activation of the ERK pathway is critical to the neurite outgrowth activity of SDF-1. Next, immunoblot analysis with anti-phospho-ERK1/2 antibody to detect ERK activation in PC12 cells by NGF or SDF-1 was conducted. NGF stimulated activation of ERK in PC12 cells, and SDF-1 also stimulated it (Figure 2B). This induction of ERK activation in PC12 cells appeared within 5 min by SDF-1 as well as NGF. However, ERK activation by SDF-1 was weaker than that by NGF (Figure 2B). These findings suggest that sustained activation of ERK is critical for SDF-1-induced neurite outgrowth of PC12 cells as well as NGF.

Chemokines mediate their biological activities through G protein-coupled cell-surface receptors [4]. Binding of chemokine to its receptor triggers the activation of several molecules and signaling pathways, including the activation of PI3K and ERK pathways [4]. Unlike most other chemokines that activate multiple receptors, SDF-1 is thought to act exclusively through its receptor CXCR4. Flow cytometry analysis revealed that CXCR4 is expressed on PC12 cells (Figure 3).

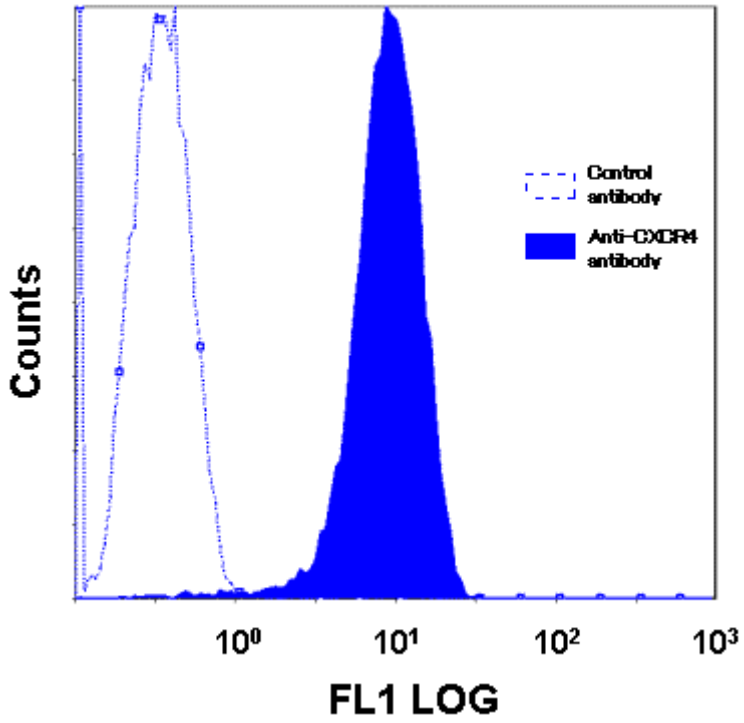

Figure 3. Expression of CXCR4 on PC12 cells. PC12 cells were analyzed by flow cytometry using rabbit polyclonal anti-CXCR4 antibody or control rabbit IgG. The experiment shown was repeated three times, with similar results.

CXCR4 is a pertussis toxin-sensitive G protein-coupled receptor, indicating that is coupled to the Gi class of heterotrimeric G proteins [4]. When PC12 cells were pretreated with pertussis toxin, the neurite outgrowth activity of SDF-1 was dramatically reduced (Figure 1A). In contrast, preincubation of pertussis toxin had no effect on NGF-induced neurite outgrowth (Figure 1A). SDF-1-induced neurite outgrowth of PC12 cells was also blocked by siRNA targeting rat CXCR4 (data not shown). These results suggest that the neurite outgrowth action of SDF-1 depends on activation of the ERK pathway through CXCR4.

Up-Regulation of CXCR4 after Rat Facial Nerve Axotomy

Axonal injury followed by retrograde degeneration of the affected nerve cells or by regeneration of axons is considered to be common process occurring in the CNS and peripheral nerve system (PNS). A rat model of facial nerve axotomy is an excellent experimental model of CNS regeneration and repair, where the events occurring in and around the affected nerve cell bodies subsequent to the axotomy can be investigated in a way separated from those directly evoked by the surgical procedure itself, because transection-site of the axons is far from the cell body of the affected nerve cells [6, 7, 8]. Quantitative RT-PCR analysis using total cellular RNA extracted from facial nucleus of the operated side or unoperated side revealed that CXCR4 expression is up-regulated 3-fold following axotomy (Figure 4). Preliminary in situ hybridization showed that CXCR4 is up-regulated by motoneurons of the rat facial nucleus seven days after axotomy (data not shown).

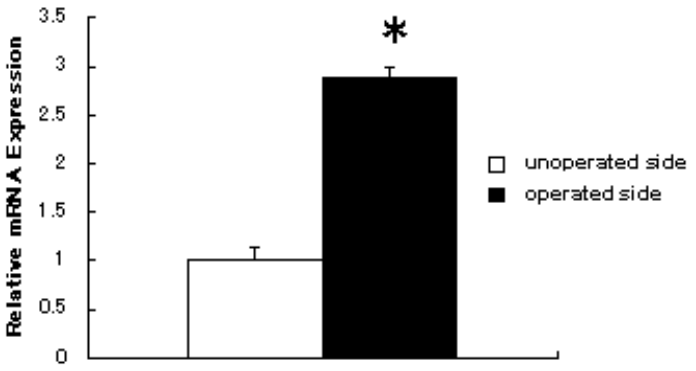

Figure 4. Axotomy- induced up-regulation of CXCR4 in rat facial nucleus. Quantitative RT-PCR was carried out using total cellular RNA derived from facial nucleus of the operated side or unoperated side. Each cDNA were analyzed in triplicate. Each value is the mean ±S.D. The experiment shown was repeated three times, with similar results. *, $p<0.001$ by student's t-test.

Discussion

The PC12 cell line was derived from a pheochromocytoma tumor of the rat adrenal medulla [2], and it has been used as a model system for neuronal differentiation and death. It has been reported that NGF and a pituitary adenylate cyclase-activating polypeptide (PACAP) induce differentiation with sympathetic neuron-like characteristics and survival of PC12 cells [1, 2, 3, 11]. These molecules have a neurotrophic effect on the CNS. NGF plays a crucial role in promoting growth, differentiation, and function in sympathetic nerve cells [1]. PACAP has neurotrophic activity on the rat cerebellar cortex during development [12]. PC12 cell line has also been used to isolate small compounds that mimic the effect of NGF [13, 14].

In order to obtain novel neurotrohic factors like NGF, the effect of over 200 small secreted proteins, such as cytokines, chemokines and bioactive peptides on PC12 cells was investigated. The result elucidated that one of the CXC chemokines, SDF-1 promotes the neuronal differentiation of PC12 cells. Chemokines are small, soluble proteins that have been recognized to regulate leukocyte migration, adhesion, and proliferation in the immune system [5]. Recent evidences suggest that chemokines and their receptors are expressed in the CNS and that their functions extend beyond their roles in inflammation [15, 16]. Extensive evidence supports SDF-1 as a key regulator for early development of the CNS. SDF-1/CXCR4 signaling is required for the migration of neuronal precursors [17], axon guidance/pathfinding [18] and maintenance of neural progenitor cells [19].

The present data show that SDF-1 increases neurite extension in PC12, which is mediated by ERK signal transduction through CXCR4, and CXCR4 mRNA level is increased in the rat model of the CNS regeneration and repair following facial nerve axotomy. In the regeneration and repair processes that occur after damage of the CNS, many genes are adequately up- or down-regulated by brain cells, such as neurons and glia, to achieve a favorable outcome [6, 7, 8]. The genes that play important roles in the regeneration and/or

repair should be significantly up-regulated after CNS damage. SDF-1 or its receptor CXCR4 may take part in regenerating damaged neurons in the CNS.

Conclusion

SDF-1/CXCR4 signaling might be participating in neurite outgrowth during nerve regeneration.

References

[1] Bibel, M and Barde, Y.A. (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* 14, 2919–2937.

[2] Greene, L. A., and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 73, 2424-2428.

[3] Greene, L. A. (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *J. Cell Biol.* 78, 747-755.

[4] Moser, B., and Loetscher, P. (2001) Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2, 123-128.

[5] Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80, 179-185.

[6] Miyake, T., Gahara, Y., Uwabe, K., Yamada, H., and Kitamura, T. (1997) Gene analysis of retrograge degeneration. *Neuropathology* 17, 145-151.

[7] Miyake, T., Gahara, Y., Nakayama, M., Yamada, H., Uwabe, K., and Kitamura, T. (1996) Up-regulation of cystatin C by microglia in the rat facial nucleus following axotomy. *Brain Res. Mol. Brain Res.* 37, 273-82.

[8] Uwabe, K., Gahara, Y., Yamada, H., Miyake, T., and Kitamura, T. (1997) Identification and characterization of a novel gene (neurorep 1) expressed in nerve cells and up-regulated after axotomy. *Neuroscience* 80, 501-509.

[9] Deutsch, P. J., and Sun, Y. (1992) The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J. Biol. Chem.* 267, 5108-5113.

[10] Barrie, A. P., Clohessy, A. M., Buensuceso, C. S., Rogers, M. V., and Allen, J. M. (1997) Pituitary Adenylyl Cyclase-activating Peptide Stimulates Extracellular Signal-regulated Kinase 1 or 2 (ERK1/2) Activity in a Ras-independent, Mitogen-activated Protein Kinase/ERK Kinase 1 or 2-dependent Manner in PC12 Cells. *J. Biol. Chem.* 272, 19666-19671.

[11] Vaudry, D., Chen, Y., Hus, C. M., and Eiden, L. E. (2002) PC12 Cells as a Model to Study the Neurotrophic Activities of PACAP. *Annu. N. Y. Acad. Sci.* 971, 491-496.

[12] Vaudry, D., Gonzalez, B. J., Basille, M., Fournier, A., and Vaudry, H. (1999) *Proc. Natl. Acad. Sci. USA.* 96, 9415-9420.

[13] Swain, C., Harper, S., Pollack, S., Smith, R., and Hefti, F. （1999）Neurotrophic factor mimetics, In Neurotrophic Factors (Hefti F, ed.), Springer-Verlag, Berlin, 281-309.

[14] Uwabe, K., Iwakawa, T., Matsumoto, M., Talkahashi, K., and Nagata, K. (2006) HU0622: a small molecule promoting GAP-43 activation and neurotrophic effects. *Neuropharmacology* 51, 727-36.

[15] Harrison, J.K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., Streit, W. J., Salafranca, M.N., Adhikari, S., Thompson, D.A., Botti, P., Bacon, K.B., and Feng, L.(1998) Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc. Natl. Acad. Sci. USA.* 95, 10896-901.

[16] Uwabe, K., Matsumoto, M., and Nagata, K. Monokine induced by interferon-gamma acts as a neurotrophic factor on PC12 cells and rat primary sympathetic neurons. (2005) *J. Biol. Chem.* 280, 34268-34277.

[17] Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi,T., and Littman, D.R. (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393,595–599.

[18] Lieberam, I., Agalliu, D., Nagasawa, T., Ericson, J., and Jessell, T.M. (2005) A CxCL12–CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. *Neuron* 47, 667–679.

[19] Imitola, J., Raddassi, K., Park, K.I., Mueller, F.J., Nieto, M., Teng, Y.D., Frenkel, D., Li, J., Sidman, R.L., Walsh, C.A., Snyder E.Y., and Khoury, S.J. (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc. Natl. Acad. Sci. USA.* 101, 18117–18122.

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Chapter 4

Innovative Strategies for Controlled Delivery and Release of NGF in Neurological Applications

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Abstract

Neurotrophic molecules have a deep influence on developmental events such as naturally occurring cell death, differentiation and process outgrowth, and could be used for treating degenerative neurological conditions and promoting neural regeneration. The neurotrophin family members (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), neurotrophins 3 (NT-3) and 4 (NT-4)) are proteins which cannot cross the blood-brain barrier and therefore need to be administered directly to their target in the brain.

Drug delivery to the nervous system remains a challenge despite advances in understanding the mechanisms involved in the development of neurodegenerative disorders and the actions of neuroactive agents. The systemic administration of neuroactive biomolecules to support neuronal regeneration has several intrinsic problems, including the toxicity and poor stability associated with many bioactive factors and access limitation by the blood-brain barrier.

The best characterized trophic molecule, NGF, has a spectrum of effects on peripheral and central neurons. It has received particular attention as a potential treatment for Alzheimer's disease because of its protective action on basal forebrain cholinergic neurons. Intraventricular infusion of NGF can prevent cholinergic cell death following fimbrial transsection in adult rodents and monkeys. Based on these data, the entrapment of NGF was performed in monolithic implantable devices.

A variety of techniques to deliver therapeutics to the CNS has been established, including osmotic pumps and silicone reservoirs. However, pumps frequently become clogged, thus limiting their ability to sustain effective concentrations; moreover these

methods are often associated to highly invasive drawbacks, including device failure and higher potentials for inflammation and infection due to their non degradable components.

This Chapter will focus on innovative strategies for delivering NGF both for *in vivo* and *in vitro* applications. Development of polymeric films, hydrogels and microparticles will be considered. Polymeric delivery systems, in fact, have the potential to maintain therapeutic levels of a drug, to reduce side effects and to facilitate the delivery of drugs with short *in vivo* half-lives. Synthetic and naturally derived polymers are widely used in controlled release devices for protein delivery. These devices are designed such that bioactive factors are released in a spatially and temporally controlled manner; for example, release can occur as the polymer degrades or by diffusion through pores in the polymer matrix. In neural applications, two types of delivery devices have been primarily used: polymer matrices and microspheres.

Particular attention will be devoted to the recent advancements in the field of neuronal tissue engineering and of neuronal regeneration. Finally, possibility to integrate NGF delivery techniques to regeneration-type (sieve) neuronal interfaces will be analysed and discussed.

1. Introduction: Drug Delivery for Neurological Application

Controlled drug delivery occurs when a polymer, both natural and synthetic, is opportunely combined with a drug or another active agent in such a way that the active agent is released from the material in a designed manner [1-2]. The release of the active agent may be constant over a long period, it may be cyclic over a long period, or it may be triggered by the environment or other external events. In any case, the purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under- and overdosing. These include situations requiring the slow release of water-soluble drugs, the fast release of low-solubility drugs, drug delivery to specific sites, delivery of two or more agents with the same formulation, and systems based on carriers that can dissolve or degrade and be readily eliminated [3-4-5]. The ideal drug delivery system should be inert, biocompatible, mechanically strong, capable of achieving high drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize.

In recent years, controlled drug delivery formulations and polymers used in these systems have become much more sophisticated. For example, current controlled-release systems can respond to changes in the biological environment and deliver drugs basing on these changes. In addition, materials have been developed that should lead to targeted delivery systems, in which a particular formulation can be directed to the specific cell, tissue, or site where the drug is to be delivered.

Drug delivery to the nervous system remains a challenge despite advances in understanding the mechanisms involved in the development of neurodegenerative disorders and the actions of neuroactive agents. Drug accessibility to the central nervous system (CNS), moreover, is limited by the blood-brain barrier. The systemic administration of neuroactive biomolecules to support neuronal regeneration has several intrinsic problems, including the toxicity and poor stability associated with many bioactive factors [6].

A variety of techniques to deliver therapeutics to the CNS has been established, including osmotic pumps [7] and silicone reservoirs [8]. However, pumps frequently become clogged, thus limiting their ability to sustain effective concentrations [9]; moreover these methods are often associated to highly invasive drawbacks, including device failure and higher potentials for inflammation and infection due to their non degradable components [10].

Polymeric delivery systems have the potential to maintain therapeutic levels of a drug, to reduce side effects and to facilitate the delivery of drugs with short *in vivo* half-lives [11].

Among different drugs and neuro-active substances, neurotrophic molecules have a profound influence on developmental events such as naturally occurring cell death, differentiation and process outgrowth and could be used for treating degenerative neurological conditions and promoting neural regeneration [12]. The neurotrophin family members (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), neurotrophins 3 (NT-3) and 4 (NT-4)) are proteins which cannot cross the blood brain barrier and therefore need to be administered directly to their target in the brain [13].

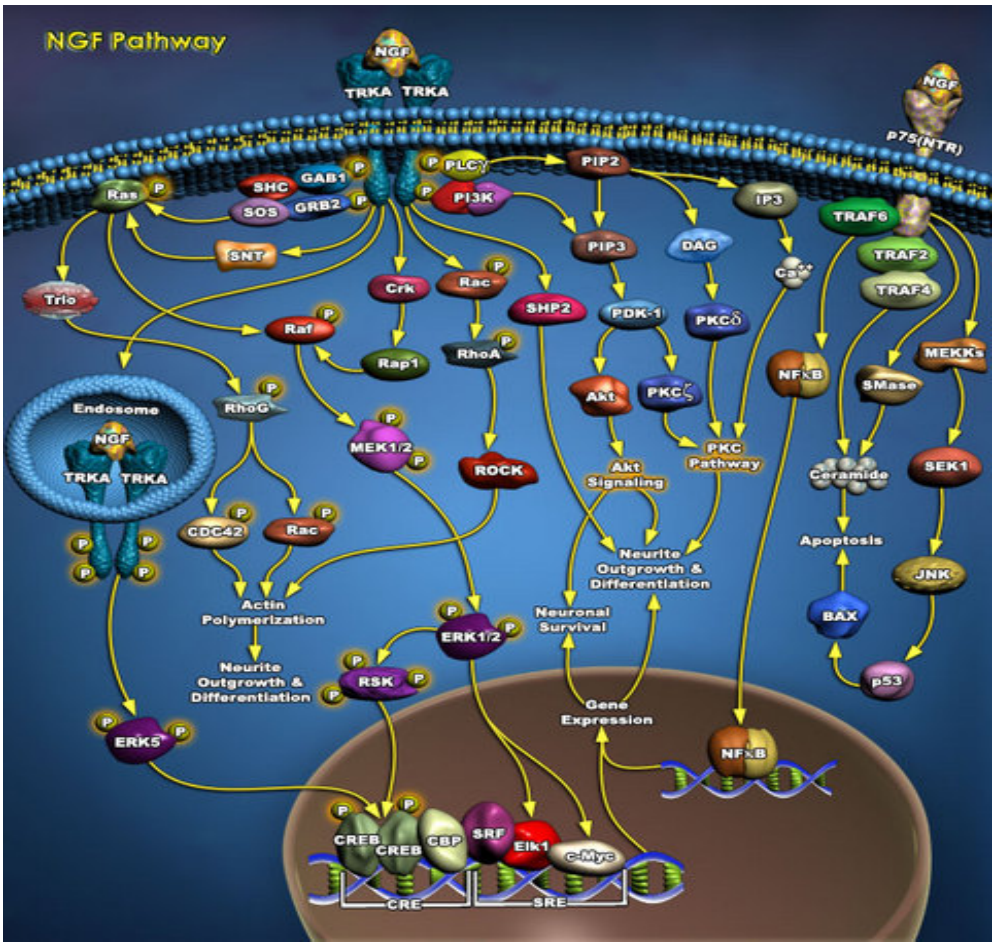

Figure 1. The NGF Pathway.

NGF is vital to the development and regeneration of the nervous system (Figure 1); consequently, NGF is the most thoroughly characterized neurotrophic factor [14]. NGF is expressed at low levels in healthy peripheral nerve and is upregulated in the distal stump upon injury [15]. Similarly, following spinal cord transection, NGF accumulates in both the distal and proximal stumps [16]. On the cellular level, NGF promotes survival, outgrowth, and branching in sensory neurons, but does not aid motor neuron regeneration [17]. Application of exogenous NGF has also been linked to increased sensory neuron regeneration from the dorsal root ganglia, through the PNS-CNS transition zone, and into the spinal cord. However, the use of NGF is not without disadvantages: the application of exogenous NGF to spinal cord injuries has been associated with significant sprouting of uninjured sensory axons. This sprouting has been linked to serious side effects, including chronic pain and inappropriate neuronal reflexes [18].

NGF, moreover, received particular attention as a potential treatment for Alzheimer's disease because of its protective action on basal forebrain cholinergic neurons. Intraventricular infusion of NGF can prevent cholinergic cell death following fimbrial transection in adult rodents and monkeys.However, one of the significant problems of the administration of protein drug such as NGF is a short half-life in plasma and poor penetration at the blood brain barrier. Hence, it is strongly wanted to develop new delivery method. Several new methods for the controlled delivery of NGF to the CNS and brain have been suggested and widely tested such as (1) controlled release from polymer matrices with NGF, (2) controlled release from biodegradable NGF-loaded microspheres, and (3) controlled delivery by cells that were genetically modified to produce NGF. Recently, the family of poly(a-hydroxy acid)s such as polyglycolide (PGA), polylactide (PLA) and its copolymer like poly(lactide-co-glycolide) (PLGA), which are among the few synthetic polymers approved for human clinical use by Food and Drug Admnistration, is extensively used or tested for the scaffolds materials as a bioerodible material due to good biocompatibility, controllable biodegradability, and relatively good processability [19]. Based on these data, the entrapment of NGF was performed in monolithic implantable devices [20]. In parallel, a few studies reported the encapsulation of NGF in PLA or PLGA microspheres to investigate the effect of its sustained release on various biological phenomena [21].

This Chapter will focus on innovative strategies for delivering NGF both for *in vivo* and *in vitro* applications. Development of polymeric films, hydrogels and microparticles will be considered. Polymeric delivery systems, in fact, have the potential to maintain therapeutic levels of a drug, to reduce side effects and to facilitate the delivery of drugs with short *in vivo* half-lives. Synthetic and naturally derived polymers are widely used in controlled release devices for protein delivery. These devices are designed such that bioactive factors are released in a spatially and temporally controlled manner. In neural applications, two types of delivery devices have been primarily used: polymer matrices and microspheres.

After this short Introduction, in the second Paragraph innovative researches, without *in vivo* testing yet, will be approached. Particular attention will be focused on the release properties of innovative systems and the strategies to tailor these properties for dedicated applications. Preliminary experiments on cell culture will be also considered.

The third Paragraph is dedicated to the systems that have already tested *in vivo*. Recent advances in the fields of polymeric microparticles, liposomes, and hydrogels will be

highlighted, with description of their applications at level of both central and peripheral nervous system. Problems related to the short half-life of NGF and to the blood brain barrier crossing will be investigated.

Application of NGF release systems in tissue engineering is the topic of the fourth Paragraph. In this section the main methods to allow a sustained release of NGF in engineered tissues will be illustrated. Particular attention will be devoted to the neuronal guidance channels. The use of biopolymers can be a practical tool to provide neurotrophic and/or cellular support while simultaneously axonal regeneration occurs. Indeed, severals natural and synthetic polymers, including poly-(a-hydroxyacids), collagen, fibronectin, and hyaluronic acid have been used as scaffolds or within scaffolds for peripheral and central nerve regeneration.

Finally, possibility to integrate NGF delivery techniques to regeneration-type neuronal interfaces will be analysed and discussed in the fifth Paragraph. This is the most challenging topic in the field of neuronal regeneration following disease or injuries, and it opens exciting perspectives in the research on human-machine interface.

Conclusion will summarize the different approaches described in this Chapter and will highlight the perspectives of the future researches.

2. Systems for in Vitro Controlled Release of NGF

Some of the most promising systems for the controlled release of peptides and proteins involve encapsulation or entrapment of proteins in biocompatible polymeric devices. These devices have found widespread use in the treatment of cancers and other life-threatening diseases [22-23-24]. Delivery systems have been designed in a variety of geometries and configurations (reservoirs, matrices, microspheres) and have been fabricated from diverse types of natural and synthetic polymers (degradable, non-degradable, see Figure 2, [25]). These devices have a common ability to control the release of bioactive proteins for extended periods of time, but the control is accomplished in different ways.

The earliest controlled delivery systems were the reservoir type, in which the drugs are surrounded by a polymer coating that regulates the rate of release (Figure 2a). Typically, the polymer membranes used to regulate the release are silicone elastomers or poly(ethylene-co-vinyl acetate) (EVAc): both are biocompatible, non-degradable polymers.

Matrix devices are fabricated by dispersing the protein within a solid continuous matrix of polymer, such as EVAc; the protein is typically in the form of solid particles, resulting in the formation of a heterogeneous micro structure within the matrix (Figure 2b, [26]). These systems may be composed of either non-degradable polymers (Fig2b.i) or biodegradable polymers (Figure 2b.ii).

Many polymers can be formed into microspheres for the controlled delivery of macromolecules (Figure 2c). Microspheres can be produced in a wide range of sizes (1–100 nm for nanospheres, and 1–100 μm for microspheres), and may be composed of non degradable (Figure 2c.i) or degradable polymers (Figure 2c.ii). One of the main advantages of microspheres is that they may be administered by injection, so that no surgical procedure is required.

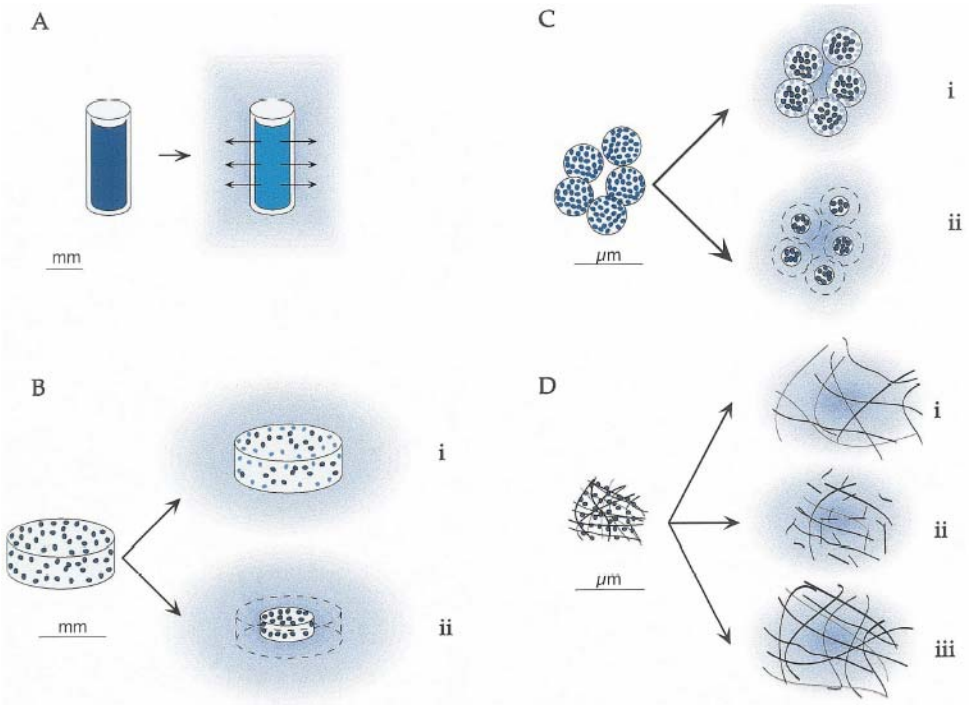

Figure 2. Devices for controlled delivery. (A) Release from a reservoir device. (B) Solid particles (dark blue) of a protein are shown suspended within a solid polymer matrix. These devices may be fabricated in the shape of slabs, pellets or disks, and may be composed of either nondegradable polymers (B.i) or biodegradable polymers (B.ii). (C) Release of proteins from both nondegradable (C.i) and biodegradable (C.ii) polymeric microspheres is shown. (D) Release from physically entangled (D.i), degradable (D.ii) and swelling (D.iii) hydrogels. From [25]. Reproduced with permission from Elsevier.

Additionally, the use of microspheres composed of biodegradable polymers eliminates the need for device removal after release of the agent. Unfortunately, these same qualities make removal of the devices difficult, in the event that therapy must be prematurely terminated. Three types of hydrogel-based protein delivery systems have been studied (Figure 2d). Physically entangled polymer systems slowly dissolve and release the protein simultaneously with polymer dissolution (Figure 2d.i). Chemically crosslinked polymer gels degrade due to hydrolysis or enzymatic digestion (Figure 2d.ii); in these delivery systems, protein is released at a rate that is dependent on the rate of polymer degradation. Hydrogels that swell after contact with water allow diffusion of macro molecules throughout the entire matrix, so that agents are released through a porous structure that expands during swelling (Figure 2d.iii). The size of the pores located within the network, which is related to the extent of crosslinking and the degree of swelling, determines the protein release rate. The rate of protein release from hydrogel networks can be modified by varying the degree of physical entanglement within the gel, by altering the number of chemical crosslinks between the polymer or by altering the interactions between the polymer matrix and the molecule of interest. For example, polymer – protein interaction can be modified by the use of ionizable groups on the polymer network, so that, oppositely charged molecules are stabilized by the matrix and like-charged molecules are excluded from the polymer matrix.

Regarding polymeric microspheres, the aim of the study of Pean and colleagues [27] was to prepare, in a reproducible manner, poly(D,L-lactide-co-glycolide) (PLGA) biodegradable microparticles with high encapsulation yield of nerve growth factor (Figure 3).

Human serum albumin (HSA) was co-encapsulated with NGF by a w:o:w emulsion solvent evaporation:extraction method to stabilize the primary emulsion and to protect NGF. The encapsulation yield optimization was first carried out with HSA alone since it was the major component in the final microspheres, as compared to NGF. The effects of ten process factors on HSA entrapment in PLGA microspheres were examined using a fractional factorial design. Four major factors were identified. The presence of carboxymethylcellulose sodium or mannitol in the internal aqueous phase, the increase of the internal aqueous volume and the concentration of HSA led to a decrease of the encapsulation yield. Two main factors, namely the internal aqueous phase volume and the proportion of acetone in the organic phase, previously identified as a predominant factor, were studied through a response surface methodology. The observed range of HSA encapsulation yield was 87.4–100%. NGF was encapsulated according to the optimal conditions found for the entrapment of HSA. The encapsulation yield was then about 97.3%.

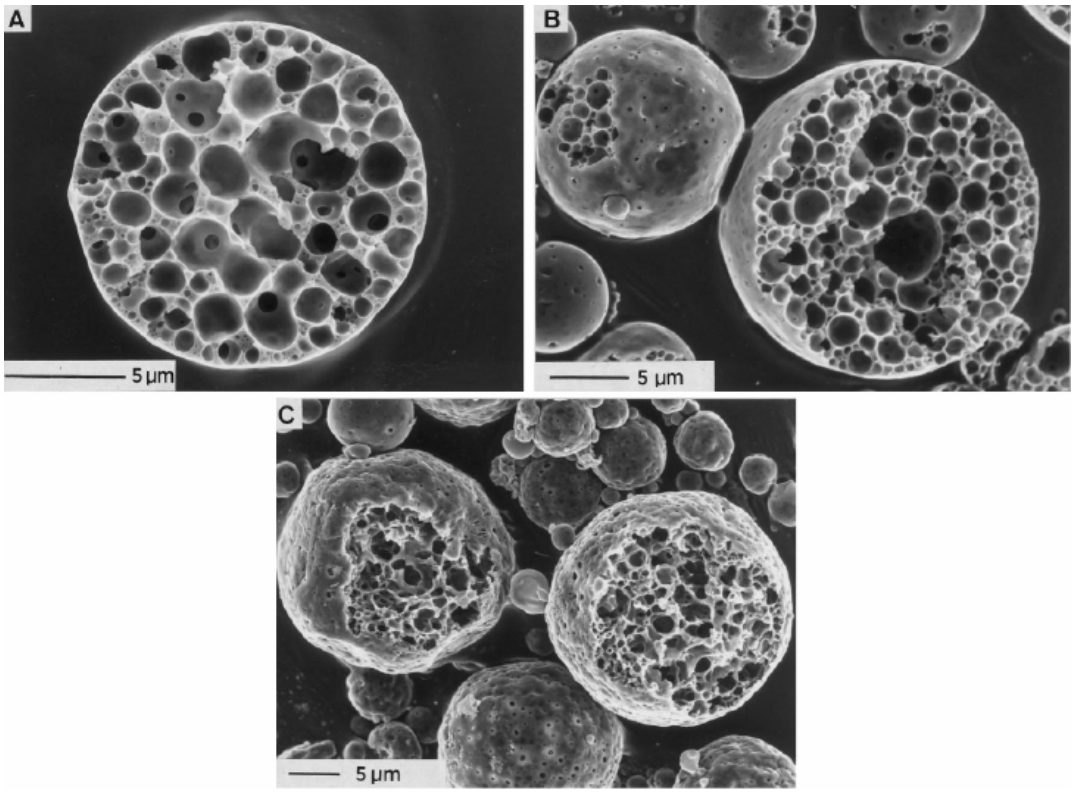

Figure 3. Photomicrographs of microparticles prepared in different experimental conditions. (A) 150 ml internal aqueous phase, 2.5 mg HSA, 2 ml organic phase among which 0.5 ml acetone (encapsulation yield-94%). (B) Formulation A+1% (w:v) carboxymethylcellulose sodium in the internal aqueous phase (encapsulation yield-21%). (C) Formulation A+1% (w:v) mannitol in the internal aqueous phase (encapsulation yield-61%). From [27]. Reproduced with permission from Elsevier.

The strategy used in this study allowed to optimize both the HSA and NGF encapsulation yields. More particularly, this study outlined the importance of the volume and the composition of the internal aqueous phase, and the proportion of acetone in the organic phase to optimize the protein encapsulation yield. In the optimized conditions, the HSA and NGF encapsulation yields were closed to 100%. Their behaviour, during the encapsulation process, was identical.

In another work [28], Pean *et al.* investigated the influence of two formulation parameters (the presence of NaCl in the dispersing phase and the type of PLGA) on the NGF release profiles and NGF stability during microencapsulation. A honey-comb-like structure characterized the internal morphology of the microspheres (see also Figure 3). The initial burst was attributed to the rapid penetration of the release medium inside the matrix through a network of pores and to the desorption of weakly adsorbed protein from the surface of the internal cavities. The non-release fraction of the encapsulated protein observed after twelve weeks of incubation was accounted for firstly by the adsorption of the released protein on the degrading microparticles and secondly by the entanglement of the encapsulated protein in the polymer chains.

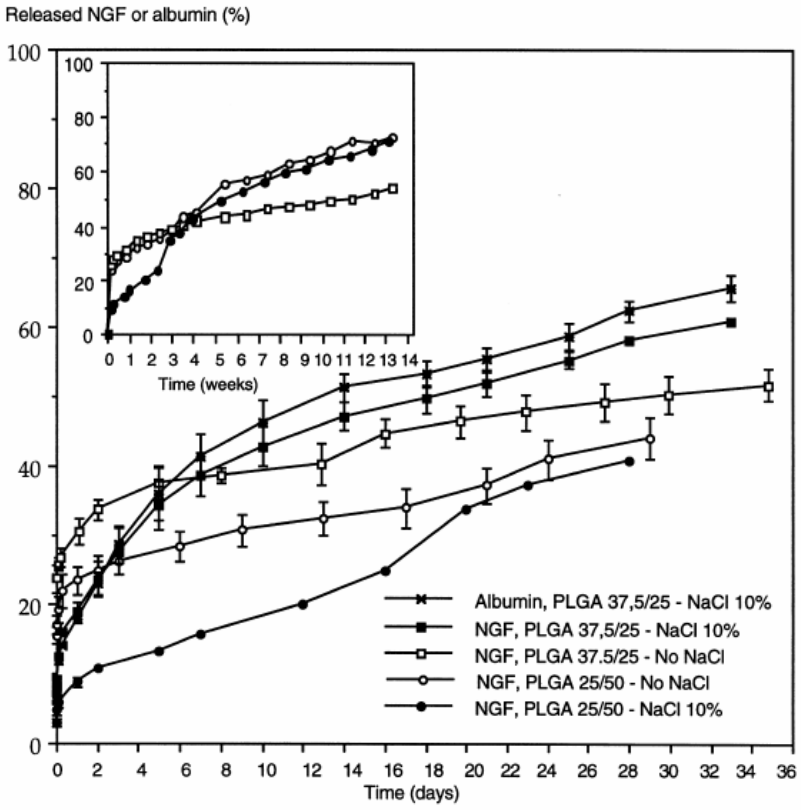

Figure 4. Effect of the polymer type and the addition of sodium chloride in the dispersing phase on the NGF release profile determined by radioactivity counting. The release profile of co-encapsulated HSA from microparticles prepared with PLGA 37.5/25 and sodium chloride is also reported. From [28]. Reproduced with permission from Elsevier.

The use of sodium chloride in the dispersing phase of the double emulsion markedly reduced the burst effect by making the microparticle morphology more compact. Unfortunately, it induced in parallel a pronounced NGF denaturation. Finally, it appeared that microparticles made from a hydrophilic uncapped PLGA 37.5/25 in the absence of salt allowed the release of intact NGF at least during the first 24 h as determined by both ELISA and a PC-12 cell-based bioassay.

Figure 4 shows the release profiles from the batches of microspheres. The addition of salt in the formulation process reduced the intensity of the burst effect: from 23% to 9% of drug release over the first 24 h for PLGA 25/50 microspheres and from 28% to 19% for PLGA 37.5/25 microspheres. After the initial burst, the amount of released NGF increased slowly with time for all the batches.

Nevertheless, in the case of PLGA 25/50 microspheres prepared with salt, a pulsed release occurred at day 16, probably due to polymer degradation. The protein recovery after four weeks of release reached between 40% to 45% for all the batches except PLGA 37.5/25 microparticles with salt which released 58% of the encapsulated drug over the same time. Over a three month period, the amount of released NGF from PLGA 37.5/25 microparticles prepared without salt, which exhibited the slowest release, was 54%. It was noticed that these PLGA 37.5/25 microparticles were severely degraded. Conversely, the amount of released NGF from PLGA 25/50 microparticles prepared with or without salt reached 68% and 72%, respectively.

In these two latter cases microparticles were still present in the medium although they appeared internally eroded. The addition of SDS to the medium for 3 h after twelve weeks of incubation resulted in the release of an additional 8% NGF (PLGA 25/50) and 11% NGF (PLGA 37.5/25). The addition of NaCl in the release medium did not induce any further release, irrespective of the process conditions. It should also be mentioned that the release profiles of the co encapsulated HSA as determined by a Bio-Rad assay and radiolabelled NGF were identical.

Also Cao and Shoichet [29] investigated a drug delivery system for NGF based on microspheres. The release profile of NGF, co-encapsulated with ovalbumin, was tailored from biodegradable polymeric microspheres using both polymer degradation and protein loading. Biodegradable polymeric microspheres were prepared from PLGA 50/50, PLGA 85/15, PCL and a blend of PCL/PLGA 50/50 (1:1, w/w), where the latter was used to further tailor the degradation rate. The amount of protein loaded in the microspheres was varied, with PCL encapsulating the greatest amount of protein and PLGA 50/50 encapsulating the least. A two-phase release profile was observed for all polymers where the first phase resulted from release of surface proteins and the second phase resulted predominantly from polymer degradation. Polymer degradation influenced the release profile most notably from PLGA 50/50 and PLGA 85/15 microspheres.

The stability of proteins at physiological conditions is limited, while that of encapsulated proteins may be further reduced by exposure to organic solvents, shear, and acidic degradation products. To assess bioactivity, the response of PC-12 cells to released NGF was monitored over a 91 d period. Since PC-12 cells respond reversibly to NGF by induction of a neuronal phenotype, a correlation exists in the amount of NGF present and neurite outgrowth. Since the positive control had 25 ng/ml of NGF supplemented to the medium, which was

significantly more NGF than was released from the microsphere samples, the results may be misinterpreted. For example, NGF-1 samples induced less differentiation than the positive control after 48 d, indicating that either the released NGF was inactive or more simply had a lower concentration than the positive control. The ELISA data clearly demonstrated that the concentration of released NGF was less than 1 ng/ml after 12 d of release. By comparing the PC-12 cell response to supplemented medium containing 1 ng/ml NGF to the NGF-1 samples, it is clear that the released NGF is active for at least 62 d; in comparison to the blank and OVA controls, NGF released from NGF-1 is active for 91 d.

In order to fabricate new sustained delivery device of nerve growth factor, Khang et al. [30] developed NGF loaded biodegradable poly(L-lactide-co-glycolide) (PLGA, the employed mole ratio of lactide to glycolide was 75:25, molecular weight: 83,000 and 43,000 g/mol, respectively) film by novel and simple sandwich solvent casting method for the possibility of the application in the neural tissue engineering. PLGA was copolymerized by direct condensation reaction and the molecular weight was controlled by reaction time. Released behaviour of NGF from NGF-loaded films was characterized by enzyme linked immunosorbent assay (ELISA) and degradation characteristics were observed by scanning electron microscopy (SEM) and gel permeation chromatography (GPC). The bioactivity of released NGF was identified using a rat pheochromocytoma (PC-12) cell based bioassay. The release of NGF from the NGF-loaded PLGA films was prolonged over 35 days with zero-order rate of 0.5~0.8 ng NGF/day without initial burst and could be controlled by the variations of molecular weight and NGF loading amount. The released NGF stimulated neurite sprouting in cultured PC-12 cells, that is to say, the remained NGF in the NGF/PLGA film at 37 °C for 7 days was still bioactive. This study suggested that NGF-loaded PLGA sandwich film is released during the desired period in delivery system and useful neuronal growth culture as nerve contact guidance tube for the application of neural tissue engineering.

A schematic diagram of the fabrication process by sandwich method is shown in Figure 5. First, 0.3 g of PLGA was dissolved in 6 ml of methylene chloride (MC). PLGA solution was cast onto Pyrex glass dish on a horizontal level in order to get 200-300 mm thickness of PLGA films.

After evaporation of MC at room temperature, 500 ng and 1 mg of NGF in 2 ml deionised water was poured onto PLGA films, then freeze dried at -56 °C using a freeze dryer. Again, same concentration amount of PLGA solution was cast onto NGF layer, that is to say, NGF layer was sandwiched between PLGA layer (total thickness; 500 mm). After evaporation of MC for 2 days at room temperature, NGF-loaded films were freeze-dried for 2 days to eliminate remained water and solvent. All samples were kept in a vacuum oven until use.

In order to assure the bioactivity of releasing NGF from the NGF sandwiched PLGA film, PC-12 cell were cultured onto the PLGA film after 7 days release. No neuritis were observed on control PLGA film, whereas significant growth of neurites was observed on NGF/PLGA films. The higher concentration of NGF, the longer neurites protruded.

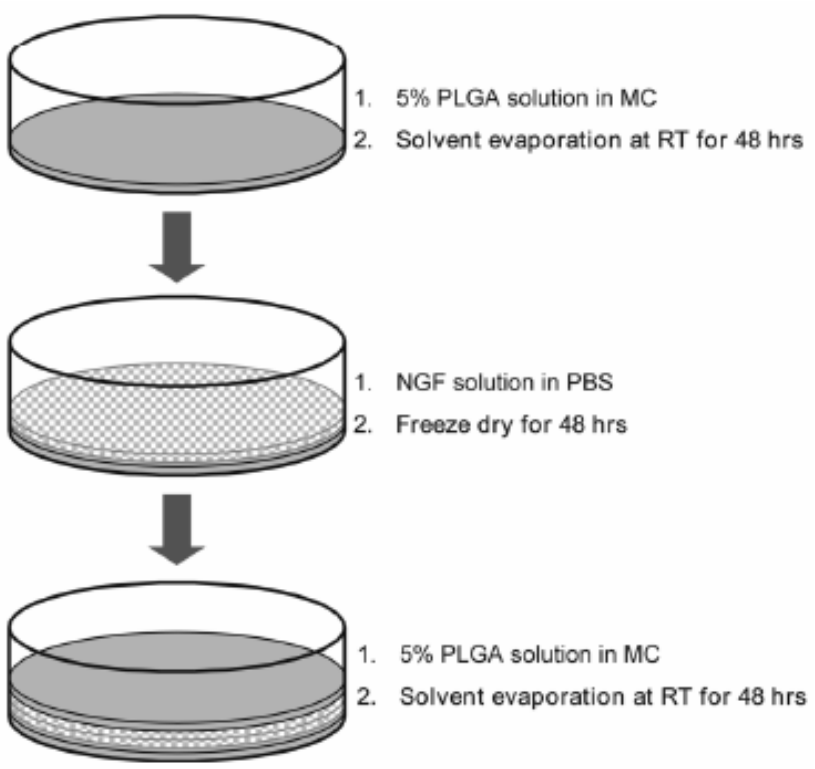

Figure 5. Schematic diagram of preparation of NGF-loaded PLGA films by sandwiched solvent casting method. From [30]. Reproduced with permission from the Polymer Society of Korea.

A general problem in using growth factors in combination with drug delivery systems resides in the inherent instability of such proteins, which tend to degrade or aggregate in solution or during processing [31]. The stability of neuronal factors during processing and formulation might be reduced when using mild processing conditions, e.g., through avoiding organic solvents, aqueous/organic interfaces, or chemical reagents.

Indeed, the fabrication of parenteral delivery systems frequently requires one or several of these stress factors either for dissolving or shaping the matrix materials or for rendering water-soluble matrix materials insoluble by cross-linking. The latter point is of particular interest for silk fibroin (SF). Thanks to its solubility in water, SF can be processed in aqueous media by freeze-drying [32] or air-drying, and subsequently be rendered insoluble by physical induction of β-sheet formation. This qualifies SF as a promising biomaterial for the fabrication of protein delivery systems, in particular long nerve guidance conduits, as it is biocompatible, of high resilience, and slowly biodegrading [33]. Silk has been used for decades as suture material and has a long-standing clinical history [34]. Moreover, silk fibers are mechanically strong and can absorb high amounts of energy before break [35].

The use of SF as a substrate for neuronal cells [36] as well as a biomaterial for the delivery of proteins [37] has been well documented as well as its biocompatibility *in vitro* and *in vivo* [38].

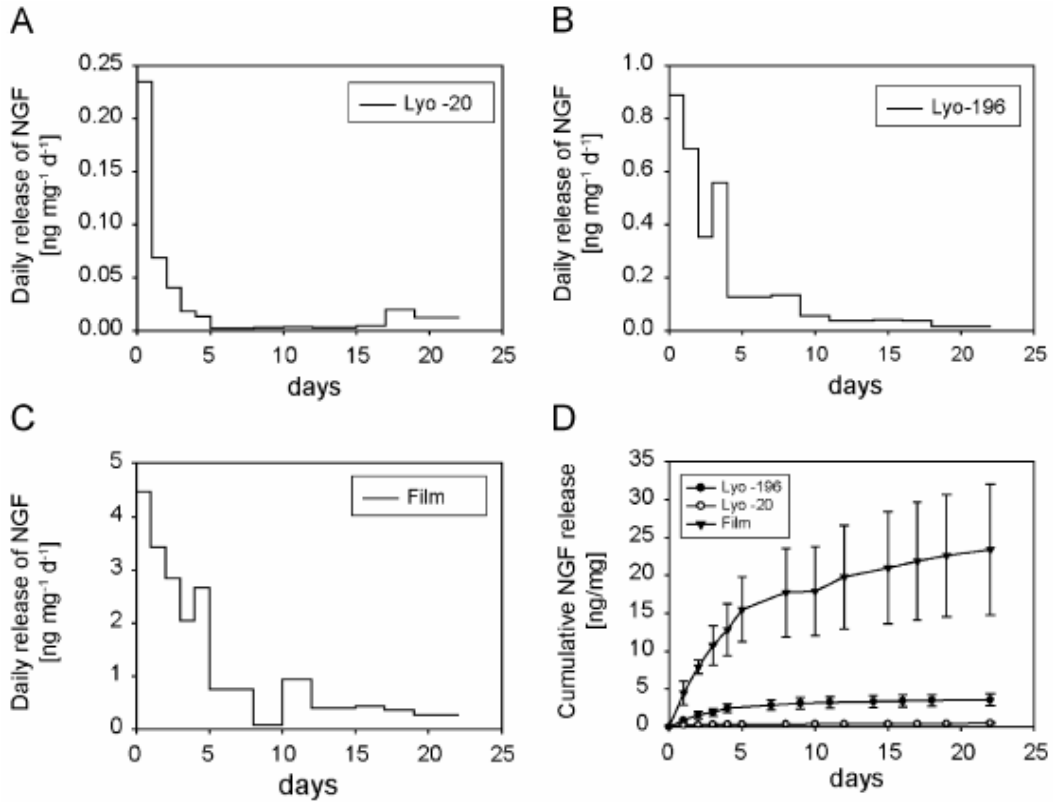

Figure 6. NGF *in vitro* release from SF matrices in acetate buffer of pH 5.5. (A–C) Daily release of NGF per mg of SF matrix prepared by freeze-drying a NGF-SF solution after slow freezing at -20 °C (A; Lyo-20), or by freeze-drying a NGF-SF solution after shock-freezing at -196 °C (B; Lyo-196), or by air-drying a NGF-SF solution (C; Film). (D) Cumulative release per mg of SF matrix prepared by the aforedescribed processes. From [39]. Reproduced with permission from Elsevier.

The study of Uebersax and colleagues [39] investigated SF derived from the silk worm *Bomyx mori* as a biomaterial for the preparation of NGF-loaded neuronal conduits (NC). The NGF-loaded SF-NC were prepared from aqueous solutions of NGF and SF and subsequent drying of this solution in suitable molds according to three different protocols (air-drying, freeze-drying after freezing at -20 °C or -196 °C). An initial experiment revealed that PC-12 cells adhered well to SF, remained metabolically active, and showed neurite outgrowth during cell differentiation when NGF was supplemented. The different processing conditions used for preparing NGF-loaded SF did not destroy the NGF bioactivity. The release of bioactive NGF over 4 weeks from both freeze-dried and air-dried formulations substantiated the potential of NGF-loaded SF materials for use in peripheral nerve defects.

The three NGF/SF matrix types released significantly different absolute amounts, but comparable relative amounts of NGF within the first 4 days (Figure 6d). During this initial phase, both freeze-dried matrices released substantially less than 1 ng NGF per mg scaffold and day (Figure 6a,b), whereas the SF film released between 2 and 4.5 ng NGF per mg scaffold and day (Figure 6c). After 5 days, the daily NGF release from all matrices remained very low, though it continued until the end of the experiment.

NGF loaded SF matrices were pre-incubated under release conditions for different time periods prior to transferring the matrix samples to adherent PC-12 cultures and assessing bioactivity. Co-incubation of matrix samples with PC-12 cells lasted for 4 days. At time zero, i.e., without pre-incubation, all matrices induced a similar extent of PC-12 differentiation (approx. 55%), which was slightly lower than that induced by 100 ng/ml NGF (approx. 70%; control). The pre-incubated air-dried NGF/SF film and freeze-dried Lyo-196 SF tube sustained sufficient NGF release during the entire 4-week period to trigger a consistently high PC-12 cell differentiation. On the contrary, the freeze-dried Lyo-20 NGF/SF tubes induced significantly less cell differentiation already after 1 week of pre-incubation, and the bioactivity of the Lyo-20 samples dropped further to 2–20% at weeks 2–4 of pre-incubation.

Nerve guidance channels (NGCs) as those proposed in this work have been shown to facilitate regeneration after transection injury to the peripheral nerve or spinal cord. Various therapeutic molecules, including neurotrophic factors, have improved regeneration and functional recovery after injury when combined with NGCs; however, their impact has not been maximized partly due to the lack of an appropriate drug delivery system. A deep investigation of the state of art of NGC and their application in neuronal tissue engineering will be extensively approached in Paragraph 4.

In this Paragraph we will describe now an interesting research by Piotrowicz and Shoichet [40] not yet tested *in vivo*.

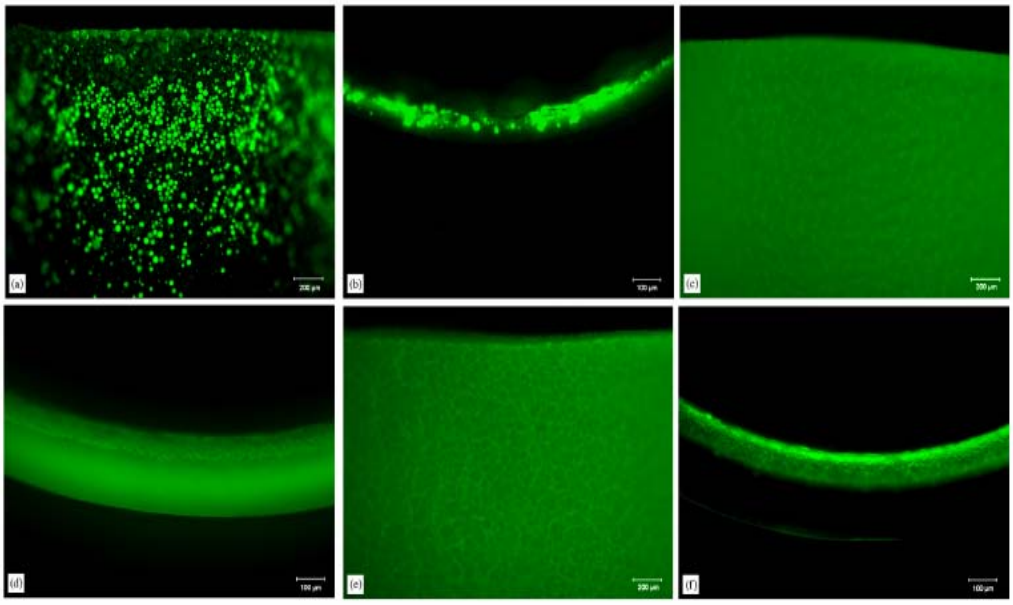

Figure 7. The distribution of protein in the three different NGC types was visualized by fluorescent microscopy using FITC-BSA: (a), (b) NGCs with FITC-BSA microspheres incorporated into an inner PHEMA layer (longitudinal and cross-sectional perspectives, respectively) had protein localized in the microspheres within the inner layer; (c), (d) NGCs with lumens soaked in FITC-BSA solution (longitudinal and cross-sectional perspectives, respectively) had protein distributed throughout the wall; and (e), (f) NGCs with FITC-BSA entrapped directly in an inner PHEMA layer (longitudinal and crosssectional perspectives, respectively) had protein localized within the inner layer. From [40]. Reproduced with permission from Elsevier.

To address some limitation related to the drug delivery system, nerve growth factor was incorporated into NGCs of poly(2-hydroxyethyl methacrylate-co-methyl methacrylate), P(HEMA-co-MMA). The NGCs were synthesized by a liquid–liquid centrifugal casting process and three different methods of protein incorporation were compared in terms of protein distribution and NGF release profile: (1) NGF was encapsulated (with BSA) in biodegradable poly(D,L-lactide-co-glycolide) 85/15 microspheres, which were combined with a PHEMA polymerization formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique; (2) pre-formed NGCs were imbibed with a solution of NGF/BSA and (3) NGF/BSA alone was combined with a PHEMA formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique. Using a fluorescently labelled model protein, the distribution of proteins in NGCs prepared with a coating of either protein-loaded microspheres or protein alone was found to be confined to the inner PHEMA layer (Figure 7).

Sustained release of NGF was achieved from NGCs with either NGF-loaded microspheres or NGF alone incorporated into the inner layer, but not from channels imbibed with NGF. By day 28, NGCs with microspheres released a total of 220 pg NGF/cm of channel whereas those NGCs imbibed with NGF released 1040 pg/cm and those NGCs with NGF incorporated directly in a PHEMA layer released 8624 pg/cm. The release of NGF from NGCs with microspheres was limited by a slow-degrading microsphere formulation and by the maximum amount of microspheres that could be incorporated into the NGCs structure.

The authors demonstrated that liquid–liquid centrifugal casting process can be used to incorporate protein, either directly or in microspheres, into the inner wall structure of P(HEMA-co-MMA) NGCs, thereby allowing for localized and sustained protein delivery to the lumen of the channels. Conversely, soaking NGCs in protein solution is not an effective means of incorporating protein into the channels for localized and sustained release applications. NGCs capable of serving a dual purpose as bridging implants and drug delivery vehicles offer a method to overcome the limitations of current delivery techniques, and are likely to be more successful in regenerating functional tissue.

As last example of *in vitro* drug delivery system for neuronal application, we would like to summarize some interesting results obtained at our laboratories employing alginate microspheres, even if not related to NGF release. This work [41] employs in fact Netrin-1 as cytokine, but could be easily adapted to release NGF.

Most components of the Netrin (“one who guides”, in Sanskrit) family are secreted proteins that govern the migration of neurons and growth cones. These proteins provide bifunctional signals that are chemoattractive for some neurons and chemorepellent for others, and can act as long or short range signals [42]. In general, Netrins act during the axons development and control a wide range of outgrowing axons and migrating neurons. To carry out their functions, Netrins interact with specific receptors that belong to two protein families: DCC and UNC-5. Netrins act either over large (a few millimetres) or short distances, depending on their local affinity for the plasma membrane and extracellular matrix [43]. Netrin-1 were incapsulated in alginate microparticle.

Alginate is a co-polymer extracted from some types of brown algae and it is made up of two uronic acids: D-mannuronic acid (M) and L-guluronic acid (G). By constituting the “skeletal” member of algae, alginate is strong and very flexible at the same time.

It can be soluble or insoluble in water, based on the type of salts that it creates. The sodium salts, the salts of ammonium and of other alkaline metals are soluble, while the salts of polyvalent ions, like calcium, are insoluble in water (except magnesium). Polyvalent cations are responsible for interchain and intrachain reticulations because they are tied to the polymer when two guluronic acid residuals are neighbours [44]. The reticulation process consists of the simple substitution of sodium ions with calcium ions. The relatively mild gelation process has enabled not only proteins, but also cells and DNA to be incorporated into alginate matrices with retention of full biological activity [45]. Furthermore, by selecting of the type of alginate and coating agent, the pore size, the degradation rate, and ultimately the release kinetics can be controlled.

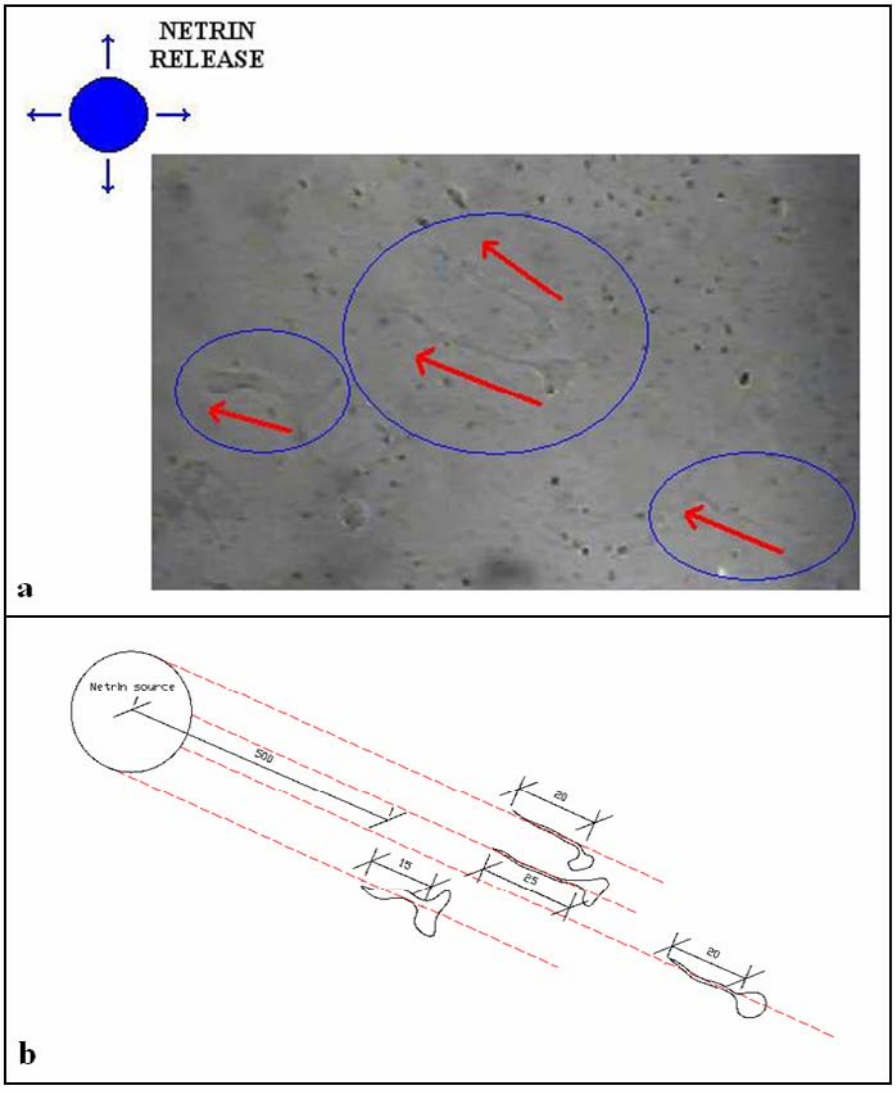

Figure 8. Axonal outgrowth directions, towards the Netrin release source. a) optical image, magnification 40X; b) graphical representation, distances expressed in μm. From [41]. Reproduced with permission from Springer.

Alginate microspheres, loaded with Netrin-1, were realized with a simple w/o emulsion and following reticulation with calcium ions. They were tailored in order to sustain a release of cytokine enough to polarize neuronal cells. The system was tested *in vitro* on embryonic hippocampal neurons of rat; the results are encouraging and have demonstrated the effectiveness of the release and the possibility to guide axons along defined trajectories. The axons have demonstrated a general lengthening as result of the treatment with the Netrin-loaded microspheres and, in many cases, an approaching towards the release source, as expected.

The photo shown in Figure 8a is particularly interesting: the direction of numerous axons is approximately parallel and directed towards the source of Netrin release. As shown by the graphical representation (Figure 8b) the distance from the Netrin source was about 500 μm, after six hours the axons were strongly oriented towards the Netrin source and the elongation was in the order of 25μm. The growth rate was therefore about 4 μm/h, while a typical growth rate, without neuronal growth factors, is about 1 – 2 μm/h [46]. The growth rate resulted lower than using a pipetting method (30 μm/h, [47]), but our method allows an active and fine control of the growth direction.

Preliminary *in vivo* experiments are currently in progress to develop an alginate based drug delivery system aimed at the regeneration and neuroprotection of the nervous system and/or providing a permissive environment and active signalling cues for guided axon growth.

3. Systems for *in Vivo* Controlled Delivery and Release of NGF

Up to last decade, long-term administration of growth factors has been limited to intraventricular infusions using cannulae or pumps. This route of administration requires repeated injections or refilling of pump reservoirs. Additionally, efficient intraparenchymatous concentrations of a neurotrophic factor, such as NGF, are difficult to sustain using current pump technology due to the degradation of the drug in solution and the low penetration of proteins from ventricular cerebrospinal fluid [48]. Moreover, due to the ventricular distribution, cells other than those targeted can respond to the factor, causing undesirable side effects [49]. Recent efforts have concentrated on the transplantation of genetically modified cells. Many neurodegenerative conditions could potentially be treated by the administration of neuroactive agents in a controlled manner through the use of genetically engineered living cells that secrete NGF. However, immortalised cells grafted into the CNS often give rise to tumor formation in the host tissue. Use of primary cells is a possible strategy to overcome this problem. Primary fibroblasts were genetically modified to produce human NGF [50] and it was determined that their intraparenchymal transplants would prevent injury-induced degeneration of cholinergic neurons in adult primate brain. More recently the use of genetically modified neural stem cell lines producing nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) has been proposed as a means to counteract neuronal degeneration in the excitotoxically lesioned striatum [51].

Cell transplants offer promise for treatment of neurological disorders but creating a local environment for transplanted brain cells that enhances this regenerative process is essential. Recently, the use of polymer-encapsulated cells was proposed. Genetically engineered rat fibroblasts producing NGF were encapsulated in alginate-polylysine-alginate gels with the objective to produce viable "minifactories" continuously producing and secreting NGF into the rat brain [52]. Biochemical analysis of microdissected brain tissues of lesioned animals treated with encapsulated NGF-secretor fibroblasts showed a significant increase in choline acetyltransferase (ChAT) activity in cortices adjacent to the lesion but not far from it (entorhinal cortex). This may indicate a gradient of concentration of the released NGF and/or differential responsivity of lesioned vs non-lesioned target tissue. In addition, experimental results demonstrated that NGF from encapsulated genetically engineered fibroblasts can be secreted enough to prevent degenerative changes of cholinergic neurons in the nucleus basalis magnocellularis.

An alternative strategy was proposed by Mahoney and Saltzman [53] which creates local and sustained levels of insoluble and soluble molecules directly at the site of cell transplantation in the brain. PLA/PGA copolymers were used to incorporate nerve growth factor (NGF) and release it at controlled rates. Cells were assembled with the cell-adhesive/controlled-release microparticles to form transplantable neo-tissues. NGF delivery by way of the synthetic microenvironment increased levels of NGF-induced biological activity over the course of 21 days *in vivo*.

Direct gene transfer to the brain has also been evaluated [54]. The trophic effect of a recombinant adenovirus encoding nerve growth factor (AdNGF) *in vivo* on basal forebrain cholinergic neurons of aged rats, a neuronal population affected during normal and pathological aging, was evaluated. Three weeks after unilateral injection of the recombinant adenovirus into the nucleus basalis magnocellularis, a significant increase in the somal areas of cholinergic neurons ipsolateral to the injection was observed. A single intracerebral injection of AdNGF produces trophic effects similar to those resulting from chronic intracerebroventricular high levels of NGF. These findings indicate that recombinant adenoviruses encoding growth factors are potentially powerful tools for improving neuronal deficits associated with degenerative processes.

All these strategies allow a prolonged and intraparenchymatous delivery of the protein, but the safety of the method and the long-term expression of the transgene still need to be determined. Moreover, the delivered doses that depend on the cell survival and the stability of the transfection could be difficult to control in a precise manner.

In the past years, some groups focused on polymer matrix, implanted by stereotaxy, for direct NGF delivery to the brain. The first polymeric devices developed were macroscopic implants also called monolithic devices [55]. These macroscopic implants are shaped as a disc, a wafer or a cylinder. But in animal design, the size was often too large for clinical design, the size and shape needed invasive surgery for implantation. The potential applications of the microencapsulation of therapeutic agents to provide local controlled drug release in the central nervous system were thus investigated. Due to their size, microparticles in suspension can be easily implanted by stereotaxy. The first system studied was a non-biodegradable pellet (ethylene-co-vinyl acetate copolymer matrix) which showed an *in vitro* controlled release of active NGF over 1 month [56] and, after implantation, a neuroprotective

effect on basal forebrain cholinergic neurons [57]. In the following years, research was focused on biodegradable microspheres. This new approach was first developed for interstitial chemotherapy of brain tumors and is now in clinical evaluation [58]. An atelocollagen matrix was also studied for NGF delivery and the protective effect of these minipellets has been demonstrated on hippocampal ischemic neuronal death [59]. Biodegradable microspheres were then developed. Due to their size, these microparticles can be easily implanted by stereotaxy in precise and functional areas of the brain, without causing damage to the surrounding tissues. This implantation avoids the inconvenient insertion of large implants by open surgery and can be repeated under local anesthesia if required. Different types of polymers such as a hyaluronane derivative [60] and alginate-polylysine [61] were used, but biodegradable microspheres made of PLGA were particularly developed. Experimental studies and the long history of the clinical use of this type of copolymer, particularly as surgical sutures, have demonstrated its excellent histocompatibility [62]. Intrastriatal implantation of NGF loaded poly(D,L-lactide-co-glycolide) microspheres has been investigated [63]. These microspheres were implanted into the rat striatum 7 days prior to infusing quinolinic acid (QA) which induces a local excitotoxic lesion and is an accepted animal model of Huntington's disease. The efficiency of the microsphere approach is demonstrated by the fact that NGF-releasing microspheres reduce the extent of the excitotoxic lesion in the striatum by 40%, compared to the control groups (Figure 9).

In addition, marked neuronal protection was noticed within the lesioned area in the animals containing NGF-releasing microspheres. This protection principally involved the cholinergic ganglia but also neuropeptide Y/somatostatin interneurons and GABAergic striatofuge neurons.

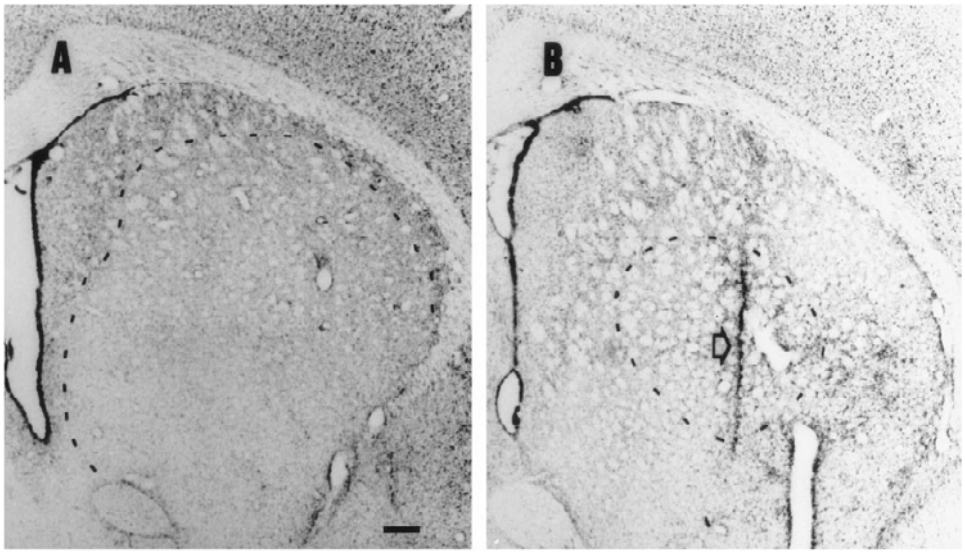

Figure 9. Coronal sections at the level of the largest lesion area after cresyl violet staining: (A) blank microsphere-receiving rat; (B) NGF-loaded microsphere-receiving rat (the arrow shows the QA injection site and the dotted line indicates the lesion border for purpose of measurement). Note significant reduction in lesion size in animal receiving NGF (bar, 250 μm). From [63]. Reproduced with permission from Elsevier.

These results indicated that implantation of biodegrable NGF-releasing microspheres can be used to protect neurons at a specific target from excitotoxin-induced lesions and this strategy may ultimately prove to be relevant for the treatment of Huntington's disease. In other studies it has been shown that supplementation of adrenal medullary grafts with NGF delivered from PLGA microspheres resulted in a marked behavioural improvement in the hemi-Parkinsonism rat model [64]. NGF-loaded microspheres prepared from a hyaluronane derivative [60] and alginate-polylysine [61] were also studied. The latter microspheres prevented neuronal degeneration which occurs in the nucleus basalis magnocellularis after cortical devascularizing lesion, in a similar way to NGF delivered via an osmotic pump for one week.

Krewson *et al*. studied the distribution of NGF entrapped in poly(ethylene-co-vinyl acetate) (EVAc) disks and poly(L-lactic acid) (PLA) microspheres within the brain interstitium. To improve NGF retention in tissue, NGF was conjugated to 70,000 molecular weight dextran and incorporated into a polymeric device. The distribution of NGF was enhanced by conjugation; comparison of NGF concentrations in the brain to a mathematical model of diffusion and elimination suggested that the elimination rate of NGF-dextran conjugate in the tissue was over seven times slower than the elimination rate of NGF. These results indicate that variation of the properties of the controlled release system may be useful in regulating the time course of NGF delivery to tissue, and that modification of the NGF itself can improve penetration and retention in the brain [65].

Among the different methods have been used to facilitate the transporting of drugs into the brain, protein or monoclonal antibody conjugated with the therapeutic drug were proposed. They showed to transport the drug into the brain much easier by receptor-mediated transcytosis, allowing to obtain better targeting efficiency [70]. In order to protect biology activity of the protein vectors, and improve the drug carrying ratio of the vectors, active targeted liposomes have been proposed [71]. The brain distribution of such NGF liposomes was studied *in vivo*. In this study RMP-7, a ligand to the B2 receptor on brain microvascular endothelial cells, was combined with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylenegly-col)]-hydroxy succinamide (DSPE-PEG-NHS) to obtain DSPE-PEG-RMP-7. Then DSPE-PEG-RMP-7 was incorporated into the liposomes' surface to target sterically stabilized liposomes (SSL-T) to the brain. The highest percent of NGF encapsulated into liposomes was about 34%, and the average size of liposomes was below 100 nm. The results indicated that the liposomes could enhance transport of NGF across the BBB. The amount of NGF in the brain was increased in the order: NGF-SSL-T > NGF-SSL + RMP-7 > NGF-SSL > NGF-L (NGF conventional liposomes). The maximum concentration of NGF was recorded in 30 min following the intravenous injection. In particular, a majority of NGF was distributed in striatum, hippocampus and cortex, and the concentration of NGF was relatively lower in olfactory bulb, cerebellum and brain stem [72].

Despite recent advances in the understanding of nerve regeneration and in surgical techniques, the complete functional recovery in a damaged nerve is rare [73]. The most common method of nerve repair is direct repair and nerve autograft, which are associated with less than optimum results [74]. One alternative method to autografting is to use a nerve guidance conduit (NGC) to connect the proximal and distal ends of the severed nerve and guide the regeneration of axons back to the appropriate target.

Table 1. Intracranially implantable NGF-loaded microspheres

Polymer	Microparticle size	Drug	Reference
Hyaluronane derivative	2–21 μm	NGF NGF+GM1	[64]
Alginate-polylysine	50 μm	NGF	[60]
Poly(DL-lactide-co-glycolide)	17.2 μm	NGF	[63-66-67]
Poly(DL-lactide-co-glycolide)	2.5 μm	GM1+NGF	[66]
–	(latex)	NGF-BDNF	[65]
Poly(DL-lactide-co-glycolide)	8–11 μm	NGF	[68]
Poly(DL-lactide-co-glycolide)	50 μm	NGF	[69]
Poly(DL-lactide-co-glycolide)	25 μm	NGF	[28]

These tubes allow the microenvironment of regeneration to be controlled by manipulating the contents of the NGC. Numerous studies have been performed to determine some of the fundamental mechanisms of regeneration by varying the conditions within such tubes.

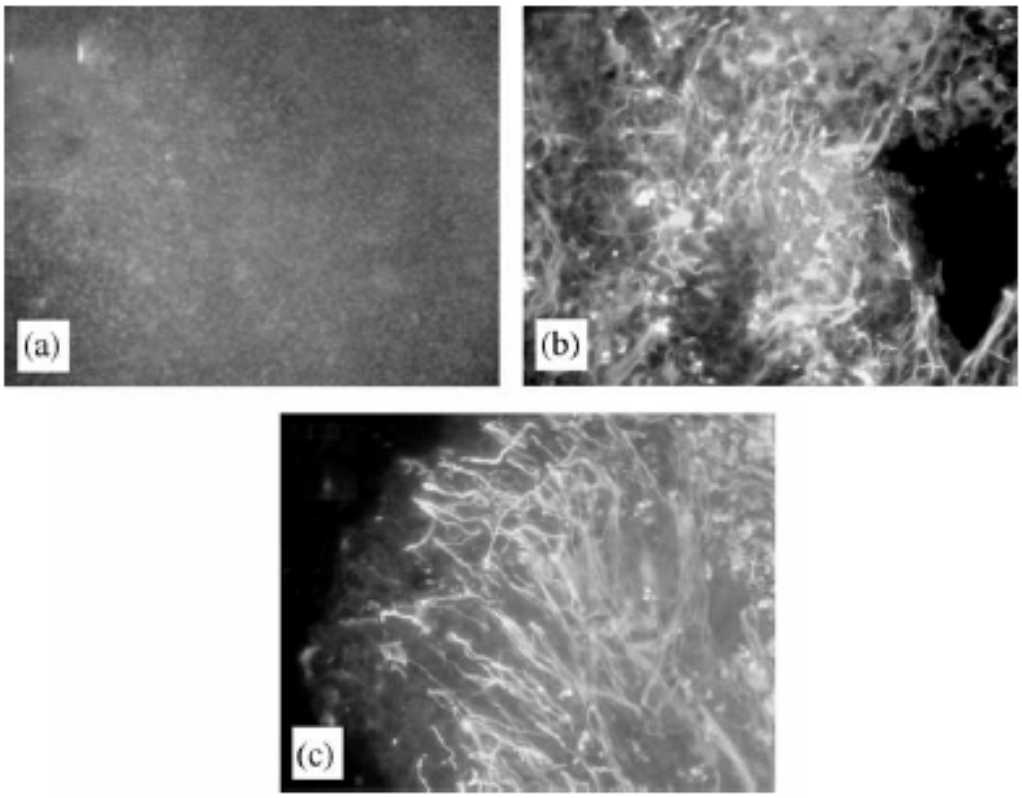

Figure 10. Effect of NGF-loaded microspheres on nerve regeneration with silicon tubes. Micrographs of sections showing tissues collected 2 weeks after implantation from a control tube loaded with BSA microspheres (A), a control tube containing 100 ng of NGF dissolved in saline (B), and the tube filled with NGF microspheres (C). Note NF68 immunoreactive axons at the distal stump in (B) and (C). Original magnification 400 X. From [79]. Reproduced with permission from Elsevier.

Neurotrophins have been shown to enhance peripheral nerve regeneration. NGF facilitates peripheral nerve regeneration in adult animals [75]. NGF was reported to protect neurons from injury-induced death in lesioned sciatic nerves [76]. Addition of NGF to saline-filled silicon tubes resulted in an increase in the number of myelinated axons in the regenerating nerve [77]. NGF has also been shown to facilitate regeneration of hippocampal neurons across a peripheral nerve bridge [78].

Controlled delivery of NGF to a target tissue by biodegradable polymer microspheres has been explored also for its potential applications in the rigeneration of the PNS. Xu *et al.* investigated the potential of polyphosphoester (PPEs) microspheres as carriers for NGF release in a rat sciatic nerve. These NGF containing microspheres were incorporated into the nerve guide conduits that were implanted to bridge a 10 mm gap in a rat sciatic nerve model. Two weeks after implantation, immunostaining with an antibody against the neurofilament protein confirmed the presence of axons at the distal end of regenerated cables within the NGF microsphere-loaded conduits ([79], Figure 10).

While the majority of growth factor delivery systems are based on diffusion of growth factors from degradable polymers, other researchers have also studied affinity based delivery systems that immobilize and release growth factors based on non-covalent interactions.

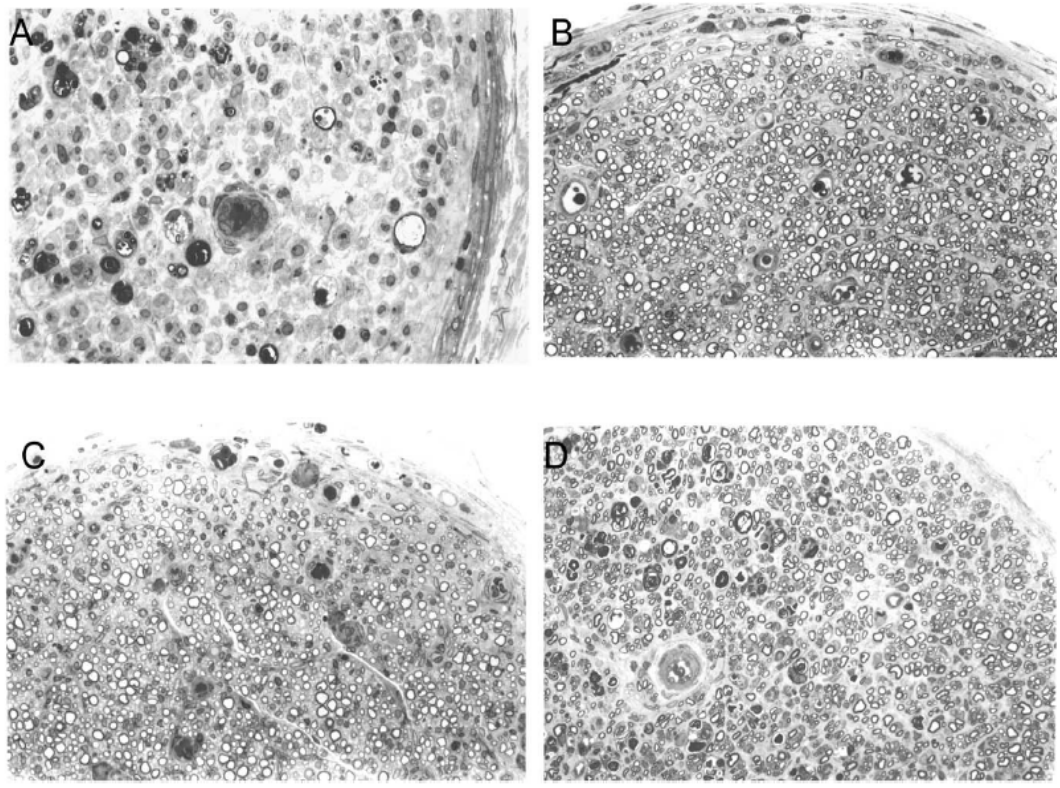

Figure 11. Histological sections of regenerating nerves at the midline of the conduit (or graft). (A) 5 ng/mL NGF + delivery system (DS); (B) 20 ng/ml NGF + delivery system; (C) 50 ng/mL NGF + delivery system; and (D) isograft (positive) control. From [80]. Reproduced with permission from Elsevier.

An example of such drug delivery system consists of heparin-binding peptides covalently immobilized within a fibrin matrix, heparin bound to the immobilized peptides, and NGF [80]. The non-covalent interactions serve to sequester growth factor by slowing its diffusion through the fibrin matrix and to allow the rate of release to be increased when the matrix is degraded by infiltrating cells during nerve regeneration. Silastic nerve guide conduits filled with the fibrin-based delivery system and of NGF were implanted in a 13-mm rat sciatic nerve lesion and the progress of nerve regeneration was examined at 6 weeks post implantation using histomorphometric analysis. Axonal regeneration in the delivery system groups revealed a marked dose-dependent effect. Results in all morphometric measurements demonstrate that an optimal NGF dose (20 and 50 ng/ml NGF), when combined with the delivery system, was not statistically different from the positive isograft control that provides the clinical "gold standard" for long gap peripheral nerve repair. Furthermore, the regeneration is comparable in terms of fiber diameter (maturity) and fiber density (at the conduit midline, Figure 11) to that observed in end-to-end (short gap) nerve repairs. These results are rather remarkable considering that the conduit gap is 13 mm in length, significantly larger than the critical defect length (10 mm) for rat sciatic nerve and that the conduit lacks both the cellular components of nerve grafts and the basal lamina, both of which are hypothesized to facilitate regeneration in nerve grafts.

4. NGF Controlled Release Systems for Tissue Engineering

Tissue engineering techniques are being used to develop constructs to replace natural nerve grafts. Tissue engineered constructs for nerve regeneration consist of one or more of the four components: (i) scaffold, usually tubular along with structural elements such as fibers, gels etc., (ii) growth factors, (iii) ECM proteins, and (iv) cells. In general, scaffolds for nerve repair should support axonal proliferation, have low antigenicity, support vascularization, be porous for oxygen diffusion and avoid long term compression. The scaffold can be made from natural or synthetic materials.

A nerve injury usually results in disruption of communication between the target organs and the neuronal cell body, and leads to Wallerian degeneration (breakdown of myelin sheath and axons). Due to cytokines released during Wallerian degeneration, Schwann cells are activated and produce neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Although many other trophic factors, including insulin-like growth factor (IGF), fibroblast growth factor (FGF) and ciliary neurotrophic factor (CNTF), have been shown to be involved in the promotion of nerve regeneration [81], it is believed that they are released from Schwann cells following mechanical damage to the cells.

NGF is produced in the target organs of sensory and sympathetic nerves in the and has been shown to stimulate and promote the survival of sensory ganglia and nerves, including spinal sensory nerves and sciatic nerves.

Many critical phenomena in tissue development, e.g,. cell patterning, motility, proliferation, aggregation and gene expression, are influenced by soluble signalling proteins like NGF. A major goal of tissue engineering is the use of the techniques of modern

biotechnology for the regeneration or replacement of lost cells and tissue. As in development, regeneration of functional tissue requires maintenance of cell viability and differentiated function, encouragement of cell proliferation, modulation of the direction and speed of cell migration, and regulation of cellular adhesion. Most applications of tissue engineering, such as the regeneration of spinal cord tissue lost through trauma, or the replacement of liver tissue lost due to disease, will require simultaneous control of complex cellular behaviours. The basic principles for regulating the assembly of cells into a functional tissue have not yet emerged, but it is clear that soluble signalling proteins provide valuable tools for the control of many of the cellular behaviours associated with tissue formation [82].

Some of the most promising systems for the controlled release of peptides and proteins involve encapsulation or entrapment of proteins in biocompatible polymeric devices. These devices have found widespread use in the treatment of cancers and other life-threatening diseases [83]. Protein growth factors provide a powerful tool for the regulation of cell behaviour. Unfortunately, the development of proteins as pharmaceuticals presents many challenges. The first generation of technology for delivery of active proteins to target cells in adequate concentrations for extended periods of time are now available for use in tissue engineering. Further development of polymeric controlled delivery techniques, such as stabilization of growth factors and delivery of gene transfer agents, should expand the opportunities for future tissue engineers.

Strategies to promote axonal extension through a site of injury include both the provision of nervous system growth factors and implantation of substrates to support axon extension, such as cellular grafts. In general, however, the growth of axons is highly random and does not extend past the lesion site and into host tissue [84]. Physically guiding the linear growth of axons across a site of injury, in addition to providing neurotrophic and/or cellular support, would help to retain the native organization of regenerating axons across the lesion site and into distal host tissue, and would potentially increase the probability of achieving functional recovery.

The use of biopolymers can be a practical tool to provide neurotrophic and/or cellular support while simultaneously guiding axonal regeneration. Indeed, several natural and synthetic polymers, including poly-(a-hydroxyacids), collagen, fibronectin, and hyaluronic acid, have been used as scaffolds or within scaffolds for peripheral and central nerve regeneration, and have been reviewed in detail [85]. Many of the fabrication technologies for these polymers are based on particulate-leaching techniques, heat compression, and extrusion [86]. However, the harsh operating conditions of these processes can limit the incorporation of bioactive proteins and cells, and residual amounts of the chemical solvents required may cause toxicity *in vivo*. Freeze/dry processing is an alternative method for producing porous scaffolds that does not require additional chemicals, relying instead on the water already present in hydrogels to form ice crystals that can be sublimated from the polymer, creating a particular micro-architecture. Because the direction of growth and size of the ice crystals are a function of the temperature gradient, linear, radial, and/or random pore directions and sizes can be produced with this methodology [87]. In the study presented by Stokols and Tuszynski [88], a novel procedure was developed for using freeze-dry processing to create nerve guidance scaffolds made from agarose, with uniaxial linear pores. Previous *in vivo* studies have identified agarose, among several candidate biopolymers, as an optimal material to use

in a nerve regeneration scaffold based on its biocompatibility, inertness, and stability in the spinal cord. The agarose scaffolds fabricated in this study, which could later be filled with extracellular matrix molecules and growth factors, were characterized for microstructure, water absorbability, *in vitro* degradation, biocompatibility, and growth factor loading. NGF was used as a model growth factor to assess scaffold growth factor loading, release, and bioactivity properties. PC-12 cell cultures were used to assay the bioactivity of NGF released from scaffolds.

Recombinant NGF protein was included in the scaffolds in one of two ways. In the first method, the pores of the scaffold were injected with polymers of extracellular matrix molecules that have previously been identified as potential substrates for axonal regeneration: collagen (3 mg/ml) or hyaluronic acid (40 mg/ml).

Prior to injection into the scaffolds, 3 ng NGF (100 ng/ml) was mixed into 30 µl of collagen or HA solution. In the second method, NGF (300 ng/ml) was added to the agarose prior to polymerization and freeze dry processing, allowing NGF incorporation into the scaffold material itself (30 ng/scaffold). Both types of NGF-containing scaffolds were placed in a sterile incubator at 37°C for 1, 2, 3 or 4 weeks. For each time point and each type of NGF-containing scaffold, a scaffold was placed inside a well containing PC-12 cells. After 24 h, neurite extension was evaluated by quantifying the number of cells extending neurites greater than one cell diameter. In the presence of NGF containing scaffolds, the proportion of PC-12 cells extending neurites (43±8.1% $p<0.001$) was indistinguishable from the proportion of cells extending neuritis when NGF protein was added directly to the medium (47±6.8%). In contrast, few PC-12 cells extended neuritis in the absence of NGF (5.0±3.1%). Freeze-dried agarose scaffolds created in this study have a number of favourable properties for potentially supporting axonal regeneration after nervous system injury. The hydrated scaffolds are soft and flexible, and contain linear guidance pores extending through their full length. Because the scaffolds are fabricated without the use of organic solvents and are stable under physiological conditions without chemical crosslinking, there is no risk of introducing toxic molecules to the site of injury. Finally, the scaffolds can be readily loaded with diffusible growth stimulating proteins (NGF).

Yang and colleagues developed an approach for fabrication of nerve conduits with single and multiple lumens (Figure 12) capable of controlled release of neurotrophic factors [89].

These conduits were fabricated from a mixture of poly(lactide-co-glycolide) (PLG) microspheres and a porogen (NaCl) that was loaded into a mold and processed by gas foaming. The porosity and mechanical properties of the constructs were regulated by the ratio of porogen to polymer microsphere. Nerve growth factor (NGF) was incorporated into the conduit by either mixing the protein with microspheres or encapsulating the protein within microspheres prior to gas foaming.

The leaching step for the conduits, which is performed to create a porous structure, resulted in losses ranging from 3% to 14% of the incorporated protein. Conduits fabricated by mixing of lyophilized NGF with the microspheres prior to foaming resulted in greater losses during the leaching step (> 11%) relative to NGF that was encapsulated into the microspheres (ranged from 3% to 8%). For the same polymer composition, NGF loss during leaching increased as the porogen to polymer ratio increased from 2:1 to 10:1.

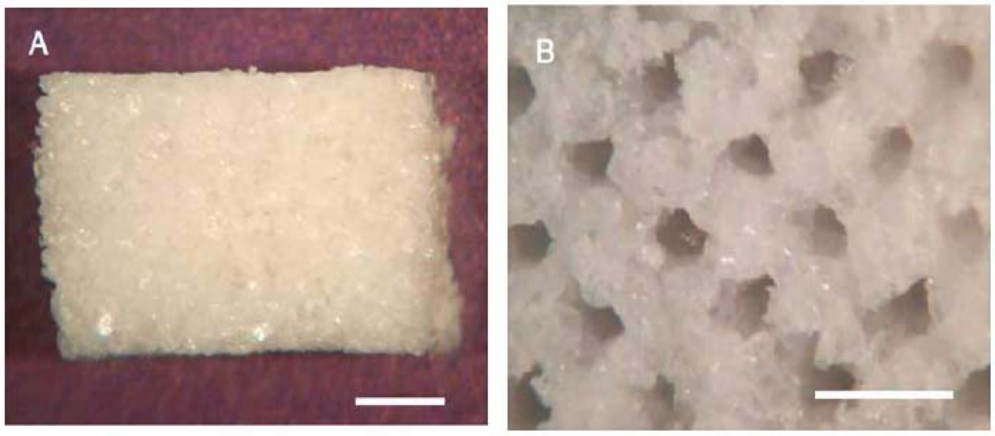

Figure 12. Multiple lumen conduits. Conduits were fabricated with HMW PLG containing 18 channels (diameter=250 μm) and visualized by light microscopy. Conduit is visualized from the top (A, scale bar=1 mm) and from the end (B, scale bar=500 μm). From [89]. Reproduced with permission from Elsevier.

A sustained release of protein from the porous conduits was observed for at least 42 days, with the release rate primarily dependent upon the mechanism of incorporation. Nerve conduits with single lumens were formed from microspheres composed of either (i) high molecular weight polymer alone, or (ii) blended high and low molecular weight polymer prior to microsphere fabrication. The release from the conduits was sustained for at least 42 days, and the percentage of incorporated protein released was statistically greater for the mixed formulation than for encapsulated microspheres. The polymer composition did not affect the release profile for conduits formed with lyophilized protein mixed with the microspheres. However, for conduits formed by protein encapsulation into microspheres, the release profile is dependent upon the polymer composition, with faster release observed for microspheres containing 25% low molecular weight polymer and 75% high molecular molecular weight polymer. Varying the porogen to polymer ratio from 2:1 to 10:1 indicated that the release is not dependent upon the porogen content. However, the absence of porogen during conduit fabrication resulted in an initial burst of protein during the initial 48 h, with no significant release for the following 36 days NGF released from porous PLG conduits (single and multiple channels) was bioactive and stimulated neurite outgrowth by DRG. NGF collected from the release medium at 1 day, 7 days, and 14 days stimulated neurite outgrowth by primary DRG (Figure 13). Furthermore, the average neurite length at 24 h of culture was not statistically different for the released NGF compared to fresh NGF, indicating that the protein retains bioactive. This bioactivity was seen for NGF released from single lumen or multiple lumen conduits. For single lumen conduits, bioactivity was observed for both mixing protein with the microspheres and encapsulating protein in the microspheres. Negative controls of release media from conduits without NGF did not elicit neurite extension.

This approach to fabricate conduits with single or multiple lumens that are capable of controlled protein delivery showed satisfactory results. The conduits have sufficient mechanical properties, controlled by porogen content, to maintain open channels that allow for tissue ingrowth *in vivo*.

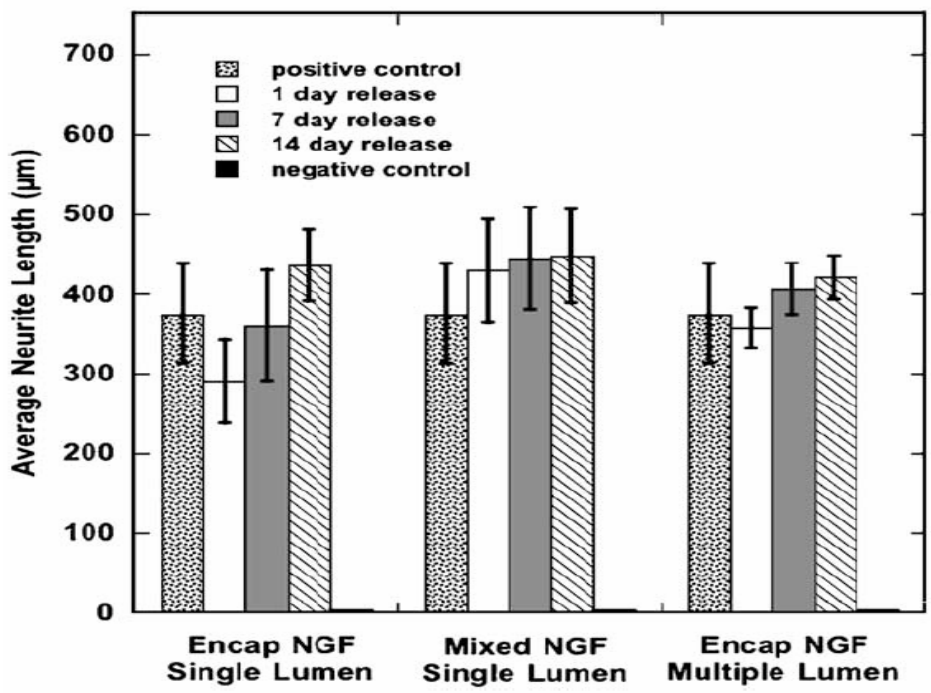

Figure 13. Bioactivity of released NGF. Conditions tested include: single lumen conduit with encapsulated NGF, single lumen conduit with mixed NGF, and multiple lumen conduit with encapsulated NGF. NGF released at different time points was assayed for the ability to stimulate neurite extension by primary DRG neurons ($n \geq 3$). No statistical difference was obtained between the experimental and control conditions ($p > 0.05$). From [89]. Reproduced with permission from Elsevier.

Protein delivery from the conduit is regulated by the mechanism of incorporation (encapsulated versus mixed), the polymer molecular weight, and the presence of porogen. The combination of a nerve conduit and controlled protein delivery has the potential to support and promote regeneration in the nervous system.

In Xu *et al.* [90] we can find an example of neural conduits embedded with NGF-loaded microspheres. In this study it was investigated whether sustained release of NGF within nerve guide conduits (NGCs) would augment peripheral nerve regeneration. NGF-containing polymeric microspheres fabricated from a biodegradable poly(phosphoester) (PPE) polymer were loaded into silicone or PPE conduits to provide for prolonged, site-specific delivery of NGF. The conduits were used to bridge a 10 mm gap in a rat sciatic nerve model. Three months after implantation, morphological analysis revealed higher values of fiber diameter, fiber population and fiber density and lower G-ratio at the distal end of regenerated nerve cables collected from NGF microsphere-loaded silicone conduits, as compared with those from control conduits loaded with either saline alone, BSA microspheres, or NGF protein without microencapsulation (Figure 14). Beneficial effects on fiber diameter, G-ratio and fiber density were also observed in the permeable PPE NGCs. Thus, the results confirm a long-term promoting effect of exogenous NGF on morphological regeneration of peripheral nerves. The tissue-engineering approach reported in this study of incorporation of a microsphere protein release system into NGCs holds potential for improved functional recovery in patients whose injured nerves are reconstructed by entubulation.

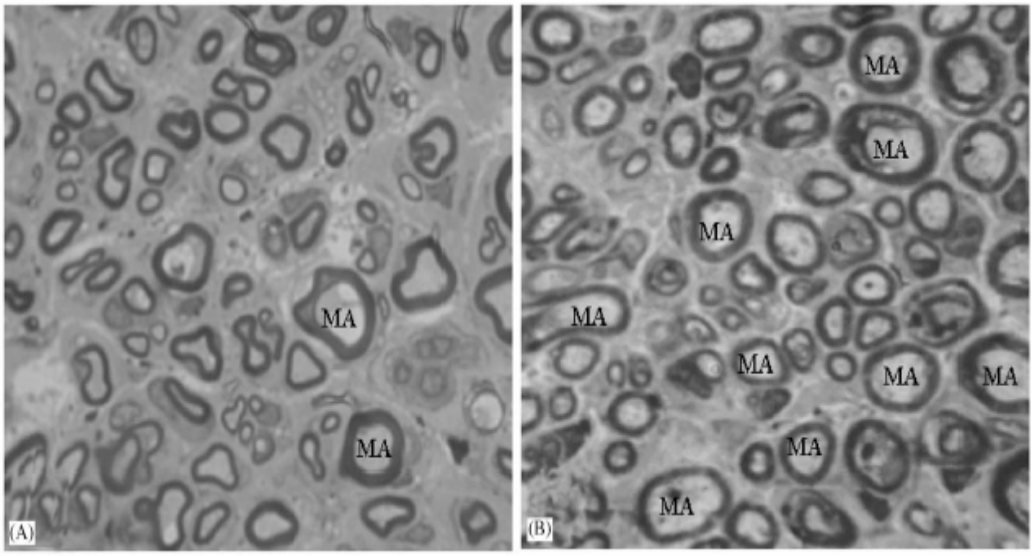

Figure 14. Effects of NGF-loaded microspheres on nerve regeneration with silicon tubes. Micrographs of semithin cross sections of distal nerve segments from rats implanted with a conduit loaded with BSA (A) and with NGF/BSA (B), respectively. Note the increased number of MAs (myelinated axons) in (B); Toluidine blue staining. Magnification 1000 X. From [90]. Reproduced with permission from Elsevier.

Previous studies of the rat sciatic nerve model have demonstrated an increase in nerve regeneration at 3 or 4 weeks following introduction of exogenous NGF in a free protein form into silicon NGCs [91]. After that period, no improvement in overall histological appearance could be seen [92], probably because of the failure in maintaining sufficient NGF concentrations over the long duration of nerve regeneration. Other studies using various nerve conduits in combination with single administration of NGF in different animal models showed basically the same finding that NGF may enhance sensory [93] and motor [94] nerve regeneration between 3 and 7 weeks. The major finding of the study of Xu *et al*. is the improved nerve regeneration for at least 3 months after applying a new tissue-engineering approach incorporating a microsphere NGF delivery system into NGCs. Understanding the features associated with our method and its application may help to develop more effective therapeutic approaches that promote functional recovery of injured nerves.

The study of Chen and colleagues [95] attempted to enhance the efficacy of peripheral nerve regeneration using gelatin-tricalcium phosphate (GTG) conduits by incorporating them with nerve growth factors and cultured Schwann cells. The nerve growth factors were covalently immobilized onto the GTG conduits (GEN) using carbodiimide. Schwann cells were harvested from neonatal Lewis rats, cultured for seven days and injected into the GEN conduits. The experiment was performed in three groups: GTG conduits, GEN conduits and GEN conduits with Schwann cells injected (GEN+Sc). The effects of different conduits (GTG, GEN and GEN with Schwann cells) on the peripheral nerve regeneration were evaluated in rat sciatic nerve repair model. 24 weeks after implantation of conduits, degradation of the conduits in all groups was illustrated by the fragmentation of the conduits. All conduits were well tolerated by the host tissue. Under microscopic evaluations,

regenerated nerve tissue with myelinated and unmyelinated axons was present in all groups. Histomorphometrically, the total nerve area of GEN+Sc group was significantly higher than GTG group. Conversely, the autonomy score evaluated 12 weeks after nerve repair showed better results for GTG group. Besides, GEN+Sc group had the highest average recovery index of compound muscle action potential, but the difference among each group did not reach statistical significance. Although the electrophysiological recovery of nerve was not significantly improved with GEN+Sc conduit, nerve repair using tissue engineered conduits still provided better histological results.

This research represent an interesting effort using the tissue engineering approach to enhance the regeneration of peripheral nerve. Although histomorphological evaluations showed the potential of GTG conduits incorporated with NGF and Schwann cells to improve nerve regeneration, recovery of CMAP (compound muscle action potential) was not significant. Furthermore, autonomy seemed to be the price paid for enhanced nerve regeneration. Therefore, further investigation with alternative neurotrophic factors and modified scaffold geometry may provide the possibility for more improvement in both nerve regeneration and functional recovery.

The goal of enhancing nerve regeneration across transection injuries by implanting tubes filled with matrices that either direct regrowing axons via contact guidance or stimulate regrowth with neurotrophic factors has been pursued extensively. These tubes are variously referred to as nerve guides, nerve conduits, nerve chambers, and entubulation chambers. Rosner et al. [96] describe an investigation of the parameters necessary to develop a contact guiding and neurotrophic nerve guide capable of sustaining NGF concentrations above 1 ng/ml for over two months: ~1 ng/ml is the approximate minimum concentration needed to sustain neurite survival and growth from DRG [97]. The contact guidance is provided by magnetically aligned collagen fibrils [98] and the sustained neurotrophism is established by the entrapment of either NGF-secreting cells or NGF-releasing polymer microspheres in the aligned collagen. The equation characterizing the microsphere release rate is incorporated into a mathematical model that describes NGF concentration in the gel as a function of position and time.

Parameters contained within the model requiring experimental determination include the diffusion coefficients of NGF in both the gel and the membrane, a source term describing the rates at which NGF is supplied to the system by the cells or the microspheres, and the limiting number of cells or microspheres which can be seeded within the construct without compromising collagen alignment. Predictions of NGF concentrations within the gel are then computed from a finite element model for the general case and from an analytical solution in the limit of a quasi-steady-state concentration gradient across the nerve guide's membrane. Predicted NGF concentrations over a two month period using entrapped microspheres are compared to those using entrapped SCs, whose release rates are based on published and experimentally determined values. In addition, the feasibility of maintaining the target NGF concentration of 1 ng/ml in the nerve guide for two months is assessed for the standard rat sciatic nerve model and a human median nerve analog.

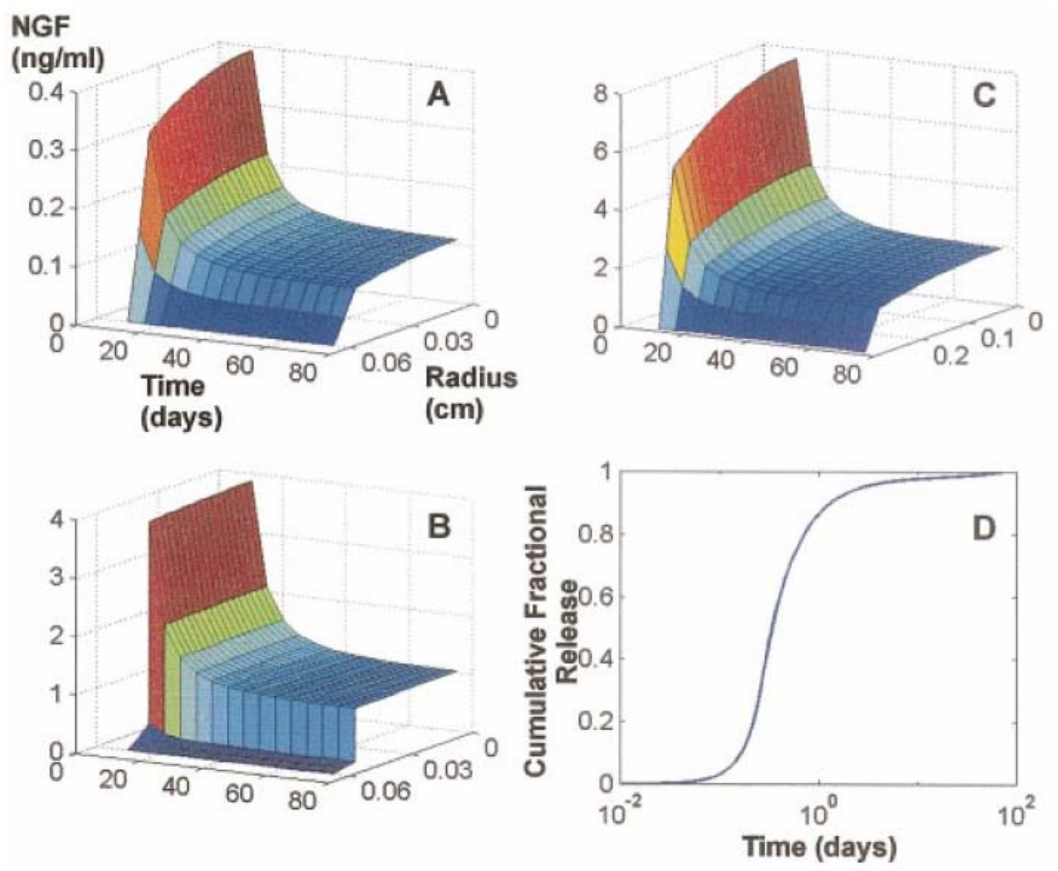

Figure 15. (a) NGF concentration as a function of radius and time in a rat sciatic nerve implant with microspheres and collagen membrane, plotted for t>10 days; (b) rat sciatic nerve implant with hypothetical membrane of permeability 1.00 10^{-6} cm/s (reduced by decreasing the partition coefficient), enabling retention of 1 ng/ml concentration; (c) human median nerve implant with microspheres and collagene membrane; (d) cumulative fractional release of NGF from microspheres. From [96]. Reproduced with permission from Springer.

Figure 15 shows the main results of these simulation, in particular when microspheres are used in the rat sciatic and human median nerve models. Computed results are only plotted from day 10 onward in order to permit detailed visualization of the post-burst phase which otherwise appears flat on the scale of the large burst. Figure 15a,c show the predicted concentration profiles when the gel is bounded by the collagen membrane for the rat and human nerve systems, respectively. Figure 15b demonstrates the effect of a hypothetical membrane of permeability 1.0 10^{-6} cm/s, which enables maintenance of the desired NGF concentration in the rat model. Figure 15d indicates the microsphere release profile.

The investigation proposed by Rosner and colleagues provides a rational and practical approach to designing a nerve guide which exploits both the mechanisms of contact guidance and neurotrophism. It demonstrates the expectation that physiologically relevant concentrations of NGF can be theoretically maintained for two months in a human median nerve analog, using either controlled-release microspheres, or NGF-secreting Schwann cells, and that similar results can be achieved in smaller constructs either with cells transfected to

hypersecrete NGF, or with microspheres and only a moderate decrease in membrane permeability.

It is however difficult to actually deliver active growth factors controllable over the entire duration of regeneration with this methods. Transplanted genetically modified cells are a possible alternative that pose advantages as a means to deliver a continual supply of active neurotrophins [6]. Furthermore, if the gene expression in the modified cells can be turned on and off, it is probable that expression could be directed in a complex manner. For example, a cascade of neurotrophin expression could lead axons to grow into a graft, switch patterns of expression, and then lead axons out of the graft.

Genetically modified fibroblasts have been a highly studied model for the delivery of neurotrophins, in particular NGF [17-99-100]. Particularly interesting is a study by Hu and colleagues [101], that employ NIH3T3-NGF for applications in engineered dermal tissue. In order to find a suitable carrier to deliver the product of gene transfection to improve the performance of bioengineered dermis, they used microencapsulation and gene transfection technology together for the first time. Recombinant nerve growth factor (pcDNA3.1+/NGF) was used to modify NIH3T3 cells genetically. Control of NIH3T3-NGF cells were encapsulated within microspheres composed of alginate-poly-L-lysine-alginate and cultivated *in vitro*.

The concentration of NGF released from the microencapsulated NIH3T3-NGF cells was confirmed using ELISA assay. NIH3T3-NGF cells, NIH3T3 cells (control) microencapsulated were co-cultivated with human keratinocytes and fibroblasts, and tested the percentage of cycle of these cells. The alkaline hydrolysis method was used to analyse the content of hydroxyproline (Hyp). Immunohistochemistry method was used to calculate the transformation efficiency from fibroblasts to myofibroblasts. The concentration of NGF released from the microencapsulated NIH3T3-NGF cells lasted about six weeks in the supernatant of bioengineered dermis *in vitro*. The proliferation of keratinocytes, as well as the concentration of Hyp in supernatant of fibroblasts, were promoted about three times. Transformation efficiency from fibroblasts to myofibroblasts was increased approximately two-fold because of the bio-effects of NGF. Two kinds of microencapsulations were seeded into collagen which contained human fibroblasts to form bioengineered skin. Microencapsulated NIH3T3-NGF cells formed a thicker dermis. The concentration of Hyp in the bioengineered skin which indicated the level of collagen synthesis was increased due to existing NGF.

Encapsulation of living cells in a protective, biocompatible, and molecular weight cut-off polymeric membrane has been proven to be an effective method for immuno-isolation of desired cells, regardless of the type of recipient involved (allograft, xenograft; [102]). In the study of Hu *et al.*, it was observed that NGF protein could release freely from the microencapsulated NIH3T3-NGF cells. After the microencapsulated NIH3T3-NGF cells were incorporated into the tissue engineered skin, the cells can still secrete NGF stably and the proliferation of fibroblasts and keratinocytes were significantly enhanced. The increased content of Hyp indicates that NGF, probably in combination with collagen, indirectly enhances the biosynthesis of collagen by directly stimulating the proliferation of the fibroblasts.

As already seen in the research of Hu and colleagues, NGF is a growth factor not only employed in neuronal tissue engineering, but also in the regeneration of other kind of tissues, such as derma and bone. In a paper of Letic-Gravilovic et al, for example, application of controlled release of NGF in a device for bone regeneration is presented [103]. In this study, a composite consisting of two well-known biomaterials, collagen hydroxyapatite (Col/HAp), was used as a drug delivery device for neurotrophin - nerve growth factor β (NGF β). This delivery device, enriched with neurogenic-osteogenic factor, was analyzed *in vitro* and *in vivo*. It was implanted into calvaria defects of 20 Wistar rats. Implants were left in place for different periods of time. Controls were as follows: (a) contralateral defect without any implant; and (b) contralateral defect implanted with composite without NGF factor. The rats were euthanized after 30 days, and the implant sites and explants were examined clinically, histologically, SEM and histomorphometrically. The results evidenced stimulation of periosteal and endocortical woven and lamellar bone formation, with increases in bone mass and decreases in bone marrow. It was found that NGF enhanced the remodeling activity in the intracortical region, and induced an increase in the intracortical cavity number and area by the end of the study. *In vitro* results were in line with *in vivo* ones.

NGF release was evaluated *in vitro*: up to 30% of the NGF was released within the first 24 h, with a large initial burst for all the NGF concentrations tested. Almost 90-100% was released at 72-96 h after loading. Biochemical results showed that during the incubation of MG63 osteoblast-like cells on the composite enriched with three different concentrations of NGF, no signs of cytotoxicity were observed. All the values detected in the material sample cultures corresponded to the control culture values. Results showed significant changes in the biochemical parameters for NGF concentrations of 2 and 4 μg. Alkaline phosphatase activity values proved to be higher, suggesting that the presence of a composite with NGF activity increases the osteogenetic function of osteoblasts. *In vivo*, the delivered osteogenic-neurogenic factor (NGF β) significantly affected and promoted bone ingrowth *in situ*.

5. Neuronal Interfaces and Controlled Release of NGF

The development of effective and usable bionic systems (e.g., exoskeletons for human augmentation/restoration, artificial limb prostheses, etc.) for the replacement and substitution of human sensory-motor function is strictly correlated with the possibility of creating a natural and intuitive link between the nervous system of the user and the artificial device. Only in this way, these new artificial devices can be easily incorporated into the natural control strategies of the subjects and can be felt as parts of their own body thanks to the sensory feedback which can be delivered from the sensors embedded in the robotic artefact to the nervous system.

In the past, several neural interfaces with the central [104] and the peripheral [105] nervous systems have been developed by several research groups in order to address this issue. In particular, peripheral nervous system (PNS) neural interfaces are very interesting because of their reduced invasinevess (when compared with central neural interfaces) and for the possibility of delivering a sensory feedback by stimulating the peripheral afferent nerves

[106] while the modifications of the central somato-sensory cortex seem to preclude this possibility centrally [107]. To this aim several interfaces have been developed during the past years by many groups. Notwithstanding the efforts carried out by different groups, the solutions developed so far suffer from several limitations which make very difficult the development of effective bionic systems. For example, cuff and epineural electrodes [108] are reliable and robust, imply a reduced invasinevess, can be used to extract interesting information [109] but suffer from a limited selectivity which is a significant drawback especially to deliver a sensory feedback.

Intraneural PNS interfaces characterized by needles to be inserted longitudinally (LIFE electrodes, [110]) or transversally (USEA electrodes, [111]) into the PNS are very interesting combining a reduced (even if not absent) invasinevess with a good selectivity but their ”bandwidth” (i.e., the amount of information which can be exchanged between the natural and artificial systems) is still limited.

Regeneration-type (or sieve) electrodes [112] are designed to interface a high number of nerve fibers by using an array of via holes, with electrodes built around them, implanted between the severed stumps of a peripheral nerve. One of the most logical and challenging applications of regenerative electrodes will be its implantation in severed nerves of an amputee limb for a bidirectional interface in cybernetic hand prostheses. Thanks to the ideal high selectivity it could be possible to record neural efferent signals to control several degrees of freedom of a mechanical prosthesis [113], and (above all) to deliver sensory feedback from tactile, position, and force sensors through stimulation of afferent nerve fibers within the residual limb [107].

However, the use of this kind of neural interface is still limited by several neurobiological issues which have to be addressed [114]. For example, motor and sensory nerves seem to compete during the regeneration and the speed and degree of regeneration are higher for sensory than for motor axons. Thus, motor fibers regenerating with delay were confined to the outer layers of the nerve and had more difficulties to find paths across the sieve perforations. For this reason, the definition of strategies to understand in a quantitative manner how regeneration is accomplished and to model and control axonal outgrowth during regeneration by exploiting the potentials of both guidance factors (e.g., intracellular signalling molecules modulators, extracellular guidance cues; [115]) and hybrid structures seem to be necessary in order to develop more effective sieve interfaces.

Neural systems research stands to benefit greatly from targeted drug delivery. The blood–brain barrier presents a challenge to systemically administered substances, often increasing the necessary dosage. In addition, neural tissues of similar cellular composition often have drastically different functions based on anatomical location. Chronically implanted electrically-based neural interface often suffer a loss of function over a period of weeks to months because the neuroinflammatory response produces a glial sheath surrounding the implant, electrically isolating the probe from the surrounding tissue. Local drug delivery to the region surrounding the implant as a means to pharmacologically intervene with this process is being actively pursued by several groups [116-117-118-119].

Figure 16 shows the concept of effect of released bioactive components on damaged neurons. Left figure shows a neuron damaged by probe insertion. Right figure shows a neuron recovered by bioactive components released by the probe.

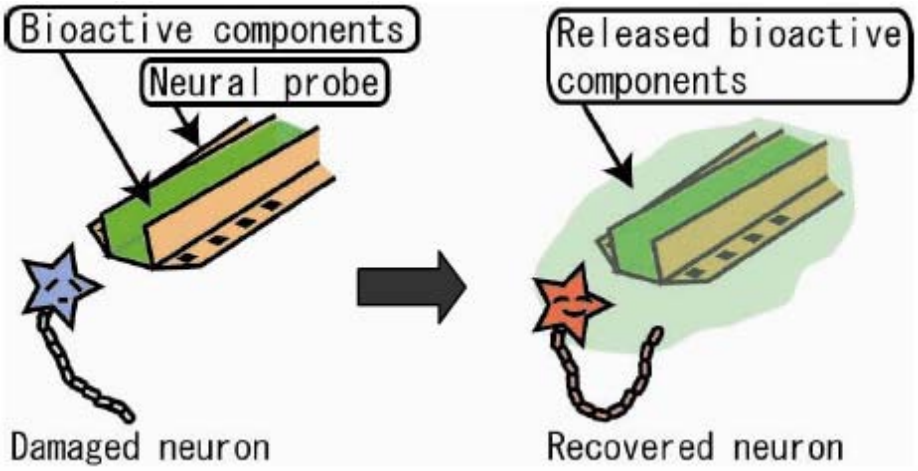

Figure 16. Concept of effect of released bioactive components on damaged neurons by a neuronal probe. From [120]. Reproduced with permission from © 2006 IEEE.

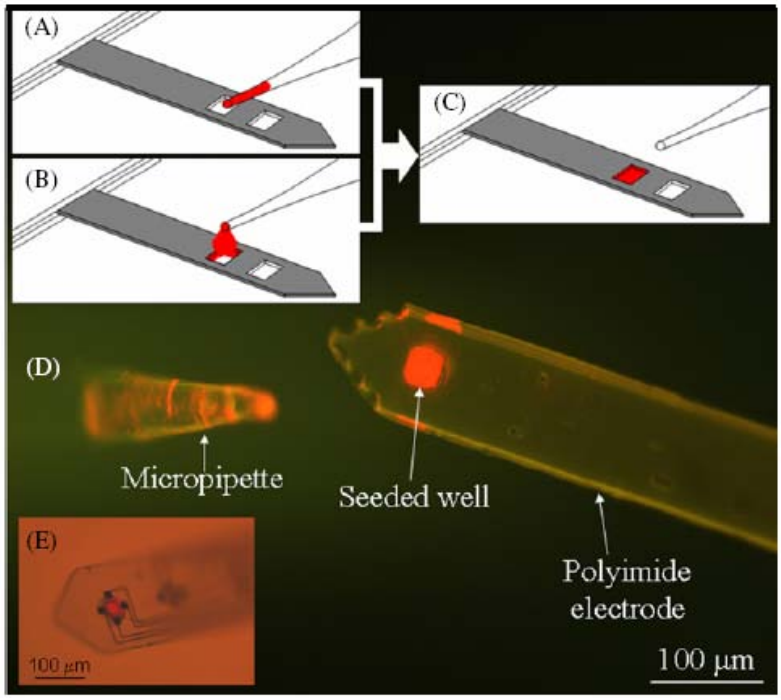

Figure 17. Illustration of electrode well seeding technique. schematics of the device and pipette depicting the injection and droplet deposition methods prior to gel loading are given in (a) and (b), respectively, (c) schematic of a device with a filled well, (d) fluorescent image of probe immediately after the gel loading and subsequent withdrawal of pipette tip. The edges of the probe appear red due to internal reflection of the fluorescing DiI emanating from the well through the polyimide substrate. The volume of gel contained within the well is approximately 9 pl based on the well dimensions and (e) shows a polyimide based electrode with a seeded well in the middle of a tetrode arrangement of recording sites. From [121]. Reproduced with permission from IOP publishing LTD.

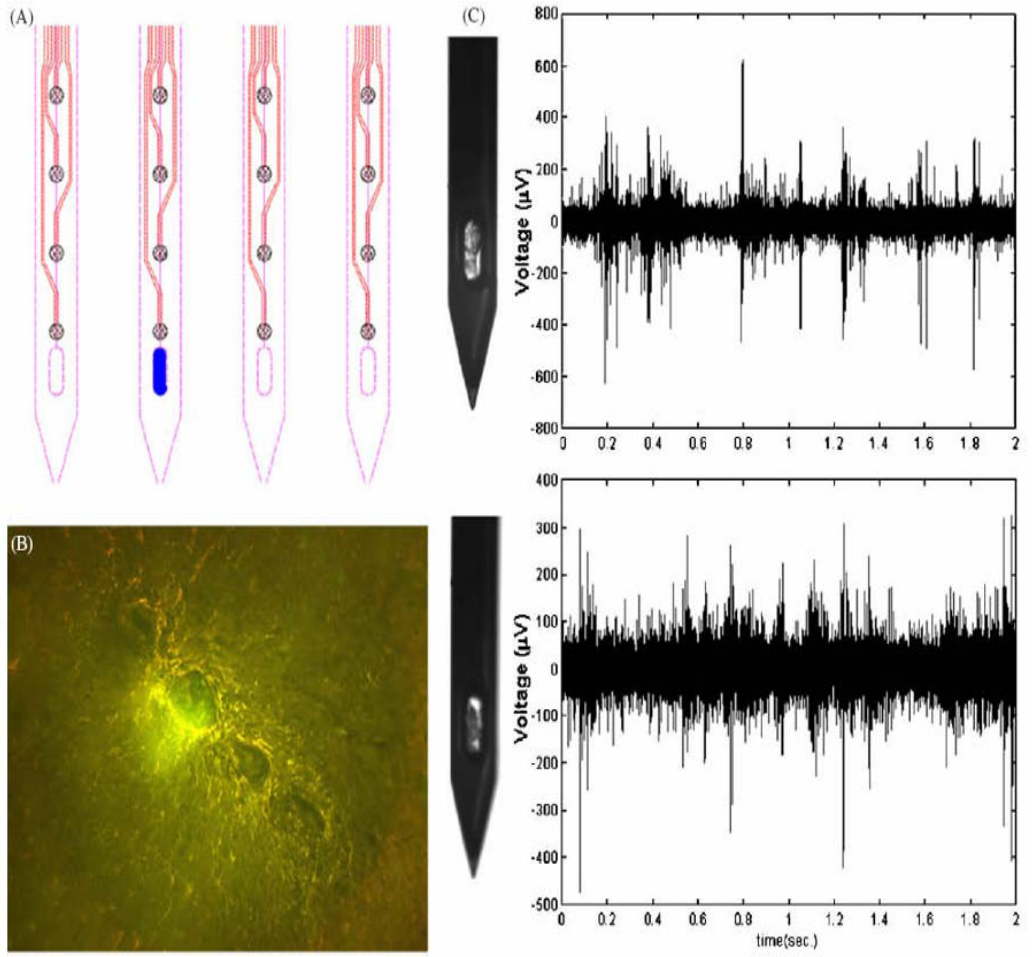

Figure 18. Wells seeded with NGF. The upper left schematic (a) illustrates the layout of an implanted microelectrode array, showing the relative position of the NGF-seeded well in relation to a recording site. In this four-shank implant, only one of the shanks has an NGF-seeded well. The implanted animal was sacrificed after 40 days, sectioned at the level of the NGF-seeded well, and immunohistochemically stained with antibodies against GFAP and the extracellular matrix protein, laminin. The lower left figure in this layout (b) is a histological image from this implant. The site that contained the NGF-seeded well (second from the left in this image) showed increased levels of matrix deposition versus the unseeded electrode shank sites. Part (c) shows the electrophysiological recordings taken from two individual electrode sites closest to two different seeded wells, each at 40-days post-implantation. The inset image to the left of each recording shows the pre-implant electrode with lyophilized NGF seeded in each well. From [121]. Reproduced with permission from IOP publishing LTD.

In general, local microscale drug delivery to the tissue surrounding neural implants has followed two basic approaches. One method involves fabricating microfluidic channels into the substrate of the probe allowing injection of drugs into the tissue immediately surrounding the opening [116]. The other technique relies on polymer surface coatings which support timed release of bioactive chemicals [118]. Both are successful, but have significant drawbacks. Integrated microfluidic channels greatly complicate probe production, leading to lower yields and more expensive fabrication costs. In addition, the channels increase the

footprint, or size, of the device, which may further injure the surrounding tissue during the implantation procedure.

Polymer surface coatings could increase the device's dimensions as well, potentially adding a layer to the surface that could range from tens to hundreds of micrometers in thickness. The high surface area-to-volume ratio of the coatings may reduce the ability for sustained delivery over long periods of time, which could minimize its potential for limiting the chronic neuroinflammatory response.

Williams *et al.* [121] present a method for targeted microscale drug delivery, based on the polymer coating method, which eliminates some of the disadvantages associated with conventional techniques (Figure 17).

In this method, micromachined "wells" are fabricated into the substrate of a MEMS (micro-electro-mechanical systems)-based chronic implant. These "wells" are holes that extend through the device's thickness and allow for the integration of matrices (i.e., hydrogels) infused with bioactive substances. Since the matrices replace substrate instead of adding to it, the device's footprint is not increased. The lower surface area-to-volume ratio lends the technique to extended time release periods. Most significantly, the release sites are localized within micrometers of individual electrodes, and each well can be loaded with a different material, leading to spatial and temporal control over diffusion profiles through the surrounding microenvironment.

To demonstrate localized delivery of a bioactive substance, Figure 18 shows the results of an implant in which the well in one shank of a four-shank probe was filled with NeuroTrace mixed with lyophilized NGF. Following a 40-day post-implant period, the animal was euthanized for histological analysis.

Qualitative observations of Figure 18b show a notable tissue response in the form of laminin deposition seen as a green 'cloud' over and adjacent to the site of NGF release from the gel. Additionally, an increased level of GFAP expression (yellow stained cells) can be seen around the implant sites. These results are typical for the sites of NGF seeding, but noticeably different from the well sites without NGF, which all showed a relatively normal response. A two-second sample of the extracellular recordings taken from two different recording sites, each located 15 μm from two different seeded wells, is given in Figure 18c. These recordings demonstrate that the sites near the NGF-seeded wells remain electrically viable following implantation. The recordings shown are typical for the sites adjacent to the NGF-seeded wells, although not statistically different from the other sites in the array. In all the animals that were implanted with functioning electrodes (n = 4, 48 hours and 40 days), neural signals were recorded from the sites adjacent to the wells that had been seeded with bioactive molecules. Qualitatively, the electrodes were able to record unit activity with acceptable signal amplitude.

Concluding, this study presents a straightforward method to achieve local, time-controlled, multi-site microscale drug delivery within a single device. Micromachined wells provide precise spatial control, decrease the volume of the implant and, unlike other techniques, do not require an increased footprint to achieve delivery of bioactive substances. The simplicity of the well filling technique makes it amenable to multiple types of probe materials and drug-infused polymers. Filling multiple wells on a single probe with different substances allows greater control of spatial and temporal drug release than is otherwise

possible. The major benefit of the work is to extend the design parameters of chronic implantable neural interface devices, giving the researcher greater control over multiple aspects of probe functionality.

Another interesting example of neuronal electrode coupled to a neuroactive molecules delivery is offered by Kato and colleagues [120]. In this preliminary study, authors designed and fabricated two types of flexible neural probes. Both of the neural probes were based on parylene C (poly-monochloro-paraxylylene), which is a flexible and biocompatible material. The type-A neural probe has a groove structure on the probe. This was designed to be seeded with a large amount of the hybrid polymer, since the small wells were limited to fill and deliver the volume of bioactive components for a long-term.

The type-B has a skeleton configuration to minimize the volume of the flexible probe. This design was aimed at providing a space with recovery for the neural tissues damaged and pressed by neural probe insertion, and flexibility to buffer injurious micromotion between the probe and the neural tissues after implantation. Biodegradable polymeric microspheres with bioactive components of nerve growth factor (NGF) and polyethylene glycol (PEG) were mixed, and seeded into the groove or coated on the skeleton-like probe.

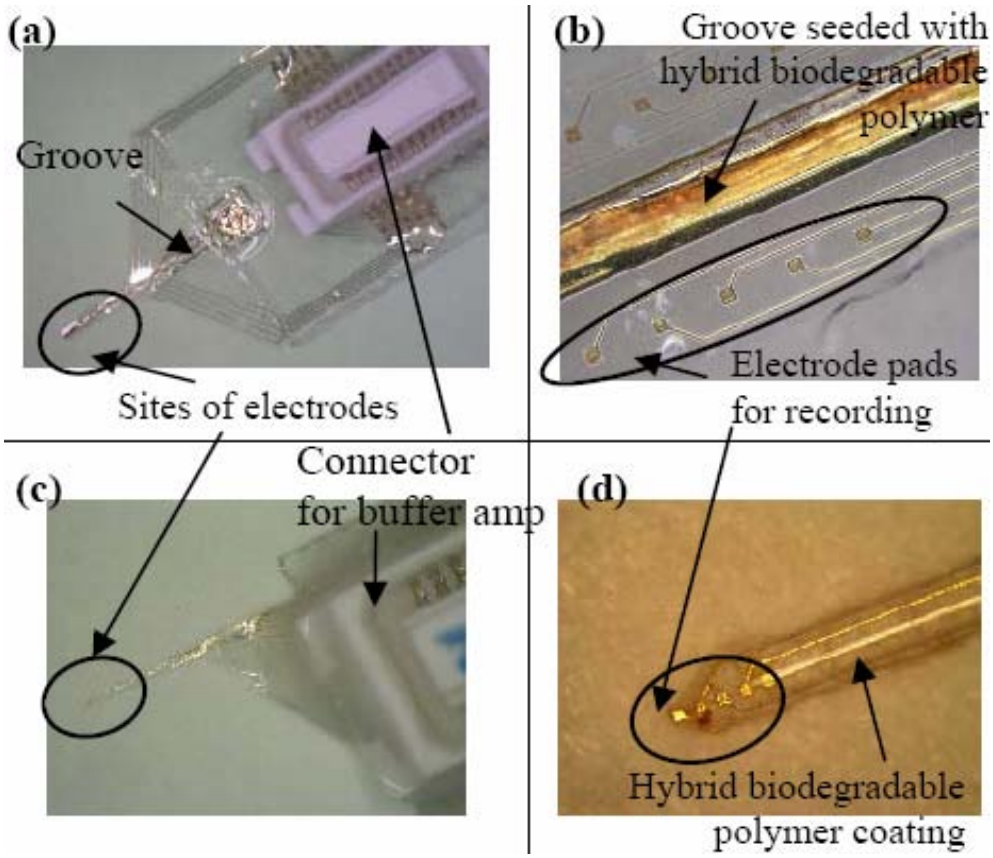

Figure 19. Photos of neural probe: (a) type-A neural probe, (b) magnified view of the electrode sites in tip of the neural probe: the hybrid biodegradable polymeric microspheres were seeded in the groove. (c) type-B neural probe, (d) magnified picture of the electrode sites and each skeleton-lie probe coated with the hybrid biodegradable polymer. From [120]. Reproduced with permission from © 2006 IEEE.

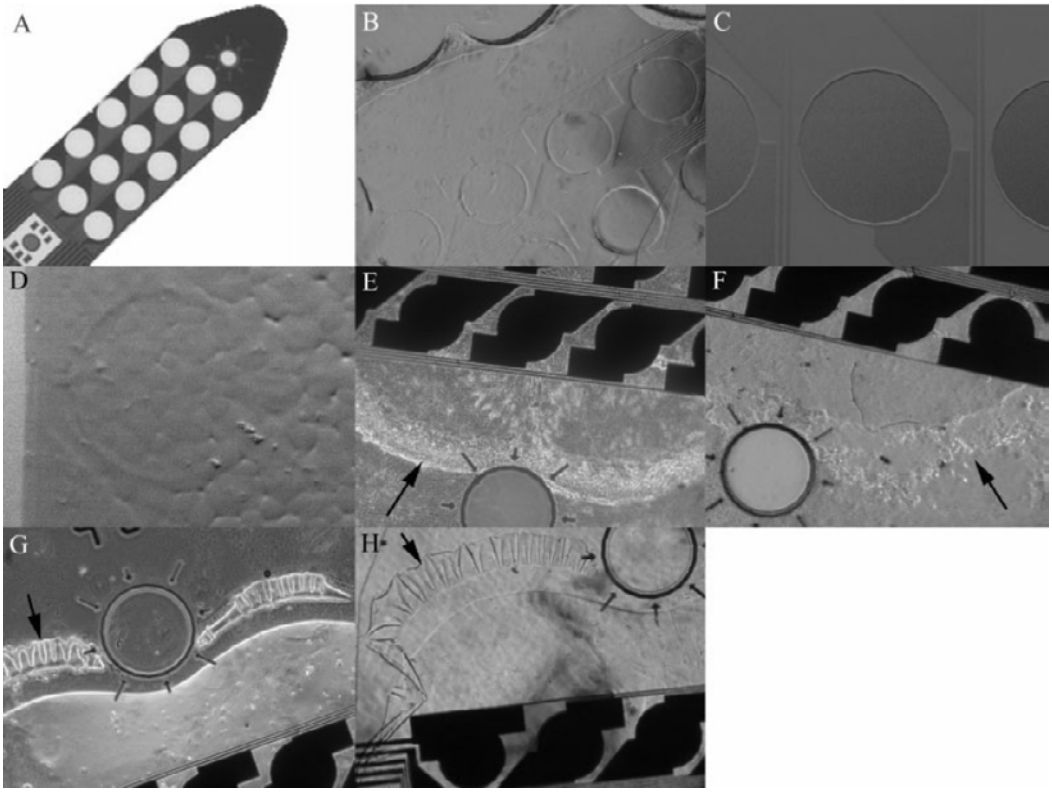

Figure 20. Adhesion of PEG-based copolymers to electrode array surfaces. (a) SEM of the distal tip of the MEA array showing 15 electrodes of 400 μm diameter in a 3 X 5 array. A small surgical suture hole is located directly above the 3 X 5 array. (b) Phase contrast optical micrograph of PEG-diacryl hydrogel bolus. PEG-diacryl boluses do not adhere to array surfaces, detaching within seconds. An impression of the electrode surface in the bolus indicates the degree of conformal coating present before detachment. (c) SEMs of untreated electrode site, and (d) same site coated with PEG1000LA2 polymer. Coverage of the electrode site is evident. (e,f) Phase contrast optical micrograph of PEG1000LA2 bolus at day 0 (e) and day 4 (f). Circular electrodes (black) and the surgical suture hole (circle ringed with arrows) are visible, along with the bolus (gray/white). Bolus adhesion (arrows) is evident on day 4 (f), and expected degradation is observed as well. (g,h) Phase contrast optical micrograph of PEG1000LA2 bolus at (g) day 0, and (h) day 11 under an agarose tissue phantom. Degradation appears reduced in the presence of the tissue phantom. Some detachment is evident in the left edge of the bolus. Scale, electrode = 400 μm diameter. From [122]. Reproduced with permission from John Wiley and Sons, Inc.

These were added with the aim of providing a route for the damaged tissue around the neural probe and improving the mechanical stiffness of the flexible neural probe for insertion. The DDS material was a biodegradable polymer of poly(lactic-co-glycolic acid) (PLGA), which was used to deliver the NGF for an optimal period. In this study, the efficacy of the two types of flexible neural probes and the biodegradable polymeric microspheres were examined.

The type-A and type-B flexible neural probes were designed and fabricated as shown in Figure 19. The hybrid bioactive degradable polymers were manually seeded in the groove for the type-A neural probe and coated on the type-B neural probe, which was aimed to promote tissue regeneration around the neural probe after implantation, and improve the mechanical

stiffness of the flexible probe for insertion. The degradable polymeric microspheres were fabricated and optimized to sizes between 10 μm and 30 μm for embedding in the groove or coating on the skeleton-like probe. It was succeeded in loading the biodegradable microspheres with bioactive components of NGF. Drug release was efficiently verified with a specific ELISA test. The bioactivity of NGF released from the microspheres was investigated with PC-12 cells, that showed clearly differentiation after an incubation of 8 days with the microspheres. Finally, in the *in vivo* experiment, inserting both of the neural probes showing sufficient stiffness, and recording neural signals from the cerebral cortex of a rat using the type-A neural probe was successfully tested.

Improved sensory and motor prostheses for the central nervous system will require large numbers of electrodes with low electrical thresholds for neural excitation. With the eventual goal of reducing stimulation thresholds, Winter *et al.* [122] investigated the use of biodegradable, neurotrophin-eluting hydrogels (i.e., poly(ethylene glycol)-poly(lactic acid), PEG-PLA) as a means of attracting neurites to the surface of stimulating electrodes (Figure 20).

PEG-PLA hydrogels with release rates ranging from 1.5 to 3 weeks were synthesized. These hydrogels were applied to multielectrode arrays with sputtered iridium oxide charge-injection sites. The coatings had little impact on the iridium oxide electrochemical properties, including charge storage capacity, impedance, and voltage transients during current pulsing. Additionally, the ability of neurotrophin-eluting PEG-PLA hydrogels to promote neurite extension *in vitro* using a PC-12 cell culture model was quantitatively examined.

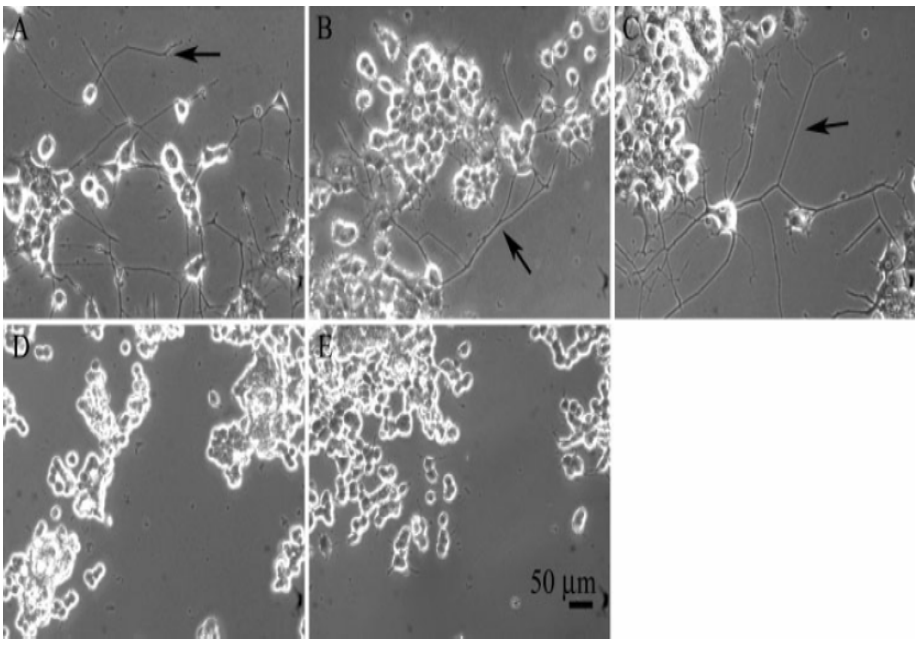

Figure 21. Representative optical micrographs of PC-12 Cells after 5 days with boluses in culture. (a) Positive control receiving 50 ng/ml NGF, (b) PEG1540LA4, and (c) PEG1000LA2 all display large numbers of neurites (arrows), whereas (d) negative control receiving 0 ng/mL NGF and (e) sham, BSA-releasing bolus display only few, short neurites. From [122]. Reproduced with permission from John Wiley and Sons, Inc.

Hydrogels released NGF for at least 1 week, with neurite extension near that of an NGF positive control and much higher than extension seen from sham, bovine serum albumin releasing boluses, and a negative control. These results show that neurotrophin-eluting hydrogels can be applied to multielectrode arrays, and suggest a method to improve neuronelectrode proximity, which could result in lowered electrical stimulation thresholds. Reduced thresholds support the creation of smaller electrode structures and high density electrode prostheses, greatly enhancing prosthesis control and function.

Hydrogel release rates were characterized using BSA as a model protein. The target release duration was 4 weeks, because this is the time period in which the acute immune and other biological responses will likely occur and it should be sufficient for nerve growth across the retinal thickness. BSA release duration varied between 1.5 weeks and 3 weeks basing on different polymeric molecular weight. Release rates did not vary significantly with increases in polymer molecular weight, but were markedly enhanced with additional lactide units.

The release profile displayed by hydrogels had an asymptotic approach to the final value, with an initial large burst. A linear release is generally preferable; however, the effects of asymmetric release profiles in diseased tissue have not been fully explored, and an initial burst may be beneficial. Additionally, BSA delivery was not complete, with molecules perhaps entrained in or associated with PEG molecules. This effect was most pronounced for slow releasing polymers. Adjustments in initial concentration of neurotrophin can be made to compensate for this effect.

To evaluate neurite extension produced by neurotrophin eluting hydrogel boluses, NGF-releasing boluses in the presence of PC-12 cells was examinated (Figure 21). PC-12 cell cultures were exposed to NGF-releasing boluses suspended in transwell inserts for 5 or 14 days, and compared these to a positive control receiving 50 ng/mL of NGF, a negative control receiving no NGF, and a sham receiving BSA-releasing boluses. Samples were evaluated for neurite extension using optical micrographs collected at 2, 5, and 7 days.

After 2 days of exposure, release from both PEG1540LA2 and PEG1000LA2 polymers (different molecular weigths) was near the target value of 50 ng/ml. Correspondingly, neurite extension was evident in both the samples. NGF-releasing boluses displayed average neurite lengths similar to those of the NGF control. For the short-releasing PEG1540LA4 polymer, neurite length were statistically insignificant from the NGF control, and statistically different from the control and sham, BSA releasing samples. Neurite length distributions for the long-releasing PEG1000LA2 polymer were different from all samples investigated, but displayed a tendency to longer neurite lengths like the NGF control, and were distinct from those of the sham, BSA releasing bolus, and the negative control.

Interestingly, at day 2, the sham BSA releasing bolus was statistically different from the negative control, which may indicate a slight increase in neurite extension as a result of BSA exposure. However, this difference did not persist at longer time points. At day 5, the supply of NGF from the short-releasing PEG1540LA4 polymer had declined, whereas release from the long-releasing PEG1000LA2 polymer remained huge. Average neurite lengths for PEG1540LA4 samples declined to values slightly above the negative control and sham values, but those of the PEG1000LA2 polymer remained near the NGF control. This decline was also reflected in the neurite length profiles.

PEG1000LA2 samples displayed a neurite length distribution that is statistically insignificant from the NGF control, indicating sustained NGF release. However, the PEG1540LA4 polymer exhibited a decline in neurite length distribution to intermediate values, statistically different from NGF, sham, and negative controls. After 5 days, experiments with the PEG1540LA4 polymer were discontinued as a result of substantial bolus degradation and NGF release. At day 7, NGF release from PEG1000LA2 boluses declined. Although average neurite length values were slightly increased with respect to those of day 5, the increase was not as significant as that evidenced in the NGF control. PEG1000LA2 sample neurite length distributions were statistically different from NGF control, sham, and control values. Neurite length on subsequent days (> day 7) could not be judged as a result of the substantial branching and networking of neurites in the positive control and cell division in the sham and negative controls. Qualitatively, neurite length continued to decline in PEG1000LA2 samples, but remained visibly different from the sham and control images even after 14 days of culture.

In vitro studies demonstrate that neurotrophin-eluting hydrogels are biocompatible and can produce neurite extension in PC-12 cells for at least 1 week. Interestingly, hydrogel release and degradation appeared to be accelerated in the presence of cells. For example, neurite extension in PEG1540LA4 polymers peaked at day 2, despite at least 5 days of release measured in PBS. Additionally, boluses appeared nearly completely dissolved by day 5 in cell culture whereas boluses persisted for at least 10 days in PBS. This enhancement may result from enzymatic enhancement of PLA degradation occurring as a result of enzymes released by the cells or contained in the serum supplemented cell culture medium. Further measurements of release rates and degradation *in vitro* are needed to approximate actual *in vivo* release rates.

The NGF releasing system described has several advantages over other methods that have been proposed to enhance neuron-device contact, the majority of which have employed passive surface coatings or the addition of biological agents. Passive surface coatings promote neuron adhesion and extension of neurites through direct contact. Given the anatomical limitations in many systems, this proximity is not always possible, and the benefits of such techniques will be lost. Factor elution overcomes this difficulty through the use of soluble, diffusible agents, which can potentially impact cells several hundred micrometers distant from the electrode surface. Similar approaches in which biological agents, including explanted cells and native tissue constructs, provide diffusible neurotrophic agents have been investigated with some success. However, avoiding adverse immune and other biological responses is a challenge. The PEG-PLA hydrogel employed in the study of Winter *et al.* comprised biocompatible polymers that have been used extensively in the clinic with a demonstrated history of safe use. In the proposed NGF-releasing polymer system, neurotrophic factors would be provided for a finite time, after which it is hoped that established connections between adjacent cells and the device could be sustained. Unfortunately, the PC-12 cell culture model is limited by retraction of neurites after NGF removal and reversion to an undifferentiated phenotype. It is not clear whether diseased or damaged neuronal tissue would respond in this way, and additional experiments with *in vivo* models will be needed to address this concern. In the event of neurite retraction, device

coatings (e.g., laminin, polylysine, collagen) could be used to enhance neurite persistence and attachment [123-124].

We would like to conclude this paragraph briefly citing an interesting study by Kim et al. [125]. In this paper an interesting application of NGF as dopant of conductive polymer is presented. The use of biologically active dopants in conductive polymers allows the polymer to be tailored for specific applications. The incorporation of nerve growth factor as a co-dopant in the electrochemical deposition of conductive polymers is evaluated for its ability to elicit specific biological interactions with neurons. The electrochemical properties of the NGF-modified conducting polymers are studied by impedance spectroscopy and cyclic voltammetry. Impedance measurements at the neurobiologically important frequency of 1 kHz revealed that the minimum impedance of the NGF-modified polypyrrole (PPy) film, 15 kΩ, is lower than the minimum impedance of peptide-modified PPy film (360 kΩ). Similar results were found with NGF-modified poly(3,4-ethylene dioxythiophene) (PEDOT). The microstructure of the conductive polymer films was characterized by optical microscopy and electron microscopy and indicates that the NGF-functionalized polymer surface topology is similar to that of the unmodified polymer film. Optical and fluorescence microscopy revealed that PC-12 cells adhered to the NGF-modified substrate and extended neurites on both PPy and PEDOT, indicating that the NGF in the polymer film is biologically active. Taken together these data indicate that the incorporation of NGF can modify the biological interactions of the electrode without compromising the conductive properties or the morphology of the polymeric film.

6. Conclusion

Neural drug delivery is the an important step beyond the traditional therapeutic approach of neuronal diseases. Drug delivery systems allow the rate of drug release to be regulated over time, which is critical for creating an environment more closely representative of *in vivo* development environments. Delivery of drugs to the nervous system remains a challenge despite advances in our understanding of the mechanisms involved in the development of neurodegenerative disorders and the actions of neuroactive agents.

A major challenge of delivering growth factors to lesions in the spinal cord, for example, is the high clearance and removal due to the high turnover rate of the cerebrospinal fluid. A way to overcome this phenomenon is to use a drug delivery system that slowly releases the growth factors over a prolonged period of time. It is also challenging to deliver growth factors to the central nervous system, because most proteins are not able to cross the blood-brain barrier and, therefore, cannot be delivered via systemic administration. The most desirable method of delivering growth factors such as nerve growth factor is locally, because only nanogram quantities are needed to supply therapeutic levels to the targeted cell population.

Neurotrophic molecules have a deep influence on developmental events such as naturally occurring cell death, differentiation and process outgrowth and they could be used for treating degenerative neurological conditions and promoting neural regeneration. This Chapter focused its attention on innovative strategies for delivering NGF both for *in vivo* and

in vitro applications, at level of both the peripheral and the central nervous system. Development of polymeric films, hydrogels and microparticles have been considered, with particular attention to the recent advancements in the field of neuronal tissue engineering and of neuronal regeneration. Because of the limited availability and functionality received from autografts, (the current gold standard for nerve regeneration and repair), recent neural tissue engineering research focused on the development of bioartificial nerve guidance conduits in order to guide axonal regrowth. The creation of artificial nerve conduits is also known as entubulation, because the nerve ends and the intervening gap are enclosed within a tube composed of biological or synthetic materials. The conduit should facilitate neurotropic and neurotrophic communication between the proximal and distal ends of the gap, block external inhibitory factors, and provide a physical guidance. The most basic objective of a nerve guidance conduit is to combine physical, chemical, and biological cues under conditions that will promote tissue formation and, in this sense, neural drug delivery is the next step beyond the basic addition of growth factors to the nerve guidance conduits.

The last paragraph of this Chapter was totally dedicated to the application of NGF delivery system in the field of neuronal interfaces. The level of understanding of the interfacing issues was improved at both system and molecular levels, and substantial technological breakthroughs have been achieved in terms of micro-design of transduction and computing elements. The field of neuro-engineering is maturing into a coherent set of methodologies and technologies that will allow to design whole new families of advanced cybernetic applications that bring together the power of the human brain and the artificial micro-devices. The development of a dynamic microenvironment resulting from a synergistic combination of chemical and physical guidance cues could allow to develop a new regeneration strategy, for example by placing several microspheres embedding chemical attractive and repulsive cues [115] into a guidance channel and between the proximal and distal parts of a peripheral broken nerve, in order to guide the movements of the axons. In this way, a correct regeneration of different types of axons could be achieved. Drug-delivery strategies like those presented in this Chapter could be merged to create a regeneration environment that offers exciting opportunities for elucidating the mechanisms behind cellular differentiation and nerve regeneration.

7. References

[1] Dash, A.K. and Cudworth, G.C. (1998). Therapeutic application of implantable drug delivery systems. *Journal of Pharmacological and Toxicological Methods 40,* 1-12.

[2] Eddington, D.T. and Beebe, D.J. (2005). Development of a disposable infusion system for the delivery of protein therapeutics. *Biomed. Microdevices 7(3),* 223-230.

[3] Kim, B. and Peppas, N.A. (2003). Poly(ethylene glycol)-containing hydrogels for oral protein delivery applications. *Biomed. Microdevices 5(4),* 333-341.

[4] Leoni, L., Boiarsky, A. and Desai, T.A. (2002). Characterization of nanoporous membranes for immunoisolation: Diffusion properties and tissue effects. *Biomed. Microdevices 4(2),* 131-139 (2002).

[5] Vert, M., Li., S. and Garreau H. (1991). More About the Degradation of LA/GA-derived Matrices in Aqueous Media. *J. Control. Release* 16, 15–26.

[6] Maysinger, D. and Morinville A. (1997). Drug delivery to the nervous system. *Trends Biotechnol. 15*, 410–418.

[7] Lewin, S.L., Utley, D.S., Cheng, E.T., Verity, A.N. and Terris D.J. (1997). Simultaneous treatment with BDNF and CNTF after peripheral nerve transaction and repair enhances rate of functional recovery compared with BDNF treatment alone. *Laryngoscope 107*, 992–999.

[8] Santos, X., Rodrigo, J., Hontanilla, B. and Bilbao, G. (1998). Evaluation of peripheral nerve regeneration by nerve growth factor locally administered with a novel system. *J. Neurosci. Meth. 85*, 119–27.

[9] Jones, L.L. and Tuszynski, M.H. (2001). Chronic intrathecal infusions after spinal cord injury cause scarring and compression. *Microsc. Res. Techniq.* 317–324.

[10] Schmidt, C.E. and Leach, LB. (2003). Neural tissue engineering: strategies for repair and regeneration. *Annu. Rev. Biomed. Eng. 5*, 293–347.

[11] Langer R. 1998. Drug delivery and targeting. *Nature 392*, 5–10.

[12] Snider, W.D. and Johson, E.M. (1989). Neurotrophic molecules. *Ann. Neurol. 26:* 489–506.

[13] Eide, F.F., Loweinstein, D.H. and Reichardt, L.F. (1993). Neurotrophins and their receptors. Current concepts and implications for neurologic disease. *Exp. Neurol. 121*, 200–214.

[14] Lundborg G. (2000). A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J. Hand Surg. [Am.]* 25, 391–414.

[15] Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M.J., Misko, T.P., Shooter, E. and Thoenen, H. (1987). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. *Proc. Natl. Acad. Sci. USA 84*, 8735–8739

[16] Murakami, Y., Furukawa, S., Nitta, A. and Furukawa, Y. (2002). Accumulation of nerve growth factor protein at both rostral and caudal stumps in the transected rat spinal cord. *J. Neurol. Sci. 198*, 63–69.

[17] Tuszynski, M.H., Gabriel, K., Gage, F.H, Suhr, S., Meyer, S., and Rosetti, A. (1996). Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. *Exp. Neurol. 137*, 157–173

[18] Romero, M.I., Rangappa, N., Garry, M.G. and Smith G.M. (2001). Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy. *J. Neurosci. 21*, 8408–8416

[19] Hollinger, J.O. and Schmitz, J.P. (1987). Restoration of bone discontinuities in dogs using a biodegradable implant. *J. Oral Maxillofac. Surg. 45*, 594-600.

[20] Krewson, C.E., Klarman, M.L. and Saltzman, W.M. (1995). Distribution of nerve growth factor following direct delivery to the brain interstitium. *Brain Res. 680*, 196–206.

[21] Krewson, C.E., Dause, R., Mak, M. and Saltzman, W.M. (1996). Stabilization of nerve growth factor in controlled release polymers and in tissue. *J. Biomater. Sci. Polym. Ed. 8(2)*, 103–117.

[22] Kuo, P.Y. and Saltzman, W.M. (1996). Novel systems for drug delivery of macromolecules. *Crit. Rev. Eukaryotic Gene Expr. 6*, 335-357.

[23] Fung, L.K. and Saltzman, W.M. (1997). Polymeric implants for cancer chemotherapy. *Adv. Drug Deliv. Rev. 26(2-3)*, 209-230.

[24] Gombotz, W.R. and Pettit, D.K. (1995). Biodegradable polymers for protein and peptide drug delivery, *Bioconj. Chem. 6(4)*, 332-351

[25] Baldwin, S.P. and Saltzman, W.M. (1998). Materials for protein delivery in tissue engineering. *Adv. Drug Deliver. Rev. 33*, 71–86

[26] Saltzman, W.M., Pasternak, S.H. and Langer, R. (1987). Quantitative image analysis for developing microstructural descriptions of heterogeneous materials. *Chem. Eng. Sci. 42*, 1989–2004.

[27] Pean, J.M., Venier-Julienne, M.C., Filmon, R., Sergent, M., Phan-Tan-Luu, R. and Benoit, J.P. (1998). Optimization of HSA and NGF encapsulation yields in PLGA Microparticles. *International Journal of Pharmaceutics 166*, 105–115.

[28] Pean, J.M., Venier-Julienne, M.C., Boury, F., Menei, P., Denizot, B. and Benoit, J.P. (1998). NGF release from poly(D,L-lactide-co-glycolide) microspheres. Effect of some formulation parameters on encapsulated NGF stability. *J. Control. Release* 56, 175–187.

[29] Cao, X. and Shoichet, M.S. (1999). Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. *Biomaterials 20*, 329-339.

[30] Khang, G., Jeon, E.K., Rhee, J.M., Lee, I., Lee, S.J. and Lee, H.B. (2003). Controlled Release of Nerve Growth Factor from Sandwiched Poly(L-lactide-co-glycolide) Films for the Application in Neural Tissue Engineering. *Macromol. Res.* 11(5), 334-340.

[31] Lam, X.M., Duenas, E.T. and Cleland, J.L. (2001). Encapsulation and stabilization of nerve growth factor into poly(lactic-co-glycolic) acid microspheres. *J. Pharm. Sci. 90(9)*, 1356–1365.

[32] Uebersax, L., Hagenmuller, H., Hofmann, S., Gruenblatt, E., Muller, R., Vunjaknovakovic, G., Kaplan, D.L., Merkle, H.P. and Meinel, L. (2006). Effect of Scaffold design on bone morphology in vitro. *Tissue Eng 12(12)*, 3417–3429.

[33] Meinel, L., Hofmann, S., Karageorgiou, V., Kirker-Head, C., McCool, J., Gronowicz, G., Zichner, L., Langer, R., Vunjak-Novakovic, G. and Kaplan, D.L. (2005). The inflammatory responses to silk films in vitro and in vivo. *Biomaterials 26(2)*, 147–155.

[34] Lange, F. (1903). Ueber die Sehnenplastik. *Verh. Dtsch Orthop. 2*, 10–12.

[35] Cunniff, P.M., Fossey, S.A., Auerbach, M.A., Song, J.W., Kaplan, D.L., Adams, W., Eby, R.K., Mahoney, D. and Vezie, D.L.. (1994). Mechanical and thermal properties of Dragline Silk from the spider Nephila cavipes. *Polym. Adv. Technol. 5*, 401–410.

[36] Uebersax, L., Fedele, D.E., Schumacher, C., Kaplan, D.L., Merkle, H.P., Boison, D., and Meinel, L. (2006). The support of adenosine release from adenosine kinase deficient ES cells by silk substrates. *Biomaterials 27(26)*, 4599–4607.

[37] Hofmann, S., Wong Po Foo, C.T., Rossetti, F., Textor, M., Vunjak-Novakovic, G., Kaplan, DL., Merkle, H.P. and Meinel, L. (2006). Silk fibroin as an organic polymer for controlled drug delivery. *J. Control. Release 111(1–2)*, 219–227.

[38] Panilaitis, B., Altman, G.H., Chen, J., Jin, H.J., Karageorgiou, V. and Kaplan, D.L. (2003). Macrophage responses to silk. *Biomaterials 24(18)*, 3079–3085.

[39] Uebersax, L., Mattotti, M., Papaloizos, M., Merkle, H.P., Gander, B. and Meinel, L. (2007). Silk fibroin matrices for the controlled release of nerve growth factor (NGF). *Biomaterials 28*, 4449–4460.

[40] Piotrowicz, A. and Shoichet, M.S. (2006). Nerve guidance channels as drug delivery vehicles. *Biomaterials* 27, 2018–2027.

[41] Ciofani, G., Raffa, V., Menciassi, A., Micera, S., Dario, P. (2007). A drug delivery system based on alginate microspheres: Mass-transport test and in vitro validation. *Biomed. Microdevices* 9, 395–403.

[42] Barallobre, M.J., Pascual, M., Del Rio, J.A. and Soriano, E. (2005). The Netrin family of guidance factors: emphasis on Netrin-1 signalling. *Brain Research Reviews 49*, 22–47.

[43] Kennedy, T.E. (2000). Cellular mechanisms of Netrin function: long-range and short-range actions, *Biochem. Cell. Biol. 78*, 569– 575.

[44] Simpson N.E. (2003). The role of the $CaCl_2$-guluronic acid interaction on alginate encapsulated bTC3 cells. *Biomaterials 25(13)*, 2603-2610.

[45] Alexakis, T., Boadi, D.K., Quong, D., Groboillot, A., O'Neill, I., Poncelet, D. and Neufeld R.J. (1995).Microencapsulation of DNA within alginate microspheres and crosslinked chitosan membranes for *in vivo* application. *Appl. Biochem. Biotechnol. 50(1)*, 93-106 (1995).

[46] Dotti, C.G., Sullivan, C.A. and Bunker G.A. (1988). The Establishment of Polarity by Hippocampal Neurons in Culture. *J. Neurosci. 81(4)*, 1454-1488.

[47] Ming, G.L., Henley, J., Tessier-Lavigne, M., Song, H.J. and Poo, M.M. (2001). Electrical Activity Modulates Growth Cone guidance by Diffusible Factors. *Neuron 29*, 441–452.

[48] Venero, J.L., Hefti, F. and Knusel, B. (1996). Trophic effect of exogenous nerve growth factor on rat striatal cholinergic neurons: comparison between intraparenchymal and intraventricular administration. *Mol. Pharmacol. 49*, 303–310.

[49] Williams, L. R. (1991). Hypophagia is induced by intracerebroventricular administration of nerve growth factor. *Exp. Neurol. 113*, 31–37.

[50] Tuszynski, M.H., Roberts, J., Senut, M.C., U, H.S. and Gage, F.H. (1996). Gene therapy in the adult primate brain: intraparenchymal grafts of cells genetically modified to produce nerve growth factor prevent cholinergic neuronal degeneration. *Gene Ther. 3*, 305–3014.

[51] Martinez-Serrano, A. and Bjorklund, A. (1996). Protection of the neostriatum against excitotoxic damage by neurotrophin producing, genetically modified neural stem cells. *J. Neurosci.* 16, 4604–4616.

[52] Maysinger, D., Piccardo, P., Liberini, P., Jalsenjak, I. andCuello, C. (1994). Encapsulated genetically engineered fibroblasts: release of nerve growth factor and

effects in vivo on recovery of cholinergic markers after devascularizing cortical lesions. *Neurochem Int. 24(5)*, 495-503.

[53] Mahoney, M.J. and Saltzman, W.M. (2001). Transplantation of brain cells assembled around a programmable synthetic microenvironment. *Nature Biotechnol. 19*, 934-939.

[54] Castel-Barthe, M. N., Jazat-Poindessous, F., Barneoud, P., Vigne, E., Revah, F., Mallet, J. and Lamour, Y. (1996). Direct intracerebral nerve growth factor gene transfer using a recombinant adenovirus: effect on basal forebrain cholinergic neurons during aging. *Neurobiol. Dis. 3*, 76–86.

[55] Langer, R. (1991). Polymer implants for drug delivery in the brain, other approaches involving the intracerebral grafting. *J. Control. Release 16*, 53–60.

[56] Powell, E.M., Sobarzo, M.R. and Saltzman, W.M. (1990). Controlled release of nerve growth factor from a polymeric implant. *Brain Res. 515*, 309–311.

[57] Hoffman, D., Wahlberg, L. and Aebischer, P. (1990). NGF released from a polymer matrix prevents loss of ChAT expression in basal forebrain neurons following a fimbria-fornix lesion. *Exp. Neurol. 110*, 39–44.

[58] Boisdron-Celle, M., Menei, P. and Benoit, J.P. (1995). Preparation and characterization of 5-fluorouracil-loaded microparticles as biodegradable anticancer drug carriers. *J. Pharm. Pharmacol. 47*, 108–114.

[59] Yamamoto, S., Yoshimine, T., Fujita, T., Kuroda, R., Irie, T., Fujioka, K. and Hayakawa, T. (1992). Protective effect of NGF atelocollagen mini-pellet on the hippocampal delayed neuronal death in gerbils. *Neurosci. Lett. 141*, 161–165.

[60] Ghezzo, E., Benedetti, L., Rochira, M., Biviano, F. and Callegaro, L. (1992). Hyaluronane derivative microspheres as NGF delivery devices: preparation methods and in vitro release characterization. *Int. J. Pharm. 87*, 21–29.

[61] Maysinger, D., Jalsenjak, V. and Cuello, A.C. (1992). Microencapsulated nerve growth factor: Effect on the forebrain neurons following devascularizing cortical lesions. *Neurosci. Lett. 140*, 71–74.

[62] Frazza, E. J. and Schmidt, E.E. (1971). A new absorbable suture. *Biomed. Mater. Res. 5*, 43–58.

[63] Menei, P., Pean J.M., Nerriere-Daguin, V., Jollivet, C., Brachet, P., Benoit, J.P. (2000) Intracerebral implantation of NGF-releasing biodegradable microspheres protects striatum against excitotoxic damage. *Experimental Neurology 161*, 259–272.

[64] Mendez, A., Camarata, P.J., Suryanarayanan, R., and Ebner, T.J.. (1994). Sustained intracerebral delivery of nerve growth factor with biodegradable polymer microspheres. *Methods Neurosci. 21*, 150–167.

[65] Riddle, D.R., Lo, D.C. and Katz, L.C. (1995). NT-4-mediated rescue of lateral geniculate neurons from effects of monocular deprivation. *Nature 378*, 189–191.

[66] Camarata, P.J., Suryanarayanan, R., Turner, D.A., Parker, R.G. and Ebner, T.J. (1992). Sustained release of Nerve Growth Factor from biodegradable polymer microspheres. *Neurosurgery 30*, 313–319.

[67] Maysinger, D., Filipovic-Grcic, J. and Cuello, A.C. (1993). Effects of coencapsulated NGF and GM1 in rats with cortical lesions. *NeuroReport 4*, 971–974.

[68] Krewson, C.E., Dause, R., Mak, M. and Saltzman, W.M. (1996). Stabilization of nerve growth factor in controlled release polymers and in tissue. *J. Biomater. Sci. Polym. Ed. 8(2),* 103–117.

[69] Cleland, J.L. and Duenas, E.T. (1997). Controlled delivery of nerve growth factor for local treatment of neuronal diseases. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater., Stoholm 24*, 823–824.

[70] Pardridge, W.M. (1999). Non-invasive drug delivery to the human brain using endogenous blood–brain barrier transports systems. *Pharm. Sci. Technol. Today 2*, 49 – 59.

[71] Huwyler, J., Wu, D.F. and Pardridge, W.M., Brain drug delivery of small molecules using immunoloposomes. *Proc. Natl. Acad. Sci. USA. 93(11)*, 14164–14169.

[72] Xie, Y., Ye, L., Zhang, X., Cui, W., Lou, J., Nagai, T. and Hou, X. (2005). Transport of nerve growth factor encapsulated into liposomes across the blood–brain barrier: In vitro and in vivo studies. *J. Control. Release 105*, 106– 119.

[73] Seckel, B.R. (1990). Enhancement of peripheral nerve regeneration. *Muscle Nerve 13*, 785–800.

[74] Lundborg, G. (1990). Nerve regeneration problems in a clinical perspective. *Restor. Neurol. Neurosci. 1*, 297–302.

[75] Jolliet-Riant, P. and Tillement, J.P. (1999) Drug transfer across the blood–brain barrier and improvement of brain delivery. *Fund. Clin. Pharmacol. 13(1)*, 16-26.

[76] Otto, D., Unsicker, K. and Grothe, C. (1987). Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transectioned sciatic nerve on neuron death in adult rat dorsal root ganglia. *Neurosci. Lett. 83*, 156–160.

[77] Rich, R., Alexander, T., Pryor, J. and Hollowell, J. (1989). Nerve growth factor enhances regeneration through silicone chambers. *Exp. Neurol. 105*, 162–170.

[78] Varon, S. and Conner, J.M. (1994). Nerve growth factor in CNS repair. *J. Neurotrauma 11*, 473–486.

[79] Xu, X., Yu, H., Gao, S., Mao, H.Q., Leong, K.W., Wang, S. (2002). Polyphosphoester microspheres for sustained release of biologically active nerve growth factor. *Biomaterials 23*, 3765–3772.

[80] Lee, A.C., Yu, V.M., Lowe, J.B., Brenner, M.J., Hunter, D.A., Mackinnon, S.E. and Sakiyama-Elberta, S.E. (2003). Controlled release of nerve growth factor enhances sciatic nerve regeneration, *Exp. Neurol. 184*, 295–303

[81] Dertinger, S.K., Jiang, X., Li, Z., Murthy, V.N. and Whitesides, G.M. (2002). Gradients of substrate-bound laminin orient axonal specification of neurons. *Proc. Natl. Acad. Sci. USA, 99*, 12542-12547.

[82] Baldwin, S.P. and Saltzman W.M. (1998). Materials for protein delivery in tissue engineering. *Advanced Drug Delivery Reviews 33*, 71–86.

[83] R. Langer. (1990). New methods of drug delivery. *Science 249*, 1527–1533.

[84] Blesch, A., Lu, P., Tuszynski, M.H. (2002). Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. *Brain Res. Bull. 57(6)*, 833–838.

[85] Geller, H.M. and Fawcett, J.W. (2002). Building a bridge: engineering spinal cord repair. *Exp. Neurol. 174(2)*, 125–136.

[86] Lin A.S., Barrows T.H., Cartmell S.H. and Guldberg R.E. (1993). Microarchitectural and mechanical characterization of oriented porous polymer scaffolds. *Biomaterials* 24(3), 481–489.

[87] Madihally, S.V. and Matthew, H.W. (1999). Porous chitosan scaffolds for tissue engineering. *Biomaterials 20(12)*, 1133–1142.

[88] Stokols, S., and Tuszynski, M.H. (2004). The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. *Biomaterials 25 (27)*, 5839-5846.

[89] Yang, Y., De Laporte, L., Rives, C.B., Jang, J.H., Lin, W.C., Shull, K.R. and Shea, L.D. (2005). Neurotrophin releasing single and multiple lumen nerve conduits. *J. Control. Release 104*, 433–446

[90] Xu, X., Yee, W.C., Hwang P.Y.K., Yu, H., Wan, A.C.A., Gao, S., Boon, K.L., Mao, H.Q., Leong, K.W. and Wang, S. (2003). Peripheral nerve regeneration with sustained release of poly(phosphoester) microencapsulated nerve growth factor within nerve guide conduits. *Biomaterials 24*, 2405–2412

[91] Rich, K.M., Alexander, T.D., Pryor, J.C. and Hollowell, J.P. (1989). Nerve growth factor enhances regeneration through silicone chamber. *Exp. Neurol. 105*, 162–170.

[92] Derby, A., Wayne, E.V., Frierdich, G.E., Neises, G., Rapp, S.R. and Roua, D.G. (1993). Nerve growth factor facilitates regeneration across nerve gaps: morphological and behavioral studies in rat sciatic nerve. *Exp. Neurol. 119*, 176–191.

[93] Pu, L.L.Q., Syed, S.A., Reid, M., Patwa, H., Goldstein, J.M., Forman, D.L. and Thomson, J.G. (1999). Effects of nerve growth factor on nerve regeneration through a vein graft across a gap. *Plast. Reconstr. Surg. 104*, 1379–1385.

[94] He, C. and Chen, Z. (1992). Enhancement of motor nerve regeneration by nerve growth factor. *Microsurgery* 13, 151–154.

[95] Chen, M.H., Chen, P.R., Chen, M.H., Hsieh, S.T., Huang, J.S. and Lin, F.H. (2006). Gelatin-tricalcium phosphate membrane modified with NGF and cultured Schwann cells for peripheral nerve repair: a tissue engineering approach. *Biomedical engineering applications, basis and communications 18(2)*, 47-54.

[96] Rosner, B.I., Siegel, R.A., Grosberg, A. and Tranquillo, R.T. (2003). Rational Design of Contact Guiding, Neurotrophic Matrices for Peripheral Nerve Regeneration. *Ann. Biomed. Eng. 31*, 1383–1401.

[97] Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.F., Miyazono, K., Urabe, A. and Takaku, F. (1989). Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell Physiol.* 140, 323–334.

[98] Ceballos, D., Navarro, X., Dubey, N., Wendelschafer-Crabb, G., Kennedy, W.R. and Tranquillo, R.T. (1999). Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration. *Exp. Neurol. 158*, 290–300.

[99] Nakahara, Y., Gage, F.H. and Tuszynski M.H. (1996). Grafts of fibroblasts genetically modified to secrete NGF, BDNF, NT-3, or basic FGF elicit differential responses in the adult spinal cord. *Cell Transpl. 5*, 191–204.

[100] McTigue, D.M., Horner, P.J., Stokes, B.T. and Gage, F.H. (1998). Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. *J. Neurosci.* 18, 5354–5365

[101] Hu, Z.H., Chen, S.Z., Jin, Y., Xiong, Y., Wang, W., Ma, X.J. and Song, M. (2006). Microencapsulated nerve growth factor-expressing NIH3T3 cells-incorporated tissue engineering skin: a preliminary study. *Singapore Med. J. 47(6)*, 504-511.

[102] Chang, T.M. (1998). Pharmaceutical and therapeutic applications of artificial cells including microencapsulation. *Eur. J. Pharm. Biopharm. 45*, 3-8.

[103] Letic-Gravilovic, A., Piattelli, A. and Abe, K. (2003). Nerve growth factor β(NGF β) delivery via a collagene / hydroxyapatite (Col/Hap) composite and its effects on new bone ingrowth. *Journal of material science: materials in medicine 14*, 95-102.

[104] Schwartz, A. (2004). Cortical neural prosthetics. *Ann. Rev. Neurosci. 27*, 487–507.

[105] Navarro, X., Lago, T., Micera, S., Stieglitz, T. and Dario, P. (2005). A critical review of interfaces with the peripheral nervous system for the control of neuroprostheses and hybrid bionic systems. *J. Periph. Nerv. System 10*, 229–258.

[106] Dhillon, G. and Horch, K., (2005). Direct neural sensory feedback and control of a prosthetic arm. *IEEE Trans Neural Sys. Rehab. Eng. 13*, 468–72.

[107] Micera, S., Carrozza, M., Vecchi, F., Beccai, L. and Dario, P. (2006). Hybrid bionic systems for the replacement of hand function. *Proc IEEE 94 (9)*, 1752-1761.

[108] Tarler, M. and Mortimer, J. (2004). Selective and independent activation of four motor fascicles using a four contact nerve-cuff electrode. *IEEE Trans Neural Sys. Rehab. Eng. 12*, 251–257.

[109] Micera, S., Jensen, W., Sepulveda, F., Riso, R.R. and Sinkjaer, T. (2001). Neuro-fuzzy extraction of angular information from muscle afferents for ankle control during standing in paraplegic subjects: An animal model. *IEEE Trans. Biomed. Eng. 48*, 787–794.

[110] Lawrence, S., Dhillon, G., Jensen, W., Yoshida, K. and Horch, K. (2004). Acute peripheral nerve recording characteristics of polymer-based longitudinal intrafascicular electrodes. *IEEE Trans. Neural Sys. Rehab. Eng. 12 (3)*, 345–348.

[111] McDonnall, D. Clark, G., Normann, R. (2004). Interleaved, multisite electrical stimulation of cat sciatic nerve produces fatigue-resistant, ripple-free motor responses. *IEEE Trans. Neural. Syst. Rehabil. Eng. 12(2)*, 208–215.

[112] Ramachandran, A, Schuettler, M, Lago, N, Doerge, T, Koch, K.P., Navarro, X, Hoffmann, K.P., and Stieglitz, T. (2006). Design, in vitro and in vivo assessment of a multi-channel sieve electrode with integrated multiplexer. *J. Neural. Eng. 3(2)*, 114-24.

[113] Edell, D. (1986) A peripheral nerve information transducer for amputees: Long-term multichannel recordings from rabbit peripheral nerves. *IEEE Trans. Biomed. Eng. 33*, 203–214.

[114] Lago, N., Ceballos, D., Rodriguez, F., Stieglitz, T. and Navarro, X. (2005). Long term assessment of axonal regeneration through polyimide regenerative electrodes to interface the peripheral nerve. *Biomaterials* 26(14), 2021–2031.

[115] Maskery, S. and Shinbrot, T. (2005). Deterministic and stochastic elements of axonal guidance. *Annual Rev. Biomed. Eng. 7*, 187–221.

[116] Retterer, S.T., Smith, K.L., Bjornsson, C.S., Neeves, K.B., Spence A.J.H., Turner J.N., Shain W. and Isaacson, M.S. (2004) Model neural prostheses with integrated microfluidics: a potential intervention strategy for controlling reactive cell and tissue responses. *IEEE Trans. Biomed. Eng. 51*, 2063–2073

[117] Shain, W., Spataro, L., Dilgen, J., Haverstick, K., Retterer, S., Isaacson, M., Saltzman, M. and Turner, J.N. (2003). Controlling cellular reactive responses around neural prosthetic devices using peripheral and local intervention strategies. *IEEE Trans. Neural Syst. Rehabil. Eng. 11*, 186–188.

[118] Cui, X.Y., Wiler, J., Dzaman, M., Altschuler, R.A. and Martin D.C. (2003). In vivo studies of polypyrrole/peptide coated neural probes. *Biomaterials 24*, 777–87

[119] Chen, J., Wise, K.D., Hetke, J.F. and Bledsoe, S.D. (1997) A multichannel neural probe for selective chemical delivery at the cellular level. *IEEE Trans. Biomed. Eng. 44*, 760–769.

[120] Kato, Y., Saito, Y., Hoshino, T., Suzuki, T. and Mabuchi, K. (2006). Preliminary Study of Multichannel Flexible Neural Probes Coated with Hybrid Biodegradable Polymer. *Proceedings of the 28th IEEE EMBS Annual International Conference, New York City, USA*, Aug 30-Sept 3, 2006

[121] Williams, J.C., Holecko, M.M., Massia, S.P., Rousche, P. and Kipke, D.R. (2005). Multi-site incorporation of bioactive matrices into MEMS-based neural probes. *J. Neural Eng. 2*, L23–L28.

[122] Winter, J.O., Cogan, S.F. and Rizzo, J.F. III. (2007). Neurotrophin-Eluting Hydrogel Coatings for Neural stimulating Electrodes. *J. Biomed. Mater. Res. Part B. Appl. Biomater. 81B*, 551–563.

[123] Moxon K.A., Kalkhoran N.M., Markert M., Sambito M.A., McKenzie J.L. and Webster J.T. (2004). Nanostructured surface modification of ceramic-based microelectrodes to enhance biocompatibility for a direct brain-machine interface. *IEEE Trans. Biomed. Eng. 51*, 881–889.

[124] He, W. and Bellamkonda, R.V. (2005). Nanoscale neuro-integrative coatings for neural implants. *Biomaterials* 26, 2983–2990.

[125] Kim, D.H., Richardson-Burns, S.M., Hendricks, J.L., Sequera, C. and Martin, D.C. (2007). Effect of Immobilized Nerve Growth Factor on Conductive Polymers: Electrical Properties and Cellular Response. *Adv. Funct. Mat. 17(1)*, 79-86.

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Chapter 5

Nerve Growth Factor: A Neurokine Orchestrating Neuroimmune-Endocrine Functions

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Abstract

Nerve growth factor (NGF) is widely recognized as a target-derived factor responsible for the survival and maintenance of the phenotype of specific subsets of peripheral neurons and basal forebrain cholinergic nuclei during development and maturation. Considerable evidence has accumulated over the last twenty years to indicate that the actions of NGF extend far beyond "classical" effects on cells of the nervous system, to encompass a role for this molecule in the interplay between the nervous, immune, and endocrine systems. NGF as well as brain-derived neurotrophic factor, another member of the protein family of neurotrophins, have been implicated in the pathophysiological mechanisms of many diseases of the nervous and the immune systems, such as multiple sclerosis, neuropathy, pain, allergic bronchial asthma and neurotrophic keratitis. The concentration of NGF is elevated in a number of inflammatory and autoimmune states in conjunction with increased accumulation of mast cells. Like NGF, brain-derived neurotrophic factor serum levels are also increased in asthmatics. Mast cells and NGF appear to be involved in neuroimmune interactions and tissue inflammation. Mast cells themselves are capable of producing and responding to NGF, suggesting that alterations in mast cell behavior may trigger maladaptive neuroimmune tissue responses, including those of an autoimmune nature. Moreover, NGF exerts a modulatory role on sensory nociceptive nerve physiology in the adult, and

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appears to correlate with hyperalgesic phenomena occurring in tissue inflammation. NGF can thus be viewed as a multifactorial modulator in the reciprocal crosstalk between neurons, immune cells and endocrine cells.

Keywords: *Nerve growth factor; neurotrophin; neuroimmune; mast cells; inflammation; hyperalgesia; pain; autoimmune disease*

Introduction

Neurotrophic factors are secreted proteins that promote neurite outgrowth, neuronal cell differentiation and survival both *in vivo* and *in vitro*. Nerve growth factor (NGF) is the founding and best characterised member of the neurotrophin family [Levi-Montalcini *et al.*, 1999] of neurotrophic polypeptides and was discovered more than half a century ago [Hamburger and Levi-Montalcini, 1949; Levi-Montalcini and Hamburger, 1951] during a search for survival factors that could explain the deleterious effects of target tissue ablation on the subsequent survival of motor and sensory neurons [Levi-Montalcini, 1987]. NGF is also present in the central nervous system (CNS) where it serves a trophic function in the development and maintenance of basal forebrain cholinergic neurons [Dreyfus, 1989].

Research over the past two decades indicates that the actions of NGF extend far beyond "classical" effects on cells of the nervous system, to encompass a role for this molecule in the interplay between the nervous, immune, and endocrine systems. Additional, NGF-responsive cells are now known to include lymphocytes [Otten *et al.*, 1994], mast cells [Horigome *et al.*, 1993], eosinophils [Solomon *et al.*, 1998], other ectodermal-derived cells such as keratinocytes [Pincelli *et al.*, 1994] and melanocytes [Yaar *et al.*, 1994], and cellular elements of the endocrine system [Polak *et al.*, 1993]. Expression of the signal-transducing NGF receptor tropomyosin-related kinase (Trk)A on cells of the nervous, immune, and endocrine systems [Meakin and Shooter, 1992; Otten *et al.*, 1994; Patterson and Childs, 1994; Chao, 2003] further strengthens the notion that NGF is a key player in interaction among these systems. This article will begin by briefly reviewing NGF and neurotrophin biology, followed by an examination of findings that lend credence to the notion of NGF as a mediator of neuroimmune-endocrine functions.

Neurotrophin Biology

NGF was discovered almost six decades ago as a diffusable agent capable of inducing neurite outgrowth in explants of sympathetic and sensory ganglia [Levi-Montalcini, 1987]. NGF is the prototypic neurotrophin that defines the properties and functions of this class of growth factors. Two unique features of NGF's actions on neurons distinguish it from growth factors on other types of cells. First, NGF regulates functions of differentiated neurons, i.e. growth as opposed to proliferation. Second, NGF is synthesized at a considerable distance from the cell body by peripheral tissues or other neurons ("targets") that are contacted by axons of the NGF-sensitive neurons. In the periphery, the tissue sources of NGF (and other

neurotrophic factors) are typically non-neuronal cells, whereas in the CNS they are synthesized predominantly by neurons under physiological conditions [Thoenen, 1995]. During development, a retrograde flow of NGF is established, transporting NGF from the target into the nerve terminal and up the axon to the cell body [Ginty and Segal, 2002]. Those neurons that establish this flow survive the period of neuronal cell death, while those that do not degenerate. Once the retrograde flow of NGF is established, it must continue for the lifetime of the neuron to develop and maintain a functionally differentiated neuronal state [Barde, 1989]. If the supply of NGF to the target is augmented, some of the neurons that would ordinarily die are rescued (Hamburger *et al.,* 1981). Studies on the expression and actions of the NGF family indicate that, in addition to target-derived factor acquisition, autocrine and non-target-derived paracrine modes of factor presentation are likely to be important [Bothwell, 1995].

The generality of the phenomenon of programmed cell death after target deprivation (axotomy) suggests that most neurons respond to and are regulated by neurotrophic factors [Oppenheim, 1991]. This hypothesis was validated by the subsequent isolation of a second neurotrophic factor, brain-derived neurotrophic factor (BDNF), capable of supporting the survival of sensory but not sympathetic neurons [Barde *et al.,* 1982]. Molecular cloning of the gene for BDNF [Leibrock et ak., 1989] revealed its structural similarity to NGF, giving rise to the concept of the neurotrophin family. Using a homology cloning approach rather than protein purification, two additional members, neurotrophin-3 (NT-3) [Ernfors *et al.,* 1990; Hohn *et al.,* 1990; Jones *et al.,* 1990; Kaisho *et al.,* 1990; Maisonpierre *et al.,* 1990; Rosenthal *et al.,* 1990; Negro *et al.,* 1994] and neurotrophin-4/5 (NT-4/5) [Berkemeier *et al.,* 1991; Hallböök *et al.,* 1991; Ip *et al.,* 1992] were later identified. The term neurotrophin-4/5 resulted from uncertainties about whether the human neurotrophin-5 [Berkemeier *et al.,* 1991] was a species homologue of the NT-4 found in *Xenopus* [Hallböök *et al.,* 1991]. NGF, BDNF, NT-3 and NT-4/5 share approximately 50% sequence identity [Hallböök *et al.,* 1991] and have been found in a wide range of vertebrates, from cartilaginous fish to mammals. The regions of sequence similarity and variation are clustered, indicating probable sections of structural and functional importance [Ibáñez, 1995]. Two novel neurotrophins from the platyfish and carp have been cloned and designated neurotrophin-6 (NT-6) [Götz *et al.,* 1994] and neurotrophin-7 (NT-7) [Lai *et al.,* 1998], respectively. These do not have orthologs in mammals or birds and appear to interact with the same receptors as the mammalian proteins. The neurotrophins exhibit actions on distinct, as well as partially overlapping, subsets of peripheral and central neurons [Eide *et al.,* 1993; Ibáñez, 1995]. Individual neurons may also be responsive to more than one neurotrophin at a given time or at subsequent times during development.

In addition to the neurotrophins, a number of polypeptide factors have been shown to possess neurotrophic activities. These include ciliary neurotrophic factor [Ip and Yancopoulos, 1996], glial cell line-derived neurotrophic factor [Lindsay and Yancopoulos, 1996], insulin-like growth factor [Doré *et al.,* 1997], and basic fibroblast growth factor [Walicke *et al.,* 1986]. Transforming growth factor-β [Farkas *et al.,* 2003] and Sonic hedgehog [Miao *et al.,* 1997] are other proteins capable of promoting survival of specific CNS neuron populations and protecting these cells from toxic insults.

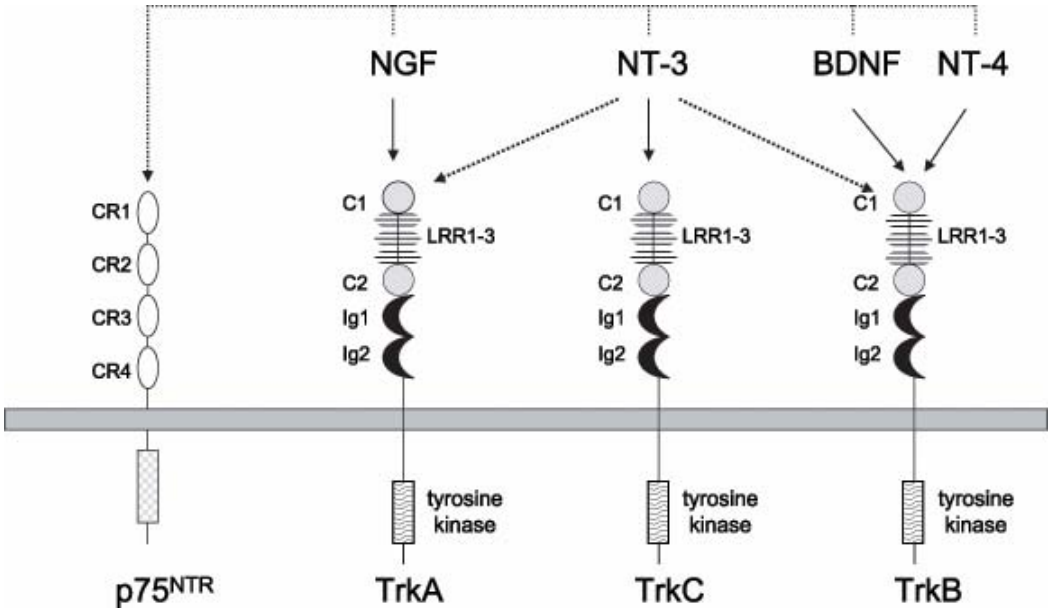

Figure 1. Neurotrophins and their receptors. The neurotrophins display specific interactions with the three Trk receptors: NGF binds TrkA, BDNF and NT-4 bind TrkB, and NT-3 binds TrkC. In some cellular contexts, NT-3 can also activate TrkA and TrkB albeit with less efficiency. All neurotrophins bind to and activate p75NTR. CR1-CR4, cysteine-rich motifs; C1/C2, cysteine-rich clusters; LRR1-3, leucine-rich repeats; Ig1/Ig2, immunoglobulin-like domains.

Diversity is also apparent in the receptors activated by neurotrophic factors. For example, the neurotrophins interact with two classes of transmembrane glycoproteins on responsive cells: protein tyrosine kinase-type receptors (members of the Trk family) and a smaller binding protein containing a short cytoplasmic tail of unknown function: the p75 low-affinity NGF or neurotrophin receptor, $p75^{NTR}$ [Meakin and Shooter, 1992]. $p75^{NTR}$ belongs to the tumor necrosis factor superfamily with an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain that includes a 'death' domain similar to those present in other members of the family [Liepinsh *et al.*, 1997; He and Garcia, 2004]. While this receptor does not contain a catalytic motif, it interacts with several proteins that relay signals important for regulating neuronal cell survival, differentiation, and synaptic plasticity. Each of the four cysteine-rich repeats of $p75^{NTR}$ participates in binding to NGF [He and Garcia, 2004] (Figure 1). The three-dimensional structure of the extracellular domain of $p75^{NTR}$ in association with an NGF dimer suggests that binding of NGF to $p75^{NTR}$ may result in dissociation of $p75^{NTR}$ multimers and propose the possibility that Trk and $p75^{NTR}$ monomers simultaneously bind the same neurotrophin monomer.

In mammals, the Trk subfamily of receptor tyrosine kinases constitutes the second major class of neurotrophin receptors. The extracellular domain of each of the Trks consists of a cysteine-rich cluster followed by three leucine-rich repeats, another cysteine-rich cluster and two Ig-like domains (Figure 1). Each receptor has a single transmembrane region that terminates in a cytoplasmic, tyrosine kinase-containing domain surrounded by several tyrosine residues that serve as phosphorylation-dependent docking sites for cytoplasmic adaptors and enzymes. The neurotrophins dimerise their cognate Trk receptor, resulting in

activation via transphosphorylation of the cytoplasmic domain kinases. Specificity of neurotrophin action is believed to be achieved in part by the selective interaction between members of the Trk family of receptors and the different neurotrophins. Thus, NGF binds to TrkA [Kaplan *et al.,* 1991a,b; Klein *et al.,* 1991a], TrkB binds BDNF and NT-4/5 with high affinity [Klein *et al.,* 1991b; Soppet *et al.,* 1991; Squinto *et al.,* 1991], and TrkC binds NT-3 [Lamballe *et al.,* 1991]. NT-3 can also interact, albeit with less efficiency, with TrkA and TrkB [Cordon-Cardo *et al.,* 1991; Soppet *et al.,* 1991; Squinto *et al.,* 1991; Ip *et al.,* 1993]. A major site at which neurotrophins interact with Trk receptors is in the membrane-proximal Ig-like domain.

The specific biological activities of the four neurotrophins on peripheral and central neurons, to some extent, correlate with their selective interaction with different members of the Trk family of receptors. Thus TrkA, which is a receptor for NGF, has been found in NGF-responsive neuronal cells, including neural crest-derived sympathetic neurons, small spinal sensory neurons of the dorsal root ganglion, and basal forebrain cholinergic neurons. TrkB is a receptor for BDNF and NT-4/5, and it is widely expressed in the peripheral nervous system and CNS, including placode-derived nodose ganglion sensory neurons and spinal cord motoneurons. Similarly, TrkC expression has been demonstrated in cells that are responsive to NT-3, including large caliber spinal sensory neurons, motor neurons, and noradrenergic neurons of the locus coeruleus. Receptor diversity is evident in that a number of isoforms of TrkB and TrkC exist which lack the tyrosine kinase domain or contain inserts in the intracellular domain that influence signaling [Guiton *et al.,* 1995; Eide *et al.,* 1966; Rose *et al.,* 2003].

Trk receptor function is modulated by $p75^{NTR}$ on several levels—by promoting ligand binding, by promoting accessibility to neurotrophins through promotion of axonal growth and target innervation, and by promoting endocytosis and retrograde transport to membrane compartments where internal engagement of neurotrophins with Trk receptors may promote signaling. For example, $p75^{NTR}$ inhibits activation of Trk receptors by non-preferred neurotrophins both in vivo and in vitro [Benedetti *et al.,* 1993; Bibel *et al.,* 1999]. The presence of $p75^{NTR}$ potentiates activation of TrkA by suboptimal concentrations of NGF, although it does not appear to potentiate the activation of other Trk receptors similarly by their ligands [Davies *et al.,* 1993; Mahadeo *et al.,* 1994], and collaborates with TrkA to form high-affinity binding sites for NGF [Esposito *et al.,* 2001b]. In addition to the promotion of binding of NGF to TrkA, $p75^{NTR}$ can promote retrograde transport of several neurotrophins [Curtis *et al.,* 1995]. $p75^{NTR}$ may reduce ligand-induced Trk receptor ubiquitination, thereby delaying Trk internalization and degradation [Makkerh *et al.,* 2005], or promote Trk receptor endocytosis through polyubiquitination and subsequent internalisation to endosomal compartments, resulting in enhanced signaling [Geetha *et al.,* 2005]. These findings each suggest a mechanism by which $p75^{NTR}$ may promote axon growth and target innervation in vivo and in vitro [Bentley and Lee, 2000; Harrison *et al.,* 2000]. Significant, albeit incomplete, sensory and sympathetic deficits (e.g. decreased pain sensitivity and cutaneous innervation) have been observed in mice lacking $p75^{NTR}$ [Lee *et al.,* 1992; Davies *et al.,* 1993; Lee *et al.,* 1994; Stucky and Koltzenburg, 1997], although the precise $p75^{NTR}$ function(s) responsible for preventing these deficits remains to be clarified.

Gene knockout experiments for the neurotrophins and the Trk receptors have proven extremely useful in understanding the roles of these ligand—receptor pairs in nervous system development and function [Snider, 1994]. Severe sensory and sympathetic neuropathies occur in mice carrying a disrupted TrkA receptor gene [Smeyne *et al.,* 1994], similar to the immunosympathectomy produced by NGF antibodies [Levi-Montalcini, 1987]. Motor neurons, whose numbers are drastically reduced in mice lacking TrkB [Klein *et al.,* 1993] are not affected in mice lacking both BDNF and NT-4/5 [Conover *et al.,* 1995; Liu *et al.,* 1995]. These results suggest that other ligands, perhaps NT-3, acts on TrkB in vivo.

Despite its long-established neuroprotective role, evidence now suggests that NGF contributes to neurodegeneration in the adult peripheral nervous system and CNS. One possible mechanism involves a change in adulthood in the predominant available form of NGF protein from the 13.5-kDa mature NGF to the 28-40-kDa precursor form (proNGF) [Seidah *et al.,* 1996; Fahnestock *et al.,* 2001], identified in recent studies as neurotoxic [Lee *et al.,* 2001] or significantly less neurotrophic [Fahnestock *et al.,* 2004] than mature NGF. ProNGF is present in high levels in several areas of adult brain [Fahnestock *et al.,* 2001], in Alzheimer's disease brain [Peng *et al.,* 2004; Pedraza *et al.,* 2005], in injured adult CNS [Harrington *et al.,* 2004], and in adult and ageing peripheral tissues [Lobos *et al.,* 2005; Bierl and Isaacson, 2007]. The neurotoxic effects of proNGF are mediated through $p75^{NTR}$ in conjunction with the sorting receptor sortilin [Nykjaer *et al.,* 2004], which is a member of the Vps family of receptors and is identical to neurotensin receptor 3 [Petersen *et al.,* 1997; Mazella *et al.,* 1998]. A new study shows that proNGF induces cell death in subpopulations of basal forebrain and peripheral sympathetic neurons of old, but not of young, adult rodents [Al-Shawi *et al.,* 2008]. Sortilin was found to play a significant part in the observed pattern of age-related proNGF-mediated neurotoxicity. In particular, survival of aged neurons was rescued by neurotensin, an alternative sortilin ligand that blocks the sortilin-mediated effects of proNGF [Al-Shawi *et al.,* 2008]. Furthermore, sortilin immunoreactivity increases markedly in ageing rodent basal forebrain and sympathetic neurons; in contrast, $p75^{NTR}$ levels are either unchanged or reduced. Selective age-related neuronal cell atrophy and neurodegeneration may thus be mediated by increased sortilin expression in neurons, together with elevated levels of proNGF expression in some targets.

NGF and Immune Cell Function

NGF displays biological activities in a broad spectrum of cell types outside the nervous system and is produced by a wide range of cell populations not normally considered targets for innervation by NGF-dependent neurons, including cells of the immune-hematopoietic lineage. For example, NGF stimulates the proliferation of both B and T lymphocytes [Thorpe and Perez-Polo, 1987; Otten *et al.,* 1989] and the production of IgM, IgA [Otten *et al.,* 1989], and IgG4 antibodies [Kimata *et al.,* 1991]. Exposure to NGF induces high-affinity interleukin-2 (IL-2) receptors on human peripheral blood mononuclear cells [Thorpe *et al.,* 1987: Brodie and Gelfand, 1992] and promotes human hematopoietic cell growth and differentiation [Matsuda *et al.,* 1988]. NGF is also involved in chemotaxis, viability, and functional properties of human polymorphonuclear neutrophils in vitro [Gee *et al.,* 1983;

Kannan *et al.,* 1991] and in vivo [Boyle *et al.,* 1985], in the differentiation of thymic stromal nonlymphoid cells [Screpanti *et al.,* 1992], and in thymic microenvironment plasticity [Marinova *et al.,* 2007]. In addition, NGF induces shape changes in platelets [Gudat *et al.,* 1981], accelerates wound healing [Matsuda *et al.,* 1998], and acts as an autocrine survival factor for memory B lymphocytes [Torcia *et al.,* 1996]. These actions of NGF are consistent with findings that human monocytes [Ehrhard *et al.,* 1993b], activated $CD4^+$ T-cell clones [Ehrhard *et al.,* 1993a; Lambiase *et al.,* 1997], lymphocytes [Santambrogio *et al.,* 1994; Melamed *et al.,* 1996], and $CD34^+$ stem cells and progenitors [Bracci-Laudiero *et al.,* 2003] express TrkA. The last two cell populations also express NGF, suggesting possible autocrine and/or paracrine actions of this neurotrophic factor in the development and regulation in immune-cell responses. A further level of diversity may be reflected by the observation of differential expression of Trk family members on lymphocyte subpopulations [Nassenstein *et al.,* 2006].

Mast cells were the first cells of the immune lineage to be recognised as a target for NGF, both in vitro [Böhm *et al.,* 1986] and in vivo [Aloe and Levi-Montalcini, 1977]. Neonatal rats given daily injections of NGF displayed a robust mast cell hyperplasia involving connective tissue mast cells in several peripheral tissues [Aloe and Levi-Montalcini, 1977], and effect later extended also to mucosal mast cells [Stead *et al.,* 1987; Marshall *et al.,* 1990]. In addition, antibodies to NGF reduced rat peritoneal mast cell numbers [Aloe, 1988]. These data point to a specific and direct action of NGF on the differentiation and maturation of mast cells and/or their precursors and are supported by the observation that NGF is capable of inducing the development of connective tissue mast cells from mouse bone-marrow cells [Matsuda *et al.,* 1991] and from newborn rat spleen cells treated with NGF [Aloe and De Simone, 1989]. Interestingly, NGF appears to act as a cofactor with IL-3 in the development of basophils or mast cells from human umbilical cord blood cells [Richard *et al.,* 1992], and can induce mast cell marker expression in the latter cell cultures [Welker *et al.,* 2000].

NGF is an extremely potent degranulating agent for cultured rat peritoneal mast cells in the presence of phosphatidylserine or its lyso derivative [Bruni *et al.,* 1982; Sugiyama *et al.,* 1985].

Table 1. Exogenous NGF regulates mast cell function

Stimulus	Net [3H]serotonin release (%)				
	None	NGF	BDNF	NT-3	NT-4/5
Antigen	9.3 ± 2.1	20.3 ± 4.2[a]	7.3 ± 3.1	6.1 ± 3.7	5.5 ± 1.8
Substance P	6.7 ± 2.9	18.9 ± 5.9[a]	5.9 ± 4.0	7.9 ± 3.5	---

Rat peritoneal mast cells were preloaded with [^{3}H]serotonin and then challenged with either a specific antigen (IgE) or substance P (30 μM), without or with the indicated recombinant neurotrophin (100 ng/ml). Net release of [^{3}H]serotonin was determined as described in Skaper *et al.* (2001). Values are means ± s.d. (three experiments). [a] $p<0.05$. NGF alone did not affect basal [^{3}H]serotonin release. [Reprinted from *Molecular Brain Research 97(2),* S.D. Skaper, M. Pollock, L. Facci, Mast cells differentially express and release active high molecular weight neurotrophins, 177-185 (Table 3), Copyright (2001), with permission from Elsevier].

Table 2. Degranulating rat peritoneal mast cells release biologically active NGF

Addition	Neurons/well			
	None	Anti-NGF	Anti-BDNF	Anti-NT-3
None	145 ± 31	---	---	---
NGF	1170 ± 72	173 ± 33	1178 ± 90	1228 ± 100
BDNF	841 ± 83	850 ± 62	158 ± 28	798 ± 51
NT-3	469 ± 53	488 ± 46	475 ± 41	133 ± 17
NGF+BDNF+NT-3	2276 ± 158	1313 ± 56	1690 ± 134	---
Mast cell medium	686 ± 58	166 ± 32	584 ± 62	---

Neurons from chicken embryonic day 9 dorsal root ganglia were cultured with the indicated recombinant neurotrophin (50 ng/ml). Medium from rat peritoneal mast cells challenged with the degranulating agent compound 48/80 (10 μg/ml) was concentrated and added to the cultures (1:10, v/v). Neutralizing neurotrophin antibodies were added at 10 μg/ml. Cultures were fixed after 48 hours incubation and numbers of surviving neurons counted. For neurons cultured with a combination of NGF, BDNF and NT-3, inclusion of all three neurotrophin antibodies reduced survival to 180 ± 42 neurons/well. Values are means ± s.d. (three experiments for neurotrophins, two experiments for mast cell conditioned medium). [Modified from Molecular Brain Research 97(2), S.D. Skaper, M. Pollock, L. Facci, Mast cells differentially express and release active high molecular weight neurotrophins, 177-185 (Table 1), Copyright (2001), with permission from Elsevier].

Table 3. Rat peritoneal mast cell degranulation releases biologically active NT-4

Addition	Neurons/well		
	None	Anti-NT-4	Anti-BDNF
None	15 ± 9	---	---
NT-4	428 ± 35	34 ± 9	416 ± 28
BDNF	426 ± 33	251 ± 33	63 ± 5
NGF	24 ± 8	---	---
Mast cell medium	254 ± 18	58 ± 21	231 ± 25

Neurons from rat embryonic day 17 nodose ganglia were cultured with the indicated recombinant neurotrophin (50 ng/ml). Medium from rat peritoneal mast cells challenged with compound 48/80 (10 μg/ml) was concentrated and added to the cultures (1:10, v/v). Neutralizing neurotrophin antibodies were added at 10 μg/ml. Cultures were fixed after 48 hours incubation and numbers of surviving neurons counted. For neurons cultured with mast cell conditioned medium and anti-NGF antibodies, survival was 269 ± 20 neurons/well. Values are means ± s.d. (two experiments). [Modified from Molecular Brain Research 97(2), S.D. Skaper, M. Pollock, L. Facci, Mast cells differentially express and release active high molecular weight neurotrophins, 177-185 (Table 2), Copyright (2001), with permission from Elsevier].

NGF can also activate mast cells through the collaborative interaction with lysophosphatidylserine expressed on the membrane surface of activated platelets [Kawamoto *et al.*, 2002]. On its own, NGF at physiological concentrations is a very poor secretagogue for rat peritoneal mast cells, while markedly enhancing antigen or other secretagogue-induced histamine release from these cells [Tomioka *et al.*, 1988; Skaper *et al.*, 2001] (Table 1). NGF may thus act as an immunomodulator in the inflammatory response through the regulation of mediator release from mast cells. Moreover, the striking cooperative effect of NGF with

platelets on mast cell activation may contribute to development of acute and chronic inflammation and wound healing processes at damaged tissues. Within the neurotrophin family NGF appears to be unique in regulating basophil functions, having a very similar sensitizing or priming effect on histamine release in mature human basophils [Bischoff and Dahinden, 1992]. In line with such findings, subcutaneous injection of NGF produces plasma extravasation [Otten *et al.,* 1984], a response consistent with histamine release caused or facilitated by NGF. Human blood-derived basophils express functional TrkA (but not TrkB or TrkC) receptors that do not require the participation of $p75^{NTR}$ [Burgi *et al.,* 1996]. NGF expression by mast cells could thus constitute an important link between mast cell activation and basophil function in the late-phase allergic reactions.

Rat peritoneal mast cells express TrkA, but not other members of the Trk family or $p75^{NTR}$ [Horigome *et al.,* 1993], and synthesis, store and release biologically active NGF [Leon *et al.,* 1993; Skaper *et al.,* 2001] (Table 2), as well as NT-4/5 [Skaper *et al.,* 2001] (Table 3). Both the human mast cell line HMC-1 and cultured human mast cells express functional TrkA and produce NGF [Nilsson *et al.,* 1997; Tam *et al.,* 1997]; HMC-1 cells express also TrkB and TrkC full-length proteins, while human lung mast cells express message for all Trks [Tam *et al.,* 1997]. Moreover, HMC-1 cells express mRNAs for NGF, BDNF, and NT-3, whereas NGF and BDNF transcripts were detectable in human umbilical cord blood mast cell preparations [Tam *et al.,* 1997]. Thus, NGF and other neurotrophins appear capable of affecting mast cell mediator release, possibly in an autocrine or paracrine fashion. A survival-promoting effect of NGF on mast cells in vitro may involve prevention of an apoptotic death mechanism [Horigome *et al.,* 1994; Kawamoto *et al.,* 1995; Bullock and Johnson, 1996] through Trk activation, in analogy to neuronal cell rescue by NGF. The actions of NGF on immune cell functions are summarised in Table 4.

Human NGF is a dimer of two identical 13 kDa subunits of 118 amino acids held together by monovalent bonds. Intriguingly, rat mast cells also contain and release high molecular weight (73 kDa) species of NGF, together with high molecular weight isoforms of NT-3 and NT-4/5, but no BDNF [Skaper *et al.,* 2001]. The use of extreme denaturing conditions failed to alter the electrophoretic pattern of the mast cell-derived proteins, suggesting that the high molecular weight bands observed were not aggregated forms of the protein [Skaper *et al.,* 2001]. Dorsal root ganglia and spinal cord of adult rat are reported to contain 53, 60 and 73 kDa proteins reactive with NGF antibodies, but not the 13 kDa NGF monomer [Reinshagen *et al.,* 2000]. These molecules likely represent multiple NGF prohormone species generated by extensive post-translational modification. Pulse-chase studies with cell lines transfected with full-length NGF cDNA have clearly documented that the NGF precursor undergoes both N-glycosylation and sulfation [Seidah *et al.,* 1996]. The presence of high molecular weight (53-73 kDa) isoforms of NGF and NT-3 have been reported in animal models of ulcerative colitis and in human inflammatory bowel disease (Crohn's disease) [Reinshagen *et al.,* 1996,1997; di Mola *et al.,* 2000]; the mature monomeric form of NGF was absent. The propensity of mast cells to accumulate at such sites of inflammation proposes these cells to represent one source of NGF which could play a role in disease pathophysiology (discussed in more detail in a subsequent section).

Locally produced NGF may play an important role in mast cell accumulation in allergic and non-allergic inflammatory conditions, given evidence that NGF may function as a chemoattactant for mast cells through both mitogen-activated protein kinase and

phosphatidylinositol 3-kinase signalling pathways [Sawada *et al.*, 2000]. NGF modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism [Marshall *et al.*, 1999], suggesting a role for prostanoid production in the regulation of local inflammatory responses and neuronal cell degeneration after tissue injury involving induction of NGF production.

Table 4. NGF Responsive Cells of the Immune-Hematopoietic Lineage

Cell type	NGF response	Reference
Mast cells	Maturation, survival, degradation, chemoattraction (NGF source)	Aloe and Montalcini (1977) Bruni *et al.* (1982) Sugiyama *et al.* (1985) Böhm *et al.* (1986) Stead *et al.* (1987) Aloe (1988) Tomioka *et al.* (1988) Aloe and De Simone (1989) Marshall *et al.* (1990) Matsuda *et al.* (1991) Richard *et al.* (1992) Leon *et al.* (1993 Horigome *et al.* (1994) Kawamoto *et al.* (1995) Bullock and Johnson (1996) Nilsson *et al.* (1997) Tam *et al.* (1997) Sawada *et al.* (2000) Welker *et al.* (2000) Skaper *et al.* (2001)
Basophils	Activation	Bischoff and Dahinden (1992) Bürgi *et al.* (1996)
Eosinophils	IL-4 production	Noga *et al.* (2002)
Lymphocytes	Proliferation, antibody Production (NGF source)	Thorpe and Perez-Polo (1987) Otten *et al.* (1989) Kimata *et al.* (1991) Ehrhard *et al.* (1993) Santambrogio *et al.* (1994) Lambiase *et al.* (1997)
Monocytes	CGRP synthesis	Bracci-Laudiero *et al.* (2005)
Peripheral blood mononuclear cells	IL-2 receptor Expression	Thorpe *et al.* (1987)
Hemopoietic cells	Growth, Differentiation	Matsuda *et al.* (1988) Brodie and Gelfand (1992)
Polymorphonuclear neutrophils	Chemotaxis, viability, Phagocytosis	Gee *et al.* (1983) Boyle *et al.* (1985) Kannan *et al.* (1991)
Platelets	Shape change	Gudat *et al.* (1981)
Memory B lymphocytes	Autocrine survival Factor	Torcia *et al.* (1996)

NGF and Endocrine-Nervous System Interaction

NGF appears to participate in the regulation of neuronal and non-neuronal cell populations involved in the control of specific neuroendocrine functions [Aloe *et al.,* 1994] and in the acquisition of male and female reproductive capacity [Lara *et al.,* 1990; Persson *et al.,* 1990]. For example, the hypothalamic content of *ngf* and NGF protein increase following stressful events [Spillantini *et al.,* 1989], while the genes encoding NGF and its receptor are expressed in the developing female hypothalamus [Ojeda *et al.,* 1991]. Biologically active NGF is found in the rat pituitary [Lathinen *et al.,* 1989], is present in both mammotroph and somatomammotroph cells [Patterson and Childs, 1994], and its release from the pituitary appears to be under neuronal control [Missale *et al.,* 1996]. Transgenic mice over-expressing the NGF gene in lactotrophs showed a marked hyperplasia of this cell type and the ability to release NGF [Borrelli *et al.,* 1992]. Thyroid and parathyroid glands of the rat express NGF message and proNGF protein [Dicou *et al.,* 1986]. NGF stimulates the pituitary-adrenocortical axis, enhancing the secretion of adrenocorticotrophic hormone and the concentration of plasma glucocorticoids [Otten *et al.,* 1979], an effect probably mediated through the hypothalamus [Scaccianoce *et al.,* 1993]. Plasma NGF levels in women during labor and lactation are elevated when compared to concentrations found at term or in controls [Luppi *et al.,* 1993], a time when plasma levels of the neurohypophyseal hormone oxytocin are high. Administration of oxytocin to female rats markedly raised hypothalamic NGF content [Luppi *et al.,* 1993]. Activation of TrkA by NGF directs the differentiation of GH3 cells, a clonal cell line related to bipotential somatomammotrophs from a prevalently growth hormone-secreting somatotroph phenotype to prolactin producing lactotrophs [Missale *et al.,* 1994], while concurrently suppressing the tumorigenicity of human prolactinomas in vivo and in vitro [Missale *et al.,* 1993]. NGF may also occur in the pituitary in a stored form and secreted into the circulation in vivo [Lathinen *et al.,* 1989]. This appears to be likely since cell lines of pituitary origin, when transfected with the NGF gene via a replication-defective retroviral vector, are able to store mature β-NGF in secretory granules and to release it upon stimulation [Wolf *et al.,* 1988]. Together, these data support a physiological role for NGF during pituitary development.

Fetal rats exposed to NGF antibodies display marked neuroendocrine deficits postnatally [Aloe *et al.,* 1981]. Not unexpectedly, such offspring have severely atrophied sympathetic and sensory ganglia, but also much smaller thyroids among endocrine organs. Deleterious effects of autoimmune NGF deprivation have also been described in rabbits and guinea pigs [Johnson *et al.,* 1983]. Maternal exposure to NGF antibodies during fetal development causes, in newborn pups, loss of body weight, sensory deficits, and lethality [Johnson *et al.,* 1980; Yip *et al.,* 1984]. Exposure to NGF antibodies during the postnatal period perhaps inactivates endogenous NGF in neuroendocrine structures, thereby provoking a widespread neuroendocrine immune-deficiency syndrome. The studies cited here, which point to a modulatory role for NGF in the hypothalamo-pituitary-adrenal axis, would support such a notion. In this regard, induction of TNBS-colitis in rats markedly increased NGF, BDNF and NT-3 expression within the adrenal cortex, suggesting that neurotrophins might act as local modulators of components of the hypothalamo-pituitary axis during peripheral inflammation [Flämig *et al.,* 2001]. Interestingly, natural autoantibodies to NGF have been detected in the

sera of some patients with autoimmune diseases [Dicou *et al.,* 1993]. This topic will be discussed in a later section.

Circulating NGF and Stress

NGF is produced and stored in large amounts in the submaxillary salivary glands of adult male mice [Levi-Montalcini, 1987], although the biological significance of this is not clear. Snake venom [Kostiza and Meier, 1996] and salivary gland of the African rat Mastomys natalensis [Burcham *et al.,* 1991] also contain substantial quantities of NGF. Aggressive interactions with conspecifics are accompanied by a massive release of NGF into the bloodstream [Aloe *et al.,* 1986]; prolonged behavior of this type leads to hypertrophy of cortical and medullary components of the adrenal gland. Intermale aggressive behavior also induces a large increase in the levels of NGF mRNA and protein in the hypothalamus [Spillantini *et al.,* 1989]. Serum levels of NGF appeared to correlate positively with the number of fighting episodes in a mouse pair [Aloe *et al.,* 1986]. The ability of NGF to elicit hypertrophy of the adrenal gland and enhanced functional activity of the medullar and cortical sections of this gland suggests this organ to be the most likely target of NGF [Maestripieri *et al.,* 1990]. NGF levels in serum consistently reach higher peaks in the subordinate rather than in the dominant partner [Maestripieri *et al.,* 1990], suggesting that NGF discharge from salivary gland to bloodstream is a combination of aggressive behavior and anxiety, which would be expected to be more acute in the subordinate mouse. Release of NGF produced by psychosocial stress is not mimicked by physical stresses. In the aged male mouse, fighting increases the expression of TrkA and TrkB in the subventricular zone of the hippocampus, and is accompanied by altered neurotrophin levels [Fiore *et al.,* 2005] – which may account for the observed increase in brain progenitor cell number. Levels of NGF in the blood increase in humans both prior to and after psychologically stressful and anxious situations, while serum levels of interleukin-1, tumor necrosis factor-α, cortisol, and adrenocorticotrophic hormone are not modified [Aloe *et al.,* 1994]. As mentioned earlier, physiological concentrations of NGF do not appear to activate mast cells and basophils directly but rather modulate their threshold to other triggering stimuli (cf. Table 1). Thus, circulating NGF could be viewed as a general alert signal used by the brain in settings of stress and anxiety to "prime" the immune system towards noxious inputs. It has also been suggested that the circulating NGF released during and following stress may serve to prevent possible deficits and/or damage linked to stress-induced sympathetic and cardiovascular activation [Manni *et al.,* 2008].

NGF, Inflammation and Mast Cells

Mast cells are a heterogeneous immune-effector cell type found in connective tissues throughout the body, occur adjacent to blood and lymphatic vessels, and are concentrated beneath mucosal surfaces [Galli, 1993; Galli *et al.,* 2005]. Morphological and functional mast cell-neuron interactions in vitro and in vivo have been observed [Roznieckі *et al.,* 1999],

within both the peripheral nervous system and CNS [Johnson and Krenger, 1992]. For example, mast cells are active in the thalamus in basal conditions and NGF has the potential to elicit long-lasting degranulation of thalamic mast cells in vivo, exerting a direct effect and/or priming these cells to react to endogenous stimuli [Florenzano and Bentivoglio, 2000].

Resting and actively degranulating mast cells are found on the brain side of the blood-brain barrier [Ibrahim, 1974; Dropp, 1976; Dimitriadou *et al.*, 1990; Theoharides, 1990; Manning *et al.*, 1994; Silverman *et al.*, 1994; Florenzano and Bentivoglio, 2000]. In the periphery, exocytosis of mast cell granules results in the release of soluble mediators and insoluble granule remnants [Lagunoff, 1973; Burwen and Satir, 1977; Wang *et al.*, 1995; Guo *et al.*, 1998]. These mast cell constituents are found in a variety of nearby cell types, e.g. macrophages [Lindahl *et al.*, 1979; Kovanen, 1991], neutrophils, and eosinophils [Baggiolini *et al.*, 1982], acquired by fusion of granule and cellular membranes or by capture of mast cell granule remnants. A recent study shows that CNS neurons can acquire mast cell products via transgranulation, a multistep process that begins with the elaboration of a mast cell pseudopodium containing granules followed by the attachment of the pseudopodium to the plasma membrane of an adjoining neuron [Wilhelm *et al.*, 2005]. Thereafter, the pseudopodium with its mast cell granules is transferred to the neuron to which it was attached.

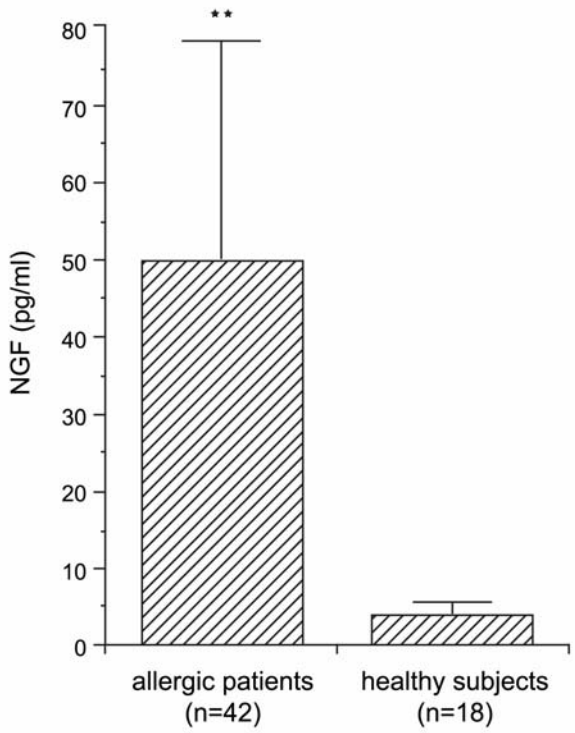

Figure 2. NGF serum levels are significantly increased (**P<0.001) in allergic patients when compared with healthy subjects. [Reprinted from *Proceedings of the National Academy of Sciences USA, 93(20),* S. Bonini, A. Lambiase, S. Bonini, F. Angelucci, L. Magrini, L. Manni and L. Aloe, Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma (Figure 1), Copyright (1996), with permission from The National Academy of Sciences of the USA].

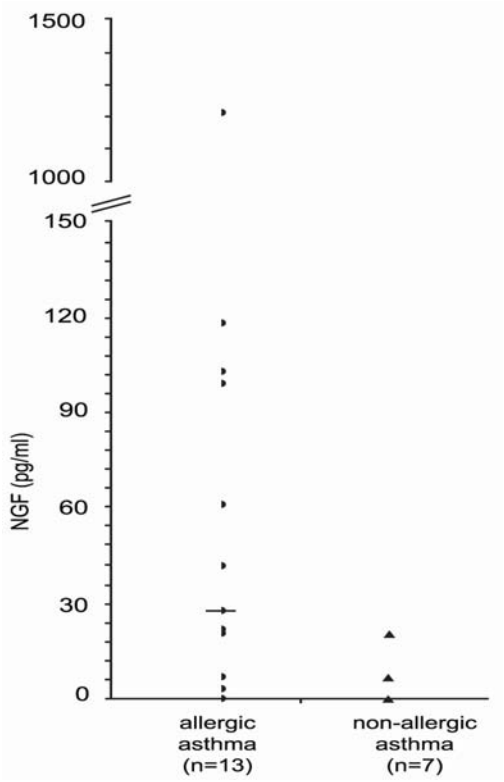

Figure 3. NGF serum levels in allergic (skin-test or RAST-positive) asthmatic patients are significantly higher than levels in non-allergic (skin-test or RAST-negative) asthmatic patients (mean = 132.1 ± 90.8 pg/ml, median = 28.0 pg/ml versus mean = 4.9 ± 2.9 pg/ml, median = 0 pg/ml, $P<0.005$). [Reprinted from Proceedings of the National Academy of Sciences USA, 93(20), S. Bonini, A. Lambiase, S. Bonini, F. Angelucci, L. Magrini, L. Manni and L. Aloe, Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma (Figure 4), Copyright (1996), with permission from The National Academy of Sciences of the USA].

Transgranulation [Greenberg and Burnstock, 1983] thus represents a novel form of intracellular communication between mast cells of the CNS and neighboring neurons. In addition to altering the neuronal microenvironment via actions on blood vessel permeability [Zhuang *et al.,* 1996; Esposito *et al.,* 2001a], mast cells can alter the internal environment/constituents of neurons that acquire mast cell products with the potential to alter the responsiveness of a neuron or by supplying products that the neuron can re-release.

Mast cells represent critical effector cells in allergic diseases and other IgE-dependent responses [Galli, 1993; Galli *et al.,* 2005]. Nervous and immunological mediators such as neuropeptides or IgE can affect the state of mast cell activation [Olsson, 1968]. Activated mast cells are capable of secreting an enormous array of cytokines and other inflammatory mediators [Gordon *et al.,* 1990; Galli, 1993]. Mast cells may thus be important messengers between the nervous, endocrine, and immune systems [Purcell and Atterwill, 1995; Silver *et al.,* 1996]. In addition to being rapid affector cells in immediate hypersensitivity reactions, mast cells appear to be involved in other pathophysiological processes including delayed-type hypersensitivity, wound healing, fibrosis, and disorders of an inflammatory nature [Gordon *et al.,* 1990; Galli, 1993; Hermes *et al.,* 2001].

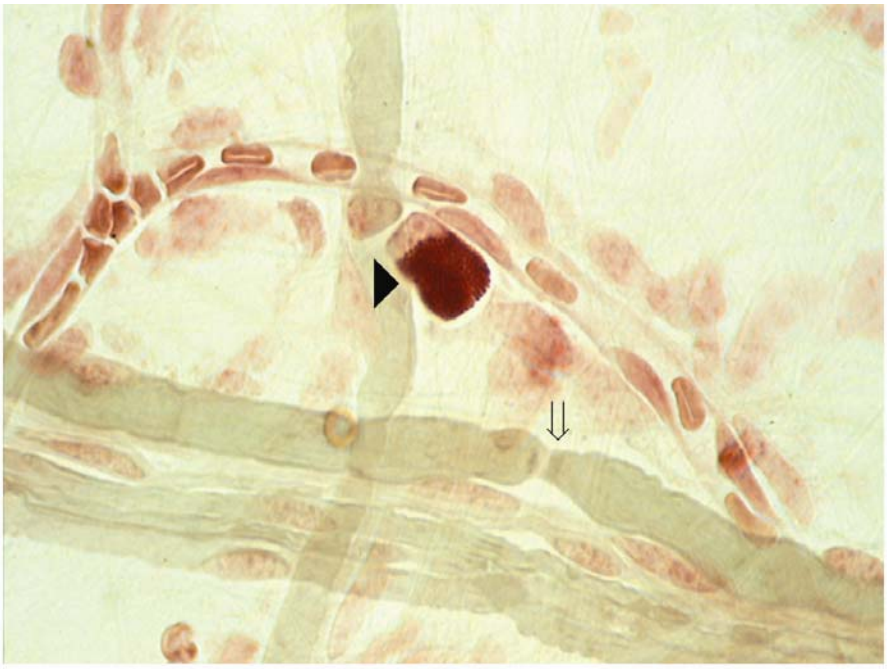

Figure 4. Intracranial mast cells are perivascular in location. Note the presence of a mast cell (arrowhead) stained red with safranin (characteristic of connective tissue-type mast cells) alongside a blood vessel. A nerve with node of Ranvier (⇓) is close by.

Altered mast cell numbers or phenotype, as well as mast cell overactivity, may underlie dysfunctional nervous-immune system interactions. Increased mast cell numbers have been reported in a variety of immunoinflammatory conditions. Mast cell numbers are elevated in the dermis of patients with early systemic sclerosis [Tuveri *et al.*, 1993] and in the synovium of rats with rheumatoid synovitis [Aloe *et al.*, 1992a]. NGF was elevated in the dermas [Tuveri *et al.*, 1993] and synovium [Aloe *et al.*, 1992a] in the latter instances, and is released from synovial fibroblasts [Manni *et al.*, 2003]. NGF synthesis and release is dysregulated in the upper airways of patients with allergic rhinitis [Sanico *et al.*, 2000; Raap *et al.*, 2008] and in lower airways of infants with respiratory syncytial virus infection [Tortorolo *et al.*, 2005], with increased circulating levels of the protein in humans with allergic diseases (Figure 2) and asthma (Figure 3) [Bonini *et al.*, 1996; Braun *et al.*, 2000] and in rats with experimental allergic bronchial airway inflammation [Stampachiacchiere *et al.*, 2006]. An increase in NGF content and nerve fiber sprouting in human allergic contact eczema has been described [Kinkelin *et al.*, 2000], and this may have a functional impact on skin-associated immune cells, in particular mast cells. Mast cells and NGF have been implicated in inflammatory bowel disease [di Mola *et al.*, 2000], Hirschsprung's disease and intestinal neuronal dysplasia [Kobayashi *et al.*, 1999], human immunodeficiency virus [Garaci *et al.*, 1999], and myeloproliferative pathologies [Simone *et al.*, 1999]. A correlation between mast cells and NGF may exist in certain inflammatory diseases, given that NGF is known to cause mast cell activation and proliferation. IgE receptor-mediated release of NGF by human mast cells further strengthens a link between NGF and mast cells in allergic inflammation [Xiang and Nilsson, 2000]. In addition, human eosinophils express functionally active TrkA, TrkB and

TrkC, and release IL-4 upon NGF stimulation [Noga *et al.,* 2002]. Eosinophils produce NGF and NT-3 and secrete NGF on immunologic stimuli [Kobayashi *et al.,* 2002], while NGF is elevated in eosinophil lysates of allergics [Noga *et al.,* 2005]. In patients with seasonal allergic rhinitis, the concentrations of NGF in nasal secretions correlate with the magnitudes of eosinophilic inflammation in the airway, suggesting a potential clinical implication of eosinophil NGF. In analogy to mast cells, these observations propose a pathologic mechanism by which eosinophils may contribute to enhanced neurologic responses in patients with allergic diseases and other eosinophilic disorders. Alternatively, eosinophils may play an important role in maintenance and restoration of homeostatic functions of mucosal tissues through the pleiotropic activities of NGF.

Mast cells produce and respond to a variety of cytokines [Gordon *et al.,* 1990]. Cytokines involved in inflammation and immune responses, such as interleukin-1β and tumor necrosis factor-α, are strong inducers of NGF synthesis [Yoshida and Gage, 1992], and differences in tissue cytokine and NGF expression may provide a positive feedback loop for autocrine/paracrine regulation of mast cell properties. NGF accumulation in acute inflammatory exudates has widely been attributed to cellular infiltrates or to up-regulation of NGF expression. The rapid appearance and amount of NGF accumulation in rat skin-blister fluid [Weskamp and Otten, 1987], however, seems more in line with NGF being released from resident tissue cells containing stores of the protein. Remodeling of intestinal mucosal nerve fibers during intestinal inflammation follows changes in mast cell density [Stead *et al.,* 1991]. Mast cells and sympathetic neurons form contacts [Blennerhasset *et al.,* 1991], proposing the existence of chemotactic NGF-like gradients between mast cell and nerve ending. The mast cell could thus represent a readily available source of NGF.

Autoimmune Diseases, NGF, and Mast Cells

The etiology and cellular mechanisms underlying autoimmune diseases remain largely undefined. Presumed autoimmune disorders like multiple sclerosis [Waksman, 1988], irritable bowel syndrome, and interstitial cystitis appear to occur with greater frequency in females [Sant *et al.,* 2007], suggesting a possible hormonal component in pathophysiology. In multiple sclerosis, plaques in human brain tissue contain mast cells [Olsson, 1968; Theoharides, 1990]. Mast cells secrete pro-inflammatory cytokines in response to myelin basic protein [Skaper *et al.,* 1996], a major suspected antigen in multiple sclerosis, which also induces peripheral [Johnson *et al.,* 1988] and central [Theoharides *et al.,* 1991] demyelination. Prolonged mast cell stimulation can damage cultured CNS neurons by the release of mast cell cytokines, which in turn induce astroglial nitric oxide synthase [Skaper *et al.,* 1996]. Mast cells, most likely of neural crest origin, have been indentified intracranially [Lambracht-Hall *et al.,* 1990a], where they show a strict perivascular location (Figure 4) [Theoharides *et al.,* 1991] and where they secrete vasoactive amines in response to mediators [Lambracht-Hall *et al.,* 1990b]. Interestingly, estradiol and myelin basic protein are reported to act synergistically in triggering mast cell activation [Theoharides *et al.,* 1993]. The endogenous immune-response system of the brain may also participate in exacerbating the fundamental pathology of Alzheimer's disease, apparently without stimulation by peripheral

inflammatory mediators or the peripheral immune system [McGeer and McGeer, 1995]. A strong inflammatory response might by autotoxic to neurons [Skaper *et al.,* 1996]. A role for mast cells in Alzheimer's disease, however, remains highly speculative.

Multiple sclerosis is accompanied by penetration of blood-borne immune cells within brain parenchyma and subsequent destruction of myelin. T-lymphocytes and monocytes are clearly involved in cellular infiltrates in areas of demyelination [Prineas and Wright, 1978; Hauser *et al.,* 1986] and have a role in experimental allergic encephalomyelitis [Sobel *et al.,* 1984] and experimental allergic neuritis. Mast cells have been reported also in multiple sclerosis plaques [Olsson, 1974; Toms *et al.,* 1990], and are activated during experimental allergic neuritis [Brosnan *et al.,* 1985]. There is some evidence to suggest that a delayed T-cell response may depend on early release of mast cell mediators [Askenase and Van Loveren, 1983] and that T-cell products can cause mast cell activation [Kaplan *et al.,* 1991c]. Mast cells themselves appear capable of presenting antigen in a major histocompatibility complex class II-restricted manner [Frandji *et al.,* 1993]. Conceiveably, myelin basic protein from demyelinated axons could provoke mast cell degranulation, leading to a feed-forward reaction. As lymphocytes and monocytes are NGF-responsive, NGF released from brain mast cells could contribute to such a cycle. In this regard, multiple sclerosis patients are reported to express increased levels of NGF in cerebrospinal fluid [Bracci-Laudiero *et al.,* 1992].

Mast cells and NGF may also participate in autoimmune diseases of extraneural origin. In the case of rheumatoid arthritis, mast cells and NGF accumulate in the synovial fluid [Mican and Metcalf, 1990; Aloe *et al.,* 1992a].

Table 5. Autoimmune and Other Inflammatory Conditions with NGF Elevation

Condition	Species	Tissue site	Reference
Multiple sclerosis	Human	Cerebrospinal fluid	Bracci-Laudiero et al. (1992)
Chronic arthritis (adult, juvenile)	Human	Synovium, plasma	Aloe et al. (1992b) Falcini et al. (1996)
Carrageenan-induced arthritis	Rat	Synovium	Aloe et al. (1992c)
Transgenic TNF-α overexpression (arthritis)	Rat	Synovium	Aloe et al. (1995)
Rheumatoid synovitis	Rat	Synovium	Aloe et al. (1992a)
Allergic diseases, asthma	Human	Nasal lavage, plasma	Bonini at al. (1996) Braun et al. (2000) Sanico et al. (2000) Raap et al. (2008
Allergic contact eczema	Human	Skin	Kinkelin et al. (2000)
Inflammatory bowel disease	Human	Intestine	Di Mola et al. (2000)
Systemic lupus erythematosus	Human	Serum	Bracci-Laudiero et al. (1993; 1996)
Systemic sclerosis	Human	Dermis	Tuveri et al. (1993)
Systemic mastocytosis	Human	Plasma	Kurosawa et al. (1999)
Carrageenan-induced pleurisy	Rat	Pleural exudate	Weskamp and Otten (1987)
Noxious thermal stimulus	Rat	Skin blister fluid	Weskamp and Otten (1987)

Increased quantities of NGF are found in several types of chronic arthritis, in systemic lupus erythematosus, and in mastocytosis [Aloe *et al.,* 1992b,c; Bracci-Laudiero *et al.,* 1993; Aloe *et al.,* 1995; Bracci-Laudiero *et al.,* 1996; Falcini *et al.,* 1996; Kurosawa *et al.,* 1999] (Table 5). Intrasynovial injection of NGF is reported to induce an increase in the local distribution of mast cells [Aloe *et al.,* 1992a]. Mast cells are frequently found at sites of tissue inflammation, and their ability to release substantial amounts of NGF proposes that these cells may contribute, both acutely and at later times, to NGF expression in inflammatory conditions.

Inflammation, Pain and NGF

A key feature of inflammation is pain and hyperalgesia. In the periphery, inflammatory mediators increase the sensitivity of high-threshold nociceptors so that a lower stimulus intensity is required to activate them; this phenomenon is called peripheral sensitisation [Treede *et al.,* 1992]. Evidence suggests that in adult animals, NGF can induce hyperalgesia, and may be an endogenous mediator in some persistent pain states. NGF levels are markedly elevated in inflamed tissues [Donnerer *et al.,* 1992; Woolf *et al.,* 1994; Koltzenburg *et al.,* 1999; Schafers *et al.,* 2003; Talhouk *et al.,* 2004; for a review see Pezet and McMahon, 2006]. This rise is secondary to the increases in levels of cytokines, specifically the interleukins and tumour necrosis factor-α [Woolf *et al.,* 1997] that are released from mast cells, phagocytic cells, and antigen-presenting cells of the immune system. Peripheral administration of NGF in the adult rat rapidly induces a decrease in the threshold of nociceptors to heat and mechanical stimuli [Lewin and Mendell, 1993; Petty *et al.,* 1994; Andreev *et al.,* 1995; Bennett *et al.,* 1998] in the absence of $p75^{NTR}$ [Bergmann *et al.,* 1998], suggesting that the TrkA receptor is sufficient to mediate the acute noxious action of NGF. Indeed, direct administration of NGF into the sciatic nerve induces hyperalgesia in rats [Ruiz *et al.,* 2004]. Hyperalgesic responses have been observed in transgenic animals overexpressing NGF in the skin [Davis *et al.,* 1993], and increased cutaneous NGF levels selectively affected the survival and functional properties of nociceptors [Stucky *et al.,* 1999]. In contrast, genetically modified rodents expressing antisense NGF mRNA exhibited hypoalgesia to mechanical stimuli [Davis *et al.,* 1993]. Animals treated with anti-NGF antibodies [Aloe *et al.,* 1980; Woolf *et al.,* 1994] or TrkA-IgG fusion proteins [McMahon *et al.,* 1995] displayed substantially reduced sensitivity to noxious mechanical and thermal stimuli, and neutralization of endogenous NGF prevented the sensitization of nociceptors supplying inflamed skin [Koltzenburg *et al.,* 1999]. Suppression of $p75^{NTR}$ in uninjured sensory neurons reduced neuropathic pain after nerve injury [Obata *et al.,* 2006], and a function-neutralizing anti-TrkA antibody reduced formalin-evoked inflammatory pain and sciatic nerve ligation-induced neuropathic pain [Ugolini *et al.,* 2007] in mice. Sympathetic postganglionic neurons appear to contribute to thermal hyperalgesia [Andreev *et al.,* 1995] but mast cells and sensory neurons are more likely to be important sites for the sustained action of NGF in producing increased sensitivity associated with tissue inflammation [Woolf *et al.,* 1996; Pezet and McMahon, 2006].

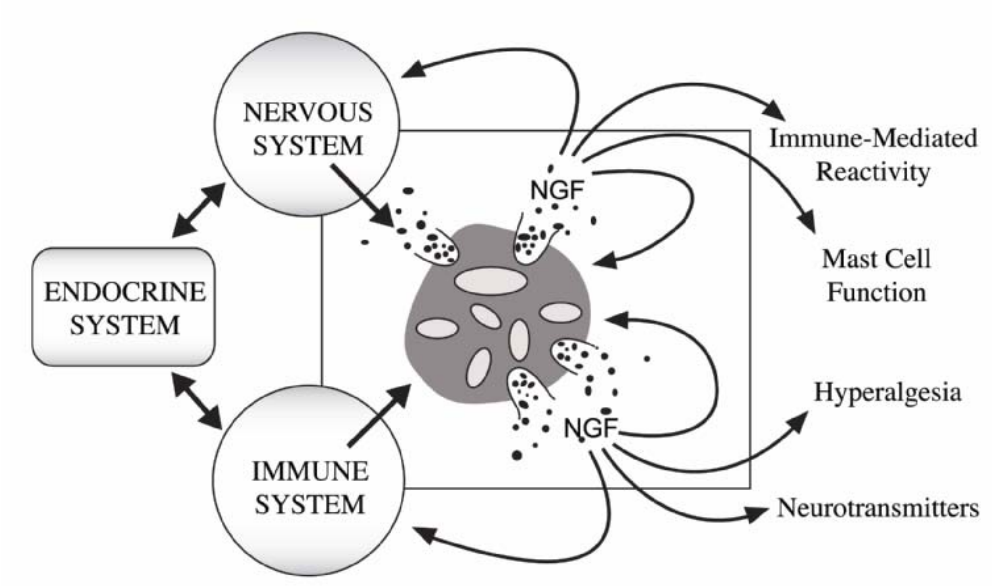

Figure 5. NGF is a modulator of nervous-immune-endocrine system interactions. Mast cell-released NGF could also function in an autocrine manner. Potential actions of mast cell-derived NGF are shown at right.

Given the range of biological actions of NGF described above, it is not surprising that pain perception in humans is affected by NGF treatment. In human volunteers, small subcutaneous or intramuscular injections of NGF give rise to pain and tenderness (hyperalgesia), at the injection site, starting within minutes and persisting for several hours. Small intravenous doses result in widespread deep pain and tenderness lasting for several days [Svensson *et al.,* 2003].

The precise mechanism of hyperalgesic induction by NGF is not clear. High levels of NGF appear to strengthen the effects of both orthodromic and antidromic sensory synaptic release by modifying amplitude and/or density of peripheral and central terminal fields, and by increasing peptidergic transmitter content and reducing the activation threshold to noxious heat and mechanical stimuli. The initial, rapid phase of NGF-induced thermal hyperalgesia seems also to be peripherally mediated by mast cells and to involve a central glutamatergic receptor-dependent mechanism [Lewin and Mendell, 1993]. NGF-induced hyperalgesia is qualitatively analogous to that occurring in tissue inflammation.

A number of ligand-gated ion channels [including vanilloid receptor 1 (TRPV1), purinergic receptor of ATP ($P2X_3$), ASIC3 and G protein-coupled receptors (including bradykinin B2 receptors and the mu opiate receptor)] are regulated by NGF, and this regulation is generally restricted to neurons bearing TrkA [Pezet and McMahon, 2006]. These receptors are normally expressed throughout the sensory neuron and are concentrated at peripheral and central arbors; therefore, NGF treatment may have functional effects on sensory transduction/transmission at both these loci. Another category of gene regulation by NGF relates to voltage-gated ion channels, including calcium and potassium [Park *et al.,* 2003], and in particular sodium channels. Functionally, both tetrodotoxin-sensitive and –resistant sodium currents are increased in sensory neurons by NGF [Okuse *et al.,* 1997;

Leffler *et al.,* 2002]. These NGF-induced changes are likely to have a major impact on the excitability of nociceptive neurons. Another functionally important action of NGF is to sensitize nociceptor responses through post-translational rather than transcriptional controls. This have been studied extensively for heat responses of nociceptive neurons in culture, which are increased acutely following NGF stimulation.The mechanism appears to be enhanced responsiveness to of TRPV1 receptors, but the intracellular cascades leading to TRPV1 sensitization are unclear, and may implicate protein kinase C, mitogen-activated protein kinases, and phosphatidylinositol 3-kinase signalling pathways [Khasar *et al.,* 1999; Ji *et al.,* 2002; Bonnington and McNaughton, 2003; Zhuang *et al.,* 2004; Zhu and Oxford, 2007].

In addition to upregulation of peptides in sensory somata, NGF also upregulates BDNF in TrkA-positive dorsal root ganglion neurons [Michael *et al.,* 1997]. At peripheral terminals another TrkB agonist, NT-4/5, can sensitize individual sensory afferents to noxious thermal stimulation, as can BDNF [Shu *et al.,* 1999]. Neurons lacking BDNF showed a profound and specific reduction in their mechanical sensitivity; postnatal treatment of $BDNF^{+/-}$ mice with BDNF completely rescued the mechanosensitivity deficit [Carroll *et al.,* 1998]. The sensitizing action of both of these neurotrophins has also been established in behavioural experiments, where NT-4/5 is more potent than BDNF [Shu *et al.,* 1999]. Mast cell depletion prevents NT-4/5 from eliciting behavioral sensitization [Shu *et al.,* 1999]. Interestingly, TrkB receptors are known to co-localise with TrkA on some mast cells [Tam *et al.,* 1997], and mast cells themselves are capable of producing and releasing NT-4/5 [Skaper *et al.,* 2001].

Chronic pancreatitis is a painful disease [Sidhu and Tandon, 1996] associated with characteristic nerve changes, including an increase in nerve numbers and enlargement of pancreatic nerves [Bockman *et al.,* 1988]. In addition, the presence and quantity of growth-associate protein-43, a marker of neuroplasticity in enlarged pancreatic nerves and pancreatic neurons, is associated with abdominal pain intensity in chronic pancreatitis [Di Sebastiano *et al.,* 1997]. These observations indicate that alterations in neural structures seem to contribute to pain generation in chronic pancreatitis. While the mechanisms that contribute to pancreatic nerve growth are unclear, activation of the NGF/TrkA pathway might influence neural morphologic changes in this disorder [Fiess *et al.,* 1999]. Another mechanism by which NGF could influence chronic pain is the regulation of transcription and synthesis of substance P and calcitonin gene-related peptide (CGRP), as well as through the release of histamine [McMahon *et al.,* 1995; McMahon, 1996; Xu and Hall, 2007]. Blocking of NGF by specific anti-NGF antibodies produces a sustained thermal and chemical hypoalgesia and a downregulation of substance P and CGRP in the rat [Aloe *et al.,* 1995]. The sensory neurotransmitters substance P and CGRP are increased in enlarged pancreatic nerves in chronic pancreatitis [Büchler *et al.,* 1992] indicating a potential regulatory interaction between both mediator systems in pain generation.

Conclusion

NGF emerges as a complex pleiotropic agent active on an unexpectedly broad array of cell types and biological functions, something which was not envisaged on the basis of earlier work establishing its trophic role for sensory and sympathetic neurons during development and adulthood. For example, high levels of NGF have been detected in the inflammatory exudates and biological fluids of patients afflicted with autoimmune diseases. Within this framework, the mast cell can be viewed as a gatekeeper between the immune and nervous systems (Figure 5). Mast cells are involved not only in hypersensitivity but also actively participate in inflammatory phenomena of various types, including those of a neurogenic nature. Mast cells synthesise and release biologically active NGF, and NGF itself has both survival and sensitizing actions on mast cells. This raises the possibility of autocrine actions that, if not properly controlled, could result in deleterious effects on surrounding tissues and lead to chronic inflammatory processes, e.g. those present in pathologies of an autoimmune nature. Thus, it has now come to be appreciated that neurological dysfunction can occur not only as a result of neurotrophic factor deficiency but also as a consequence of excess. An important goal of future studies will be to more fully understand the involvement of NGF in disorders resulting from dysregulation of tissue homeostasis, which may occur, for example, in chronic inflammatory diseases like multiple sclerosis.

References

Aloe, L. and Levi-Montalcini, R. (1977) Mast cells increase in tissues of neonatal rats injected with nerve growth factor. *Brain Research 133,* 358-366.

Aloe, L., Cozzari, C., Calissano, P. and Levi-Montalcini, R. (1981) Somatic and behavioral postnatal effects of fetal injections of nerve growth factor antibodies in the rat. *Nature 291,* 413-415.

Aloe, L., Alleva, E., Böhm, A. and Levi-Montalcini, R. (1986) Aggressive behavior induces release of nerve growth factor from mouse salivary gland into the bloodstream. *Proceedings of the National Academy of Sciences USA 83,* 6184-6187.

Aloe, L. (1988) The effect of nerve growth factor and its antibody on mast cells in vivo. *Journal of Neuroimmunology 18,* 1-12.

Aloe, L. and De Simone, R. (1989) NGF primed spleen cells injected in brain of developing rats differentiate into mast cells. *International Journal of Developmental Neuroscience 7,* 565-573.

Aloe, L., Tuveri, M.A. and Levi-Montalcini, R. (1992a) Nerve growth factor and distribution of mast cells in the synovium of adult rats. *Clinical and Experimental Rheumatology 10,* 203-204.

Aloe, L., Tuveri, M.A., Carcassi, U. and Levi-Montalcini, R. (1992b) Nerve growth factor in the synovial fluid of patients with chronic arthritis. *Arthritis and Rheumatism 35,* 351-355.

Aloe, L., Tuveri, M.A. and Levi-Montalcini, R. (1992c) Studies on carrageenan-induced arthritis in adult rats: presence of nerve growth factor and role of sympathetic innervation. *Rheumatology International 12,* 213-216.

Aloe, L., Bracci-Laudiero, L., Alleva, E., Lambiase, A., Micera, A. and Tirassa, P. (1994) Emotional stress induced by parachute jumping changes blood nerve growth factor levels and the distribution of nerve growth factor receptors in lymphocytes. *Proceedings of the National Academy of Sciences USA 91,* 10440-10444.

Aloe, L., Skaper, S.D., Leon, A and Levi-Montalcini, R. (1994) Nerve growth factor and autoimmune diseases. *Autoimmunity 19,* 141-150.

Aloe, L., Probert, L., Kollias, G., Micera, A. and Tirassa, P. (1995) Effect of NGF antibodies on mast cell distribution, histamine and substance P levels in the knee joint of TNF-arthritic transgenic mice. *Rheumatology International 14,* 249-252.

Al-Shawi, R., Hafner, A., Olson, J., Chun, S., Raza, S., Thrasivoulou, C., Lovestone, S., Killick, R., Simons, P. and Cowen, T. (2008) Neurotoxic and neurotrophic roles of proNGF and the receptor sortilin in the adult and ageing nervous system. *European Journal of Neuroscience 27,* 2103-2114.

Andreev, N.Y., Dimitrieva, N., Koltzenburg, M. and McMahon, S.B. (1995) Peripheral administration of nerve growth factor produces a thermal hyperalgesia that requires the presence of sympathetic post-ganglionic neurones. *Pain 63,* 109-115.

Askenase, P.W. and Van Loveren, H. (1983) Delayed-type hypersensitivity activation of mast cells by antigen-specific T-cell factors initiates the cascade of cellular interactions. *Immunology Today 4,* 259-264.

Baggiolini, M., Horisberger, U. and Martin, U. (1982) Phagocytosis of mast cell granules by mononuclear phagocytes, neutrophils and eosinophils during anaphylaxis. *International Archives of Allergy and Applied Immunology 67,* 219-226.

Barde, Y.-A. (1989) Trophic factors and neuronal survival. *Neuron 2,* 1525-1534.

Barde, Y.-A., Edgar, D. and Thoenen, H. (1982) Purification of a new neurotrophic factor from mammalian brain. *EMBO Journal 1,* 549-553.

Benedetti, M., Levi, A. and Chao, M.V. (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proceedings of the National Academy of Sciences USA, 90,* 7859-7863.

Bennett, G., al-Rashed, S., Hoult, J.R. and Brain, S.D. (1998) Nerve growth factor induced hyperalgesia in the rat hind paw is dependent on circulating neutrophils. *Pain 77,* 315-322.

Bentley, C.A. and Lee, K.-F. (2000) p75 is important for axon growth and Schwann cell migration during development. *Journal of Neuroscience 20,* 7706-7715.

Bergmann, I., Reiter, R., Toyka, K.V. and Koltzenburg, M. (1998) Nerve growth factor evokes hyperalgesia in mice lacking the low-affinity neurotrophin receptor p75. *Neuroscience Letters 255,* 87-90.

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nicolics, K., Goeddel, D.V. and Rosenthal, A. (1991) Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron 7,* 857-866.

Bierl, M.A. and Isaacson, L.G. (2007) Increased NGF proforms in aged sympathetic neurons and their targets. *Neurobiology of Ageing 28,* 1144-1159.

Bischoff, S.C. and Dahinden, C.A. (1992) Effect of nerve growth factor on the release of inflammatory mediators by mature human basophils. *Blood 79,* 2662-2669.

Bibel, M., Hoppe, E. and Barde, Y.-A. (1999) Biochemical and functional interactions between the neurotrophin receptors *trk* and $p75^{NTR}$. *EMBO Journal 18,* 616-622.

Blennerhasset, M.G., Tomioka, M. And Bienenstock, J. (1991) Formation of contacts between mast cells and sympathetic neurons *in vitro. Cell and Tissue Research 265,* 121-128.

Bockman, D.E., Büchler, M.W., Malfertheiner, P. and Beger, H.G. (1988) Analysis of nerves in chronic pancreatitis. *Gastroenterology 94,* 1459-1469.

Böhm, A., Aloe, L. and Levi-Montalcini, R. (1986) Nerve growth factor enhances precocious differentiation and numerical increase in mast cells in cultures of rat splenocytes. *Accademia Nazionale Lincei 80,* 1-6.

Bonini, S., Lambiase, A., Bonini, S., Angelucci, F., Magrini, L., Manni L. and Aloe, L. (1996) Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proceedings of the National Academy of Sciences USA 93,* 10955-10960.

Bonnington, J.K. and McNaughton, P.A. (2003) Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor. *Journal of Physiology 551 (Part 2),* 433-446.

Borrelli, E., Sawchenko, P.E. and Evans, R.M. (1992) Pituitary hyperplasia produced by ectopic expression of nerve growth factor. *Proceedings of the National Academy of Sciences USA 89,* 2764-2768.

Bothwell, M. (1995) Functional interactions of neurotrophins and neurotrophin receptors. *Annual Review of Neuroscience 18,* 223-253.

Boyle, M.D.P., Lawman, M.J.P., Gee, A.P. and Young, M. (1985) Nerve growth factor: a chemotactic factor for polymorphonuclear leukocytes in vivo. *Journal of Immunology 134,* 564-568.

Bracci-Laudiero, L.B., Aloe, L., Levi-Montalcini, R., Buttinelli, C., Schilter, D., Gillessen, S. And Otten, U. (1992) Multiple sclerosis patients express increased levels of beta-nerve growth factor in cerebrospinal fluid. *Neuroscience Letters 147,* 9-12.

Bracci-Laudiero, L., Aloe, L., Levi-Montalcini, R., Galaezzi, M., Schilter, J.L., Scully, J.L. and Otten, U. (1993) Increased levels of NGF in sera of systemic lupus erythematosus patients. *NeuroReport 4,* 563-565.

Bracci-Laudiero, L., Lundeberg, T., Stenfors, C., Theodorsson, E., Tirassa, P. and Aloe, L. (1996) Modification of lymphoid and brain nerve growth factor levels in systemic lupus erythematosus mice. *Neuroscience Letters 204,* 13-16.

Bracci-Laudiero, L., Celestino, D., Starace, G., Antonelli, A., Lambiase, A., Procoli, A., Rumi, C., Lai, M., Picardi, A., Ballatore, G., Bonini, S. and Aloe, L. (2003) CD34-positive cells in human umbilical cord blood express nerve growth factor and its specific receptor TrkA. *Journal of Neuroimmunology 136,* 130-139.

Bracci-Laudiero, L., Aloe, L., Caroleo, M.C., Buanne, P., Costa, N., Starace, G. and Lundeberg, T. (2005) Endogenous NGF regulates CGRP expression in human monocytes, and affects HLA-DR and CD86 expression and IL-10 production. *Blood 106,* 3507-3514.

Braun, A., Lommatzsch, M. and Renz, (2000) The role of neurotrophins in allergic bronchial asthma. *Clinical and Experimental Allergy 30,* 178-186.

Brodie, C. and Gelfand, E.W. (1992) Functional nerve growth factor receptors on human B lymphocytes: interaction with IL2. *Journal of Immunology 148,* 171-178.

Brosnan, C.F., Lyman, W.D., Tansey, F.A. and Carter, T.H. (1985) Quantitation of mast cells in experimental allergic neuritis. *Journal of Neuropathology and Experimental Neurology 44,* 196-203.

Bruni, A., Bigon, E., Boarato, E., Mietto, L., Leon, A. and Toffano, G. (1982) Interaction between nerve growth factor and lysophosphatidylserine on rat peritoneal mast cells. *FEBS Letters 138,* 190-192.

Büchler, M., Weihe, E., Friess, H.,Malfertheiner, P., Bockman, E., Müller, S., Nohr, D. and Beger, H.G. (1992) Changes in peptidergic innervation in chronic pancreatitis. *Pancreas 7,* 183-192.

Bullock, E.D. and Johnson, E.M. Jr. (1996) Nerve growth factor induces the expression of certain cytokine genes and bcl-2 in mast cells. Potential role in survival promotion. *Journal of Biological Chemistry 271,* 27500-27508.

Burcham, T.S., Sim, I., Bolin, L.M. and Shooter, E.M. (1991) The NGF complex from the African rat Mastomys natalensis. *Neurochemical Research 16,* 603-612.

Bürgi, B., Otten, U.H., Ochensberger, B., Rihs, S., Heese, K., Ehrhard, P.B., Ibanez, C.F. and Dahinden, C.A. (1996) Basophil priming by neurotrophic factors. Activation through the trk receptor. *Journal of Immunology 157,* 5582-5588.

Burwen, S. and Satir, B. (1977) A freeze-fracture study of early membrane events during mast cell degranulation. *Journal of Cell Biology 73,* 660-671.

Carroll, P., Lewin, G.R., Koltzenburg, M., Toyka, K.V. and Thoenen, H. (1998) A role for BDNF in mechanosensation. *Nature Neuroscience 1,* 42-46.

Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nature Reviews Neuroscience, 4,* 299-309.

Conover, J.C., Erickson, J.T., Katz, D.M., Bianchi, L.M., Poueymirou, W.T., McClain, J., Pan, L., Helgren, M., Ip, N.Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A.C., DeChiara, T.M. and Yancopoulos, G.D. (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature, 375,* 235-238.

Cordon-Cardo, C., Tapley, P., Jing, S.Q.,, Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L.F. and Barbacid, M. (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell 66,* 173-183.

Curtis, R., Adryan, K.M., Stark, J.L., Park. J.S., Compton, D.L., Weskamp, G., Huber, L.J., Chao, M.V., Jaenisch, R., Lee, K.-F., Lindsay, R.M. and DiStefano, P.S. (1995) Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. *Neuron 14,* 1201-1211.

Davies, A.M., Lee, K.F. and Jaenisch, R. (1993) p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron 11,* 565-575.

Davis, B.M., Lewin, G.R., Mendell, L.M., Jones, M.E. and Albers, K.M. (1993) Altered expression of nerve growth factor in the skin of transgenic mice leads to changes in response to mechanical stimuli. *Neuroscience 56,* 789-792.

Dicou, E., Lee, J. and Brachet, P. (1986) Synthesis of nerve growth factor mRNA and precursor protein in the thyroid and parathyroid glands of the rat. *Proceedings of the National Academy of Sciences USA 83,* 7084-7088.

Dicou, E., Hurez, D. and Nerrière, V. (1993) Natural autoantibodies against nerve growth factor in autoimmune diseases. *Journal of Neuroimmunology 47,* 159-168.

Dimitriadou, V., Lambracht-Hall, M., Reichler, M. and Theoharides, T.C. (1990) Histochemical and ultrastructural characteristics of rat brain perivascular mast cells stimulated with compound 48/80 and carbachol. *Neuroscience 39,* 209-224.

di Mola, F.F., Friess, H., Zhu, Z.W., Koliopanos, A., Bley, T., Di Sebastiano P., Innocenti, P., Zimmermann, A. and Büchler, M.W. (2000) Nerve growth factor and Trk high affinity receptor (TrkA) gene expression in inflammatory bowel disease. *Gut 46,* 670-678.

Di Sebastiano, P., Fink, T., Weihe, E., Friess, H., Innocenti, P., Beger, H.G. and Büchler, M.W. (1997) Immune cell infiltration and growth-associated protein 43 expression correlate with pain in chronic pancreatitis. *Gastroenterology 112,* 1762-1765.

Diogenes, A., Akopian, A.N. and Hargreaves, K.M. (2007) NGF up-regulates TRPA1: implications for orofacial pain. *Journal of Dental Research 86,* 550-555.

Donnerer, J., Schuligoi, R. and Stein, C. (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. *Neuroscience 49,* 693-698.

Doré, S., Kar, S. and Quirion, R. (1997) Rediscovering an old friend, IGF-1: potential use in the treatment of neurodegenerative diseases. *Trends in Neurosciences 20,* 326-331.

Dreyfus, C.F. (1989) Effects of nerve growth factor on cholinergic brain neurons. *Trends in Pharmacological Sciences 10,* 145-149.

Dropp, J.J. (1976) Mast cells in the mammalian brain. *Acta Anatomica 94,* 1-21.

Ehrhard, P.B., Erb, B., Graumann, U. and Otten, U. (1993a) Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proceedings of the National Academy of Sciences USA 90,* 10984-10988.

Ehrhard, P.B., Ganter, U., Bauer, J. and Otten, U. (1993b) Expression of functional *trk* protooncogene in human monocytes. *Proceedings of the National Academy of Sciences USA 90,* 5423-5427.

Eide, F.F., Lowenstein, D.H. and Reichardt, L.F. (1993) Neurotrophins and their receptors--current concepts and implications for neurologic disease. *Experimental Neurology 121,* 200-214.

Eide, F.F., Vining, E.R., Eide, B.L., Zang, K., Wang, X.-Y. and Reichardt, L.F. (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. *Journal of Neuroscience 16,* 3123-3129.

Ernfors, P., Ibáñez, C.F., Ebendal, T., Olson, L. and Persson, H. (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proceedings of the National Academy of Sciences USA 87,* 5454-5458.

Esposito, P., Gheorghe, D., Kandere, K., Pang, X., Connolly, R., Jacobson, S. and Theoharides, T.C. (2001a) Acute stress increases permeability of the blood-brain barrier through activation of brain mast cells. *Brain Research 888,* 117-127.

Esposito, D., Patel, P., Stephens, R.M., Perez, P., Chao, M.V., Kaplan, D.R. and Hempstead, B.L. (2001b) The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. *Journal of Biological Chemistry 276,* 32687-32695.

Fahnestock, M., Michalski, B., Xu, B. and Coughlin, M.D. (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. *Molecular and Cellular Neuroscience 18,* 210-220.

Fahnestock, M., Yu, G., Michalski, B., Mathew, S., Colquhoun, A., Ross, G.M. and Coughlin, M.D. (2004) The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. *Journal of Neurochemistry 89,* 581-592.

Falcini, F., Matucci Cerinic M., Lombardi, A., Generini, S., Pignone, A., Tirassa, P., Ermini, M., Lepore, L., Partsch, G. and Aloe, L. (1996) Increased circulating nerve growth factor is directly correlated with disease activity in juvenile chronic arthritis. *Annals of the Rheumatic Diseases 55,* 745-748.

Farkas, L.M., Dünker, N., Roussa, E., Unsicker, K. and Krieglstein, K. (2003) Transforming growth factor-βs are essential for the development of midbrain dopaminergic neurons *in vitro* and *in vivo. Journal of Neuroscience 23,* 5178-5186.

Fiore, M., Amendola, T., Triaca, V., Alleva, E. and Aloe, L. (2005) Fighting in the aged male mouse increases the expression of TrkA and TrkB in the subventricular zone and in the hippocampus. *Behavioural Brain Research 157,* 351-362.

Flämig, G., Engele, J., Geerling, I., Pezeshki, G., Adler, G. and Reinshagen, M. (2001) Neurotrophin and GDNF expression increases in rat adrenal glands during experimental colitis. *NeuroEndocrinology Letters 22,* 461-466.

Florenzano, F. and Bentivoglio, M. (2000) Degranulation, density, and distribution of mast cells in the rat thalamus: a light and electron microscopic study in basal conditions and after intracerebroventricular administration of nerve growth factor. *Journal of Comparative Neurology 424,* 651-669.

Frandji, P., Oskeritzian, C., Cacaraci, F., Lepeyre, J., Peronet, R., David, J.G. and Mecheri, S. (1993) Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-restricted T cell hybridoma. *Journal of Immunology 151,* 6318-6328.

Friess, H., Zhu, Z.-W., di Mola, F., Kulli, C., Graber, H.U., Andren-Sandberg, Å., Zimmermann, A., Korc, M., Reinshagen, M. and Büchler, M.W. (1999) Nerve growth factor and its high-affinity receptor in chronic pancreatitis. *Annals of Surgery 230,* 615-624.

Galli, S.J. (1993) New concepts about the mast cell. *New England Journal of Medicine 328,* 257-265.

Galli, S.J., Nakae, S. and Tsai, M. (2005) Mast cells in the development of adaptive immune responses. *Nature Immunology 6,* 135-142.

Garaci, E., Caroleo, M.C., Aloe, L., Aquaro, S., Piacentini, M., Costa, N., Amendola, A., Micera, A., Caliò, R., Perno, C.-F. and Levi-Montalcini, R. (1999) Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. *Proceedings of the National Academy of Sciences USA 96,* 14013-14018.

Gee, A.P., Boyle, M.D.P., Munger, K.L., Lawman, M.J.P. and Young, M. (1983) Nerve growth factor: stimulation of polymorphonuclear leukocyte chemotaxis in vitro. *Proceedings of the National Academy of Sciences USA 80,* 7215-7218.

Geetha, T., Jiang, J. and Wooten, M.W. (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Molecular Cell 20,* 301-312.

Ginty, D.D. and Segal, R.A. (2002) Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr. Opin. Neurobiol. 12,* 268-274.

Gordon, J.R., Burd, P.R. and Galli, S.J. (1990) Mast cells as a source of multifunctional cytokines. *Immunology Today 11,* 458-464.

Götz, R., Köster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M. and Thoenen, H. (1994) Neurotrophin-6 is a new member of the nerve growth factor family. *Nature 372,* 266-269.

Greenberg, G. and Burnstock, G. (1983) A novel cell-to-cell interaction between mast cells and other cell types. *Experimental Cell Research 147,* 1-13.

Gudat, F., Laubscher, A., Otten, U. and Pletscher, A. (1981) Shape changes induced by biologically active peptides and nerve growth factor in blood platelets of rabbits. *British Journal of Pharmacology 74,* 533-538.

Guiton, M., Gunn-Moore, F.J., Glass, D.J., Geis, D.R., Yancopoulos, G.D. and Tavare, J.M. (1995) Naturally occurring tyrosine kinase inserts block high affinity binding of phospholipase Cγ and Shc to TrkC and neurotrophin-3 signaling. *Journal of Biological Chemistry 270,* 20384-20390.

Guo, Z., Turner, C. and Castle, D. (1998) Relocation of the t-SNARE SNAP-23 from lamellopodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell 94,* 537-548.

Hallböök, F., Ibáñez, C.F. and Persson, H. (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron 6,* 845-858.

Hamburger, V. and Levi-Montalcini, R. (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *Journal of Experimental Zoology 111,* 457-501.

Hamburger, V., Brunso-Bechthold, J.K. and Yip, J.W. (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *Journal of Neuroscience 1,* 60-71.

Harrington, A.W., Leiner, B., Blechschmitt, C., Arevalo, J.C., Lee, R., Mori, K., Meyer, M., Hempstead, B.L., Yoon, S.O. and Giehl, K.M. (2004) Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. *Proceedings of the National Academy of Sciences USA 101,* 6226-6230.

Harrison, S.M.W., Jones, M.E., Uecker, S., Albers, K.M., Kudrycki, K.E. and Davis, B.M. (2000) Levels of nerve growth factor and neurotrophin-3 are affected differentially by the presence of p75 in sympathetic neurons in vivo. *Journal of Comparative Neurology 424,* 99-110.

Hauser, S.L., Bhan, A.K., Gilles, F., Kemp, M., Kerr, C. and Weiner, H.L. (1986) Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Annals of Neurology 19,* 578-587.

He, X.-E. and Garcia, K.C. (2004) Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science 304,* 870-872.

Hermes, B., Welker, P., Feldmann-Böddeker, I., Krüger-Krasagakis, S., Hartmann, K., Zuberbier, T. and Henz, B.M. (2001) Expression of mast cell growth modulating and chemotactic factors and their receptors in human cutaneous scars. *Journal of Investigative Dermatology 116,* 387-393.

Hohn, A., Leibrock, J. Bailey, K. and Barde, Y.-A. (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature 344,* 339-341.

Horigome, K., Pryor, E.D., Bullock, E.M. and Johnson, E.M. Jr. (1993) Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. *Journal of Biological Chemistry 268,* 14881-14887.

Horigome, K., Bullock, E.D. and Johnson, E.M. Jr. (1994) Effects of nerve growth factor on rat peritoneal mast cells. Survival promotion and immediate-early gene induction. *Journal of Biological Chemistry 269,* 2695-2702.

Ibáñez, C.F. (1995) Neurotrophic factors: from structure-function studies to designing effective therapeutics. *Trends in Biotechnology 13,* 217-227.

Ibrahim, M.Z.M. (1974) The mast cells of the central nervous system. Part I. Morphology, distribution, and histochemistry. *Journal of Neurological Sciences 21,* 431-478.

Ip, N.Y., Ibáñez, C.F., Nye, S.H., McClain, J., Jones, P.F., Gies, D.R., Belluscio, L., Le Beau, M.M., Espinosa, R. III, Squinto, S.P., Persson, H. and Yancopoulos, G.D. (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proceedings of the National Academy of Sciences USA 89,* 3060-3064.

Ip, N.Y. and Yancopoulos, G.D. (1996) The neurotrophins and CNTF: two families of collaborative neurotrophic factors. *Annual Review of Neuroscience, 19,* 491-515.

Ji, R.-R., Samad, T.A., Jin, S.-X., Schmoll, R. and Woolf, C.J. (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron 36,* 57-68.

Johnson, D., Seeldrayers, P.A. and Weiner, H.L., (1988) The role of mast cells in demyelination. I. Myelin proteins are degraded by mast cell proteases and myelin basic protein and P_2 can stimulate mast cell degranulation. *Brain Research 444,* 195-198.

Johnson, E.M., Gorin, P.D., Brandeis, L.D. and Pearson, J. (1980) Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science 210,* 916-918.

Johnson, E.M. Jr., Osborne, P.A., Rydel, R.E., Schmidt, R.E. and Pearson, J. (1983) Characterization of the effects of autoimmune nerve growth factor deprivation in the developing guinea pig. *Neuroscience 8,* 631-642.

Johnson, D. and Krenger, W. (1992) Interactions of mast cells with the nervous system-recent advances. *Neurochemical Research 9,* 939-951.

Jones, K.R. and Reichardt, L.F. (1990) Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proceedings of the National Academy of Sciences USA 87,* 8060-8064.

Kaisho, Y., Yoshimura, K. and Nakahama, K. (1990) Cloning and expression of a cDNA encoding a novel human neurotrophic factor. *FEBS Letters 266,* 187-191.

Kannan, Y., Ushio, H., Koyama, H., Okada, M., Oikawa, M., Yoshihara, T., Kaneko, M. and Matsuda, H. (1991) 2.5S Nerve growth factor enhances the survival, phagocytosis, and superoxide production of murine neutrophils. *Blood 77,* 1320-1325.

Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V. and Parada, L.F. (1991a) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science 252,* 554-558.

Kaplan, D.R., Martin-Zanca, D. and Parada, L.F. (1991b) Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. *Nature 350,* 158-160.

Kaplan, A.P., Reddigari, S., Baeza, M. and Kuna, P. (1991c) Histamine releasing factors and cytokine-dependent activation of basophils and mast cells. *Advances in Immunology 50,* 237-260.

Kawamoto, K., Okada, T., Kannan, Y., Ushio, H., Matsumoto, M. and Matsuda, H. (1995) Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the trk proto-oncogene receptor. *Blood 86,* 4638-4644.

Kawamoto, K., Aoki, J., Tanaka, A., Itakura, A., Hosono, H., Arai, H., Kiso, Y. and Matsuda, H. (2002) Nerve growth factor activates mast cells through the collaborative interaction with lysophosphatidylserine expressed on the membrane surface of activated platelets. *Journal of Immunology 168,* 6412-6419.

Khasar, S.G., Lin, Y.-H., Martin, A., Dadgar, J., McMahon, T., Wang, D., Hundle, B., Aley, K.O., Isenberg, W., McCarter, G., Green, P.G., Hodge, C.W., Levine, J.D. and Messing, R.O. (1999) A novel nociceptor signaling pathway revealed in protein kinase C ε mutant mice. *Neuron 24,* 253-260.

Kimata, H., Yoshida, A., Ishioka, C., Kusunoki, T., Hosoi, S. and Mikawa, H. (1991) Nerve growth factor specifically induces human IgG4 production. *European Journal of Immunology 21,* 137-141.

Kinkelin, I., Mötzing, S., Koltzenburg, S. and Bröcker, E.-B. (2000) Increase in NGF content and nerve fiber sprouting in human allergic contact eczema. *Cell and Tissue Research 302,* 31-37.

Klein, R., Jing, S.Q., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991a) The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell 65,* 189-197.

Klein, R., Nanduri, V., Jing, S.A., Lambelle, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K.R., Reichardt, L.F. and Barbacid, M. (1991b) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell 66,* 395-403.

Klein, R., Smeyne, R.J., Wurst, W., Long, L.K., Auerbach, B.A., Joyner, A.L. and Barbacid, M. (1993) Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell 75,* 113-122.

Klöppel, G. (1990) Pathology of chronic pancreatitis and pancreatic pain. *Acta Chirurgica Scandinavica 156,* 261-265.

Kobayashi, H., Yamataka, A., Fujimoto, T., Lane, G.J. and Miyano, T. (1999) Mast cells and gut nerve development: implications for Hirschsprung's disease and intestinal neuronal dysplasia. *Journal of Pediatric Surgery 34,* 543-548.

Kobayashi, H., Gleich, G.J., Butterfield, J.H. and Kita, H. (2002) Human eosinophils produce neurotrophins and secrete nerve growth factor on immunologic stimuli. *Blood 99,* 2214-2220.

Koltzenburg, M., Bennett, D.L. and McMahon, S.B. (1999) Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. *European Journal of Neuroscience 11,* 1698-1704.

Kostiza, T. and Meier, J. (1996) Nerve growth factors from snake venoms: chemical properties, mode of action and biological significance. *Toxicon 34,* 787-806.

Kovanen, P.T. (1991) Mast cell granule mediated uptake of low density lipoproteins by macrophages: a novel carrier mechanism leading to the formation of foam cells. *Annals of Medicine 23,* 551-119.

Kurosawa, M., Inamura, H., Amano, H., Kanbe, N., Nagata, H., Nagai, H. (1999) Nerve growth factor release with mast-cell-derived mediators in a patient with systemic mastocytosis after middle-wave ultraviolet irradiation. *Allergy 54,* 994-998.

Lagunoff, D. (1973) Membrane fusion during mast cell secretion. *Journal of Cell Biology 57,* 252-259.

Lai, K.-O., Fu, W.-Y., Ip, F.C.F. and Ip, N.Y. (1998) Cloning and expression of a novel neurotrophin, NT-7, from carp. *Molecular and Cellular Neuroscience 11,* 64-76.

Lamballe, F., Klein, R. and Barbacid, M. (1991) *trk*C, a new member of the *trk* family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell 66,* 967-979.

Lambiase, A., Bracci-Laudiero, L., Bonini, S., Bonini, S., Starace, G., D'Elios, M.M., De Carli, M. and Aloe, L. (1997) Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. *Journal of Allergy and Clinical Immunology 100,* 408-414.

Lambracht-Hall, M., Dimitriadou, V. and Theoharides, T.C. (1990a) Migration of mast cells in the developing rat brain. *Developmental Brain Research 56,* 151-159.

Lambracht-Hall, M., Konstantinidou, A.D. and Theoharides, T.C. (1990b) Serotonin release from rat brain mast cells *in vitro. Neuroscience 30,* 199-207.

Lara, H.E., McDonald, J.K. and Ojeda, S.R. (1990) Involvement of nerve growth factor in female sexual development. *Endocrinology 126,* 364-375.

Lathinen, T., Soinila, S. and Lakshmanan, J. (1989) Biological demonstration of nerve growth factor in the rat pituitary gland. *Neuroscience 30,* 165-170.

Lee, K.-F., Li, E., Huber, L.J., Landis, S.C., Sharpe, A.H., Chao, M.V. and Jaenisch, R. (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral nervous system. *Cell 69,* 737-749.

Lee, K.F., Bachman, K., Landis, S. and Jaenisch, R. (1994) Dependence on p75 for innervation of some sympathetic targets. *Science 263,* 1447-1449.

Lee, R., Kermani, P., Teng, K.K. and Hempstead, B.L. (2001) Regulation of cell survival by secreted proneurotrophins. *Science 294* 1945-1948.

Leffler, A., Cummins, T.R., Dib-Hajj, S.D., Hormuzdiar, W.N., Black, J.A. and Waxman, S.G. (2002) GDNF and NGF reverse changes in repriming of TTX-sensitive Na+

currents following axotomy of dorsal root ganglion neurons. *Journal of Neurophysiology 88,* 650-658.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Theonen, H. and Barde, Y.-A. (1994) Molecular cloning and expression of brain-derived neurotrophic factor. *Nature 341,* 149-152.

Leon, A., Buriani, A., Dal Toso, R., Fabris, M., Romanello, S., Aloe, L. and Levi-Montalcini, R. (1993) Mast cells synthesize, store, and release nerve growth factor. *Proceedings of the National Academy of Sciences USA 91,* 3739-3743.

Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. *Science 237,* 1154-1162.

Levi-Montalcini, R. and Hamburger, V. (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool. 116,* 321-361.

Levi-Montalcini, R., Skaper, SD., Aloe, L. and Leon, A. Nerve growth factor. In: Adelman G, Smith BH editors. *Encyclopedia of Neuroscience,* Second Edition. Amsterdam: Elsevier Science B.V; 1999; 1297-1299.

Lewin, G.R. and Mendell, L.M. (1993) Nerve growth factor and nociception. *Trends in Neuroscience 16,* 353-359.

Liepinsh, E., Ilag, L.L., Otting, G. and Ibáñez, C.F. (1997) NMR structure of the death domain on the p75 neurotrophin receptor. *EMBO Journal 16,* 4999-5005.

Lindahl, U., Pertoft, H. and Seljelik, R. (1979) Uptake and degradation of mast cell granules by mouse peritoneal macrophages. *Biochemical Journal 182,* 189-193.

Lindsay, R.M. and Yancopoulos, G.D. (1996) GDNF in a bind with known orphan: accessory implicated in new twist. *Neuron 17,* 571-574.

Liu, X., Ernfors, P., Wu, H. and Jaenisch, R. (1995) Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. *Nature, 375,* 238-241.

Lobos, E., Gebhardt, C., Kluge, A. and Spanel-Borowski, K. (2005) Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. *Endocrinology 146,* 1922-1929.

Luppi, P., Levi-Montalcini, R., Bracci-Laudiero, L., Bertolini, A., Arletti, R., Tavernari, D., Vigneti, E. and Aloe, L. (1993) NGF is released into plasma during human pregnancy: an oxytocin-mediated response? *NeuroReport 4,* 1063-1065.

Maestripieri, D., De Simone, R., Aloe, L. and Levi-Montalcini, R. (1990) Social status and serum levels after antagonistic encounters in mice. *Physiology and Behavior 47,* 161-164.

Mahadeo, D., Kaplan, L., Chao, M.V. and Hempstead, B.L. (1994) High affinity nerve growth factor binding displays a faster rate of association than $p140^{trk}$ binding. *Journal of Biological Chemistry 269,* 6884-6891.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science 247,* 1446-1451.

Makkerh, J.P.S., Ceni, C., Auld, D.S., Vaillancourt, F., Dorval, G. and Barker, P.A. (2005) p75 neurotrophin receptor reduces ligand-induced Trk receptor ubiquitination and delays Trk receptor internalization and degradation. *EMBO reports 6,* 936-941.

Manni, L., Lundeberg, T., Fiorito, S., Bonini, S., Vigneti, E. and Aloe, L. (2003) Nerve growth factor release by human synovial fibroblasts prior to and following exposure to tumor necrosis factor-α, interleukin-1β and cholecystokinin-8: inflammatory response. *Clinical and Experimental Rheumatology 21,* 617-624.

Manni, L., Di Fausto, V., Fiore, M. and Aloe, L. (2008) Repeated restraint and nerve growth factor administration in male and female mice: effect on sympathetic and cardiovascular mediators of the stress response. *Current Neurovascular Research 5,* 1-12.

Manning, K.A., Pienkowski, T.P. and Uhlrich, D.J. (1994) Histaminergic and non-histamine-immunoreactive mast cells within the cat lateral geniculate complex examined with light and electron microscopy. *Neuroscience 63,* 191-206.

Marinova, T., Philipov, S. and Aloe, L. (2007) Nerve growth factor immunoreactivity of mast cells in acute involuted human thymus. *Inflammation 30,* 38-43.

Marshall, J.S., Stead, R.H., McSharry, C., Nielsen, L. and Bienenstock, J. (1990) The role of mast cell degranulation products in mast cell hyperplasia. I. Mechanism of action of nerve growth factor. *Journal of Immunology 144,* 1886-1892.

Marshall, J.S., Gomi, K., Blennerhassett, M.G. and Bienenstock, J. (1999) Nerve growth factor modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism. *Journal of Immunology 162,* 4271-4276.

Matsuda, H., Coughlin, M.D., Bienenstock, J. and Denburg, J.A. (1988) Nerve growth factor promotes human hemopoietic colony growth and differentiation. *Proceedings of the National Academy of Sciences USA 85,* 6508-6512.

Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H. and Kitamura, Y. (1991) Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *Journal of Experimental Medicine 174,* 7-14.

Matsuda, H., Koyama, H., Sato, H., Sawada, J., Itakura, A., Tanaka, A., Matsumoto, M., Konno, K., Ushio, H. and Matsuda, K. (1998) Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice. *Journal of Experimental Medicine 187,* 297-306.

Mazella, J., Zsürger, N., Navarro, V., Chabry, J., Kaghad, M., Caput, D., Ferrara, P., Vita, N., Gully, D., Maffrand, J.-P. and Vincent, J.-P. (1998) The 100-kDa neurotensin receptor is gp95/sortilin, a non-G protein-coupled receptor. *Journal of Biological Chemistry 273,* 26273-26276.

McGeer, P.L. and McGeer, E.G. (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Research Reviews 21,* 195-218.

McMahon, S.B., Bennett, D.L., Priestley, J.V. and Shelton, D.L. (1995) The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nature Medicine 1,* 774-780.

McMahon, S.B. (1996) NGF as a mediator of inflammatory pain. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 351,* 431-440.

Meakin, S.O. and Shooter, E.M. (1992) The nerve growth factor family of receptors. *Trends in Neuroscience 15,* 323-331.

Melamed, I., Kelleher, C.A., Franklin, R.A., Brodie, C., Hempstead, B., Kaplan, D. and Gelfand, E.W. (1996) Nerve growth signal transduction in human B lymphocytes is mediated by gp140trk. *European Journal of Immunology 26,* 1985-1992.

Miao, N., Wang, M., Ott, J.A., D'Alessandro, J.S., Woolf, T.M., Bumcrot, D.A., Mahanthappa, N.K. and Pang, K. (1997) Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult *in vitro*. *Journal of Neuroscience 17,* 5891-5899.

Mican, J.M. and Melcalfe, D.D. (1990) Arthritis and mast cell activation. *Journal of Allergy and Applied Immunology 86,* 677-683.

Michael, G.J., Averill, S., Nitkunan, A., Rattray, M., Bennett, D.L., Yan, Q. and Priestley, J.V. (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectivity in TrkA-expressing dorsal root ganglion cells and in the central terminations within the spinal cord. *Journal of Neuroscience 17,* 8476-8490.

Missale, C., Boroni, F., Losa, M., Giovanelli, M., Zanellato, A., Dal Toso, R., Balsari, A. and Spano, P. (1993) Nerve growth factor suppresses the transforming phenotype of human prolactinoma. *Proceedings of the National Academy of Sciences USA 90,* 7961-7965.

Missale, C., Boroni, F., Sigala, S., Zanellato, A., Dal Toso, R., Balsati, A. and Spano, P. (1994) Nerve growth factor directs differentiation of the bipotential cell line GH-3 into the mammotroph phenotype. *Endocrinology 135,* 290-298.

Missale, C., Boroni, F., Sigala, S., Buriani, A., Fabris, M., Leon, A., Dal Toso, R. and Spano, P. (1996) Nerve growth factor in the anterior pituitary: localization in mammotroph cells and cosecretion with prolactin by a dopamine-regulated mechanism. *Proceedings of the National Academy of Sciences USA 93,* 4240-4245.

Nassenstein, C., Möhring, U.H., Luttmann, W., Virchow, J.C. Jr. and Braun, A. (2006) Differential expression of the neurotrophin receptors p75NTR, TrkA, TrkB and TrkC in human peripheral blood mononuclear cells. *Experimental and Toxicologic Pathology 57,* 55-63.

Negro, A., Tavella, A., Grandi, C. and Skaper, S.D. (1994) Production and characterization of recombinant rat brain-derived neurotrophic factor and neurotrophin-3 from insect cells. *Journal of Neurochemistry 62,* 471-478.

Nilsson, G., Forsberg-Nilsson, K., Xiang, Z., Hallböök, F., Nilsson, K. and Metcalfe, D.D. (1997) Human mast cells express functional TrkA and are a source of nerve growth factor. *European Journal of Immunology 27,* 2295-2301.

Noga, O., Englmann, C., Hanf, G., Grützkau, A., Guhl, S. and Kunkel, G. (2002) Activation of the specific neurotrophin receptors TrkA, TrkB and TrkC influences the function of eosinophils. *Clinical and Experimental Allergy 32,* 1348-1354.

Noga, O., Hanf, G., Görges, D., Dinh, Q.T., Groneberg, D.A., Suttorp, N. and Kunkel, G. (2005) Regulation of NGF and BDNF by dexamethasone and theophylline in human peripheral eosinophils in allergics and non-allergics. *Regulatory Peptides 132,* 74-79.

Nykjaer, A., Lee, R., Teng, K.K., Jansen, P., Madsen, P., Nielsen, M.S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T.E., Hempstead, B.L. and Petersen, C.M. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature 427,* 843-848.

Obata, K., Katsura, H., Sajurai, J., Kobayashi, K., Yamanaka, H., Dai, Y., Fukuoka, T. and Noguchi, K. (2006) Suppression of the p75 neurotrophin receptor in uninjured sensory

neurons reduces neuropathic pain after nerve injury. *Journal of Neuroscience 26,* 11974-11986.

Ojeda, S.R., Hill, D.F. and Katz, K.H. (1991) The genes encoding nerve growth factor and its receptor are expressed in the developing female rat hypothalamus. *Molecular Brain Research 9,* 47-55.

Okuse, K., Chaplan, S.R., McMahon, S.B., Luo, Z.D., Calcutt, N.A., Scott, B.P., Akopian, A.N. and Wood, J.N. (1997) Regulation of expression of the sensory neuron-specific sodium channel SNS in inflammatory and neuropathic pain. *Molecular and Cellular Neuroscience 10,* 196-207.

Olsson, Y. (1968) Mast cells in the nervous system. *International Reviews of Cytology 24,* 27-70.

Olsson, Y. (1974) Mast cells in plaques of multiple sclerosis. *Acta Neurologica Scandanavia 50,* 611-618.

Oppenheim, R.W. (1991) Cell death during development of the nervous system. *Annual Review of Neuroscience 14,* 451-501.

Otten, U., Baumann, J.B. and Girard, J. (1979) Stimulation of the pituitary-adrenocortical axis by nerve growth factor. *Nature 282,* 413-414.

Otten, U., Baumann, J.B. and Girard, J. (1984) Nerve growth factor induces plasma extravasation in rat skin. *European Journal of Pharmacology 106,* 199-201.

Otten, U., Ehrhard, P. and Peck, R. (1989) Nerve growth factor induces growth and differentiation of human B lymphocytes. *Proceedings of the National Academy of Sciences USA 86,* 10059-10063.

Otten, U., Scully, J.L., Ehrhard, P.B. and Gadient, R.A. (1994) Neurotrophins: signals between the nervous and immune systems. *Progress in Brain Research 103,* 293-305.

Patterson, J.C. and Childs, G.V. (1994) Nerve growth factor in the anterior pituitary: regulation of secretion. *Endocrinology 135,* 1697-1704.

Pearce, F.L. and Thompson, H.L. (1986) Some characteristics of histamine secretion from rat peritoneal mast cells stimulated with nerve growth factor. *Journal of Physiology 372,* 379-393.

Pedraza, C.E., Podlesniy, P., Vidal, N., Arévalo, J.C., Lee, R, Hempstead, B., Ferrer, I., Iglesias, M. and Espinet, C. (2005) Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR. *American Journal of Pathology 166,* 533-543.

Peng, S., Wuu, J., Mufson, E.J. and Fahnestock, M. (2004) Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer disease. *Journal of Neuropathology and Experimental Neurology 63,* 641-649.

Persson, H., Ayer-Le Lievre, C., Söder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M. and Hökfelt, T. (1990) Expression of β-nerve growth factor receptor mRNA in Sertoli cells downregulated by testosterone. *Science 247,* 704-707.

Petersen, C.M., Nielsen, M.S., Nykjaer, A., Jacobsen, L., Tommerup, N., Rasmussen, H.H., Roigaard, H., Gliemann, J., Madsen, P. and Moestrup, S.K. (1997) Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *Journal of Biological Chemistry 272,* 3599-3605.

Petty, B.G., Cornblath, D.R., Adornato, B.T., Chaudhry, V., Flexner, C., Wachsman, M., Sinicropi, D., Burton, L.E. and Peroutka, S.J. (1994) The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. *Annals of Neurology 36*, 244-246.

Pezet, S. and McMahon, S.B. (2006) Neurotrophins: mediators and modulators of pain. *Annual Review of Neuroscience 29*, 507-538.

Pincelli, C., Sevignani, C., Manfredini, R., Grande, A., Fantini, F., Bracci-Laudiero L., Aloe, L., Ferrari, S., Cossarizza, A. and Giannetti, A. (1994) Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes. *Journal of Investigative Dermatology 103*, 13-18.

Polak, M., Scharfmann, R., Seilheimer, B., Eisenbarth, G., Dressler, D., Verma, I.M. and Potter, H. (1993) Nerve growth factor induces neuron-like differentiation of a insulin-secreting pancreatic beta cell line. *Proceedings of the National Academy of Sciences USA 90*, 5781-5785.

Prineas, J.W. and Wright, R.G. (1978) Macrophages, lymphocytes, and plasma cells in the perivascular compartment in chronic multiple sclerosis. *Laboratory Investigation 38*, 409-421.

Purcell, W.M. and Atterwill, C.K. (1995) Mast cells in neuroimmune function: neurotoxicological and neuropharmacological perspectives. *Neurochemical Research 20*, 521-532.

Raap, U., Fokkens, W., Bruder, M., Hoogsteden, H., Kapp, A. and Braunstahl, G.-J. (2008) Modulation of neurotrophin and neurotrophin receptor expression in nasal mucosa after nasal allergen provocation in allergic rhinitis. *Allergy 63*, 468-475.

Reinshagen, M., Geerling, I., Lakshmanan, J., Tirpitz, C.V., Rohm, H. and Adler, G. (1996) Localisation of nerve growth factor prohormone isoforms in the inflamed gut of patients with Crohn's disease and ulcerative colitis. *Society for Neuroscience Abstract 22*, 298.

Reinshagen, M., Rohm, H. and Lakshmanan, J. (1997) Expression of neurotrophin-3 prohormone in different animal models of ulcerative colitis and in human inflammatory bowel disease. *Gastroenterology 112*, A1070.

Reinshagen, M., Geerling, I., Eysselein, V.E., Alder, G., Huff, K.R., Moore, G.P. and Lakshmanan, J. (2000) Commercial recombinant human β-nerve growth factor and adult rat dorsal root ganglia contain an identical molecular species of nerve growth factor prohormone. *Journal of Neurochemistry 74*, 2127-2133.

Richard, A., McColl, S.R. and Pelletier, G. (1992) Interleukin-4 and nerve growth factor can act as cofactors for interleukin-3-induced histamine production in human umbilical cord blood in serum-free culture. *British Journal of Haematology 81*, 6-11.

Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramee, G.R., Nikolics, K. and Winslow, J.W. (1990) Primary structure and biological activity of a novel human neurotrophic factor. *Neuron 4*, 767-773.

Rose, C.R., Blum, R., Pichler, B., Lepier, A., Kafitz, K.W. and Konnerth, A. (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature 426*, 74-78.

Rozniecki, J.J., Dimitriadou, V., Lambracht-Hall, M., Pang, X. and Theoharides, T.C. (1999) Morphological and functional demonstration of rat dura mater mast cell-neuron interactions in vitro and in vivo. *Brain Research 849*, 1-15.

Ruiz, G., Ceballos, D. and Baños, J.-E. 92004) Behavioral and histological effects of endoneurial administration of nerve growth factor: possible implications in neuropathic pain. *Brain Research 1011,* 1-6.

Sanico, A.M., Stanisz, A.M., Gleeson, T.D., Bora, S., Proud, D., Bienenstock, J., Koliatsos, V.E. and Togias, A. (2000) Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. *American Journal of Respiratory and Critical Care Medicine 161,* 1631-1635.

Sant, G.R., Kempuraj, D., Marchand, J.E. and Theoharides, T.C. (2007) The mast cell in interstitial cystitis: role in pathophysiology and pathogenesis. *Urology 69 (Supplement 4A)*, 34-40.

Santambrogio, L., Benedetti, M., Chao, M.V., Muzaffar, R., Kulig, K., Gabellini, N. and Hochwald, G. (1994) Nerve growth factor production by lymphocytes. *Journal of Immunology 153*, 4488-4495.

Sawada, J., Itakura, A., Tanaka, A., Furusaka, T. and Matsuda, H. (2000) Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Blood 95*, 2052-2058.

Scaccianoce, S., Cigliana, G., Nicolai, R., Muscolo, L.A., Porcu, A., Navarra, D., Perez-Polo, J.R. and Angelucci, L. (1993) Hypothalamic involvement in the activation of the pituitary-adrenocortical axix by nerve growth factor. *Neuroendocrinology 58*, 202-209.

Schafers, M., Sorkin, L.S. and Sommer, C. (2003) Intramuscular injection of tumor necrosis factor-α induces muscle hyperalgesia in rats. *Pain 104*, 579-588.

Screpanti, I., Meco, D., Scarpa, S., Morrone, S., Frati, L., Gulino, A. and Modesti, A. (1992) Neuromodulatory loop mediated by nerve growth factor and interleukin 6 in thymic stromal cell cultures. *Proceedings of the National Academy of Sciences USA 89*, 3209-3212.

Seidah, N.G., Benjannet, S., Pareek, S., Savaria, D., Hamelin, J., Goulet, B., Laliberte, J., Lazure, C., Chrétien, M. and Murphy, R.A. (1996) Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. *Biochemical Journal 314*, 951-960.

Shu, X.Q., Llinas, A. and Mendell, L.M. (1999) Effects of trkB and trkC neurotrophin receptor agonists on thermal nociception: a behavioral and electrophysiological study. *Pain 80*, 463-470.

Sidhu, S. and Tandon, R.K. (1996) Chronic pancreatitis: diagnosis and treatment. *Postgraduate Medical Journal 72*, 327-333.

Silver, R., Silverman, A.-J., Vitkovic, L. and Lederhendler, I.I. (1996) Mast cells in the brain: evidence and functional significance. *Trends in Neuroscience 19*, 25-31.

Silverman, A.J., Millar, R.P., King, J.A., Zhuang, X. and Silver, R. (1994) Mast cells with gonadotropin releasing hormone-like immunoreactivity in the brain of doves. *Proceedings of the National Academy of Sciences USA 91*, 3695-3699.

Simone, M.D., De Santis, S., Vigneti, E., Papa, G., Amadori, S. and Aloe, L. (1999) Nerve growth factor: a survey of activity on immune and hematopoietic cells. *Hematology and Oncology 17,* 1-10.

Skaper, S.D., Facci, L., Romanello, S. and Leon, A. (1996) Mast cell activation causes delayed neurodegeneration in mixed hippocampal cultures via the nitric oxide pathway. *Journal of Neurochemistry 66,* 1157-1166.

Skaper, S.D., Pollock, M. and Facci, L. (2001) Mast cells differentially express and release active high molecular weight neurotrophins. *Molecular Brain Research 97,* 177-185.

Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A. and Barbacid, M. (1994) Sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature 368,* 246-249.

Snider, W.D. (1994) Functions of the neurotrophins during development: what the knockouts are teaching us. *Cell 77,* 627-638..

Sobel, R.A., Blanchette, B.W., Bhan, A.K. and Colvin, R.B. (1984) The immunopathology of experimental allergic encephalomyelitis: quantitative analysis of inflammatory cells *in situ. Journal of Immunology 132,* 2392-2401.

Solomon, A., Aloe, L., Pe'er, J., Frucht-Pery, J., Bonini, S. and Levi-Schaffer, F. (1998) Nerve growth factor is preformed in and activates human peripheral blood eosinophils. *Journal of Allergy and Clinical Immunology 102,* 454-460.

Soppet, D., Escandón, E., Maragos, J., Middlemas, D.S., Reid, S.W., Blair, J., Burton, L.E., Stanton, B.R., Kaplan, D.R., Hunter, T., Nikolics, K. and Parada, L.F. (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell 65,* 895-903.

Spillantini, M.G., Aloe, L., Alleve, E., De Simone, R., Goedert, M. and Levi-Montalcini, R. (1989) Nerve growth factor mRNA and protein increase in hypothalamus in a mouse model of aggression. *Proceedings of the National Academy of Sciences USA 86,* 8555-8559.

Squinto, S.P., Stitt, T.N., Aldrich, T.H., Davis, S., Bianco, S.M., Radziejewski, C., Glass, D.J., Masiakowski, P., Furth, M.E., Valenzuela, D.M., DiStefano, P.S. and Yancopoulos, G.D. (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell 65,* 885-893.

Stead, R.H., Bienenstock, J. and Stanisz, A.M. (1987) Neuropeptide regulation of mucosal immunity. *Immunological Reviews 100,* 333-359.

Stampachiacchiere, B., Aloe, L., Micera, A. and Bonini, S. (2006) Allergic bronchial airway inflammation in nerve growth factor (NGF)-deprived rats: evidence suggesting a neuroimmunomodulatory role of NGF. *Experimental Lung Research 32,* 305-320.

Stead, R.H., Kosecka-Janiszewska, U., Oestreicher, A.B., Dixon, M.F. and Bienenstock, J. (1991) Remodelling of B-50 (GAP-43)- and NSE-immunoreactive mucosal nerves in the intestines of rats with *Nippostrongylus brasiliensis. Journal of Neuroscience 11,* 3809-3821.

Stucky, C.L. and Koltzenburg, M. (1997) The low-affinity neurotrophin receptor p75 regulates the function but not the selective survival of specific subpopulations of sensory neurons. *Journal of Neuroscience 17,* 4398-4405.

Stucky, C.L., Koltzenburg, K.M., Schneider, M., Engle, M.G., Albers, K.M. and Davis, B.M. (1999) Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. *Journal of Neuroscience 19*, 8509-8516.

Sugiyama, K., Suzuki, Y. and Furuta, H. (1985) Histamine-release induced by 7S nerve growth factor of mouse submandibular salivary glands. *Archives of Oral Biology 30,* 93-95.

Svensson, P., Cairns, B.E., Wang, K. and Arendt-Nielsen, L. (2003) Injection of nerve growth factor into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia. *Pain 104,* 241-247.

Talhouk, R.S., Saade, N.E., Mouneimne, G., Masaad, C.A. and Safieh-Garabedian, B. (2004) Growth hormone releasing hormone reverses endotoxin-induced localized inflammatory hyperalgesia without reducing the upregulated cytokines, nerve growth factor and gelatinase activity. *Progress in Neuropsychopharmacology and Biological Psychiatry 28,* 625-631.

Tam, S.-Y., Tsai, M., Yamaguchi, M., Yano, K., Butterfield, J. and Galli, S.J. (1997) Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. *Blood 90,* 1807-1820.

Theoharides, T.C. (1990) Mast cells: the immune gate to the brain. *Life Sciences 46,* 607-617.

Theoharides, T.C., Baloyannis, S.J. and Manolidis, L.S. (1991) Activated rat peritoneal mast cells can cause syngeneic brain demyelination *in vitro. International Journal of Immunopathology and Pharmacology 4,* 137-144.

Theoharides, T.C., Dimitriadou, V., Letourneau, R., Rozniecki, J.J., Vliagoftis, H. and Boucher W. (1993) Synergistic action of estradiol and myelin basic protein on mast cell secretion and brain myelin changes resembling early stages of demyelination. *Neuroscience 57,* 861-871.

Thoenen, H. (1995) Neurotrophins and neuronal plasticity. *Science 270,* 593-598.

Thorpe, L.W. and Perez-Polo, J.R. (1987) The influence of nerve growth factor on the in vitro proliferative response of rat spleen lymphocytes. *Journal of Neuroscience Research 18,* 134-139.

Thorpe, L.W., Werrbach-Perez, K. and Perez-Polo, J.R. (1987) Effects of nerve growth factor expression on interleukin-2 receptors on cultured human lymphocytes. *Annals of the New York Academy of Sciences 496,* 310-311.

Tomioka, M., Stead, R.H., Nielsen, L., Coughlin, M.D. and Bienenstock, J. (1988) Nerve growth factor enhances antigen and other secretagogue-induced histamine release from rat peritoneal mast cells in the absence of phosphatidylserine. *Journal of Allergy and Clinical Immunology 82,* 599-607.

Toms, R., Weiner, H.L. and Johnson, D. (1990) Identification of IgE-positive cells and mast cells in frozen sections of multiple sclerosis brains. *Journal of Neuroimmunology 30,* 169-177.

Torcia, M., Bracci-Laudiero, L., Lucibello, M., Rubartelli, A., Cozzolino, F., Aloe, L. and Garaci, E. (1996) Nerve growth factor is an autocrine factor for memory B lymphocytes. *Cell 85,* 1-20.

Tortorolo, L., Langer, A., Polidori, G., Vento, G., Stampachiacchere B., Aloe, L. and Piedimonte, G. (2005) Neurotrophin overexpression in lower airways of infants with

respiratory syncytial virus infection. *American Journal of Respiratory Critical Care Medicine 172,* 233-237.

Treede, R.D., Meyer, R.A., Raja, S.N. and Campbell, J.N. (1992) Peripheral and central mechanisms of cutaneous hyperalgesia. *Progress in Neurobiology 38,* 397-421.

Tuveri, M.A., Passiu, G., Mathieu, A. and Aloe, L. (1993) Nerve growth factor and mast cell distribution in the skin of patients with systemic sclerosis. *Clinical and Experimental Rheumatology 11,* 319-322.

Ugolini, G., Marinelli, S., Covaceuszach S., Cattaneo, A. and Pavone, F. (2007) The function neutralizing anti-TrkA antibody MNAC13 reduces inflammatory and neuropathic pain. *Proceedings of the National Academy of Sciences USA 104,* 2985-2990.

Waksman, B.H. (1988) Autoimmunity in demyelinating diseases. *Annals of the New York Academy of Sciences 540,* 13-24.

Walicke, P., Cowan, W.M., Ueno, N., Baird, A. and Guillemin, R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proceedings of the National Academy of Sciences USA 83,* 3012-3016.

Wang, Y., Lindstedt, K.A. and Kovanen, P.T. (1995) Mast cell granule remnants carry LDL into smooth muscle cells of the synthetic phenotype and induce their conversion into foam cells. *Arteriosclerosis, Thrombosisand Vascular Biology 15,* 801-810.

Welker, P., Grabbe, J., Gibbs, B., Zuberbier, T. and Henz, B.M. (2000) Nerve growth factor-β induces mast-cell marker expression during *in vitro* culture of human umbilical cord blood cells. *Immunology 99,* 418-426.

Weskamp, G. and Otten, U. (1987) An enzyme-linked immunoassay for nerve growth factor (NGF): a tool for studying regulatory mechanisms involved in NGF production in brain and peripheral tissues. *Journal of Neurochemistry 48,* 1779-1786.

Wilhelm, M., Silver, R. and Silverman, A.J. (2005) Central nervous system neurons acquire mast cell products via transgranulation. *European Journal of Neuroscience 22,* 2238-2248.

Wolf, D., Richter-Landsberg, C., Short, M.P., Cepko, C. and Breakefield, X.O. (1988) Retrovirus-mediated transfer of beta-nerve growth factor into mouse pituitary line AcT-20. *Molecular Biology and Medicine 5,* 43-59.

Woolf, C.J., Safieh-Garabedian, B., Ma, Q.P., Crilly, P. and Winter, J. (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensibility. *Neuroscience 62,* 327-331.

Woolf, C.J., Ma, Q.P., Allchorne, A. and Poole, S. (1996) Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. *Journal of Neuroscience 16,* 2716-2723.

Woolf, C.J., Allchorne, A., Safieh-Garabedian, B. and Poole, S. (1997) Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. *British Journal of Pharmacology 121,* 417-424.

Wu, X.Q., Myers, A.C., Goldstone, A.C., Togias, A. and Sanico, A.M. (2006) Localization of nerve growth factor and its receptors in the human nasal mucosa. *Journal of Allergy and Clinical Immunology 118,* 428-433.

Xiang, Z. and Nilsson, G. (2000) IgE receptor-mediated release of nerve growth factor by mast cells. *Clinical and Experimental Allergy 30,* 1379-1386.

Xu, P. and Hall, A.K. (2007) Activin acts with nerve growth factor to regulate calcitonin gene-related peptide mRNA in sensory neurons. *Neuroscience 150,* 665-674.

Yaar, M., Eller, M.S., DiBenedetto, P., Reenstra W.R., Zhai, S., McQuaid T., Archambault, M. and Gilchrest, B.A. (1994) The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes. *Journal of Clinical Investigation 94,* 1550-1562.

Yip, H.K., Rich, K.M., Lampe, P.A. and Johnson, E.M. Jr. (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. *Journal of Neuroscience 4,* 2986-2992.

Yoshida, K. and Gage, F.H. (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. *Brain Research 569,* 14-25.

Zhu, W. and Oxford, G.S. (2007) Phosphoinositide-3-kinase and mitogen activated protein kinase signaling pathways mediate acute NGF sensitization of TRPV1. *Molecular and Cellular Neuroscience 34,* 689-700.

Zhuang, X., Silverman, A.J. and Silver, R. (1996) Brain mast cell degranulation regulates the blood brain barrier. *Journal of Neurobiology 31,* 393-403.

Zhuang, Z.-Y., Xu, H., Clapham, D.E. and Ji, R.-R. (2004) Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. *Journal of Neuroscience 24,* 8300-8309.

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Chapter 6

The in Vivo Role of NGF as an Intermediate in Neuroendocrine, Immune, and Redox Regulation following Peripheral or Central Inflammation Implicating Neurotransmitter Regulation

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A major consequence of severe chronic infection is the often massive loss of body weight (=cachexia), which is directly linked to increased morbidity and reduced survival, similarly large increases in body weight leading to obesity are also associated with reduced survival. Animal models must be used to determine the mechanisms involved and this may led to the development of appropriate therapy. Our use of chronic murine infection models and brain inflammatory models suggests that *in vivo* the site of initiation of the inflammatory process be it peripheral or central is of prime importance to the subsequent pathology. Neuroendocrine interactions with the immune system have recently been suggested to be of importance in determining the degree of pathology due to dysregulation of the immune system. In particular peripheral hormones involved in energy balance, such as leptin and ghrelin have been shown to be important in regulating immune function and susceptibility to infection. We observed that following permanent middle cerebral artery occlusion (pMCAO) or in acute/chronic infection models all of which induce changes in circulating ghrelin. These changes in ghrelin are associated with altering nerve growth factor (NGF) and redox

regulatory factors such as monoamine oxidase (MAO), uncoupling protein 2 (UCP2) and glutathione (GSH). Our studies suggest that NGF can play a role in modifying neuroendocrine systems involving peripheral/central MAO activity, UCP2 and GSH. We also have evidence that NGF may play a specific role in food intake regulation following inflammation. In this review we will focus on our *in vivo* studies demonstrating regulatory circuits involving NGF modulation by ghrelin and leptin. Furthermore we will also show a role for NGF in modifying central MAO activity, UCP2, cytokine production and glutathione levels. Finally we will discuss the role of NGF on food intake and energy expenditure based on our studies. The role of NGF as a integrator of neuroendocrine system, immune function and physiological systems will be evaluated.

Background

Severe body weight loss or body weight gain, are both associated with reduced survival (Schlep 1998; Dorn Et al, 1997; Seidell et al, 1996). An understanding of the mechanisms involved in body weight regulation are therefore of importance for the treatment of obese or cachectic individuals so that they may improve their body weight. Furthermore dramatic changes in body weight are associated with changes in immunity and cell redox levels in particular involving glutathione (GSH) (Yang et al, 2000; Arsenijevic et al, 2005A). GSH has been implicated in regulating energy balance homeostasis and immune responses (Arsenijevic et al, 2006; Arsenijevic et al, 2007A; Arsenijevic et al, 2007C; Hernadfalvi et al, 2007). As a specific example of redox state and metabolic interactions, the reactive oxygen species regulator the uncoupling protein 2 (UCP2) is involved in production of insulin (Zhang et al, 2001). Changes in basal immune function can result in possible enhanced anorexia in response to inflammation or infection (Arsenijevic et al, 1998).

Body weight regulation may be considered in terms of energy balance equation, in that food intake and energy expenditure will determine if the individual is gaining or losing weight. The regulation of energy balance is controlled by the brain and factors from the periphery. Peripheral factors can act directly on the brain usually in areas of reduced blood brain barrier such as the circumventricular regions rather than actual entry into the brain or by peripheral nerves such as the vagus (Andrews et al, 1985; Ahima and Flier, 2000; Berthoud and Neuhuber 2000; Tschop et al, 2000; Timofeeva et al, 2005). Peripheral factors such as ghrelin (produced by the stomach) and leptin (produced by adipocytes) have been implicated to act on the brain where they can alter both food intake and energy expenditure (Attele A.S. et al, 2002). Ghrelin is believed to promote increases in appetite and lower energy expenditure resulting in increased body weight. Leptins' action is the opposite, it increases energy expenditure and reduces appetite. Actually, data indicates that for both leptin and ghrelin when given individually either peripherally or centrally their action on energy balance may differ (Takeda et al 2003; Tschop et al, 2000). Specifically during obesity, leptin may have a reduced anorexic effect peripherally but may still maintain its potent activity when given centrally (Scarpace and Tumer, 2001). Ghrelin when given continuously in the periphery increased body weight by decreasing metabolic rate, whereas when give centrally it increases food intake and decreases metabolic rate (Tschop et al, 2000). It should be noted

that disease states can alter the blood brain barrier permeability and may therefore alter normal homeostatic mechanisms (Arsenijevic et al, 2000A; Banks, 2006).

Energy expenditure and food intake can be regulated by various factors inside the brain, which includes hormones, neuropeptides, neurotransmitters, eicosanoids and cytokines. These five different mediators interact with members found within each group and between the groups. The resulting pathways involved in food intake and energy expenditure are therefore complex. Furthermore neurons interact with each other and are considered to be the main effects for energy balance regulation (Berthoud, 2002). Although recent studies suggest that glia may also play a role, since they are capable of metabolising neurotransmitters (Barnes and Sharp, 1999). Neuropeptides be they anorexigenic or orexigenic in nature can be regulated by nutrients and by protein regulatory pathways such as transcription factors, as we have recently shown to be the case for neuropeptide Y regulation by peoxisome proliferator activated receptor beta (PPARβ) (Arsenijevic et al, 2006). Although PPARβ is involved in obesity and energy balance (Evans et al, 2004), to date no studies have been carried out to determine if a relation exists between PPARβ and neurotransmitters.

Neurotransmitters, like neuropeptides can be orexigenic (dopamine – DA) or anorexigenic (serotonin – 5HT). Neurotransmitter effects such as serotonin (5HT) can be regulated at may levels, this includes limiting their formation substrates such as tryptophan for 5HT. Enhanced degradation of tryptophan by indoleamine dioxygenase (IDO) / tryptophan dioxygenase (TDO) in the brain and other tissue can reduce 5HT levels (Salter et al, 1995; Dang et la, 2000). Interestingly IDO elevation and reduced 5HT have been show to occur in obese individuals (Brandacher et al, 2006). Inactivation of neurotransmitters can occur by a variety of neurotransmitter degrading enzymes such as monoamine oxidase A / B (Shih et al, 1999) and catechol-O-methyl-transferase – COMT (Mannisto and Kaakkola, 1999). Monoamine oxidase A (MAO-A) shows more specificity in degrading 5HT than other neurotransmitters compared to monoamine oxidase B (MAO-B) which catabolizes catecholamines (Glover and Sandler, 1986). Under physiological conditions MAO-A is found in catecholeamine neurones (Shih et al, 1999) however in pathological circumstances it can be induced in glia (Kennedy et al, 2003). The degradation of neurotransmitters by the MAO occurs in the mitochondria outer membrane (Shih et al, 1999). MAO-A and COMT have been implicated in obesity by genetic studies (Need et al, 2006; Ben-Dor, 2002), food intake studies (Banchelli et al, 2001) and also in energy expenditure (Gordan and Duncan, 1994).

The efficiency of neurotransmitter signalling, such as for serotonin, will also depend on receptors, serotonin for example has 14 subtypes, some of these receptors can act as autoreceptors or receptors on post synaptic neurons (Barnes and Sharp, 1999). The use of agonist and the generation of knockout mice for some of these receptors indicate that 5HT may play a role in obesity/anorexia in contrasting ways. 5HT1A and 5HT3 receptors are involved in hyperphagia, whereas 5HT1B, 5HT2C, 5HT4 and 5HT6 are implicated in reducing appetite. Interestingly, one has to consider that these receptors involved in energy expenditure regulation (5HT1A, 5HT1B and 5HT2 receptors) and appetite regulation (5HT1B and 5HT2 receptors) also appears to depend on circadian rhythms (Bovetto and Richard, 1995; Bovetto et al, 1996). Concerning energy balance catecholamines (adrenaline - AD, noradrenaline - NA, dopamine - DA) have been shown to play a role in both energy expenditure and food intake regulation (Wellman, 1992). 5HT has been show to regulate food

intake and energy expenditure (Leibowitz and Alexander, 1998) by mechanisms involving the hypothalamus and brain stem, sites were leptin and ghrelin are known to act and have receptors. Data suggests that normal homeostatic regulation of energy balance involving 5HT may differ in pathological circumstances where a 5HT receptor subtype may actually gain in importance (Hrupka and Langhans, 2001; Von Meyenburg et al, 2003; Compan et al, 2004).

It is known that the uncoupling protein 1 (UCP1) found in the mitochondria of brown adipocytes can regulate energy expenditure in rodents and that the hormones, leptin, ghrelin, thyroid hormones (T4/T3), cholecystokinin and enterostatin can modulate it (Rippe et al, 2000; Bianco et al, 2005; Tsubone et al, 2005), 5HT is believed to be involved in these pathways (Nonogaki et al, 2006; Morrison, 2004). Although 5HT1A and 5HT2C receptors have been implicated in energy changes in response to ghrelin / leptin, no studies have compared the role of theses receptors in peripheral vs central mechamisms, which may show differences, as previously discussed. The role for MAO-A and IDO in 5HT receptor mediated changes in appetite and energy expenditure following leptin or ghrelin treatments have not been studied but merits further investigation particularly since MAO-A and IDO are implicated in obesity.

Although UCP1 involvement in energy expenditure was previously thought to be significant only in rodents, it has recently been show to be activated in human after cold exposure and may therefore represent an important mechanism in humans (Garcia et al, 2006; Nedergaard et al, 2007). The role of UCPs other than UCP1 in energy balance remains to be convincingly demonstrated. The above mentioned hormones have been shown to modify UCP2 levels in various tissues (Ricquier and Bouillaud, 2000; Arsenijevic et al, 2005A; Sun et al, 2006) suggesting a role for UCP2 in body weight regulation. Currently UCP2s' main function is believed to be to regulate mitochondrial reaction oxygen species and immune responses (Negre-Salvayre et al, 1997; Arsenijevic et al, 2000b; Paradis et al, 2003; de Bilbao et al, 2004; Krauss et al, 2005). However, UCP2 is expressed in various nuclei in the brain involved in energy balance regulation and some studies suggest that it may regulate insulin and food intake (Richards et al, 1998; Zhang et al, 2001; Arseniejvic et al, 2006). Furthermore double labeling of UCP2 has been shown associated with neurons with leptin receptors in the arcuate and other nuclei (Horvath et al, 1999). Ghrelin receptors are also found in the arcuate but no studies so far have investigated the relationship with UCP2. In hypophagia during chronic toxoplasmosis infection, we have suggested that UCP2 may be involved in energy balance regulation involving the arcuate nucleus (Arsenijevic et al, 2007B). UCP2 is known to be co-expressed with neuropeptide Y (Horvath et al, 1999), corticotroph releasing hormone, POMC (Diano et al, 2000) and agouti related protein - Agrp (Richard et al, 2001), neuropeptides known to be involved in energy balance. UCP2 has also been implicated in energy balance involving thyroid hormones in brain centres implicated in energy balance (Coppola et al, 2007). UCP2 and MAO-A similar distribution patterns in the brain in rodents and they are also both mitochondrial proteins (Luque et al 1995; Richard et al, 1998). Interestingly MAO-A KO mice have increased brain 5HT levels and a 10% reduction in body weight and altered thermoregulation compared to wildtype controls (Evard et al, 2002; Holscheider, 2001).

Different peripheral hormones that induce hypophagia or hyperphagia may not use the same 5HT receptors (ie 5HT1B, 5HT2C, 5HT3, 5HT4) suggesting that there are multiple

pathways for 5HT to induce alterations in appetite. Our studies indicate that food intake regulation after permenant middle cerebral artery occlusion (pMCAO) may differ from regular homeostatic food intake regulation (Arsenijevic et al, 2006). A similar finding has been found in 5HT4 receptor knockout mice, which do not show difference in basal food intake compared to their wildtype counterparts, but differences in food intake occur in response to stress (Compan et al, 2004). The role of 5HT4 receptor merits to be studied in pathological conditions – in particularly its role in pMCAO induced hyperphagia, since pMCAO involves a lesion in the cortex and the 5HT4 receptor in the raphe can be regulated from the cortex (Lucas et al, 2006). The raphae nucleus is known to play an important role in food intake and energy expenditure regulation, UCP2 is expressed in this region (Richard et al, 1998). Our studies have shown an association between brain stem and arcuate UCP2 mRNA expression and food intake in MCAO (Arsenijevic et al, 2005B) and toxoplasmosis infection (Arsenijevic et al, 2007B). Interestingly we have observed that UCP2 decreases in some brain nuclei but is not affected in other nuclei (Arsenijevic et al, 2007B), similar to reports involving the regulation of MAO-A (Jahng et al, 1998) in response to altered food intake. These findings, further supports comparing UCP2 KO and MAO-A mice in the regulation of energy balance and a possible role of the serotonergic systems.

We have demonstrated that PPARβ is a positive regulator of UCP2 (Arsenijevic et al, 2006) and MAO-A activity (unpublished data) induction in the brain of mice. In that same study PPARβ KO mice did not show a transient hyperphagia phase following MCAO finding similar to the UCP2 KO response. So PPARβ agonists / antagonist could not only regulate UCP2 and MAOA, but may have effects on the 5HT and 5HT receptors. Such a study would provide evidence for the first time that PPARβ regulates neurotransmitters.

Problems of Studying the Role of Cytokines in Infection and Cachexia

A major consequence of severe infection is the often severe loss of body weight (=cachexia), which is directly linked to increased morbidity and reduced survival (Schelp, 1998). Chronic cachexia may also render the host more susceptible to secondary infections (bacterial lipopolysaccharide) (Arsenijevic et al, 1998). Contrastingly in the same chronic infection model we observe that brain lesion size is reduced after pMCAO (Arseniejvic et al, 2007C), this difference in susceptibility of infected mice to lipopolysaccharide or pMCAO is associated with tissue specific immune regulation and redox status. Cytokines are produced by immune cells (but also by other cells) and have been implicated as potential endogenous mediators of cachexia following infection as well as in tissue destruction. Cytokine injections have been shown to induce anorexia and hypermetabolism (Plata-Salaman 1999), and are also involved in the neuroendocrine control of metabolism. Yet, such injections do not necessarily trigger the same physiological responses as an infection.

In different models of cachexia, antibodies to cytokines or cytokine knockout mice result in attenuation of body weight loss and improved morbidity (Arsenijevic et al, 2000A). Yet experimental infection in these knockout or anti-cytokine treatments can result in rapid cachexia and death (Yap and Sher, 1999). Cytokines therefore are required for resistance

against infection and can potentially also induce cachexia. The complexity of cachexia can not be addressed by cytokine injection experiments. Rather, suitable infection models are needed for thorough analysis.

Body weigh loss after infection cannot always be explained by underfeeding (Arseniejvic et al, 1996), tissues can also be differentially affected some showing weight loss while others show no change in weight or even weight gain. Moreover, complex interactions between cytokines and other neuroendocrine regulatory factors involved in energy balance exist. For example, factors such as ghrelin, leptin and corticosterone interact with the immune system and can influence energy balance and survival directly or indirectly (Mandrup-Poulsen et al, 1995). Therefore, regulators that are upstream and downstream of cytokines in the regulation of energy balance must also be considered to get a complete picture. Again, suitable infection models provide a much better tool to unravel these complex interactions than cytokine injections.

Finally, in response to infection, hypermetabolism and anorexia often occur simultaneously. This makes it difficult to identify the regulatory cytokine(s) and the mechanisms specific for each individual response. Moreover, the use of most infection models to study the behavioural and metabolic consequence of sepsis is limited due to their transient nature. Some infection models are of a very rapid onset and lethal (Cooper et al, 1989**;** Moore et al, 1977**;** Reiss 1958), while other models do not permit to study steady states in chronic infection (Samuels and Baracos, 1992**;** Mutwiri et al, 1995). The problem with models using dynamic phases of infection such as those encountered with bacterial lipopolysaccharide (LPS) models, to understand cachexia is that variables such as cytokines are continually changing (Pearson et al, 1990). All these shortcomings do not apply to our model of *Toxoplasma gondii* infection in Swiss Webster mice (Me49 strain). *Toxoplasma gondii* is an obligate intracellular protozoan parasite which can potentially infect all nucleated cells. This model possesses the following qualities: slow onset of responses, low mortality, separation of acute and stable chronic phases of infection as well as intervals in which the anorexia and the hypermetabolic components are separated (Arsenijevic et al, 1997). This is therefore a very useful model to study the mechanisms of infection–induced anorexia, hypermetabolism, and other metabolic changes possibly mediated by cytokines. Both female and male mice in this model show weight loss in the late acute phase, and this is followed by a variable weight recovery in the chronic phase of infection: Approximately 50% of infected mice show a partial weight recovery - which we call Gainers (G) – whereas the remaining animals maintain and stabilise their lower body weights – the Non–Gainers (NG). This model therefore not only permits the study of factors influencing hypermetabolism and anorexia separately, but also to investigate variable weight recovery following infection (see Figure 1) (Arsenijevic et al, 1997; Henderson et al, 1993**;** Duggan and Milner, 1986).

Our studies with murine toxoplasmosis indicate that hypermetabolism is associated with a decrease in the mitochondrial thermogenic uncoupling protein 1 (UCP1) expression (90 % reduction in UCP1 by northern blot in infected mice) and increased lipid peroxidation in the lung, spleen, and sera (200 % increase) and this is not related to mitochondrial lipid oxidation (Arsenijevic et al, 2001). Mice that had undergone a permanent focal cerebral ischemic injury (pMCAO) showed a similar response (Arsenijevic et al, 2001). Using these two different models we were able to show that the changes in lipid metabolism observed following

toxoplasmosis are not specific for that model. Thus, having different infection/inflammation models which result in negative energy balance is very useful to determine whether mechanisms are specific for a particular model or are a general response. It should also be noted that cerebral ischemic injury is a prevalent pathology (associated with stroke) and is intensively studied, yet little has been done in relation to the effects on whole body energy balance.

An alternative approach to determine the role of cytokines and/or other factors in septic cachexia could be to study infections that result in body weight gain and/or hyperphagia. Such a model would nicely complement the cachexia models and allow to identify factors involved in the regulation of body fat and body protein in response to infection. Mice or rats infected with the plerocercoid of *Spirometra mansonoides* provides such a rare model (*Mueller, 196; Phares 1996; Yang, 2006*), the observed increased body weight is due to increase fat and lean tissue mass. In this model the increase in body weight is due to enhanced energy efficiency and not due to hyperphagia (see Figure 2). For the rest of this discussion infection with plerocercoids of *Spirometrea mansonoides* will be referred to as *Spirometra* infection. The growth hormone/insulin-like growth factor axis and sex hormones appears to play an important role in this infection. Both models (*Spirometra* and *Toxoplasma gondii* infection) result in stable chronic infections, are not virulent to mice, and have a high survival rate. This makes them the ideal candidates for comparison.

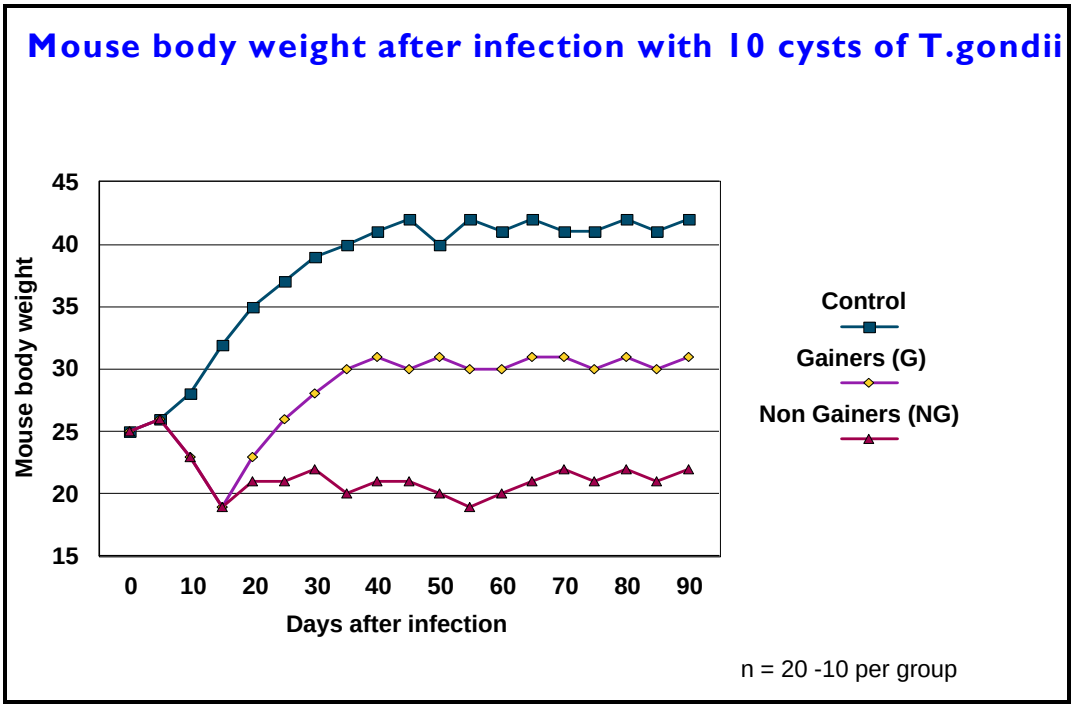

Figure 1. Body weight following *Toxoplasma gondii* infection During the first week all infected mice are hypermetabolic. In the chronic phase only NG remain hypermetabolic (compared to weight matched controls). In the chronic phase of infection all infected mice maintain an anorexic component. Note the stable body weight in the chronic phase of the infection.

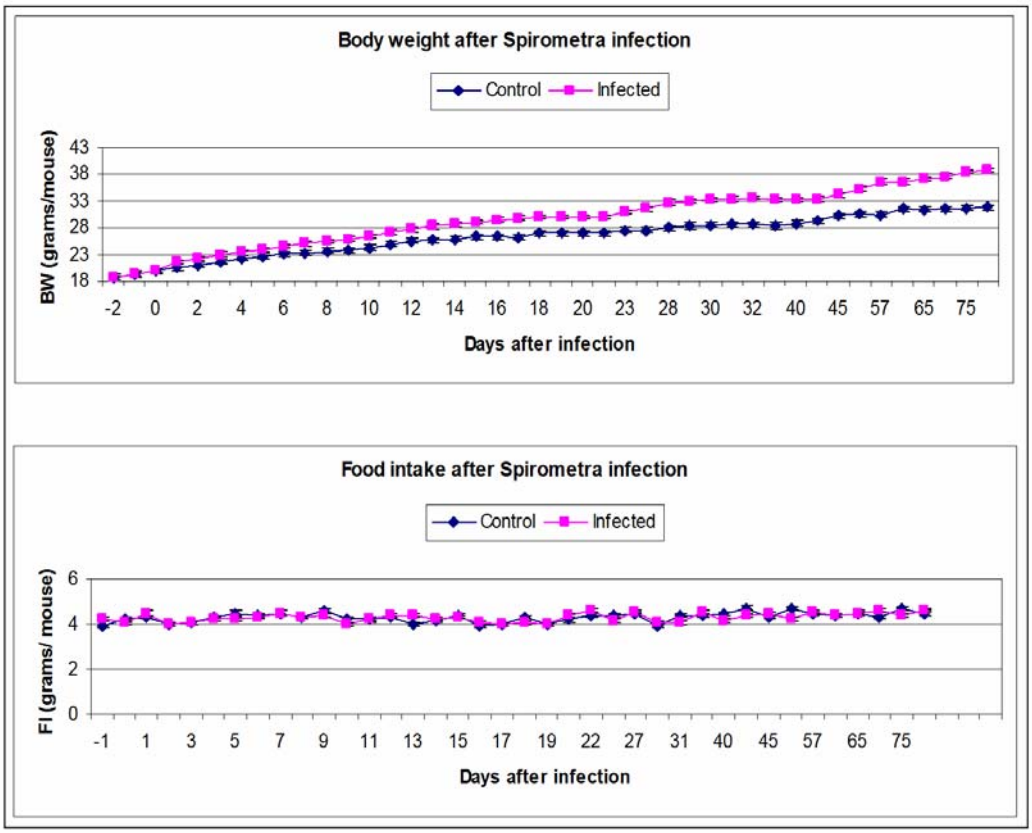

Figure 2. Body weight and food intake following subcutaneous Spirometra infection Body weight prior to infection between the two groups of mice did not differ, strikingly 2 days after infection Spirometra infected mice body weight was significantly increased and body weight increase continued throughout the period under study. Note that the increase in body weight is not due to hyperphagia. (n=20 mice for each group).

Another advantage of this model of infection-induced weight gain is that the Spirometra infection is an exclusively peripheral infection whereas *Toxoplasma gondii* is an obligate intracellular parasite, the latter also infects all types of cells including the brain. This is of importance since body weight is regulated by peripheral and/or central mechanisms, therefore the role of central verses peripheral cytokines is of particular importance. It has been shown that peripheral cytokines differ in their mechanism of action on food intake regulation compared to central cytokine action (Langhans and Hrupka, 1999).

Thus comparison of potentially causative factors for body weight regulation in these two models should also yield valuable information concerning this peripheral-central distinction. To complement the peripheral – central components we also use a brain specific inflammatory model of permanent middle cerebral artery occlusion (pMCAO) model for stroke. This model results in a transient phase of hypophagia (day of lesion) and a hyperphagia phase lasting for 4-6 days (see Figure 3). Thus we are able to compare the role of cytokines and other regulators of body weight and food intake regulation due to brain specific immune activation and determine whether general regulatory mechanisms apply.

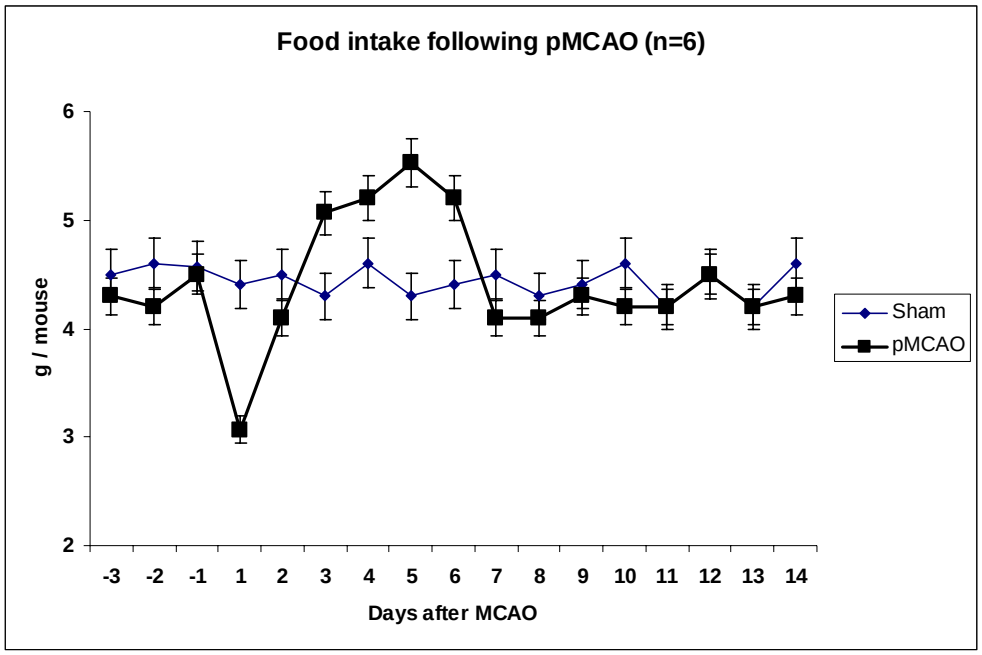

Figure 3. Food intake following permanant middle cerebral artery occlusion (pMCAO), note that ischemia results in hypophagia followed by transient hyperphagia.

In our models of infection we have observed that the cytokine interferon-gamma (IFNγ) play a major role in energy expenditure following toxoplasmosis (Arsenijevic et al, 2001), in food intake regulation following pMCAO (Arsenijevic et al, 2006) and we observed suppressed IFNγ in Spirometra infected mice which show increased body weight (Table 1A and 1B).

Infection / inflammation leads to the induction of cytokines which show temporal specificity (Arsenijevic et al, 1996), however in the chronic phase of toxoplasmosis (day 14 to day 90) the Gainers and Non-Gainers have a stable cytokine levels that remain constant over this period of time, similarly for Spirometra infection we do not observe major changes in TNFα, IL10, NGF and IFNγ from day 7 to 90 post infection (unpublisned data), this stable cytokine levels therefore allow us to try and draw conclusions about cytokine regulatory mechanisms during stable infection phases. As can be seen from the summary of the three infection models in Table 1A and Table 1B, leptin cannot explain food intake and energy expenditure levels in these models, since leptin is considered to be anorexic and hypermetabolic. Although ghrelin may explain in part the weight gain in Spirometra infected mice.

In all our models we observe an increase in nerve growth factor (NGF). The relation with weight loss is seen by a reduced NGF/IFNγ ratio (toxoplasmosis) whereas an increase in the ratio is associated induced weight gain (Spirometra) (Table 1A and 1B). Initially NGF has been show to be involved in neuron survival, but a few rare studies have shown it to regulate immune responses (Manning et al, 1985) and food intake (Berger et al, 1973). NGF has previously been shown in vitro to be antagonistic to the Th1 (IFNγ) cell cytokine production (Brodie, 1996, Arsenijevic, 2007C).

Table 1A. Summary of the changes in food intake, energy expenditure, body temperature and body fat and lean body mass, in the three models compared to control mice. Note that in the pMCAO group we are in the anorexic phase which is the day of performing the pMCAO. Concerning the Toxoplasma group this is the data of the Non-Gainers 30 days after infection. In the Spirometra group this is representative of mice infected for more than 14 day.

	Food intake	Energy expenditure	Body temperature	Body fat	Lean body mass
pMCAO	↓	↑	↔	↔	↓↓
Toxoplasma	↓↓	↑↑	↓	↓↓	↔
Spirometra	↔	↓↓	↓	↑↑	↑

Table 1B. Summary of changes in ghrelin, leptin, NGF, IFNγ, GSH and UCP2 mRNA levels following pMCAO, Toxoplasma and Spirometra. The groups represent the same time phase as described in Table 1A.

	Ghrelin	Leptin	NGF	IFNγ	GSH Brain Liver	UCP2 Brain
pMCAO	↑↑	↓	↑	↑↑	↓ ↓	↑↑↑
Toxoplasma	↑↑↑	↓↓↓	↑↑↑	↑↑↑↑	↑↑ ↓↓	↑↑↑
Spirometra	↑	↑↑↑↑	↑↑↑	↓↓	↑ ↑	↓

The different NGF/IFNγ ratios are therefore associated with both food intake changes and energy expenditure. Interestingly ip injection of NGF results in a lowering of body temperature in non-infected mice (unpublished data), other studies have reported that NGF alter sensitivity to body temperature regulation (Obata et al, 2005). We have also recently shown that the degree of lipopolysaccharide (LPS) induced anorexia is associated with the NFG/IFNγ ratio is related to tissue glutathione levels in particular, and that it may be the GSH rather than cytokine (or NGF) that determines the cytokine production and its effects (Arsenijevic et al, 2007C; Haddad and Harb, 2005). Our studies also suggested that GSH regulation may be tissue specific. Just like NGF, ip injection of GSH can lower body temperature (unpublished data). Compatible with tissue specific NGF regulation of GSH and inflammatory tissue damage can be seen in the *Toxoplasma gondii* model where the enhanced resistance to cerebral ischemia in infected mice can be overcome by using depletors of GSH (Arsenijevic et al, 2007A). Interestingly we have shown that following infection alterations in glutathione levels occur at the tissue level but also at the mitochondrial levels (Hernadfalvi et al, 2007). The role of NGF in altering mitochondria properties is suggested by modifying anti-oxidants in particular Mn superoxide dismutase (MnSOD) (Guegan et al, 1998), we have show that in our models (Toxoplasmosis, pMCAO) that there are increases in the mitochondrial uncoupling protein 2, which acts to reduce reactive oxygen species production (Arsenijevic et al, 2000B; de Bilbao et al, 2004). Using mice that overexpress NGF we have shown that NGF decreases basal neuronal UCP2 mRNA levels in the paraventricular hypothalamic region (see Figure 4), this suggests that UCP2 regulation does not only occur in the presence of inflammation and may have some other functions. We have also shown that NGF transgenic mice have increased brain GSH levels (Arsenijevic et al, 2007C). Thus NGF can modify redox state by modifying several anti-oxidant systems.

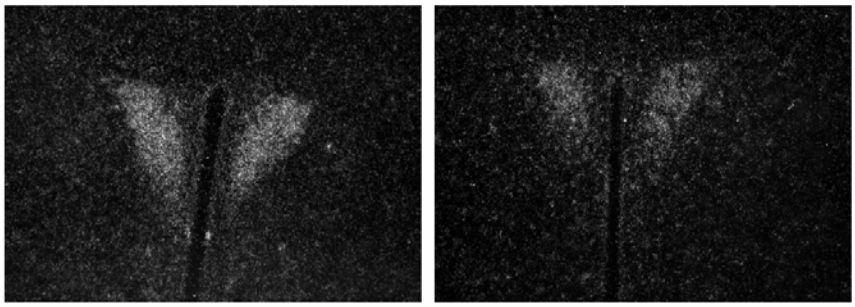

Figure 4. UCP2 mRNA expression is decreased in PVN of NGF transgenic mice (right photo) compared to WT control (left photo).

Furthermore we observe that other potential sources of ROS produced by mitochondria such as the monoamine oxidases (MAOA, MAOB and nonA/B amine oxidases) are altered in our infection models (Figure 5). Monoamine oxidase activity changes appear to be specific for infection in brain and liver of intracellular parasite *Toxoplasma gondii* (Tox) and *Neospora caninuim* (Neo), this is consistent with the lack of observed weight loss in the *Neospora* infected animals. *Neospora* is an obiligate intracellular parasite similar to *Toxoplasma gondii* in appearance but not in pathology in mice. The actual consequences of altered MAO remains to be determined.

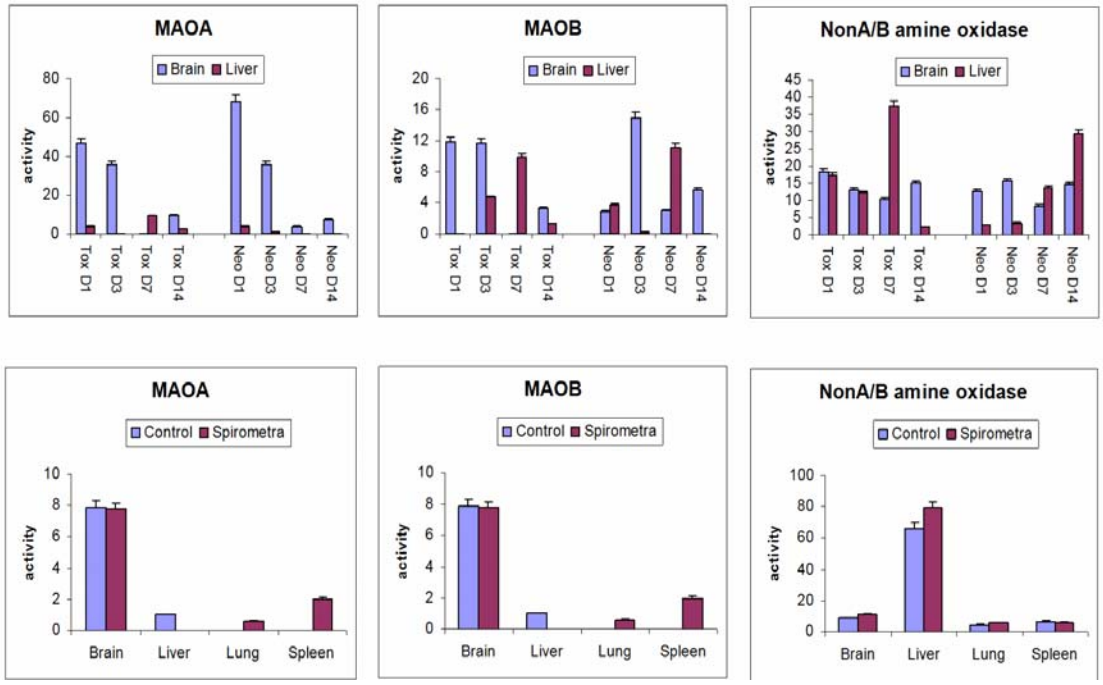

Figure 5. The level of monoamine oxidase in the brain and liver of Toxoplasma gondii infected mice on day 1 (Tox D1), day 3, day 7 and day 14 post-infection and Neospora caniniun infection (Neo), it should be noted that during chronic infection MAO activity was similar to day 14 values (n=4 mice). MAO activity in brain, liver, lung and spleen in Spirometra infected mice 30 days after infection (n=4 mice).

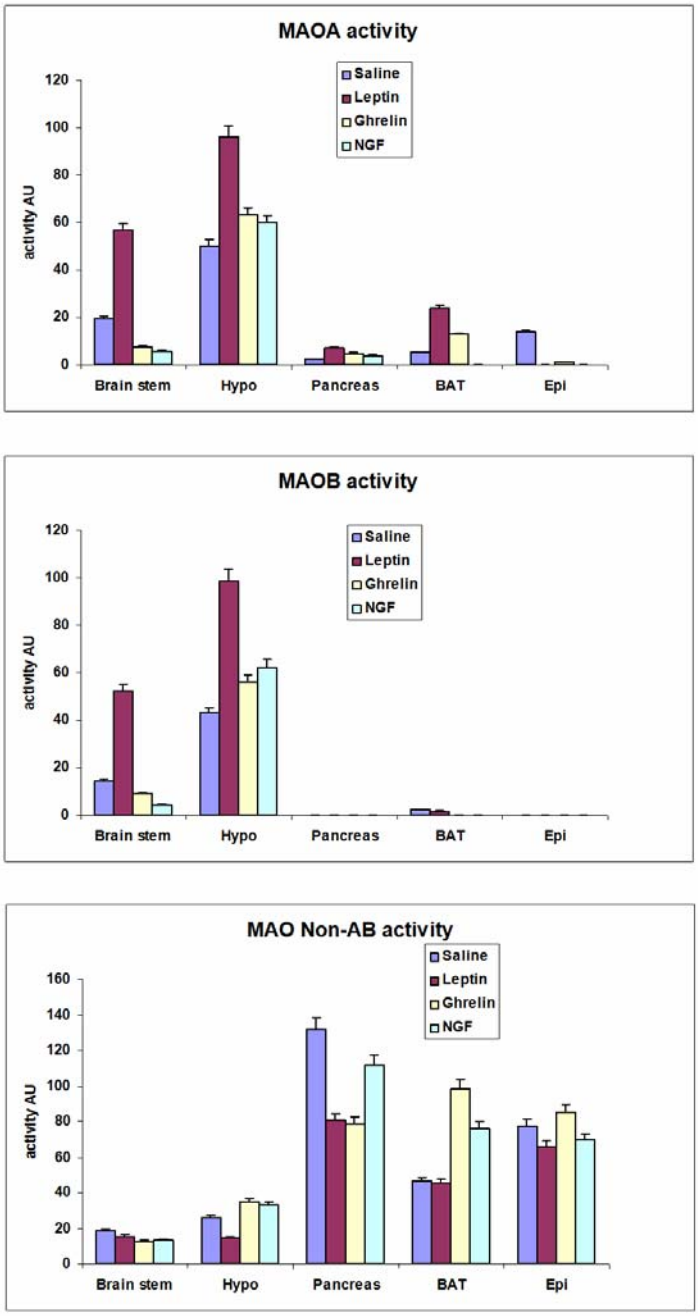

Figure 6. Monoamine oxidase isoform activity in brain stem, hypothalamus (hypo), pancreas, brown adipose tissue (BAT) and epididymal(Epi) fat 30 minutes after injection of each hormone.

So we have shown that NGF can modify anti-oxidant and oxidant systems, which may also have effects on immune responses (*Arseniejvic et al, 2007C*). In particular the antagonistic Th1 (IFNγ) and Th2 (IL10) immune system responses have been suggest to involve changes in phagocyte GSH (*Murata et al, 2002*) and/or MAOA (*Chaitidis et al, 2002*). Interesting in this context is that NGF can modify UCP2 and UCP2 KO mice have

been shown to have a Th1 cytokine profile (*Rousset et al, 2006*). We have observed that UCP2 KO mice have a 30% increase in their brain NGF compared to wildtype mice. Both acute NGF injection and NGF transgenic mice results in ablated MAOA activity in the brain (unpublished data). We also observe that leptin regulates MAOA and MAOB in an antagonist manner to ghrelin and NGF in the brain stem and the hypothalamus. NonA/B MAO also appears to be regulated in a tissue specific manner. Basically we observe that leptin may counter regulate the effects of ghrelin/NGF on MAO activity in a tissue specific manner as shown in Figure 6.

Apart from MAO data in the brain and liver we have begun to test MAO activity in other tissues, we observe that infection modifies it in adipose and skeletal muscle following infection (unpublished data). These changes in MAO activity and mRNA levels has also recently been shown to occur in other pathological circumstances (Rybaczyk et al, 2008). Since we observe changes in body composition in Toxoplasma (Arsenijevic et al, 1996) and Spirometra (Table 1A and 1B) model, that cannot be explained by changes in food intake alone and that tissue remodelling occurs, we observe that fat is lost in chronic toxoplasmosis whereas fat / lean body mass is gained in Spirometra infection, this may be consistent with the decease in MAOA in Toxoplasma and increased in MAOA in Spirometra fat tissue, as catecholamines are know to alter these tissues. Concerning other tissue metabolic effects we also measure mRNA involved in metabolic regulation, non-infected mice response to ip NGF injection was accessed, basically we observed that NGF could induce changes in PPAR genes, fatty acid synthase (FAS), glucose transported 4 (Glut4) and UCP2 in a tissue specific manner (Table 2), this would confirm previous studies that showed that NGF could alter glucose and lipid metabolism (Nogogaki and Iguchi, 1997; Bullo et al, 2007). As previously mentioned leptin could not explain the changes in body fat in both Toxoplasma and Spirometra (Table 1A and 1B), so we have to look for other mechanisms, of interest is the MAOs as they have been implicated in altered glucose and lipid metabolism (Morin et al, 2002; Visentin et al, 2003). Although it is too early to have a definitive answer, our studies implicate that changes in both immune regulation and body weight regulation are associated with tissue specific changes in MAO activity.

Table 2. The effect of in vivo ip injection of nerve growth factor (200ng) on liver, skeletal muscle and fat pad gene expression of peroxisome proliferator activated receptors isoforms (PPARs), uncoupling protein-2 (UCP2), fatty acid synthase (FAS) and Glut4

	Liver	Skeletal Muscle	Epididymal fat
PPARα	↑↑	↑	↑↑
PPARβ	↑↑↑	↑	↑↑
PPARγ1	↑	↑	↑
PPARγ2	↑		↑
UCP2	↑↑	↑	↑↑
FAS	↑↑	↑	↑↑
Glut4	↑↑	↑	↑↑

It has recently been documented that in cancer MAO activities are also changed in tissues (Rybaczyk et al, 2008), this finding complements our observations that MAO activity may be of importance in tissue regulatory processes during disease states.

Interestingly factors that normally do not play an evident role in body weight regulation may become important in infection or pathological circumstance, as is seen in food intake regulation following pMCAO. The hyperphagic phase seen after pMCAO lesion (see Figure 3) is absent in PPARβ knockout mice and UCP2 knockout mice. Although the lack of these factors does not have a marked effect on basal food intake, in PPARβ study we showed that NGF plays a role in food intake regulation after pMCAO (Arsenijevic et al, 2006). Finally since we observed that NGF was increased in our three models and that ghrelin was also increased, we measured the effect of injecting ghrelin subcutaneously over a 6 day period at 2.4 μmol / kg of body weight per day (Tschop M et al, 2000), brain levels of NGF and GSH, were markedly increase in the ghrelin treated group (Figure 7). Since the dose used did not induce hyperphagia over the 6 day period we can exclude a confounding effect of increased food intake on the increased NGF and GSH.

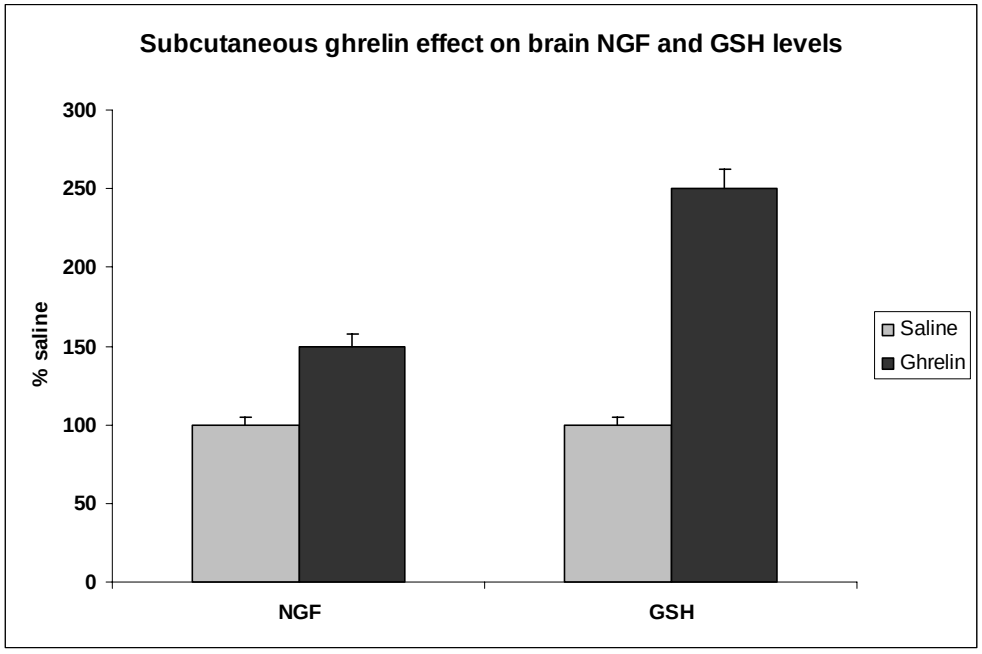

Figure 7. Subcutaneous ghrelin injection (2.4 μmol / kg of body weight) over a six day period resulted in increase brain NGF and GSH levels (n=6 mice).

Conclusions

Using our chronic murine models for infection/inflammation we show that body weight changes cannot be simply attributed to changes in catabolic hormones (leptin) or anabolic hormone (ghrelin). Weight changes cannot be directly attributed to proinflammatory cytokines (TNFα, IFNγ) nor anti-inflammatory cytokines (NGF, IL10). However NGF/IFNγ

ratios may be of use in relating to the degree of weight change. Our studies suggest that ghrelin levels may regulate tissue NGF levels and NGF in turn may modulate anti-oxidant status (GSH, UCP2) and oxidant status (MAOA activity) and subsequently these factors may be effectors on diverse systems such as the immune system, and metabolic regulatory systems - involving food intake, energy expenditure, body temperature regulation as well lipid and glucose metabolism. Further studies are required to elucidate the role of the ghrelin – NGF – MAOA/UCP2/GSH in the regulation of infection specific changes in both immune function and metabolic regulation, as it may lead to therapies involving solving the nefast effects and presently unresolved problems due to infection/cancer cachexia or obesity. The finding that MAO activity can be regulated by ghrelin/NGF and that leptin can have antagonistic action suggests that MAO activity may play a role in energy repartitioning and regulation of tissue metabolism. The role of interactions between MAO and other regulatory factors such as UCP2 remain to be shown but from our data it would be of interest to determine if UCP2 KO mice also show changes in these 5HT systems and have behavioural changes comparable to MAO-A KO mice. This could clarify whether some of the phenotypes of the UCP2 KO mice (feeding behaviour, altered insulin regulation, reactive oxygen species production, altered immune response) are due to MAO-A alterations involving changes in catecholeamines or indoleamines. These studies imply that mitochondrial activity involving MAO and UCP2 are important in body weight and immune regulation. Basically our studies suggests that leptin/ghrelin (and cytokines) can modify NGF which in turn may modify tissue monoamine oxidase activity in a tissue specific manner and this may explain changes in body weight changes during infection, suggesting an important role for catecholamines and indoleamines in central / peripheral tissue metabolism regulation.

References

Ahima R.S. and Flier J.S. (2000) Leptin. *Ann. Rev. Nutr.* 62, 413-437.

Andrews P.L.R., Rothwell N.J. and Stock M.J. (1985) Effect of subdiaphramatic vagotomy on energy balance and thermogenesis in the rat. *J. Physiol.* 362, 1-12.

Arsenijevic D., Girardier L., Seydoux J., Chang H.R., and Dulloo A.G. (1997) Altered energy balance and cytokine gene expression in a murine modle of chronic infection with Toxoplasma gondii. *Am. J. Physiol.* 272, E908-E917

Arsenijevic D., Girardier L., Seydoux J., Pechere J.C., Garcia I., Lucas R., Chang H.R., and Dulloo A.G. (1998) Metabolic cytokine responses to a secondary immunological challenge with LPS in mice with T.gondii infection. *Am. J. Physiol.* 274, E439-E445.

Arsenijevic D., Garcia I., Vesin C., Vesin D., Arsenijevic Y., Seydoux J., Girardier L., Ryfell B. and Richard D. (2000A) Differential roles of tumor necrosis factor-α and interferon-γ in mouse hypermetabolic and anorectic responses induced by LPS. *Eur Cytokine Netw*. 11 : 662-668

Arsenijevic D., Onuma H., Pecqueur C. *et al.* (2000B) Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat. Genet.* 26, 435-439.

Arsenijevic D, de Bilbao F, Giannakopoulos P, Girardier L, Samec S, and Richard D. (2001) A role for interferon-gamma in the hypermetabolic response to murine toxoplasmosis. *Eur Cyt Netw.* 12: 518-527

Arsenijevic D., Gallmann E., Moses W., Lutz T., Erlanson-Albertsson C. and Langhans W. (2005a) Enterostatin decreases postprandial pancreatic UCP2 mRNA levels and decreases plasma insulin and amylin. *Am. J. Physiol.* 289, 40-45.

Arsenijevic D., Clavel S., Langhans W. and Richard D. (2005b) Absence of hyperphagia following middle cerebral artery occlusion (MCAO) in uncoupling protein knockout mice. *Appetite* 44, 330

Arsenijevic D., de Bilbao F., Plamondon J., Paradis E., Vallet P., Richard D., Langhans W. and Giannakopoulos P. (2006) Increased infarct size and lack of hyperphagic response after focal cerebrtal ischemia in peroxisome proliferator-activated receptor β-deficient mice. *J. Cereb. Blood Flow Met.* 26, 433-445.

Arsenijevic D, de Bilbao F, Vallet P, Hemphill A, Gottstein B, Richard D., Giannakopoulos P, Langhans W. (2007A) Decreased infarct size after focal cerebral ischemia in mice chronically infected with Toxoplasma gondii. *Neuroscience* 150, 537-546.

Arsenijevic D, Clavel S, Sanchis S, Plamondon S, Huang Q, Ricquier D, Rouger L, Richard D (2007B) Induction of UCP2 expression in brain phagocytes and neurons following murine toxoplasmosis an essential role for IFN-γ and an association with negative energy balance. *Journal of Neuroimmunology* 186, 121-132

Arsenijevic D., Hernadfalvi N.,Ontienente B., Richard D. and Langhans W. (2007C) Role of nerve growth factor in the in vivo regulation of glutathione in response to LPS in mice. *European Cytokine Network* 18, 47-55.

Attele A.S., Shi Z.Q. and Yuan C.S. (2002) Leptin, gut and food intake. *Biochem. Pharmacol.* 63, 1579-1583.

Banchelli G., Ghelardini C., Raimondi L., Galeotti N. and Pirisino R. (2001) Selective inhibition of amine oxidases differentially potentiate the hypophagic effect of benzylamine in mice. *Eur. J. Pharmacol.* 413, 91-99

Banks W.A. (2006) The blood brain barrier as a regulatory interface in the gut-brain axes. *Physiol. Behav.* 89: 472-478

Barnes N.M. and Sharp T. (1999) A review of central 5-HT receptors and their function. *Neuropharmacol.* 38, 1083-1152.

Ben-Dor D.H., Laufer N., Apter A., Frisch A. and weizman A. (2002) Heritability, genetics and association findings in anorexia nervosa. *Isr. J. Psychiatry Relat. Sci*. 39, 262-70.

Berger BD, Wise CD and Stein L (1973) Nerve growth factor: enhanced recovery of feeding after hypothalamic damage. *Science* 180, 506-508.

Berthoud H.R. and Neuhuber W.L. (2000) Functional and chemical anatomy of the afferent vagal system. *Auton. Neurosci.: Basic and Clinical* 85, 1-17.

Berthoud H.R. (2002) Multiple neural systems controlling food intake and body weight. *Neurosciences and biobehavioral rev.* 26, 393-428.

Bianco A.C., Maia A.L., da Silva W.S. and Christoffolete M.A. (2005) Adaptive activation of thyroid hormone and energy expenditure. *Biosci Report*. 25, 191-208.

Bovetto S. and Richard D. (1995) Functional assessment of the 5-HT 1A, 1B, 2A/2C and 3 receptor subtypes on food intake and metabolic rate in rats. *Am. J. Physiol.* 268, R14-20

Bovetto S, Roullard C. and Richard D. (1996) Role of CRH in the effects of 5HT-receptor agonists on food intake and metabolic rate. *Am. J. Physiol.* 271, R1231-R1238

Brandacher G., Winkler C., Aigner F., Schwelberger H., Schroecksnadel K., Margreiter R., Fuchs D. and Weiss H.G. (2006) Bariatric surgery cannot prevent tryptophan depletion due to chronic immune activation in morbidly obese patients. *Obesity Surgery*. 16, 541-548.

Brodie C. (1996) Differential effects of Th1 and Th2 derived cytokines on NGF synthesis by mose astrocytes. *FASEB J.* 394: 117.120

Bullo M, Peeraully MR, Trayhurn P, Folch J and Salas-Salvado J. (2007) Circulating nerve growth factor levels in relation to obesity and the metabolic syndrome. *Eur. J. Endocrinol.* 157: 303-310

Chaitidis P, Billett EE, O'Donnell VB, Fajardo AB, Fitzgerald J, Kuban RJ, Ungethuem U and Kuhn H. (2004) Th2 response of human peripheral monocytes involves isoform specific induction of monoamine oxidase-A. *J. Immunol.* 173: 4821-4827.

Clemett D.A., Cockett M.I., Marsden C.A. and Fone K.C. (1999) Antisense oligonucleotide induced reduction in 5-hydroxtryptamine receptors in the rat hypothalamus without alteration in exploratory behaviour and neuroendocrine function. *J. Neurochem*. 71, 1271-1279.

Compan V., Charnay Y., Dusticier N., Dezuta A., Hen R. and Bockaert J. (2004a) Feeding disorders in 5HT4 receptor knockout mice. *J. Soc. Biol.* 198, 37-49.

Company V., Zhou M., Grailhe R., Gazzara R.A., Gingrich J., Dumuis A., Brunner D., Bockaert J. and Hen R. (2004b) Attenuated response to stress and novelty and hypersensitivity to seizures in 5HT4 receptor knockout mice. *J. Neurosci.* 24, 412-419.

Cooper A.L., Fitzgeorge R.B., Baskerville A., Little R.A. and Rothwell N.J. (1989) Bacterial infection (Legionella pneumophilla) stimulates fever, metabolic rate and brown adipose tissue activity in the guinea pig. *Life Sciences* 45 : 843-847

Coppola A, Liu ZW, Andrews ZB, Paradis E, Roy MC, Friedman JM, Ricquier D, Richard D, Horvath TL, Gao XB and Diano S. (2007) A central thermogenic like mechanism in feeding regulation: An interplay between arcuate nucleus T3 and UCP2. *Cell* 5: 21-33

Dang Y., dale W.E. and Brown O.R. (2000) Comparative effects of oxygen on indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase of the kynurenine pathway. *Free Rad. Biol. Med.* 28, 615-624.

de Bilbao F., Arsenijevic D., Vallet P. *et al.* (2004) Resistance to cerebral ischemic injury in UCP2 knockout mice: evidence for a role of UCP2 as a regulator of mitochondrial glutathione levels. *J. Neurochem.* 89, 1283-1292.

Dorn J.M., Schisterman E.F., Winkelstein W Jr. and Trevisan M. (1997) Body mass index and mortality in a general population sample of men and women. The Buffalo health study. *Am J. Epidem*. 146, 919-931.

Duggan M.B. and Milner R.D.G. (1986) Composition of weight gain by Kenyan children during recovery from measles. *Human Nutrition : Clin. Nutr.* 40C: 173-183

Evans R.M., Barish G.D. and Wang Y.X. (2004). PPARs and the complex journey to obesity. *Nat. Med.* 10, 355-361.

Evrard A., Malagie I., Laporte A.M., Boni C., Hanoun N., trillat A.C., Seif I., De Maeyer E., Gardier A., Hamon M. and Adrien J. (2002). Altered regulation of the 5-HT system in the brain of MAO-A knockout mice. *Eur. J. Neurochem.* 15, 841-851

Faure C., Mnie-Filali O., Scarna H., Debonnel G. and Haddjeri N. (2006) Effects of 5HT7 receptor antagonist SB-269970 on rat hormonal and temperature responses to the 5HT1A/7 receptor agonist 8-OH-DPAT. *Neurosci. Lett.* 404, 122-126.

Fisas A., Codony X., Romero G., Dordal A., Giraldo J., Merce R., Holenz D., Buschmann H. and Pauwels P.J. (2006) Chronic 5HT6 receptor modulation by E-6837 induces hypophagia and substained weight loss in diet induced obese rats. *Brit. J. Pharmacol.* 148, 973-983.

Garcia CA, Van Nostrand D, Atkins F, Acio E, Butler C, Esposito G, Kulkarni K and Madj M. (2006) Reduction of brown fat 2-deoxy-2-(F-18) fluoro-D-glucose uptake by controlling environmental temperature prior to positron emission tomography scan. *Mol. Imaging Biol*. 8: 24-29.

Glover V. and Sandler M. (1986) Clinical chemistry of monoamine oxidase. *Cell Biochem. Function.* 4, 89-97.

Gordon C.J. and Duncan W.C. (1994) Autonomic and behavioural thermoregulation in golden hamster during subchronic admistration of clorgyline. *Pharamacol. Biochem. Behav.* 48, 119-125.

Guegan C, Ceballos-Picot I, Nicole A, Kato H, Onteniente B and Sola B. (1998) Recruitment of several neuroprotective pathways after permanent focal ischemia in mice. *Exp Neurology* 154: 371-380.

Haddad JJ and Harb HL. (2005) L-γ-Glutamyl-L-cysteinyl-glycine (glutathione; GSH) and GSH-related enzymes in the regulation of pro- anti-inflammatory cytokines: a signalling transcriptional scenario for redox(y) immunologic sensor(s). *Molecular Immunology* 42: 987-1014.

Henderson G.S., Nix N.A., Montesano M.A., Gold D., Freeman Jr G.L., McCurley T.L. and Colley D.G. (1993) Two distinct pathological syndromes in male CBA/J inbred mice with chronic Schistosoma mansoni infections. *Am. J. Pathol*. 142: 703-714.

Hernadfalvi N, Langhans W, Ontienente B., Richard D. and Arsenijevic D. (2007) Role of glutathione in hyposensitivity of LPS-preteated mice to LPS anorexia. *European Cytokine Network* 18: 39-45.

Hrupka B.J. and Langhans W. (2001) A role for serotonin in lipopolysaccharide induced anorexia in rats. *Pharmacol, Biochem, Behav.* 68, 355-362.

Holschneider D.P., Chen K., Seif I. and Shih J.C. (2001) biochemical, behavioral, physiological and neurodevelopmental changes in mice deficient in monoamine oxidase A or B. *Brain Res. Bull.* 56, 453-462.

Jahng J.W., Houpt T.A., Joh T.H. and Son J.H. (1998) Differential expression of monoamine oxidase A, serotonin transporter, tyrosine hydroxylase and norepinephrine transporter mRNA by anorexia mutation and food deprivation. *Develop. Brain Res.* 107, 241-248

Kennedy B.P., Ziegler M.G., Alford M., Hansen L.A., Thal L.J. and Masliah E. (2003) Early and persistent alterations in prefrontal cortex MAO A and B in Alzheimer's disease. *J. Neural Transm.* 110, 789-801.

Kong E.K., Peng L., Chen Y., Yu A.C. and Hertz L. (2002) Upregulation of 5HT2B receptor density and receptor mediated glycogenolysis in mouse astrocytes by long termfluoxetine administration. *Neurochem. Res.* 27, 113-120.

Krauss S., Zhang C.Y. and Lowell B.B. (2005) The mitochondrial uncoupling-protein homologues. *Nature Rev. Mol. Cell Biol.* 6, 248-261.

Leibowitz S.F. and Alexander J.T. (1998) Hypothalamic serotonin in control of eating behaviour, meal size, and body weight. *Biol. Psychiatry.* 44, 851-864.

Lucas G., Compan V., Charnay Y., Neve R.L., Nestler E.J., Bockaert J., Barrot M. and Debonnel G. (2005) Frontocortical 5HT4 receptor exert positive feedback on serotonergic activity : viral transfections, subacute and chronic treatments with 5HT4 agonsits. *Biol. Psychiatry.* 57, 918-925.

Luque J.M., Kwan S.W., Abell C.W., da Prada M. and Richards J.G. (1995) Cellular expression of mRNAs encoding monoamine oxidase A and B in the rat central nervous system. *J. Comp.. Neurol.* 363, 665-680.

Mandrup-Poulsen T., Nerup J., Reimers J.L., Pociot F., Andersen H.U., Karlsen A., Bjerre U. and Bergholdt R. (1995) Cytokines and the endocrine system. 1. The immunoendocrine network. *Eur. J. Endocrinol.* 133: 660-671.

Manning PT, Russell JH, Simmons B and Johnson EM Jr (1985) Protection from guanethidine induced neuronal destruction by nerve growth factor: effect of NGF on immune function. *Brain Res.* 340, 61-69.

Mannisto P.T. and Kaakkola S. (1999) Catechol-O-methyltransferase (COMT): Biochemistry, molecular biology, pharmacology and clinical efficacy of the new selective COMT inhibitors. *Pharmacol. Rev.* 51, 593-628.

Moore R.N., Johnson B.A. and Berry L.J. (1977) Nutritional effects of salmonellosis *Am. J. Clin. Nutr*. 30: 1289-1293.

Morin N, Visetin V, Calise D, Marti L, Zorzano A, Testar X, Vallet P, Fischer Y and Carpene C (2002) Tyramine stimulates glucose uptake in insulin sensitive tisse in vitro and in vivo via its oxidation by amine oxidases. *J. Pharmacol. and Exp. Therap.* 303, 1238-1247.

Morrison S.F. (2004) Activation of 5HT1A receptors in raphe pallidus inhibits leptin evoked increases in brown adipose tissue thermogenesis. *Am. J. Physiol.* 286, R832-R837.

Mueller J.F. (1965) Further studies on parasitic obesity in mice, deer mice and hamsters *J. Parasitology* 51: 523-531.

Murata Y, Shimamura T and Hamuro J. (2002) The polarization of Th1/Th2 balance is dependent on the intracellular thiol redox status of macrophages due the distinctive cytokine production. *Int. Immunology* 14: 201-212.

Mutwiri G.K., Butler D.G., Rosendale S. and Woodward B. (1995) The role of restricted food intake in pathogenesis of cachexia in sevre combined immunodeficient beige mice infected with Mycobacterium paratuberculosis *Can. J. Vet. Res.* 59 : 40-45.

Need A.C., Ahmadi K.R., Spector T.D. and Goldstein D.B. (2006) Obesity is associated with genetic variants that alter dopamine availability. *Ann. Human Genetics.* 70, 293-303.

Nedergaard J, Bengtsson T, and Cannon B. (2007) Unexpected evidence for active brown adipose tissue in adult humans. *Am. J. Physiol.* 293: E444-E452.

Negre-Salvayre A, Hirtz C, Carrera G, Cazenaye R, Troly M, Salvayre R, Penicaud L, and Casteilla L. (1997) A role for uncoupling protein 2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J.* 11: 809-815.

Nonogaki K and Iguchi A. (1997) Stress, acute hyperglycemia, and hyperlipidemia: Role of the autonomic nervous system and cytokines. *Trends Endocrinol Metab*. 8: 192-197.

Nonogaki K., Memon R.A., Grunfeld C., Feingold K.R. and Tecott L.H. (2002) Altered gene expression involved in energy expenditure in 5HT2C receptor mutant mice. *BBRC.* 295, 249-254.

Nonogaki K., Ohashi-Nozue K. and Oka Y. (2006) A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice. *BBRC*. 341, 203-207.

Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuola T, Tokunaga A, Tominaga M and Noguchi K. (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J. Clin. Invest.* 115: 2394-2401.

Paradis E., Clavel S., Bouillaud F., Ricquier D and Richard D. (2003) Uncoupling protein 2: a novel player in neuroprotection. *Trend in Mol. Med.* 9, 522-525.

Pearson R.D., Cox G., Evans T., Smith D.L., Weidel D. and Castracane J. (1990) Wasting and macrophage production of tumor necrosis factor / cachectin and interleukin 1 in experimental visceral leishmaniasis *Am. J. Trop. Med. Hyg.* 43 : 640-649.

Phares K. (1996) An unusual host-parasite relationship: the growth hormone-like factor from plerocercoids of spirometrid tapeworms. *Int. J. Parasitol.* 26: 575-588.

Plata-Salaman C.R. (1999) Curt P. Richter Award. Brain mechanisms in cytokine induced anorexia. *Psychoneuroendrocrinology* 24 : 25-41.

Reiss E. (1958) Protein metabolism in infection. 1. Changes in certain visceral proteins studied with glycine-N15. *Metab. Clin. Exptl.* 8 : 151-159.

Ricquier D. and Bouillaud F. (2000) The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem. J.* 345, 161-179.

Richard D., Rivest S., Huang Q., Bouillaud F., Sanchis D., Champigny O. and Riquier D. (1998) Distribution of the uncoupling protein 2 mRNA in the mouse brain. *J. Comp. Neurology* 397: 549-560.

Rippe C., Berger K., Boiers C., Ricquier D. and Erlanson-Albertsson C. (2000) Effect of high fat diet, surrounding temperature and enterostatin on uncoupling protein gene expression. *Am. J. Physiol.* 279, E293-E300.

Rousset S, Emre Y, Join-Lambert O, Hurtaud C, Ricquuier D, Cassard-Doulcier AM. (2006) the uncoupling protein 2 modulates the cytokine balance in innate immunity. *Cytokine* 35: 135-142.

Rybaczyk LA, Bashaw MJ, Pathak DR and Huang K (2008) An indicator of cancer: Downregulation of monoamine oxidase-A in multiple organs and species. *BMC Genomics* 9: 134 Epub.

Salter M., Hazelwood R., Pogson C.I., Iyer R. and Madge D.J. (1995) The effect of a novel and selective inhibitor of tryptophan 2,3-dioxygenase on tryptophan and serotonin metabolism in the rat. *Biochem. Pharmacol.* 49, 1435-1442.

Samuels S.E. and Baracos V.E. (1992) Catch-up growth following Escherichia coli infection in weanling rats. *Metabolism* 41: 208-215.

Scarpace P.J. and Tumer N. (2001) Peripheral and hypothalamic leptin resistance with age–related obesity. *Physiol. Behav.* 74, 721-727.

Schelp F.P. (1998) Nutrition and infection in tropical countries – implications for public health intervention – personal perspectives. *Nutrition.* 14, 217-222.

Seidell JC, Verschuren WM, van Leer EM, Kromhout D. (1996) Overweight, underweight and mortality. A prospective study of 48,287 men and women. *Arch Intern Med.* 156, 958-963.

Shih J.C., Chen K. and Ridd M.J. (1999) Monoamine oxidase: From genes to behaviour. *Ann. Rev. Neurosci.* 22, 197-217.

Sugimoto Y., Noma T., Yoshikawa T. and Yamada J. (2000) Effeects of a nitric oxide synthase inhibitor in hypophagia induced by peripheral 5-HT receptor agonist alpha-methyl-5-hydroxytryptamine and 5-carbboxamidotryptamine in rats. *Biol. Pharm. Bull.* 23, 1514-1516.

Sun Y., Asnicar M., Saha P.K., Chan L. and Smith R.G. (2006) Ablation of ghrelin improves diabetic but not obese phenotype of ob/ob mice. *Cell Metabol.* 3, 379-386.

Takeda S., Elefteriou F. and Karsenty G. (2003) Common endocrine control of body weight, reproduction and bone mass. *Ann. Rev. Nutr.* 23, 403-411.

Tecott L.H., Sun L.M., Akana S.F., Strack A.M., Lowenstein D.H., Dallman M.F. and Julius D. (1995) Eating disorder and epilepsy in mice lacking 5HT2C serotonin receptors. *Nature*. 374, 542-546.

Timofeeva E., Baraboi E.D. and Richard D. (2005) Contribution of the vagus nerve and amina terminalis to brain activation induced by refeeding. *Eur. J. Neurosci.* 22, 1489-1501.

Tschop M., Smiley D.L. and Heiman M.L. (2000) Ghrelin induces adiposity in rodents. *Nature*. 407, 908-913.

Tsubone T., Masaki T., Katsuraagi I., Tanaka K., Kakuma T. and Yoshimatsu H. (2005) Ghrelin regulates adiposity in white adipose tissue and UCP1 mRNA expression in brown adipose tissue in mice. *Regulatory Peptides.* 130, 97-103.

Visentin V, Prevot D, Marti L and Carpene C. (2003) Inhibition of rat fat cell lipolysis by monoamine oxidase and semicarbazide sensitive amine oxidase substrates. *Eur. J. Pharmacol* 466, 235-243.

Von Meyenburg C., Langhans W. and Hrupka B.J. (2003) Evidence for a role of the 5HT2C receptor in central lipopolysaccharide, interleukin 1β and leptin induced anorexia. *Pharmacol, Biochem, Behav.* 74, 1025-1031.

Wellman P.J. (1992) Overview of adrenergic anorectic agents. *Am. J. Clin. Nutr.* 55, 193S-198S.

Williams D.L., Grill H.J., Cummings D.E. and Kaplan J.M. (2003) Vagotomy dissociates short- and long-term controls of circulating ghrelin. *Endocrinology.* 144: 5184-5187.

Woolley M.L., Marsden C.A. and Fone K.G. (2004) 5HT6 receptors. *Curr. Drug Targets CNS Neurol. Disord.* 3, 59-79.

Yang HJ (2006) Feminization and reduction of testicular weight in mouse sparaganosis. *Kor J Parasitol.* 44: 167-169.

Yang S.Q., Zhu H., Li Y., Lin H.Z., Gabrielson K., Trush M.A. and Diehl A.M. (2000) Mitochondrial adaptations to obesity-related oxidant stress. *Arch. Biochem. Biophys.* 378, 259-268.

Yap GS and Sher A (1999) Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (IFN)-γ and tumor necrosis factor (TNF)-α dependent host resistance to the intracellular pathogen, Toxoplasma gondii. *J. Exp. Med.* 189: 1083-1091.

Zhang C.Y., Baffy G., Perret P., Krauss S., Peroni O., Grujic D., Hagen T., Vidal-Puig A.J., Boss O., Kirn Y.B., Zheng X.X., Wheeler M.B., Shulman G.I., Chan C.B. and Lowell B.B. (2001) Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, b cell dysfunction, and type 2 diabetes. *Cell* 105, 745-755.

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Chapter 7

Nerve Growth Factor Signaling in Neural Cancer and Metastasis

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Abstract

Nerve growth factor (NGF), the founder member of the neurotrophin family, plays an essential role in the development and functioning of the vertebrate nervous system. It regulates survival and function of different neuron populations of the central and peripheral nervous system through two different membrane-associated receptors: the p75 common neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor superfamily, and the tyrosine kinase receptor TrkA. NGF is not only active in the nervous system, but also in other different cell types including tumor cells. Changes in the neurotrophin signaling system are significant for the pathogenesis of malignancies at the initiation stage as well as during subsequent tumor progression steps. NGF and its receptors can affect malignant cells and tissues in different ways, acting on cell proliferation, cell maintenance and survival, apoptosis, and metastasis regulation. Differences in mechanisms and outcomes of NGF action depend on the cell and tissue type in which this neurotrophin works, as well as the ratio $p75^{NTR}$/TrkA present in the cell. In this chapter, we summarize the current information on the NGF signaling network in various neural tumors and demonstrate its contribution to the disease course. Furthermore, we show that nuclear translocation of the intracellular domain of $p75^{NTR}$ ($p75^{ICD}$) induced by NGF in RN22 schwannoma cells, reduces the proliferative capacity of these cells. This provides a novel mechanism by which $p75^{NTR}$ can act as a tumor suppressor gene.

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Introduction

Nerve growth factor (NGF) was the first member of the neurotrophin family to be described in the early 1950s (Levi-Montalcini and Hamburger, 1951), being initially purified from sarcoma cells (Cohen et al., 1954). Several decades later, three additional growth factors structurally related to NGF: brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT3) (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Jones and Reichardt, 1990), and neurotrophin-4/5 (NT4/5) (Hallböök et al., 1991; Berkemeier et al., 1991) were identified. All neurotrophins were shown to be small (14 kDa) basic proteins containing three cysteine bonds, which play a crucial role in survival, differentiation, and maintenance of the vertebrate nervous system.

A major role of NGF is to protect against cell death in particular neuronal subsets of the peripheral nervous system (PNS) (Lewin and Barde, 1996). Nevertheless, this molecule has also been involved in several other functions, including cell death induction in particular neuron types from both the PNS and the central nervous system (CNS), axonal outgrowth and neuronal differentiation, induction of cell movements, regulation of synaptic plasticity, and control of cell cycle progression (Frade and Barde, 1998; López-Sánchez and Frade, 2002; Reichardt, 2006). Most of the opposed effects of NGF are due to cell-specific signaling cascades initiated from two different receptors, either by themselves or acting synergistically.

The Receptors of the NGF Family

NGF is able to interact with, and activate two different membrane proteins that transduce its signals: the tropomyosin-related kinase A (TrkA) receptor and the common neurotrophin receptor p75 ($p75^{NTR}$). TrkA, the first member of the Trk (tropomyosin-related kinase) family of receptor tyrosine kinases to be identified was initially discovered from a colon carcinoma-derived oncogene, in which the first seven exons of tropomyosin were fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase protein (Martín-Zanca et al., 1986). The normal cellular counterpart of this chimeric protein is a single pass transmembrane molecule that is highly expressed in the developing nervous system. In 1991, TrkA was identified as a signaling receptor for NGF (Kaplan et al., 1991; Klein et al., 1991a). Two other related receptors were subsequently identified, TrkB and TrkC, which were described as the neurotrophin receptors for BDNF/NT4/5 and NT3, respectively (Lamballe et al., 1991; Klein et al., 1991b; 1992). Trk receptors dimerize in response to ligand (Jing et al., 1992), leading to rapid phosphorylation of cytoplasmic tyrosine residues and activation of the tyrosine kinase domain (Klein et al., 1991a). The phosphorylated tyrosine residues provide attachment sites for adaptor proteins containing PTB or SH2 domains and mediating initiation of intracellular signaling cascades (Schlessinger and Ullrich, 1992). Previous work using TrkA mutants defective in association sites for intracellular effector molecules has indicated the existence of at least three major signal transduction cascades used by the neurotrophins to mediate different biological responses: the Ras/MAPK pathway, which promotes either neuronal differentiation including neurite outgrowth or cell proliferation; the PI3K/Akt pathway, which induces survival; and the PLC-γ1 pathway, resulting in activation

of Ca^{2+}- and protein kinase C-regulated pathways that promote synaptic plasticity (for a review of Trk signaling see Kaplan and Miller, 2000; Segal, 2003; Reichardt, 2006) (See Figure 1).

In a similar manner to other receptor tyrosine kinases, Trk receptors can provide signals to stimulate proliferation, or cell cycle withdrawal and differentiation, depending on the cellular context in which the transduction pathway is activated (Marshall, 1995; Grewal et al., 1999; Meakin et al., 1999). The molecular basis that determines whether exposure to neurotrophins results in proliferation or cell cycle arrest seems to depend on whether there is a transient or prolonged activation of the ERK pathway (Marshall, 1995; Patapoutian and Reichardt, 2001). In each case, the recruitment of different adaptor proteins to the phosphotyrosine Y_{490} in the human sequence initiates defined signal transduction cascades.

Signals triggering cell cycle progression upon Trk activation seem to depend on Shc recruitment to Y_{490}. This interaction is subsequently translated into the recruitment of the Grb-2 and Ras exchange factor Son of Sevenless (SOS), which in turn promotes transient activation of Ras (Borrello et al., 1994; York et al., 1998), thereby triggering the c-Raf/ERK pathway and proliferation (York et al., 1998). Alternatively, the prolonged activation of ERK can be initiated by recruitment of the adaptor protein fibroblast growth factor receptor substrate (FRS)-2 to Y_{490} (Meakin et al., 1999).

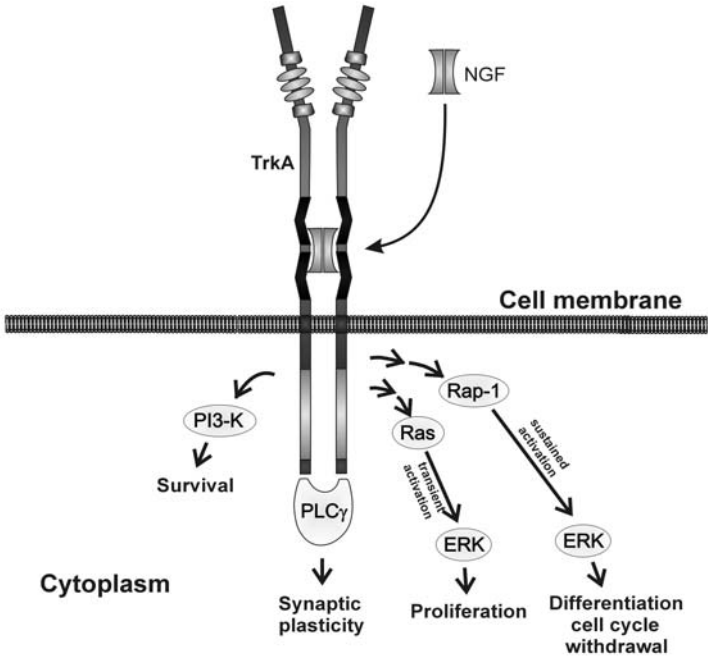

Figure 1. Structure of the TrkA receptor and its signal transduction pathways regulating survival, synaptic plasticity, proliferation, and neuronal differentiation. Trk receptors are transmembrane proteins containing three tandem leucine-rich motifs (ovals) flanked by two cysteine clusters (small rectangles) and two immunoglobulin segments (black segments) in the extracellular region. They have a single transmembrane motif and one tyrosine kinase domain (light grey segments) in the intracellular domain. Upon binding to NGF, TrkA dimerizes and activates its tyrosine kinase domain, thus becoming autophosphorylated on specific tyrosine residues. Depending on the adaptor proteins, several signaling pathways become activated (see text for details).

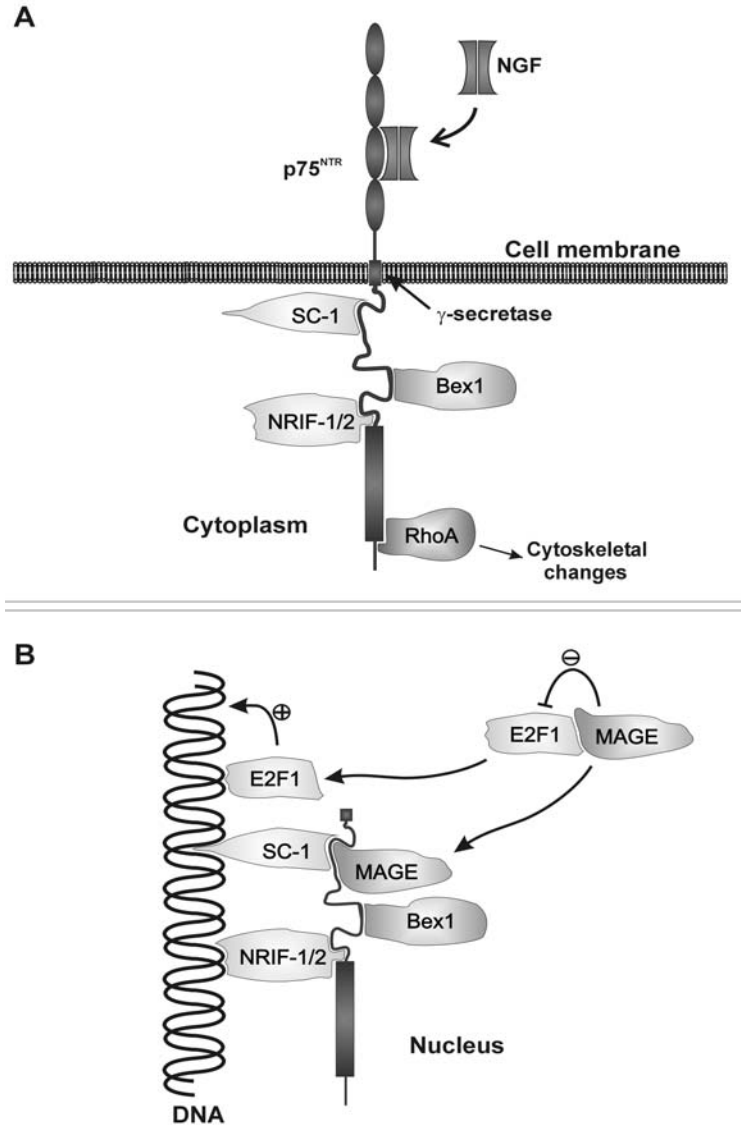

Figure 2. Structure of $p75^{NTR}$ and its most representative interactor proteins able to regulate the cell cycle and cytoskeletal changes. A. $p75^{NTR}$ is a transmembrane receptor characterized by a extracellular domain containing four cysteine repeats (ovals), a single transmembrane domain (small rectangle), and an intracellular domain containing a highly conserved juxtamembrane domain (grey line) and a class II death domain (large rectangle). Binding of NGF to $p75^{NTR}$ can induce γ-secretase-dependent release of its intracellular domain ($p75^{ICD}$) and stimulate different signaling pathways depending on the cellular context. Several proteins can interact with the intracellular tail of $p75^{NTR}$ that are involved in the regulation of cell cycle progression and apoptosis, as well as modulate cytoskeletal changes triggering axonal outgrowth and, likely, cellular movements (see text for details). B. $p75^{ICD}$ can translocate to the nuclear compartment and interact with DNA-binding proteins, thus regulating cell cycle progression. It interacts with transcription factors such as SC1 and NRIF1/2, thus providing a possible mechanism for the regulation of cell cycle progression by $p75^{NTR}$. The MAGE proteins are known to bind to the E2F1 transcription factor and inhibit the function of the latter during G1/S-phase progression. The presence of $p75^{ICD}$ can sequester the MAGE proteins and facilitate E2F1 activity.

FRS-2 is then phosphorylated, thereby creating docking sites for adaptor proteins such as Crk and Src-homology protein kinase phosphatase (SH-PTP)-2 (Meakin et al., 1999). Once bound to FRS-2, Crk binds and activates the guanine nucleotide exchange factor C3G (Nosaka et al., 1999), which in turn activates the small G protein Rap-1. Rap-1 activates B-raf and ERK signaling in a prolonged manner (York et al., 1998; 2000), thus promoting cell cycle withdrawal and differentiation. Prolonged activation of ERK seems also to be facilitated by other proteins like SH-PTP-2 (Wright et al., 1997).

A further degree of complexity to be added to the control of cell cycle via Trk receptors relates to the integrity of their tyrosine kinase homology region. Thus, splice variants of TrkC with amino acid insertions in the kinase domain do not mediate the mitogenic activity of NT3 in fibroblasts (Valenzuela et al., 1993; Guiton et al., 1995). The presence of these insertions is manifested by the lack of high affinity binding of Shc and phospholipase C-γ to the TrkC variants, and this interferes with the biological response to NT3 in fibroblasts (Guiton et al., 1995). Additionally, the signaling triggered by Trk receptors in terms of cell cycle regulation is also dependent on functional cross-talk between Trk and $p75^{NTR}$.

$p75^{NTR}$ is a member of the tumor necrosis factor family of receptors, which can bind with similar affinity all neurotrophins (Rodríguez-Tébar et al., 1990; Rodríguez-Tébar et al., 1992). This receptor participates in at least three different signaling platforms, in collaboration with different co-receptors (Barker, 2004). Thus, in collaboration with Sortilin, $p75^{NTR}$ is able to transduce proapoptotic signals initiated by proNGF or proBDNF (Schweigreiter, 2006). Furthermore, $p75^{NTR}$ regulates axonal outgrowth when interacting with NogoR and LINGO-1 (Yamashita et al., 2005); and $p75^{NTR}$ has been shown to enhance Trk responses to preferred ligands (e.g., NGF for TrkA, BDNF for TrkB) while attenuating responses to non-preferred ligands (e.g., NT3 for TrkA) (Barker, 2007).

Apart from all these functions, $p75^{NTR}$ can also be considered as a bona fide tumor suppressor gene (Khwaja and Djakiew, 2003; Dimaras and Gallie, 2008), able to interact with different proteins that regulate the cell cycle and apoptosis, many of which contain DNA interacting domains in their sequence or are able to interact with transcription factors (Figure 2A). The recent realization that $p75^{NTR}$ can release and translocate its intracellular domain, $p75^{ICD}$, to the nucleus in response to neurotrophin binding (Frade, 2005; Kenchappa et al., 2006; Podlesniy et al., 2006) suggest that most of the actions of $p75^{NTR}$ on cell cycle regulation can be mediated by the interaction of $p75^{ICD}$ with these DNA interacting proteins (see below and Figure 2B).

Among these $p75^{ICD}$ interacting proteins able to regulate the cell cycle are the neurotrophin receptor interacting factor (NRIF)1 and NRIF2. These proteins contain a zinc finger domain (Casademunt et al., 1999; Benzel et al., 2001). In the C5/BL6 mouse strain, null mutants of the *Nrif1* gene results in significantly smaller embryos which do not survive beyond E12, a phenotype that is consistent with cell cycle arrest (Benzel et al., 2001). Furthermore, when either NRIF1 or NRIF2 are transiently expressed in mammalian cells, the rate of proliferation is markedly decreased (Benzel et al., 2001), suggesting that these proteins can interfere with cell cycle progression.

Another zinc finger protein that interacts with the intracellular tail of $p75^{NTR}$ and has potential effects on the cell cycle is SC-1. This protein contains six zinc finger motifs and one positive regulatory (PR) domain, previously identified as a common domain in several transcription factors including the tumor suppressor, retinoblastoma-interacting zinc finger

(RIZ) protein. SC-1 interacts with $p75^{ICD}$ and in response to the specific activation of this receptor by NGF, it translocates to the nucleus in COS cells, an effect that is blocked by the expression of TrkA (Chittka and Chao, 1999). The presence of SC-1 in the nucleus correlates with the loss of BrdU incorporation, indicating that this protein is a clear candidate for mediating the anti-proliferative effects triggered by the activation of $p75^{NTR}$. SC-1 has been shown to act as a transcriptional repressor able to bind to the promoter and prevent the expression of the promitotic gene cyclin E (Chittka et al., 2004).

Another $p75^{ICD}$ interacting protein able to modulate the cell cycle is brain-expressed X-linked 1 (Bex1). This protein was identified as a small adaptor-like protein whose levels oscillate during the cell cycle (Vilar et al., 2006) and participates in neurotrophin-dependent cell cycle arrest and differentiation in PC12 cells, whereas its over-expression reduced neuronal differentiation of precursors isolated from the brain subventricular zone.

A further $p75^{NTR}$ interacting protein implicated in cell cycle regulation is the neurotrophin-receptor-interacting MAGE homolog, NRAGE (Salehi et al., 2000). This protein is a member of the melanoma antigen (MAGE) family of proteins, initially characterized as precursors of a number of cell-surface antigens expressed by tumor cells. NRAGE induces cell cycle arrest when over-expressed in human embryonic kidney 293 cells. Within the developing CNS, NRAGE co-localizes with $p75^{NTR}$ in the mantle zone, a region where neurons are born, suggesting that NRAGE might participate in the mechanisms that control the arrest of growth that takes place during neurogenesis. Interestingly, TrkA seems to compete with NRAGE for the same binding site in $p75^{NTR}$. Thus, over-expression of NRAGE blocks the physical association of $p75^{NTR}$ with TrkA (Salehi et al., 2000), again emphasizing the opposing interactions between these two receptors. Other members of the MAGE protein family have been shown to interact with $p75^{ICD}$ (Tcherpakov et al., 2002; Kuwako et al., 2004; López-Sánchez et al., 2007a). Interestingly, these MAGE proteins can interact with, and prevent the transcriptional activity of E2F1 (Kuwako et al., 2004; López-Sánchez et al., 2007a), which is crucial for the G1/S transition, thus giving a direct link between $p75^{ICD}$ and the cell cycle. Indeed, the presence of $p75^{ICD}$ has been shown to inhibit the chick MAGE protein CMAGE and the mammalian MAGE protein Necdin, thus favoring E2F1 activity and cell cycle re-activation in differentiated neuroblastoma cells (López-Sánchez et al., 2007a).

Relationship of NGF and its Receptors with Tumor Biology

NGF and the rest of neurotrophins are well known for their role in the development and functioning of the nervous system. Nevertheless, their relevance to the field of tumor biology is not sufficiently appreciated by most neuroscientist yet. This is surprising, given that NGF was originally isolated from a sarcoma (Cohen et al., 1954), that its neurotrophic receptor TrkA was discovered in a human colon carcinoma (Martin-Zanca et al., 1986), and that $p75^{NTR}$ was initially purified from a human melanoma cell line (Marano et al., 1987).

Table 1. Participation of NGF and its receptors in the physiology of tumors of non-neural origin

Tumor	Function	References
Prostate cancer	$p75^{NTR}$ is a tumor suppressor gene	(1),(2),(3),(4)
	$p75^{NTR}$ enhances apoptosis	(5), (6)
	NGF modulates growth and metastasis through $p75^{NTR}$/TrkA	(7),(8), (9),(10)
Gastric cancer	$p75^{NTR}$ suppresses proliferation, invasion and metastasis	(11), (12)
Breast cancer	NGF induces proliferation through TrkA and survival through TrkA/Ku70 and $p75^{NTR}$	(13),(14),(15),(16), (17),(18)
	NGF favors metastasis	(18)
Thyroid carcinoma	NGF inhibits proliferation and invasion	(19)
Pancreatic cancer	NGF modulates cell growth through the TrkA/$p75^{NTR}$ ratio	(20), (21), (22), (23)
	Through TrkA, NGF enhances invasion and resistance to chemotherapy in vitro	(24), (25)
Myosarcomas	NGF inhibits apoptosis and favors growth through TrkA. $p75^{NTR}$ induces apoptosis.	(26), (27)
Lung cancer	NGF inhibits proliferation and invasion	(28),(29),(30)
Bladder tumor	$p75^{NTR}$ induces apoptosis	(31),(32)
Melanoma	NGF induces cell migration	(33),(34),(35),(36), (37),(38),(39),(40)
	$p75^{NTR}$ induces survival	(41)
Promyelocytic leukemia	NGF induces cell growth through TrkA	(27), (42)
Hodgkin lymphoma	NGF induces cell growth through TrkA	(42)
Esophageal cancer	Loss of NGF/TrkA function occurs during tumour progression	(43)

(1) Pflug et al., 1992; (2) Weeraratna et al., 2000; (3) Krygier and Djakiew, 2001; (4) Sigala et al., 2002; (5) Khwaja et al., 2006; (6) Quann et al., 2007; (7) Krygier and Djakiew, 2002; (8) Khwaja and Djakiew, 2003; (9) Nalbandian and Djakiew, 2006; (10) Festuccia et al., 2007; (11) Jin et al., 2007b; (12) Jin et al., 2007a; (13) Descamps et al., 1998; (14) Descamps et al., 2001b; (15) Descamps et al., 2001a; (16) Davidson et al., 2004a; (17) Com et al., 2007; (18) Adriaenssens et al., 2008; (19) Páez-Pereda et al., 2000; (20) Zhu et al., 2001; (21) Zhu et al., 2002; (22) Ketterer et al., 2003; (23) Zhang et al., 2005; (24) Okada et al., 2004; (25) Liu et al., 2007; (26) Astolfi et al., 2001; (27) Rende et al., 2006; (28) Ricci et al., 2001; (29) Fiorentini et al., 2002; (30) Davidson et al., 2004b; (31) Tabassum et al., 2003; (32) Khwaja et al., 2004; (33) Marchetti et al., 1993; (34) Herrmann et al., 1993; (35) Menter et al., 1994-1995; (36) Nicolson et al., 1994; (37) Kanik et al., 1996; (38) Iwamoto et al., 1996; (39) Walch et al., 1999; (40) Shonukan et al., 2003; (41) Marchetti et al., 2004; (42) Renné et al., 2008; (43) Zhu et al., 2000.

Recent research demonstrates that $p75^{NTR}$ and TrkA participate in the physiology of tumor cells from several non-neural tissues (see Table 1), being $p75^{NTR}$ particularly highly

expressed in an extensive large number of tumors (Fanburg-Smith and Miettinen, 2001). Within the nervous system, NGF and its receptors have been linked to different types of neoplasias and cancer, including neuroblastomas, medulloblastomas, retinoblastomas, gliomas, and schwannomas. Moreover, this neurotrophin and its receptor $p75^{NTR}$ have been shown to regulate melanoma cell invasion and metastasis in the nervous system. In this chapter we will focus in what is currently known about the effects of NGF and its receptors on neural cancer and metastasis.

NGF and Neuroblastoma

Neuroblastoma is the most frequently occurring extracranial solid cancer in infants, with an incidence of 1.3 cases per 100,000 children aged 0-14 years. Neuroblastomas develop from embryonal neural crest-derived sympathetic precursors, cells known to be responsive to neurotrophins (Verdi and Anderson, 1994), which fail to maturate (Schor, 2002). For many years, it has been known that expression by neuroblastomas of TrkA, the truncated form of TrkB (lacking the kinase domain), and TrkC generally impart good prognosis, while full-length TrkB imparts poor prognosis to neuroblastoma patients (Nakagawara et al., 1993, 1994; Matsumoto et al., 1995; Yamashiro et al., 1996). Therefore, despite the known effect of NGF/TrkA in survival of normal sympathetic neurons (Misko et al., 1987), signaling triggered by this receptor in neuroblastoma cells seems to be deleterious for these cells. The basis for TrkA to be harmful in neuroblastoma cells seems to be related to several mechanisms including its capacity to prevent the expression of pro-angiogenic factors by neuroblastoma cells (Eggert et al., 2000a; 2002), the functional association between TrkA expression and mitotic arrest or differentiation of neuroblastoma cells (Lucarelli et al., 1997; Lachyankar et al., 2003; Woo et al., 2004), and the induction of apoptosis by this receptor via a p53-dependent mechanism (Lavoie et al., 2005; Stephan et al., 2008).

The mechanisms used by TrkA to induce neuronal differentiation is of major interest in the biology of neuroblastoma, as differentiation to more benign ganglioneuromas is an important clinical event in a subset of neuroblastomas. NGF activation of TrkA-expressing neuroblastoma cells promotes morphological differentiation and neurite outgrowth (Matsushima and Bogenmann, 1990; Lavenius et al., 1995; Poluha et al., 1995; Lucarelli et al., 1997). Although several redundant pathways are present downstream, activation of the RAS/MAPK signaling pathway seems to be of major importance for Trk-mediated differentiation of neuroblastoma cells, whereas inhibition of the PI3K survival pathway has no influence on differentiation signaling (Eggert et al., 2000b). The antiproliferative effect of NGF/TrkA in neuroblastoma cells has been shown to be mediated by decreasing MYCN, E2Fs and cyclin E kinase activity as well as increasing binding of $p27^{Kip1}$ to cyclin E kinase (Woo et al., 2004).

The rule that TrkA expression by neuroblastoma cells is a general marker for good prognosis has exceptions since a substantial number of advanced neuroblastomas that express TrkA are known to show poor clinical outcome (Schwab et al., 2003). One explanation for this paradox is that NGF can act either as mitogen or inducer of cell differentiation, depending on the tumor from which cells are derived (Bogenmann et al., 1998). Another

explanation for the poor prognosis of TrkA in certain neuroblastoma tumor cells can rely on a novel splice variant of TrkA, referred to as TrkAIII, that can be expressed by neuroblastoma cells. TrkAIII lacks part of the extracellular domain of TrkA, encoded by exons 6, 7, and 9, which results in its inability to bind NGF. This TrkA variant acts therefore as a ligand-independent, constitutively active receptor analogous to many oncogenes (Tacconelli et al., 2004), thus suggesting that it could participate in situations of poor prognosis. The expression of TrkAIII was found to be induced by hypoxia, a condition often encountered within tumors.

Another explication for the paradox that TrkA expression can result in poor prognosis of neuroblastoma patients comes from the fact that $p75^{NTR}$ and TrkA can be expressed at variable levels by these tumor cells (Dominici et al., 1997; Stephan et al., 2008). Indeed, the effect of NGF on the proliferation rate and survival of neuroblastoma cells in culture depends critically on the ratio of $p75^{NTR}$ to TrkA (Yan et al., 2002).

In the absence of TrkA expression, $p75^{NTR}$ can mediate either proliferative or antiproliferative signals depending of the neuroblatoma cell line (Evangelopoulos et al., 2004), thus suggesting that the signaling cascade used by $p75^{NTR}$ in neuroblastoma cells is variable and depends on the different interactors that can be expressed by these tumor cells.

Facilitation of cell cycle exit in differentiating neuroblastoma cells may prevent apoptosis induced by conflicts in the regulation of the cell cycle in these cells. In agreement with this notion, NGF binding to $p75^{NTR}$ is both necessary and sufficient for the abrogation of apoptosis in neuroblastoma cells treated with antimitotic agents (Cortazzo et al., 1996), thus suggesting that $p75^{NTR}$ facilitates cell cycle exit and prevents apoptosis in this particular paradigm. A study performed by Lachyankar et al. (2003) demonstrated that TrkA kinase initiates differentiation in LAN5 neuroblastoma cells, while $p75^{NTR}$ enhances differentiation of these cells by rescuing them from apoptosis via the PI3K pathway, thus indicating that in this particular paradigm, activation of $p75^{NTR}$ by NGF can act as an antiapoptotic agent. Differentiation and mitotic arrest seem to be dissociable from one another, and $p75^{NTR}$ signaling appears necessary for the latter but not for the former in rat pheochromocytoma cells (Ito et al., 2002).

In contrast, ligand-dependent and ligand-independent activity of $p75^{NTR}$ can also induce apoptosis in neuroblastoma cells lacking TrkA expression (Bunone et al., 1997; Kuner and Hertel, 1998; Bono et al., 1999; Eggert et al., 2000c), further stressing the concept that $p75^{NTR}$ signaling is highly pleiotropic in neuroblastoma cells.

NGF and Medulloblastoma

Medulloblastomas are primary neuroectodermal tumors originated from cerebellar granule cells and represent the most common malignant brain tumor of childhood. As in the case of other neural tumor cells, neurotrophins play an important role in the development of these tumors. It has been demonstrated that the expression of endogenous TrkC by medulloblastoma cells generally impart good prognosis, while patients whose tumors had little or no TrkC show an approximately fivefold greated risk of death than children with tumors expressing high levels of TrkC (Segal et al., 1994; Grotzer et al., 2000). Accordingly, analysis of biopsies showed that TrkC expression in individual tumor cells is strongly

correlated with apoptosis, and functional analyses of medulloblastoma cell lines found that the TrkC ligand NT3 induces apoptosis via activation of TrkC in vitro. In vivo, over-expression of TrkC inhibits the growth of intracerebral xenografts of TrkC-transfected medulloblastoma cells in nude mice (Kim et al., 1999). The responsible downstream pathways involved in this proapoptotic response to NT3 seem to depend on the immediate early genes c-jun and c-fos. Other neurotrophins and neurotrophin receptors have been shown to be expressed by medulloblastoma cells (Eberhart et al., 2001), but no prognosis predictive value has been established. Interestingly, NGF is known to induce apoptosis in TrkA-expressing medulloblastoma cells undergoing S-phase (Muragaki et al., 1997), suggesting that alterations of cell cycle progression can trigger apoptosis in these tumor cells. Bühren et al. (2000) reported that $p75^{NTR}$ expression is characteristic of a subvariant of medulloblastoma, the desmoplastic/nodular medulloblastoma, and that $p75^{NTR}$ is predominantly found in proliferative areas.

Neurotrophin-regulated heparanase (HPSE) is a unique extracellular matrix-degrading enzyme known to be associated with tumor progression in a wide variety of cancers, including medulloblastomas (Sinnappah-Kang et al., 2005). Although the latter authors found that the activation of TrkC or TrkC/$p75^{NTR}$ by NT-3 inhibited heparanase activity and cell-invasive properties of medulloblastoma cells in vitro, recent studies have demonstrated a correlation between HPSE and $p75^{NTR}$ expression in medulloblastoma (Marchetti et al., 2007), thus suggesting that activation of $p75^{NTR}$ by neurotrophins other than NT3 may favor the invasive pathways used by medulloblastoma cells.

NGF and Retinoblastoma

Retinoblastoma is a relatively uncommon tumor that arises in the retina and affects 1 in 15,000 to 20,000 live births. Although retinoblastoma may occur at any age, it most often occurs in younger children, usually before age 2 years, and accounts for about 3% of the cancers occurring in children younger than 15 years. As in other examples of neural tumors, all Trk receptors are known to be expressed by most retinoblastoma cells (Stephan et al., 2008). Expression of TrkB and its ligand, BDNF, was most pronounced, suggesting TrkB to be the major Trk receptor involved in retinoblastoma biology. In contrast, these authors found that $p75^{NTR}$ expression was substantially reduced in a subset of tumors and cell lines, when compared to its expression in normal retina. Indeed, the loss of $p75^{NTR}$ expression has been shown to accompany malignant progression to human and murine retinoblastoma (Dimaras et al., 2006), in agreement with the recent finding that $p75^{NTR}$ receptor behaves as a tumor suppressor in retinoblastoma development (Dimaras and Gallie, 2008).

As in other paradigms, the effect of $p75^{NTR}$ seems to counteract the known proliferative function of Trk receptors in retinoblastoma cells. Indeed, the inhibition of Trk signaling by K252a has been shown to result in marked growth inhibition of retinoblastoma cells in vitro (Stephan et al., 2008), and the addition of NGF to the Y-79 retinoblastoma cell line is known to result in a TrkA-dependent mitotic response, in agreement with the known expression of TrkA in these cells (Wagner et al., 2000).

NGF and Gliomas

Gliomas are the most common primary tumors of the CNS in adults, which arise from glial cells, with glioblastomas, astrocytomas and oligodendrogliomas representing the most frequent subtypes (Reifenberger et al., 2004). Although gliomas are mainly detected in the brain, they can also affect the spinal cord or any other part of the CNS, such as the optic nerves.

Astrocytomas and glioblastomas, but not oligodendrogliomas, are known to express Trk receptors (Wang et al., 1998; Singer et al., 1999), and Trk expression seems to be limited to the astrocytic component in mixed gliomas (oligoastrocytomas). The level of expression of Trk receptors is variable in astrocytomas. Thus, high expression of Trks has been found in low-grade astrocytoma (I and II), while no expression of Trk receptors can be detected in high-grade gliomas (giant cell glioblastoma and glioblastoma multiforme) (Wadhwa et al., 2003). This indicates that TrkA and TrkB may play a role in the early stage tumor pathophysiology. Some human glioblastoma cell lines (U251, U87, and U373) are responsive to NGF, with NGF exerting a mitogenic effect via TrkA receptors (Singer et al., 1999). However, it has been reported that Trk receptor expression is rapidly lost when cell lines are being established from primary astrocytoma, preventing the examination of the signaling pathways of endogenous Trk receptors in these astrocytoma cells (Wang et al., 1998).

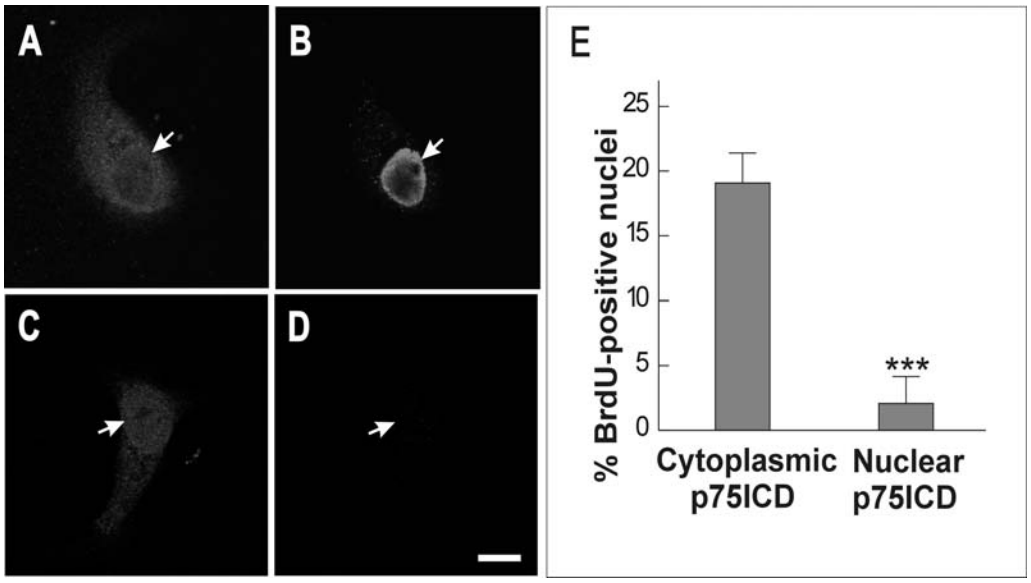

Figure 3. Nuclear translocation of $p75^{ICD}$ prevents cell cycle progression. RN22 Schwannoma cells were cultured for 24 h, pulse labeled (30 min) with BrdU, fixed and subjected to double immunocytochemistry using the [9992] antiserum (A,C), and an anti-BrdU mAb (B,D). Cells containing high levels of $p75^{ICD}$ showed reduced capacity to incorporate BrdU. E. The proportion of BrdU-positive nuclei after a 30 min pulse was estimated in RN22 Schwannoma cells containing low levels (Cytoplasmic p75ICD) or high levels (Nuclear p75ICD) of $p75^{ICD}$ in their nuclei. Note the reduction of BrdU-positive nuclei in cells with nuclear-enriched $p75^{ICD}$. ***$p<0.001$ (Student's t-test). Bar: 10 μm.

NGF and Schwannoma

Schwannomas are tumors originated from the Schwann cells, the glial cells giving rise to the myelin sheath that covers peripheral nerves. Schwannomas are relatively slow growing, usually being benign (less than 1% degenerate into malignant neoplasms of the nerve sheath). Interestingly, whereas benign Schwannomas express high levels of $p75^{NTR}$ and neurotrophins (Bonetti et al., 1997; Frade, 2005), malignant scwhannoma cells show either low levels or absence of $p75^{NTR}$ expression (Bonetti et al., 1997). This suggests that $p75^{NTR}$ acts as a tumor suppressor gene in these cells, as described in other paradigms (see above). Activation of $p75^{NTR}$ by NGF or other neurotrophins could result in reduced proliferative capacity of schwannoma cells, thus explaining their lack of malignity.

In neurons, signaling transduction through $p75^{NTR}$ can be mediated by ligand-dependent release of its intracellular domain, $p75^{ICD}$, mediated by the γ-secretase activity (Kenchappa et al., 2006; Podlesniy et al., 2006). $p75^{ICD}$-dependent signal transduction could also take place in schwannoma cells, where activation of $p75^{NTR}$ by NGF induces the nuclear translocation of the cytoplasmic tail of $p75^{NTR}$ (Frade, 2005). We therefore decided to analyze whether activation of $p75^{NTR}$ and nuclear translocation of $p75^{ICD}$ in RN22 schwannoma cells could modulate cell cycle progression in these tumor cells, thus providing a mechanism by which $p75^{NTR}$ can act as a tumor suppressor gene.

In the absence of fetal calf serum, RN22 schwannoma cells proliferate (López-Sánchez et al., 2007b; Figure 3A,B), and some of these cells contain nuclear $p75^{ICD}$ immunolabeling due to endogenous production of neurotrophins (Frade, 2005). Interestingly, those cells whose nuclei contain high levels of $p75^{ICD}$ incorporate less BrdU (Figure 3C-E) and, accordingly, nuclear translocation of $p75^{ICD}$ in response to NGF treatment over 24 h reduces the proliferation of RN22 Schwannoma cells, as measured by BrdU incorporation (Figure 4A).

Reduced proliferative capacity of RN22 schwannoma cells in response to $p75^{NTR}$ activation is dependent on the concentration of NGF, half-maximal inhibition being achieved with around 5-10 ng/ml NGF (Figure 4B), a value similar to the dissociation constant of the binding of NGF to $p75^{NTR}$ (Rodríguez-Tébar et al., 1990). The reduction in proliferation is dependent on $p75^{NTR}$ since it can be prevented by the presence of the anti- $p75^{NTR}$-specific antiserum [9651] in the culture medium (Figure 4A). Moreover, the activity of γ-secretase is also required for the reduction of proliferation of RN22 schwannoma cells since BrdU incorporation is not affected by NGF when the cells are also exposed to the γ-secretase-specific inhibitor DAPT (Figure 4A). In the absence of NGF, DAPT had no effect on the incorporation of BrdU (Figure 4A). A further proof that the nuclear accumulation of $p75^{ICD}$ in RN22 Schwannoma cells reduces their proliferative capacity is the decreased proportion of cells that contained the mitotic marker phospho-Histone H3 when cultured for 24 h in the presence of NGF (Figure 4C), being this effect suppressed by the presence of DAPT as well (Figure 4C). Cell death does not account for the reduced accumulation of cell cycle markers in the presence of NGF, since the proportion of TUNEL labeled nuclei was not significantly different under any of the experimental conditions (control: 11.69±0.92%; NGF: 11.44±0.98%; DAPT: 10.87±0.66%; DAPT/NGF: 10.07±0.97%; n=4). Hence, these data provide further physiological evidence that $p75^{NTR}$ can act as a tumor suppressor gene by reducing the proliferative capacity of RN22 schwannoma cells in response to NGF.

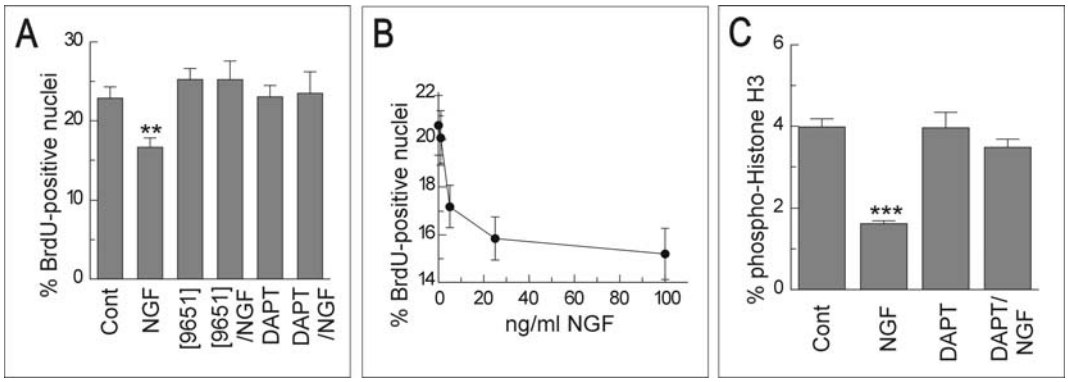

Figure 4. NGF reduces the proliferative capacity of RN22 Schwannoma cells. A. Addition of 100 ng/ml NGF to RN22 Schwannoma cells for 24 h reduced the capacity to incorporate BrdU (compare NGF with Control, Cont). Addition of the $p75^{NTR}$ blocking antiserum [9651] at a dilution of 1/500 prevented the reduction in the incorporation of BrdU induced by NGF (compare [9651] with [9651]/NGF), thereby demonstrating that this effect was specifically mediated by $p75^{NTR}$. The presence of the γ-secretase-specific inhibitor DAPT did also block the reduction of proliferative capacity of RN22 Schwannoma cells in the presence of NGF, thus indicating that that effect required the release of its intracellular domain of $p75^{NTR}$. B. RN22 Schwannoma cells cultured for 24 h in the presence of increasing concentrations of NGF were pulse labeled (30 min) with BrdU, fixed and subjected to immunocytochemistry with an anti-BrdU mAb. The proportion of BrdU-positive nuclei was then estimated. Note the dose-response effect of NGF on this effect. C. RN22 Schwannoma cells cultured for 24 h with vehicle (Cont), NGF, DAPT, or both (NGF/DAPT) were fixed and subjected to immunocytochemistry using an anti-phospho-Histone H3 antiserum to reveal nuclei undergoing Mitosis. The proportion of phospho-Histone H3-positive nuclei was then estimated in the different conditions. Addition of NGF to RN22 Schwannoma cells for 24 h induced a reduction in the proportion of cells with immunoreactivity for phospho-Histone H3, which was prevented by treatment with DAPT, thus indicating that the release of $p75^{ICD}$ is crucial for this activity of NGF. $^{**}p<0.05$, $^{***}p<0.001$ (Student's t-test).

Brain Metastases and NGF

Brain metastases occur in 20% to 40% of cancer cases, and represent a major cause of death in cancer patients. Lung, breast and skin (melanoma) are the commonest sources of brain metastases, and in up to 15% of patients the primary site remains unknown (Soffietti et al., 2002).

Melanomas are neural crest derived neoplasms and considered among the most aggressive tumors known in humans. Malignant melanoma metastasizes to the brain and spinal cord with one of the highest frequencies of any cancer capable of colonizing the CNS. This results in severe and debilitating neurological complications (Sawaya et al., 1996; Soffietti et al., 2002). Marchetti et al (2003; 2004) performed in vitro experiments using melanoma cell lines to address the role of neurotrophins in these cells and found that NGF/$p75^{NTR}$ signaling promotes the survival of melanoma cells. These authors also found TrkC expression in these cells. Interestingly, they observed the presence of NGF and NT-3 in tumor-adjacent tissues at the invasive front of melanoma brain metastases, which might indicate a paracrine activation of $p75^{NTR}$ and TrkC on melanoma cells by NGF and NT-3

produced by nearby glial cells. Besides promoting melanoma cell survival, NGF and other neurotrophins also induce the expression of heparanase by melanoma cells, an enzyme important for local invasion and metastasis by cleaving heparan sulfate chains of proteoglycans and thus modifying the extracellular matrix surrounding tumor cells (Marchetti et al., 1993; Menter et al., 1994-1995; Nicolson et al., 1994; Walch et al., 1999). This effect on heparanase expression has been shown to be mediated by $p75^{NTR}$ (Herrmann et al., 1993; Kanik et al., 1996; Papandreou et al., 1996; Iwamoto et al., 1996; 2001; Walch et al. 1999). Moreover, it has been established that neurotrophins are chemotactic for melanoma cells, triggered by $p75^{NTR}$-mediated dephosphorylation of the actin bundling protein fascin (Shonukan et al., 2003). All the above evidence demonstrates that NGF and its receptors fulfill important roles in melanoma-derived brain metastases.

Conclusion

The NGF receptors TrkA and $p75^{NTR}$ are known to be differentially expressed in several types of nerve tumor cells, and they respond to endogenous sources of NGF. The activation of these receptors results in various outcomes, including modulation of cell cycle progression, induction or repression of apoptosis, and facilitation of cell migration. Therefore, the activity of these receptors can modulate the invasive and/or proliferative capacities of different neural tumors, thus providing a therapeutic target for intervention. The signaling pathways initiated by TrkA and, particularly by $p75^{NTR}$, are currently under analysis. It is foreseen that in the near future we will have a clear picture of the mechanisms used by NGF and other neurotrophins to regulate neural tumor progression and invasion.

Material and Methods

Primary antibodies. BrdU was visualized with the monoclonal antibody (mAb) G3G4 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) diluted 1/4,000. The rabbit polyclonal antiserum [9992] against $p75^{ICD}$ and the rabbit polyclonal antiserum [9651] against $p75^{ECD}$ (kindly provided by Moses Chao, New York University School of Medicine) were used at a dilution of 1/800 for immunocytochemistry. The anti-phospho-Histone H3 rabbit polyclonal antiserum (Upstate Biotechnology, Cambridge, UK), a well-characterized mitotic marker (Gurley et al., 1978), was used at a dilution of 1/1,000.

Cell culture. RN22 Schwannoma cells were maintained at 37 ºC in Dulbecco-Modified Eagle Medium (DMEM, Gibco BRL) containing 10 % FCS (Gibco BRL). For most experiments, RN22 Schwannoma cells were initially cultured for 24 h in DMEM deprived of FCS on 12-mm coverslips (Menzel-Gläser) coated with 500 μg/ml poly (D-L) ornithine (Sigma) prior to the addition of any factor. The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl-L-alanyl]-S-phenylglycine-t-butyl ester (DAPT, Calbiochem) was stored at 1 mM in DMSO and used at a final concentration of 1 μM. Human recombinant nerve growth factor-β (Sigma) was stored at 10 ng/μl in PBS containing 0.5% bovine serum albumin (BSA, Sigma), and used at 100 ng/ml unless otherwise stated. Control cultures

contained PBS/0.5% BSA diluted 1/100 plus 0.1% DMSO. BrdU (Roche) was prepared in PBS (0.5 mg/ml) and applied at 0.5 µg/ml. Cell death was determined by TUNEL (In Situ Cell Death Detection Kit, Roche) following the manufacturer's instructions.

Immunocytochemistry. Cells were fixed for 15 min with 4 % paraformaldehyde (Merck) at room temperature, and permeabilized for 30 min with 0.05% Triton X-100 (Sigma) in PBS containing 10 % Normal Goat Serum (NGS; PBTx). The cells were then incubated for 1 h with PBTx containing 1 % NGS and the appropriate primary antibody. Following five washes in PBTx, the cells were incubated for an additional 1 h in PBTx containing 1 % NGS and a 1/800 dilution of Cy2-conjugated Affinipure Goat Anti-Rabbit IgG (H+L; Jackson Immunoresearch). Immunostaining of cultures previously treated with BrdU for 30 min was performed as described by Murciano et al. (2002).

Cell counting and statistical analysis. Cultures were examined under a Nikon E80i microscope using an oil immersion 60× objective with phase contrast and epifluorescence illumination. An average of 500 cells were analyzed per coverslip. Quantitative data are the mean ± s.e.m. of at least three independent experiments. Statistical differences were analyzed using the Student's t-test.

References

Adriaenssens, E., Vanhecke, E., Saule, P., Mougel, A., Page, A., Romon, R., Nurcombe, V., Le Bourhis, X. and Hondermarck, H. (2008). Nerve growth factor is a potential therapeutic target in breast cancer. *Cancer Res.*, 68, 346-351.

Astolfi, A., Nanni, P., Landuzzi, L., Ricci, C., Nicoletti, G., Rossi, I., Lollini, P.L. and De Giovanni, C. (2001). An anti-apoptotic role for NGF receptors in human rhabdomyosarcoma. *Eur. J. Cancer*, 37, 1719-1725.

Barde, Y.A., Edgar. D. and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.*, 1, 549-553.

Barker, P.A. (2004). $p75^{NTR}$ is positively promiscuous: novel partners and new insights. *Neuron*, 42, 529-533.

Barker, P.A. (2007). High affinity not in the vicinity? *Neuron*, 53, 1-4.

Benzel, I., Barde, Y.A. and Casademunt, E. (2001). Strain-specific complementation between NRIF1 and NRIF2, two zinc finger proteins sharing structural and biochemical properties. *Gene*, 281, 19-30.

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V. and Rosenthal, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron*, 7, 857-866.

Bogenmann, E., Peterson, S., Maekawa, K. and Matsushima H. (1998). Regulation of NGF responsiveness in human neuroblastoma. *Oncogene*, 17, 2367-2376.

Bonetti, B., Panzeri, L., Carner, M., Zamboni, G., Rizzuto, N. and Moretto, G. (1997). Human neoplastic Schwann cells: changes in the expression of neurotrophins and their low-affinity receptor p75. Neuropathol. *Appl. Neurobiol.*, 23, 380-386.

Bono, F., Lamarche, I., Bornia, J., Savi, P., Della Valle, G. and Herbert, J.M. (1999). Nerve growth factor (NGF) exerts its pro-apoptotic effect via the $p75^{NTR}$ receptor in a cell cycle-dependent manner. *FEBS Lett.*, 457, 93-97.

Borrello, M.G., Pelicci, G., Arighi, E., De Filippis, L., Greco, A., Bongarzone, I., Rizzetti, M., Pelicci, P.G., Pierotti, M.A. (1994). The oncogenic versions of the Ret and Trk tyrosine kinases bind Shc and Grb2 adaptor proteins. *Oncogene*, 9, 1661-1668.

Bühren, J., Christoph, A.H., Buslei, R., Albrecht, S., Wiestler, O.D. and Pietsch, T. (2000). Expression of the neurotrophin receptor $p75^{NTR}$ in medulloblastomas is correlated with distinct histological and clinical features: evidence for a medulloblastoma subtype derived from the external granule cell layer. J. Neuropathol. *Exp .Neurol.*, 59, 229-240.

Bunone, G., Mariotti, A., Compagni, A., Morandi, E. and Della Valle, G. (1997). Induction of apoptosis by p75 neurotrophin receptor in human neuroblastoma cells. Oncogene, 14, 1463-1470.

Casademunt, E., Carter, B.D., Benzel, I., Frade, J.M., Dechant, G. and Barde, Y.A. (1999). The zinc finger protein NRIF interacts with the neurotrophin receptor $p75^{NTR}$ and participates in programmed cell death. *EMBO J.*, 18, 6050-6061.

Chittka, A., Arevalo, J.C., Rodríguez-Guzmán, M., Pérez, P., Chao, M.V. and Sendtner, M. (2004). The $p75^{NTR}$-interacting protein SC1 inhibits cell cycle progression by transcriptional repression of cyclin E. *J. Cell Biol*., 164, 985-996.

Chittka, A. and Chao, M.V. (1999). Identification of a zinc finger protein whose subcellular distribution is regulated by serum and nerve growth factor. *Proc. Natl. Acad. Sci. USA*, 96, 10705-10710.

Cohen, S., Levi-Montalcini, R. and Hamburger, V. (1954). A nerve growth-stimulating factor isolated from sarcomas 37 and 180. *Proc. Natl. Acad. Sci. USA*, 40, 1014-1018.

Com, E., Lagadec, C., Page, A., El-Yazidi-Belkoura, I., Slomianny, C., Spencer, A., Hammache, D., Rudkin, B.B. and Hondermarck, H. (2007). Nerve growth factor receptor TrkA signaling in breast cancer cells involves Ku70 to prevent apoptosis. *Mol. Cell. Proteomics*., 6, 1842-1854.

Cortazzo, M.H., Kassis, E.S., Sproul, K.A. and Schor, N.F. (1996). Nerve growth factor (NGF)-mediated protection of neural crest cells from antimitotic agent-induced apoptosis: the role of the low-affinity NGF receptor. *J. Neurosci*., 16, 3895-3899.

Davidson, B., Reich, R., Lazarovici, P., Flørenes, V.A., Nielsen, S. and Nesland, J.M. (2004a). Altered expression and activation of the nerve growth factor receptors TrkA and p75 provide the first evidence of tumor progression to effusion in breast carcinoma. *Breast Cancer Res. Treat*., 83, 119-128.

Davidson, B., Reich, R., Lazarovici, P., Flørenes, V.A., Risberg, B., Nielsen, S., Sert, B. and Bedrossian, C. (2004b). Expression of the nerve growth factor receptors TrkA and p75 in malignant mesothelioma. *Lung Cancer*, 44, 159-165.

Descamps, S., Lebourhis, X., Delehedde, M., Boilly, B. and Hondermarck, H. (1998). Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells. *J. Biol. Chem.*, 273, 16659-16662.

Descamps, S., Pawlowski, V., Révillion, F., Hornez, L., Hebbar, M., Boilly, B., Hondermarck, H. and Peyrat, J.P. (2001a). Expression of nerve growth factor receptors and their prognostic value in human breast cancer. *Cancer Res*., 61, 4337-4340.

Descamps, S., Toillon, R.A., Adriaenssens, E., Pawlowski, V., Cool, S.M., Nurcombe, V., Le Bourhis, X., Boilly, B., Peyrat, J.P. and Hondermarck, H. (2001b). Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. *J. Biol. Chem*., 276, 17864-17870.

Dimaras, H., Coburn, B., Pajovic, S. and Gallie, B.L. (2006). Loss of p75 neurotrophin receptor expression accompanies malignant progression to human and murine retinoblastoma. *Mol Carcinog*., 45, 333-343.

Dimaras, H. and Gallie, B.L. (2008). The $p75^{NTR}$ neurotrophin receptor is a tumor suppressor in human and murine retinoblastoma development. *Int. J. Cancer*., 122, 2023-2029.

Dominici, C., Nicotra, M.R., Alemà, S., Bosman, C., Castello, M.A., Donfrancesco, A., Gallo, P., McDowell, H. and Natali, P.G. (1997). Immunohistochemical detection of p140trkA and p75LNGFR neurotrophin receptors in neuroblastoma. *J. Neurooncol*., 31, 57-64.

Eberhart, C.G., Kaufman, W.E., Tihan, T. and Burger, P.C. (2001). Apoptosis, neuronal maturation, and neurotrophin expression within medulloblastoma nodules. *J. Neuropathol. Exp. Neurol*., 60, 462-469.

Eggert, A., Grotzer, M.A., Ikegaki, N., Liu, X.G., Evans, A.E. and Brodeur, G.M. (2002). Expression of the neurotrophin receptor TrkA down-regulates expression and function of angiogenic stimulators in SH-SY5Y neuroblastoma cells. *Cancer Res.*, 62, 1802-1808.

Eggert, A., Grotzer, M.A., Ikegaki, N., Liu, X.G., Evans, A.E. and Brodeur, G.M. (2000a). Expression of neurotrophin receptor TrkA inhibits angiogenesis in neuroblastoma. *Med. Pediatr.Oncol.*, 35, 569-572.

Eggert, A., Ikegaki, N., Liu, X., Chou, T.T., Lee, V.M., Trojanowski, J.Q. and Brodeur, G.M. (2000b). Molecular dissection of TrkA signal transduction pathways mediating differentiation in human neuroblastoma cells. *Oncogene*, 19, 2043-2051.

Eggert, A., Sieverts, H., Ikegaki, N. and Brodeur, G.M. (2000c). p75 mediated apoptosis in neuroblastoma cells is inhibited by expression of TrkA. Med. Pediatr. *Oncol.*, 35, 573-576.

Evangelopoulos, M.E., Weis, J. and Kruttgen, A. (2004). Neurotrophin effects on neuroblastoma cells: correlation with trk and $p75^{NTR}$ expression and influence of Trk receptor bodies. *J. Neurooncol*., 66, 101-110.

Fanburg-Smith, J.C. and Miettinen, M. (2001). Low-affinity nerve growth factor receptor (p75) in dermatofibrosarcoma protuberans and other nonneural tumors: a study of 1,150 tumors and fetal and adult normal tissues. *Hum. Patho*l., 32, 976-983.

Festuccia, C., Muzi, P., Gravina, G.L., Millimaggi, D., Speca, S., Dolo, V., Ricevuto, E., Vicentini, C. and Bologna, M. (2007). Tyrosine kinase inhibitor CEP-701 blocks the NTRK1/NGF receptor and limits the invasive capability of prostate cancer cells in vitro. Int. *J. Oncol*. 30, 193-200.

Fiorentini, C., Facchetti, M., Finardi, A., Sigala, S., Páez-Pereda, M., Sher, E., Spano, P. and Missale, C. (2002). Nerve growth factor and retinoic acid interactions in the control of small cell lung cancer proliferation. *Eur. J. Endocrinol*., 147, 371-379.

Frade, J.M. (2005). Nuclear translocation of the p75 neurotrophin receptor cytoplasmic domain in response to neurotrophin binding. *J. Neurosci*., 25, 1407-1411.

Frade, J.M. and Barde, Y.A. (1998). Nerve growth factor: two receptors, multiple functions. *Bioessays*, 20, 137-145.

Grewal, S.S., York, R.D. and Stork P.J.S. (1999). Extracellular-signal-regulated kinase signalling in neurons. *Curr. Op. Neurobiol*., 9, 544-553.

Grotzer, M.A., Janss, A.J., Fung, K., Biegel, J.A., Sutton, L.N., Rorke, L.B., Zhao, H., Cnaan, A., Phillips, P.C., Lee, V.M. and Trojanowski, J.Q. (2000). TrkC expression predicts good clinical outcome in primitive neuroectodermal brain tumors. *J. Clin. Oncol.*, 18, 1027-1035.

Guiton, M., Gunn-Moore, F.J., Glass, D.J., Geis, D.R., Yancopoulos, G.D. and Tavare J.M. (1995). Naturally occurring tyrosine kinase inserts block high affinity binding of phospholipase C gamma and Shc to TrkC and neurotrophin-3 signaling. *J. Biol. Chem*., 270, 20384-20390.

Gurley, L.R., D'Anna, J.A., Barham, S.S., Deaven, L.L. and Tobey, R.A. (1978). Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur. J. Biochem*., 84, 1-15.

Hallböök, F., Ibáñez, C.F. and Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron*, 6, 845-858.

Herrmann, J.L., Menter, D.G., Hamada, J., Marchetti, D., Nakajima, M. and Nicolson, G.L. (1993). Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: melanoma p75 functions independently of trkA. *Mol. Biol. Cell*., 4, 1205-1216.

Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature*, 344, 339-341.

Ito, H., Nomoto, H. and Furukawa, S. (2002). Role of low-affinity p75 receptor in nerve growth factor-inducible growth arrest of PC12 cells. *J. Neurosci. Res*., 69, 653-661.

Iwamoto, S., Odland, P.B., Piepkorn, M. and Bothwell, M. (1996). Evidence that the p75 neurotrophin receptor mediates perineural spread of desmoplastic melanoma. *J. Am. Acad. Dermatol*., 35, 725-731.

Jin, H., Pan, Y., He, L., Zhai, H., Li, X., Zhao, L., Sun, L., Liu, J., Hong, L., Song, J., Xie, H., Gao, J., Han, S., Li, Y. and Fan, D. (2007a). p75 neurotrophin receptor inhibits invasion and metastasis of gastric cancer. *Mol. Cancer Res*., 5, 423-433.

Jin, H., Pan, Y., Zhao, L., Zhai, H., Li, X., Sun, L., He, L., Chen, Y., Hong, L., Du, Y. and Fan, D. (2007b). p75 neurotrophin receptor suppresses the proliferation of human gastric cancer cells. *Neoplasia*, 9, 471-478.

Jing, S., Tapley, P. and Barbacid, M. (1992). Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron*, 9, 1067-1079.

Johnston, AL., Lun, X., Rahn, J.J., Liacini, A., Wang, L., Hamilton, M.G., Parney, I.F., Hempstead, B.L., Robbins, S.M., Forsyth, P.A. and Senger, D.L. (2007). The p75 neurotrophin receptor is a central regulator of glioma invasion. *PLoS Biol*., 5, e212.

Jones, K.R. and Reichardt, L.F. (1990). Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA*, 87, 8060-8064.

Kanik, A.B., Yaar, M. and Bhawan, J. (1996). p75 nerve growth factor receptor staining helps identify desmoplastic and neurotropic melanoma. *J. Cutan. Pathol.*, 23, 205-210.

Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V. and Parada, L.F. (1991). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science*, 252, 554-558.

Kaplan, D.R. and Miller, F.D. (2000). Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol*., 10, 381-391.

Kenchappa, R.S., Zampieri, N., Chao, M.V., Barker, P.A., Teng, H.K., Hempstead, B.L. and Carter, B.D. (2006). Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. *Neuron*, 50, 219-232.

Ketterer, K., Rao, S., Friess, H., Weiss, J., Büchler, M.W. and Korc, M. (2003). Reverse transcription-PCR analysis of laser-captured cells points to potential paracrine and autocrine actions of neurotrophins in pancreatic cancer. *Clin. Cancer Res.*, 9, 5127-5136.

Khwaja F, Allen J, Lynch J, Andrews P, Djakiew D. (2004) Ibuprofen inhibits survival of bladder cancer cells by induced expression of the $p75^{NTR}$ tumor suppressor protein. *Cancer Res.*, 64, 6207-6213.

Khwaja, F. and Djakiew, D. (2003). Inhibition of cell-cycle effectors of proliferation in bladder tumor epithelial cells by the $p75^{NTR}$ tumor suppressor. *Mol. Carcinog.*, 36, 153-160.

Khwaja, F., Tabassum, A., Allen, J. and Djakiew, D. (2006). The $p75^{NTR}$ tumor suppressor induces cell cycle arrest facilitating caspase mediated apoptosis in prostate tumor cells. *Biochem. Biophys. Res. Commun*., 341, 1184-1192.

Kim, J.Y., Sutton, M.E., Lu, D.J., Cho, T.A., Goumnerova, L.C., Goritchenko, L., Kaufman, J.R., Lam, K.K., Billet, A.L., Tarbell, N.J., Wu, J., Allen, J.C., Stiles, C.D., Segal, R.A. and Pomeroy, S.L. (1999). Activation of neurotrophin-3 receptor TrkC induces apoptosis in medulloblastomas. *Cancer Res*., 59, 711-719.

Klein, R., Jing, S.Q., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991a). The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell*, 65, 189-197.

Klein, R., Nanduri, V., Jing, S.A., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K.R., Reichardt, L.F. and Barbacid, M. (1991b). The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell*, 66, 395-403.

Klein, R., Lamballe, F., Bryant, S. and Barbacid, M. (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron*, 8, 947-956.

Krygier, S. and Djakiew, D. (2001). Molecular characterization of the loss of $p75^{NTR}$ expression in human prostate tumor cells. *Mol. Carcinog*., 31, 46-55.

Krygier, S. and Djakiew, D. (2002). Neurotrophin receptor $p75^{NTR}$ suppresses growth and nerve growth factor-mediated metastasis of human prostate cancer cells. *Int. J. Cancer*, 98, 1-7.

Kuner, P. and Hertel, C. (1998). NGF induces apoptosis in a human neuroblastoma cell line expressing the neurotrophin receptor $p75^{NTR}$. *J. Neurosci. Res*., 54, 465-474.

Kuwako, K., Taniura, H. and Yoshikawa, K. (2004) Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor. *J. Biol. Chem*., 279, 1703-1712.

Lachyankar, M.B., Condon, P.J., Daou, M.C., De, A.K., Levine, J.B., Obermeier, A. and Ross, A.H. (2003). Novel functional interactions between Trk kinase and p75 neurotrophin receptor in neuroblastoma cells. *J. Neurosci. Res.*, 71, 157-172.

Lamballe, F., Klein, R. and Barbacid, M. (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell*, 66, 967-979.

Lavenius, E., Gestblom, C., Johansson, I., Nånberg, E. and Påhlman, S. (1995). Transfection of TRK-A into human neuroblastoma cells restores their ability to differentiate in response to nerve growth factor. *Cell Growth Differ*., 6, 727-736.

Lavoie, J.F., Lesauteur, L., Kohn, J., Wong, J., Furtoss, O., Thiele, C.J., Miller, F.D. and Kaplan, D.R. (2005). TrkA induces apoptosis of neuroblastoma cells and does so via a p53-dependent mechanism. *J. Biol. Chem*., 280, 29199-29207.

Levi-Montalcini, R. and Hamburger, V. (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool*., 116, 321-361.

Lewin, G.R. and Barde, Y.A. (1996). Physiology of the neurotrophins. *Annu. Rev. Neurosci*., 19, 289-317.

Liu, D., Zhang, Y., Dang, C., Ma, Q., Lee, W. and Chen, W. (2007). siRNA directed against TrkA sensitizes human pancreatic cancer cells to apoptosis induced by gemcitabine through an inactivation of PI3K/Akt-dependent pathway. *Oncol. Rep*., 18, 673-677.

López-Sánchez, N. and Frade, J.M. (2002). Control of the cell cycle by neurotrophins: lessons from the p75 neurotrophin receptor. *Histol. Histopathol.*, 17, 1227-1237.

López-Sánchez, N., González-Fernández, Z., Niinobe, M., Yoshikawa, K. and Frade, J.M. (2007a). Single mage gene in the chicken genome encodes CMage, a protein with functional similarities to mammalian type II Mage proteins. *Physiol. Genomics*, 30, 156-171.

López-Sánchez, N., Rodríguez, J.R. and Frade, J.M. (2007b). Mitochondrial c-Jun NH2-terminal kinase prevents the accumulation of reactive oxygen species and reduces necrotic damage in neural tumor cells that lack trophic support. *Mol. Cancer Res.*, 5, 47-60.

Lucarelli, E., Kaplan, D. and Thiele, C.J. (1997). Activation of trk-A but not trk-B signal transduction pathway inhibits growth of neuroblastoma cells. *Eur. J. Cancer.*, 33, 2068-2070.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990). Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science*, 247, 1446-1451.

Marchetti, D., Aucoin, R., Blust, J., Murry, B. and Greiter-Wilke, A. (2004). p75 neurotrophin receptor functions as a survival receptor in brain-metastatic melanoma cells. *J. Cell Biochem.*, 91, 206-215.

Marchetti, D., Denkins, Y., Reiland, J., Greiter-Wilke, A., Galjour, J., Murry, B., Blust, J. and Roy, M. (2003). Brain-metastatic melanoma: a neurotrophic perspective. *Pathol. Oncol. Res*., 9, 147-158.

Marchetti, D., Menter, D., Jin, L., Nakajima, M. and Nicolson, G.L. (1993). Nerve growth factor effects on human and mouse melanoma cell invasion and heparanase production. *Int. J. Cancer*, 55, 692-699.

Marchetti, D., Mrak, R.E., Paulsen, D.D. and Sinnappah-Kang, N.D. (2007). Neurotrophin receptors and heparanase: a functional axis in human medulloblastoma invasion. *J. Exp. Clin. Cancer Res.*, 26, 5-23.

Martin-Zanca, D., Hughes, S.H. and Barbacid, M. (1986). A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. *Nature*, 319, 743-748.

Marano, N., Dietzschold, B., Earley, J.J. Jr., Schatteman, G., Thompson, S., Grob, P., Ross, A.H., Bothwell, M., Atkinson, B.F. and Koprowski, H. (1987). Purification and amino terminal sequencing of human melanoma nerve growth factor receptor. *J. Neurochem.*, 48, 225-232.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, 80, 179-185.

Matsumoto, K., Wada, R.K., Yamashiro, J.M., Kaplan, D.R. and Thiele, C.J. (1995). Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cells. *Cancer Res.*, 55, 1798-1806.

Matsushima, H. and Bogenmann, E. (1990). Nerve growth factor (NGF) induces neuronal differentiation in neuroblastoma cells transfected with the NGF receptor cDNA. *Mol. Cell. Biol.*, 10, 5015-5020.

Meakin, S.O., MacDonald, J.I., Gryz, E.A., Kubu, C.J. and Verdi J.M. (1999). The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. *J. Biol. Chem.*, 274, 9861-9870.

Menter, D.G., Herrmann, J.L., Marchetti, D. and Nicolson, G.L. (1994-1995). Involvement of neurotrophins and growth factors in brain metastasis formation. *Invasion Metastasis.*, 14, 372-384.

Misko, T.P., Radeke, M.J. and Shooter, E.M. (1987). Nerve growth factor in neuronal development and maintenance. *J. Exp. Biol.*, 132, 177-190.

Muragaki, Y., Chou, T.T., Kaplan, D.R., Trojanowski, J.Q. and Lee, V.M. (1997). Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express TrkA receptors. *J. Neurosci.*, 17, 530-542.

Murciano, A., Zamora, J., López-Sánchez, J. and Frade, J.M. (2002). Interkinetic nuclear movement may provide spatial clues to the regulation of neurogenesis. *Mol. Cell. Neurosci.*, 21, 285-300.

Nakagawara, A., Arima-Nakagawara, M., Scavarda, N.J., Azar, C.G., Cantor, A.B. and Brodeur, G.M. (1993). Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N. Engl. J. Med.*, 328, 847-854.

Nakagawara, A., Azar, C.G., Scavarda, N.J. and Brodeur, G.M. (1994). Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol. Cell. Biol.*, 14, 759-67.

Nalbandian, A. and Djakiew, D. (2006). The $p75^{NTR}$ metastasis suppressor inhibits urokinase plasminogen activator, matrix metalloproteinase-2 and matrix metalloproteinase-9 in PC-3 prostate cancer cells. *Clin. Exp. Metastasis*, 23, 107-116.

Nicolson, G.L., Menter, D.G., Herrmann, J., Cavanaugh, P., Jia, L., Hamada, J., Yun, Z., Nakajima, M. and Marchetti, D. Tumor metastasis to brain: role of endothelial cells, neurotrophins, and paracrine growth factors. *Crit. Rev. Oncog.*, 5: 451-471.

Nosaka, Y., Arai, A., Miyasaka, N. and Miura, O. (1999). CrkL mediates ras-dependent activation of the Raf/ERK pathway through the guanine nucleotide exchange factor C3G in hematopoietic cells stimulated with erythropoietin or interleukin-3. *J. Biol. Chem.*, 274, 30154-30162.

Okada, Y., Eibl, G., Guha, S., Duffy, J.P., Reber, H.A. and Hines, O.J. (2004). Nerve growth factor stimulates MMP-2 expression and activity and increases invasion by human pancreatic cancer cells. Clin. Exp. Metastasis., 21, 285-292.

Páez-Pereda, M., Missale, C., Grübler, Y., Arzt, E., Schaaf, L. and Stalla, G.K. (2000). Nerve growth factor and retinoic acid inhibit proliferation and invasion in thyroid tumor cells. *Mol. Cell. Endocrinol*., 167, 99-106.

Papandreou, C., Bogenrieder, T., Loganzo, F., Chao, M.V., Nanus, D.M. and Albino, A.P. (1996). Mutation and expression of the low affinity neurotrophin receptor in human malignant melanoma. *Melanoma Res*., 6, 373-378.

Patapoutian, A. and Reichardt, L.F. (2001). Trk receptors: mediators of neurotrophin action. *Curr. Op. Neurobiol*., 11, 272-280.

Pflug, B.R., Onoda, M., Lynch, J.H. and Djakiew, D. (1992). Reduced expression of the low affinity nerve growth factor receptor in benign and malignant human prostate tissue and loss of expression in four human metastatic prostate tumor cell lines. *Cancer Res.*, 52, 5403-5406.

Podlesniy, P., Kichev, A., Pedraza, C., Saurat, J., Encinas, M., Perez, B., Ferrer, I. and Espinet, C. (2006). Pro-NGF from Alzheimer's disease and normal human brain displays distinctive abilities to induce processing and nuclear translocation of intracellular domain of $p75^{NTR}$ and apoptosis. *Am. J. Pathol*., 169, 119-131.

Poluha, W., Poluha, D.K. and Ross, A.H. (1995). TrkA neurogenic receptor regulates differentiation of neuroblastoma cells. *Oncogene*, 10, 185-189.

Quann, E.J., Khwaja, F., Zavitz, K.H. and Djakiew, D. (2007). The aryl propionic acid R-flurbiprofen selectively induces $p75^{NTR}$-dependent decreased survival of prostate tumor cells. *Cancer Res*., 67, 3254-3262.

Reichardt, L.F. (2006). Neurotrophin-regulated signalling pathways. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, 361, 1545-1564.

Reifenberger, G. and Collins, V.P. (2004). Pathology and molecular genetics of astrocytic gliomas. *J. Mol. Med*., 82, 656-670.

Rende, M., Pistilli, A., Stabile, A.M., Terenzi, A., Cattaneo, A., Ugolini, G. and Sanna, P. (2006). Role of nerve growth factor and its receptors in non-nervous cancer growth: efficacy of a tyrosine kinase inhibitor (AG879) and neutralizing antibodies antityrosine kinase receptor A and antinerve growth factor: an in-vitro and in-vivo study. *Anticancer Drugs*, 17, 929-941.

Renné, C., Minner, S., Küppers, R., Hansmann, M.L. and Bräuninger, A. (2008). Autocrine NGFbeta/TRKA signalling is an important survival factor for Hodgkin lymphoma derived cell lines. *Leuk. Res.*, 32, 163-167.

Ricci, A., Greco, S., Mariotta, S., Felici, L., Bronzetti, E., Cavazzana, A., Cardillo, G., Amenta, F., Bisetti, A. and Barbolini, G. (2001). Neurotrophins and neurotrophin receptors in human lung cancer. *Am. J. Respir. Cell. Mol. Biol*., 25, 439-446.

Rodríguez-Tébar, A., Dechant, G. and Barde, Y.A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron*, 4, 487-492.

Rodríguez-Tébar, A., Dechant, G., Götz, R. and Barde, Y.A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.*, 11, 917-922.

Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramee, G.R., Nikolics, K. and Winslow, J.W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron*, 4, 767-773.

Salehi, A.H., Roux, P.P., Kubu, C.J., Zeindler, C., Bhakar, A., Tannis, L.L., Verdi, J.M. and Barker, P.A. (2000). NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. *Neuron*, 27, 279-288.

Sawaya, R., Ligon, B.L., Bindal, A.K., Bindal, R.K. and Hess, K.R. (1996). Surgical treatment of metastatic brain tumors. *J. Neurooncol.*, 27, 269–277.

Segal, R.A., Goumnerova, L.C., Kwon, Y.K., Stiles, C.D. and Pomeroy, S.L. (1994). Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. *Proc. Natl. Acad. Sci. USA*, 91, 12867-12871.

Sigala, S., Tognazzi, N., Rizzetti, M.C., Faraoni, I., Missale, C., Bonmassar, E. and Spano, P. (2002). Nerve growth factor induces the re-expression of functional androgen receptors and p75(NGFR) in the androgen-insensitive prostate cancer cell line DU145. *Eur. J. Endocrinol*., 147, 407-415.

Singer, H.S., Hansen, B., Martinie, D. and Karp, C.L. (1999). Mitogenesis in glioblastoma multiforme cell lines: a role for NGF and its TrkA receptors. *J. Neurooncol*., 45, 1-8.

Schlessinger, J. and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. *Neuron*, 9, 383-391.

Schor, N.F. (2002). Neurology of neuroblastoma: neuroblastoma as a neurobiological disease. Boston, MA: Kluwer Academic Publishers.

Schwab, M., Westermann, F., Hero, B. and Berthold, F. (2003). Neuroblastoma: biology and molecular and chromosomal pathology. *Lancet Oncol.*, 4, 472-480.

Schweigreiter, R. (2006). The dual nature of neurotrophins. *Bioessays*, 28, 583-594.

Segal, R.A. (2003). Selectivity in neurotrophin signaling: theme and variations. *Annu. Rev. Neurosci.*, 26, 299-330.

Shonukan, O., Bagayogo, I., McCrea, P., Chao, M. and Hempstead, B. (2003). Neurotrophin-induced melanoma cell migration is mediated through the actin-bundling protein fascin. *Oncogene*, 22, 3616-3623.

Sinnappah-Kang, N.D., Kaiser, A.J., Blust, B.E., Mrak, R.E. and Marchetti, D. (2005) Heparanase, TrkC and $p75^{NTR}$: their functional involvement in human medulloblastoma cell invasion. *Int. J. Oncol*., 27, 617-626.

Stephan, H., Zakrzewski, J.L., Bölöni, R., Grasemann, C., Lohmann, D.R. and Eggert, A. (2008). Neurotrophin receptor expression in human primary retinoblastomas and retinoblastoma cell lines. Pediatr. *Blood Cancer*., 50, 218-222.

Soffietti, R., Ruda, R. and Mutani, R. (2002). Management of brain metastases. *J. Neurol.*, 249, 1357–1369.

Tabassum, A., Khwaja, F. and Djakiew, D. (2003). The $p75^{NTR}$ tumor suppressor induces caspase-mediated apoptosis in bladder tumor cells. *Int. J. Cancer*, 105, 47-52.

Tacconelli, A., Farina, A.R., Cappabianca, L., Desantis, G., Tessitore, A., Vetuschi, A., Sferra, R., Rucci, N., Argenti, B., Screpanti, I., Gulino, A. and Mackay, A.R. (2004). TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma. *Cancer Cell.*, 6, 347-360.

Tcherpakov, M., Bronfman, F.C., Conticello, S.G., Vaskovsky, A., Levy, Z., Niinobe, M., Yoshikawa, K., Arenas, E. and Fainzilber, M. (2002). The p75 neurotrophin receptor interacts with multiple MAGE proteins. *J. Biol. Chem.*, 277, 49101-49104.

Valenzuela, D.M., Maisonpierre, P.C., Glass, D.J., Rojas, E., Nunez, L., Kong, Y., Gies, D.R., Stitt, T.N., Ip, N.Y. and Yancopoulos G.D. (1993). Alternative forms of rat TrkC with different functional capabilities. *Neuron*, 10, 963-974

Verdi, J.M. and Anderson, D.J. (1994). Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron*, 13, 1359-1372.

Vilar, M., Murillo-Carretero, M., Mira, H., Magnusson, K., Besset, V. and Ibáñez, C.F. (2006). Bex1, a novel interactor of the p75 neurotrophin receptor, links neurotrophin signaling to the cell cycle. *EMBO J.*, 25, 1219-1230.

Walch, E.T., Albino, A.P. and Marchetti, D. (1999). Correlation of overexpression of the low-affinity p75 neurotrophin receptor with augmented invasion and heparanase production in human malignant melanoma cells. *Int. J. Cancer*, 82, 112-120.

Wadhwa, S., Nag, T.C., Jindal, A., Kushwaha, R., Mahapatra, A.K. and Sarkar, C. (2003) Expression of the neurotrophin receptors Trk A and Trk B in adult human astrocytoma and glioblastoma. *J. Biosci*., 28, 181-188.

Wagner, N., Wagner, K.D., Sefton, M., Rodríguez-Tébar, A. and Grantyn, R. (2000). An abnormal response of retinoblastoma cells (Y-79) to neurotrophins. *Invest. Ophthalmol. Vis. Sci.*, 41, 1932-1939.

Walch, E.T., Albino, A.P. and Marchetti, D. (1999). Correlation of overexpression of the low-affinity p75 neurotrophin receptor with augmented invasion and heparanase production in human malignant melanoma cells. *Int. J. Cancer*., 82, 112-120.

Wang, Y., Hagel, C., Hamel, W., Müller, S., Kluwe, L. and Westphal, M. (1998). Trk A, B, and C are commonly expressed in human astrocytes and astrocytic gliomas but not by human oligodendrocytes and oligodendroglioma. *Acta Neuropathol*., 96, 357-364.

Weeraratna, A.T., Arnold, J.T., George, D.J., DeMarzo, A. and Isaacs, J.T. (2000). Rational basis for Trk inhibition therapy for prostate cancer. *Prostate*, 45, 140-148.

Woo, C.W., Lucarelli, E. and Thiele, C.J. (2004). NGF activation of TrkA decreases N-myc expression via MAPK path leading to a decrease in neuroblastoma cell number. *Oncogene*, 23, 1522-1530.

Wright, J.H., Drueckes, P., Bartoe, J., Zhao, Z., Shen, S.H. and Krebs, E.G. (1997), A role for the SHP-2 tyrosine phosphatase in nerve growth- induced PC-12 cell differentiation. *Mol. Biol. Cell*, 8, 1575-1585.

Yamashiro, D.J., Nakagawara, A., Ikegaki, N., Liu, X.G. and Brodeur, G.M. (1996). Expression of TrkC in favorable human neuroblastomas. *Oncogene*, 12, 37-41.

Yamashita, T., Fujitani, M., Yamagishi, S., Hata, K. and Mimura, F. (2005). Multiple signals regulate axon regeneration through the Nogo receptor complex. *Mol. Neurobiol*., 32, 105-111.

Yan, C., Liang, Y., Nylander, K.D., Wong, J., Rudavsky, R.M., Saragovi, H.U. and Schor, N.F. (2002). p75-nerve growth factor as an antiapoptotic complex: independence versus cooperativity in protection from enediyne chemotherapeutic agents. *Mol. Pharmacol.*, 61, 710-719.

York, R.D., Yao, H., Dillon, T., Ellig, C.L., Eckert, S.P., McCleskey, E.W. and Stork, P.J. (1998). Rap 1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature*, 393, 622-626.

York, R.D., Mollivar, D.C., Grewal, S.S., Steinberg, P.E., McCleskey, E.W. and Stork P.J. (2000). Role of phosphoinositide 3-kinase and endocytosis in NGF induced extracellular signal-regulated kinase activation via Ras and Rap 1. *Mol. Cell. Biol.*, 20, 18069-18083.

Zhang, Y., Dang, C., Ma, Q. and Shimahara, Y. (2005). Expression of nerve growth factor receptors and their prognostic value in human pancreatic cancer. *Oncol. Rep*. 14, 161-171.

Zhu, Z., Kleeff, J., Kayed, H., Wang, L., Korc, M., Büchler, M.W. and Friess, H. (2002). Nerve growth factor and enhancement of proliferation, invasion, and tumorigenicity of pancreatic cancer cells. *Mol. Carcinog.*, 35, 138-147.

Zhu, Z.W., Friess, H., Wang, L., Bogardus, T., Korc, M., Kleeff, J. and Büchler, M.W. (2001). Nerve growth factor exerts differential effects on the growth of human pancreatic cancer cells. *Clin. Cancer Res*., 7, 105-112.

Zhu, Z.W., Friess, H., Wang, L., Di Mola, F.F., Zimmermann, A. and Büchler, M.W. (2000). Down-regulation of nerve growth factor in poorly differentiated and advanced human esophageal cancer. *Anticancer Res*., 20, 125-132.

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Chapter 8

The Role of Neurotrophins in Affective Disorders Study of Blood and Brain BDNF and NT-3 and cAMP-PKA-Regulated BDNF Expression

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Abstract

The neurotrophins - nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4 - represent a family of proteins essential for neuronal survival and plasticity. Recent reports have suggested that these molecules, particularly BDNF, play an important role in neuropsychiatric disorders such as major depression (MDD), suicide, bipolar disorder (BD) and eating disorders (ED). A comprehensive study of the biology of BDNF is an important step in developing its clinical relevance.

Following the neuroplastic hypothesis of affective disorders we have led an investigation of BDNF's biology and related molecules. Blood and brain BDNF and NT-3 levels were measured in rodents and in psychiatric subjects. These studies aimed to associate the dysregulation of neurotrophic factors and psychiatric pathologies. We also investigated the regulation of BDNF mRNA expression in drug-treated cells through cAMP transduction pathways. Results showed that, in major depression disorder (MDD), subjects are characterized by decreased serum BDNF levels, and that the decrease was correlated with the severity of illness. Moreover, the decrease was reversed after an antidepressant treatment. We also observed that, during the postnatal maturation period in the rat, changes in serum and brain BDNF protein levels followed the same development scheme and were positively correlated. Neurotrophin protein levels (BDNF

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and NT-3) have also been measured in postmortem human brains. The data indicated a significant decrease in BDNF and NT-3 levels in both the hippocampus and prefrontal cortex of drug-free depressed suicide subjects compared to non-depressed controls. The decrease was observed in different suicide groups whatever the diagnosis, indicating either a suicide-specific trait or a trait common to other psychiatric diagnoses. Subjects who were under drug treatment at the time of death did not differ from non-suicide controls. By using lymphoblasts (from BD patients), we assessed the drug-manipulated BDNF expression as downstream target of the cAMP-Protein kinase A (PKA) and the transcription factor CREB signaling axis. It was observed that, in BD-derived cells, BDNF mRNA expression was decreased, but that the decrease was masked by an upregulated PKA-CREB signaling pathway, resulting in normalization of BDNF mRNA levels. In conclusion, these studies on neurotrophins have identified BDNF and NT-3 as potential biological markers of neuroplasticity in the affective disorders. Given the reported morphological deficits associated with affective disorders, the study of neurotrophins could be a promising track for drug development.

A. Introduction

Since the discovery of nerve growth factor (NGF) in the 1950s (Montalcini and Hamburger, 1951) followed by that of brain-derived neurotrophic factor (BDNF) in the 1980s (Barde, 1982), a great deal of evidence has highlighted the role played by neurotrophins in development, physiology and pathology. Particularly important have been the roles played by BDNF in neural development and cell survival, and in the molecular mechanisms of synaptic plasticity and stress-related psychopathologies (for recent reviews: Schmidt and Dumn 2007; Pittenger and Duman 2007; Lipski and Marini, 2007 Skaper 2008). During the last decade, a new theory of mood disorders has emerged that emphasises deficits in plasticity-induced networks (Duman et al. 1996; Manji et al., 2000; Fuchs et al. 2004). The involvement of neurotrophic factors and their receptors has been the subject of intense research (Friedman and Greene, 1999; Lipsky and Marina, 2007). These studies have led to the formulation of the neurotrophic hypothesis of major depression, which suggests that brain BDNF levels are reduced in depressed subjects, whereas application of BDNF to animal brain produces antidepressant-like effects (Shirayama et al., 2002; Altar et al., 2003). The biochemistry and biology of neurotrophins have been thoroughly reviewed elsewhere and the reader is refered to these excellent reviews for in-depth coverage (Castrén 2004; Duman and Monteggia, 2006). This chapiter is intented to provide a brief, but comprehensive review of our recent research on BDNF and NT-3 regulation in affective disorders, particularly in major depression disorder (MDD) and in bipoar disorder (BD). The studies have particularly focused on dysregulation of peripheral (serum, whole blood and plasma) and brain BDNF and NT-3 levels in major depressive patients. We also investigated the antidepressant effects on serum and *postmortem* BDNF levels in depressed subjects and persons who died after a long period of psychotropic drug treatment respectively. In parallell to these human studies, BDNF levels were assessed in rat brain and serum samples, to determine the relationship between BDNF measures on the one hand, and to investigate postnatal changes in neurotrophin levels on the other. Lastly, an *in vitro* study on bipolar depressed-derived lymphoblast cells aimed to manipulate pharmacologically BDNF mRNA expression, by

targeting a cAMP-signal transduction axis, thus mimicking what could be the *in vivo* hormone-regulated BDNF expression in affective disorders. Considered as a whole, all these studies help to add weight to the neurotrophin hypothesis of affective disorders by demonstrating that BDNF and NT-3 play critical roles in the pathophysiology of these diseases and in the activity of therapeutic agents. Modulation of neurotrophin action holds significant potential for novel therapies for a variety of neurological and psychiatric disorders.

As an introduction to this chapiter, we give a short reminder of the nature of neurotrophins and their receptors and of the roles they play in various pathologies, including psychopathologies. We also present the reports on neuroplasticity and mood disorders from which the neurotrophic hypothesis of affective disorders has emerged.

1. The Nature of Neurotrophins

The neurotrophins (NT) are the best understood trophic factors in the nervous system. They are a group of proteins structurally and functionally related to NGF and consist of four proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4 (NT-4/5). Neurotrophins are a unique family of polypeptide growth factors that influence many aspects of neuronal development and function, including cell proliferation, differentiation, synapse formation, membrane trafficking, neuronal survival and death (Reichardt, 2006). During the last decade, these molecules, which signal through receptor-associated tyrosine kinase, have been shown to regulate almost all aspects of neuronal development and function. It was demonstrated early on in a model of cellular interactions that NGF, for instance, is internalized by a receptor-dependent process and transported along axons by a cytosqueletal process (Thoenen and Barde, 1980). These molecules are essential for the health and well-being of the nervous system, but also mediate higher-order activities such as learning, memory and behavior (Lipsky and Marini, 2007). The neurotophins and their genes share homologies in sequence and structure and the organisation of the genome segments adjacent to these genes is also the same (see reference in Maness et al., 1994). These observations provide compelling evidence that NT genes have arisen through successive duplications of a portion of the genome from ancestral chordate (Hallbook et al., 1999; Reichardt, 2006). Initially synthesized as precursors or pro-neurotrophins, neurotrophins are cleaved to release the mature active proteins (Reichardt, 2006). The latter form a stable, non-covalently linked homodimer with a molecular wheight (MW) of approxiamately 28kDa. It has been demonstrated that many of the mechanisms through which neurotrophins control neuronal development are shared by their receptors, the typrosine kinase family.

NGF

NGF was the first to be described in the early 1950s because of its trophic effect on sensory and sympathetic neurons (Levi-Montalcini and Hamburger, 1951). It was further found to support the survival and neurotransmitter synthesis of cholinergic neurons in the central nervous system (CNS). In the brain, it is primarily synthesized in cholinergic target

tissues such as the cortex, hippocampal pyramidal layer and striatum (Gall and Isackson, 1989; Rylett and Williams, 1994). Basal levels of NGF in the hippocampus are very low, but NGF expression levels are regulated by activity and increase following kindling (Bengzon, et al., 1992). NGF has a restricted target population. In the peripheral nervous sytem, NGF acts on the sympathetic neurons as well as the sensory neurons involved in nociception and temperature sensation. In the CNS, it promotes the survival and function of neurons in the basal ganglia (Chen et al., 1997). These neurons project to the hippocampus and are believed to be important for memory processes, which are specifically affected in Alzheimer's disease (AD),

BDNF

Purified from pig brain in 1982, BDNF was the second neurotrophin to be described, and was immediately shown to promote the survival of a subpopulation of peripheral neurons not responsive to NGF (Barde et al., 1982). The amino acid sequence of BDNF was found to have close homology with NGF. BDNF mRNA and protein are widely distributed in the CNS (Cornner et al., 1997), including the limbic forebrain and neocortex, and are abundant in all the principal neurons of the hippocampus (Ernfors et al., 1990; Hofer et al., 1990). The principal receptor for BDNF is TrkB, which is found in both catalytic and truncated forms in the adult forebrain (Fryer et al., 1996). TrkB mRNA is mainly found in the hippocampus (Altar et al., 1994). Studies performed in animals after targeted removal of genes or "knock-outs" have shown that, when one copy of the BDNF gene is deleted and the animals are therefore heterozygous for BDNF (BDNF+/-), they are severely deficient in their ability to produce LTP (a paradigm linked to the process of learning and memory) in hippocampus slices (Korte et al., 1995) and are unable to migrate in a Morris water maze (Linnarson et al., 1997). The hippocampus shows decreased neurogenesis and is reduced in size (Lee et al., 2002). A deficit in BDNF is also associated with a variety of neurochemical and behavioral alterations, including decreases in serotonin (5-HT) in association with the development of aggressiveness and hyperphagia (Lyons et al., 1999).

NT-3

There is a substantial degree of sequence between BDNF and NGF, and it was their conserved regions that helped greatly in the identification of a third novel neurotrophic molecule, the neurotrophin-3 (NT-3) in several species (Enfors et al., 1990; Jones and Reichardt 1990). The molecule was described for the first time by Maisonpierre and coworkers (1990), and subsequently the protein was found to be widely distributed in the adult brain (Zou and Rush, 1994; Katoh-Semba et al., 1996). Like BDNF, NT-3 is involved in synaptic transmission and neural excitability (Thoenen, 1995). Addition of NT-3 to hippocampal slices enhances synaptic strength at Schaffer collateral-CA1 synapses (Kang and Schuman, 1995). NT-3 also enhances the survival and differentiation of neural progenitor cells (Barnabe-Heider and Miller, 2003). It was recently shown that developmental loss of NT-3 *in vivo* results in reduced levels of myelin-specific proteins, reduced myelination and increased apoptosis of Schwann cells (Woolley et al., 2006). Elevated neuronal activity, either in LTP-inducing stimuli or ischemia, enhances the expression of NT-3 mRNA in the denatate gyrus (Lindvall et al. 1992). Reports have indicated that, whereas NGF and BDNF

levels increase after seizures, NT-3 levels are reduced in dentate gyrus granules neurons (Mudo et al., 1996; Kim et al., 1998), suggesting that the role of NT-3 in epileptic seizures is probably different (according to Binder, 2007). In NT-3 +/- knockout mice, which exhibit 30% reduction in basal NT-3 mRNA, amygdala kindling was markedly retarded, while chronic intraventricular infusion of NT-3 retards the development of behavioral seizures (Elmer et al., 1997; Xu et al., 2002). In mossy fibers, chronic infusion of NT-3 inhibits kindling-associated sprouting (Xu et al., 2002). Lastly, direct evidence for the role of endogenous NT-3 in the proliferation, survival or differentiation of hippocampal progenitor cells has been recently provided (Shimazu et al., 2008).

NT-4/5

While analyzing the evolutionary conservation of aminoacid sequences among the three neurotrophins, a fourth related factor was detected (Hallbook et al., 1991). The new NT isolated from *Xenopus* and viper was refered to as neurotrophin-4 (Hallbook et al., 1991; Ip et al., 1992). Shortly afterwards, another factor designated NT-5 was isolated from both humans and rats, but the aminoacid sequences were shown to be identical (Berkmeier et al., 1991; Ip et al. 1992). This observation has promoted a growing feeling that the molecules are similar enough to be designed NT-4/5. This new neurotrophin was soon revealed to offer neuroprotection to hippocampal and cortical neurons against injury and to be endowed with antiapoptotic ability in hippocampal granule cells (Cheng et al., 1994; Qiao et al., 1996). However, in contrast to BDNF knockouts, homozygotous knockout mice (NT-4/5 -/-)) are normal and long-lived with no obvious neurological deficits (Conover et al., 1995). Moreover, in an amygdala kindling phenotype of NT-4 mice, no aspect of development was found to be affected in knockout mice (He et al., 2006). Brain levels of NT4/5 are very low at baseline and are not increased by seizures (Timmusuk et al., 1993; Mudo et al. 1996). Nevertheless, application of NT-4 enhances excitatory synaptic transmission in cultured hippocampal neurons (Lessmann et al., 1994). In the cat visual cortex, it blocks the effect of monocular deprivation during the critical deprivation period (Gillespie et al., 2000)

2. Neurotrophins Signal through Trk and $p75^{NTR}$ Receptor Family

There has been a wealth of recent findings concerning the identification and function of neurotrophin receptors (Binder 2007; Reichardt, 2006). The neurotrophins are unique in that they exert their effects through two classes of receptors: the tropomyosin-related kinase (Trk) receptor tyrosine kinase and $p75^{NTR}$. Through these receptors, their intracellular mechanisms are connected with signal transduction pathways in cells (such as MAPK kinase cascade or cAMP cascade). Three members of the Trk receptor family have been identified; TrkA, TrkB and TrkC. Each NT binds one or more of the Trk receptors (Maness et al., 1994; Ciao, 2003). This specificity in binding, conferred *via* the juxtamembrane Ig-like domain in the extracellular portion of the receptor ,has the following pattern: TrkA binds NGF (with low affinity for NT-3 in some systems); TrkB binds BDNF and NT-4/5 with low affinity binding by NT-3 and TrkC binds NT-3 (Barbacid, 1994; Maness et al., 1994). Trk B and C receptors exist in both a full length isoform and a truncated form, as a result of differential splicing,

which generates isoforms lacking the kinase domain (Fryer et al., 1997; Reichardt, 2006). Activation of Trk signaling by neurotrophins follows the general canonical scheme established for receptor tyrosine kinase: ligand-induced dimerisation, initial activation of kinase activity and phosphorylation of tyrosines in the activation loop, full kinase activation and autophopsphorylation of tyrosine outside the loop and binding of the signaling molecules to the latter, eventually resulting in synaptic plasticty (Reichardt, 2006). In addition to the Trk tyrosine kinase receptors, which marked the differences in specificity for particular neurotrophins, an additional receptor, related to proteins of tumor necrosis factor (TNFR) and termed $p75^{NTR}$, binds all members of the neurotrophin family with low and similar affinity (Frade and Barde, 1998). In some cases, the precursor of NGF binds to this p75 receptor with high affinity (Lee et al., 2001). The $p75^{NTR}$ serves as a pro-apoptotic receptor during developmental cell death and after injury to the nervous system (Roux et al., 2002). NT binding to $p75^{NTR}$ is linked to several intracellular signal transduction pathways and increases in JUN, NF-kB and ceramide have been reported (Roux et al., 2002; Reichardt, 2006).

3. Affective Disorders and Neuroplasticity

Nowadays, the brain is considered to be a dynamic system that owes its structural and functional capacity to the interaction of genetic and environmental factors. The neural circuits and connections are subject to lifelong modification and reorganisation in response to external and internal stimuli. The brain is permanently subjected to functional adaptations because of the adaptive capacity of neural systems, brain nuclei, synapses and receptors (Kendler et al., 1999; Post, 2000). This dynamic system is referred to as neuroplasticity. In the adult brain, it is manifested at cellular level by modification of dendritic growth, axonal sprouting, synaptic remodelling and the creation of new synapses and is linked with neurogenesis (Muselman, 1999; Piitenger and Duman 2007). Mood disorders represent a collection of psychological, behavioral and physiological symptoms whose frequency and chronicity constitute a recognizable clinical condition (DSM-IV, 1994). Among the most potent factors known to trigger or induce depressive episodes are stressful life events (Kendler et al., 1999; Paykel, 2001).

Neuroimaging and postmortem studies have demonstrated selective structural changes in various circuits in the brain of depressed individuals (Fussati et al., 2004; Drevets et al., 2000; Rajkowska, 2000). Preclinical studies demonstrate that exposure to stress leads to atrophy and cell loss in the hippocampus as well as decreased expression of neurotrophic/growth factors, and that antidepressant administration reverses or blocks the effects of stress (Nibuya et al., 1995; Shirayama et al., 2002). Accumulating evidence suggests that altered neurogenesis in the adult hippocampus mediates the action of antidepressants. Chronic antidepressant administration upregulates neurogenesis in the adult hippocampus and this cellular response is required for the effects of antidepressants in certain animal models of depression (Gould et al., 1995).

A number of authors have reviewed cellular (e.g. adult neurogenesis) and behavioral studies that support the neurotrophic/neurogenic hypothesis of depression and antidepressant action (Fuchs et al., 2004; Lipsky and Marini, 2007). Aberrant regulation of neuronal

plasticity, including neurogenesis, in the hippocampus and other limbic nuclei may result in the maladaptive changes in neural networks that underlie the pathophysiology of MDD. Affective disorder (MDD and BD) were therefore characterized by structural and neurochemical changes in limbic and non limbic structures, including the hippocampus, that regulate mood and cognitive functions. Hippocampal atrophy is repeatedly observed in patients with depression and this effect is blocked or reversed by antidepressant treatments (Sheline et al., 2003).

4. Neurotrophins and Mood Disorders

Several human studies have provided data that are suggestive of the roles played by neurotrophins in the pathophysiology of MDD and BD (Altar 1999; Duman et al., 2002; Hashimoto et al., 2005; Walz et al., 2007; Monteleone et al., 2008). Following reports that chronic, but not acute, administration of pharmacological antidepressant treatments enhances hippocampal neurogenesis, it seemed likely that adaptations that arise in response to treatment underlie the trophic influence of antidepressants. These treatments may converge on specific signaling cascades and candidate genes to stimulate an adaptative form of neuronal plasticity. Two of the candidate molecules that could mediate such a trophic effect are the neurotrophins, BDNF and NT-3. As stated above, BDNF and NT-3 are known to exert an influence on development and plasticity within the immature and adult brain. Studies have subsequently indicated that BDNF expression serves as a target for antidepressants.

Preclinical and clinical studies of altered BDNF expression during chronic stress and increased BDNF and TrkB activity during antidepressant treatment confirm the role of BDNF in the pathogenesis of depression. Peripheral BDNF measures have also contributed to reinforce this view (Hishimoto et al., 2004). BDNF has also currently been found in human blood, where almost 99% is stored in the platelets and then released into the serum (Radka et al., 1996; Fukujura et al., 2002). It has also been reported that BDNF can cross the blood-brain-barrier (Pan et al., 1995) suggesting therefore that brain and blood BDNF may interact. Similar dysregulation in both brain and serum BDNF has been hypothesized and demonstrated in rodents (Karege et al., 2002b). These measures were carried out to find out an accessible tool to monitor brain BDNF. Subsequently, numerous independent reports have reported decreased BDNF values, compared with control subjects, in both serum and plasma of MDD patients (Karege et al., 2002a; Shimazu et al., 2003; Gunil et al., 2005; Montelone et al., 2008). Normalization of BDNF values in antidepressant-treated subjects was also reported by independent laboratories including our group (Gervasoni et al., 2005; Shimazu et al., 2003; Gunul et al., 2005). In bipolar disorder, reports have also shown that BDNF is altered in both serum and plasma (Cunha et al., 2006; Machado-Viera et al., 2007). In the two types of bipolar disorder, I and II, serum BDNF was also found to be reduced compared with controls (Montelone et al., 2008). Serum NT-3 has been assessed in BD but, in contrast to BDNF, results indicated increased serum NT-3 levels in both manic and depressed BD, compared with euthymic states (Walz et al., 2007). The molecular genetic studies focused on associations between BDNF gene polymorphisms and bipolar disorder or cognitive

function disturbances. Two independent studies have shown an association between BDNF gene polymorphic variants in BD (Sklar et al., 2002; Neves-Perreira et al., 2002).

When considered as a whole, these findings clearly indicate an alteration of BDNF and NT-3 in affective disorders and that these neurotrophins could play a role in the mechanism of antidepressant drug treatment.

5. The Neurotrophic Hypothesis

One hypothesis to emerge from the above mentioned studies is that mood disorders reflect failed function of critical neuronal networks, whereas a gradual network recovery through activity-dependent neuronal plasticity induces the antidepressant effect (Fuchs et al., 2004; Fossati et al., 2004). The neurotrophin hypothesis of depression assumes that the disease states result from the effects of decreased availability of neurotrophins and/or of their receptors on affected neurons and/or decreased neuronal survival. These assumptions, which are largely based on correlations between stress or antidepressant treatment and down- or upregulation of neurotrophins (BDNF and NT-3) respectively, have been confirmed in neuroimagined and postmortem studies. However, there is no indication that neurotrophic factors themselves actually control mood. They probably act as necessary tools in the activity-dependent modulation of networks, the physiological function of which determines how a plastic change influences mood. Disruption of the signaling pathways involving neurotrophins and receptors does not seem to cause depressive behaviors, but does hamper the effect of antidepressant drugs. Thus, neurotrophins may be the main target of antidepressants.

6. Neurotrophins in other Psychopathologies

Besides mood disorders, neurotrophins may play roles in various somatic and neurodegenerative pathologies (Manes et al., 1994; Chao et al., 2006). A complete review of all pathologies in which neurotrophins are involved is beyond the scope of this paper. However, we should like to mention briefly their role in distant pathologies like asthma (Noga et al., 2001), eating disorders (Lyons et al., 1999; Nakazo et al., 2003), cognitive deficits and memory disorders (..), neurodenenerative diseases (Murer et al., 2001; Spires et al., 2004), schizophrenia (Toyota et al, 2002; Weickert et al.), epilepsy (Ernfors et al., 1991; Binder 2007) and traumatic brain injury (TBI) (Royo et al., 2007). Traumatic brain injury (TBI) causes selective hippocampal cell death, which is believed to be associated with the cognitive impairment observed in both clinical and experimental settings. Measurements of NT-4 in rats subjected to TBI revealed two to threefold increases in the injured cortex and hippocampus in the acute period (1-3 days) following brain injury (Royo et al. 2007). Neurotrophin deficiency was observed in infantile hypertrophic pyloric stenosis (IHPS), where the quantity of total NGF, NT-3 and BDNF was found to be significantly lower than in controls (Guarino et al., 2006). Like BDNF, NT-3 levels were reported in human biceps brachii tissue of amyotrophic lateral sclerosis (Küst et al., 2002). In addition, recent studies

have revealed a diversity of roles for these factors outside the nervous system, most notably in cardiac development, neovascularisation and immune system function (Donovan et al., 2000; Lin et al., 2000; Coppola et al., 2004; Kermani et al., 2005). Finally, several human genetic diseases have been associated with mutations in neurotrophins or their receptors. Several variant types of the TrkA gene have been identified in the congenital insensitivity to pain found in anhydrosis (CIPA) patients (Indo, 2001). A mutation in TrkB kinase signaling was reported in a patient with hyperphagic obesity and severe impairments in nociception, learning and memory (Yeo et al., 2004). Lastly a polymorphism in the *bdnf* gene that impairs the transport of BDNF protein has been associated with impaired hippocampal function and episodic memory (Egan et al., 2003). The variant observed in the pro-domain of BDNF (BDNFval-met) was further found to be associated with various psychiatric (depression and bipolar disorder) and neuropsychiatric disorders (Alzeihmer's disease and Parkinson's disease) (Momose et al., 2002; Sen et al., 2003; Ribases et al., 2003; Sklar et al., 2002). These genetic mutations show the role played by the neurotophins and their receptors in higher order CNS functions and how they can lead to neuropsychiatric and neurodegenerative diseases.

7. Neurotrophins and Postnatal Development

There is considerable evidence to suggest that BDNF plays an important role in the development of the nervous system and influences many aspects of neuronal function in the adult brain (Lewin and Barde, 1996; Maisonpierre et al., 1990). Interest in BDNF and aging was aroused after reports of its involvement in age-related pathologies such as Alzheimer's disease or age-associated impairment of cognitive performance (Connor et al., 1997; Croll et al., 1998). This neurotrophin has been extensively studied in the mammalian prenatal phase and during the neurogenesis phase, but relatively few reports have addressed the question of its expression in the postnatal period (Katoh-Semba et al., 1998). The aim of our study was to assess the developmental profile of brain and serum BDNF in order to establish whether there are similar or different changes between BDNF levels in brain and blood. Assuming that regulation of brain BDNF expression is affected by development, it is possible that similar alteration occurs in other peripheral tissues. Therefore, following the previously demonstrated passage of BDNF across the blood-brain-barrier (BBB) (Pan et al., 1998a; 1998b), it was possible to hypothesize that parallel changes occur in brain and serum BDNF levels, characterized by increased expression during the postnatal development and maturation phases.

B. Methods

The methods used for BDNF and NT-3 extraction and analysis are summarized in the following paragraphs. The subjects were either major depressed subjects and age and sex-matched normal controls (for blood samples), *postmortem* brain tissue (from suicide or non-suicide deceased persons) or transformed lymphocytes from bipolar depressed patients.

Table 1. Serum BDNF levels, age, MADRS scores, gender distribution in major depressed and control subjects

	Controls (N)	Patients (N)	t-test	P
BDNF (ng±sd/ml)				
Tot. pupulation	26.5±7 (30)	22.6±3 (30)	2.72	0.008
Male subjects	26.0±7 (15)	24.1±3 (15)	1.21	NS
Female subjects	26.0±6 (15)	21.2±3 (15)	2.74	0.009
Age (years				
Tot population	37.9±9 (30)	36.8±8 (30)	0.51	NS
Male subjects	37.9±9 (15)	36.8±8 (15)	0.52	NS
Female subjects	36.0±8 (15)	36.0±8 (15)	0.02	NS
MADRS				
Tot population	-	34±5	-	-
Male subjects		32±4 36±4*		
Female subjects	-		-	-
Drug history	no	drug-naive	-	-

*) Male vs Female patients : t-test = 2.33, P<0.02 ; NS, son significant.

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Table 2. Serum and Plasma BDNF and PF4 levels, and whole blood BDNF levels as well, from drug-free major depressed patients and control subjects

	Controls (N)	Patients (N)	t-test
Serum BDNF (ng/ml)*	12.20±2.4 (34)	10.07±2.3 (43)	p<0.006
Blood BDNF (ng/ml)	16.47±3.0 (34)	16.31±3.9 (43)	NS
Serum-to-Blood ratio (%)	74.0±13 (34)	64.0±15 (43)	p<0.002
Plasma BDNF (pg/ml)	2165±349 (12)	1685±243 (12)	p<0.001
Plasma (IU/ml)**	18.4±2.5(12)	24.3±2.1 (12)	p<0.001
Serum (IU/ml)	108.4±19 (12)	104.8+25 (12)	NS

* serum values were adjusted to the serum-whole blood ratio. Before adjustment BDNF levels in serum were similar to our previous data (Table 2). Values are mean±sd. BDNF, Brain-derived Neurotrophic Factor ; ** Plasma PF4 values (±sd) reported in litterature range from 5 IU/ml (Kuijpers et al 2002) to 57.6 IU/ml (Serebruany et al 2003) and depend on the degree of in vitro platelet activation. PF4, Platelet Factor 4.

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1. Serum, Plasma and whole Blood BDNF: Subjects and Sampling Methods

Major depressed patients diagnosed according to DSM-IV criteria (American Psychiatric Association) participated after giving written consent. The Institutional Review Board of

Geneva University approved all procedures. The patients were either drug-naive or had been drug-free for several days before blood collection. Healthy controls, who also participated in the studies after giving written consent, were sex and age-matched. The inclusion criteria for control subjects were good physical health and no history of mental disorder, neurological disease or drug abuse. Demographic and clinical characteristics are given in Table 1. The severity of depression was measured by experienced psychiatrists using the Montgomery-Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979). The methods used for BDNF extraction and measurement were published (Karege et al., 2002; Karege et al., 2005). Briefly, blood was sampled in anticoagulant-free tubes and kept at room temperature for 1 hr followed by 1 hr at 4^{o}C (for platelet activation) before serum was isolated (centrifugation at 2000 g x10 min at 4^{o}C). All serum was carefully collected and kept at –20^{o}C before assaying BDNF content. According to numerous authors, serum BDNF represents the fraction of whole blood BDNF released by platelets during their activation (Radka et al., 1996; Fujimura et al., 2002). For plasma BDNF, poor-platelet plasma was carefully prepared from blood collected in tubes containing an appropriate anticoagulant solution and centrifuged at 2,000g for 10 min at 4^{o}C. In the case of whole blood BDNF, a second collection was made in tubes containing anticoagulant solution (Table 2).

Table 3. Serum and brain (hippocampus and cortex) BDNF levels in four groups of rats of different ages

Sample	Age groups			
	One-week	3-weeks	2-months	2-years old
Serum (pg/ml)	$1174^{\pm}318$	$2463^{\pm}545^{c}$	$4828^{\pm}753^{d,e}$	$4336^{\pm}1119^{e,f}$
Hippocampus (pg/g)	$1303^{\pm}180$	$1583^{\pm}200^{c}$	$2330^{\pm}269^{g}$	$1737^{\pm}286^{g}$
Frontal cortex (pg/g)	$131^{\pm}35$	$206^{\pm}39^{c}$	$652^{\pm}44^{g,g}$	$742^{\pm}125^{g}$

a). BDNF levels were assayed with the Elisa (Emax BDNF immunoassay system from Promega Corp.) at different ages of the animal.

b). ANOVA test for serum data: $F(3,16)=25.7$; $p<0.001$; ; ANOVA test for brain data: $F(3,16)=6.1$.$P<0.001$ and $F(3,16)=88.1$, $P<0.001$ for hippocampus and cortex, respectively .

c). Mann-Whitney post-hoc analysis, with respect to one week-old group: $p<0.05$;

d). Mann-Whitney U-test, with respect to one week-old group : $p<0.001$;

e). Mann-Whitney U-test, with respect to one week-old group : $P<0.01$;

f). Mann-Whitney U-test, with respect to one week-old group: $p<0.01$;

g). Mann-Whitney post-hoc analysis, with respect to one week-old group: $p<0.01$

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Table 4. Characteristics of suicide victims and control subjects

Subject	Age	Sex	PMD	Diagnosis	Drugs	MOD
Group 1 : Suicide victims with depression diagnosis and negative toxicology.						
1	53	M	23	BD, depr	none	drowining
2	34	F	34	MD	none	hanging
3	33	M	96	MD	none	gunshot
4	62	F	45	MD	none	jump
5	25	M	28	MD	none	jump
6	20	M	48	MD	none	hanging
7	54	F	20	MD	none	asphixia
8	45	M	15	MD	none	asphixia
9	59	F	40	BD+SA	none	CO
10	62	M	24	MD	none	hanging
Group 2 : Suicide victims with other diagnosis and negative toxicology						
1	33	F	14	alcohol abuse	none[a]	overdose
2	80	F	14	agoraphobia+PD	none	jump
3	34	M	10	schizophrenia	none	gunshot
4	57	M	27	schizoaffectif	none	overdose
5	86	F	7	Alzheimer's disease	none	hanging
6	47	M	15	schizoaffectif	none	MT
7	32	M	18	anxiety disorder	none	CO
8	75	M	18	Alzheimer's disease	none	drowning
9	52	M	15	alcohol abuse	none[a]	overdose
10	22	M	8	SA	none[a]	hanging
Group 3 : Suicide victims with depression and positive toxicology						
1	65	F	19	MD	trazadone	gunshot
2	37	M	48	MD	citalopram	MT
3	25	M	35	MD	fluvox/parox	hanging
4	35	M	9	MD	citalopram	overdose
5	32	M	48	BD	quetiapine	jump
6	40	F	24	MD+ SA	fluoxetine	overdose
7	56	M	3	MD	clomipramine	hanging
8	73	M	18	MD	fluoxetine	MT
9	59	F	40	MD	citalopram	overdose
10	35	F	9	BD, depressed	olanazepine	gunshot
Control group : drug-free non suicide subjects (number, age, gender distribution and PMD)						
n=24 ;	45±14	19M/11F	28±21	none	none	

Notes: Abbreviations: MOD; mode of death ; PMD : postmortem delay; MD, major depression; BD, bipolar disorder; SA: suicide attempt; PD: personality disorder; CO: carbon monoxide poisoning, MT: multiple trauma. a) These patients presented traces of alcohol (1.1-1.54 g/l).

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Blood was homogenized by sonication in 1% Triton-100 Tris-HCl solution containing antiproteases (1µM leupeptin, 1mM PMSF, 10µg/ml aprotinin, 1%NP40) centrifuged at 2000 g for 10 min at 4°C and the supernatant was isolated. Serum BDNF was assayed using an Elisa Kit (*Emax ImmunoAssay system* kit; Catalys AG, Wallisellen, CH), after dilution with the Block and Sample solution (provided with the kit). A microplate reader (Anthos Labtec Instrument, Chatel-St-Denis, CH) set at 450 nm, was used to determine serum BDNF values (intra-assay and inter-assay variations were less than 5% and 8% respectively).

2. Subjects and *Postmortem* Brain Sampling

Human brains were collected at autopsy from the Institute of Forensic Medicine, Geneva, Switzerland. This study was approved by the Research and Ethics Review Board of the Department of Psychiatry, Faculty of Medecine in Geneva. The brains were collected in accordance with the legal procedures in force in the République et Canton de Genève, which, in some cases, require family consent. After the brain had been removed from the cranium, it was examined for neuropathologic abnormalities and then three brain areas were isolated from the right hemisphere by forensic physicians and skilled technicians. The right hemisphere was used because of the reported association with suicidal tendancies and with functional insufficiency (Weinberg, 2000). The isolated blocks of tissues were stored at –80°C and included the ventral prefrontal cortex (PFC) (Broadman's area 11), the hippocampal area (CA zones) and the entorhinal cortex (Broadman's area 28). Table 4 shows the characteristics of the subjects (including controls) and their different groups. On the day of fine dissection, blocks of tissue were thawed from -80°C to -20°C (for about 30 min) for convenient cutting up and then specimens were carefully dissected on a glass surface with dry ice in accordance with the Brodman Atlas. Only grey matter samples (0.5-1-cm coronal slices) were carefully isolated. No white matter was included. Then samples were either stored at -80oC until used or immediately homogenized in appropriate buffers using a tissue grinder.

3. Laboratory Assays: Brain BDNF and NT-3 Levels

Brain BDNF protein levels were assessed using the Western blot technique and NT-3 was analyzed using the Elisa method. In brief, coronal slices (*approx.* 50 mg fresh weight) were homogenized in a lysis buffer [100mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium decolate, 0.1% SDS, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 10µg/ml aprotinin, 1µg/ml leupeptin and 1µg/ml pepstatin]. Homogenates were incubated for 20 min at 4°C with shaking and then centrifuged. The supernatant was used for the assay samples. The loading buffer contained 0.125 mM Tris (pH 6.8), 20% glycerol, 10% mercaptoethanol, 4% SDS and 0.02% bromophenol blue. Samples were heated at 95°C for 10 min before gel loading. Protein concentrations were previously determined using the BCA kit (Pearse Chemical, Rockford, USA). Equal amounts of soluble fraction of brain proteins (25-

50 µg) were electrophoresed on 15% (w/v ratio) SDS-polyacrylamide gel using a mini protean system (Bio Rad) with molecular weight standards. After electrophoresis, the samples were electrotransferred (*approx.* 2h) onto PDVF membranes (Amersham Pharmacia). Samples were incubated overnight at 4ºC with primary antibody for BDNF (1:500 dilution) (sc546; Santa-Cruz Biotechnology, Santa Cruz, CA) or ß-actin antibody (Abcam, UK). After washing, membranes were incubated with an anti-rabbit IgG labeled with HRP (Amersham Pharmacia) for 1 h at room temperature. The membranes were washed and developed using Chemilumescence ECL western blotting (Amersham), followed by exposition of the membranes with radiographic films (Hyperfilm ECL, Amersham Pharmacia). Immunoreactivity quantification was performed by densitometric scanning using an image analysis system (Molecular Analyst, BioRad). The optical density (OD) obtained from each band was normalized against the corresponding ß-actin band.

4. Assay of Brain BDNF and NT-3 Using the ELISA Method

For brain BDNF and NT-3 assay, the ELISA technique was performed using the BDNF and NT-3 *Emax ImmunoAssay system* kits (Promega Inc, Madison, WI) respectively. In brief, Nunc Maxsorb 96-well plates (Nunc, CH) were coated with the monoclonal antibody (anti-BDNF or anti-NT-3) diluted in the carbonate coating buffer and incubated overnight at 4ºC. The coating buffer was then emptied and a blocking solution (provided with the kit) added for 1 hour. 5 washes were performed before adding samples of diluted solution containing known levels of neurotrophin standards (for a standard curve, in appropriate wells). Afterwards, specific polyclonal antibody was added and incubated at room temperature (2h for BDNF or 3h for NT-3) and then washed 5 times before further incubation with a secondary anti-IgY antibody conjugated to HRP (2h for BDNF or 2h30 for NT-3). After careful washing with TBST, a peroxidase substrate (tetramethylbenzine:TMB) was added for 1h to produce color and, finally, samples were acidified by adding 1mM phosphoric acid. A microplate reader (Anthos Labtec Instrument, Chatel St-Denis, CH), set at 450 nm, was used to determine brain BDNF and NT-3 levels in homogenates. The method was able to detect levels as low as 15 pg/ml of neurotrophins.

5. Assay of Plasma PF4 Levels

PF4 is an indicator of platelet activation. PF4 levels were assayed in randomly selected subgroups of 24 subjects composed of 12 patients (6 male and 6 female) and 12 control subjects (6 male, 6 female) using a commercial ELISA kit (Asserachrom PF4; Diago, France). Precautions were taken to avoid *in vitro* platelet stimulation. In these conditions, basal levels of PF4 were found in plasma. Changes in basal levels indicate a change in platelet reactivity.

6. Rat Serum and Brain BDNF Protein and Platelet BDNF mRNA

For the serum BDNF's study, male Wistar rats (Arbresle, France) were used and four age groups were examined (5 rats per group): newborns of one week (NB-1 of 14-16g) and three weeks (NB-3 of 82-86g); two month-old mature rats (M: 200-250g) and two year-old rats (A: 450-500g). Animals were decapitated and the brain was rapidly removed from the skull onto dry ice and then either immediately dissected or stored at –80° C before use within two weeks. For dissection, deep-frozen brains were sectioned manually with a razor blade under the microscope and two hippocampus areas (dentate gyrus and CA nuclei) were cut and mixed before homogenization. For serum BDNF assay, blood was collected in anticoagulant-free tubes and kept at room temperature for one hour and then at 4^{o}C for a second hour before centrifugation at 2000 g x10 min. Serum was then stored at –20^{o}C until use. Alternatively, blood was collected on anticoagulant tubes for platelet-rich plasma isolation and platelets were counted.

Brain BDNF protein was extracted from brain tissue by homogenizing the structures using ultrasonication in 20mM Tris-HCl buffer (pH 8.0) containing protease inhibitors. The homogenates were centrifuged at 14,000 g x 10 min at 4^{o}C and the supernatant was collected. Brain and serum BDNF protein were assayed using an immunoassay kit (ELISA kit from Promega Corp. Madison, WI) and absorbance was read at 450 nm.For the RNA extraction and measurement, platelet count was adjusted by appropriate dilution (approx. 300,000/µl), and then pelleted at 2000 g x 10 min for RNA extraction. For newborns, only 3-week old animals were used for RNA extraction. Samples were collected from 3 rats because of low blood volume. Total RNA was extracted from either frozen brain tissue (hippocampus and frontal cortex) or platelet pellet by using the NucleoSpin RNA II kit (Macherey-Nagel Ltd., Oensingen, Switzerland).

7. Bipolar Disorder-Derived Cells

Lymphocytes were obtained from bipoalr disorder (BD), euthymic patients and 12 healthy control (CT) subjects. Both patient and control populations were balanced with respect to age (37$^{\pm}$6 vs. 40$^{\pm}$7 yrs for BD and CT respectively). The inclusion criteria for BD were at least one manic episode. No comorbidity was revealed in BD and the CT subjects had no known psychiatric or neurological history. The cells were isolated using the Ficoll-Hypaque kit (Sigma, Buchs, CH) and transformed with the Epstein-Barr virus supernatant (Walls et al., 1995). Immortalized cells were cultured at 37^{o}C in humidified atmosphere at 5% CO_2 on RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100µg/ml streptomycin). The cell lines were subcultured every 3-5 days depending on confluence and harvesting was carried out between 8 and 20 passages.

8. Cell Culture and Stimulation of the cAMP-Dependent Protein Kinase (PKA)

Six million cells ($6x10^6$) were transferred into medium with low serum content (2%) and the following drugs were added 24h before cell harvesting: 10 µM Sp-8-Br-cAMPS or 10 µM Rp-8-Br-cAMPS. These drugs are cAMP-PKA activator and inhibitor respectively. Twenty-four hours after adding the drugs, culture was stopped, cells were counted and then rinsed in phosphate buffer (PBS, pH 6.5) and finally homogenized in 0.5 ml ice-cold 25 mM Tris-EDTA buffer, pH 7.4 (containing 1µM IBMX and 100 µM leupeptin, phosphodiesterase and protease inhibitor respectively). The cAMP-PKA enzymatic activity was assayed in cytosol, the PepTag© kit from Promega Corp (Madison, WI). This method has been published in detail in Karege et al., 2001 and 2004.

9. RNA Extraction and Quantitative RT-PCR Assay

Total RNA was extracted from a fixed number ($6x10^6$) of lymphoblasts using the RNeasy kit (Qiagen, Basel, CH) and cDNA was synthesized from 2 µg of tRNA using the RT Omniscript enzyme in 20 µl mixtures, following Qiagen's instructions. For PCR assay, a fragment of 594 bp was amplified from 2 µl of the above cDNA mixture (sequence corresponding to exon 5; Genebank Accession XM-006027) in 50 µl mixtures containing 0.4 µM of each BDNF primer (F-primer: 5'-*agagtgatgaccatccttttcc*-3' and R-primer: 5-*gcagccttctttgtgtaacc*-3'), PCR buffer and 2.5 U of Taq DNA polymerase completed to 50 µl with nuclease-free water. The PCR program was 2 min at 94°C followed by an amplification phase of 32 cycles (94 °C for 1 min, 55 °C for 30 s and 72°C for 1 min) and completed by 5 min at 72°C. A 848 bp sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in 28 cycles (F-primer: 5'-*atcaatggaaatcccatcacc*-3' and R-primer: *tctcttcctcttgtgctcttgc*-3') using the above PCR parameters to act as a control. The PCR products were quantitated using the fluorescent PicoGreen® dye (Molecular Probes, Eugene, OR), a fluorochrome that selectively binds dsDNA (Karege et al., 2004). A standard curve with serial dilutions of commercial dsDNA had been previously established. The results, expressed in ng/µl of BDNF cDNA synthesized in 32 cycles, were normalized to GAPDH PCR products of the same samples and finally expressed as a ratio of BDNF/GAPDH values. The control value in drug-naive culture was used as reference (100 %)

10. Statistical Analyses

All statistical analyses were performed using the StatView IV software from BrainPower Inc., Calabasas, CA). The ANOVA test and ANCOVA were performed for most of the statistical analyses, after the distribution was tested for skewness., Cochran's C test for homogeneity was also performed in some cases. A *post-hoc* Student's test or Mann-Whitney U-test (small population or transformed values) were used to detect significant changes

between groups. Regression analysis (Pearson product moment) was carried out. Statistical significance was set at $p<0.05$ for ANOVA or 0.01 for some *post-hoc* tests (i.e. *Bonferroni test*).

In human blood, serum volume varies from one subject to another. In view of the fact that serum BDNF is released from its platelet stores into the serum (Fujimura et al., 2002), three parameters were analysed: 1) serum BDNF levels were assessed in total serum but expressed with respect to the whole blood volume; this measure represents the fraction of platelet BDNF released into serum (referred to adjusted serum BDNF levels); 2) whole blood BDNF levels were assessed in the blood volume, which represents the total platelet BDNF content and 3) the ratio of serum content to whole blood BDNF content was calculated, which represents platelet capacity to release BDNF into serum.

For brain measures, ANCOVA was used with PMD and age as covariates. The *Bonferroni test* was used for multiple comparisons and significance was accepted at $p<0.01$. To test the effect of gender on various measures, a 2-way ANOVA (gender and diagnosis) was used. The effect of mode of suicide on BDNF/NT-3 levels was tested by comparing violent with non-violent mode of death. An unpaired t-test was used and significance was set at $p<0.05$.

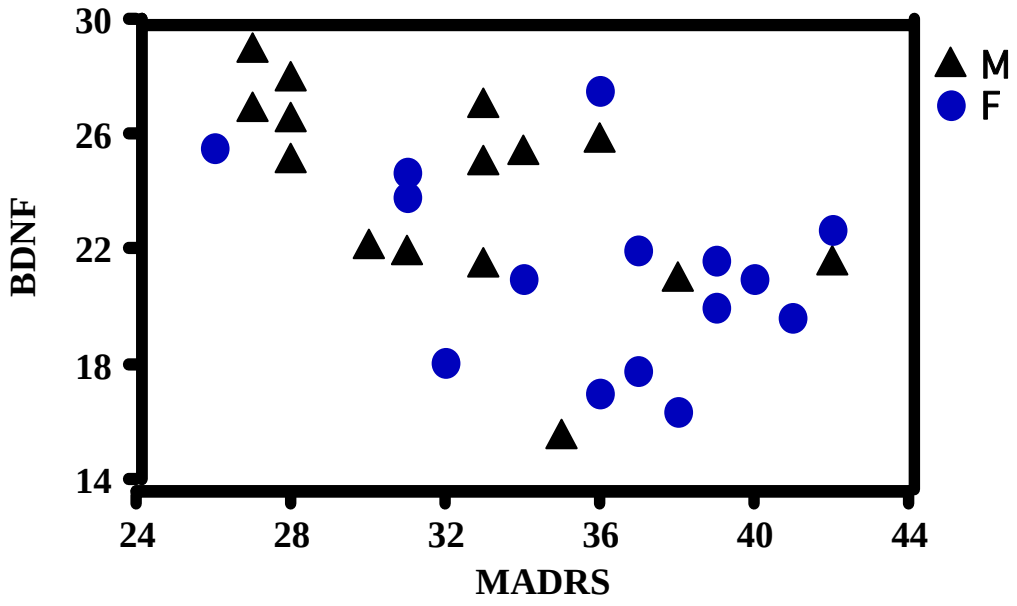

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Figure 1. Correlation between MADRS values and serum BDNF levels in major depressed patients. Thirty (15F + 15 M) major depresed patients were assessed with respect to their severity of depression with the MADRS interview, and their serum BDNF levels were measured with an Elisa kit. The regression analysis yields a significant correlation coefficient (r=-0.55; N=30; p<0.01).

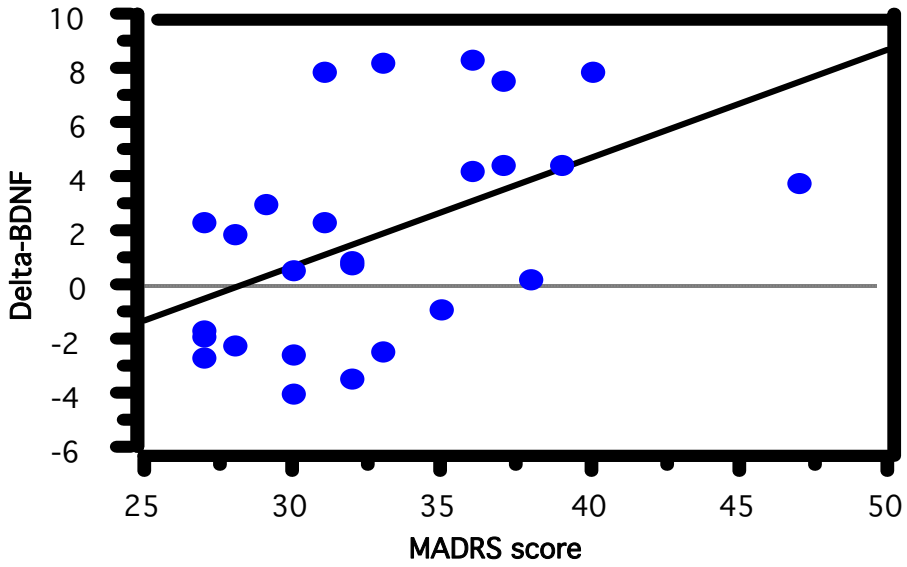

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Figure 2. Correlation between pretreatment MADRS scores and the change in serum levels during treatment. R=0.51, p=0.008.

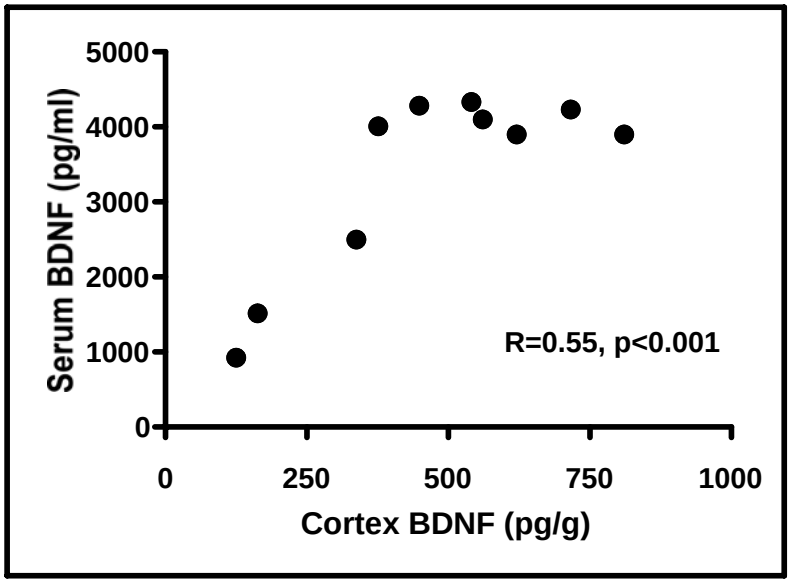

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Figure 3. Correlation of BDNF contents between serum and frontal cortex in rats. To obtain different BDNF content samples, ten rats of different ages (raging from one-week to mature rats) were selected. Cortex and serum BDNF were measured as in the above experiments (see Methods). Of note is the curve slope in low BDNF values (newborns) which became flat in high BDNF levels (mature rats), indicating that there is no more interaction between the two compartments. Regression analysis yields r=0.81, P<0.01.

C. Results

1. Serum BDNF in Drug-Free Depressed and Healthy Subjects

Table 1 gives the mean values (±sd) for serum BDNF in both patient and control subjects. Mean values ± standard deviation are 22.6±3.6 and 26.4±7 ng/ml for patients and controls, respectively (t-test = 2.72; df = 58; p=0.008). Regarding the gender difference, BDNF levels were lower in depressed females than in males (t-test = 2.3; df = 28; p<0.009). The mean value for MADRS score in the patient population was $34^{\pm}5$. Women were more depressed than men: MADRS mean values were 36±4 and 32±4 for women and men respectively (t-test = 2.33; df = 28; p<0.02). A significant negative correlation was observed between patients' BDNF levels and the MADRS scores (r = -0.55; *p<0.02*) (Figure 1). Analysis of covariance (gender and MADRS as independent variables *vs* BDNF content as dependent variable) indicated that depression severity, rather than sex, accounted for the negative correlation between BDNF and depression (F-ratio was 7.60 and 1.67, *p=0.01* and *p=0.20* for severity and gender respectively).

2. Serum BDNF Levels in Antidepressant-Treated Patients

After a four-week period of drug treatment, mean levels of BDNF were significantly increased: BDNF-1=22.6±3.6 ng/ml (pretreatment); BDNF-2=24.4±3.6 ng/ml (post-treatment) [*ANOVA F(1,24)=4.46, p=0.04*]. However, some patients had decreased BDNF levels after treatment. When patients were split into two groups (increased and decreased BDNF levels), there was a statistically significant difference in their preteatment MADRS scores. The group with decreased BDNF level had a low pretreatment MADRS score (mean±sd of MADRS values was *X±sd=29.9±2.8*) compared with the group who had increased their BDNF levels (mean±sd MADRS values were *X±sd=33.4.6±5.2*). *ANOVA* yielded: [*F(1,24)=6.8, p=0.015*]. A positive correlation *(r=0.51, p=0.008*) was observed between pretreatment MADRS scores and the difference in serum before and after antidepressant treatment. The more severe the depressive episode, the more substantial the increase in BDNF level (Figure 2).

3. Whole Blood, Plasma BDNF and Plasma PF4 Levels in MDD

In another group of patients and controls, BDNF was assessed simultaneously in plasma, serum and blood. The aim was to establish BDNF changes in different blood compartments. As serum volume varies individually, serum BDNF concentrations were adjusted as a function of the blood fraction occupied by serum. Table 2 shows the mean values (±SD) for BDNF levels in serum, plasma and whole blood lysate and the fraction of BDNF (percentage) released in serum. Compared with control subjects, a significant decrease was found in patients' serum (*10.07±2.3 vs 12.2±2.4 ng/ml; t test=-2.8; p<0.006*) and plasma BDNF contents (*1685±342 vs 2165±349 pg/ml*; t test =-3.8*; p<0.001*). No difference was found in

BDNF levels between the two groups in whole blood lysate (*16.3±4 vs 16.4±3* ng/ml for patients and controls respectively, *t-test =-0.195; NS*). The fraction (percentage) of blood BDNF released in vitro (serum/blood BDNF ratio) was lower in patients than in control subjects (*64%±15% vs 74%±13%* respectively; *t test = -3.3; p<0.002*). Serum BDNF levels were negatively correlated with the severity of depression (*r=-0.63; p<0.001*) but blood BDNF levels were not (r=0.019; NS). In comparison, BDNF levels found in the above group, because of the adjustment to whole blood volume, the serum values presented in Table 2 are lower than those of Table 1. The plasma PF4 levels assayed in 12 patients and 12 controls are also presented in Table 2. The patients display elevated plasma levels (*24.3±2 vs 18.4±2* IU/ml, for patients and controls repectively). This difference was statistically significant (*z=-3.82; p<0.001*) in plasma, but not in serum values *(NS*).

4. BDNF Levels in Rat Brain and Serum

Table 3 gives rat serum, hippocampal and cortical BDNF levels, which show gradual and significant changes at different ages (ANOVA: $F_{(3,16)}$ =25.7, *P*<0.001). Low BDNF levels were found in one-week old rat serum (mean values: 1174±318 pg/ml), but rapidly increased (up to two-fold) in the three-week old rats (mean values±sd =2463±545 pg/ml), and were four-fold higher in two-month old rats (means±sd= 4828±753 pg/ml). Two-year old rats had nearly the same BDNF values in serum (X=4336±1119) as young adult animals. Similar changes were observed in brain samples, with a gradual increase from the postnatal period to maturation and aging phases both in hippocampus and frontal cortex (ANOVA $F_{(3,16)}$ = 6.1; *P<0.005*; $F_{(3,16)}$ =88.1; *P*<0.001, for hippocampus and cortex respectively). Hippocampal values (range: 1329 to 2207 pg/g of brain tissue) were higher than cortical levels (range 130 to 742 pg/g of brain tissue) at all the five ages examined (Table 3). In old rats, there was a slight decrease in hippocampus (*P*<0.05) but not in cortex BDNF levels (Table 3). The relative value (RV) of BDNF mRNA expression was assessed by comparing the amplified sequences of BDNF from different samples (platelet and hippocampus) to the newborn values (3-weeks old), by using the same total RNA level (figure not shown). Compared to newborns, brain sample values changed in mature and old rats (RV for newborn, mature and old rats: 100%, 260% and 140%, respectively) but the platelet sample did not (RV for newborn, mature and aged rats: 100%, 97% and 102%, respectively). Serum and cortical BDNF levels for rats of different ages were compared in a regression analysis curve. Figure 3 presents a positive correlation between serum and cortex BDNF values (r=0.55, *P*<0.01).

5. Brain BDNF and NT3 in Depressed Suicide Subjects

Table 4 gives the characteristics of the subjects and their different groups. The suicide victims consisted of a well defined population of thirty subjects (19M/11F) carefully determined by forensic physicians. The mean age was 47±17 *years* and the postmortem delay (PMD) from death to storage was 24±19 *hours*. The causes of death are shown in Table 4. The control group consisted of twenty-four non-suicide subjects (14 M /10 F) who died from

a determined cause. Mean age was 45±14 years and postmortem delay (PMD) was 28±21 hours. Subjects with a documented history of neuropsychiatric or neurological disorder or positive toxicology were excluded from this control group. The causes of death varied and included myocardial infarction (n=5), pulmonary embolism (n=2), homicide (n=2), road traffic accident (n=7), various somatic diseases (n=7) and accidental drowning (n=1).

Figure 4 gives mean values (±sd) for optical density (OD) (BDNF/ßactin ratio). To test the effect of diagnosis and drugs, the *ANOVA* yields for hippocampus measures ($F_{3,50}$ *= 6,15; p<0.001),* for PFC measures ($F_{3,50}$*=16,1, p<0.00),* and for entorhinal cortex measures ($F_{3,50}$=0.66; NS) are given. Compared to the control group, Bonferroni *post hoc* tests showed that BDNF levels were reduced in drug-free depressed (*p<0.001*) and non-depressed suicide victims (*p<0.001*), but not in drug-treated suicide victims (*NS*), both in the hippocampus and PFC. There was no significant difference between depressed and non-depressed groups, either in the hippocampus (NS) or PFC of drug-free subjects. No significant change was observed in entorhinal cortex, whatever the suicide group. In the three brain areas, subjects who were drug-treated were not statistically different from controls. To test the effect of age and PMD, the latter were used as covariates and no significant effect on BDNF levels was observed.

ELISA assays in small groups of randomly selected subjects (Table 5) were performed to validate the western blot assay. Likewise, *ANOVA* test yields in the hippocampus and PFC were $F_{3,26}$*=12.4; p<0.001* and $F_{3,26}$*=6.92; p<0.002 respectively*. In the hippocampus, the Bonferroni *ad hoc* test indicated a significant decrease in the drug-free suicide group (with or without depression; *p<0.003* and *p<0.004* respectively), but not in the drug-treated group, compared to the control group. In the PFC, decrease in BDNF levels was significant in drug-free suicide vicitims (with or without depression, *p<0.02* and *p<0.002* respectively). In the entorhinal cortex, no change was found regardless of diagnostic group.

Table 6 shows the NT-3 measures in the respective brain areas. To test the effect of diagnosis and drug treatments, ANOVA yields the following for hippocampus measures: $F_{3,50}$ = 4.65, *p<0.006*. Compared to the control group, the Bonferroni test yields significant decreases in drug-free depressed (*p<0.001*) and non-depressed suicide subjects (*p<0.004*). No change was observed in the drug-treated group (Bonferroni test:NS). In the PFC and entorhinal cortex, there was no change in all suicide victim groups. (ANOVA: NS).

Table 5. BDNF levels in postmortem brain areas of suicide and non-suicide control subjects (assay with the ELISA method)

Brain areas	Subjects (number)			
	Controls (8)	Sui/dep (7)	Sui/other (8)	Sui/drugs (7)
Hippocampus (ng/g)	24.5+3.6	17.7+2.9**	16.8+3.1**	23.3+2.2
Prefront. cortex (ng/g)	17.5+3.0	13.8+2.6*	12.7+2.6**	17.9+2.9
Entorhinal cortex (ng/g)	13.4+2.5	14.1+2.1	13.4+2.4	12.9+2.3

Mann-Whitney test: * P<0.01 ; **P<0.005.

Table 6. NT-3 levels in *postmortem* brain areas of suicide and non-suicide control subjects (assay with the ELISA method)

Brain areas	Subjects (number)			
	Controls (24)	Sui/dep (10)	Sui/other (10)	Sui/drugs (10)
Hippocampus (ng/g)	10.5+2.8	7.6+2.6*	7.3+3.4**	10.3+2.2
Prefront. cortex (ng/g)	9.2+2.3	8.9+2.4	9.6+2.7	8.9+2.9
Entorhin. cortex (ng/g)	7.8+2.1	6.9+2.6	7.3+2.7	7.7+3.4

Student t-test *P<0.01; **: P<0.005.

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Confounding effects of age and PMD were tested. The latter were used as covariates in ANCOVA and yielded non-significant effects on BDNF levels, in both the hippocampus (*p=0.613, NS*) and PFC (*p=0.762*, NS).

The effect of gender on BDNF and NT-3 levels was analyzed by using either a two-way ANOVA (gender and diagnosis) or unpaired t-test of independant variables and no effect of gender was observed on neurotrophin values (*p=0.429* and *p=0.521* for hippocampal BDNF and NT-3 values respectively).

6. BDNF mRNA Expression in cAMP-PKA Activated Cells from BD

Lymphoblast BDNF levels were assessed in drug-manipulated cAMP-PKA activity. A time-course of PKA activity from 3 h to 72 h culture of control cells has indicated that, after 24 h drug treatment, cAMP-PKA activity was significantly increased by cAMP analogs (Sp-8-Br-cAMPS) and decreased by the Rp-8-Br-cAMPS (Karege et al. 2004). Then, cAMP-PKA activity and BDNF levels were measured after 24h in cells treated with enzyme activator and inhibitor respectively. Figure 5 gives mean values for PKA activity. In all groups, the Friedman test indicated significant differences among groups (*Chi-2=35.9; df=5, p<0.001*). *Post-hoc* analyses using the Mann-Whitney U-test showed that cells extracted from bipolar disorder (BD) show significantly higher PKA activity, either in drug-naive (*Z=-2.31; p<0.02*) or in Sp-treated PKA activity, than controls (*Z=-2.19 p<0.01*). The Sp-isomer increased PKA activity both in BD (Z=-3.41; *p*<0.01) and in CT-derived cells (Z=-2.68; *p*<0.02). The Rp-isomer decreased PKA activity in both groups of populations compared to their respective naive conditions: BD cells (*Z=-3.00; p<0.01*) and control group cells (*Z=-2.16 p<0.02*). The relative levels of BDNF mRNA expressed as ratios of GAPDH mRNA levels are shown in Figure 6. The Friedman test for analysis of variance indicated significant changes among groups (*Chi-2=39.3; p<0.001*), while no change was observed in GAPDH mRNA (not shown). Mann-Whitney *post-hoc* analyses of BDNF levels yielded no difference between BD *vs* respective CT cells, either in drug-naive (*NS*) or in Sp-treated cells (*NS*). The Sp-isomer drug induced an increase in BDNF levels in BD (*Z=-3.81; p<0.001*) and in CT cells (*Z=-3.31; p<0.001*). The Rp-isomer decreased BDNF expression in BAD cells (Z=-3.84; *p*<0.001) but not in CT subjects (*Z=-1.1; NS*). In Rp-isomer culture, BD subjects express significantly less BDNF than CT subjects (*Z=-2.8; p<0.02*).

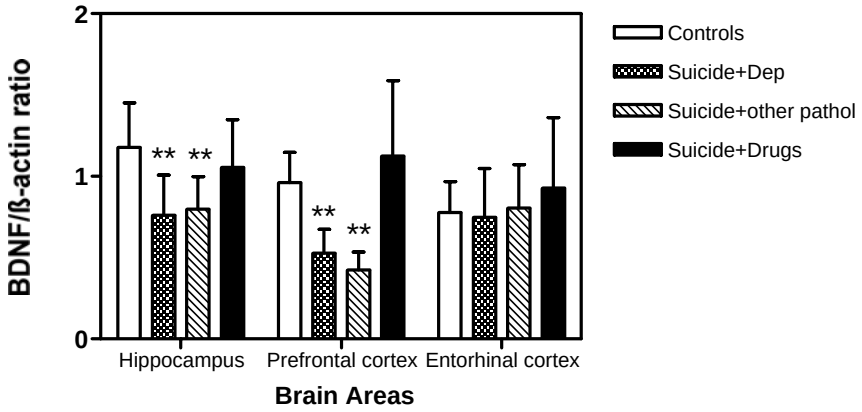

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Figure 4. Mean values (±) of BDNF protein in soluble fraction of the hippocampus, prefrontal and entorhinal cortex from non-suicide controls and 3 groups of suicide victims : drug-free suicide victims either with depression (group 1) or without depression but with various psychiatric diseases (group 2) and drug-treated suicide victims with depression (group 3). The values are expressed as a ratio of the optical density (OD) of BDNF and ß-actin proteins. The statistical differences were analyzed with ANOVA: -in hipppocampus : F3,50=6,15, p<0.001. Bonferroni for multiples comparions: significance was accepted at 0.01 : with respect to controls:drug-free major depression group: p<0.001; drug-free with other psychiatric diseases: p<0.001; drug-treated major depressed group: p<0.03; NS.-in PFC: F3,50 : 16,1, p<0.001. Bonferroni for multiples comparisons: significance was accepted at p<0.01: drug-free major depression group: p<0.001; drug-free with other psychiatric diseases : p<0.001; drug-treated major depressed group: p<0.06; NS. -in entorhinal cortex: F3,50= 0.66, NS. Bonferroni for multiple comparisons yields no significant difference between groups.

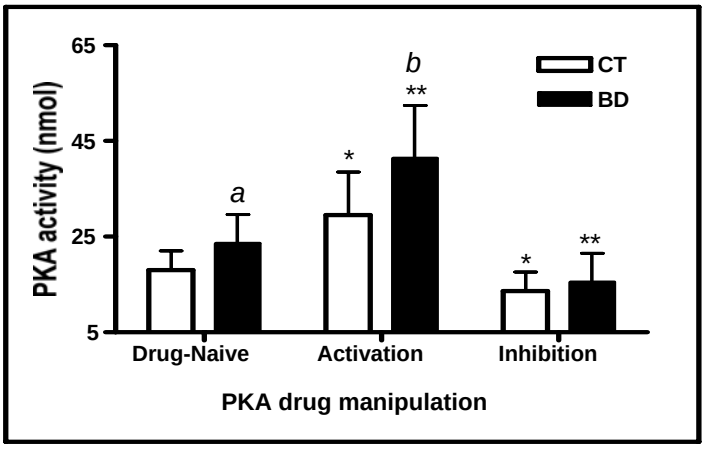

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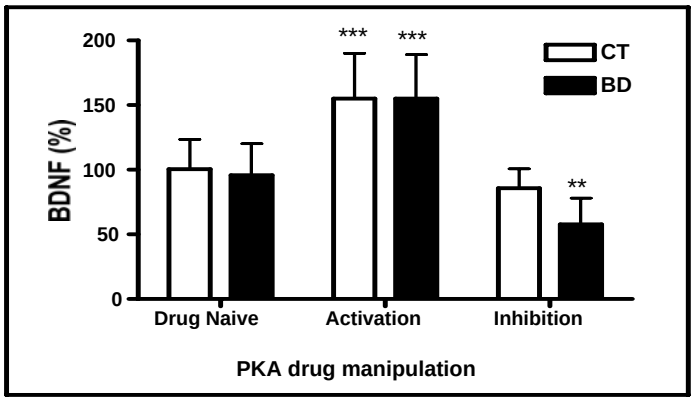
Figure 5. Cyclic-AMP-dependent PKA activity in drug-treated cells. Values (+sd) from 12 bipolar- and 12 control-derived cells cultured either in drug-naive, in 10 µM Sp-8-bromo-cAMPS) or in 10 µM Rp-8-bromo-cAMPS for 24 h. Results are expressed in nmol/min/mg prot of phospho-kemptide levels. Friedman test's analysis of variance yielded: Chi=35.9 ; df=5, p<0.001. Comparison between bipolar and control cells with non-parametric Mann-Whitney U-test : ¶¶: p<0.01 and ¶ : p<0.02. Comparison between drug-treated and naive cells ** ; p<0.01 and * p<0.02.



(reproduced from Karege et al 2004, with the agreement of Neuropsychopaharmacology.org).

Figure 6. BDNF gene expression in lymphoblasts from 12 control (open) and 12 bipolar (closed) subjects, cultured either in drug-naive (N : circle), in PKA stimulator (Sp-isomer: triangle) or inhibitor (Rp-isomer : diamond-shaped) conditions. Results were expressed in ratio of BDNF/GAPDH PCR products. Friedman test yielded significant changes ($p<0.001$) and Mann-Whitney test: drug-treatment vs control (CT-N): a) $p<0.001$; comparison between BAD-Rp and CT-Rp: ¶) $p<0.02$.

D. Discussion

The neurotrophin hypothesis of affective disorders has prompted various studies on the regulation of BDNF and NT-3, both of central and peripheral origin. This chapiter has presented studies that we have undertaken in humans, animals and cell cultures to explore this hypothesis. The first investigation was conducted on serum BDNF of drug-free normal healthy and depressed patients. Patients were subsequently given drug treatment for 4 weeks, and BDNF levels were again assessed. In a second study, we investigated whether, like serum, whole blood BDNF levels are also altered in mood disorders. As previously demonstrated, almost all blood BDNF levels are stored in blood platelets and, upon activation, released into serum (Radka et al., 1996; Fujimura et al., 2002). However given that all platelet BDNF is not releasable into serum, how does the non-released BDNF behave? The aim of this second study was to assess the relationship between serum BDNF (released) and whole blood BDNF (both releasable and non-releasable) in affective disoders. Information on the relationship between serum and brain BDNF is also of fundamental importance. The study was performed on rat blood and brain cortex during the postnatal maturation period. The study also aimed to disclose the postnatal development profile of blood and brain BDNF. The fourth study was conducted on *postmortem* human brains of depressed suicide and non-suicide subjects. In this study both brain BDNF and NT-3 were assessed. The role of antidepressant drugs on human brain and hippocampal BDNF levels was also investigated. The last study, about the expression of BDNF in bipolar disorder, was conducted in BD-derived lymphoblast cells pharmacologically treated to manipulatate the second messengers that control the gene expression of BDNF. The results from these various studies are dicussed below.

1. Serum, whole Blood and Plasma BDNF.

Globally, the results from human serum show that depressed patients were characterized by a statistically significant decrease in serum BDNF levels and that this decrease was correlated with the severity of depression, as measured by the MADRS scores. Women patients presented the largest decrease in BDNF levels and relatively higher MADRS scores than men. Previous data based on animal models and confirmed in depressed subjects have indeed indicated an association of platelet BDNF with mood (Radka et al., 1997; Duman et al., 1997; Altar, 1999; D'Sa and Duman, 2002; Surayama et al., 2000). Blood platelets have been extensively used in studies of mood disorders and changes in some components of the 5-HT system (5HT transporter, platelet 5HT and 5HT2a receptors) have been proposed as peripheral markers of depression (Mann et al., 1992; Nemeroff et al., 1994; Hirdina et al., 1995). In addition, platelets and neurons develop from a common embryonic precursor in the neural crest (Pearse, 1980). The negative correlation previously observed between platelet 5HT content and the severity of depression should be noted (Mann et al., 1992). For all these reasons, platelets are thought to offer an index of brain 5HT function. Our findings suggest that platelet BDNF may also be a peripheral marker of depression. The data provide additional and specific evidence of a possible role played by BDNF in the pathophysiology of depression. In line with Duman's cellular hypothesis of depression (Duman et al., 1997), these findings lend further support to the neuronal morphologic approach to depression, which proposes that alteration in the expression of neurotrophic factor could be responsible for disturbed neural plasticity (Duman et al., 1997; Sheline et al., 1996; Rajkowska et al., 2000). It is interesting to note that similar findings have been reported in schizophrenic patients with respect to neurotrophic factors. Low plasma nerve growth factor (NGF) was found in schizophrenic patients, while abnormal expression of BDNF and its cognate receptor was observed in the cortico-limbic system (Bersani et al., 1999; Takahashi et al., 2000).

2. Post-Treatment Serum BDNF

After drug treatment, mean levels in patients increased significantly, compared with pre-treatment levels. Results suggest that, in this popualtion, an increase in peripheral plasma BDNF level can be measured in a subgroup of treated subjects in remission. However, a decreasing trend in the evolution of serum BDNF levels before and after antidepressant treatment can be observed in a subgroup, and statistical analysis indicates that this group has a lower MADRS score and thus seems to be less severely depressed. There is a correlation between the severity of depression before treatment and the size of the change in serum BDNF levsls. Shimizu and coworkers have reported comparable results, and concluded that drug treatment may increase serum BDNF levels (Shimizu et al., 2003). These findings lend further support to the cellular hypothesis and the mecahnisms through which antidepressants react with cellular mechanisms. However, we must be cautious before drawing any firm conclusion. We are aware that platelet cells are not brain cells, and the possibility of an epiphenomenon can not be excluded. Moreover, considering the multidimensional aspect of affective disorder, an alternative explanation for this association could be supplied by other

components of depression such as anxiety, agitation, depression-associated motor slowing or other symptomatic dimensions of this disease (Pandey et al., 1999; Russo-Neurstadt et al., 1999; Oliff et al., 1998).

When asessing both whole blood BDNF levels and the fraction of blood BDNF released into the serum, we found that the latter decreased in major depression, while whole blood BDNF levels remained unchanged in the same population. The decrease in released BDNF, but not in total BDNF, was correlated with the severity of depression. The study also indicates that, in contrast to serum BDNF, plasma levels of PF4 increase in subjects with major depression as compared with control subjects. This study provides evidence that the alteration in serum BDNF levels in depression is unrelated to change either in whole blood BDNF levels or in the platelet release of the activation marker, PF4.

By assessing whole blood BDNF levels, this study aimed to investigate the origin of that decrease. The study accounts for an absence of change in whole blood BDNF levels in depressed patients, together with a change in serum BDNF, thus suggesting that the two blood compartments may be regulated differently. The decrease in serum BDNF levels may be due to a defect in platelet release, since there is evidence that serum BDNF is released from platelet stores (Fujimura et al., 2002). However, following this decrease in platelet release, a decrease in other platelet elements, such as PF4, would be expected. Curiously, assay of plasma PF4 levels revealed an elevation in these levels, indicating elevated platelet reactivity. Elevated platelet reactivity has been consistently posited in depressed patients, as was indicated by increased plasma levels either of PF4 and β-TG or increased expression of procoagulant platelet surface receptors (Markovitz et al., 2000; Nemeroff and Musselman, 2000; Kuijpers et al., 2002). How could we reconcile the fact that two proteins that are supposed to be stored in the same α-granules have inverse regulations of release? Are there different subcellular localisations for these proteins? Our observations, suggest different mechanisms of BDNF secretion and PF4 release. The profile of platelet BDNF release brings the release of platelet serotonin to mind. Firstly, a correlation has been shown between platelet 5HT release and BDNF release (Radka et al., 1996). Secondly, like BDNF, platelet 5HT levels are negatively correlated with the severity of depression in major depression (Mann et al., 1992) and with the severity of anxiety in post-traumatic stress disorder (Spivak et al., 1999). Lastly, any study of the mechanisms of BDNF secretion should begin by identifying the elements of platelet exocytosis machinery in order to examine the regulation of proteins involved in vesicle docking and fusion. Early studies have indicated that some of the proteins, which, it has been hypothesized, provide vesicle trafficking in neuronal release, are also present in platelets (Bahler et al., 1990, Lemons et al., 1997, Flaumenhaft et al., 1999). Platelet membranes were shown to contain SNARE and other transport proteins (e.g. NSF, SNAPs., syntaxin 2 and 4), thus indicating that platelet exocytosis could use a molecular mechanism similar to other secretory cells (Bahler et al., 1990; Lemons et al., 1997). Moreover, in neuron cultures, intracellular trafficking/processing and secretion of BDNF were shown to be complex and to depend on BDNF gene polymorphism and subcellular locations (Egan et al., 2003). Abnormalities in SNARE proteins were reported in the brains of depressed patients (Honer et al., 2002). Although this conclusion may be speculative at the current stage of our work, platelet trafficking proteins are interesting

candidate molecules for further studies that could test how platelet BDNF release is altered in major depression.

3. BDNF Levels in Rat Serum and Brain: The Maturation Profile

The relationship between serum and brain BDNF is a fundamental problem. We have addressed this issue by assessing blood and brain cortex BDNF levels in rats during the postnatal maturation period. Thus, the experiment has also disclosed the postnatal developement profile of blood and brain BDNF. Our findings showed age-associated changes in BDNF levels, both in serum and in central structures. The changes in brain and serum BDNF protein levels were fairly similar, as were changes in hippocampal BDNF protein. When plaletet BDNF mRNA was measured, a dissociation was observed between mRNA and protein levels. Platelet mRNA levels were too low and not age-associated, while platelet protein levels were abundant and age-related, which suggests an external origin for a major part of platelet BDNF protein. In a report from Nakahashi et al., (2000), vascular endothelial cells were shown to synthesize BDNF, which is then secreted into the circulatory system. Lastly, a positive correlation was observed between cortical and serum BDNF contents, especially in newborn rats (serum BDNF less than 3000 pg/ml), indicating that platelet and cortex BDNF levels express similar and parallel development profiles in the rat.

From a developmental viewpoint, this issue of blood BDNF has recently assumed added importance following studies of a correlation between peripheral BDNF levels and autism and mental retardation (Nelson et al., 2001). Futhermore, in schizophrenia, another development-linked pathology, low expression of neurotrophins was shown in the CNS and blood samples (Bersani et al., 1999; Takahashi et al., 2000). The fact that both serum and brain levels rise in some regions during development and then fall in aging means that there are similar developmental profiles, probably driven by a similar mechanism. It is legitimate, therefore, to seek the cause underlying this mechanism. An interaction between blood and brain BDNF was hypothesized on the basis of reports on BDNF passage from blood to CNS and *vice-versa* (Pan et al., 1998a; 1998b). Indeed, BDNF was shown to cross the BBB and a direct permeation into the CNS through the spinal cord or brain was demonstrated for other neurotrophins (Pan et al., 1998a, 1998b). Neurotrophins can enter the CNS by retrograde axonal transport (DiStephano et al., 1992) and a high-capacity transport to the CNS, as well as an efflux from brain to blood of unmodified BDNF have been reported, suggesting the involvement of specific carrier systems (Pan et al., 1998a, 1998b. The interaction between blood and brain BDNF has proved to be a hypothesis well worth investigation.

4. Postmortem Brain BDNF and NT-3 in Depressed Suicide Victims

The study of brain neurotrophins has demonstrated a significant decrease in BDNF levels in both the hippocampus and ventral prefrontal cortex, but not in the entorhinal cortex, of suicide victims compared with non-suicide subjects. The decrease was observed in all drug-free suicide victims, whatever the diagnosis, but not in drug-treated subjects. A decrease in

NT-3 levels was also observed in the drug-free hippocampus, but not in drug-free PFC or entorhinal cortex, regardless of diagnosis.

There are very few studies, however, on neurotrophins and suicide, and data on NT-3 are even scarcer. In the case of BDNF, a pioneer postmortem study has reported an antidepressant drug-associated upregulation of BDNF expression in major depression (Chen et al., 2001). Another study has demonstrated that levels of BDNF, as well as of its receptor TrkB, are decreased in the prefrontal and hippocampus of suicide victims, regardless of whether they were depressed or not (Dwivedi et al., 2003). The present data provide novel evidence that BDNF and NT-3 play a role in the pathophysiology of suicidal behavior. The decrease in neurotrophins was observed not only in suicide victims with documented depression but also in those with non-documented depression, but diagnosed with other mental disorders such as schizophrenia, anxiety, Alzheimer's disease, alcohol abuse and panic disorder. Whether these changes were related to the above diseases or to suicide *per se* is as yet unclear.

The data also show that subjects who were on drug treatment before death, have "normal" values for BDNF and NT-3, suggesting a drug-induced "normalization" of their levels. An early study reported increased BDNF immunoreactivity in the hippocampus of subjects treated by AD drugs when compared with non-treated subjects (Castrén et al., 2004). Moreover, expression of the high affinity BDNF receptor, Trk-B mRNA, is increased in drug-on subjects compared with drug-off subjects. When considered as a whole, these results suggest that neurotrophins may be involved in the drug's effects (Altar et al., 2003; Castrén et al., 2004). Importantly, it was recently shown that AD drugs activate the release of endogenous BDNF and induce its signal transduction (Saarelainen et al., 2003). BDNF and NT-3 signal through specific high affinity receptors of the tyrosine kinase type, trkB and trkC repectively. After BDNF has bound to the full-length form of its receptor, trkB dimerizes, auto-phosphorylates and promotes activation of several intracellular pathways, *via* several classes of enzymatic effectors (i.e. PLCγ, MAPK, PI-3K) and transcription factors (i.e. CREB) (Schinder and Poo, 2000). However, it has been demonstrated that transgenic mice with reduced trkB signaling or BDNF knockout ($BDNF^{+/-}$) are insensitive to the behavioral effects of AD drugs (Saarelainen et al., 2003). This suggests, therefore, that neurotrophins are sufficient and necessary for antidepressant-produced behavioral effects. However, the observation that normalizing BDNF levels has not prevented the act of suicide, suggests that changes in neurotrophin levels may precede the clinical or behavioral effect, which slowly develops after trophic process have been initiated. An acute change in neurotrophins levels, therefore, should not be critical to their mode of action. These molecules would rather play the role of mediators for the underlying pathologies of psychiatric illnesses. It is important to remember that suicide is not merely a psychosocial setback but that it always occurs in the context of psychiatric illness. Over 90% of cases are associated with significant psychiatric disease (mood disorder accounts for 60%) (Kamali et al., 2001; Mann et al., 2001), although most patients with psychiatric illness do not commit suicide, suggesting that some patients have a vulnerability to suicidal behavior.

The changes in neurotrophin levels were found in the hippocampus and ventral prefrontal cortex. The hippocampus is a limbic area involved in affective and cognitive functions. In affective disorders, the volume of the hippocampus was shown to be decreased, which

suggests neuroplastic impairment (Bremmer et al., 2000]. In a previous meta-analysis, BDNF was shown to be one of the very few abnormal molecules (together with reelin, SNAP-25, complexin, all of which are measures of synaptic density or neuronal plasticity) to be reduced in the hippocampus of psychiatric patients (Knable et al., 2004). Our observations of deficiency of BDNF and NT-3 in the hippocampus are consistent, therefore, with the possibility that the neuronal plasticity of the hippocampus is impaired.

With regard to the prefrontal cortex, there is evidence that, in humans, PFC may be involved in behavioral inhibition, decision making and the expression of emotion (Bechara et al., 2000). Lesions of the PFC are commonly associated with the development of depression or aggression. In attempting to identify the area of the prefrontal cortex that may influence the risk of suicide, Arango and coworkers found that the reduction in $5HT_{1a}$ receptors was most prononced in the ventral PFC, and concluded that suicide victims have an abnormality in the serotonin system predominantly involving the ventral PFC (Arango et al., 1995).

5. cAMP-PKA Signaling-Regulated BDNF Expression

Basal and drug-manipulated lymphoblast PKA activity can be performed by cAMP analogs. Treatment with a PKA activator, Sp-8-Br-cAMPS, increased activity in both BD and CT, but did not affect PKA Cα subunit levels. Conversely, the use of a PKA inhibitor, Rp-8-Br-cAMPS, also resulted in decreased activity and protein levels. In basal conditions, there was no difference in BDNF expression between BD and CT subjects. Activation of PKA increased BDNF expression, while PKA inhibition decreased it, specifically in the BD population. Thus, when PKA activity and protein levels are lowered, BD patients express less BDNF compared with control subjects. Expression could be reduced in bipolar affective disorder, therefore, by slowing down PKA activity and protein levels. We have previously observed that PKA activity is upregulated in BD without any change in BDNF levels compared with controls (Karege et al., 2003). Moreover, in a report from Chen et al. (2001) on the human postmortem brain, no difference was observed in hippocampal BDNF levels between BD and CT subjects.BDNF levels were decreased in major depression with respect to controls, although BDNF expression was found to be increased in antidepressant-treated major depression subjects, without changes in ATD-treated BD.

Among the multiple cell signal transduction pathways, cAMP signaling has attracted particular interest (Tardito et al., 2000; Stewart et al., 2001; Bezchlibnyk and Young, 2002) and hyperfunction of cAMP and downstream targets has been postulated for the etiopathology of BD (Young et al., 1993; Perez et al., 1999). The first cAMP signal target is protein kinase A (PKA), an important phosphorylating enzyme that activates the transcription factors, *i.e*. the cAMP responsive element binding (CREB) protein, and controls the expresssion of critical genes such as brain-derived neurotrophic factor (BDNF) (Shieh and Ghosh, 1999). Elevated PKA activity was reported both in discrete postmortem brain regions (Fields et al., 1999; Chang et al., 2003) and in peripheral cells from BAD patients (Perez et al., 1995; 1999; Tardito et al., 2003; Karege et al., 2003). The present data, compared to studies on major unipolar depression, suggest a different dysfunction of the cAMP signal and its target, BDNF. It is worth mentioning that PKA activity was generally reported to be

decreased in major depression (Shelton et al., 1996; Perez et al., 2001), whereas BDNF levels were found to be decreased either in the postmortem brain tissue (Chen et al., 2001) or in the serum of major depression patients (Karege et al., 2002a; Shimizu et al., 2003). In addition, antidepressant drugs induce an increase in cAMP signal and BDNF levels (Duman et al., 1997) while lithium, the most commonly used mood stabilizer, causes a decrease in cAMP signal (reviewed by Li et al., 2000). Our findings, therefore, combined with those reports, provide new insights, which may explain some of the pharmacological features found in the different diagnostic groups of depressive subjects.

6. Conclusion

Discovered more than half a century ago (at least for the first of them, NGF), the neurotrophins have not yet ended to amaze the scientific community. The small NGF-related family of neurotrophic factors, grown now up to four members with BDNF, NT-3 and NT-4, still remains enigmatic molecules, with a wide range of roles, including higher order CNS functions. The past decade has seen their emergence in the pathophysiology and psychopharmacology of affective disorders.

Our studies presented in this chapiter, together with reports from other laboratories, have contributed to demonstrate that the expression of neurotrophins is altered, both in peripheral cells and CNS of patients with mood disorders; that this alteration is rectified by drug effective treatments, and that BDNF expression is regulated by the cAMP signaling pathway. Working on genetic variations, complementary studies from independent laboratories have shown for BDNF, risk variants (BDNFval/met) for bipolar disorder and cognitive functions. Moreover, last past decade has also seen a surge of neuroimaging studies, which are now imposing a new view of psychopatholgy of affective disorders and treatment's action. Emerging from research performed directly from human brains, this new view emphasizes defects in information processing within critical networks as the mechanisms underlying mood disorders, as well as the role of plasticity induced network recovery drug action.

Mechanistic studies on various neurotrophin signaling pathways (thanks to the characterization of Trk recepetor and $p75^{NRT}$, and their intracellular effectors) have tremenndiously advanced. Important breakthroughs have been achieved: a number of outstanding reports consider affective disorder as being due to transduction mechanisms abnormaly regulated, associated with impairment of structural plasticity and cellular resilience. Being given their role in cell survival, axon growth, synapse formation, and with the help of this neuroplastic approach, the role of neurotrophin in affective disorders studies and therapies, will surely increase and better appreciated in the close future.

However the challenge will be the translation of this improved understanding into better clinical strategies for the treatment and prevention of mood disorders. The recent description of "BDNF mimetics" having partial agonit's actions, and the possibility of developing small molecules that activate Trk receptors acting on G-protein-coupled recptors to activate neuroprotective pathways, prove however, that therapeutic use could be a reality.

References

Altar, CA (1999). Neurotrophins and depression. *Trends in Pharmacological Sci*. 20, 59-61.

Altar CA, Whitehead RE, Chen R, Wörtwein G, Madsen, TM(2003): Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. *Biol. Psychiatry* 54 :703-709.

Arango, A, Underwood,MD, Gubbi AV, Mann, JJ (1995). Localized alterations in Pre-and postsynaptic serotonin binding sites in the ventrolateral prefrontal Cortex of suicide victims, *Brain Res.* 688: 121-133.

Bahler M, Cesura, AM, Fisher G, Kuhn, H, Klein, RL, Da Prada M (1990). Serotonin organelles of rabbit platelets contain synaptophysin. *Eur. J. Biochem.* 194 : 825-29.

Barbacid M (1994). The Trk family of neurotrophin receptors. *J. Neurobiol*. 25: 1386-1403.

Barde YA, Edgar D, Thoenen H (1982). Purification of a new neurotophic factor from mammalian brain. *EMBO J.* 1: 549-553.

Barnabe-Heider F and Miller FD (2003). Endegenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathway. *J. Nerurosci*. 23 : 5149-5160.

Bengzon J, Soderstrom S, Kokaia Z, Kokaiai M, Enfors P et al (1992). Widespread increase of nerve growth factor protein in the rat forebrain after kindling-induced seizures. *Brain Res.* 587 : 338-342.

Burbach GJ, Hellweg R, Haas CA, Del Turco D, et al (2004). Induction of BDNF in plque-associated glial cells of aged APP23 transgenic mice. *J. Neurosci.* 24: 2421-2430.

Bechara A, Damasio H, Damasio AR (2000). Emotion, decision making and the orbitofrontal cortex, Cereb. *Cortex* 10: 295-307.

Binder DK (2007). Neurotrophins in the dentate gyrus. *Progress in Brain Research* 163: 371-397

Bremmer, JD, Narayan M, Anderson ER, Staib LH, Miller L, Charney DS (2000). Hippocampal volume reduction in major depression. *American Journal of Psychiatry* 157 , 115-118.

Bersani G, Iannitelli A, Maselli P, Pancheri P, Aloe L, Angelucci F, Alleva E (1999). Low nerve growth factor plasma levels in schizophrenic patients: a preliminary study. *Schizophrenia Research* 37, 201-203.

Bezchlibnyk Y and Young LT (2002). The neurobiology of bipolar disorder : focus on signal transduction pathways and the regulation of gene expression. *Can J. Psychiatry* 47:135-148.

Castrén E (2004): Neurotrophins as mediators of drug effect on mood. Addiction, and Neuroprotection. *Mol. Neurobiol*. 29: 289.

Chang A, Li PP, Warsh JJ (2003). Altered cAMP-dependent protein kinase subunit immunolabeling in postmortem brain from patients with bipolar affective disrder. *J. Neurochem.* 84: 781-791.

Cheng B, Goodman Y, Begley JG, and Mattson MP (1994). Neurotrophin-4/5 protects hippocampal and cortical neurons against energy depreivation and excitatory amino acids-induced injury. *Brain Res*. 650 : 331-335.

Chao, M. V. and Hempstead, B. L. (1995). p75 and trk: A two receptor system. *Trends Neurosci.* 19, 321–326.

Chen B, Dowlatshahi D, MacQueen, GM, Wang, J-F., Young, LT (2001). Increased hippocampal BDNF immunoreactivity in subjects treated with anitdepressant medication. *Biol. Psychiatry* 50, 260-265.

Chao MV, (2003). Neurotrophins and their receptors; a convergence point for many signalling pathways. *Nat. Rev. Neurosci.* 4 : 299-309.

Chao MV and Lee FS (2004): Neurotrophin survival signaling mechanisms. *J. Alzheimer's Dis*. 6: S7-11.

Chao MV, Rajagopal R, Lee FS (2006). Neurotrophin signaling in health and desease. *Clin. Science* 100: 167-173.

Chen ZY, Patel PD, Sant, G et al. (2004) Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *J. Neurosci.* 24, 4401–4411

Chen K, Nishimura, M, Armanini, M., Crowley C, Spencer, S and Phillips H. (1997). Disruption of a single allele of the nerve growth factor gene results in atrophy of basal forebrain cholinergic neurons and memory deficits. *J. Neurosci.* 17, 7288–7296

Conner JM, Lauterborn JC, Yan Q, Gall CM and Varon S (1997). Distribution of BDNF protein and mRna in the normal adult rat CNS-evidence for anterograde axonal transport. *J. Neurosci.* 17: 2295-2313.

Connor B,Young D, Yan Q, Faull, RM, Syenek B, Dragaunow M, (1997). Brain-derived neurotrophic factor is reduced in Alzheimer's disease, *Mol. Brain Res.* 49: 71-81.

Conover JC, Erickson JT, Katz DM, Bianchi LM et al (1995). Neuronal deficits, not involving motor neurons in mice lacking BDNF and/or NT4. *Nature* 375: 235-238.

Coppola V, Kucera J, Palko M.E(2001). Dissection of NT3 functions in vivo by gene replacement strategy. *Development* 2001;128:4315–4327.

Coppola V et al. (2004). Ablation of TrkA function in the immune system causes B cell abnormalities. *Development* 131:5185–5195.

Croll, S.D., Ip, N.Y., Lindsay, R.M. , Wiegand, S.J (1998). Expression of BDNF and trk B as a function of age and cognitive performance, *Brain Res*., 812: 200-208.

DiStefano, P.S., Friedman, B., Radzejewski, C., Alexander, C., Boland, P., Schick, C.M., Lindsay, R.M., Wiegand, S.J., The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons, *Neuron*, 8 (1992) 983-993.

Donovan M.J, et al. Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development* 2000;127:4531–4540.

Duman RS, Heninger GR., Nestler EJ (1997). A molecular and cellular theory of depression. *Arch Gen. Psychiatry* 54, 597-605.

Duman RS (2002). Synaptic plasticity and mood disorders. *Mol. Psychiatry* 7: S29-S34.

Durany N, Michel T, Kurt J, Cruz-Sanchez FF, Cervas-Navarro J, Riederer P (2000). Brain-derived neurotrophic factor and neurotrophin-3 levels in Alzheimer's disease brains. *Int. J. Dev. Neurosci*. 18: 807-813.

Dwivedi Y, Rizavi HS, Conley RR, Tamminga CA, Pandey GN (2003) : Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Arch Gen. Psychiatry* 60: 804-815.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Weinberger DR (2003) : The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112: 257-269.

Elmer E, Kokaia M, Ernfors P et al (1997). Suppressed kindling epileptogenesis and perturbed BDNF and Trk B gene regulation in NT-3 mutant mice. *Exp. Naeurol* 145: 93-103.

Emamghoreishi M, LI PP, Schlichter L, Sugar V, Parikh SV Cooke R, Warsh JJ (2000). Associated disturbances in calcium homeostasis and G-protein-mediated cAMP signaling in bipolar I disorder. *Biol. Psychiatry* 48: 665-673.

Enfors P, Ibanez C, Ebendal T, Olson L, Peterson H (1990). Molecular cloning and neurotrophic activities of a protein with structural similairties to nerve growth factor : development and topographical expression in the brain. *PNAS USA* 87: 5454-5458.

Fields A, Li PP, Kish SJ, Warsh JJ (1999). Increased cyclic AMP-dependent protein kinase activity in postmortem brain from patients with bipolar affective disorder. *J. Neurochem.* 73: 1704-1710.

Flaumenhaft R, Groce K, Chen E, Furie B, Furie BC (1999) : Proteins of the exocytotic core complex mediate platelet α-granule secretion. Role of vesicule-associated membrane protein, SNAP-23, and synthaxin-4. *J. Biol. Chem.* 274 :292-2501.

Fossati P, Radtchenko A, Boyer P (2004) : Neuroplasticity : from MRI to depressive symtoms. *Eru. Neuropsychophramacol.* S503-510.

Fryer RH, Kaplan DR, Feinstein SC, Redeke MJ et al (1996). Development and mature expression of full length and truncated TrkB receptors in the rat forebrain. *J. Compar. Neurol.* 374: 21-40.

Fujimura H, Altar CA, Chen R, Nakamura T, Nakahashi T, Kambayashi J-i, Sun B, Tandon, NN (2002): Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thromb Haemost* 87: 728-34.

Gall CM and Isacjson PJ (+989). Limbic seizure increase neuronal production of messager RNA for nerve growth factor. *Science* 245: 225-245.

Gervasoni N, Aubry JM, Bondolfi G, Osiek C, Schwald M, Bertschy G, Karege F (2005). Partial Normalization of serum Brain-derived Naurotrophic Factor in remitted Patients after a Major Depressive Episode. *Neuropsychobiol*: 51: 234-238.

Gillespie DC, Crair MC, Stryker MP (2000). Neurotrophin-4/5 alters responses and blocks the effect of monocular deprivation in cat visual cortex during the critical period. *J. Neurosci.* 20 : :9174-86

Gonul AS, Akdenz F, Taneli, Donat O, Eker C, Vahip S (2003) : The effect of treatment on serum brain-derived neurotrophic factor in depressed patients. *Eur. Neuropsycho-pharmacol.* 13 (suppl 4): S203.

Greco A, Villa R, Fusetti L, Orlandi R and Pierotti M.A (2000). The Gly571Arg mutation, associated with the autonomic and sensory disorder congenital insensitivity to pain with

anhidrosis, causes the inactivation of the NTRK1/nerve growth factor receptor. *J. Cell Physiol*. 182: 127–133

Hallbook F (1999). Evolution of the vertebrate neurotrophin and Trk receptor gene families. *Curr. Opin. Neurobiol*. 9:616–621.

Hallbook F, Ibanez CF, Peterson H (1991) : Evolutionnary studies of the nerve growth factor family abundantly expressd in Xenopus ovary. *Neuron* 6: 845-58.

He XP, Buttler L, Liu X and McNamara JO(2006). The Trk B ligand, neurotrophin-4, is no n required for either epileptogenesis or Trk B activation in the kindling model. *Neuroscience*, 141: 515-520.

Hempstead B (2002): The many faces of $p75^{NTR}$. *Curr. Opin. Neurobiol*. 12 : 260–267.

Holn A, Leibrock J, Bailey K, Barde YA (1990). Identification and charcterisation of a member of the nerve growth factor/brain-derived neurotrophix factor family. *Nature* 344: 339-341.

Honer WG, Falkai P, Bayer TA, Xie J, Hu L, Li HL, Arango V, Mann JJ, Dwork AJ, Trimbale W (2002) : Abnormaties of SNARE mechanism proteins in anterior frontal cortex in severe mental illness: *Cer. Cortex* 12: 349-356.

Hrdina PD, Bakish D, Chudzik J, Ravindram A, Lapierre YD (1995) : Serotoninergic markers in platelets of patients with major depression: Upregulation of $5\text{-}HT_2$ receptors. *J. Psychiatr. Neurosci*.20: 11-19.

Huang EJ, Reichardt LF (2001): Neurotrophins: role in neuronal development and function. *Annu. Rev. Neurosci*. 24: 677-736.

Indo Y(2001). Molecular basis of congenital insensitivity to pain with anhidrosis (CIPA): mutations and polymorphisms in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. *Hum. Mutatations* 18:462–471.

Indo Y, Tsuruta, M., Hayashida, Y. et al. (1996) Mutations in the TrkA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat. Genet.* 13, 485–488

Ip NY, Ibanez CF, Vye SH, et al 1992). Mammalian eurotrophin-4: structure, chromosomal location, tissue distribution and receptor specificity. *PNAS USA* 89: 390-3064.

Jones KR, Reichardt LF (1990). Molecular cloning of a human gene that is a member of the nerve growth factor family. *PNAS USA* 87: 8060-8064.

Kamali, M, Oquendo, MA, Mann, JJ (2001): Understanding the neurobiology of suicidal behavior, *Depression and Anxiety* 14 :164-176.

Kang H and Schuman EM (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* 267: 1658-62.

Kaplan KL, Owen J (1981) : Plasma levels of b-thromboglobulin and platelet factor 4 as indices of platelet activation in vivo. *Blood* 57: 199-202.

Karege F, Schwald M, Lambercy C, Murama JJ, Cissé M, Malafosse A (2001). A non radioactive assay for the cAMP-dependent protein kinase activity in rat brain homogenates and age-related changes in hippocampus and cortex. *Brain Res*. 903: 86-93.

Karege F, Perrez G, Bondolfi G, Schwald M, Bertschy G, Aubry J-M (2002a) : Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psych. Res.* 109: 143-48.

Karege F, Schwald M, Cissé M (2002b). Postnatal development profile of brain-derived neurotrophic factor in rat brain and platelets. *Neurosci. Let.* 328: 261-264.

Karege F., Schwald M., Papadimitriou P, Lachausse C., Cisse M (2003). The cAMP-dependent protein kinase A and brain-derived neurotrophic factor expression in lymphoblast cells of bipolar affective disorder. *J. Affect Disorder* 79: 187-192

Karege F, Schwald M. El Kouassi R (2004). Drug-induced Decrease of Protein Kinase A activity reveals alteration in BDNF Expression of Bipolar Affective disorder. *Neuropsychopharmacol* 29:805-812.

Karege F, Bondolfi G, Gervasoni N, Schwald, M Aubry JM, Bertschy G (2005a). Low Brain-derived neurotrophic Factor (BDNF) levels in serrum of depressed patients probably results from lowered platelet BDNF release unirelated to platelet reactivity. *Biol. Psychiatry* 57: 1068-1072.

Karege F, G. Vaudan G, Schwald M, Perroud N, La Harpe R (2005b). Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotrophic drugs. *Mol. Brain Research* : 136 : 29-37.

Katoh-Semba R., Semba R, Takeuchi I.K, Kato K.(1998). Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3, *Neurosci. Res*. 31: 227-234.

Katoh-Samba R, Ichisaka S, Hata, Y, Tsumoto T (2003). NT-4 protein is localized in neuronal cells in the brain stem as well as the dorsal root ganglion of embryonic and adult rats. *J. Neurochem*. 66: 330-337.

Kermani P et al. (2005). Neurotrophins promote revascularization by local recruitment of TrkB+ endothelial cells and systemic mobilization of hematopoietic progenitors. *J. Clin. Invest.* 115:653–663.

Kim SY, Smith MA, Post RM, and Rosen JB (1998). Attenuation of kindling-induced decreases in NT-3 mRNA by thyroid hormone depletion. *Epilepsy Res.* 29: 211-220.

Knable, B, Barci, BM, Webster, MJ et al (2004). Molecular abnormalities of hippocampus in severe Psychiatric illness: Post-mortem findings from the Stanley Neuropathology Consortium, *Mol. Psychiatry* 9 609-20.

Korte M, Caroll P, Wolf E, Brem G, Thoenen H and Bonhoeffer T (1995). Hippocampal longterm potentiation is imapired in mice lacking BDNF. *PNAS USA* 92: 8856-8860.

Kuijpers P, Hamulyak K, Strik J, Wellens H, Honig A (2002) : Beta-thromboglobulin and platelet factor 4 levels in post-myocardial infarction patients with major depression. *Psychiatry Res*. 109: 207-210.

Kunugi, H., Hashimoto, R., Yoshida, M., Tatsumi, M. and Kamijima, K. (2004) A missense polymorphism (S205L) of the low-affinity neurotrophin receptor p75NTR gene is associated with depressive disorder and attempted suicide. *Am. J. Med. Genet. B: Neuropsychiatry Genet.* 129, 44–46

Lee R, Kermani P, Teng KK and Hempstead B L (2001). Regulation of cell survival by secreted proneurotrophins. *Science* 294, 1945–1948

Lee J, Duan W and Mattson MP (2002). Evidence that BDNF is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. *J. Neurochem*. 82: 1367-1375.

Lemons PP, Chen D, Bernstein AM, Bennett MK, Whiteheart SW (1997). Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood* 90: 1490-1500.

Lessmann V, Gottmann K, Heumann R (1994). BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. *Neuroreport* 30: :21-5.

Levi-Montalcini R and Hamburger V (1953). A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscerain the chick embryo. *J. Exp. Zool.* 123: 233-288Lewin GR, Barde YA (1996). Physiology of neurotrophins. *Annu. Rev. Neurosci.* 19:289-317.

Levi-Montalcini R and Hamburger (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool*. 116: 321-61

Li PP, Andreopoulos A, Warsh JJ (2000). Signal transduction abnormalities in bipolar affective disorder. In Cerebral Signal Transduction : From First to Fourth messengers (Reith M.E.A. ed) pp 283-309. *Human Press, Totowa*.

Lin MI, Das I, Schwartz GM, Tsoulfas P, Mikawa T, Hempstead BL (2000). Trk C receptor signaling regulates cardiac myocyte proliferation during early heart development in vivo. *Dev. Biol*. 226:180–191.

Linnarsson S, Björklund A, Ernfors P (1997). Learning deficit in BDNF mutant mice. *Eur. J. Neurosci.* 9:2581-7.

Lipski AH and Marini AM 2007 : Brain-derived neurotrophic factor in Neuronal survival and Behavior-related plasticity. *Ann. NY Acad. Sci.* 1122: 130.143.

Lou H, Kim SK, Zaitsev E, Snell CR, Lu B, Loh YP (2005). Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase. *Neuron* 45: 245–255.

Lu B, Pang PT, Woo NH (2205). The yin and yang of neurotrophin action. *Nat. Rev. Neurosci.* 6:603–614.

Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW et al (1999). Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *PNAS USA*. 96: 15239-44.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth, ME. Lindsay R, Yancopoulos, GD (1990). NT-3, BDNF and NGF, in the development of rat nervous system: parallel as well as reciprocal patterns of expression, *Neuron* 5: 501-509.

Maness LM, Kastin AJ, Weber JT, Banks WA et al (1994). The neurotrophins an their receptors : structure, Function and Neuropathology. *Neurosci. Biobehav. Rev*. 18: 143-159.

Manji HK, Moore GJ, Rajkowska G, Chen G (2000). Neuroplasticity and cellular resilience in mood disorders. *Mol. Psychiatry* 5: 578-593.

Mamounas LA, Blue, ME, Siuciak JA., Altar CA (1995). Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J. Neuroscience* 15, 7929-7939.

Mann JJ (1997). Neurobiology of suicidal behaviour, *Nature Rev. Neuroscience* 4 (2003) 819-828.

Mann JJ, McBride PA, Anderson GM, Mieczkowski TA (1992). Platelet and whole blood serotonin content in depressed inpatients : correlations with acute and life-time psychopathology. *Biol. Psychiatry* 32, 243-257.

Mann, JJ, Brent, DA, Arango, V (2001). The neurobiology and genetics of suicide and attempted suicide: a focus on the serotonergic system, *neuropsychopharmacology* 24 467-77.

Markovitz JH, Shuster JL, Chitwood WS, May RS, Tolbert LC (2000). Platelet activation in depression and effects of sertraline treatment: an open-label study. *Am. J. Psychiatry* 157: 1006-1008.

Mayrs B and Montminy M (2001). Transcriptional regulation by the phosphorylation-dependent factor CREM. *Nature Rev. Mol. Cell Biol*. 2: 599-609.

Miura, Y, Mardy, Awaya, Y et al. (2000). Mutation and polymorphism analysis of the TRKA (NTRK1) gene encoding a high-affinity receptor for nerve growth factor in congenital insensitivity to pain with anhidrosis (CIPA) families. *Hum. Genet*. 106, 116–124

Momose Y, Murata M, Kobayashi K et al. (2002). Association studies of multiple candidate genes for Parkinson's disease using single nucleotide polymorphisms. *Ann. Neurol.* 51, 133–136

Montgomery SA, Asberg MA (1979). A new depression scale designed to be sensitive to change. *British Journal of Psychiatry* 134, 382-389

Mudò G, Jiang XH, Timmusk T, Bindoni M, Belluardo N (1996). Change in neurotrophins and their receptor mRNAs in the rat forebrain after status epilepticus induced by pilocarpine. *Epilepsia* 37:198-207.

Musselman DL, Torner A, Manatunga AC, Knight BT, Porter MR et al (1996) . Exaggerated platelet reactivity in major depression. *Am. J. Psychiatry* 153 : 1313-1317.

Nelson B, Grether JK, Croen LA, Dambrosia JM, Dickens BF, Jelliffe LL, Hansen RL, Phillips TM (2001) : Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation. *Ann. Neurol*. 49: 597-606.

Nemeroff CB, Knight, DL, Franks J, Craighead WE., Krishnan RR (1994). Further studies on platelet serotonin transporter binding in depression. *Am. J. Psychiatry* 151,:1623-1625.

Nemeroff CB, Musselman, DL (2000). Are platelets the link between depression and ischemic heart disease ? *Am. Heart J*. 140: S57-S62

Neves-Pereira M, Mundo E, Muglia P, King N, Macciardi F, Kennedy JL (2002). The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder :evidence from a family-based association study. *Am. J. Hum. Genet* 71: 651-655.

Nibuya, M., Morinabu, S., Duman S. (1995). Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *Journal of Neuroscience* 15, 7539-7547.

Oliff HS, Bertchtold, NC, Isackson P, Cotman, CW (1998). Exercise-induced regulation of brain-derived neurotrophic factor (BDNF) transcripts in the rat hippocampus. *Mol. Brain Research* 61, 147-153.

Pan W, Banks, WA, Fasold MB, Bluth J, Kastin AJ (1998a). Transport of brain-derived neurotrophic factor across the blood-brain barrier, *Neuropharmacol.* 37: 1553-1561.

Pan W, Banks WA, Kastin, AJ (1998b). Permeability of the blood-brain barrier to neurotrophins, *Brain Res*. 788: 87-94.

Pandey, GN (1997). Altered serotonin function in suicide. Evidence from platelet and neuroendocrine studies. *PNAS* 836: 182-183.

Pandey SC, Zhang D, Mittal N, Nayyar D (1999) : Potential role of the gene transcription factor cyclic AMP-responsive element binding protein in ethanol withdrawal-related anxiety. *J. Exp. Therap.* 288: 866-878.

Pearse HGE (1980). The APUD concept and hormone production. *Clin. Endocrinol. Met.* 9:211-222.

Perez J, Zanardi R, Mori S, Gasperini M, Smeraldi E, Racagni G (1995). Abnormalties of cAMP-dependent endogenous phosphorylation in platelets from patients with bipolar disorder. *Am. J. Psychiatry* 152: 1204-1206.

Perez J, Tardito D, Mori S, Racagni G, Smeraldi E, Zanardi R (1999). Abnormalities of cyclic adenosine monophosphate signaling in platelets from untreated patients with bipolar disoder. *Arch Gen. Psychiatry* 56:248-253.

Perez J, Tardito D, Smeraldi E, Racagni G, Zanardi R (2001). Protein kinase A and Rap 1 levels in platelets of untreated patients with major depression. *Mol. Psychiatry* 6:44-49.

Pittenger C and Duman RS (2008). Stress, Depression and Neuroplasticity : A convergence of mechanisms. *Neuropsychopharmacol. Rev.* 33: 88-109.

Poo MM (2001). Neurotrophins as synaptic modulators. *Nat. Rev. Neurosci*. 2:24-32.

Qiao X, Hughes PE, Venero JL, Dugich-Djordjevic MM, Nichols NR, Hefti F, Knusel B (1996). NT-4/5 protects against adrenalectomy-induced apoptosis of rat hippocampal granule cells. *Neuroreport* 7:682-6.

Radka, SF, Holst, PA, Fristche, M, Altar, CA (1996) Presence of brain-derived neurotrophic factor in brain and human and rat but not mouse serum detected by a sensitive and specific immunoassay. *Brain Research* 709, 122-130.

Rajkowska G, Michel-Hidalgo JJ, Wei J, Dille G, Pittman, SD, Meltzer HY (1999). Morphologic evidence for neuronal and glyal prefrontal cell pathology in major depression. *Biological Psychiatry* 45,:1085-1098.

Rajkowska G (2000). Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. *Biol. Psychiatry* 48, 766-777.

Rylett RJ, Williams LR (1994). Role of neurotrophins in cholinergic-neurone function in the adult and aged CNS.*Trends Neurosci.* 17:486-90.

Ribases M, Gratacos, M, Armengol L et al. (2003). Met66 in the brain-derived neurotrophic factor (BDNF) precursor is associated with anorexia nervosa restrictive type. *Mol. Psychiatry* 8: 745–751

Russo-Neustadt A, Beard C, Cotman CW (1999). Excercise, antidepressant medications, and enhanced brain-derived neurotrophic factor expression. *Neuropsychopharmacol.* 21, 679-682.

Roux, P and Barker P (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Prog. Neurobiol.* 67, 203–233

Royo NC, Conte V, Saatman KE, Shimizu S et al (2001). Neurotrophin-mediated neuroprotection of hippocampal neurons following traumatic brain injury is not associated with acute recovery of hippocampal function. *J. Pediatr Surg.* 36:1280-4

Russo-Neustadt A (2003). Brain-derived neurotrophic factor, behavior, and new directions for the treatment of mental disorders. *Semin. Clin. Neuropsychiatry* 2: 109-118.

Saarelainen, T, Hendolin, P, G. Lucas, G et al (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioural effects. *J. Neurosci.* 23: 349-57.

Sen S, Nesse R, Stoltenberg S et al. (2003). A BDNF coding variant is associated with the neo personality inventory domain neuroticism, a risk factor for depression. *Neuropsychopharmacol*. 28, 397–401

Sheline, Y.I., Wang, P.W., Gado, M.H., Csernansky JG, Vannier MM. (1996). Hippocampal atrophy in recurrent major depression. *Proceedings of National Academy of Science* 93, 3908-3913.

Shelton RC (2000). Cellular mechanisms in the vulnerability to depression response to antidepressants. *The Psychiatric Clinics of North America* 23: 713-729.

Schinder AF and Poo M (2000). The neurotrophin hypothesis for synaptic plasticity, *Trends Neuroscience* 23 : 639-45.

Shelton RC, Manier DH, Sulzer F (1996). cAMP-dependent protein kinase activity in Major Depression. *Am. J. Psychiatry* 153 : 1037-1042.

Shieh PB and Ghosh A (1999). Molecular mechanisms underlying cAMP activity-dependent regulation of BDNF expression. *J. Neurobiol*. 41: 127-134.

Shimizu E, Hashimoto K, Okamura N, Koike K. Komatsu N, Kumakiri C *et al*. (2003). Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol. Psychiatry* 54: 70-75.

Schmidt HD and Duman RS (2007). The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. *Bahav. Pharmacol.* 18: 391-418.

Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS (2002). Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J. Neurosci.* 22:3251-61.

Siuciak JA, Lewis D, Wiegand, SJ (1997). Antidepressant-like effect of brain-derived neurotrphic factor (BDNF). *Pharmacological and Bioch. Behavior* 56, 131-137.

Sklar P, Gabriel SB, McInnis, MG, Bennett P, Lim YM, Tsan G, Schaffner S, Kirov G, Jones I, Owen M, Craddock N, DePaulo JR, Lander ES (2002) : Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. *Mol. Psychiatry* 7: 579-593.

Spivak B, Vared Y, Graff E, Blum I, Mester R, Weizman A (1999): Low platelet-Poor plasma concentration in Patients with Combat-related Posttraumatic Stress Disorder. *Biol. Psychiatry* 45: 840-845.

Stewart RJ, Chen B, Dowlatshahi D, MacQueen GM, Young LT (2001). Abnormlities in the cAMP signaling pathway in postmortem brain tissue from the Stanley neuropathology consortium. *Brain Res. Bull.* 55: 625-629.

Suter U, Heymach J and Shooter E (1991). Two conserved domains in the NGF polypeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. *EMBO J*. 10, 2394–2400

Takahashi M, Shirakawa O, Toyooka K, Kitamura N, Hashimoto T, Maeda K., Koizumi S (2000). Abnormal expression of brain-derived neurotrophic factor and its receptor in the corticolimbic system of schizophrenic patients. *Mol. Psychiatry* 5: 293-300.

Tardito D, Tura GB, Bocchio L, Bognotti S, Piolo L, Racagni G, Perez J (2000). Abnormal levels of cAMP-dependent protein kinase regulatory subunits in platelets from schizophrenic patients. *Neuropsychopharmacol.* 23: 216-219.

Teng HK, et al (2005) : ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *J. Neurosci*. 25:5455–5463.

Timmusk T, Belluardo N, Metsis M, Persson H (1993). Widespread and developmentally regulated expression of neurotrophin-4 mRNA in rat brain and peripheral tissues. *Eur. J. Neurosci*. 5(6):605-13

Toyooka K, Asama K, Watanabe Y, Muratake T, Makoto T, Someya T, Nawa H (2002) : Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients. *Psychiatry Res.* 110: 249-257.

Watanabe Y, Gould E, Daniels DC, Cameron H, McEwen BS (1992). Tianeptine attenuates stress-induced morphological changes in the hippocampus. *Eur. J. Pharmacol.* 222, 157-162.

Weinberg, I (2000). The prisoners of despair: right hemisphere deficiency and suicide, *Neurosci. Biobehav. Rev*. 24: 799-815.

Xu B, Michalski B, Racine RJ, Fahnestock M (2002).. Continuous infusion of neurotrophin-3 triggers sprouting, decreases the levels of TrkA and TrkC, and inhibits epileptogenesis and activity-dependent axonal growth in adult rats. *Neuroscience* 115:1295-308.

Yamamoto H, Gurney M.E (1990). Human platelets contain brain-derived neurotrophic factor, *J. Neurosci.* 10: 3469-3478.

Yeo GS, Connie Hung CC, Rochford J et al. (2004) : A *de novo* mutation affecting human TrkB associated with severe obesity and developmental delay. *Nat. Neurosci.* 7: 1187–1189

Young LT, Li PP, Kish SJ, Siu KP, Kamble A, Hornykiewicz O, Warsh JJ (1993). Cerebral cortex Gαs protein levels and forskolin-stimulated cAMP formation are increased in bipolar affective disorder. *J. Neurochem.* 61: 890-898.

Zhou XF and Rush RA (1994). Localization of neurotrophin-3-like immunoreactivity in the rat central nervous system. *Brain Res.* 643:162-72.

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Chapter 9

Neurotrophin in Female Reproductive System

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Abstract

The neurotrophins (NT) including nerve growth factor (NGF) are a family of related growth factors and their respective receptor tyrosine kinases that are of major importance in the regulation of neuronal survival and differentiation. Since Rita Levi Montalcini and Stanley Cohen received Nobel Prize for their pioneering work on NGF, its role in female reproductive system has been reinforced in last two decades. While role of NGF in mast cell-mediated egg implantation and inhibition of rejection were primary concern at their time, in the ovary they can help in the differentiation process by which ovarian follicles become responsive to gonadotrophins. They help in follicular maturation, steroid secretion and ovulation in the ovary, by inducing the FSH receptor (FSHR). Due to the pleiotropism, NGF is mandatory for the success of pregnancy, while progesterone helping to maintain local levels of NGF in utero. Perimenopausal ovarian surface epithelium (OSE) can also express FSHR. NGF deregulates expression of FSHR in OSE and secretion of FSH from the pituitary and also has the ability to increase VEGF expression. This phenomenon strongly suggests an autocrine role of NGF in epithelial ovarian cancer (EOC). From endometriosis to EOC, NGF is implicated in a variety of female reproductive disorder. Thus its study will infuse new insight in health and disease of female reproductive system.

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Introduction

In 1934 Viktor Hamburger, Nobel laureate Rita Levi Montalcini's friend in Washington, replaced the amphibian larva with that of the chick embryo to study the effects of limb bud extirpation on spinal motor neurons and sensory nerve cells innervating the limbs (Hamburger 1934). This effort signed the beginning of a long series of investigations centered on the analysis of related experimental systems in avian embryos. Their joint work on selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo paved the way for materializing the nerve growth-stimulating factor (Levi-Montalcini and Hamburger 1951). However, one point needs to be mentioned here that in a 1948 article 'a former student of Viktor Hamburger' Elmer Bueker, described grafting of fragments of mouse sarcoma 180 into the body wall of three-day chick embryos and showed that sensory nerve fibers emerging from adjacent dorsal root ganglia had gained access into the neoplastic tissue while no motor nerve fibers entered into the tumor (Bueker 1948). With this first hint of its existence in sarcoma in Washington University in St. Louis revelation of this growth factor came in a theatrical and grand way in 1954, as if spurred by the bright atmosphere of the explosive and exuberant manifestation of life that is the Carnival in Rio de Janeiro, when upon approval and invitation by Professor Chagas from the University of Brazil, Levi-Montalcini (1975) boarded a plane for Rio, carrying in her handbag two mice bearing transplants of mouse sarcomas 180 and 37 and worked in Hertha Meyer's tissue culture unit of the University in Rio de Janeiro. In 1954 nerve growth-stimulating factor could be isolated from sarcomas 37 and 180. This experiment was reported jointly by Levi-Montalcini, Hamburger along with her another friend and her joint Nobel awardee of 1986, Stanley Cohen (Cohen *et al* 1954). The Nerve Growth Factor (NGF) was officially established in 1964 by an article in single authorship of Professor Levi-Montalcini in the annals of New York academy of Science (Levi-Montalcini 1964).

Gradually neurotrophins (NTs), which include NGF and many like proteins and their receptors, came into existence. Tremendous ramification of the subject is palpable by nearly 30000 pubmed entries till date. Neurotrophins induce the survival of neurons signaling particular cells to survive, differentiate, or grow within both the central and peripheral nervous system. Neurotrophic factors are secreted by target tissue. They prevent the associated neuron from initiating programmed cell death. Neurons then can survive by target-derived trophic action of this factor. Four structurally related factors are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Another novel neurotrophin-1 (NNT1), structurally unrelated to NGF, BDNF, NT-3 and NT-4 is also described. Among receptors, p75 is a low affinity neurotrophin receptor, to which all neurotrophins bind. It is a member of the tumour necrosis super family. The Tyrosine kinases receptors, Trk family include TrkA, TrkB, and TrkC, and will only bind with specific neurotrophins, but with a much higher affinity.

As for its significance in reproductive system Professor Levi-Montalcini herself visualized that NGF could participate in fertilization mechanisms by cytoskeletal-mediated activation of spermatozoa locomotion much in the same way as in neurite outgrowth, or by favoring egg implantation, via inhibition of rejection through the immune system. The latter

hypothesis was assessed seeing the effect of NGF on uterine mast cells, which, through histamine release, are postulated to initiate the local immune reaction (Beer *et al* 1984). Though female reproductive system was not touched in this respect, relevance of this subject has not only been updated over all the years but newer developments and hypotheses have made it really a vast field which now very well includes it.

Romantic Love

Before proceeding to the original subject of NGF or Neurotrophin's role in female reproductive system we can talk about a subject that is not very remotely related to reproductive biology. This is romantic love in human, which has a bearing with NGF. In view of the complex nature of a sentiment like human love, it is quite expected that a diversity of biochemical mechanisms could be involved in the mood changes of the initial stage of a romance. Emanuele *et al* (2006) examined whether the early romantic phase of a loving relationship bears any relationship with changes in circulating levels of NTs. Plasma levels of NGF, BDNF, NT-3 and NT-4 were measured in subjects who had recently fallen in love and compared with those of two control groups, consisting of subjects who were either single or were already engaged in a long-lasting relationship. NGF level was significantly higher in the subjects in love [mean (SEM): 227 (14) pg/ml] than in either the subjects with a long-lasting relationship [123 (10) pg/ml] or the subjects with no relationship [149 (12) pg/ml]. Significant positive correlation was noticed between levels of NGF and the intensity of romantic love as assessed with the passionate love scale ($r = 0.34$; $p = 0.007$). The concentrations of other NTs did not differ much and was without any significance. They opined that raised NGF levels in the bloodstream might lead to some behavioural and/or psychological features associated with falling in love.

Ovary

Mouse sarcoma was first detected source of NGF. Snake venom, which was primarily used for its degrading enzyme, phosphodiesterase to degrade the nucleic acid of sarcoma fraction, accidentally proved to be having lot of NGF in it. Later the salivary gland of the mouse seemed to be unique in that the corresponding glands of the rat, guinea pig, cow, pig, rabbit and man contain no NGF. No other clearly characterized source of NGF in mammalian non-neuronal tissue in vivo had been reported by that time. However, NGF was detected first not in female but in male reproductive system. As Rita Levi-Montalcini showed in her Nobel lecture, NGF's role in spermatozoa locomotion much in the same way as in neurite outgrowth, NGF-like immunoreactivity was found in guinea pig (Chapman *et al* 1979, Harper *et al* 1979) and rabbit prostate, and in bull semen and seminal vesicle. Furthermore, in the mouse testis, immunoreactivity was shown to be present in germ cells, and a possible role for NGF in sperm maturation or motility was hypothesized (Ayer-LeLievre *et al* 1988). Mouse NGF cDNA probe for in situ hybridization and RNA blot analysis was used to reveal the identity and distribution of cells synthesizing NGF in rodent male genital organs. The

presence of NGF protein in the same tissues was studied by immunohistochemistry; male mouse germ cells synthesize both NGF mRNA and protein. In addition, NGF mRNA-containing cells were also found in the epithelium of convoluted ducts of corpus epididymidis suggesting a role for NGF in maturation or motility of spermatozoa or both.

Differences in NGF concentrations between men and women (in both phases of the menstrual cycles) are statistically significant (Martocchia *et al* 2002). We now know that the difference of serum NGF concentrations between the follicular and luteal phase in each woman is also statistically significant. A Japanese group first showed effect of NGF on ovary in as early as 1970 (Akasu *et al* 1970). But, Ojeda and his groups vastly study regulation of ovarian function over last four decades. However, they could start relate the subject with NGF in as late as 1990. We can summarize their work, which will give us a unique picture of evolution of the role of NGF in ovary and ovarian function.

In early days they worked on prostaglandins (PGs). Support to the hypothesis that PGs play a physiological role in the control of gonadotropin secretion was obtained by using inhibitors of PG synthesis administered at high doses that can inhibit LH release in the rat. But, this effect was mainly due to a direct effect of the drug or drugs on the central nervous system (Ojeda *et al* 1975).

Then they worked on Vasoactive intestinal polypeptide (VIP). It was first isolated from the porcine duodenum by Said and Mutt (1970), and noted to produce vasodilation in anesthetized dogs. VIP provokes vaginal lubrication in normal women. VIP showed an array of activity right from the effects of VIP on the uterine vasculature and neurogenic relaxation in the isthmus of the human fallopian tube leading to inhibitory effect of VIP on spontaneous oviductal contractility. When NGF research was of highest acclaim and joint Nobel Prize was given for it in 1986, Ojeda and his group was considering potentiality and role of VIP and Substance P in ovarian function (Dees *et al* 1986). Nevertheless, their journey to reach NGF was interesting and quite relevant to our topic. VIPergic nerves appeared to be involved in the developmental regulation of ovarian steroidogenesis. Afterward VIP and norepinephrine (NE), two of the neurotransmitters contained in ovarian nerves, were found to be present in the ovary before the gland becomes responsive to gonadotropins. They proposed that ovarian nerves, acting via neurotransmitters coupled to the cAMP generating system, contribute to the differentiation process by which newly formed primary follicles acquire FSH receptors and responsiveness to FSH. Follicles that begin to grow in more densely innervated ovarian regions, may have a selective advantage over those not exposed to neurotransmitter-activated, cAMP-dependent signals and, thus, may become more rapidly subjected to gonadotropin control (Mayerhofer *et al* 1986).

Nerve fibers containing substance P (SP) were localized in ovaries from juvenile and peripubertal rats by immunofluorescence (Ojeda *et al* 1985). The peripubertal animals seemed to have a greater concentration of ovarian SP than the juvenile animals. SP is not directly involved in regulating steroidogenesis. Instead, SP may be a component of the so-called sensory innervation of the ovary, and among other undisclosed functions it may contribute to the regulation of ovarian blood flow system. In the same year they also produced proof for another interesting proposal that neonatal release of gonadotropins and in particular FSH, may be involved in the acquisition of FSH receptors by the developing ovary system (Smith *et al* 1986). Neuropeptide Y (NPY)-like immunoreactivity was then found in

nerves that innervate the rat ovary and the profuse network of NPY-containing fibers strongly implied a physiological involvement of NPY in the regulation of ovarian function (McDonald et al 1987).

Calcitonin gene-related peptide (CGRP) was found in nerves that innervate the rat ovary (Calka *et al* 1988). Studies revealed coexistence of CGRP and substance P in several axons. Neither tyrosine hydroxylase nor neuropeptide Y-immunoreactivity was co-localized in CGRP-containing fibers. CGRP had no effect on the basal or follicle-stimulating hormone-stimulated release of estradiol or progesterone from ovaries of pregnant mare's serum gonadotropin-treated rats or from cultured granulosa cells from hypophysectomized, diethylstilbestrol-treated rats. They showed that though CGRP-containing nerves enter the ovary via the plexus nerve but are probably involved in the regulation of vasomotor function rather than ovarian function.

Treatment of neonatal rats with antibodies to nerve growth factor (NGF Ab) resulted in failure of the sympathetic (noradrenergic and neuropeptide-Y) nerves to develop (Lara *et al* 1990). Plasma LH levels were elevated, and LH pulsatility was enhanced, suggesting primary ovarian failure. Partial loss of sensory innervation, represented by absence of calcitonin gene-related peptide fibers, was also observed. Follicular growth was stunted, and production of androgens and estradiol was reduced. The timing of first ovulation was delayed, estrous cyclicity was disrupted, and fertility was compromised. A normal appearance of tyrosine hydroxylase-, LHRH-, and neuropeptide-Y-immunoreactive neurons in the hypothalamus, as determined by immunocytochemistry, suggested that neonatal immunosympathectomy did not directly affect hypothalamic reproductive function. In vitro release of LHRH from median eminence nerve terminals in response to prostaglandin E2 was, however, reduced in NGF Ab-treated rats. Normalization of the response by prior in vivo exposure of the animals to physiological estradiol levels, suggested that the diminished LHRH output was due at least in part to estrogen deficiency. Although ovarian dysfunction induced by immunosympathectomy may be related to alterations in vascular tone, the striking loss of perifollicular noradrenergic innervation caused by NGF Ab suggested that the absence of the nonvascular norepinephrine stimulus to follicular steroidogenesis is a primary factor responsible for the alterations observed. It was proved that development of the sympathetic innervation of the ovary is NGF dependent and that NGF, by supporting the differentiation and survival of the innervating neurons, contributes to the acquisition of mature ovarian function.

Guanethidine-mediated destruction of ovarian sympathetic nerves disrupts ovarian development and function in rats. Immature rat ovary synthesizes NGF, and that interference of NGF actions by immunoneutralization during neonatal life prevents development of the ovarian sympathetic innervation and delays follicular maturation. Since the actions of NGF are exerted via binding to specific cell surface receptors, the presence of NGF receptors in the developing rat ovary was studied. NGF interacts with two classes of NGF receptors. The most abundant is low affinity form (later came to be known as p75NTR) expressed in the central nervous system and peripheral tissues. This receptor was encoded by a single 3.8-kilobase mRNA species. Cross-linking of [^{125}I] NGF to ovarian membranes followed by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography showed the presence of an approximately 90-kilodalton molecular species,

which corresponds in size to the predominant NGF receptor species, cross-linked to its ligand (Dissen *et al* 1990). Ovarian NGF receptors may be of neuronal origin and reach the gland exclusively by anterograde axonal transport. RNA blot hybridization demonstrated that the ovary expresses NGF receptors mRNA species that encodes p75NTR and, thus, implicated the ovary itself as a site of NGF receptors synthesis. NGF receptors mRNA levels decreased abruptly after the first ovulation, suggesting that NGF receptors may be synthesized in growing follicles and that this capacity is lost after follicular rupture and luteinization.

NGF and NT-3, two members of the neurotrophin family, was then identified in the rat ovary and one of its receptors (p75NTR) had been localized to the innervation and thecal cells of developing follicles. Although NGF supports the sympathetic innervation of the gland, the extent to which follicles are innervated appears to be defined by the differential expression of NGF receptors in the theca of developing follicles. The presence of NGF receptors in steroid-producing cells suggests a direct involvement of neurotrophins in the regulation of gonadal endocrine function (Ojeda *et al* 1992). The mature mammalian ovary has now been shown to synthesize several neurotrophins, including NGF, NT-3, and NT-4/5. The ovary also expresses some of the neurotrophin receptors, including p75NTR, trkB (the receptor for NT-4/5 and BDNF) and trkA (the NGF receptor) (Dissen *et al* 1995). Developmental pattern of expression related to the completion of folliculogenesis revealed that ovarian content of both NGF and trkA mRNA decreases at the time of folliculogenesis and NT-4 mRNA levels increased at the time of follicular assembly, coinciding with the abrupt appearance of trkB mRNA. Substantial, but unchanging, levels of NT-3 mRNA and the mRNA encoding trkC, the preferred NT-3 receptor, were detected throughout the perinatal period. Very low and invariable levels of BDNF were also detected.

Primate ovary contains an intrinsic network of neuron-like cells (Dissen *et al* 1996). Because such an intrinsic neuronal network has not been detected in rodents or other non-primate species, it would appear that its presence in the primate ovary might have evolutionary significance. The remarkably narrow time frame of trkA gene activation at the completion of follicular growth suggests that NGF acting as a neuroendocrinotrophic factor in a developmentally restricted manner contributes to the acute cytodifferentiation process that leads to the first ovulation in mammals which in turn may be mediated by the activation of trkA in mammalian ovulation. During the hour preceding follicular rupture, a marked increase in trkA and NGF gene expression occurs in thecal-interstitial cells of the ovary. Immunoneutralization of NGF actions or pharmacological blockade of trkA transducing activity inhibits ovulation, suggesting that activation of the NGF-trkA complex in nonneural cells of the periovulatory follicle is a physiological component of the ovulatory cascade. Gap junctions functionally couples thecal cells of Graafian follicles, and the ovulatory rupture requires dissociation of thecal cell-cell communication, NGF-induced activation of trkA receptors in isolated ovarian thecal cells disrupts cell to cell communication by affecting the functional integrity of gap junctions. Thus, cell-specific activation of trkA receptors in periovulatory follicles may provide one of the signals involved in inducing the cellular dissociation of the follicular wall that precedes ovulatory rupture (Mayerhofer *et al* 1996).

Later, in total at least four of the five known neurotrophins, including NGF, BDNF, NT-3, and NT-4, and their receptors (p75 NTR, trkA, trkB and trkC) were seen to be present in the developing ovary. Null mutations study of the genes encoding neurotrophins (NGF, NT-

4, BDNF) or the receptor that mediates the actions of NT-4 and BDNF (trkB) also shows that neurotrophins are required for the growth of primordial follicles. However, null mutation of the NT-4 gene failed to affect either folliculogenesis or follicular development. Formation of primary and secondary follicles was compromised in mice carrying a null mutation of both the NT-4 and BDNF genes, suggesting compensation of function by BDNF in NT-4 knockouts. Support for this concept is provided by the similar deficiency in follicular growth observed in animals carrying a null mutation of the gene encoding trkB, the receptors mediating NT-4 and BDNF actions. Formation and development of mammalian follicles requires the concerted action of genes originally thought to be only involved in cell differentiation/survival of neuronal cells, and genes that may control the growth, differentiation, and cell-cell interactions of somatic and germ cells in the ovary (Ojeda *et al* 2000). The contribution of NGF to the ovulatory process also includes a stimulatory effect on steroidogenesis, PGE2 formation, and proliferative activity of thecal compartment cells. NGF-null (-/-) mutant animals, analyzed after completion of ovarian histogenesis, exhibited a markedly reduced population of primary and secondary follicles in the presence of normal serum gonadotropin levels, and an increased number of oocytes that failed to be incorporated into a follicular structure (Dissen *et al* 2001). The delay in follicular growth observed in NGF (-/-) mice may be related to the loss of a proliferative signal provided by NGF to the nonneural endocrine component of the ovary. In addition to the extrinsic innervation, the ovaries of nonhuman primates and a strain of rats contain a discrete population of intrinsic neurons. Later, presence of these intrinsic neurons in the human ovary, similar to those previously found in other species, proved that they might be engaged in regulating common, phylogenetically conserved, ovarian functions. It also raised the possibility that their dysfunction may contribute to the manifestation of particular ovarian pathologies (Anesetti *et al* 2001).

The growth of primordial follicles is retarded in the absence of NGF, indicating that NGF contributes to facilitating early follicular development. One of the functions of NGF in the developing ovary is to facilitate the differentiation process by which early growing follicles become gonadotropin-dependent during postnatal life, and it does so by increasing the synthesis of FSHRs (Romero *et al* 2002).

Implantation

While NGF were not detected in any stage of the ovine preimplantation embryo (Watson *et al* 1994) NGF and its receptors (TrkA and p75NTR) were expressed in granulosa cells, theca cells, interstitial cells and lutein cells in ovaries. Immunoreactions for NGF, TrkA and p75NTR were also detectable in epithelial cells and muscle cells of the ampulla and isthmus of the oviduct, and in epithelial cells and uterine glands of the uterus. These results strongly suggest autocrine and paracrine regulation of reproductive function by NGF in the reproductive tract (Ren *et al* 2005). Long back, isolation of human NGF from placental tissue by using the chicken embryo dorsal root ganglia assay showed highest levels of NGF activity in placental cotyledons amongst amnion, placental cotyledons, cord serum, fetal serum, and maternal serum (Goldstein *et al* 1978). Eventually large-scale isolation of human NGF of

placental origin, and its immunological characterization was possible in 1990 (Bigon *et al* 1990). Using reverse transcriptase-polymerase chain reaction (RT-PCR), trophoblast, amnion/chorion and maternal decidua showed the expression of NGF mRNA both in early gestation and at term. By immunohistochemistry, the immunoreactive NGF was found in the cyto and syncytial trophoblast cells, chorionic mesodermal cells. Vessel endothelial cells were stained in maternal compartments, while fetal vessels were unstained. Thus, human placenta is a potent neuroendocrine organ throughout gestation (Toti *et al* 2006).

NGF isolated from mouse submandibular gland or from snake venom was shown to produce a dose-dependent release of histamine from isolated rat peritoneal mast cells, the response being almost totally dependent on the presence of extracellular calcium ions requiring phosphatidylserine or its lyso-derivative (Pearce *et al* 1986). At high concentrations, strontium ions could substitute for calcium. The process was non-cytotoxic, relatively slow, pH dependent and blocked by polyclonal antibodies to NGF. Binding of NGF to the mast cell was not dependent on added calcium. The release of histamine was unaffected by low molecular weight glucose polymers or specific quaternary ammonium salts. Secretion of histamine from rat mast cells may be by interaction with a specific receptor on the plasma membrane, possibly similar to that present on sensory and sympathetic neurons. Nerves and mast cells may constantly communicate and provide a structural and conceptual framework whereby the central nervous system may communicate with inflammatory events (Bienenstock *et al* 1987). Interleukin-4 (IL-4) and NGF significantly enhanced IL-3-promoted histamine production in serum-free cultures. Both IL-4 and NGF can function as cofactors with IL-3 in the support of histamine production in human umbilical cord blood cells (Richard *et al* 1992). Release of histamine from mast cells after stimulation with NGF is regulated by tyrosine kinase, phospholipse C (PLC), phosphatidyl inositol-3kinase (PI-3K) and protein kinase C (PKC), but not by MAP kinases (Stempelj *et al* 2005). Stress and substance P (SP) injection significantly increased the abortion rate and up-regulated decidual NGF and TrkA expression compared with the control. Stress, elicited by environmental and social conditions, is known to affect the homeostasis of the nervous, endocrine and immune systems. In pregnancy, perceived stress results in a predomination of inflammatory abortion-associated Th1 (helper T cell 1) cytokines over immunosuppressive, pregnancy-protective-associated Th2 cytokines, putatively via neuropeptide substance P (SP). NGF being an important trophic factor for sympathetic neurons has been implicated in the responsiveness of immune-competent cells through its functional receptor, TrkA. NGF enhances local mast cell number in decidua proving a cross talk with nerve in decidualisation. Dendritic cell expressing trkA and p75NTR may have some role to play through inflammation. Monocyte and resultant macrophages may also act in this pathway (Tometten *et al* 2004).

Strong evidence indicates that stress-triggered abortion is mediated by adhesion molecules, i.e., intercellular adhesion molecule 1 (ICAM1) and leukocyte function associated molecule 1, now being referred to as integrin alpha L (ITGAL), which facilitate recruitment of inflammatory cells to the feto-maternal interface. The neurotrophin beta (NGFβ), which has been shown to be upregulated in response to stress in multiple experimental settings including in the uterine lining (decidua) during pregnancy, increases ICAM1 expression on endothelial cells. Subsequently, decidual cytokines are biased toward a proinflammatory and

abortogenic cytokine profile. Additionally, a decrease of pregnancy protective CD8alpha (+) decidual cells is also noticed. Strikingly, all such uterine stress responses are abrogated by NGFβ neutralization. Hence, NGFβ acts as a proximal mediator in the hierarchical network of immune rejection by mediating an abortogenic environment comprised of classical signs of neurogenic inflammation (Tometten *et al* 2006). NGF is also mandatory for the success of pregnancy via inhibition of paternal major histocompatibilty antigen, MHC II molecule expression on trophoblast cells. This is supported by published evidence on progesterone, the hormone of pregnancy, which maintains local levels of NGF (Tometten *et al* 2005). Maternally derived NGF may play a role in placentation by promoting the giant-cell transformation of trophoblast cells through p75NGFR. Along with macrophages they may promote apoptosis or programmed cell death. High tryptophan and/or low indoleamine 2,3-dioxygenase through NGF may contribute to a deleterious effect on pregnancy (Kanai-Azuma *et al* 1997). Thus, delicate balance of NGF and other factors decisively helps in progress of early pregnancy.

Pregnancy

In the pregnant uterus, a pronounced transient decrease of autonomous innervation evidenced by a marked axonal degeneration of myometrial and perivascular adrenergic fibers has been reported in animal models and in humans. The physiological significance of the uterine denervation during pregnancy is thought to be a reduction of myometrial contractility and the prevention of preterm labor. The mechanisms underlying this transient and reversible uterine denervation remain largely unknown. However, several factors have been implicated in the pregnancy-related uterine denervation: 1) the mechanical stretch induced by the growing fetus 2) the high levels of circulating ovarian steroids during pregnancy and 3) a dilution of neurotrophic factors in the pregnant uterus. The density of sympathetic innervation in effector organs is directly correlated to the expression of neurotrophins especially NGFß. But previous studies have analyzed the presence and fluctuations of NGFß in the pregnant uterus with conflicting results (Lobos *et al* 2005).

The physiological denervation occurring in the uterus and uterine artery during pregnancy is related to a decrease in the availability of NGF by nerve fibers, and to the impossibility to mediate its effect due to a remarkable decrease in the signal-transducing TrkA receptor (Naves *et al* 1998). In the guinea pig, pregnancy is associated with a generalised depletion of noradrenaline in uterine sympathetic nerves and, in the areas of the uterus surrounding the foetus, by a complete degeneration of sympathetic nerve fibres. These pregnancy-induced changes have been interpreted as a selective effect of placental hormones on the system of short sympathetic fibres arising from the paracervical ganglia. An alternative explanation is that pregnancy affects the neurotrophic capacity of the uterus. NGF-protein levels in the guinea pig uterine horn, tubal end and cervix was measured at early pregnancy, late pregnancy and early postpartum, using a two-site enzyme-linked immunosorbent assay. For comparative purposes the distribution and relative density of noradrenaline-containing sympathetic nerve fibres were assessed histochemically, and tissue levels of noradrenaline were measured biochemically, using high-performance liquid chromatography with

electrochemical detection. In all the uterine regions analysed, NGF-protein levels showed a decline at term pregnancy, but in no case was this change statistically significant. After delivery, NGF-protein levels showed a marked increase in the cervix as well as in both the fertile and empty horns. So it was thought that alterations in NGF-protein do not account for the impairment of uterine sympathetic innervation during pregnancy, but may contribute to their recovery after delivery (Brauer *et al* 2000). This was supported by another contemporary experiment in turkey. NGF and NGF mRNA assayed in the uterus of non-pregnant and pregnant rats showed decrease during middle and late pregnancy. Strangely, when values were corrected for the increase of uterine weight and total RNA yield during pregnancy, NGF content and mRNA per horn increased during middle and late pregnancy. Similar, but less pronounced, changes were observed in the cervix. By seven days postpartum, both parameters returned to near normal (Varol *et al* 2000).

These authors concluded that alterations in NGF content do not account for the impairment of sympathetic uterine innervations. These results are in contrast to studies that showed that the survival of sympathetic neurons are dependent of NGF-ß produced by target tissues and that NGF-ß synthesized and secreted by smooth muscle cells is crucial for the development and maintenance of vascular and bladder innervation and that the transient degenerative and regenerative changes in the myometrial innervation in the pregnant rat uterus offer a convenient model for studying the mechanisms involved in the neuronal plasticity of the peripheral nervous system.

To solve this dilemma NGF metabolism was carefully scrutinized. It was seen that NGF is synthesized as a precursor protein (proNGF) that is processed posttranslationally into mature 13.5-kDa NGF-ß. The processing of proNGF results also in a series of high-molecular-weight intermediate forms whose biological roles are not well understood. High-molecular weight glycosylated proNGF forms have been detected in a wide variety of cells in vitro and in vivo. ProNGF forms and not mature NGF-ß are the predominant forms in human and rat brains and several peripheral tissues. ProNGF and its proteolytically processed protein products may differentially activate pro- and antiapoptotic cellular responses through preferential activation of TrkA or p75NTR. Therefore, it was suggested that the balance between cell death and survival may be determined by the ratio of secreted proNGF and mature NGF-ß (Chao and Bothwell 2000).

Alterations in NGF isoforms during pregnancy, accumulation of proNGF, and decreased ratios of mature NGF-ß to proNGF in the pregnant rat uterus coinciding with the transient uterine axonal degeneration of the pregnant myometrium then corroborated the results reported by previous studies. Under certain circumstances when the balance between a proneurotrophin and mature neurotrophin favors the proneurotrophins, it may cause degeneration of neurons (Krizsan-Agbas *et al* 2000) and this happens in pregnant uterus.

It is worthwhile to discuss hormonal influence on such denervation. Sympathetic innervation of the adult rodent uterus undergoes cyclic remodelling. Terminal sympathetic axons degenerate when estrogen levels rise and regenerate when estrogen levels decline. There seems to be a unique role of neurotrophins in estrogen-mediated uterine sympathetic nerve remodelling. Oestrogen injection of ovariectomized female rats did not affect uterine NT-3 levels 24 h postinjection, and increased endometrial NGF protein, indicating that reduced NGF or NT-3 is not responsible for the estrogen-induced denervation. Estrogen also

raised BDNF protein and mRNA in myometrium and endometrium. To assess whether increased BDNF affects uterine receptivity to sympathetic outgrowth, sympathetic ganglion explants were co-cultured with myometrium (Krizsan-Agbas *et al* 2000). Myometrium from ovariectomized rats induced neuritogenesis in estrogen-free conditions, and this was abolished when BDNF was added to the medium. Neuritogenesis induced by ovariectomized myometrium was suppressed by estrogen, and restored by a BDNF function-blocking antibody. To determine if target BDNF synthesis is required for estrogen to suppress sympathetic neurite outgrowth, uteri from wild-type mice and mice homozygous or heterozygous for recombinant mutations of the BDNF gene were cultured with rat sympathetic ganglia. Neuritogenesis induced by wild-type uteri was diminished by estrogen. Neurite formation in the presence of homozygous BDNF mutant uteri was not affected by oestrogen, but was lower than that of wild-type mice. Uteri from mice heterozygous for the BDNF mutation, who have reduced BDNF synthesis, showed normal neuritogenic properties, but were not affected by estrogen. Estrogen alters neuritogenic properties of uterus by regulating BDNF synthesis, which inhibits sympathetic neurite outgrowth.

The human cervix in nonpregnant, early pregnant and term pregnant women was by far more densely innervated than the rest of the uterus. No obvious difference in nerve density was observed between nonpregnant, early pregnant and term pregnant women, and still during labor the innervation appeared morphologically preserved. It is suggested that the segmental difference in innervation of the human uterus (corpus versus cervix) may have specific importance for myometrial and cervical contractility during pregnancy and parturition (Bryman *et al* 1987). Women display markedly decreased BDNF serum levels before and after childbirth. This phenomenon might reflect an increased risk for the development of mood disorders in the perinatal period. However, the individual serum concentration of BDNF alone did not predict maternal depression (Lommatzsch *et al* 2006).

The presence of biologically active NGF in the peripheral circulation of women during pregnancy, labour and lactation was investigated. Using a sensitive immunoenzymatic assay (ELISA), an approximately five-fold increase in plasma NGF levels was found during labour and lactation compared with the concentrations found at the term of gestation or in control healthy women (Luppi *et al* 1993). Since labour and lactation are characterized by activation of the hypothalamo-pituitary-adrenal axis and by high plasma levels of the neurohypophyseal hormone oxytocin, and since the intravenous injection of oxytocin in female rats causes a 176% increase in the hypothalamic levels of NGF, it is possible that the increased amount of circulating NGF is correlated with one or both of these events.

Intrauterine Growth Restriction

Intrauterine growth restricted (IUGR) fetuses are those with estimated weight <10th customized centile, displaying signs of chronic malnutrition and hypoxia leading to brain sparing effect. IUGR is associated with perinatal mortality and with neurologic damage from intraventricular hemorrhage (IVH). Neurotrophins, [NGF, BDNF, NT-3 and NT-4] are important for pre- and post-natal brain development. Circulating NGF, BDNF, NT-3 and NT-4 levels in IUGR and appropriate for gestational age (AGA) full term fetuses and neonates

(day-1 [N1] and day-4 [N4]) and in their mothers was measured. While no statistically significant differences existed between IUGR and AGA maternal, fetal and neonatal levels of BDNF, NT-3 and NT-4, NGF was significantly higher in AGA than IUGR maternal ($p=0.007$), fetal ($p=0.01$), neonatal day 1 ($p=0.043$) and 4 ($p=0.003$) plasma. NGF positively correlated with the infants' centiles and birthweights proving brain sparing effect of BDNF, NT-3 and NT-4, in contrast to NGF levels, which are higher in the AGA group (Malamitsi-Puchner *et al* 2007).

S100B, a neural protein found in high concentrations after cell injury in the nervous system, is increased in serum of women whose pregnancies are complicated by IUGR and whose newborns develop IVH (Gazzolo *et al* 2006). The S100B mRNA expression in the amnion of pre-eclamptic patients and patients with pre-eclampsia with IUGR was significantly higher than that in the control. The amniotic fluid S100B protein concentration of the pre-eclampsia and normotensive IUGR cases was also significantly higher than that of the control. This study shows that amnion could be a source responsible for the increased concentration of S100B in amniotic fluid. In pre-eclampsia, reactive oxygen species (ROS) are generated by oxidative stress. Some pathological conditions that develop during pregnancy and are related to hypoxic stress can affect the elevation of S100B concentration in the amnion (Tskitishvili *et al* 2006). To relate this with NGF, effects of forced expression of S100B in the neuronal cell line pheochromocytoma PC12 was studied. PC12 was chosen because they normally do not express S100B mRNA or protein and they cease to proliferate and differentiate into a neuronal-like cell type on exposure to the NGF. Over expression of S100B results in enhanced cell survival under stress conditions, increased cell proliferation, a reduced extent of apoptosis, and decreased responsiveness to the differentiating effect of NGF to the benefit of condition prevailing in IUGR (Arcuri *et al* 2005).

Endometriosis

Possible role for nerve growth factor (NGF) in the mechanism of pain and hyperalgesia induced by deep adenomyotic nodules and other forms of endometriosis was investigated. It was hoped that this might clarify the relationship between endometriotic lesions and the surrounding nerves also (Anaf *et al* 2002). Immunohistochemistry with antibodies against NGF, NGF specific tyrosine-kinase receptor (Trk-A) and S-100B protein was performed in endometriotic lesions (deep adenomyotic nodules, peritoneal endometriosis, ovarian endometriosis) in two groups (group 1: patients with a deep adenomyotic nodule; group 2: patients with peritoneal and/or ovarian endometriosis but without deep adenomyotic nodule) and eutopic endometrium. Results were expressed as mean H-scores +/- SD, and correlated with the presence of hyperalgesia. The percentage of patients presenting hyperalgesia at physical examination was significantly higher in group 1 (96%) than in group 2 (11%) ($P < 0.001$). NGF expression was significantly stronger in deep adenomyotic nodules (DAN) than in ovarian (OE) and peritoneal endometriosis (PE), both in the proliferative phase in the glands and in the stroma. NGF expression in DAN is also significantly stronger than in OE and PE in the secretory phase in the glands and in the stroma. Perineurial and intraneurial invasion by endometriotic lesions were found only in deep adenomyotic nodules and not in

the other forms of endometriosis. The specific receptor for NGF (Trk-A) is expressed in all the nerves that were included in the biopsies. The strong expression of the NGF-TrkA pathway in deep adenomyotic nodules could explain why this type of lesion infiltrates in richly innervated anatomical sites. Thus it is proposed that NGF may normally regulate the differentiation of the mesenchyme into uterine myocytes through paracrine mechanisms and that an early disturbance of this process plays a key role in the subsequent development of adenomyosis (Green *et al* 2003).

In a study to compare the effects on uterine development of the selective estrogen receptor modulators, tamoxifen, toremifene, and raloxifene with estradiol, they were given orally to female mice on days 2 to 5 after birth. Uterine adenomyosis was found in all (14 of 14) mice dosed with tamoxifen and most mice (12 of 14) treated with toremifene, but in none of the vehicle-dosed controls, in only one animal treated with raloxifene at 42 and 90 days after dosing and in none of the mice treated with estradiol at 42 days. At 6 days, the uterus in the groups that developed a high incidence of adenomyosis showed histological evidence of disturbed differentiation of the myometrium. Gene-expression XY-scatterplots using Clontech mouse 1.2 Atlas mouse cDNA expression arrays analyzing total uterine RNA showed nerve growth factor-alpha, preadipocyte factor-1, and insulin-like growth factor-2 were key genes differentially modified by tamoxifen or toremifene treatment, relative to the controls. As these genes may play an important role in regulating differentiation and development of the myometrium, adenomyosis may be caused primarily by defects in the formation of the myometrium (Parrott *et al* 2001). Tamoxifen can upregulate *NGF* gene that in turn may be responsible for the development of adenomyosis. Tamoxifen is contraindicated during pregnancy but many births have been reported in breast cancer patients taking this drug and numbers might be expected to increase with FDA approval of tamoxifen for risk reduction in women at high risk of breast cancer. In another similar expriment, the neonatal mouse, exquisitely sensitive to xenobiotic estrogens, has been used to investigate the effects of short-term oral dosing with tamoxifen (1 mg/kg on days 2-5 after birth) on long-term changes in uterine pathology and gene expression. Increased adenomyosis incidence and severity was evident in the tamoxifen-treated mice with increasing age. Uterine weights in treated mice remained lower than the corresponding controls up until 9 months, after which they became greater but during life-time studies (up to 36 months), there was no development of uterine tumours. Pathological examination of uterine tissues showed there to be extensive down-growth of endometrial glands and stroma into thickened, abnormal myometrium that had disorganised fascicles of smooth muscle and increased interstitial collagen deposition. In advanced cases, the endometrial epithelium showed mild degrees of focal hyperplasia and squamous metaplasia but no atypical cytology suggestive of premalignant change. Microarray analysis of uterine RNA taken at 1.5, 3, 6, 9 and 12 months showed from 4500 ESTs, only 12 genes were continuously over-expressed by tamoxifen treatment over this time, while none was continuously down-regulated. Up-regulated genes include those for nerve growth factor (NGFa), cathepsin B (Ctsb), transforming growth factor beta induced (Tqfbi) and collagens (Colla1, Colla2). Results provide a basis for understanding the mechanism for tamoxifen induced tissue remodelling and the development of adenomyosis (Green *et al* 2001).

In an experiment to investigate the topographical relationship between nerve fibers and peritoneal endometriotic lesions and to determine the origin of endometriosis-associated nerve fibers in a retrospective nonrandomized study, immunohistochemistry was used to study the expression of neurofilament, substance P, smooth muscle actin, von Willebrand factor, growth-associated protein 43, nerve growth factor, and neutrophin-3 in peritoneal endometriotic lesion samples from women with symptomatic endometriosis and in peritoneal samples from women without endometriosis in premenopausal women with histologically confirmed endometriosis, Peritoneal endometriotic lesions and unaffected peritoneal biopsies from patients without endometriosis. Pain-conducting substance-P-positive nerve fibers were found to be directly colocalized with human peritoneal endometriotic lesions in 74.5% of all cases. The endometriosis-associated nerve fibers are accompanied by immature blood vessels within the stroma. Endometriotic cells express NGF and NT-3. Growth-associated protein 43, a marker of neural outgrowth and regeneration, is expressed in endometriosis-associated nerve fibers but not in existing peritoneal nerves. The data provide the first evidence of direct contact between sensory nerve fibers and peritoneal endometriotic lesions. This implies that the nerve fibers play an important role in the etiology of endometriosis-associated pelvic pain (Mechsner *et al* 2007). Moreover, emerging evidence suggests that peritoneal endometriotic cells exhibit neurotrophic properties.

Peritoneal endometriotic lesions and normal peritoneum were prepared from women with and without endometriosis and endosalpingiosis lesions. These sections were stained immunohistochemically with antibodies against protein gene product 9.5, neurofilament (NF), NGF, p75NTR, substance P, calcitonin gene-related peptide (CGRP), acetylcholine (ACh) and tyrosine hydroxylase (TH) to demonstrate myelinated, unmyelinated, sensory, cholinergic and adrenergic nerve fibres. There was significantly more nerve fibres identified in peritoneal endometriotic lesions than in normal peritoneum or endosalpingiosis lesions. These nerve fibres were SP, CGRP, ACh or TH immunoreactive. Many of these markers were co-localized. There was an intense NGF immunoreactivity near endometriotic glands, and p75NTR immunoreactive nerve fibres were present near endometriotic glands and blood vessels in the peritoneal endometriotic lesions. Thus, peritoneal endometriotic lesions were innervated by sensory A delta, sensory C, cholinergic and adrenergic nerve fibres. These nerve fibres may play an important role in the mechanisms of pain generation in this condition (Tokushige *et al* 2006). They also tried to see how hormonal treatment can change nerve fiber density and tried to identify types of nerve fibers in endometrium and myometrium in women with endometriosis. The nerve fiber density in the functional and the basal layers of endometrium and myometrium from women with hormonally treated endometriosis was much lower than that of endometrium and myometrium from women with untreated endometriosis. Nerve growth factor and nerve growth factor receptor p75 expression was also significantly reduced in women with hormonally treated endometriosis compared with women with untreated endometriosis. Thus, hormonal treatment can significantly reduce nerve fiber density in endometrium and myometrium in women with endometriosis (Tokushige *et al* 2007).

Endometriosis is produced in cycling rats by autotransplanting pieces of uterus onto abdominal arteries where they develop into cysts. The surgery induces abdominal muscle hyperalgesia, whose severity is greatest in proestrus and nearly absent in estrus. The cysts

contain growth factors and cytokines and develop their own sympathetic and sensory C- and A delta-fiber innervation. Quantitative immunostaining and protein array analyses were performed to test the hypothesis that the innervations and growth factor/cytokine content of the cysts, but not uterine horn, contribute to proestrous-to-estrous changes in hyperalgesic severity. If so, these characteristics in the cysts, but not the uterine horn, should change with estrous stage. In cysts, the density of sympathetic (but not sensory) neurites and amounts of NGF and VEGF proteins (but not cytokines IL-1, IL-6, IL-10, or TNF-alpha) were greater in proestrus than estrus. These changes were accompanied by vascular changes. Both sympathetic and sensory fibers in both stages co labeled with TrkA, indicating that changes in NGF could act on both afferent and efferent fibers. In contrast with the cysts, no changes occurred in the uterine horn between proestrus and estrus. Coordinated proestrous-to-estrous changes in innervation and vascularization of the cysts contribute to similar changes in hyperalgesic severity showing endometriosis as a neurovascular condition (Zhang *et al* 2008). Endometriosis is associated with low follicular-fluid BDNF levels, and diminished ovarian reserve is associated with increased follicular-fluid NGF levels. Follicular-fluid neurotrophin levels were determined in women undergoing assisted reproductive techniques for different etiologies of infertility in a prospective observational study. Women with a history of endometriosis have significantly lower follicular-fluid BDNF levels compared with women with male-factor (control) infertility. Women with diminished ovarian reserve have significantly higher levels of nerve growth factor (NGF) compared with women with male-factor infertility. Follicular-fluid BDNF levels tend to be lower in patients with endometriosis and diminished ovarian reserve and to be higher in patients with polycystic ovarian syndrome. Interestingly, NGF concentrations follow the opposite trend. But, follicular-fluid NT-3 concentrations are similar in women with different etiologies of infertility (Buyuk *et al* 2008).

Polycystic Ovaries

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine and metabolic disorder recognized as the primary cause of infertility in women of the reproductive age. The syndrome is associated with ovulatory dysfunction, abdominal obesity, hyperandrogenism, and profound insulin resistance.

The precise etiology of the disease is unknown, even though the disturbances detected in PCOS has been attributed to primary defects in the hypothalamus-pituitary-adrenal (HPA) axis, the ovarian microenvironment, the adrenal gland, and the insulin/insulin-like growth factor (IGF)-I metabolic regulatory system. Several investigators have suggested that the sympathetic nervous system may be a primary factor in the development and maintenance of PCOS.

Even though it is impossible to reproduce human PCOS in an animal model, murine models may provide important leads. Studies on adult normal cycling rats found that a single intramuscular (i.m.) injection of estradiol valerate (EV) causes acyclicity and formation of PCO. The EV-induced rat PCO model reflects some endocrinological and morphological characteristics of human PCOS, and it is assumed that activity in the ovarian sympathetic

nerves is higher than in normal rats. This is evidenced by an early increase in ovarian levels of norepinephrine (NE), an enhanced release of NE from ovarian nerve terminals, an increased activity of the catecholamine synthesis-limiting enzyme tyrosine hydroxylase (TH), and down-regulation of β2-adrenoceptors (ARs) in theca-interstitial cells (Manni *et al* 2006). Anti-NGF treatment in the PCO group restored all changes in mRNA and protein content, except that of alpha1b-AR and TrkA mRNAs, to control levels.

An increase in sympathetic outflow to the ovary proceeds, by several weeks, the appearance of cysts, suggesting the involvement of a neurogenic component in the pathology of this ovarian dysfunction. This change in sympathetic tone is related to an augmented production of ovarian NGF, and this abnormally elevated production of NGF contributes to the formation of ovarian cysts induced by EV (Lara *et al* 2000). Intraovarian injections of the retrograde tracer fluorogold combined with in situ hybridization to detect tyrosine hydroxylase (TH) messenger RNA-containing neurons in the celiac ganglion revealed that these changes in NGF/p75 NGFR synthesis are accompanied by selective activation of noradrenergic neurons projecting to the ovary. The levels of RBT2 messenger RNA, which encodes a beta-tubulin presumably involved in slow axonal transport, were markedly elevated, indicating that EV-induced formation of ovarian cysts is preceded by functional activation ofceliac ganglion neurons, including those innervating the ovary. Intraovarian administration of a neutralizing antiserum to NGF in conjunction with an antisense oligodeoxynucleotide to p75 NGFR, via Alzet osmotic minipumps, restored estrous cyclicity and ovulatory capacity in a majority of EV-treated rats. These functional changes were accompanied by restoration of the number of antral follicles per ovary that had been depleted by EV and a significant reduction in the number of both precystic follicles and follicular cysts. The hyperactivation of ovarian sympathetic nerves seen in EV-induced PCO is related to an overproduction of NGF and its low affinity receptor in the gland. Activation of this neurotrophic-neurogenic regulatory loop is a component of the pathological process by which EV induces cyst formation and anovulation in rodents. A similar alteration in neurotrophic input to the ovary might contribute to the etiology and/or maintenance of the PCO syndrome in humans.

Selective blockade of NGF actions and p75NTR synthesis in the ovary can restore estrous cyclicity and ovulatory capacity in estradiol valerate-treated rats, suggesting that an increase in NGF-dependent, p75NTR -mediated actions within the ovary contributes to the development of cystic ovarian disease. Dissen *et al* (2000) tested this hypothesis by grafting NGF-producing neural progenitor cells into the ovary of juvenile rats that have been induced to ovulate precociously by a single injection of pregnant mare serum gonadotropin, PMSG. The NGF-producing cells, detected by their content of immunoreactive p75NTR material, were found scattered throughout the ovary with some of them infiltrating the granulosa cell compartment of large, precystic follicles. Ovarian NGF content was 2-fold higher than in the ovary of rats receiving control cells. Estrous cyclicity was disrupted, with the animals showing prolonged periods of persistent estrus, and an almost continuous background of vaginal cornified cells at other phases of the estrous cycle. Morphometric analysis revealed that the presence of NGF-producing cells neither reduced the total number of corpora lutea per ovary nor significantly increased the formation of follicular cysts. However, the ovaries receiving these cells showed an increased incidence of precystic follicles, accompanied by a

reduced number of healthy antral follicles, and an increased size of both healthy and atretic follicles. These changes in follicular dynamics were accompanied by a selective increase in serum androstenedione levels. Abnormally elevated production of NGF within the ovary suffices to initiate several of the structural and functional alterations associated with the development of follicular cysts in the rat ovary.

NGF is known to serve as a neurotrophin for both the sympathetic and the sensory nervous systems and to enhance the activity of catecholaminergic and possibly other neuron types. Electro-acupuncture (EA) is known to reduce hyperactivity in the sympathetic nervous system. For these reasons, a model was used to investigate the effects of EA (12 treatments, approximately 25 min each, over 30 days) by analyzing NGF in the central nervous system and the endocrine organs, including the ovaries. The points chosen for stimulation were bilateral in biceps femoris and erector spinae, in somatic segments corresponding to the innervation of the ovaries. Significantly higher concentrations of NGF were found in the ovaries and the adrenal glands in the rats in the PCO model than in the control rats that were only injected with the vehicle (oil or NaCl). Repeated EA treatments in PCO rats resulted in concentrations of NGF in the ovaries that were significantly lower than those in non-EA-treated PCO rats but were within a normal range that did not differ from those in the untreated groups. The results provided support for the theory that EA inhibits hyperactivity in the sympathetic nervous system (Stener-Victorin *et al* 2000).

The hypothesis that repeated EA treatments modulates sympathetic nerve activity in rats with PCO was tested by analysing endothelin-1 (ET-1), a potent vasoconstrictor involved in ovarian functions, as well as NGF and NGF mRNA expression involved in the pathophysiological process underlying steroid-induced PCO (Stener-Victorin *et al* 2003). Concentrations of ET-1 in the ovaries were significantly lower in the PCO group receiving EA compared with the healthy control group ($p < 0.05$). In the hypothalamus, however, ET-1 concentrations were found to be significantly higher in the PCO group receiving EA than in the healthy control group ($p < 0.05$). So, EA modulates the neuroendocrinological state of the ovaries, most likely by modulating the sympathetic nerve activity in the ovaries, which may be a factor in the maintenance of steroid-induced PCO. Distribution of proteins of α1a-, α 1b-, α 1d-, and β2-adrenoceptors (ARs), as well as the low-affinity neurotrophin receptor (p75NTR) as measured by mRNA expression shows a significantly lower expression of β2-ARs mRNA expression in PCO rats. EA normalizes most of the EV-induced changes in ovarian ARs. Furthermore, EA was able to prevent the EV-induced up regulation of p75NTR, probably by normalizing the sympathetic ovarian response to NGF action. Possible role of EA in the regulation of ovarian responsiveness to sympathetic inputs depicts a possible complementary therapeutic approach to overcoming sympathetic-related anovulation in women with PCOS (Manni *et al* 2005, Bai *et al* 2004).

Denervation of ovarian sympathetic nerves restores ovulatory disruption in EV-induced PCO in rats. 5 weeks of voluntary exercise influence ovarian morphology and the expression of sympathetic markers in the EV-induced PCO rat model. The effect of exercise on (i) ovarian morphology; (ii) mRNA and protein expression of NGF; and (iii) mRNA and number of ovarian-expressing cells for the NGF receptor (p75NTR and the adrenergic receptors (ARs) in rats was evaluated against some control. The results obtained indicated that ovarian morphology was almost normalised in the PCO exercise group; NGF mRNA and protein

concentrations were normalised in the PCO exercise group; high numbers of NGF receptor expressing cells in PCO ovaries were lowered by exercise. Beneficial effect of regular exercise, as a modulator of ovarian sympathetic innervation was observed in the prevention and treatment of human PCOS (Manni *et al* 2005).

Pak et al (2005) investigated the effect of Korean red ginseng total saponins (GTS) on ovarian morphology and NGF expression in the ovaries, pituitary, and hippocampus in PCO rat model induced by EV in thirty sexually mature female Sprague-Dawley rats weighing 190-210 g. Increased expression of NGF was noted in the ovaries and the brain of rats with PCO. GTS administration attenuated NGF expression in the ovaries but not in the brain as deed two other herbal formulas, Changbudodam-Tang (cang fu dao tan tang) and Yongdamsagan-Tang (long dan xie gan tang) (Lee *et al* 2003). Thus NGF has become a gold standard in measuring PCO treatment efficacy in animal model.

Breast Cancer

NGF is able to stimulate the proliferation of breast cancer cells (MCF-7 and MDA-MB-231 cell lines). But, it is unable to stimulate growth of normal breast epithelial cells (NBEC) (Descamps *et al* 1998). In cells expressing low levels of TrkA such as breast carcinoma cells, NGF must recruit other overexpressed receptors such as p185 (HER2) in order to generate a biological signal that can induce breast cancer cell growth (Tagliabue *et al* 2000). The antiestrogen drug tamoxifen (TAM) inhibited NGF-induced MCF-7 cell proliferation and trkA (NGFR) phosphorylation in a concentration-related fashion. The effect of TAM seemed to be estrogen receptor-independent, because the pure estrogen receptor antagonist ICI 182.780 was unable to block NGF-induced trkA phosphorylation (Chiarenza *et al* 2000). Two distinct signaling pathways are required for NGF activity and confirm the roles played by p75NTR and NF-kappaB in the activation of the survival pathway in breast cancer cells (Descamps *et al* 2001). NGF receptors and signaling are thus looking increasingly promising as potential drug targets for breast cancer. The constitutive growth of breast cancer cells was strongly inhibited by NGF-neutralizing antibodies or K-252a, a pharmacological inhibitor of NGF receptor TrkA, indicating the existence of an NGF autocrine loop. Physiological relevance of NGF in breast cancer and its potential interest as a marker and therapeutic target is fast gaining ground (Dollé *et al* 2003).

That targeting NGF in breast cancer may have therapeutic ramifications is also shown in a recent study where analysis of a series of biopsies revealed widespread expression of NGF, in the majority of human breast tumors, with anti-NGF immunoreactivity concentrated in the epithelial cancer cells (Adriaenssens *et al* 2003). Moreover, immunodeficient mice xenografted with human breast cancer cells and treated with either anti-NGF antibodies or small interfering RNA against NGF displayed inhibited tumor growth and metastasis. NGF can be targeted in breast cancer to inhibit tumor cell proliferation, survival, and metastasis. Such treatments directed against NGF can induce a decrease in cell proliferation with a concomitant increase in apoptosis of breast cancer cells and an inhibition of tumor angiogenesis.

Serous Ovarian Carcinoma

Correlation between expression and activation of the high-affinity nerve growth factor (NGF) receptor TrkA, cell cycle protein expression, and disease outcome in serous ovarian carcinoma was analyzed by Davidson *et al* (2003) in malignant effusions and corresponding solid tumors in serous ovarian carcinoma. Coexpression of NGF with molecules involved in angiogenesis and phospho-TrkA expression in endothelial cells suggested that the proangiogenic role attributed to NGF in vitro and in vivo might be relevant in clinical cancer. Expression of poly (ADP-ribose) polymerase (p85-PARP) as a marker of apoptosis and cytoplasmic expression of TrkA (probably representing nonglycosylated receptor) predict better outcome, whereas phospho-TrkA activation correlates with poor outcome in advanced stage serous ovarian carcinoma.

In the ovary, NT including NGF can help in follicular maturation and ovulation by inducing the FSH receptor (FSHR). Current literature shows that perimenopausal ovarian surface epithelium (OSE) can also express FSHR. By G protein link, this FSHR is capable of precipitating neoplasia of OSE, which is the commonest in the ovary. NTs may be implicated as the cause of this aberrant expression of FSHR in OSE. By central action NT can lower serum FSH, as is found in postmenopausal ovarian cancer. Thus, NGF deregulates expression of FSHR in OSE and secretion of FSH from the pituitary. This phenomenon may hold the key to the hitherto unexplained carcinogenic process of sporadic epithelial ovarian cancer (Bose 2005).

Actually TrkA mRNA levels were over-expressed in ovarian cancer compared to normal ovarian samples, whereas NGF mRNA levels remained unchanged. NGF and trkA proteins were absent or found in very low levels in normal ovarian surface epithelium (OSE), whereas they were highly expressed in epithelial cells of EOC. Additionally, NGF stimulated the expression of VEGF isoforms in cancer explants. The effect was dose-dependent and inhibited by a NGF antibody and by K252a, a trk receptor inhibitor. The abundance of NGF and trkA receptors in epithelial cells of EOC, together with the ability of NGF to increase VEGF expression strongly suggests an autocrine role of NGF in EOC (Campos *et al* 2007). Thus, blocking neurotrophin action could be a therapeutic target in treating ovarian cancer.

Epidermal growth factor receptor, EGFR and TrkB crosstalk each other in response to EGF and BDNF, leading to cell survival pathway activation in ovarian cancer cells. A combination of inhibitors of both receptors with cell survival pathway inhibitors may provide a better outcome in the clinical treatment of ovarian cancer (Qiu *et al* 2006). Evidence of clinical role of p-TrkA in ovarian carcinoma is provided by the expression of biologically active p-TrkA receptor at the cell membrane is up regulated along tumor progression in ovarian carcinoma, whereas p75 expression remains unaltered. NGF receptors probably signal via MAPK-independent pathways in ovarian carcinoma (Ødegaard *et al* 2007). Follitropin receptor knockout (FORKO) mice are sterile and have age-dependent abnormalities including increased ovarian tumor incidence. To explore why atrophic ovaries of FORKO mice become tumorigenic later in life, gene expression profile was done by microarray at different ages showing unexpected ovarian expression of glial cell line-derived neurotrophic factor, GDNF and its bimodal regulation (Aravindakshan *et al* 2006). Though

evidence for GDNF up-regulation in GC tumors and a potential role for androgen were suggested, typical epithelial ovarian cancer might have different pathology.

Conclusion

Using sophisticated in vivo and in vitro techniques it is becoming possible to search for many NGF target cells including those of reproductive system. With multimodal approach NGF target cells are located in the immune system, neuroendocrine and hematopoietic cell lines thus revealing other as yet uncovered roles of this growth factor. Using gene transcription or translation techniques valuable information will be available on some of the still unexplored, hidden areas of the NGF mystery land. Alternate post-transcriptional or post-translational pathways leading to NGFs with a different structure and function and other proteins or peptides having the trophic, chemotactic and/or differentiative activity of NGF, but coded by other genes will gradually be discovered. Pharmacological agents that modify NGF gene expression or processing acting on the same path as those involved in the regulation of the synthesis and release of NGF could come out in near future by which it will be possible to interfere many endocrinal, immunological and other biological processes to our advantage.

References

Adriaenssens, E; Vanhecke, E; Saule, P; Mougel, A; *et al.* Nerve growth factor is a potential therapeutic target in breast cancer. *Cancer Res.* 2008 Jan 15; 68(2), 346-51.

Akasu, F; Tagawa, Y; Shimada, H. Promoting effects of the combined administration of nerve growth promoting factor (NGF) and human chorionic gonadotropin (HCG) on the ovaries and uteri of immature mice. *Acta Obstet Gynaecol Jpn.* 1970 Jul; 17(3), 179-85

Anaf, V; Simon, P; El Nakadi, I; Fayt, I; *et al.* Hyperalgesia, nerve infiltration and nerve growth factor expression in deep adenomyotic nodules, peritoneal and ovarian endometriosis. *Hum. Reprod*. 2002 Jul; 17(7),1895-900.

Anesetti, G; Lombide, P; D'Albora, H; Ojeda SR. Intrinsic neurons in the human ovary. *Cell Tissue Res*. 2001 Nov; 306(2), 231-7.

Aravindakshan, J; Chen, XL; Sairam, MR. Age-dependent bimodal GDNF regulation during ovarian tumorigenesis in follitropin receptor mutant mice. : *Biochem. Biophys. Res. Commun.* 2006 Dec 15; 351(2), 507-13.

Ayer-LeLievre C, Olson L, Ebendal T, Hallböök F, Persson H.. Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. *Proc. Natl. Acad. Sci. USA* 1988 Apr;85(8):2628-32.

Bai, YH; Lim, SC; Song, CH; Bae, CS; *et al.* Electro-acupuncture reverses nerve growth factor abundance in experimental polycystic ovaries in the rat. *Gynecol. Obstet Invest.* 2004; 57(2), 80-5.

Beer, DJ; Matloff, SM; Rocklin, RE. The influence of histamine on immune and inflammatory responses.*Adv. Immunol.* 1984; 35, 209-215.

Bianchi, R; Brozzi, F; and Donato, R. Increases Proliferation in PC12 Neuronal Cells and Reduces Their Responsiveness to Nerve Growth Factor via Akt Activation. *J. Biol. Chem.* 2005, 280 (6), Feb 11, 4402–4414,

Bienenstock, J; Tomioka, M; Matsuda, H; Stead, RH; *et al*. The role of mast cells in inflammatory processes: evidence for nerve/mast cell interactions. *Int. Arch Allergy Appl. Immunol*. 1987, 82(3-4), 238-43.

Bigon, E; Soranzo, C; Minozzi, C; Skaper, SD; Callegaro, L. Large scale purification and immunological characterization of human placental nerve growth factor. *Neurochem. Res*. 1990 Dec, 15(12), 1197-202.

Bose, CK. Nerve growth factor, follicle stimulating hormone receptor and epithelial ovarian cancer. *Med. Hypotheses*. 2004, 63(5), 917-8.

Bose, CK. Role of nerve growth factor and FSH receptor in epithelial ovarian cancer. *Reprod. Biomed. Online*. 2005 Aug, 11(2), 194-7.

Brauer, MM; Shockley, KP; Chávez, R; Richeri, A; *et al*. The role of NGF in pregnancy-induced degeneration and regeneration of sympathetic nerves in the guinea pig uterus. *J. Auton Nerv Syst*. 2000 Feb 14, 79(1), 19-27

Bryman, I; Norström, A; Dahlström, A; Lindblom, B. Immunohistochemical evidence for preserved innervation of the human cervix during pregnancy. *Gynecol. Obstet Invest*. 1987, 24(2), 73-9.

Bueker, ED. Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbosacral nervous system. *Anat. Rec.* 1948 Nov, 102 (3), 369-89.

Buyuk, E; Seifer, DB. Follicular-fluid neurotrophin levels in women undergoing assisted reproductive technology for different etiologies of infertility. *Fertil Steril.* 2008 Jan 25 [Epub ahead of print]

Calka, J; McDonald, JK; Ojeda, SR. The innervation of the immature rat ovary by calcitonin gene-related peptide. *Biol. Reprod.* 1988 Dec, 39(5), 1215-23.

Campos, X; Muñoz, Y; Selman, A; Yazigi, R; *et al*. Nerve growth factor and its high-affinity receptor trkA participate in the control of vascular endothelial growth factor expression in epithelial ovarian cancer. *Gynecol. Oncol.* 2007 Jan, 104(1), 168-75.

Chao, MV; Bothwell, M. Neurotrophins: to cleave or not to cleave. *Neuron* 2002, 33, 9–12

Chapman, CA; Banks, BE; Carstairs, JR; Pearce, FL; Vernon, CA. The preparation of nerve growth factor from the prostate of the guinea-pig and isolation of immunogenically pure material from the mouse submandibular gland. *FEBS Lett.* 1979 Sep 15, 105(2), 341-4.

Chiarenza, A; Lazarovici, P; Lempereur, L; Cantarella, G; *et al*.Tamoxifen inhibits nerve growth factor-induced proliferation of the human breast cancerous cell line MCF-7. *Cancer Res.* 2001 Apr 1, 61(7), 3002-8.

Chiarenza, A; Lazarovici, P; Lempereur, L; Cantarella, G; *et al*. Tamoxifen inhibits nerve growth factor-induced proliferation of the human breast cancerous cell line MCF-7. *Cancer Res*. 2001 Apr 1, 61(7), 3002-8.

Cohen, S; Levi-Montalcini, R; Hamburger V. A nerve growth-stimulating factor isolated from sarcomas 37 and 180. *Proc. Natl. Acad. Sci. USA* 1954 Oct, 40(10), 1014-8.

Davidson, B; Reich, R; Lazarovici, P; Nesland, JM; *et al*. Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. *Clin. Cancer Res*. 2003 Jun, 9(6), 2248-59.

Dees, WL; Ahmed, CE; Ojeda SR. Substance P- and vasoactive intestinal peptide-containing fibers reach the ovary by independent routes. *Endocrinology* 1986 Aug, 119(2), 638-41

Descamps, S; Lebourhis, X; Delehedde, M; Boilly, B; Hondermarck, H. Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells. *J. Biol. Chem*. 1998 Jul 3, 273(27), 16659-62

Descamps, S; Toillon, RA; Adriaenssens, E; Pawlowski, V; *et al*. Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. *J. Biol. Chem*. 2001 May 25, 276(21), 17864-70.

Dissen, GA; Hill, DF; Costa, ME; Les Dees, CW; Lara HE, Ojeda SR.A role for trkA nerve growth factor receptors in mammalian ovulation. *Endocrinology* 1996 Jan, 137(1), 198-209.

Dissen, GA; Hill, DF; Costa, ME; Ma, YJ; Ojeda, SR. Nerve growth factor receptors in the peripubertal rat ovary. *Mol. Endocrinol*. 1991 Nov, 5(11), 1642-50.

Dissen, GA; Hirshfield, AN; Malamed, S; Ojeda, SR. Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. *Endocrinology* 1995 Oct, 136(10), 4681-92.

Dissen, GA; Romero, C; Hirshfield, AN; Ojeda, SR. Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology* 2001 May, 142(5), 2078-86.

Dollé, L; El Yazidi-Belkoura, I; Adriaenssens, E; Nurcombe, V; Hondermarck, H; Nerve growth factor overexpression and autocrine loop in breast cancer cells. *Oncogene* 2003 Aug 28, 22(36), 5592-601.

Emanuele, E; Politi, P; Bianchi, M; Minoretti, P; *et al*. Raised plasma nerve growth factor levels associated with early-stage romantic love. *Psychoneuroendocrinology* 2006 Apr, 31(3), 288-94.

Gazzolo, D; Marinoni E, Di Iorio, R; Lituania, M; *et al*. High maternal blood S100B concentrations in pregnancies complicated by intrauterine growth restriction and intraventricular hemorrhage. *Clin. Chem.* 2006 May;52(5):819-26.

Goldstein, LD; Reynolds, CP; Perez-Polo, JR. Isolation of human nerve growth factor from placental tissue. *Neurochem. Res.* 1978 Apr, 3(2), 175-83.

Green, AR; Edwards, RE; Greaves, P; White, IN. Comparison of the effect of oestradiol, tamoxifen and raloxifene on nerve growth factor-alpha expression in specific neonatal mouse uterine cell types using laser capture microdissection. *J. Mol. Endocrinol*. 2003 Feb, 30(1), 1-11.

Green, AR; Styles, JA; Parrott, EL; Gray, D; *et al*. Neonatal tamoxifen treatment of mice leads to adenomyosis but not uterine cancer. *Exp. Toxicol. Pathol*. 2005 Mar; 56(4-5): 255-63

Hamburger V. (1934). Effects of wing bud extirpation on the development of the central nervous system in chick embryos. *J. Exp. Zool*. 68, 449-494.

Harper, GP; Barde, YA; Burnstock, G; Carstairs, JR; *et al*. Guinea pig prostate is a rich source of nerve growth factor. *Nature* 279, 160 - 162 (10 May 1979).

Kanai-Azuma, M; Kanai, Y; Matsuda, H; Kurohmaru, M; *et al*. Nerve growth factor promotes giant-cell transformation of mouse trophoblast cells in vitro. *Biochem. Biophys. Res. Commun.* 1997 Feb 13, 231(2), 309-15.

Krizsan-Agbas, D; Pedchenko, T; Hasan, W; Smith, PG. Oestrogen regulates sympathetic neurite outgrowth by modulating brain derived neurotrophic factor synthesis and release by the rodent uterus. *Eur. J. Neurosci.* 2003 Nov, 18(10), 2760-65.

Lara, HE; Dissen, GA; Leyton, V; Paredes, A; *et al*. An increased intraovarian synthesis of nerve growth factor and its low affinity receptor is a principal component of steroid-induced polycystic ovary in the rat. *Endocrinology* 2000 Mar, 141(3), 1059-72.

Lara, HE; McDonald, JK; Ojeda, SR. Involvement of nerve growth factor in female sexual development. *Endocrinology* 1990 Jan, 126(1), 364-75.

Lee, JC; Pak, SC; Lee, SH; Lim, SC; *et al*. The effect of herbal medicine on nerve growth factor in estradiol valerate-induced polycystic ovaries in rats. *Am. J. Chin. Med.* 2003, 31(6), 885-95.

Levi-Montalcini, R; Hamburger, V. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool*. 1951 Mar, 116(2), 321-61.

Levi-Montalcini R. The Nerve Growth Factor. *Ann. N Y Acad. Sci.* 1964 Oct 9, 118, 149-70.

Levi-Montalcini R., in The Neurosciences: Paths of Discovery, F. G. Worden, J. P. Swayzey, G. Adelman, Eds. (MIT Press, 1975), pp. 245-265.

Lobos, E; Gebhardt, C; Kluge, A; and Spanel-Borowski, K. Expression of Nerve Growth Factor (NGF) Isoforms in the Rat Uterus during Pregnancy: Accumulation of Precursor proNGF. *Endocrinology* 2005, 146 (4), 1922-1929

Lommatzsch, M; Hornych, K; Zingler, C; Schuff-Werner, P; *et al*. Maternal serum concentrations of BDNF and depression in the perinatal period. *Psychoneuroendocrinology* 2006 Apr, 31(3), 388-94

Luppi, P; Levi-Montalcini, R; Bracci-Laudiero, L; Bertolini, A; *et al*. NGF is released into plasma during human pregnancy: an oxytocin-mediated response? *Neuroreport* 1993 Aug, 4(8), 1063-5.

Malamitsi-Puchner, A; Nikolaou, KE; Economou, E; Boutsikou, M; *et al*. Intrauterine growth restriction and circulating neurotrophin levels at term. *Early Hum. Dev.* 2007 Jul, 83(7), 465-9.

Manni, L; Cajander, S; Lundeberg, T; Naylor, AS; *et al*. Effect of exercise on ovarian morphology and expression of nerve growth factor and alpha(1)- and beta(2)-adrenergic receptors in rats with steroid-induced polycystic ovaries. *J. Neuroendocrinol*. 2005 Dec;17(12):846-58. .

Manni, L; Holmäng, A; Cajander, S; Lundeberg, T; *et al*. Effect of anti-NGF on ovarian expression of alpha1- and beta2-adrenoceptors, TrkA, p75NTR, and tyrosine hydroxylase in rats with steroid-induced polycystic ovaries. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2006 Mar, 290(3), 826-35

Manni, L; Lundeberg, T; Holmäng, A; Aloe, L; Stener-Victorin, E. Effect of electro-acupuncture on ovarian expression of alpha (1)- and beta (2)-adrenoceptors, and p75 neurotrophin receptors in rats with steroid-induced polycystic ovaries. *Reprod. Biol. Endocrinol*. 2005 Jun 7, 3, 21

Martocchia, A; Sigala, S; Proietti, A; D'Urso, R; *et al*. Sex-related variations in serum nerve growth factor concentration in humans. *Neuropeptides* 2002 Dec, 36(6), 391-5.

Mayerhofer, A; Dissen, GA; Costa, ME; Ojeda, SR. A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. *Endocrinology* 1997 Aug, 138(8), 3320-9.

Mayerhofer, A; Dissen, GA; Parrott, JA; Hill, DF; *et al*. Involvement of nerve growth factor in the ovulatory cascade: trkA receptor activation inhibits gap junctional communication between thecal cells. *Endocrinology* 1996 Dec, 137(12), 5662-70.

McDonald, JK; Dees, WL; Ahmed, CE; Noe, BD; Ojeda, SR. Biochemical and immunocytochemical characterization of neuropeptide Y in the immature rat ovary. *Endocrinology* 1987 May, 120(5), 1703-10

Mechsner, S; Schwarz, J; Thode, J; Loddenkemper, C; *et al*. Growth-associated protein 43-positive sensory nerve fibers accompanied by immature vessels are located in or near peritoneal endometriotic lesions. *Fértil. Steril.* 2007 Sep, 88(3), 581-7.

Naves, FJ; Vázquez, MT; José, IS; Martínez-Almagro, A; Vega, JA. Pregnancy-induced denervation of the human uterine artery correlates with local decrease of NGF and TrkA. *Ital. J. Anat. Embryol*. 1998,103(4 Suppl 1), 279-90.

Ødegaard, E; Staff, AC; Abeler, VM; Kopolovic, J; *et al*. The activated nerve growth factor receptor p-TrkA is selectively expressed in advanced-stage ovarian carcinoma. *Hum. Pathol*. 2007 Jan, 38(1), 140-6.

Ojeda, SR; Costa, ME, Katz, KH; Hersh, LB. Evidence for the existence of substance P in the prepubertal rat ovary. I. Biochemical and physiologic studies. : *Biol. Reprod.* 1985 Sep, 33(2), 286-95.

Ojeda, SR; Dissen, GA; Junier, MP. Neurotrophic factors and female sexual development. *Front Neuroendocrinol.* 1992 Apr, 13(2), 120-62.

Ojeda, SR; Harms, PG; McCann, SM. Effect of inhibitors of prostaglandin synthesis on gonadotropin release in the rat. *Endocrinology* 1975 Oct, 97(4), 843-54.

Ojeda, SR; Romero, C; Tapia, V; Dissen, GA. Neurotrophic and cell-cell dependent control of early follicular development. *Mol. Cell Endocrinol*. 2000 May 25, 163(1-2), 67-71.

Pak, SC; Lim, SC; Nah, SY; Lee, J; Hill, JA; Bae, CS. Role of Korean red ginseng total saponins in rat infertility induced by polycystic ovaries. *Fertil Steril.* 2005 Oct, 84 Suppl 2, 1139-43.

Parrott, E; Butterworth, M; Green, A; White, IN; Greaves, P. Adenomyosis--a result of disordered stromal differentiation. *Am. J. Pathol.* 2001 Aug, 159(2), 623-30.

Pearce, FL; Thompson, HL. Some characteristics of histamine secretion from rat peritoneal mast cells stimulated with nerve growth factor. *J. Physiol*. 1986 Mar, 372, 379-93.

Qiu, L; Zhou, C; Sun, Y; Di, W; *et al*. Crosstalk between EGFR and TrkB enhances ovarian cancer cell migration and proliferation. *Int. J. Oncol.* 2006 Oct, 29(4), 1003-11

Ren, L; Medan, MS; Weng, Q; Jin, W; *et al*. J Immunolocalization of nerve growth factor (NGF) and its receptors (TrkA and p75LNGFR) in the reproductive organs of Shiba goats. *Reprod. Dev*. 2005 Jun, 51(3), 399-404.

Richard, A; McColl, SR; Pelletier, G. Interleukin-4 and nerve growth factor can act as cofactors for interleukin-3-induced histamine production in human umbilical cord blood cells in serum-free culture. *Br. J. Haematol.* 1992 May, 81(1), 6-11.

Romero, C; Paredes, A; Dissen, GA; Ojeda, SR. Nerve growth factor induces the expression of functional FSH receptors in newly formed follicles of the rat ovary. *Endocrinology* 2002 Apr, 143(4), 1485-94.

Said, SI; Mutt, V. Polypeptide with broad biological activity: Isolation from small intestine. *Science* 1970, 160, 1217-1218.

Smith, SS; Ojeda, SR. Neonatal release of gonadotropins is essential for development of ovarian follicle- stimulating hormone receptors. *Biol. Reprod*. 1986 Feb, 34(1), 219-27

Stempelj, M; Ferjan, I. Signaling pathway in nerve growth factor induced histamine release from rat mast cells. *Inflamm. Res*. 2005 Aug, 54(8), 344-9.

Stener-Victorin, E; Lundeberg, T; Cajander, S; Aloe, L; *et al*. Steroid-induced polycystic ovaries in rats: effect of electro-acupuncture on concentrations of endothelin-1 and nerve growth factor (NGF), and expression of NGF mRNA in the ovaries, the adrenal glands, and the central nervous system. *Reprod. Biol. Endocrinol.* 2003 Apr 8, 1, 33.

Stener-Victorin, E; Lundeberg, T; Waldenström, U; Manni, L; *et al*. Effects of electro-acupuncture on nerve growth factor and ovarian morphology in rats with experimentally induced polycystic ovaries. *Biol. Reprod.* 2000 Nov, 63(5), 1497-503.

Tagliabue, E; Castiglioni, F; Ghirelli, C; Modugno, M; *et al*. Nerve growth factor cooperates with p185(HER2) in activating growth of human breast carcinoma cells. *J. Biol. Chem.* 2000 Feb 25, 275(8), 5388-94.

Tokushige, N; Markham, R; Russell, P; Fraser, IS. Nerve fibres in peritoneal endometriosis. *Hum. Reprod*. 2006 Nov, 21(11), 3001-7.

Tokushige, N; Markham, R; Russell, P; Fraser, IS. Effects of hormonal treatment on nerve fibers in endometrium and myometrium in women with endometriosis. *Fertil Steril.* 2007 Nov 30 [Epub ahead of print]

Tometten, M; Blois, S; Arck, PC. Nerve growth factor in reproductive biology: link between the immune, endocrine and nervous system? *Chem. Immunol. Allergy* 2005, 89, 135-48

Tometten, M; Blois, S; Kuhlmei, A; Stretz, A; Klapp, BF; Arck, PC. Nerve growth factor translates stress response and subsequent murine abortion via adhesion molecule-dependent pathways. *Biol. Reprod.* 2006 Apr, 74(4), 674-83.

Tometten, M; Klapp, BF; Joachim, R; Fest, S; *et al*. Nerve growth factor and its functional receptor TrkA are up-regulated in murine decidual tissue of stress-triggered and substance P-mediated abortion. *Am. J. Reprod. Immunol.* 2004 Jan;51(1):86-93.

Toti, P; Ciarmela, P; Florio, P; Volpi, N; *et al*. Human placenta and fetal membranes express nerve growth factor mRNA and protein. *J. Endocrinol. Invest*. 2006 Apr, 29(4), 337-41.

Tskitishvili, E; Komoto, Y; Temma-Asano, K; Hayashi, S; Kinugasa, Y; *et al*. S100B protein expression in the amnion and amniotic fluid in pregnancies complicated by pre-eclampsia. *Mol. Hum. Reprod.* 2006 Dec;12(12):755-61.

Varol, FG; Duchemin, AM; Neff, NH; Hadjiconstantinou, M. Nerve growth factor (NGF) and NGF mRNA change in rat uterus during pregnancy. *Neurosci. Lett.* 2000 Nov 10, 294(1), 58-62.

Watson, AJ; Watson, PH; Arcellana-Panlilio, M; Warnes, D; *et al*. A growth factor phenotype map for ovine preimplantation development. *Biol. Reprod.* 1994 Apr, 50(4), 725-33

Zhang, G; Dmitrieva, N; Liu, Y; McGinty, KA; Berkley, KJ. Endometriosis as a neurovascular condition: estrous variations in innervation, vascularization, and growth factor content of ectopic endometrial cysts in the rat. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2008 Jan, 294(1), R162-71.

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Chapter 10

Nerve Growth Factor and Its Receptors in the Human Ovary

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Abstract

The nerve growth factor (NGF) receptor-signaling system is composed of the neurotrophin receptor p75 and the tyrosine kinase A (TrkA) receptor. Recent studies suggest that NGF plays important roles in both the normal development and function of the human ovary as well as with the promotion of ovarian cancer.

NGF and its receptors were investigated in human ovaries from fetuses, girls and women and in granulosa cells (GCs) and oocytes from in vitro fertilization (IVF) cycles. The NGF protein was identified in all GCs, and in oogonia and oocytes from preantral follicles. The TrkA protein was expressed mainly in oocytes, but also in GCs and in theca cells of antral follicles. The mRNA transcripts of NGF and TrkA were also identified. The p75 protein and mRNA transcripts were predominantly localized in stroma cells surrounding the oogonia/oocytes of fetuses aged up to 22 gestational weeks, but also in neuronal-like cells or fibers among blood vessels, in neuronal bodies, and in theca cells from fetuses and babies. The proteins and mRNA transcripts for NGF and TrkA were identified in cultured GCs and unfertilized mature oocytes from IVF cycles. NGF treatment of these GCs resulted in an increase in 17-β estradiol (E2) production and

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a decrease in progesterone levels; the addition of NGF together with FSH, induced higher E2 secretion. NGF also promoted an increase in FSH receptor mRNA; this action was blocked by a Trk inhibitor (K252a).

Studies indicate that p75 might be involved in follicular assembly during normal fetal ovarian development, as it is expressed mainly around this period. NGF, by promoting E2 production and reducing progesterone levels, prevents early luteinization and induces follicular acquisition of the FSH receptors required for their growth.

NGF and its two receptors have also been identified in various ovarian cancers. The NGF and TrkA proteins as well as TrkA mRNA were overexpressed in epithelial ovarian cancer, and the expression of TrkA was found to be a prediction of better outcome in the advanced-stage serous epithelial ovarian cancer. NGF probably promotes postmenopausal epithelial ovarian cancer by reducing FSH levels and by stimulating the expression of FSH receptors on the ovarian surface epithelium. NGF also stimulates angiogenesis in surface epithelium cancer directly or by promoting the production of vascular endothelial growth factor. Studies have shown that this increase is inhibited by the addition of an anti-NGF antibody and by K252a. Therefore, blocking NGF action could be a therapeutic target in ovarian cancer.

Introduction

Relevant Aspects of Human Oogenesis and Folliculogenesis

This section briefly discusses the *in vivo* development of germ cells and follicles from fetal to adult life, focusing on topics essential for the understanding of later sections.

The female is born with a complete pool of germ cells [Faddy and Gosden, 1996; Gougeon, 1996]. Their number declines gradually from birth (about one million) to menopause, mostly by atresia but also by ovulations. Although proliferating primordial germ cells have been detected in the ovaries and bone marrow of adult mice [Johnson *et al.*, 2004; 2005], their presence in women is unlikely [Liu *et al.*, 2007], owing to menopause [Faddy and Gosden, 1996; Gougeon, 1996] and the apparent inability of human bone marrow to produce new oocytes, as shown in studies of bone marrow transplantation for cancer therapy [Feigin *et al.*, 2008].

During fetal development, human primordial germ cells arrive from the yolk sac to the gonad from day 26 of pregnancy, and are then termed *oogonia* [Gosden, 1995; Abir *et al.*, 2006]. Thereafter, three events induce the development of the female fetal germ cells in the gonad (ovary): mitotic division cycles of the oogonia; meiotic division; and follicular assembly. The number of female germ cells in the fetal ovary peaks at about seven million in mid-pregnancy, and then drops dramatically during the third trimester. Meiotic division usually commences gradually in the third month of gestation, and the diplothen stage is achieved within weeks of its initiation. At this point, the oogonia increase in size and acquire more intracellular organelles, and their nuclei become round and large. These germ cells are now termed *oocytes*. Just before birth, the oocytes are arrested at the diplotene stage of the prophase of the first meiotic division. They do not undergo any additional nuclear maturation until puberty, when menstrual cycles are initiated.

Follicular formation in humans begins during the fourth month of gestation. During follicular assembly, there is a rapid proliferation of the nearby cells, and the oocytes become surrounded by a single layer of flattened somatic cells, termed *granulosa cells* (GCs) [Gougeon, 1996; Abir *et al*., 2006]. These cellular complexes, called *primordial follicles*, measure 30-50 μm in diameter and can be identified in humans from around 22 gestational weeks (GW). Most of the follicles in human fetal as well as in adult ovaries remain in the quiescent primordial form. Their growth regulation is not hormonal, and the exact factors that stimulate their development are unknown [Abir *et al*., 2006]. Primordial follicles are activated when their GCs become cuboidal, and they are then termed *primary follicles* (50-80 μm in diameter).

Primary follicles develop to *secondary follicles* (80 μm -0.2 mm in diameter) with an increase in the GC proliferation rate and consequent formation of a multilaminar layer [Gougeon, 1996]. In humans, a definitive *theca layer* is created from the stroma cells surrounding the secondary follicle. Steroid hormones such as 17β estradiol (E2) and progesterone are synthesized through complex interactions between the GCs and theca cells. The final follicular stage consists of the development of *antral follicles* (early antral follicle: 0.2-0.4 mm in diameter) containing a fluid-filled cavity within several layers of cuboidal GCs; the innermost layers surrounding the oocytes are termed *cumulus cells*, and the rest of the GCs are defined as *mural GCs*. Follicles preceding the antral stage (primordial, primary and secondary) are termed, collectively, *preantral* follicles.

The cyclic secretion of the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), is initiated at puberty [Speroff *et al*., 1994]. FSH levels rise in the first half of the menstrual cycle before ovulation, inducing follicular development and growth from the secondary stages (*follicular phase*) [Speroff *et al*., 1994; Abir *et al*., 2006]. A primordial follicle reaches the ovulatory antral size (18-20 mm in diameter) within six to nine months [Gougeon, 1996]. Ovulation occurs at the mid-menstrual cycle and is stimulated by a drastic increase in baseline LH levels (*LH surge*) [Speroff *et al*., 1994]. During ovulation, the first meiotic division is completed, with the extrusion of the first polar body, forming a mature oocyte. The ovarian site at which ovulation occur develops into a gland (*corpus luteum*), secreting high levels of progesterone and also E2. The remaining days until the end of the cycle are termed the *luteal phase*. Follicular depletion at menopause results in a drastic decrease in circulating steroid hormones, promoting high gonadotropin secretion.

Nerve Growth Factor: Structure, Functions and Receptor-Signaling System

Nerve growth factor (NGF) is the founder member of the neurotrophin family [Chao, 1992]. Neurotrophins are best known for their role in the development and maintenance of the peripheral and central nervous system, but they are also important in other non-neuronal cells [Snider, 1994], such as ovarian cells [Dissen *et al.*, 2001]. All neurotrophins share a structural and functional similarity, with a sequence identity of 50%, and numerous signaling pathways, some overlapping one another [Snider, 1994].

Structurally the NGF monomer is elongated, and its central portion is formed by two pairs of antiparallel β-strands [Wiesmann and de Vos, 2001]. One molecule end contains

three hairpin loops, and the other a cysteine-knot motif, which stabilizes the fold and preserves its conformation. The biologically active form of NGF contains two monomers arranged in parallel to form a close-packed homodimer. NGF transmits signals via the activation of two distinct cell-surface- expressed receptors: the high affinity tyrosine kinase A (TrkA) receptor, which is specific to NGF; the low affinity p75 receptor, which is shared by all neurotrophins [Chao and Hempstead, 1995; Wiesmann and de Vos, 2001].

The TrkA receptor is distinguished by an extracellular portion which consists of one cysteine rich domain, a second domain with three leucine-rich repeats, a third domain which is also cysteine-rich, and two immunoglobulin-like domains [Schneider and Schweiger, 1991]. The extracellular portion is linked by a single transmembrane helix to the intracellular kinase domain. The p75 receptor belongs to the tumor necrosis factor family. It has an extracellular sequence with four distinct cysteine-rich domains that are responsible for ligand binding [Chao and Hempstead, 1995].

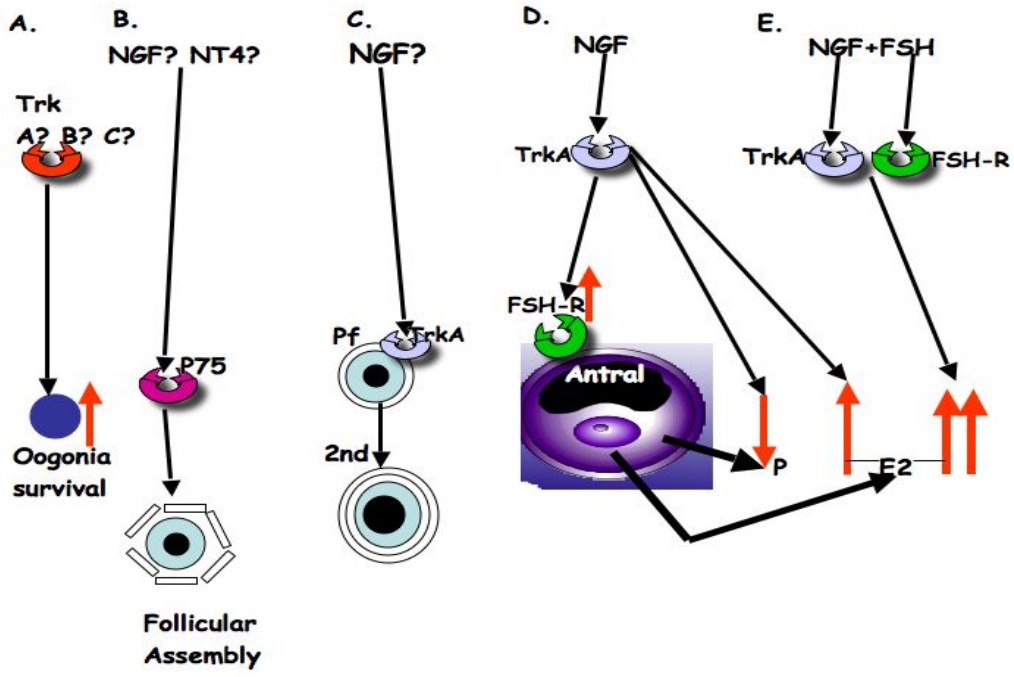
The complex interactions between TrkA and p75 are responsible for the specific cellular affinity of NGF. Because p75 is a universal receptor, it has a wider distribution than TrkA in various cell types, including Schwann cells, motor cells, meningeal cells, dental pulp cells, hair follicle cells, and cerebellar Purkinje cells. Moreover, during development, TrkA expression in the nervous system is restricted to the sensory and sympathetic neurons in the peripheral system and to cholinergic neurons in the basal forebrain. Most NGF-responsive cells express both p75 and TrkA, but the expression of TrkA is usually limited. Therefore, most NGF binds to p75. Furthermore, because p75 binding is characterized by rapid association and dissociation, and TrkA binding, by very slow dissociation, overall NGF has a lower affinity for TrkA. However, when p75 and TrkA are co-expressed, a high-affinity site is formed by the two receptors, yielding a 25-fold increase in NGF binding compared to the TrkA receptor alone. Changes in levels of p75 and TrkA can also influence the overall binding affinities of NGF. NGF transmits its cell survival signals through the TrkA receptor and its cell death signals through the p75 receptor [Wiesmann and de Vos, 2001]. Nevertheless, NGF-induced-apoptosis is initiated only in TrkA-negative neurons (cells), whereas even low NGF levels can promote cell survival if p75 is co-expressed with TrKA on the cell surface [Yano and Chao, 2000].

The NGF System and Normal Human Ovarian Development

Figure 1 illustrates the main functions and developmental events in the normal human ovary in which NGF and its receptors are involved. Immunohistochemical studies have identified the expression of NGF protein in primordial, primary, secondary [Abir *et al.*, 2005; Salas *et al.*, 2006], and antral [Salas et al., 2006] ovarian follicles from human fetuses at 19-33 GW (Figure 2A) and from women/girls (age 13-45 years) (Figure 2B) [Abir *et al.*, 2005; Salas *et al.*, 2006]. The fetal oocytes/oogonia exhibited full cytoplasmic staining without nuclear staining, (Figure 2A) [Abir *et al.*, 2005], and the oocytes from the women/girls exhibited partial cytoplasmic staining as well as nuclear staining (Figure 2B). NGF was also detected in GCs of follicles from primordial stages onward and in some of the stroma cells

from both fetuses and women/girls (Figures 2A and B). On reverse transcriptase polymerase chain reaction (RT-PCR) studies, NGF mRNA transcripts were identified in all ovarian extracts from fetuses and women/girls (Figure 3).

Others investigated GCs [Salas *et al*., 2006; Seifer *et al*., 2006] and mature unfertilized human oocytes [Seifer *et al*., 2006] obtained from *in vitro* fertilization (IVF) programs [Salas *et al*., 2006; Seifer *et al*., 2006]. The NGF protein was identified immunocytochemically in both mural and cumulus GCs, with higher expression in nuclei than in the cytoplasm. NGF staining was also detected in the nuclei and cytoplasm of the oocytes [Seifer *et al*., 2006]. On RT-PCR, NGF mRNA transcripts were identified in the GCs [Salas *et al*., 2006]. Moreover, when GCs were incubated with NGF, the NGF protein and mRNA transcripts were expressed throughout the culture period.



Note: Red arrows pointing up represent an increase; double red arrows pointing up represent a higher increase than single up-pointed red arrows; red arrows pointing down represent a decrease; Pf=primordial follicles, 2nd=secondary follicles; P=progesterone

A. Promotion of oogonia survival through the Trk receptors. It is unclear which of the Trk receptors and neurotrophins are involved in this process. Modified from Spears *et al*., 2003.

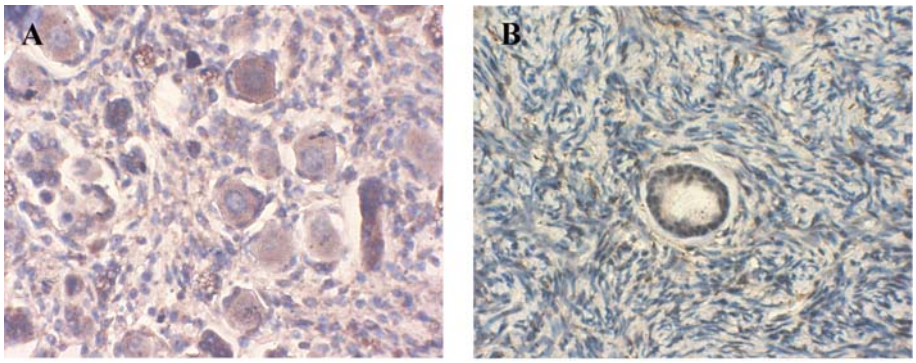
B. Promotion of follicular assembly in the human fetal ovary through the p75 receptor. Modified from Anderson *et al*., 2002; Abir *et al*., 2005.

C. Activation of primordial follicles to secondary follicles by possible interaction of NGF with the GCs expressing TrkA receptor. Modified from Abir *et al*., 2005.

D. Increase in GCs FSH-R expression consequent to NGF stimulation through its TrkA receptor, an increase in E2 secretion and a decrease in P secretion affected by the same pathway. Modified from Salas *et al*., 2006.

E. Higher E2 secretion stimulated by NGF with FSH than by NGF alone. Modified from Salas *et al*., 2006.

Figure 1. Schematic illustration of the main functions and developmental events of the normal human ovary in which NGF and its receptors are involved.



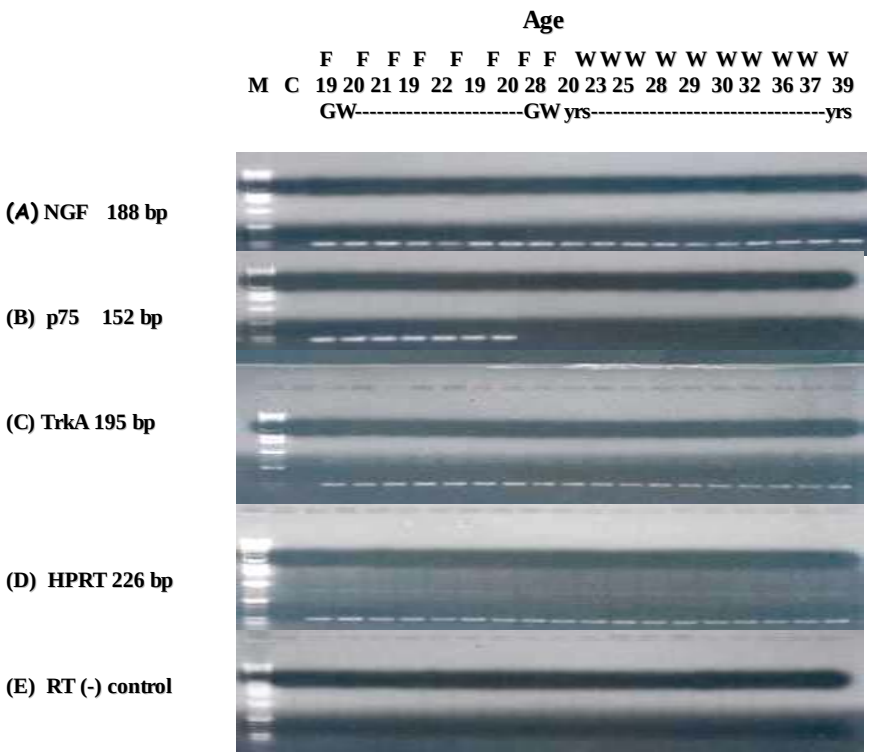
A. Section of human ovary from a 27-GW-old fetus. Note the primordial follicles, the full brown cytoplasmic staining without nuclear staining, and the brown staining in a portion of the GCs and in the stroma cells, indicating NGF expression. Original magnification X400.

B. Section of human ovary from a 38-year-old woman. Note the secondary follicle and the brown staining its GCs, the pale brown partial oocyte cytoplasmic staining and the staining in a portion of the stroma cells, indicating NGF expression. Original magnification X400.

Figure 2. Immunohistochemical photographs of NGF staining.

In further culture studies, GCs were exposed to various doses of NGF with testosterone as the aromatase substrate, with and without polyclonal antibodies against NGF. Measurement of E2 and progesterone in spent media samples, revealed an NGF-stimulated increase in E2 production in parallel with inhibition of progesterone secretion (Figure 1D). The presence of neutralizing antibodies against NGF prevented these effects. NGF also promoted the acquisition of high levels of FSH receptors (FSH-R) (Figure 1D). Semiquantitative and real-time PCR revealed that the action of NGF was dose dependent, and that the increase in FSH-R was obliterated on exposure to a Trk inhibitor (K252a), indicating that NGF functioned through its TrkA receptor (Figures 1D and E). These findings were in line with rat studies in which the treatment of primordial follicles with NGF increased FSH-R expression and the ovarian capacity to respond to FSH [Romero *et al*., 2002]. Moreover, human GCs from IVF cycles, first primed with NGF and then with FSH, promoted higher E_2 production than those exposed to NGF alone (Figure 1E) [Salas *et al*., 2006]. Thus, in the human ovary, NGF can diminish GC differentiation into progesterone producing lutein cells by enhancing E_2 production either directly or by increasing FSH-R levels. However, the lack of change in levels of ki67, a cell proliferating marker, indicates that NGF is unable to promote human GC proliferation. By contrast, in the rat, NGF stimulated GC proliferation from unilaminar to secondary stages (Figure 1C) [Romero *et al*., 2002].

Immunohistochemistry studies also revealed that the protein for the TrkA receptor is expressed in ovaries of human fetuses at 19-33 GW [Abir *et al*., 2005] and women/girls (13-45 years) [Abir *et al*., 2005; Salas *et al*., 2006]. TrkA staining was demonstrated in primordial, primary, secondary [Abir *et al*., 2005], and antral [Salas *et al*., 2006] ovarian follicles from both fetuses (Figure 4A) [Abir *et al*., 2005] and women/girls (Figure 4B) [Abir *et al*., 2005; Salas *et al*., 2006] mainly in oogonia/oocytes and also in some stroma cells [Abir *et al*., 2005]. The majority of fetal oocytes/oogonia showed full cytoplasmic staining without nuclear staining (Figure 4A), and the remainder showed partial cytoplasmic staining.



W: Women; F: Fetus; GW: gestational weeks; M: 100 base pair (bp) ladder
(A) NGF RT-PCR product 188 bp
(B) p75 152 bp detected in fetal ovarian samples up to 22 GW
(C) TrkA 195 bp
(D) HPRT 226 bp (constitutively expressed gene-positive control for the RT reaction)
(E) RT negative control [RT (-)].

Figure 3. Representative gel from the RT-PCR products for NGF, p75, TrkA and hypoxanthine phosphoribosyl transferase (HPRT) from human adult and fetal ovaries.

From Abir *et al*., 2005 (Molecular Human Reproduction, 11, 229-36).
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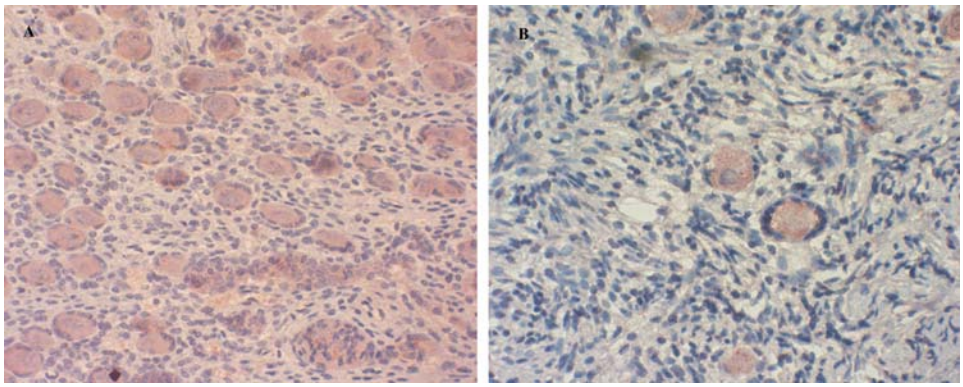
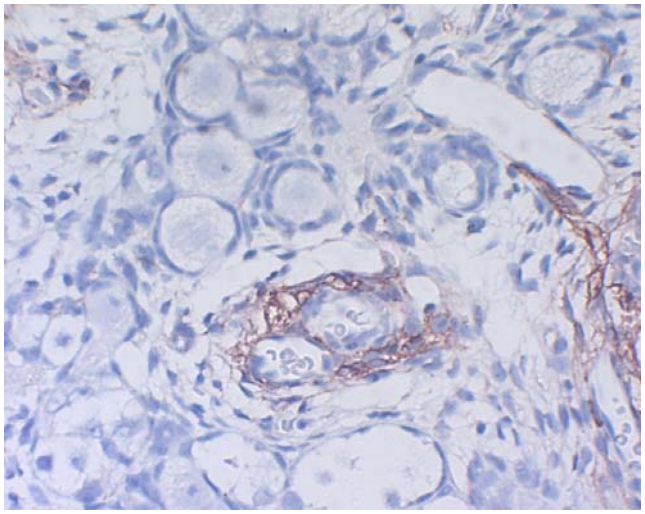
Most oocytes from women/girls showed partial cytoplasmic staining with nuclear staining (Figure 4B); full cytoplasmic oocyte staining was identified in the remainder. TrkA staining was also identified in GCs in 35% of the fetal samples and 50% of the samples from women/girls, regardless of follicular class [Abir *et al*., 2005; Salas *et al*., 2006], and in theca cells of antral follicles [Salas *et al*., 2006]. TrkA mRNA transcripts were detected by RT-PCR in ovaries from fetuses aged 13-33 GW (Figure 3) [Anderson *et al*., 2002; Abir *et al*., 2005] as well in ovarian extracts from women/girls (Figure 3) [Abir *et al*., 2005]. Accordingly, culture of human ovaries from normal fetuses aged 13-16 GW with K252a reduced the oogonia number [Spears *et al*., 2003]. However, it remained unclear which of the three Trk receptors [Chao and Hempstead, 1995] and their activating neurotrophins were essential for oogonia survival (Figure 1A).



A. Section of human ovary from 31-GW-old fetus. Note the primordial follicles and the full red-brown cytoplasmic oocyte staining with nuclear staining, and the staining in a portion of the GCs and in stroma cells, indicating TrkA expression. Original magnification X400.

B. Section of human ovary from 20-year-old woman. Note the primordial and primary follicles, the full red-brown cytoplasmic staining without nuclear oocyte staining, and the staining in a portion of their GCs and in stroma cells, indicating TrkA expression. Original magnification X400.

Figure 4. Immunohistochemical photographs of TrkA expression.



Section of human ovary from a 20-GW-old fetus. Note the forming primordial follicles and the brown staining in some of the stroma cells especially those surrounding the oocytes, indicating p75 expression. Staining is absent in GCs and oocytes. Original magnification X400.

Figure 5. Immunohistochemical expression of p75.

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The protein for TrkA was also identified immunocytochemically in both mural and cumulus GCs from IVF programs [Salas *et al.,* 2006; Seifer *et al*., 2006] as well as in the cytoplasm and nuclei of mature unfertilized oocytes [Seifer *et al*., 2006]. TrkA mRNA

transcripts were identified by RT-PCR in these GCs [Salas *et al*., 2006]. The TrkA protein and mRNA transcripts were also expressed in GCs incubated with NGF, although the TrkA levels decreased during culture.

Anesetti *et al.* [2001] investigated the expression of the p75 protein by immunohistochemistry and immunohistofluorescence-confocal microscopy in human ovaries from fetuses aged 24-37 GW and from infants aged 13 days to 10 months after sudden death syndrome. They identified a population of p75 immunoreactive cells with a neuronal appearance mostly in the ovarian medulla among blood vessels, but also in the ovarian cortex in fibers derived from extrinsic nerves located in the adventitia of blood vessels and in some neuronal bodies. More importantly, theca cells of growing follicles were p75-positive.

In two other studies [Anderson *et al*., 2002; Abir *et al.*, 2005], the expression of the p75 protein was detected by immunohistochemical and immunoblotting studies in a portion of the stroma cells of human ovaries from fetuses aged 13-21 GW, specifically in stroma cells surrounding oocytes before or during follicular assembly (Figure 5). The mRNA transcripts were identified by RT-PCR in ovaries of fetuses aged 19-21 GW (Figure 3) [Abir *et al.*, 2005], but not in samples from older fetuses or women (Figure 1B). These findings are in line with studies in rodents [Dissen *et al*., 1995, 2001; Ojeda *et al*., 2000] wherein p75 was expressed in ovaries of infantile mice during follicular assembly [Dissen *et al*., 2001] and in rat fetal ovarian mesenchymal cells that transformed, by the end of gestation, into pocket-like epithelial structures surrounding oocyte clusters. Their postnatal accumulation paralleled follicular formation [Dissen *et al*., 1995; Ojeda *et al*., 2000]. There was no p75 expression in GCs or oocytes (Ojeda *et al*., 2000). Thus, p75 may play a role in follicular assembly in humans (Figure 1B) [Anderson *et al*., 2002; Abir *et al*., 2005] as well as other mammals [Dissen *et al*., 1995, 2001; Ojeda *et al*., 2000]. Given that in the rat, NGF and TrkA levels decreased at the time of follicular assembly [Ojeda *et al*., 2000] and that NGF transmits apoptotic signals through the p75 receptor [Wiesmann and de Vos, 2001], it is unlikely that follicular formation is promoted by NGF activation of p75, unless both NGF receptors are co-expressed [Yano and Chao, 2000]. This assumption is supported by findings in rats [Dissen *et al*., 1995; Ojeda *et al*., 2000] and humans [Anderson *et al*., 2002] of an increase in neurotrophin 4 (NT-4) levels around the time of follicular assembly [Dissen *et al*., 1995; Ojeda *et al*., 2000; Anderson *et al*., 2002].

The NGF System and Ovarian Cancer

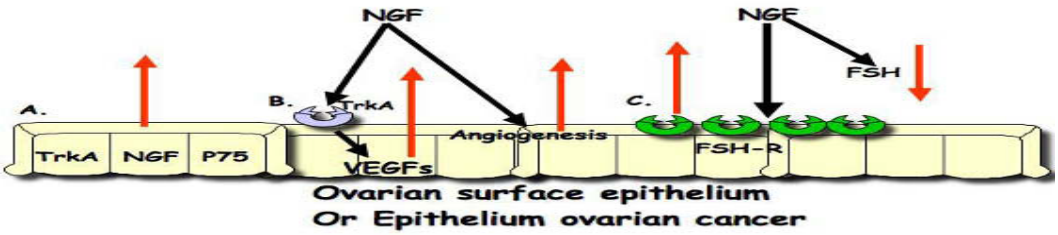
Each year, more women die from ovarian cancer than from any other gynecological malignancy [Campos *et al.*, 2007]. The lifetime risk for a woman to be affected by ovarian cancer is 1.7%, and the annual incidence approaches 61.3 per 100,000 in women aged 75-79 years [Ries *et al.*, 1998]. Ovarian cancer is an insidious and deadly disease because it is asymptomatic until the malignancy metastasizes beyond the ovaries. Unfortunately, screening for early detection of ovarian malignancies has not proven effective [Campos *et al.*, 2007].

Most cases of ovarian cancer are assumed to result from neoplastic transformation of post-ovulation surface epithelium. The integrity of the cellular DNA in the area of the ovarian rupture site is compromised, leading to clonal expansion of the damaged epithelial cells [Mok

et al., 1992]. However, the multifactorial sequence of events that leads to ovarian cancer is not adequately understood. It is possible that a local increase in growth factors promotes angiogenesis and proliferation of ovarian surface epithelial cells, initiating cancer progression [Campos *et al.*, 2007].

Several studies from the last decade suggested that NGF and its two receptors might be involved in the etiology of ovarian cancer (Figure 6) [Koizumi *et al.*, 1998; Davidson *et al.*, 2001 ,2003; Campos *et al.*, 2007]. Findings included elevated levels of NGF and TrkA in epithelial ovarian cancer (45 patients with serous ovarian carcinomas and five with mucinous ovarian carcinomas) (Figure 6A) [Campos *et al.*, 2007], and a high expression of TrkA and p75 receptors in effusions and in primary and metastatic tumors from patients with serous ovarian carcinoma (Figure 6A) [Koizumi *et al.*, 1998; Davidson *et al.*, 2001 ,2003]. However, cytoplasmic expression of TrkA predicted a better outcome only in cases of advanced-stage serous epithelial ovarian cancer [Davidson *et al.*, 2003].

Angiogenesis and the production of angiogenic factors are essential for tumor development and metastasis [Campos *et al.*, 2007]. NGF may play a dual role in this process during cancer transformation, acting as a direct vascular promoter in endothelial cells of serous ovarian carcinoma as well as a stimulator of vascular endothelial growth factor (VEGF) expression in epithelial ovarian cells (Figure 6B) [Calza *et al.*, 2001; Cantarella *et al.*, 2002; Campos *et al.*, 2007]. VEGF, also called VEGF-A or vascular permeability factor, is the most important regulator of blood vessel formation [Geva and Jaffe, 2000; Holmes and Zachary, 2005; Kaczmarek *et al.*, 2005]. It is secreted by epithelial ovarian tumors and is involved in tumor progression and maintenance [Cantarella *et al.*, 2002; Campos *et al.*, 2007].



Note: Red arrows pointing up represent an increase; red arrows pointing down represent a decrease

A. Elevated levels of NGF and its two receptors, TrkA and p75, in epithelial ovarian cancer. Modified from Koizumi et al., 1998**;** Davidson et al., 2001 **,**2003**;** Campos et al., 2007.

B. Effects of NGF on angiongenesis during epithelial ovarian cancer transformation. NGF acts as a direct vascular promoter and via TrkA stimulation of VEGF production. Modified from Calza et al., 2001; Cantarella et al., 2002**;** Campos et al., 2007.

C. NGF promotion of an increase in FSH-R levels in epithelial ovarian cancer and ovarian surface epithelium and of a decrease in FSH circulating levels in menopausal women. Modified from Wang et al., 2003; Bose, 2005.

Figure 6. Schematic illustration of the main interactions between NGF and its receptors that contribute to epithelial ovarian cancer promotion.

The VEGF gene in humans is composed of eight exons [Geva and Jaffe, 2000]. Alternative splicing results in at least six isoforms encoding different-length polypeptides [Geva and Jaffe, 2000; Holmes and Zachary, 2005; Kaczmarek *et al*., 2005]. The two major isoforms, secreted as covalently linked homodimeric proteins, are the lower potency and weakly acidic polypeptide $VEGF_{121}$ and the biologically active, abundant, glycosylated $VEGF_{165}$ (46kDa). $VEGF_{165}$ also has a functional role in ovarian cyst formation associated with ovarian cancer [Duyndam *et al.*, 2002]. The incubation of epithelial ovarian cancer explants with increasing doses of NGF resulted in a significant increase in mRNA (RT-PCR) and protein (immunohistochemistry) levels of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (Figure 6B). These findings were confirmed by the identification of the $VEGF_{121}$ and $VEGF_{165}$ proteins also in the spent culture media samples on ELISA. The NGF-induced increase in VEGF mRNA was blocked by an antibody against NGF or by K252a, confirming that the VEGF increase was specifically promoted by NGF activation through its TrkA receptor (Figure 6B) [Campos *et al.*, 2007].

NGF normally stimulates the increase in FSH-R expression in GCs that is essential for FSH-induced follicular proliferation [Romero *el al.*, 2002; Salas *et al*., 2006]. However, FSH-Rs have also been identified in abnormal locations, such as postmenopausal ovarian surface epithelium and epithelial ovarian cancer (Figure 6C) [Wang *et al.*, 2003]. In these cases, an elevation in ovarian or circulating NGF levels (due to various causes), in absence of sufficient levels of sex hormone regulators, can induce aberrant FSH-R expression in ovarian surface epithelium in parallel with a significant decrease in FSH menopausal levels in the epithelial ovarian cancer (Figure 6C) [Bose, 2005]. Therefore, NGF directly disregulates FSH-R ovarian signaling and promotes growth disorder and neoplasia in the ovarian surface epithelium.

Conclusion

NGF and its receptors might be involved in various normal processes in the human ovary (Figure 1) [Ojeda *et al*., 2000; Abir *et al.*, 2005], as indicated by findings of NGF-stimulated activation of primordial follicles in the rat [Romero *et al*., 2002], and the presence of the TrkA receptor protein in GCs of human primordial follicles (Figure 1C) [Abir *et al*., 2005]. However, considering that TrkA was expressed only in a portion of the human GCs, and that NGF was unable to induce proliferation of human GCs from IVF cycles [Salas *et al*., 2006], it seems more likely that other growth factors [Abir *et al*., 2006] including neurotrophins [Harel *et al*., 2006], participate to a greater degree in this process. Be that as it may, NGF at least indirectly affects human unilaminar follicular development by increasing FSH-R levels in GCs (Figure 1D) [Salas *et al*., 2006], and by the acquisition of their ability to respond to FSH, essential for GC proliferation from secondary stages.

NGF plays other important roles in later stages of human folliculogenesis, such as promotion of steroid production (Figure 1D and E). NGF enhances E2 production, delays the differentiation of GCs from preovulatory follicles into progesterone-secreting lutein cells. Its effect on steroid production is achieved both directly and via the ability of NGF to increase FSH-R levels by signaling through its TrkA receptor (Figure 1D and E).

The universal neurotrophin receptor p75 seems to be involved in follicular assembly in mammals, including humans, as it is expressed in ovaries mainly around the period of follicular formation (Figure 1B) [Ojeda *et al*., 2000; Anderson *et al*., 2002; Abir *et al*., 2005]. However, other neurotrophins such as NT-4, seem to be more promising candidates for this role [Ojeda *et al*., 2000; Anderson *et al*., 2002]. By contrast to NGF, NT-4 was shown to be expressed at high levels during follicular formation in the rat [Ojeda *et al*., 2000] and human [Anderson *et al*., 2002]. Moreover, NGF can promote apoptosis through the p75 receptor [Wiesmann and de Vos, 2001].

Our understanding of the role of NGF in ovarian cancer has only started gaining ground (Figure 6) [Wang *et al.,* 2003; Bose 2005; Campos *et al*., 2007]. An elevation in NGF levels can induce prominent FSH-R expression in abnormal locations such as postmenopausal ovarian surface epithelium and epithelial ovarian cancer (Figure 6C) [Wang *et al.,* 2003], in parallel with a significant decrease in FSH levels (Figure 6C) [Bose, 2005], thereby disregulating FSH ovarian signaling and promoting ovarian epithelial growth disorders. It is, therefore, possible that low levels of FSH and high levels of NGF, in the blood circulation of postmenopausal women, might provide an early marker for ovarian cancer. Together, NGF's direct angiogenic activity, its ability to increase VEGF (Figure 6B), and the abundance of NGF and its two receptors in epithelial ovarian cancer cells (Figure 6A) suggest that blocking NGF action could be a therapeutic target in treating ovarian cancer [Campos *et al*., 2007]. Elevated expression levels of NGF or its receptors (Figure 6A) in the ovarian surface epithelium might also provide a future novel prognostic marker for the diagnosis of surface epithelium ovarian cancer. However, further studies are needed to determine whether the initiation of TrkA expression in normal ovarian surface epithelium is relevant to their transformation into malignant cells.

Acknowledgments

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References

[1] Abir, R, Fisch, B, Jin, S, Barnett, M, Ben Haroush, A, Felz, C, Kessler-Icekson, G, Feldberg, D, Nitke, S and Ao, A. (2005) Presence of NGF and its receptors in ovaries from human fetuses and adults. *Mol. Hum. Reprod*, *11*, 229-36.

[2] Abir, R, Nitke, S, Ben-Haroush, A and Fisch, B. (2006) In vitro maturation of human primordial ovarian follicles-Clinical significance, progress and methods for growth evaluation, *Histol. Histopathol., 21*, 887-98.

[3] Anderson, RA, Robinson, LLL, Brooks, J and Spears, N. (2002) Neurotropins and their receptors are expressed in the human fetal ovary. *J. Clin. Endocrinol. Metab, 87*, 890-7.

[4] Anesetti, G, Lombide, P, D'Albora, H and Ojeda, SR. (2001) Intrinsic neurons in the human ovary. *Cell Tissue Res., 306,* 231-7.

[5] Bose, CK. (2005) Role of nerve growth factor and FSH receptor in epithelial ovarian cancer. *RBM Online, 11,* 194-7.

[6] Calza, L, Giardino, L, Guiliani, A, Aloe, L and Levi-Montalcini, R. (2001) Nerve growth factor control of neuronal expression of angiogenic and vasoactive factors. *Proc. Natl. Acad. Sci., 98,* 4160–5.

[7] Campos, X, Munoz, Y, Selman, A, Yazigi, R, Moyano, L, Weinstein-Oppenheimer, C, Lara, HE and Romero, C. (2007) Nerve growth factor and its high-affinity receptor trkA participate in the control of vascular endothelial growth factor expression in epithelial ovarian cancer. *Gynecol. Oncol, 104,* 168-75.

[8] Cantarella G, Lempereur L, Presta M, Ribatti D, Lombardo G, Lazarovici P, Zappala, G, Pafumi, C and Bernardini, R. (2002) Nerve growth factor-endothelial cell interaction leads to angiogenesis in vitro and in vivo. *FASEB J., 16,* 1307–9.

[9] Chao, MV. (1992) Neurotrophin receptors: a window into neuronal differentiation. *Neuron,* 9, 583–93.

[10] Chao, MV and Hempstead, BL. (1995) p75 and Trk: a two receptor system. *Trends Neurosci, 18,* 321-6.

[11] Davidson, B, Lazaroviei, P, Ezersky, A, Nesland, JM, Risberg, B, Trope, CG, Kristensen, GB, Goscinski, H, Van de Putte, G and Reich, R. (2001) Expression levels of the NGF receptors trkA and p75 in effusions and solid tumors of serous ovarian carcinoma patients. *Clin. Cancer Res, 7,* 3457-64.

[12] Davidson, B, Reich, R, Lazaroviei, P, Nesland, JM, Skrede, M, Risberg, B, Trope, CG and Florenes, VA. (2003) Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. *Clin. Cancer Res, 9,* 2248-59.

[13] Dissen, GA, Hirshfield, AN, Malamd, S and Ojeda, SR. (1995) Expression of neurotropins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogensis. *Endocrinol, 136,* 4681-92.

[14] Dissen, GA, Romero, C, Newman Hirshfield, A and Ojeda, SR. (2001) Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinol, 142,* 2078-86.

[15] Duyndam, MCA, Hilhorst, MCGW, Schluper, HMM, Verheul, HMW, van Diest, PJ and Kraal, G. (2002) Vascular endothelial growth factor-165 overexpression stimulates angiogenesis and induces cyst formation and macrophage infiltration in human ovarian cancer xenografts. *Am. J. Pathol, 160,* 537–48.

[16] Faddy, MJ and Gosden, RG. (1996) A model confirming the decline in follicle numbers to the age of menopause in women. *Hum. Reprod, 11,* 1484-6.

[17] Feigin, E, Freud, E, Fisch, B, Orvieto, R, Kravarusic, D, Avrahami, G and Abir, R. (2008) Fertility preservation in female adolescents with malignancies. In: Moorland MT, Ed, Cancer in Female Adolescents. Hauppauge, NY, USA: Nova Science Publishers Inc., Chapter 3, pp 103-38.

[18] Geva, E and Jaffe RB. (2000) Role of vascular endothelial growth factor in ovarian physiology and pathology. *Fertil Steril, 74,* 429-38.

[19] Gosden RG. (1995) Oocyte development throughout life. In: Grudzinshes JG, Yovici JL, eds. Gametes-The Oocyte. Cambridge, UK: Cambridge University Press, 119-49.

[20] Gougeon, A. (1996) Regulations of ovarian follicular development in primates: facts and hypotheses. *Endocrinol. Rev, 17*, 121-54.

[21] Harel, S, Jin, S, Fisch, B, Feldberg, D, Krissi, H, Freimann, S, Tan, SL, Ao, A and Abir R. (2006) Tyrosine kinase B receptor and its activated neurotrophins in ovaries from human fetuses and adults. *Mol. Hum. Reprod, 12*, 357-65.

[22] Holmes, DIR and Zachary, I. (2005) The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. *Genome Biol, 6*, 209.1-10.

[23] Johnson, J, Canning, J, Kaneko T, Pru JK and Tilly JL. (2004) Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature*, *428*, 145-50.

[24] Johnson, J, Bagley, I, Skznik-Wikiel, M, Lee, HJ, Adams, GB, Niikura, Y, Tschudy, KS, Tilly, JC, Cortes, ML, Forkert, R, Spitzer, T, Iacomini, I, Scadden, DT and Tilly, JL. (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell, 122*, 303-15.

[25] Kaczmarek, MM, Schams, D and Ziecik, AJ. (2005) Role of vascular endothelial growth factor in ovarian physiology-an overview. *Reprod. Biol, 5*, 111-36.

[26] Koizumi, H, Morita, M, Mikami, S, Shibayama, E and Uchikoshi T. (1998) Immunohistochemical analysis of TrkA neurotrophin receptor expression in human non-neural carcinomas. *Pathol. Int, 48*, 93-101.

[27] Liu, Y, Wu, C, Qifeng, L, Yang, D, Albertini, DF, Keefe, DL and Liu, L. (2007) Germline stem cells and neo-oogenesis in the adult human ovary. *Dev. Biol, 306*, 112-20.

[28] Mok, CH, Tsa, SW, Knapp, RC, Fishbaugh, PM and Lau, CC. (1992) Unifocal origin of advanced human epithelial ovarian cancers. *Cancer Res, 52*, 5119–22.

[29] Ojeda, SR, Romero, C, Tapia, V and Dissen GA. (2000) Neurotrophic and cell-cell dependent control of early follicular development. *Mol. Cell Endocrinol, 163*, 67-71.

[30] Ries, LA, Kosary, CL, Hankey, BF, Miller, BA and Edwards, BK. (1998) SEER Cancer Statistics. Review 1973–1995. In: Ries LA, Kosary CL, Hankey BF, Miller BA, Edwards, BK, eds. Bethesda, MD, USA: National Cancer Institute.

[31] Romero, C, Parades, A, Dissen, GA and Ojeda SR. (2002) Nerve growth factor induces the expression of functional FSH receptors in newly formed follicles of the rat ovary. *Endocrinol, 143*, 1485-94.

[32] Salas, C, Julio-Pieper, M, Vallandares, M, Pommer, R, Vega, M, Mastronardi, C, Kerr, B, Ojeda, SR, Lara, HE and Romero, C. (2006) Nerve growth factor-dependent activation of trkA receptors in the human ovary results in synthesis of follicle-stimulating hormone receptors and estrogen secretion. *J. Clin. Endocrinol. Metab, 91*, 2396-2403.

[33] Schneider, R. and Schweiger M. (1991) A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors. *Oncogene, 6*, 1807–11.

[34] Seifer, DB, Feng, B and Shelden RM. (2006) Immunocytochemical evidence for the presence and location of the neurotrophin-Trk receptor family in adult human preovlatory follicles. *Am. J. Obstet Gynecol, 194*, 1129-36.

[35] Snider, WD. (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell*, 77, 627–38

[36] Spears, N, Molinek, MD, Robinson, LLL, Fulton, N, Cameron, H, Shimoda, K, Telfer, EE, Anderson, RA and Price, DJ. (2003) The role of neurotropin receptors in female germ-cell survival in mouse and human. *Development, 130*, 5481-91.

[37] Speroff, L, Glass, RH and Kase, NG. (1994) The Ovary-Embryology and Development. In: Speroff L, Glass RH, Kase NG, eds. Clinical Gynecologic Endocrinology and Infertility. Baltimore, Maryland, USA: Wiliams and Wilkins, 93-152.

[38] Wang, J, Lin, L, Farkash, V, Schwartz, PE, Lauchlan, SC and Zheng, W. (2003) Quantitative analysis of follicle stimulating hormone receptor in ovarian epithelial tumors: a novel approach to explain the tiled effect of ovarian cancer development in secondary Mullerian systems. *Intl. J. Cancer, 103*, 328-34.

[39] Wiesmann, C and de Vos, AM. (2001) Nerve growth factor: structure and function. *Cel. Mol. Life Sci*, *58*, 748-59.

[40] Yano, H and Chao, M (2000) Neurotrophin receptor structure and interactions. *Pharm. Acta Helv*, *74*, 253–60.

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Chapter 11

Nerve Growth Factor and Alzheimer's Disease: Where Do We Stand Today and Where Should We Go?

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Abstract

Nerve growth factor (NGF) and other members of the neurotrophin family are critical for the survival and differentiation of neurons in the central nervous system (CNS) and have been implicated in the pathophysiology of numerous disease states. Several studies have sought to demonstrate that neurodegeneration during disease and in old age is associated with reduced support of neurotrophins. Because NGF maintains the magnocellular cholinergic neurons that are damaged in Alzheimer's disease (AD), over the past decade, this neurotrophin has gained attention as a candidate therapeutic agent for the disease. However, because NGF does not cross the blood-brain barrier, inconvenient pharmacokinetics and adverse side-effect profiles have limited its clinical usefulness and therapeutic potential. Nevertheless, in recent years, alternative strategies have been developed with particular emphasis on small molecules able to modulate NGF function in AD. Compounds that mimic NGF signaling and overcome the pharmacokinetic and side-effect barriers may have therapeutic potential.

Here, we review the past and recent preclinical studies and advances into clinical development on the therapeutic effect of NGF in AD and illustrate additional strategies to target (modulate) NGF production and/or its signal transduction pathways. Moreover, since inflammation has been proposed as a possible pathogenic mechanism of AD and NGF is a key mediator of neuronal and immune cross talk, the potential involvement of

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immune system on NGF-based therapeutic approaches are also discussed. The development of studies on the role of immune-mediated mechanisms in AD and in the regulation of NGF expression can open the door to new frontiers for therapeutic intervention in AD.

The Nerve Growth Factor (NGF)

Nerve growth factor (NGF), the first member of the neurotrophin family to be characterized, was discovered during a search for such survival factors (cf review Levi-Montalcini, 1987). It is considered the prototypical growth factor, and its discovery by Rita Levi-Montalcini and Stanley Cohen was rewarded with a Nobel Prize in 1986. This molecule exists as a dimer of two identical polypeptide chains, each of 118 amino acid residues (McDonald and Blundell, 1991). NGF was initially purified as a factor able to support survival of sympathetic and sensory spinal neurons in culture (Levi-Montalcini, 1987). However, successively other relevant functions of NGF in the peripheral and central nervous system were evidenced by Levi-Montalcini herself (Levi-Montalcini et al., 1996; Levi-Montalcini, 2004).

NGF Receptors

The biological actions of NGF are mediated by two classes of membrane receptors (cf reviews Barbacid, 1995; Bothwell, 1995; Chao et al., 1998; Dechant, 2001): the Trk family of receptor tyrosine kinases and a protein named as NT receptor p75 (p75NTR), a member of the TNF receptor superfamily.

The mechanisms of transduction mediating the biological effects of p75NTR in neurons are poorly understood. On the one hand, p75NTR can modulate cellular responses to NGF, by interacting with their high-affinity TrkA. Modulation of TrkA interaction with NGF has been considered as the main p75NTR mechanism of action since the discovery of Trk receptors (Barbacid, 1995; Bothwell, 1996; Chao and Hempstead, 1995). However, it was clearly established that p75NTR can induce cellular responses in the absence of Trk receptors, such as cell death, when it is activated by the immature form of NGF (Pro-NGF) (Beattie et al., 2002; Harrington et al., 2004).

The mature form of NGF binds to the receptor TrkA with high-affinity (Kaplan and Miller, 1997; Klein, 1994). Through this receptor, NGF activates many signalling pathways, including those mediated by RAS and members of the cdc-42/ras/rho G protein families, and the MAP kinase, PI-3 kinase, and Jun kinase cascades. Within neural precursors and neurons, the pathways regulated by Trk receptors include survival and differentiation, axonal and dendritic growth and remodelling, assembly of the cytoskeleton, membrane trafficking and fusion, and synapse formation and function.

NGF-Producing Cells

Anti-NGF injections demonstrated that this factor is important in maintaining survival of sympathetic neurons *in vivo* as well as *in vitro*. In the peripheral nervous system (PNS), NGF is synthesized and secreted by sympathetic and sensory target organs (cf review Korsching 1993). From these sources, it is captured in nerve terminals by receptor-mediated endocytosis and is transported through axons to neuronal cell bodies where it acts to promote neuronal survival and differentiation. Within the target organs, synthesis of NGF is associated with peripheral tissues such as cutaneous tissues, internal organs and hair follicles, which become innervated by sensory and sympathetic neurons.

In contrast to the periphery, NGF expression in the CNS is much more restricted; NGF is produced in the CNS during development and throughout adult life with the hippocampus providing the single largest source in the entire CNS (Korsching et al, 1985). Most of NGF-producing cells are neurons, including pyramidal neurons, in the cortical and hippocampal target regions of basal forebrain cholinergic neurons.

In the hippocampus, NGF mRNA and protein are expressed by the principal excitatory (glutamate) neurons, as well as by a subset of T-aminobutyric acid (GABA)-containing inhibitory neurons (Rocamora et al, 1996). These hippocampal target cells receive rich innervations from ascending neurons with their cell bodies in the basal forebrain.

In the hippocampal formation, pyramidal and dentate granule neurons express NGF, as do subpopulations of GABAergic interneurons (French et al., 1999; Gall and Isackson, 1989; Pascual et al., 1998). NGF expression in hippocampus is regulated by neuronal activity; increases are caused by glutamatergic and cholinergic neurotransmission, and decreases are caused by GABAergic neurotransmission (Berzaghi et al., 1993; Knipper et al., 1994; French et al., 1999). In striatum, NGF is produced by a subpopulation of small interneurons (Bizon et al., 1999). Among glial cells, NGF is produced throughout the CNS by astrocytes and microglia (Elkabes et al., 1996). Increased NGF levels in the injured CNS suggest that astrocytes and microglial cells could serve as local sources of NGF for injured neurons and other NGF responsive cell types (Arendt et al., 1995; Micera et al., 1998).

NGF Receptor Expressing Cells

p75NTR gene expression in the CNS is widespread, especially during development. However, p75NTR expression is more restricted in the adult. Several neuronal populations, including cholinergic neurons of the caudate-putamen and cranial nerve nuclei of the brainstem, show markedly reduced or no expression at the adult stage (Koh and Higgins, 1991). Cerebellar Purkinje neurons, hippocampal pyramidal neurons, and retinal ganglion neurons also downregulate expression to undetectable levels in adults but start again expressing p75NTR after injury (Brann et al., 1999; Eckenstein, 1998; Martinez-Murillo et al., 1998; Yamashita et al., 1999). The majority of p75NTR-expressing neurons do not express TrkA.

However, developing horizontal cells and amacrine cells of the retina express TrkA and potentially p75NTR (Karlsson et al., 1998), whereas cholinergic neurons of the septal-basal forebrain complex express both TrkA and p75NTR during development and throughout adult

life (Holtzman et al., 1992). Expression of both TrkA and p75NTR in forebrain neurons is upregulated by NGF (Gage et al., 1989; Holtzman et al., 1992).

Adult cholinergic neurons of the extended striatal complex (caudate, putamen, accumbens, etc) express mainly TrkA; however, p75NTR is upregulated to detectable levels, and TrkA expression is increased by local tissue injury or NGF infusions (Gage et al., 1989; Holtzman et al., 1995). Adult neurons that express TrkA, but not p75NTR, are found in the thalamic paraventricular nuclei, rostral and intermediate subnuclei of the interpeduncular nucleus, and various other brain regions (Holtzman et al., 1995; Venero et al., 1994), and also in the spinal cord in regions associated with regulation of the autonomic outflow (Michael et al., 1997). Some hippocampal pyramidal neurons may also express very low levels of TrkA (Cellerino, 1995), and immunocytochemical studies points to the presence of TrkA and p75NTR proteins in pyramidal cells of the somatosensory cortex of the mature rat (Pitts and Miller 2000).

Among glial cells, very low levels of p75NTR are present in many mature astrocytes. p75NTR and TrkA are also expressed by astrocytes *in vitro*, particularly after exposure to NGF or inflammatory cytokines (Hutton and Perez-Polo, 1995; Kumar et al., 1993). Oligodendrocytes express p75NTR (Casaccia-Bonnefil et al., 1996; Kumar et al., 1993). Microglia have the capacity to express p75NTR and TrkA, and expression levels are modulated by inflammatory stimuli, such as cytokines and bacterial lipopolysaccharide (Elkabes et al., 1998).

NGF and Cholinergic Neurons in Animal Models

In the CNS, the cortex and hippocampus are innervated by cholinergic neurons originating from the nbM (basal forebrain nuclei) and memory function is impaired following either lesions that interrupt this cholinergic innervation or administration of anticholinergics (Ridley et al., 1986).

Numerous experimental evidences indicate that NGF is a potent trophic factor for cholinergic neurons. *In vitro* studies have shown that NGF increases survival rate of cholinergic neurons and activity of the enzyme ChAT (Gnahn et al., 1983; Hatanaka et al., 1988). *In vivo*, in rats, lesion of the fimbria-fornix, the fibres connecting the septum and the hippocampus, prevents retrograde transport of NGF and results in cholinergic cell loss and atrophy, whereas intracerebroventricular (ICV) administration of NGF eliminates this degeneration and resultant cognitive deficits (Hefti, 1986; Kromer, 1987). This retrograde transport of NGF from the hippocampus to the septum is virtually abolished in aged rats (Cooper et al., 1994). However aged rats, with reduced cholinergic function and behavioural deficits, when treated intracerebroventricularly with NGF, have increased cholinergic function and improved spatial memory (Fischer et al., 1987).

Other studies on transgenic mice have also unequivocally demonstrated the importance of NGF for cholinergic functions. In NGF- or TrkA-null mice and heterozygote phenotype, cholinergic function is markedly reduced in the cortex, hippocampus and basal forebrain (Chen et al., 1997; Smeyne et al., 1994), and this loss correlates with deficits in spatial memory function (Chen et al., 1997). Transgenic mice producing antibodies to NGF (AD11)

show an expected cholinergic deficit in the basal forebrain and exhibit behavioural deficits; however, surprisingly they also show cortical cell loss, amyloid plaques and hyperphosphorylated tau in cortical and hippocampal neurons (Capsoni et al., 2000). Since NGF does not easily cross the blood brain barrier (BBB), the same group tried to administer NGF to AD11 mice through less invasive ways, as compared to ICV injections. The data clearly demonstrated that intranasal administration of NGF, as well as intraperitoneal injection of the cholinesterase inhibitor galantamine, was able to restore cholinergic function and reduce numbers of amyloid plaques (Capsoni et al., 2002). Moreover, intranasal NGF was able to rescue memory deficits in the anti-NGF transgenic mice (De Rosa et al., 2005).

Other studies used NGF conjugated to a transferrin receptor antibody that can carry NGF across the BBB (Albeck et al., 1999, Friden et al., 1993, Granholm et al., 1994; Kordower et al., 1993). The conjugated NGF administration not only restored memory, but also increased NGF levels in the basal forebrain in the aged rat (Albeck et al., 2003).

Taken together, these studies showed that NGF is able to promote survival and repair of cholinergic neurons and is essential for their phenotypic maintenance. However, until recently it was not known what effects exogenous NGF might have on the dysfunctional endogenous NGF system during aging or in AD.

NGF and Alzheimer's Disease in Human Studies

Alzheimer's Disease is a chronic neurodegenerative disorder and the most frequent form of dementia among elderly people. It causes severe brain atrophy, due to pronounced cell loss, as a consequence of long lasting pathogenic processes within the brain. The disease is associated with an age-dependent, progressive and devastating impairment of cognitive and behavioural functions, accompanied by deterioration in the ability to function independently. In particular, a sporadic, heterogeneous and multifactorial form of AD with unknown aetiology accounts for the large majority of AD cases, generally beginning after age 65. Considering the progressive gain of life expectancy in the population, AD is going to increasingly represent a major cost for both society and families. However, although in the last decades considerable progress has been made in understanding the molecular mechanisms of AD neurodegeneration, the comprehension of etiological mechanisms and, consequently, the availability of disease-modifying therapies have been somewhat limited.

From a pathological point of view, AD is accompanied by characteristic hallmarks, such as the progressive deposition within the brain of extracellular senile plaques, the accumulation of intracellular neurofibrillary tangles and the massive neuronal loss mainly located in hippocampal and other cortical and subcortical areas (Selkoe, 2001). The principal constituents of senile plaques are the filamentous forms of amyloid beta protein (Aβ), such as the fibrillogenic and neurotoxic peptide Aβ 1-42, product of an altered processing of the Amyloid Precursor Protein (APP). A huge body of evidence indicates that the abnormal generation and accumulation of Aβ peptides is an early event and plays a pivotal role in the pathogenesis of AD, being itself the cause of a cascade of neurotoxic effects leading to neurodegeneration (Hardy and Selkoe, 2002; LaFerla and Oddo, 2005; Selkoe, 2001).

In AD early memory loss also coincides with the early loss of cholinergic function (Bowen et al., 1976; Davies et al., 1976). Cholinergic function, as measured by markers such as ChAT (choline acetyltransferase), choline uptake at terminals or acetylcholine synthesis all showed a marked decline at a very early stage in the disease (Sims et al., 1983). By contrast, acetylcholinesterase inhibitors, which inhibit the breakdown of the neurotransmitter acetylcholine, act as a therapeutic solution for some AD patients.

Contrary to expectation, target tissue (i.e. cortex and hippocampus) levels of NGF were not found to be reduced in AD (Allen et al., 1999; Crutcher et al., 1993), although NGF levels in the basal forebrain were decreased in AD patients (Mufson et al., 1999). However, more recently, there was a reported reduction in NGF (Hellweg et al., 1998) in the frontal cortex of undemented patients with senile plaques. In addition, a significant decrease was seen in serum NGF in patients with mild cognitive impairment compared with AD patients (Schaub et al., 2002). The authors suggested that the availability of NGF might be reduced at the onset of neurodegenerative processes.

Measurements of NGF, by means of ELISA, will in most cases not be able to distinguish between mature NGF protein and the uncleaved or pro- form. It has been shown that, in human brain, the predominant form of NGF is that of pro-NGF, and this has now been reported to be increased in AD (Fahnestock et al., 2001) and binds preferentially to the p75NTR (Chao and Bothwell, 2002). Since there is evidence to show that pro-NGF binding to p75NTR may, under certain circumstances, be associated with apoptosis (Nykjaer et al., 2005), the apparent increased levels in AD may have implications in terms of cholinergic vulnerability. In addition, p75NTR has been shown to bind Aβ (Yaar et al., 2002), again implying a negative effect on cell survival.

Therapeutic Approaches of Alzheimer' Disease with NGF

Exogenous NGF Administration in Human Trials

The initial animal studies on NGF and cholinergic neurons showed that it was possible to restore function to the endogenous trophic system when NGF is administered to the appropriate brain region at least in animal models. However, NGF is a protein and it will not cross the blood–brain barrier. Therefore, in order to be used as a therapeutic, it would need to be administered directly into the brain. Clinical trials related to NGF in AD patients were performed in Sweden (Eriksdotter Jonhagen et al., 1998; Jonhagen, 2000; Olson et al., 1992). Patients were given purified mouse NGF into the cerebral ventricles for up to 3 months continuously. Two patients were given up to 6.6 mg in total. Results were variable, but some improvement was seen in scores on some of the cognitive tests. The two patients administered the higher dose also showed increased [11C]nicotine binding in the cortex (Eriksdotter Jonhagen et al., 1998; Jonhagen, 2000; Nordberg, 1999). However, these studies also demonstrated that direct administration of NGF into the lateral ventricle leads to intolerable side-effects, most prominently to back pain associated with the NGF infusion and weight loss. The NGF-induced pain was dose-dependent and disappeared after cessation of drug treatment. Further studies in rats (Day-Lollini et al., 1997; Winkler et al., 1997) and primates (Winkler et al., 1997) with ventricular administration of rhNGF (recombinant

human NGF) have since revealed a dose-related (non-malignant) Schwann cell hyperplasia, an effect due to the presence of p75NTR, since these hyperplasic cells are immunoreactive for p75NTR but not TrkA (Day-Lollini et al., 1997). This was reversible, was reduced after 8 weeks and was completely abolished after 52 weeks (Day-Lollini et al., 1997). Ways to circumvent this problem have been explored: for instance, intranasal application of NGF has been considered. Following injection of 125I-labelled NGF into the olfactory bulb of rats, radiolabelled NGF is retrogradely transported to basal forebrain cholinergic cells (Altar and Bakhit, 1991) and also following nasal administration (Thorne and Frey, 2001). Although this route of administration seems to be less invasive, its efficiency and side effects need to be further evaluated.

Tissue Grafts that Produce NGF

These studies in humans showing that systemic or ICV administration may give rise to serious side effects, have led researchers to develop alternative drug delivery systems for NGF. Grafting studies of fetal hippocampal tissue to the basal forebrain showed that after a cholinergic lesion, fetal grafts could restore cholinergic innervation to the hippocampus and restore physiological function (Tuszynski et al., 1990). Therefore it was hypothesized that transplants of brain tissue producing NGF placed near the basal forebrain may restore the loss of NGF and survive for a long period of time (Curtis et al., 1995; Doering et al., 1991). However, given the ethical issues accompanying stem cell and fetal tissue, these modalities may not be a viable option for conventional AD treatment (Dalrymple-Alford, 1994). Therefore, research has recently focused on other methods of delivering NGF such as genetically engineered cells or biomaterials that produce NGF.

Gene Therapy

Gene therapy with NGF producing cells involves establishing a cell line *in vitro*, introducing a transgene, and transplanting the cell line to the site of need, in this case the basal forebrain. A large body of work has been carried out using fibroblasts engineered to express NGF. These were able to rescue cholinergic function in fimbria-fornix-lesioned rats (Blesch et al., 2001) and in aged rhesus monkeys with atrophy in the cholinergic basal forebrain (Smith et al., 1999). These and other studies have shown that such fibroblasts were able to sustain NGF production for at least 18 months (Blesch et al., 2001; Tuszynski et al., 2005).

One of the first clinical trials for gene therapy in AD was recently performed on eight individuals with mild AD. Patients received autologous fibroblast transplants genetically modified to produce NGF into the basal forebrain region (Tuszynski et al., 2005). The first two subjects received unilateral injections, while the other six received bilateral grafts. The fibroblasts were obtained from skin biopsies and were modified to produce and secrete human NGF via retroviral vectors as previously described in animal models by the same research groups (Chen and Gage, 1995; Tuszynski and Blesch, 2004; Tuszynski et al., 1998).

The first two subjects were only sedated and, due to movement in surgery, suffered subcortical haemorrhage. Both suffered hemiparesis, and one subject died. Subsequent operations were carried out successfully under general anaesthesia. Subjects were monitored for 18–24 months and no weight loss or pain was seen in the six patients. Slowing down of

decline in cognition was seen using both the MMSE (Mini-Mental Status Examination) and ADAS-cog (Alzheimer's Disease Assessment-Scale-Cognitive). Histopathology carried out on the brain of the one subject who died 5 weeks after surgery revealed that the patient had Lewy body disease, with numerous plaques, tangles and Lewy bodies in the brain stem, substantia nigra and cortex. The differentiated fibroblast cells were still evident with robust sprouting of cholinergic fibres into the fibroblast grafts. Despite the intrusive and hazardous nature of this type of operation, it would appear that, at least for up to 2 years, these NGF-producing implants provide therapeutic potential. It will be interesting to see if a longer time frame in a larger trial will provide a robust positive response.

Delivering NGF with Biomaterials or Transfected Cells in Animals Models

In the 1980s an 1990s, scientists began using polymer biomaterials to produce spheres or rods that could deliver growth factors for a number of weeks or months when grafted to the brain or spinal cord of injured animals (Camarata et al., 1992; Mahoney and Saltzman, 1999). One of the most common polymer materials is poly-(dl-lactide)co-glycolide, which is usually manufactured as microspheres containing the growth factor of choice, which may be released continuously for as long as 8–10 months after implantation (Emerich et al., 1999). Other investigators have demonstrated that polymer pellets secreting NGF must be placed at least within 1–2 mm of the cells in order to have effects (Mahoney and Saltzman, 1999). Because the human forebrain is of irregular shape and divided into several subnuclei, using the polymer microspheres may not be the most efficient way to replace NGF in AD patients. Therefore, to our knowledge, this type of biomaterials has not been tested in humans to date.

Some investigators have opted to utilize encapsulated cells modified to secrete NGF (Lindner et al., 1996; Schinstine et al., 1995). Schinstine and collaborators found that an encapsulated Schwannoma cell line (SCT-1 cells) transfected with human NGF cDNA, protected adult rat cholinergic neurons from fimbria-fornix lesion-induced degeneration. Other investigators have found that delivery of NGF transfected cells can improve cognition of aged rats (Lindner et al., 1996). One definite advantage with the encapsulation is that it is possible to retrieve the grafted cells if the brain reacts abnormally or if there are severe side effects.

NGF, Inflammation, and Alzheimer's Disease

Several mechanisms have been postulated to explain AD pathogenesis, such as beta-amyloid toxicity, cholinergic dysfunction, Tau hyper-phosphorylation, oxidative damage, and synaptic dysfunction. All these theories fit well with a neurotrophin deficit (particularly NGF) hypothesis of AD pathogenesis. However, during the past few years, it has become evident that the cholinergic neurons in AD do not only die because of trophic factor deprivation (i.e., axotomy, target loss, etc.), even though one of the earliest pathological alterations occurring is a decrease in TrkA receptors in the basal forebrain cholinergic neurons (see, e.g., Counts et al., 2004). An increasing set of evidence has supported the major role of deregulation of the interaction patterns between glial cells and neurons in the pathway toward neuronal degeneration.

Glial cells (astrocytes and microglia) are also the major producers of inflammatory mediators, and cytotoxic activation of glial cells is linked to several neurodegenerative diseases. Whether inflammation is a consequence or a cause of neurodegeneration is still unclear. Even more intriguing is the fact that an inflammatory process in the brain is a double-edged event that may result in tissue injury, but can also be beneficial in promoting homeostasis and repair. Indeed, robust evidence indicates that deregulated inflammation may play an important role in mediating glial alterations and neurodegeneration that occur in AD patients. A current hypothesis considers that an extracellular insult to neurons could trigger an elevated production of inflammatory cytokines by glial cells. These cytokines, such as interleukin (IL)-1β, TNF-α, IL-6 and IL-18, could affect the normal behavior of neuronal cells (Akiyama et al, 2000; Arai et al., 1990; Bossù et al 2008; Griffin et al., 2002; Licastro et al., 2003; Perry et al., 2001; Sala et al., 2003). The neurotoxic processes mediated by these cytokines may include direct neuronal death, decreased synaptic function as indicated by inhibition of long-term potentiation, and inhibition of hippocampal neurogenesis.

Among glial cells, NGF is produced throughout the CNS by astrocytes and microglia, and NGF expression in both cell types is markedly upregulated by local tissue injury, inflammation, cytokines, and bacterial lipopolysaccharide (both *in vivo* and *in vitro*) (Arendt et al., 1995; Elkabes et al., 1996, 1998; Heese et al., 1998; Yoshida and Gage, 1992). Increased NGF levels in the injured CNS suggest that astrocytes and microglial cells could serve as local sources of NGF for injured neurons and other NGF responsive cell types.

At the same time NGF may also act as a modulator of many immune cells. It is known to promote survival, proliferation and differentiation of lymphocytes and mast cells, stimulate mast cell degranulation, enhance survival of eosinophils, monocytes and neutrophils and induce release of mediators from T-cells, macrophages, basophils and eosinophils (Hamada et al., 1996; Solomon et al., 1998; Tal and Liberman, 1997). TrkA has been reported to be expressed in several immune cell types, including basophils, mast cells, monocytes and B- and T-cells. In the human lung, TrkA immunoreactivity has been detected in alveolar cells and mucous glands (Shibayama and Koizumi, 1996) and mast cells from bronchial biopsies (Kassel et al., 2001). In addition, TrkA mRNA has been detected in human lung mast cells (Tam et al., 1997). Other observations, more relevant for their possible application in AD, indicated that NGF can induce chemotaxis of microglial cells through the activation of TrkA receptor (De Simone et al., 2007).

It is interesting that microglia cells are functionally and phenotipically similar to another type of immune cells, the dendritic cells (DCs) which are crucial actors in the initiation and regulation of immune response and inflammation, and likely to play a pivotal role in neurodegenerative and neuroinflammatory diseases (Town et al., 2005). Recently, a possible contribution of DCs to AD pathogenesis has also been suggested (Ciaramella et al., 2007). Interestingly, DCs can produce NGF (Noga et al., 2008) and, upon NGF treatment, they can easier maturate in response to LPS (Jiang et al., 2007) and release either pro- or anti-inflammatory cytokines, accordingly to the (allergic or healthy, respectively) state of the donors (Noga et al., 2007). Altogether, these data indicate that NGF may have a role in both the early and late phases of inflammatory responses, probably playing as an autocrine pro- or anti-inflammatory factor produced in the brain.

Despite this evidence, no studies reporting possible association between inflammation and NGF expression in AD patients have been performed, so far. Nevertheless, it seems plausible that, whilst in healthy subjects an increased production of NGF after a brain insult can suppress inflammation by switching the immune response to an anti-inflammatory mode and promote cellular repair, in AD patients a perpetuated inflammatory state can cause neuronal loss through a reduction of NGF support by glial cells. Moreover, a lack of glial NGF may further contribute to the deregulation of immune response and alteration of glial/neuronal pathway.

New Directions for Advances in NGF-Based Therapies of Alzheimer's Disease

Due to non-specific side effects observed in the early animal and clinical trials of NGF, many debated whether NGF treatment was a viable option for AD patients. The increased knowledge of NGF-signal transduction and of the pathogenetic mechanism of Alzheimer's disease have raised the question of whether therapeutic approaches of AD based on increase availability of NGF may actually accelerate the neuropathology associated with plaques and tangles, and even lead to apoptotic events in basal forebrain cholinergic neurons. In addition, if there are other biological mechanisms at play, NGF-based therapies would merely delay the process and not necessarily halt the progression of cholinergic loss. Nevertheless, even though we do not fully understand the cause of AD, neurotrophic therapy using NGF may have significant beneficial effects for the patient. Future studies and current clinical trials will certainly determine whether local intraparenchymal transplantation of NGF producing cells and/or other neuroprotective measures may halt the progression of AD.

Another novel pharmacological strategy for AD is the development of small molecules that more easily cross the BBB than NGF, and limit biological activity to only the wanted effects and minimize the unwanted side-effects. This field of investigation is very promising and may provide future "smart drugs" for AD and other neurodegenerative diseases. For example, L-753,000, a derivative of the staurosporine family of protein kinase inhibitors often used for cancer treatment has been shown to potentiate the signaling of NGF to TrkA (Pollack et al., 1999; Wilkie et al., 2001).

Gene therapy delivering NGF to discrete regions of the brain is also a promising future therapy for AD. Hopefully, control of gene expression required for dose-specific or time-specific therapeutic treatment schedules will be achieved by characterization of NGF gene promoters, such as the zinc-inducible metallothionein promoter that has recently been described (Wyman et al., 1999).

Evidence supports the notion that damage caused by inflammation is not only a primary cause of neurodegeneration through the induction of glial dysfunction but also an inducer for the accumulation of Aβ in the brain of AD patients. Assuming that inflammation is a key event in the development of AD, then the administration of anti-inflammatory drugs should alleviate or prevent AD symptoms. Indeed a series of studies have shown that Nonsteroidal Anti-inflammatory Drugs (NSAIDs) may potentially provide some benefit in *in vitro* AD models of investigations by reducing Aβ toxicity (see for review Rojo et al., 2008). However,

the precise mechanism of action of NSAIDs in humans is still unclear. Moreover, clinical studies indicate that NSAIDs cannot improve the clinical symptoms in mild cognitive impairment or advanced cases of AD patients but they seem to show relative efficacy in lowering the risk of developing AD. Altogether these evidences indicate that anti-inflammatory drugs might be promising therapeutic agents for AD. However, it appears that some factors are missing in this model/investigation. In particular, microglia and DCs are promising candidates to be further studied since they seem to have a crucial role in orchestrating immune responses and neuro-immune cross talk involved in AD pathogenesis. In fact, given the immunological properties of NGF and related neurotrophins, and also their role in the CNS, it would be of relevance that future studies will characterize the role of NGF in inflammation related to AD. This will hopefully further elucidate the pathogenesis of AD and provide the knowledge to develop new immune-based therapeutic strategies.

References

Albeck, D., Mesches, M.H., Juthberg, S., Browning, M., Bickford, P.C., Rose, G.M. and Granholm, A.C. (2003). Exogenous NGF restores endogenous NGF distribution in the brain of the cognitively impaired aged rat. *Brain Research, 967,* 306–310.

Albeck, D.S., Backman, C., Veng, L., Friden, P., Rose, G.M. and Granholm, A. (1999). Acute application of NGF increases the firing rate of aged rat basal forebrain neurons. *European Journal of Neuroscience, 11,* 2291–2304.

Allen, S.J., MacGowan, S.H., Treanor, J.J., Feeney, R., Wilcock, G.K. and Dawbarn, D. (1991). Normal *β*-NGF content in Alzheimer's disease cerebral cortex and hippocampus. *Neuroscience Letters, 131,* 135–139

Altar, C.A. and Bakhit, C. (1991). Receptor-mediated transport of human recombinant nerve growth factor from olfactory bulb to forebrain cholinergic nuclei. *Brain Research, 541,* 82–88

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L., Finch, C.E., Frautschy, S., Griffin, W.S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I.R., McGeer, P.L., O'Banion, M.K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F.L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G. and Wyss-Coray T. (2000). Inflammation and Alzheimer's disease. *Neurobiology of Aging, 21,* 383-421.

Arai, K.I., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Annual Review of Biochemistry, 59,* 783-836.

Arendt, T., Briickner, M.K., Krell, T., Pagliusi, S., Kruska, L. and Heumann, R. (1995). Degeneration of rat cholinergic basal forebrain neurons and reactive changes in nerve growth factor expression after chronic neurotoxic injury. IL Reactive expression of the nerve growth factor gene in astrocytes. *Neuroscience, 65,* 647-659.

Beattie, M.S., Harrington, A.W., Lee, R., Kim, J.Y., Boyce, S.L., Longo, F.M., Bresnahan, J.C., Hempstead, B.L. and Yoon, S.O. (2002). ProNGF Induces p75-Mediated Death of Oligodendrocytes following Spinal Cord Injury. *Neuron, 36,* 375-386.

Bizon, J.L., Lauterborn, J.C. and Gall, C.M. (1999). Subpopulations of striatal interneurons can be distinguished on the basis of neurotrophic factor expression. *Journal of Comparative Neurology, 408,* 283-298.

Barbacid, M. (1995). Structural and functional properties of the TRK family of neurotrophin receptors. *Annals of the New York Academy of Sciences, 766,* 442-458.

Blesch, A., Conner, J. M. and Tuszynski, M.H. (2001). Modulation of neuronal survival and axonal growth *in vivo* by tetracycline-regulated neurotrophin expression. *Gene Therapy, 8,* 954–960

Bossù, P., Ciaramella, A., Salani, F., Bizzoni, F., Varsi, E., Di Iulio, F., Giubilei, F., Gianni, W., Trequattrini, A., Moro, M.L., Bernardini, S., Caltagirone, C. and Spalletta, G. (2008). Interleukin-18 produced by peripheral blood cells is increased Alzheimer's disease and correlates with cognitive impairment. *Brain, Behavior and Immunity, 22,* 487-492.

Bothwell, M. (1995). Functional interactions of neurotrophins and neurotrophin receptors. *Annual Review of Neuroscience, 18,* 223-253.

Bothwell, M. (1996). p75NTR: a receptor after all. *Science, 272,* 506-507.

Bowen, D.M., Smith, C.B., White, P. and Davison, A.N. (1976). Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain, 99,* 459–496.

Camarata, P.J., Suryanarayanan, R., Turner, D.A., Parker, R.G. and Ebner, T.J. (1992). Sustained release of nerve growth factor from biodegradable polymer microspheres. *Neurosurgery, 30,* 313–319.

Capsoni, S., Giannotta, S. and Cattaneo, A. (2002). Nerve growth factor and galantamine ameliorate early signs of neurodegeneration in anti-nerve growth factor mice. *Proceedings of the National Academy of Sciences USA, 99,* 12432–12437.

Capsoni, S., Ugolini, G., Comparini, A., Ruberti, F., Berardi, N. and Cattaneo, A. (2000). Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice. *Proceedings of the National Academy of Sciences USA, 97,* 6826–6831.

Cellerino, A. (1995). Expression of messenger RNA coding for the nerve growth factor receptor trkA in the hippocampus of the adult rat. *Neuroscience, 70,* 613-616.

Chao, M.V. and Bothwell, M. (2002). Neurotrophins: to cleave or not to cleave. *Neuron, 33,* 9–12

Chao, M.V. and Hempstead, B.L. (1995). p75 and Trk: a two-receptor system. *Trends in Neuroscience, 18,* 321-326.

Chao, M., Casaccia-Bonnefil, P., Carter, B., Chittka, A., Kong, H. and Yoon, S.O. (1998). Neurotrophin receptors: mediators of life and death. *Brain Research Review, 26,* 295-301.

Chen, K.S. and Gage, F.H. (1995). Somatic gene transfer of NGF to the aged brain: behavioral and morphological amelioration. *Journal of Neuroscience, 15,* 2819–2825.

Chen, K.S., Nishimura, M.C., Armanini, M.P., Crowley, C., Spencer, S.D. and Phillips, H.S. (1997). Disruption of a single allele of the nerve growth factor gene results in atrophy of

basal forebrain cholinergic neurons and memory deficits. *Journal of Neuroscience, 17,* 7288–7296.

Ciaramella, A., Sanarico, N., Bizzoni, F., Moro, M.L., Salani, F., Scapigliati, G., Spalletta, G., Caltagirone, C. and Bossù P. (2007). Amyloid beta peptide promotes differentiation of pro-inflammatory human myeloid dendritic cells. *Neurobiology of Aging,* [Epub ahead of print].

Cooper, J.D., Lindholm, D. and Sofroniew, M.V. (1994). Reduced transport of [125I]nerve growth factor by cholinergic neurons and down-regulated TrkA expression in the medial septum of aged rats. *Neuroscience, 62,* 625–629.

Counts, S.E., Nadeem, M., Wuu, J., Ginsberg, S.D., Saragovi, H.U. and Mufson, E.J. (2004). Reduction of cortical TrkA but not p75(NTR) protein in earlystage Alzheimer's disease. *Annals of Neurology, 56,* 520–531.

Crutcher, K.A., Scott, S.A., Liang, S., Everson, W.V. and Weingartner, J. (1993). Detection of NGF-like activity in human brain tissue: increased levels in Alzheimer's disease. *Journal of Neuroscience, 13,* 2540–2550.

Curtis, M., Bergman, H., Price, M.L., Srivastava, N. and Granholm, A.C. (1995). Hypothalamic tissue stimulates hippocampal pyramidal neuron survival during development: evidence from intraocular double transplants. *Hippocampus, 5,* 584–594.

Dalrymple-Alford, J.C. (1994). Behavioral effects of basal forebrain grafts after dorsal septo-hippocampal pathway lesions. *Brain Research, 661,* 243–258.

da Penha Berzaghi, M., Cooper, J., Castrén, E., Zafra, F., Sofroniew, M., Thoenen, H. and Lindholm D. (1993). Cholinergic regulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) but not neurotrophin-3 (NT-3) mRNA levels in the developing rat hippocampus. *Journal of Neuroscience, 13,* 3818-3826.

Davies, P. and Maloney, A. J. (1976). Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet ii,* 1403.

Day-Lollini, P.A., Stewart, G.R., Taylor, M.J., Johnson, R.M. and Chellman, G.J. (1997). Hyperplastic changes within the leptomeninges of the rat and monkey in response to chronic intracerebroventricular infusion of nerve growth factor. *Experimental Neurology, 145,* 24–37.

Dechant, G. (2001). Molecular interactions between neurotrophin receptors. *Cell and Tissue Research, 305,* 229-238.

De Rosa, R., Garcia, A.A., Braschi, C., Capsoni, S., Maffei, L., Berardi, N. and Cattaneo, A. (2005). Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice. *Proceedings of the National Academy of Sciences USA, 102,* 3811-3816.

De Simone, R., Ambrosiani, E., Carnevale, D., Ajmone-Cat, M.A. and Minghetti, L. (2007). NGF promotes microglial migration through the activation of its high affinity receptor: modulation by TGF-beta. *Journal of Neuroimmunology 190,* 53-60.

Doering, L.C., Eriksdotter-Nilsson, M. and Olson, L. (1991). Spatial distributions of cytoskeletal proteins and the nerve growth factor receptor in septal transplants in oculo: protection from abnormal immunoreactivity by hippocampal co-grafts. *Neuroscience, 44,* 381–392.

Elkabes, S., DiCicco-Bloom, E. and Black, I. (1996). Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. *Journal of Neuroscience, 16*, 2508-2521.

Elkabes,. S., Peng, L. and Black, I.B. (1998). Lipopolysaccharide differentially regulates microglial trk receptor and neurotrophin expression. *Journal of Neuroscience Research, 54*, 117-122.

Emerich, D.F., Tracy, M.A., Ward, K.L., Figueiredo, M., Qian, R., Henschel, C. and Bartus, R.T. (1999). Biocompatibility of poly(DL-lactide-co-glycolide) microspheres implanted into the brain. *Cell Transplantation, 8*, 47–58.

Eriksdotter Jonhagen, M., Nordberg, A., Amberla, K., Backman, L., Ebendal, T., Meyerson, B., Olson, L., Seiger, A., Shigeta, M., Theodorsson, E., Viitanen, M., Winblad, B. and Wahlund, L.O. (1998). Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders, 9*, 246–257.

Fahnestock, M., Michalski, B., Xu, B. and Coughlin, M.D. (2001). The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. *Molecular and Cellular Neuroscience, 18*, 210–220.

Fischer, W., Wictorin, K., Bjorklund, A., Williams, L. R., Varon, S. and Gage, F.H. (1987). Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature (London), 329*, 65–68

French, S.J., Humby, T., Horner, C.H., Sofroniew, M.V. and Rattray, M. (1999). Hippocampal neurotrophin and trk receptor mRNA levels are altered by local administration of nicotine, carbachol and pilocarpine. *Molecular Brain Research, 67*, 124-136.

Friden, P.M., Walus, L.R., Watson, P., Doctrow, S.R., Kozarich, J.W., Backman, C., Bergman, H., Hoffer, B., Bloom, F. and Granholm, A.C. (1993). Blood–brain barrier penetration and in vivo activity of an NGF conjugate. *Science, 259*, 373–377.

Gall, C.M. and Isackson, PJ. (1989). Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. *Science, 245*, 758-761.

Gnahn, H., Hefti, F., Heumann, R., Schwab, M. E. and Thoenen, H. (1983). NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? *Brain Research, 285*, 45–52.

Granholm, A.C., Backman, C., Bloom, F., Ebendal, T., Gerhardt, G.A., Hoffer, B., Mackerlova, L., Olson, L., Soderstrom, S., Walus, L.R. and Friden, P.M. (1994). NGF and anti-transferrin receptor antibody conjugate: short and long-term effects on survival of cholinergic neurons in intraocular septal transplants. *Journal of Pharmacology and Experimental Therapeutics, 268*, 448–459.

Griffin, W.S. and Mrak, R.E. (2002). Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. *Journal of Leukocyte Biology, 72*, 233–238.

Hamada, A., Watanabe, N., Ohtomo, H. and Matsuda, H. (1996). Nerve growth factor enhances survival and cytotoxic activity of human eosinophils. *British Journal of Haematology, 93*, 299–302.

Hardy J. and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road of therapeutics. *Science, 297,* 353-356.

Harrington, A.W., Leiner, B., Blechschmitt, C., Arevalo, J.C., Lee, R., Mörl, K., Meyer, M., Hempstead, B.L., Yoon, S.O. and Giehl, K.M. (2004). *Proceedings of the National Academy of Sciences · USA, 101,* 6226.

Hatanaka, H., Nihonmatsu, I. and Tsukui, H. (1988). Nerve growth factor promotes survival of cultured magnocellular cholinergic neurons from nucleus basalis of Meynert in postnatal rats. *Neuroscience Letters, 90,* 63–68.

Hefti, F. (1986). Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *Journal of Neuroscience, 6,* 2155–2162

Hellweg, R., Gericke, C. A., Jendroska, K., Hartung, H. D. and Cervos-Navarro, J. (1998). NGF content in the cerebral cortex of non-demented patients with amyloidplaques and in symptomatic Alzheimer's disease. *International Journal of Developmental Neuroscience, 16,* 787–794

Heese K., Fiebich B.L., Bauer J. and Otten, U. (1998). NF-kappaB modulates lipopolysaccharide-induced microglial nerve growth factor expression. *Glia, 22,* 401-407.

Jiang, Y., Chen, G., Zhang, Y., Lu, L., Liu, S. and Cao, X. (2007). Nerve growth factor promotes TLR4 signaling-induced maturation of human dendritic cells in vitro through inducible p75NTR 1. *Journal of Immunology, 179,* 6297-6304.

Jonhagen, M.E. (2000). Nerve growth factor treatment in dementia. *Alzheimer Diseaseand Associated Disorders,14 (Suppl. 1),* S31–S38.

Kaplan, D.R. and Miller, F.D. (1997). Signal transduction by the neurotrophin receptors. *Current Opinion in Cell Biology, 9,* 213-221.

Kassel, O., de Blay, F., Duvernelle, C., Olgart C., Israel-Biet D., Krieger P., Moreau L., Muller C., Pauli G. and Frossard, N. (2001). Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated inhalation of allergen at low-dose. *Clinical and Experimental Allergy, 31,* 1432–1440.

Klein R. (1994). Role of neurotrophins in mouse neuronal development. *FASEB Journal, 8,* 738-744.

Kordower, J.H., Mufson, E.J., Granholm, A.C., Hoffer, B. and Friden, P.M. (1993). Delivery of trophic factors to the primate brain. *Experimental Neurology, 124,* 21–30.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. *Journal of Neuroscience, 13,* 2739-2748.

Korsching, S., Auburger, G., Heumann, R., Scott, J. and Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO Journal, 74,* 1389-1393.

Knipper, M., Berzaghi, M.P., Blochl, A., Breer, H., Thoenen, H. and Lindholm, D. (1994). Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived growth factor in the rat hippocampus. *European Journal of Neuroscience, 6,* 668-671.

Kromer, L. F. (1987). Nerve growth factor treatment after brain injury prevents neuronal death. *Science, 235,* 214–216

LaFerla, F.M. and Oddo, S. (2005). Alzheimer's disease: a beta, tau and synaptic dysfunction. *Trends in Molecular Medicine, 11,* 170–176.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science, 237,* 1154-1162.

Levi-Montalcini, R. (2004). The nerve growth factor and the neuroscience chess board. *Progress in Brain Research, 146,* 525-527.

Levi-Montalcini, R., Skaper, S.D., Dal Toso, R., Petrelli, L. and Leon, A. (1996). Nerve growth factor: from neurotrophin to neurokine. *Trends in Neurosciences 19,* 514-520.

Lindner, M.D., Kearns, C.E., Winn, S.R., Frydel, B. and Emerich, D.F. (1996). Effects of intraventricular encapsulated hNGF-secreting fibroblasts in aged rats. *Cell Transplantation, 5,* 205–223.

Licastro, F., Grimaldi, L.M., Bonafe, M., Martina, C., Olivieri, F., Cavallone, L., Giovanetti, S., Masliah, E. and Franceschi, C. (2003). Interleukin-6 gene alleles affect the risk of Alzheimer's disease and levels of the cytokine in blood and brain. *Neurobiology of Aging, 24,* 921-926.

Mahoney, M.J. and Saltzman, W.M. (1999). Millimeter-scale positioning of a nerve-growth-factor source and biological activity in the brain. *Proceedings of the National Academy of Sciences USA, 96,* 4536–4539.

McDonald, N.Q. and Blundell, T.L. (1991). Crystallization and characterization of the high molecular weight forni of nerve growth factor (7 S NGF). *Journal of Molecular Biology, 219,* 595-601.

Micera, A., Vigneti, E., and Aloe, L. (1998). Changes of NGF presence in nonneuronal cells in response to experimental allergie encephalomyelitis in lewis rats. *Experimental Neurology, 154,* 41-46.

Mufson, E.J., Kroin, J.S., Sendera, T.J. and Sobreviela, T. (1999). Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative diseases. *Progress in Neurobiology, 57,* 451–484.

Noga, O., Peiser, M., Altenähr, M., Knieling, H., Wanner, R., Hanf, G., Grosse, R. and Suttorp, N. (2007). Differential activation of dendritic cells by nerve growth factor and brain-derived neurotrophic factor. *Clinicaland Experimental Allergy, 37,* 1701-1708.

Noga, O., Peiser, M., Altenähr, M., Schmeck, B., Wanner, R., Dinh, Q.T., Hanf, G. and Suttorp, N. (2008). Selective induction of nerve growth factor and brain-derived neurotrophic factor by LPS and allergen in dendritic cells. *Clinicaland Experimental Allergy, 38,* 473-479.

Nordberg, A. (1999). PET studies and cholinergic therapy in Alzheimer's disease. *Revue Neurologique, (Paris) 155,* S53–S63.

Nykjaer, A., Willnow, T. E. and Petersen, C.M. (2005). p75NTR: live or let die. *Current Opinion in Neurobiology, 15,* 49–57.

Olson, L. (1993). NGF and the treatment of Alzheimer's disease. *Experimental Neurology, 124,* 5–15.

Pascual, M., Rocamora, N., Acsàdy, L., Freund, T.F. and Soriano, E. (1998). Expression of nerve growth factor and neurotrophin-3 mRNAs in hippocampal interneurons: morphological characterization, levels of expression, and colocalization of nerve growth factor and neurotrophin-3. *Journal of Comparative Neurology, 395,* 73-90.

Perry, R.T., Collins, J.S., Wiener, H., Acton, R. and Go, R.C. (2001). The role of TNF and its receptors in Alzheimer's disease. *Neurobiology of Aging, 22,* 873-83.

Pollack, S., Young, L., Bilsland, J., Wilkie, N., Ellis, S., Hefti, F., Broughton, H. and Harper, S. (1999). The staurosporine-like compound L-753,000 (NB-506) potentiates the neurotrophic effects of neurotrophin-3 by acting selectively at the TrkA receptor. *Molecular Pharmacology, 56,* 185–195.

Ridley, R.M., Murray, T.K., Johnson, J.A. and Baker, H.F. (1986). Learning impairment following lesion of the basal nucleus of Meynert in the marmoset: modification by cholinergic drugs. *Brain Research, 376,* 108–116.

Rocamora, N., Pascual, M., Acsady, L., de Lecea, L., Freund, T.F. and Soriano, E. (1996). Expression of NGF and NT3 mRNAs in hippocampal interneurons innervated by the GABAergic septohippocampal pathway. *Journal of Neuroscience, 16,* 3991-4004.

Rojo, L.E., Fernández, J.A., Maccioni, A.A., Jimenez, J.M. and Maccioni, R.B. (2008). Neuroinflammation: implications for the pathogenesis and molecular diagnosis of Alzheimer's disease. *Archives of Medical Research, 39,* 1-16.

Sala, G., Galimberti, G., Canevari, C., Raggi, M.E., Isella, V., Facheris, M., Appollonio, I. and Ferrarese, C. (2003). Peripheral cytokine release in Alzheimer patients: correlation with disease severity. *Neurobiology of Aging, 24,* 909-914.

Schaub, R.T., Anders, D., Golz, G., Gohringer, K. and Hellweg, R. (2002). Serum nerve growth factor concentration and its role in the preclinical stage of dementia. *American Journal of Psychiatry, 159,* 1227–1229.

Schinstine, M., Fiore, D.M., Winn, S.R. and Emerich, D.F. (1995). Polymer-encapsulated Schwannoma cells expressing human nerve growth factor promote the survival of cholinergic neurons after a fimbria-fornix transection. *Cell Transplantation, 4,* 93–102.

Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiological Reviews, 81,* 741-766.

Shibayama, E. and Koizumi, H. (1996). Cellular localization of the Trk neurotrophin receptor family in human non-neuronal tissues. *American Journal of Pathology, 148,* 1807–1818.

Sims, N.R., Bowen, D.M., Allen, S.J., Smith, C.C., Neary, D., Thomas, D.J. and Davison, A.N. (1983). Presynaptic cholinergic dysfunction in patients with dementia. *Journal of Neurochemistry, 40,* 503–509.

Smeyne, R. J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A. and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature (London), 368,* 246–249.

Smith, D.E., Roberts, J., Gage, F.H. and Tuszynski, M.H. (1999) Age-associated neuronal atrophy occurs in the primate brain and is reversible by growth factor gene therapy. *Proceedings of the National Academy of Sciences USA, 96,* 10893–10898.

Solomon, A., Aloe, L., Pe'er, J., Frucht-Pery, J., Bonini, S., Bonini, S. and Levi-Schaffer, F. (1998). Nerve growth factor is preformed in and activates human peripheral blood eosinophils. *Journal of Allergy and Clinical Immunology, 102,* 454–460.

Tam, S.Y., Tsai, M., Yamaguchi, M., Yano, K., Butterfield, J. H. and Galli, S.J. (1997). Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. *Blood, 90,* 1807–1820.

Tal, M. and Liberman, R. (1997). Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. *Neuroscience Letters, 221,* 129–132.

Thorne, R.G. and Frey, W.H. (2001). Delivery of neurotrophic factors to the central nervous system: pharmacokinetic considerations. *Clinical Pharmacokinetics, 40,* 907–946.

Town, T, Nikolic, V. and Tan, J. (2005). The microglial "activation" continuum: from innate to adaptive responses. *Journal of Neuroinflammation 2,* 24.

Tuszynski, M.H., Buzsaki, G. and Gage, F.H. (1990). Nerve growth factor infusions combined with fetal hippocampal grafts enhance reconstruction of the lesioned septohippocampal projection. *Neuroscience, 36,* 33–44.

Tuszynski, M.H. and Blesch, A. (2004). Nerve growth factor: from animal models of cholinergic neuronal degeneration to gene therapy in Alzheimer's disease. *Progress in Brain Research, 146,* 441–449.

Tuszynski, M.H., Smith, D.E., Roberts, J., McKay, H. and Mufson, E. (1998). Targeted intraparenchymal delivery of human NGF by gene transfer to the primate basal forebrain for 3 months does not accelerate beta-amyloid plaque deposition. *Experimental Neurology, 154,* 573–582.

Tuszynski, M.H., Thal, L., Pay, M., Salmon, D.P., U.H.S., Bakay, R., Patel, P., Blesch, A., Vahlsing, H.L., Ho, G., Tong, G., Potkin, S.G., Fallon, J., Hansen, L., Mufson, E.J., Kordower, J.H., Gall, C. and Conner, J. (2005). A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nature Medicine, 11,* 551–555.

Wilkie N., Wingrove P.B., Bilsland J.G., Young L., Harper S.J., Hefti F., Ellis S. and Pollack S.J. (2001). The non-peptidyl fungal metabolite L-783,281 activates TRK neurotrophin receptors, *Journal of Neurochemistry, 78,* 1135–1145.

Winkler, J., Ramirez, G.A., Kuhn, H.G., Peterson D.A., Day-Lollini P.A., Stewart G.R., Tuszynski M.H., Gage F.H. and Thal L.J. (1997). Reversible Schwann cell hyperplasia and sprouting of sensory and sympathetic neurites after intraventricular administration of nerve growth factor. *Annals of Neurology, 41,* 82–93.

Wyman, T., Rohrer, D., Kirigiti, P., Nichols, H., Pilcher, K., Nilaver G. and Machida, C. (1999). Promoter-activated expression of nerve growth factor for treatment of neurodegenerative diseases, *Gene Therapy, 6,* 1648–1660.

Yaar, M., Zhai, S., Fine, R.E. Eisenhauer, P.B., Arble, B.L., Stewart, K.B. and Gilchrest, B.A. (2002). Amyloid β binds trimers as well as monomers of the 75-kDa neurotrophin receptor and activates receptor signaling. *Journal of Biological Chemistry, 277,* 7720–7725.

Yoshida, K. and Gage, F.H. (1992). Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. *Brain Research, 569,* 14-25.

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Chapter 12

Cellular Effects of the Neurotrophic Factor S100B Following Experimental Traumatic Brain Injury

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Abstract

Objective: The neurotrophic factor S100B has been promoted as a clinical marker of brain damage and high serum levels are considered to correlate with the severity of injury and a poor prognosis. However, experimental research demonstrated increased cerebral levels of S100B to enhance hippocampal neurogenesis and to improve cognitive recovery following traumatic brain injury (TBI). The purpose of the present study was to elucidate the cellular effects of S100B and thus to differentiate beneficial and detrimental ones.

Methods: Following lateral fluid percussion injury in male rats (n=32), we infused S100B (50ng/hr) or vehicle into the lateral ventricle for 7 days using an osmotic micro-pump. The animals were sacrificed on day 5 or 5 weeks post-injury, and 5μm sections, 100μm apart (bregma -3.3 to -5.6mm) were analysed histologically. Cell death was assessed using TUNEL and hematoxylin-eosin staining, axonal damage and microglial activation by APP and ED1 immunostaining, and gliosis applying the glial markers GFAP and S100B.

Results: TUNEL-positive cells were present directly beneath the lesion site in vehicle and S100B-treated animals on day 5 post-injury (238±6 and 234±24 cells/mm^2,

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respectively, n.s.), but not after 5 weeks. The intraventricular S100B infusion did not significantly affect the early (TBI p=0.004, TBI+S100B p=0.036) or late (TBI p=0.039, TBI+S100B p=0.002) axonal injury, but resulted in an unspecific microglial activation opposite to the injury site as documented by an increased ED1 expression (TBI+S100B p=0.001). After 5 weeks, both injury and S100B treatment resulted in an increased number of GFAP expressing cells in the corpus callosum (TBI p=0.005, TBI+S100B p=0.003, sham+S100B p=0.005), while in the hippocampal granular cell layer (GCL) this effect was only present in non-injured control animals (p=0.048). The S100B expression in the GCL was increased by a S100B treatment after 5 weeks, both in injured and non-injured animals (p=0.017 and p<0.001).

Conclusion: In our model, the exogenous application of S100B did not exert an effect on early cell death or late axonal injury. The significance of some delayed APP and ED1 accumulation and stimulation of reactive astrocytosis following injury and S100B treatment has to be clarified by long-term experiments in order to exclude any participation in neurodegenerative processes.

Keywords*: S100B, neurotrophic factor, hippocampus, neuronal cell damage, gliosis, traumatic brain injury, rat*

Introduction

Following traumatic brain injury (TBI), endogenous repair mechanism can be associated with an up-regulation of mitogenic and neurotrophic factors within the injured brain. Hippocampal astrocytes are a cellular source for these mitogenic/neurotrophic factors. One factor actively secreted by astrocytes is S100B, a low molecular weight (9 to 13 kD) calcium-binding protein exerting intracellular and extracellular regulatory activities [1]. Furthermore, following astrocytic release into the extracellular compartment, S100B acts in an autocrine and paracrine manner on astrocytes, neurons and microglia [2].

Increased S100B serum levels have been found in a variety of acute and chronic brain pathologies including TBI, ischemic insult and neurodegenerative diseases [review [3]]. In vitro, S100B has been demonstrated to convey neuroprotective and neurotrophic properties at nanomolar concentrations [4], and the S100B induced upregulation of the anti-apoptotic factor Bcl-2 supports the notion that S100B promotes neuronal repair during the very early phase following brain insults and might contribute to neuronal differentiation during development [5]. However, a deleterious effect of S100B has been proposed to occur during late phases following brain insults and S100B has been associated with an overexpression of β-amyloid precursor protein (APP) in neurodegenerative diseases [6]. In vivo, astrocytosis occurred in S100B transgenic mice demonstrated by an increased immunoreactivity to the classical marker for astrogliosis, the glial fibrillary acid protein [GFAP, [7]]. Persistent activation of the receptor of advanced glycation end products (RAGE) in neurons by high doses of S100B causes neuronal death by apoptosis as a result of increased production of reactive oxygen species [5]. Furthermore, S100B stimulates the nitric oxide (NO) production in a BV-2 microglial cell line although this effect depends on the presence of other factors such as pro-inflammatory cytokines [8].

Thus, in the diseased central nervous system, astrogliosis is accompanied by microglial activation. Depending on the context of their activation, reactive astrocytes are involved in neuronal survival and regeneration in an either a protective or an impedimental way. Since we previously demonstrated an improvement in cognitive function resulting from an intraventricular S100B infusion following lateral fluid percussion injury [9], we sought to determine the underlying mechanisms of S100B on cellular responses initiated following TBI. Specifically, we assessed the effect of a S100B treatment on (i) cell death and apoptosis by TUNEL staining, on [10] axonal injury by APP immunohistochemistry, on (iii) microglial activation by ED1 immunohistochemistry, and on (iv) astrocytosis by GFAP and S100B immunohistochemistry.

Materials and Methods

Animals and Surgical Procedure. The studies were conducted under approval of the Institutional Animal Care and Use Committee and according to NIH guidelines. Experiments were carried out on 250 to 300 g adult male Sprague-Dawley rats (n=60, Harlan, Indianapolis, IN). Rats were housed with a 12:12 hour light/dark cycle, and at 22±1°C with 60% humidity, pellet food and water ad libitum. Surgery was performed after intubation under isoflurane anaesthesia and controlled ventilation (3% isoflurane in 70% N_2 and 30% O_2). End-tidal CO_2 was monitored throughout anesthesia and kept at 35 to 45 mmHg. Rectal temperature was maintained at 37±0.2ºC using a heating blanket. A 4.9 mm craniotomy was trephined half way between lambda and bregma over the left hemisphere for fluid percussion injury. Two holes were made rostral and lateral to the craniotomy. Screws were inserted into the holes for stability and a Luer-Loc hub made from the 3 mm plastic end of a 20G needle was cemented into the craniotomy with dental cement.

A fluid percussion pulse of 2.09±0.05 atm was administered through the craniotomy onto the intact dura by a fluid percussion injury device [11]. Sham animals received all procedures except the percussion pulse. After the percussion pulse or sham procedure, the Luer-loc hub was removed and the animals were placed in a stereotactic frame and fitted with a brain infusion cannula (Alzet brain infusion kit 3-5 mm, Durect Corp., Cupertino, CA). The cannula was implanted according to the atlas of Paxinos and Watson (1986) with the tip inserted into the lumen of the left lateral ventricle (stereotactic coordinates 0.8 mm behind bregma, 1.5 mm lateral to midline, 3 to 4 mm beneath the surface of the skull). The correct placement of the infusion cannula was verified by the measurement of the intracranial pressure demonstrating a drop by the passage from the brain parenchyma into the ventricle. The cannula was secured with dental cement to 2 stainless steel screws inserted into the holes made before the percussion pulse. A micro-osmotic pump (Alzet model 1007D, Durect Corp., Cupertino, CA) filled with 90 µl infusion volume kept at 37ºC, was implanted subcutaneously in the neck and connected to the infusion cannula. After sutures were completed, anaesthesia was turned off and the animals returned to the animal facility. After a week, when the total volume of S100B or vehicle solution was delivered, the infusion cannula and osmotic pump were removed under anaesthesia.

Study Protocol. The objective of these experiments was to assess the cellular effects of an intraventricular S100B infusion after fluid percussion injury. The animals were randomly assigned to an intraventricular S100B or vehicle infusion group, following injury or sham procedure. Purified bovine S100B protein (Calbiochem La Jolla, CA USA) was added to a vehicle solution containing phosphate buffered saline (PBS) and 0.1 mg rat serum albumin per ml. We infused S100B intraventricularly at a rate of 0.5 µl per hour (2.5 nM S100B infusion per hour) up to day 7 in order to reach a S100B CSF concentration of approximately 10 nM [12]. We choose this dose because it has been found that this concentration of S100B causes a proliferative response by astrocytes [13] and is within the range (below 50 nM) shown to cause an increased proliferative activity on melanoma cell lines [14] and rat C6 glioma cells [15]. All animals tolerated the operative procedure and intraventricular infusion without obvious side effects or infections, no morbidity or mortality occurred. The animals were sacrificed by an overdose of pentobarbital either on day 5 to assess early post-traumatic cell damage, or after 5 weeks to quantify neurodegeneration (n=4 per group and time point).

Histological assessment. The animals were deeply anesthetized with 4% halothane in N2O/O2 (70%/30%) and transcardially perfused with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in 0.1 M phosphate buffer (pH 7.25). The brains were then removed, postfixed in the same fixative for 4 hours at room temperature, blocked for coronal sections and paraffin embedded for microtome sectioning. Series of coronal sections (5 µm) were taken from the dorsal and rostral blade of the dentate gyrus (Bregma -1.6 mm) to the habenular comissure (Bregma -4.8 mm) to assess the cell damage and glial response throughout the area below the maximum percussion impulse and the hippocampus. The sections were taken using a rotary microtome, and 5 adjacent sections were collected every 100 µm. Serial sections were then processed for hematoxylin and eosin (HandE) and TUNEL staining as well as for immunohistochemistry against different antigens.

Tissue staining. Immunohistochemistry was performed applying the following antibodies: chromatin fragmentation was assessed using the TUNEL technique (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany); a mouse monoclonal antibody against amyloid precursor protein (APP; clone 22C11) (Chemicon; www.chemicon.com); a mouse monoclonal antibody against macrophages/activated microglia (ED1, Serotec, Oxford, United Kingdom); a rabbit polyclonal antibody against glial fibrillary acid protein (GFAP; Dako, www.dakogmbh.de); a rabbit polyclonal antibody against S100B (Abcam; www.abcam.com).

Sections were deparaffinized and rehydrated, subjected to microwave pretreatment in 10mM citric acid buffer (in case of ED1 and APP antibodies), and blocked for 10 minutes with 10% FCS in PBS. This was followed by incubation with primary antibodies at concentrations of 1:3000 for APP, 1:500 for ED1, 1:1000 for S100B, and 1:1000 for GFAP, diluted in 10% FCS/PBS, overnight at 4°C. The following day, sections were washed with PBS plus 0.1% Tween 20 (PBST) and incubated with an appropriate biotinylated secondary antibody. Antibody binding was visualized by using a standard avidin-biotin-peroxidase method with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen. Control sections were incubated with isotype control antibodies, rabbit non-immune serum or without primary antibodies. All immunostained sections were counterstained with hematoxylin before they were coverslipped with DePex® (Serva; www.serva.de).

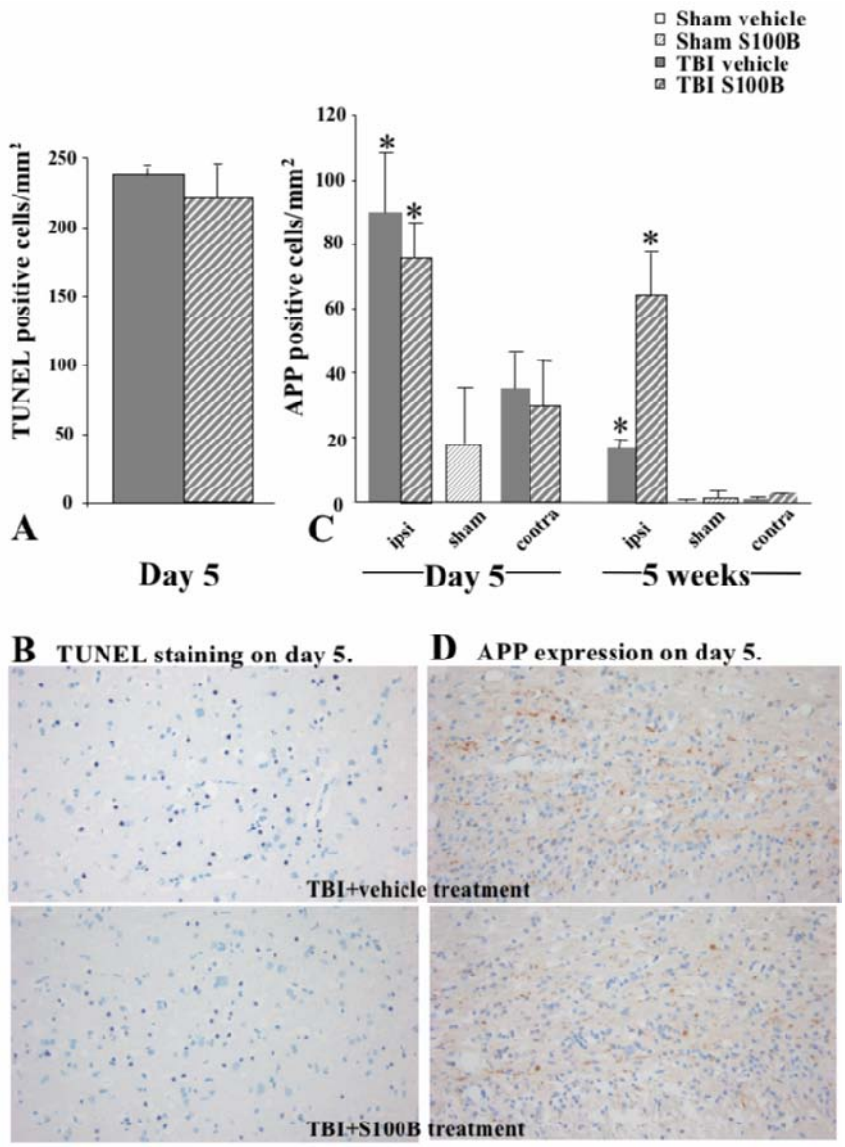

Figure 1. Cell death and axonal damage following lateral fluid percussion injury and an intraventricular S100B infusion. The injury induced cell damage around the lesion as assessed by TUNEL staining on day 5 post-injury was not altered by an intraventricular S100B infusion (A+B). Some residual APP expression below the lesion is present at 5 weeks following injury. The intraventricular infusion of S100B resulted in a significantly increased APP immunoreactivity in this area but although on the contralateral side 5 weeks after injury. In the non injured control group, S100B induced no relevant long-term APP immunoreactivity. (C+D). Asterisk represents statistical significance compared to the sham vehicle group.

Quantitative morphometry. Numbers of nuclear morphologies, TUNEL- and immunopositive cells in the cortical area below the primary percussion impulse, the corpus callosum, the granular cell layer of the dentate gyrus, and the hilus and CA3 region of the hippocampus were determined both ipsilateral to the lesion and in the corresponding contralateral areas. Ipsi- and contralateral sides were identified on the basis of the extent of

gliosis in the corpus callosum. The number of cells residing in each region was determined in every third section in a series of 5 μm coronal sections throughout the rostro-caudal extent of these regions. The areas to be counted were selected using a systematic random sampling scheme and resulted in the sampling of 10 microscopic fields at x20 magnification per region using an ocular morphometric grid. The values represent the number of immunoreactive cells per mm^2. An analysis of variance was performed using SPSS software, and significance was accepted at $p < 0.05$.

Results

Effect of an Intraventricular S100B Infusion on the Injury Induced Cell Death

To determine the extent of cell death following TBI and an intraventricular S100B infusion, we applied TUNEL staining and assessed morphological features and TUNEL-positivity on day 5 and 5 weeks post-injury. 5 days after injury, in the cortex immediately adjacent to the lesion site numerous neurons, but also some glial cells were TUNEL positive indicating DNA fragmentation (TBI 236.4±13.9 cells/mm², TBI+S100B 236.4±60.7 cells/mm²; Figure 1A+B). Some TUNEL-positive neurons were also detected in the basal ganglia. In the corpus callosum few oligodendrocytes showed DNA fragmentation. Within the hippocampus only very few apoptotic cells were present as demonstrated by the TUNEL staining. However, some hippocampal neurons beneath the lesion demonstrated a fine granular chromatin fragmentation indicative of necrosis but were not stained TUNEL-positive [16]. There was no difference of the number of apoptotic cells between S100B and vehicle infused animals on day 5 post-injury. Five weeks after injury, very few cells remained to be stained TUNEL positive, among them some macrophages. There was no difference of the number of apoptotic cells between S100B or vehicle infused injured and non-injured animals.

Effect of an Intraventricular S100B Infusion on the Injury Induced Axonal Injury

To determine axonal injury following TBI and an intraventricular S100B infusion, we performed APP immunohistochemistry and assessed the number of APP-positive axonal spheroids on day 5 and 5 weeks post-injury (Figure 1C+D). Early after injury, the number of APP positive axons in the ipsilateral corpus callosum was significantly increased both, following vehicle (90.3±43.4 per mm² compared to 18.3±18.1 cells/mm² in sham, p=0.004), and, although less pronounced, following S100B infusion (76.3±25.2 cells/mm², p=0.036 compared to sham). Conversely, 5 weeks after injury the number of APP positive axons remained significantly increased both, following S100B treatment (64.5±31.0 cells/mm² compared to 0.1±0.3 cells/mm² in sham, p=0.002), and, although less pronounced, following vehicle infusion (16.8±6.8 cells/mm², p=0.039 compared to sham).

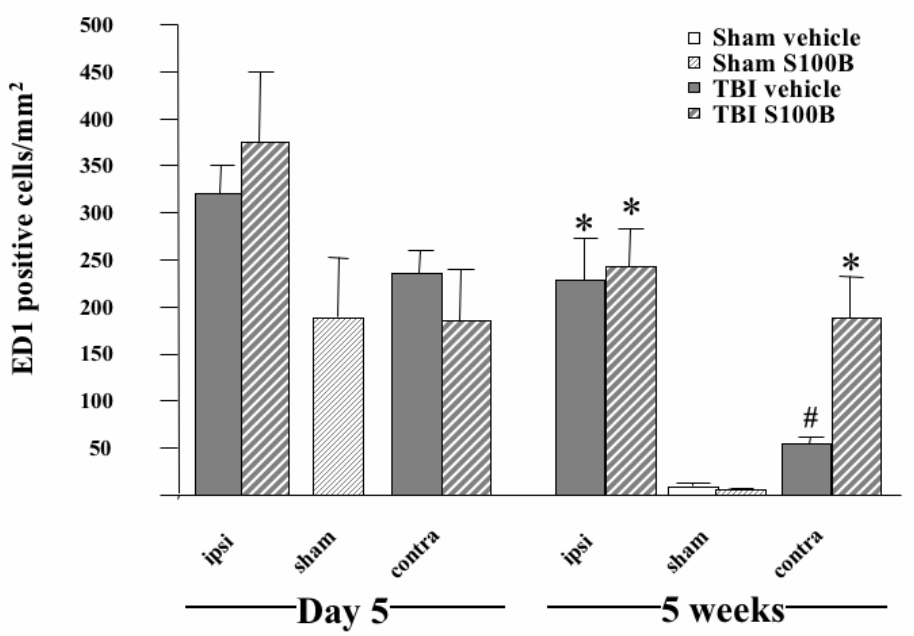

Figure 2. Microglial activation in the corpus callosum following lateral fluid percussion injury and an intra-ventricular S100B infusion. The early injury induced microglial activation is most pronounced in the subcortical area, and declined thereafter a little over 5 weeks. The intraventricular S100B infusion endorsed this effect substantially, and also on the non-lesioned side. Asterisk represents statistical significance compared to the sham vehicle group. GCL granular cell layer.

Effect of an Intraventricular S100B infusion on the Injury Induced Microglial Activation

To determine the effect of an intraventricular S100B infusion following TBI on microglial activation, we quantified the number of ED1 positive cells in the corpus callosum and the hippocampus (and assessed morphological features of these cells) on day 5 and 5 weeks post-injury. Early after injury, there was an injury induced microglial activation as demonstrated by an increased ED1 expression pronounced in the subcortical area (320±31 cells/mm^2 ipsilateral, 236±24 cells/mm^2 contralateral, per mm^2) as well as after an intraventricular S100B infusion in injured (376±59 cells/mm^2 ipsilateral, 185±54 cells/mm^2 contralateral) and non-injured animals (157±52 cells/ mm^2, Figure 2). Five weeks post-injury, the ED1 expression remained significantly increased in the ipsilateral corpus callosum (TBI 228±116 cells/mm² as compared to 9.3±5.1 in non-injured vehicle infused animals, $p<0.001$, TBI+S100B 243±41 cells/mm², $p<0.001$) and slightly decreased on the contralateral side (TBI 75±55 cells/mm²). However, S100B treatment following injury increased the contralateral ED1 expression significantly (188±102 cells/mm², $p<0.001$).

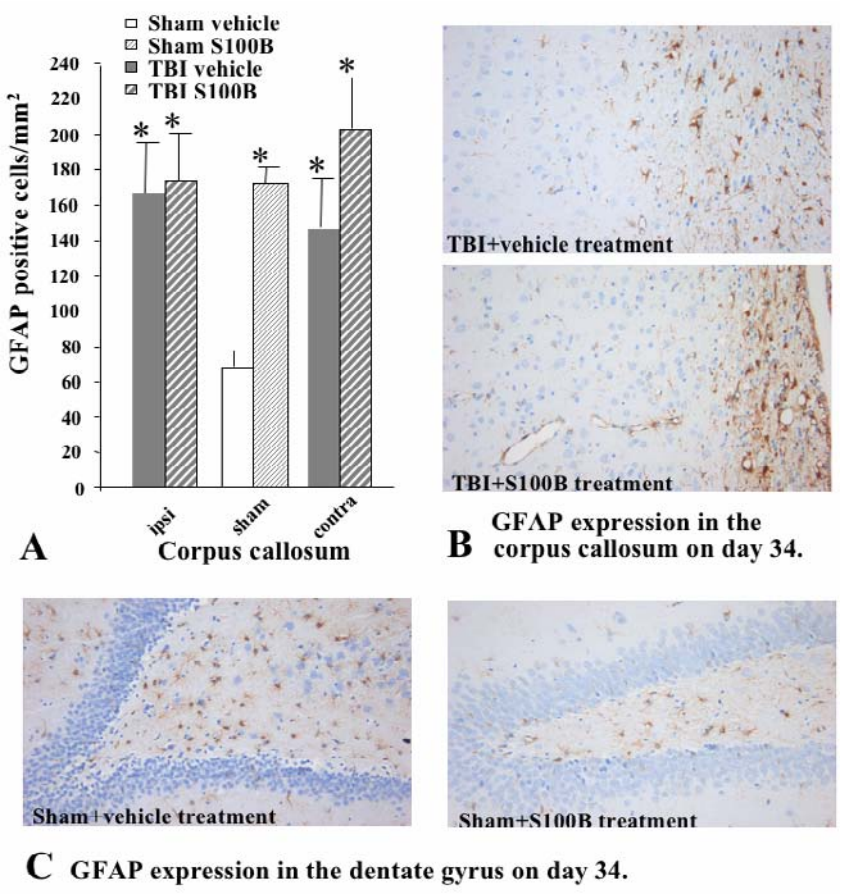

Figure 3. Glial reaction 5 weeks following lateral fluid percussion injury and intraventricular S100B infusion. Gliosis at 5 weeks post injury localized to the subcortical matter below the lesion and corpus callosum and the laterally reversed region (A+B). In the dentate hilus and the CA3 region, an intraventricular S100B infusion enhanced the astrocyte reactivity, both in uninjured control animals and following injury. Asterisk represents statistical significance compared to the sham vehicle group. GCL granular cell layer.

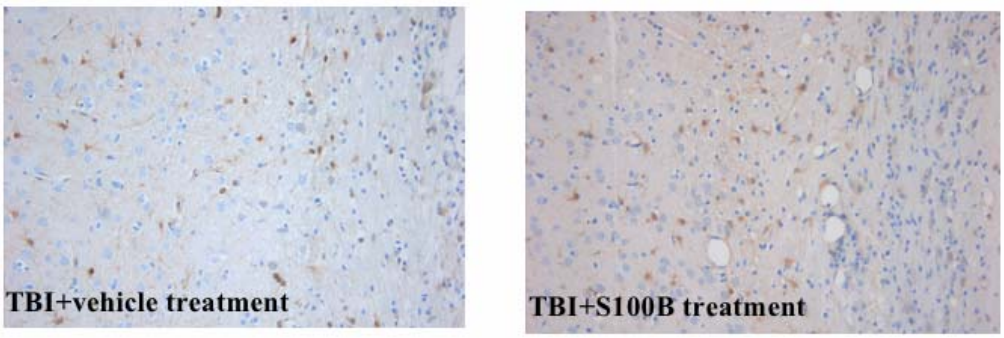

Figure 4. S100B expression following lateral fluid percussion injury and intraventricular S100B infusion. At 5 days post-injury, the injury-induced increased S100B expression is significantly enhanced by an intraventricular S100B infusion around the injury site.

Effect of an Intraventricular S100B Infusion on the Injury Induced GFAP Expression

To determine the effect of an intraventricular S100B infusion following TBI on GFAP expression, we quantified the number of GFAP positive cells in the corpus callosum and the hippocampus and assessed morphological features of GFAP-immunopositive cells on day 5 and 5 weeks post-injury (Figure 3). Early after injury, there was no relevant difference of GFAP immunoreactivity between the different groups.

In the corpus callosum, the GFAP expression was increased bilaterally by injury *per se* after 5 weeks (166.9±56.5 cells/mm^2 ipsilateral, p=0.005, 146.7±54.9 cells/mm^2 contralateral, p=0.027), as compared to vehicle infused non-injured animals (68.3±8.5 cells/mm²). An intraventricular S100B infusion did not add a relevant effect in injured animals (173.3±53.9 cells/mm^2 ipsilateral, p=0.003, 202.7.0±57.1 cells/mm^2 contralateral, p<0.001), while in non-injured animals the GFAP expression following S100B treatment reached the levels of the injured animals (171.2±9.6 cells/mm², p=0.005).

Effect of an Intraventricular S100B Infusion on the Injury Induced S100B Expression

To determine the effect of an intraventricular S100B infusion following TBI on S100B expression, we quantified the number of S100B positive cells in the corpus callosum and the hippocampus (and assessed morphological features of these cells) on day 5 and 5 weeks post-injury. Early after injury, the number of S100B positive cells was significantly increased on the infusion site in the ipsilateral corpus callosum compared to vehicle infused animals (30.3±5.4 versus 21.5±4.7 per mm², p=0.038, Figure 4). In the corpus callosum, this effect was lost 5 weeks after injury. However, in the germinative area of the hippocampus, the GCL, the effect of an increase in S100B immunoreactivity following a S100B treatment started to occur after the 5th day, and was significant after 5 weeks (17.0±0.4 cells/mm² in sham+S100B compared to 9.6±0.4 cells/mm² in sham+vehicle, p=0.048, and 12.8±1.3 cells/mm² in TBI S100B compared to 8.2±0.9 cells/mm² in TBI vehicle, p=0.017).

Conclusion

In humans, learning and memory impairment are common sequelae of TBI and may persist for many years regardless of injury severity, and trauma may accelerate cognitive decline associated with normal aging [17]. Clinical studies also suggest that TBI may induce neurodegenerative changes typically associated with Alzheimer's disease [18-20]. Indeed, diffuse deposits of ß amyloid (Aß) peptide, the central component of Alzheimer's senile plaques, have been detected in human brains following a single incident of head injury [21]. Although no Aß deposits have been detected in the rat brain after experimental injury, APP or APP-like proteins accumulate in damaged axons following fluid percussion injury in the

rat [22]. The accumulation of APP appears to be one of the earliest markers of axonal swelling following clinical brain injury [20, 23, 24].

The lateral fluid percussion injury is a well characterized and widely used model of brain injury due to trauma, and has been shown to replicate many of the relevant clinical and neuropathological features seen in man [22, 25-28]. Previous morphological studies emphasised the nature and distribution of damage to the cortex and subcortical white matter and associated inflammatory cell response in animals surviving up to one month post injury [29-31]. Axonal injury and dysfunction of the blood-brain-barrier are also features of the model [22, 29, 30]. Further contributions to the overall pattern of structural and functional change following lateral fluid percussion may occur through various mechanisms that include retrograde and/or anterograde degeneration, deafferentiation, or trophic factor withdrawal [32].

While several lines of evidence indicate that the neurotrophic protein S100B participates in some of the cellular events following injury, the specific role of S100B is ambiguous and may depend on the effective concentration and duration of exposure. Thus, we determined the effect of an intraventricular S100B infusion on cellular mechanisms at 5 days and 5 weeks following fluid percussion injury in the rat.

Cell death and apoptosis. Previous morphological studies have emphazised the nature and distribution of damage in animals surviving up to one month post injury [29-31]. We determined the mode and extent of cell death following fluid percussion injury on the basis of cell morphology and TUNEL staining, and found that most of the cell death occurred in a non-apoptotic manner, and was localized in the cortex immediately adjacent to the injury site.

In B 104 neuroblastoma cells, the addition of S100B at high concentrations (60 µg/ml respectively 3µM) resulted in changes of cell morphology consistent with dying cells like swelling of the soma and shorter, less robust neurites, degeneration and loss of cells [4]. Interestingly, these effects required the presence of astrocytes as the incubation of B 104 cells alone failed to display these changes in cell morphology (Hu 1997). The evaluation of the pattern of neuronal cell death in response to S100B treatment in vitro revealed both, a necrotic and an apoptotic cell death [4]. In our study, we found a S100B treatment at 200 nM not to induce neuronal necrosis or apoptosis, both in non-injured rats and after fluid percussion injury.

Axonal injury. APP immunohistochemistry is a standard method to demonstrate axonal injury in man [20, 23, 29], and has been used in various laboratory models of acute and chronic brain injury. In the early 1980's, the underlying pathology of diffuse axonal injury has been described and recognised as a widespread damage of axons [33], but may reflect also potentially reversible disturbances of axonal transport. This unifying concept was rapidly appreciated to be the common denominator of various groups of human trauma, the short surviving patients characterised by evidence of widespread damage of axons (axonal swelling and bulbs) to be gradually replaced by microglia and macrophages (microglial clusters) and in patients surviving for months or years, loss of myelinated axons in ascending and descending fibre tracts (Wallerian degeneration).

In neurodegenerative diseases, like Alzheimers's disease, S100B overexpression has been implicated in stimulation of neuronal ßAPP expression and subsequent dystrophic neurite formation and deposition of amyloid plaques [6]. Although increased levels of soluble

APP have been shown to protect neurons against excitotoxic, metabolic and oxidative injury in vitro [review [34], APP also induces microglial activation with increased inducible NO synthase expression [35].

Following severe fluid percussion injury (2.7 to 3.1 atm) in the rat, APP immunohistochemistry revealed ongoing axonal damage in the ipsilateral striatum and corpus callosum of injured brains up to one year and in the thalamus up to 6 months post-injury [36]. The observed APP immunoreactivity was considerably decreased compared with that reported at time points earlier than one month post injury [22, 37]. Taken together with the continual expansion of the cortical cavity [28] it appears that neuronal degeneration following experimental brain injury is an ongoing process which does not subside following the acute post injury period [36]. Paralleling these immunohistochemical changes are persistent cognitive and motor deficits for a prolonged time periods after injury [36].

In our study, beside the early axonal injury we found some residual APP expression below the lesion and in the corpus callosum at 5 weeks following a moderate fluid percussion injury (mean 2.09 atm). At that time point, the group receiving an intraventricular infusion of S100B at 200nM demonstrated more APP-positive cells in this area, although this difference was not significant. In the non-injured control group, the S100B infusion induced no relevant long-term APP immunoreactivity. Thus, a moderate concentration of S100B does not protect against, but possibly even promotes a delayed APP accumulation in axons reflecting disturbance of axonal transport following injury. Since we previously demonstrated the S100B treated animals to perform better in a learning and memory task, the increased APP expression does not impair the cognitive performance of these animals and may reflect (transient) ongoing disturbances in axonal transport.

Astrocytosis. Reactive astrocytes exhibit major differences in their morphological appearance and in their expression of glial markers following fluid percussion injury [38]. The spatial differences may be due to the pattern of neuronal cell loss. In vitro experiments have demonstrated that the expression of several glial proteins is regulated by neuronal interactions [39]. When astrocytes are cultured in the absence of neurons they proliferate rapidly and express increased levels of GFAP, and the addition of neurons into the culture arrest these changes. Following fluid percussion injury, astrogliosis was found to be closely associated spatially with the regions of neuronal cell loss, and observed first in the cortical area surrounding the primary lesion after 1 to 2 days [38]. Three days after injury, reactive astrocytes were also present in the hippocampus and thalamus. One months post injury, gliosis appeared to localize only to regions that contained extensive and progressive neuronal cell loss. Regions where neuronal cell loss had subsided by one month, such as the dentate hilus and adjacent cortex, showed a reduction in astrocyte reactivity [38].

Following experimental cerebral ischemia, an increased hippocampal GFAP expression was found probably due to synaptic remodelling [40], in congruence to the reported increased GFAP expression following deafferentiation injury in the dentate gyrus [41] and denervation in the motor cortex [42]. The increased GFAP expression by activated astrocytes was further enhanced by housing in a complex environment [40]. Thus reactive astrocytes may play a role in enhancing the regenerative capabilities of the central nervous system [43].

In our study, we found a significant reactive astrocytosis at 5 weeks post-injury to localize to areas where axonal disturbances occur, i.e. the subcortical white matter below the

lesion, the corpus callosum and the laterally reversed region [40]. On the other hand, in the dentate hilus and the CA3 region, where neuronal loss had subsided by one month after injury [38], we found a decreased GFAP immunoreactivity compared to non-injured animals. An intraventricular S100B infusion enhanced generally the astrocyte reactivity, both in uninjured control animals and following fluid percussion injury, in accordance to the notion that S100B transgenic mice demonstrated an increased GFAP immunoreactivity [7].

S100B immunoreactivity. The possible beneficial effect of reactive astrocytes has been speculated to result from enhancing astrocyte-neuron metabolic interactions through the release of neurotrophic or growth factors. Among others, for example an increased fibroblast growth factor expression has been demonstrated following experimental ischemia in the rat [40]. Following fluid percussion injury, a two-fold increase of the neurotrophic protein S100B in all reactive astrocytes has been demonstrated [38]. S100B was found to increase in astrocytes at the same time they looked to be reactive, and began to decrease in astrocytes as soon as their reactivity was subsiding [38]. These changes have been attributed to the regulation of the cytoskeleton by S100B [38].

In our study, we found the S100B expression to be significantly enhanced by an intraventricular S100B infusion in the germinative area of the hippocampus, the GCL, of the lesion side at 5 weeks post injury, and is in accordance to the participation of S100B in injury-induced cell proliferation. Interestingly, the S100B infusion in non-injured control rats provoked an astrocytosis and symmetrically increased S100B expression in both GCL after 5 weeks.

The supposed contrasting effects of S100B, the beneficial one in acute injury like TBI, and the detrimental one in chronic injury and neurodegeneration like Alzheimer's disease, have been speculated to be due to a variation of the concentration and duration of exposure of S100B or result from a modulation by additional factors. Furthermore, the role of S100B in neurodegenerative diseases may be either a causative one or a compensatory one. Likewise, depending on the context of their activation, reactive astrocytes are involved in neuronal survival and regeneration in an either protective or impedimental way. Even activated microglia following injury of the brain might support the onset of astrogliosis on the one hand, but might delay or reduce subsequent glial scar formation on the other hand [44]. Accordingly, although we were able to demonstrate S100B to improve cognitive function 5 weeks following fluid percussion injury in the rat, in the present study we found S100B to promote delayed APP and ED1 accumulation as well as astrocytosis. Whether these findings represent an enhancement of repair by S100B or result in a promotion of delayed neurodegeneration, has to be clarified by additional long-term experiments.

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References

[1] Heizmann, C. W., Fritz, G. and Schafer, B. W. (2002). S100 proteins: structure, functions and pathology. *Front Biosci*, 7, d1356-68.

[2] Van Eldik, L. J. and Wainwright, M. S. (2003). The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. *Restor Neurol. Neurosci*, 21, 97-108.

[3] Rothermundt, M., Peters, M., Prehn, J. H. and Arolt, V. (2003). S100B in brain damage and neurodegeneration. *Microsc. Res. Tech*, 60, 614-32.

[4] Hu, J., Ferreira, A. and Van Eldik, L. J. (1997). S100beta induces neuronal cell death through nitric oxide release from astrocytes. *J. Neurochem*, 69, 2294-301.

[5] Huttunen, H. J., Kuja-Panula, J., Sorci, G., Agneletti, A. L., Donato, R. and Rauvala, H. (2000). Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J. Biol. Chem*, 275, 40096-105.

[6] Li, Y., Wang, J., Sheng, J. G., Liu, L., Barger, S. W., Jones, R. A., Van Eldik, L. J., Mrak, R. E. and Griffin, W. S. (1998). S100 beta increases levels of beta-amyloid precursor protein and its encoding mRNA in rat neuronal cultures. *J. Neurochem*, 71, 1421-8.

[7] Reeves, R. H., Yao, J., Crowley, M. R., Buck, S., Zhang, X., Yarowsky, P., Gearhart, J. D. and Hilt, D. C. (1994). Astrocytosis and axonal proliferation in the hippocampus of S100b transgenic mice. *Proc. Natl. Acad. Sci. USA*, 91, 5359-63.

[8] Adami, C., Bianchi, R., Pula, G. and Donato, R. (2004). S100B-stimulated NO production by BV-2 microglia is independent of RAGE transducing activity but dependent on RAGE extracellular domain. *Biochim. Biophys. Acta*, 1742, 169-77.

[9] Kleindienst, A., McGinn, M. J., Harvey, H. B., Colello, R. J., Hamm, R. J. and Bullock, M. R. (2005). Enhanced hippocampal neurogenesis by intraventricular S100B infusion is associated with improved cognitive recovery after traumatic brain injury. *J. Neurotrauma*, 22, 645-55.

[10] Savola, O., Pyhtinen, J., Leino, T. K., Siitonen, S., Niemela, O. and Hillbom, M. (2004). Effects of head and extracranial injuries on serum protein S100B levels in trauma patients. *J. Trauma*, 56, 1229-34; discussion 1234.

[11] Dixon, C. E., Lyeth, B. G., Povlishock, J. T., Findling, R. L., Hamm, R. J., Marmarou, A., Young, H. F. and Hayes, R. L. (1987). A fluid percussion model of experimental brain injury in the rat. *J. Neurosurg*, 67, 110-9.

[12] Kleindienst, A., Harvey, H. B., Rice, A. C., Muller, C., Hamm, R. J., Gaab, M. R. and Bullock, M. R. (2004). Intraventricular infusion of the neurotrophic protein S100B improves cognitive recovery after fluid percussion injury in the rat. *J. Neurotrauma*, 21, 541-7.

[13] Goncalves, D. S., Lenz, G., Karl, J., Goncalves, C. A. and Rodnight, R. (2000). Extracellular S100B protein modulates ERK in astrocyte cultures. *Neuroreport*, 11, 807-9.

[14] Klein, J. R., Hoon, D. S., Nangauyan, J., Okun, E. and Cochran, A. J. (1989). S-100 protein stimulates cellular proliferation. *Cancer Immunol. Immunother*, 29, 133-8.

[15] Selinfreund, R. H., Barger, S. W., Pledger, W. J. and Van Eldik, L. J. (1991). Neurotrophic protein S100 beta stimulates glial cell proliferation. *Proc. Natl. Acad. Sci. USA*, 88, 3554-8.

[16] Muller, G. J., Stadelmann, C., Bastholm, L., Elling, F., Lassmann, H. and Johansen, F. F. (2004). Ischemia leads to apoptosis--and necrosis-like neuron death in the ischemic rat hippocampus. *Brain Pathol*, 14, 415-24.

[17] Stuss, D. T., Stethem, L. L., Picton, T. W., Leech, E. E. and Pelchat, G. (1989). Traumatic brain injury, aging and reaction time. *Can. J. Neurol. Sci*, 16, 161-7.

[18] Geddes, J. F., Vowles, G. H., Robinson, S. F. and Sutcliffe, J. C. (1996). Neurofibrillary tangles, but not Alzheimer-type pathology, in a young boxer. *Neuropathol. Appl. Neurobiol*, 22, 12-6.

[19] Mortimer, J. A., van Duijn, C. M., Chandra, V., Fratiglioni, L., Graves, A. B., Heyman, A., Jorm, A. F., Kokmen, E., Kondo, K., Rocca, W. A. and et al. (1991). Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODEM Risk Factors Research Group. *Int. J. Epidemiol*, 20 Suppl 2, S28-35.

[20] Gentleman, S. M., Nash, M. J., Sweeting, C. J., Graham, D. I. and Roberts, G. W. (1993). Beta-amyloid precursor protein (beta APP) as a marker for axonal injury after head injury. *Neurosci. Lett*, 160, 139-44.

[21] Roberts, G. W., Gentleman, S. M., Lynch, A., Murray, L., Landon, M. and Graham, D. I. (1994). Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry*, 57, 419-25.

[22] Pierce, J. E., Trojanowski, J. Q., Graham, D. I., Smith, D. H. and McIntosh, T. K. (1996). Immunohistochemical characterization of alterations in the distribution of amyloid precursor proteins and beta-amyloid peptide after experimental brain injury in the rat. *J. Neurosci*, 16, 1083-90.

[23] Sherriff, F. E., Bridges, L. R., Gentleman, S. M., Sivaloganathan, S. and Wilson, S. (1994). Markers of axonal injury in post mortem human brain. *Acta Neuropathol.* (Berl), 88, 433-9.

[24] Blumbergs, P. C., Scott, G., Manavis, J., Wainwright, H., Simpson, D. A. and McLean, A. J. (1994). Staining of amyloid precursor protein to study axonal damage in mild head injury. *Lancet*, 344, 1055-6.

[25] Bramlett, H. M., Green, E. J. and Dietrich, W. D. (1999). Exacerbation of cortical and hippocampal CA1 damage due to posttraumatic hypoxia following moderate fluid-percussion brain injury in rats. *J. Neurosurg*, 91, 653-9.

[26] Hicks, R. R., Smith, D. H., Lowenstein, D. H., Saint Marie, R. and McIntosh, T. K. (1993). Mild experimental brain injury in the rat induces cognitive deficits associated with regional neuronal loss in the hippocampus. *J. Neurotrauma*, 10, 405-14.

[27] McIntosh, T. K., Vink, R., Noble, L., Yamakami, I., Fernyak, S., Soares, H. and Faden, A. L. (1989). Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. *Neuroscience*, 28, 233-44.

[28] Smith, D. H., Chen, X. H., Pierce, J. E., Wolf, J. A., Trojanowski, J. Q., Graham, D. I. and McIntosh, T. K. (1997). Progressive atrophy and neuron death for one year following brain trauma in the rat. *J. Neurotrauma*, 14, 715-27.

[29] Cortez, S. C., McIntosh, T. K. and Noble, L. J. (1989). Experimental fluid percussion brain injury: vascular disruption and neuronal and glial alterations. *Brain Res*, 482, 271-82.

[30] Soares, H. D., Hicks, R. R., Smith, D. and McIntosh, T. K. (1995). Inflammatory leukocytic recruitment and diffuse neuronal degeneration are separate pathological processes resulting from traumatic brain injury. *J. Neurosci*, 15, 8223-33.

[31] Toulmond, S., Duval, D., Serrano, A., Scatton, B. and Benavides, J. (1993). Biochemical and histological alterations induced by fluid percussion brain injury in the rat. *Brain Res*, 620, 24-31.

[32] Povlishock, J. T., Erb, D. E. and Astruc, J. (1992). Axonal response to traumatic brain injury: reactive axonal change, deafferentation, and neuroplasticity. *J. Neurotrauma*, 9 Suppl 1, S189-200.

[33] Adams, J. H., Graham, D. I., Murray, L. S. and Scott, G. (1982). Diffuse axonal injury due to nonmissile head injury in humans: an analysis of 45 cases. *Ann. Neurol*, 12, 557-63.

[34] Mattson, M. P. (1997). Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiol. Rev*, 77, 1081-132.

[35] Barger, S. W. and Harmon, A. D. (1997). Microglial activation by Alzheimer amyloid precursor protein and modulation by apolipoprotein E. *Nature*, 388, 878-81.

[36] Pierce, J. E., Smith, D. H., Trojanowski, J. Q. and McIntosh, T. K. (1998). Enduring cognitive, neurobehavioral and histopathological changes persist for up to one year following severe experimental brain injury in rats. *Neuroscience*, 87, 359-69.

[37] Lewen, A., Li, G. L., Nilsson, P., Olsson, Y. and Hillered, L. (1995). Traumatic brain injury in rat produces changes of beta-amyloid precursor protein immunoreactivity. *Neuroreport*, 6, 357-60.

[38] Hill, S. J., Barbarese, E. and McIntosh, T. K. (1996). Regional heterogeneity in the response of astrocytes following traumatic brain injury in the adult rat. *J. Neuropathol. Exp. Neurol*, 55, 1221-9.

[39] Hatten, M. E. (1985). Neuronal regulation of astroglial morphology and proliferation in vitro. *J. Cell Biol*, 100, 384-96.

[40] Briones, T. L., Woods, J., Wadowska, M., Rogozinska, M. and Nguyen, M. (2006). Astrocytic changes in the hippocampus and functional recovery after cerebral ischemia are facilitated by rehabilitation training. *Behav. Brain Res*, 171, 17-25.

[41] Steward, O., Kelley, M. S. and Torre, E. R. (1993). The process of reinnervation in the dentate gyrus of adult rats: temporal relationship between changes in the levels of glial fibrillary acidic protein (GFAP) and GFAP mRNA in reactive astrocytes. *Exp. Neurol*, 124, 167-83.

[42] Bury, S. D., Eichhorn, A. C., Kotzer, C. M. and Jones, T. A. (2000). Reactive astrocytic responses to denervation in the motor cortex of adult rats are sensitive to manipulations of behavioral experience. *Neuropharmacology*, 39, 743-55.

[43] Ridet, J. L., Malhotra, S. K., Privat, A. and Gage, F. H. (1997). Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci, 20, 570-7.

[44] Rohl, C., Lucius, R. and Sievers, J. (2006). The effect of activated microglia on astrogliosis parameters in astrocyte cultures. *Brain Res.*

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Chapter 13

Information Processing by NGF-Activated Signaling Networks

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Abstract

Cell biology faces a sharp rise in the amount of experimental data and thus, it's difficult to interpret those data into an integral functional plan of a living cell. There is a demand for adequate tools for the analysis of highly interconnected signaling networks at different levels of complexity. This would allow an understanding of how to do signaling network process multiple receptor inputs to produce adoptive physiological responses both at the single cell level and in the a multicellular organism. Here we discuss the use of Boolean networks for the interpretation of experimental results on converging signaling pathways induced by nerve growth factor (NGF) and extracellular matrix (ECM) in neurons. We consider Boolean logics as a convenient tool for the systematization of causal connections between signaling events. As an illustration, a Boolean analysis of NGF-Src signaling is performed that triggers axonal growth. High-throughput quantitative studies of signaling events allows a more detailed understanding of signaling network connectivity based on the modular response analysis and other mathematical methods. A possible structural mechanism of NGF- and ECM-induced signal integration is then discussed at the level of the Src-FAK molecular complex. We hypothesize that FAK mediates alternative signaling pathways for axonal growth in the presence versus the absence of NGF. Finally, the importance of quantification of subcellular targeting of signaling events is emphasized for the analysis of signaling networks.

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Introduction

Over the past decades, NGF was shown to regulate a wide range of cell functions in the nervous system. To reveal the molecular mechanism of this regulation, NGF-activated intracellular signaling pathways was extensively studied. Accumulation of experimental data brought into focus the problem of the information exchange between different pathways. We need to understand how the genetically encoded molecular machinery executes coordination between different cell functions. We also need to understand how signals from different types of receptors are collected and processed together inside a cell in order to control each particular cell function. These problems bring cell biology to a significantly new level of complexity of the studied phenomena, namely to the level of signaling networks.

A living cell can be understood as a complicated machine which consists of multiple molecular components. The machine is driven by a combination of 1) genetically encoded programs and 2) environmental signals received by receptors. The major problem is that we have very fragmentary knowledge of how molecular components are connected with one another (Figure 1). As a result, we do not know all signaling consequences caused by a receptor activation (the question of signal divergence). We also do not know how many different types of regulatory inputs a signaling molecule receives from other signaling molecules (the question of signal convergence). Moreover, present cell biology has limited technical tools to address these questions adequately. Three methods for finding signaling connections within a limited set of molecules – Boolean networks, Bayesian networks and modular response analysis - are discussed later in this chapter.

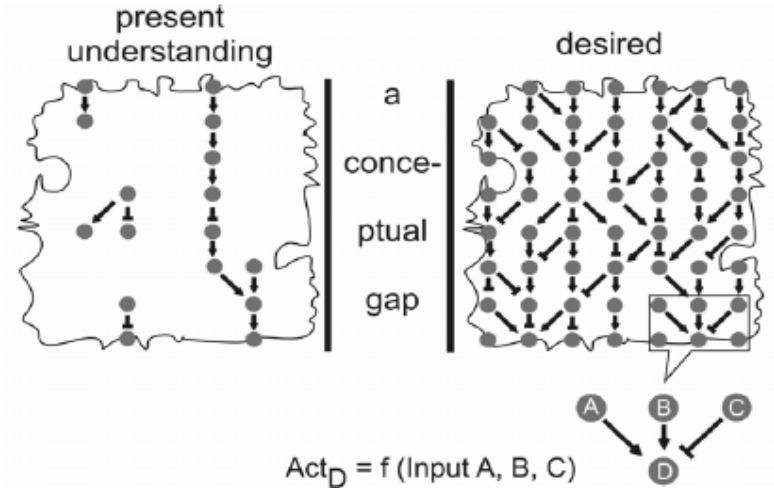

Figure 1. At present, studies on signaling pathways take into account a small portion of signaling connections which potentially exist between molecules in the network. Ideally, detailed prediction and manipulation of cell behaviour would require a full map of causal signaling connections within a cell. This would allow the expression of a signaling molecule as a function of all regulatory inputs to this molecule. There is a conceptual gap both in experimental methods and analytic tools between present fragmentary knowledge of signaling pathways and the desired understanding of the whole-cell signaling network.

Current experimental data on signaling pathways indicate complex behaviour and emergent phenomena in regulation of cell physiological functions (Bhalla, 2003; Katz and Clemens, 2001; Papin et al., 2005; Weng et al., 1999). Kinetic modelling has been used extensively to describe and predict signal propagation through signaling pathways and networks (Bhalla et al., 2002; Wolkenhauer et al., 2005). The bottleneck of mathematical modelling in cell biology is the ability to combine theoretical and experimental techniques to address a complex phenomenon which is otherwise difficult to understand intuitively. Such "model - experiment coupling" exploits experimental verification of model predictions and model-based design of further experiments for model refinement, as an example, please see (Swameye et al., 2003). Construction and experimental verification of realistic signaling network models requires experimental data on activation state and kinetic parameters of signaling molecules. An important technical limitation here is the availability of simultaneous measurements of the activation state of multiple signaling molecules in the same experiment. In the next section, some experimental approaches will be discussed to address this problem.

Activity Measurements on Multiple Network Components

Western blot analysis of protein phosphorylation on specific sites remains the most widely used method to study activation of signaling proteins. A significant advantage of this method compared to cell-based immunostaining methods is the direct estimation of the molecular weight of detected proteins. This provides verification of the antibody specificity that remains a crucial problem in antibody-based methods for measuring protein phosphorylation at specific sites. Quantitative studies of signaling networks require quantification of Western blot results (Schilling et al., 2005). Santos and co-authors recently used quantitative Western blot analysis of phosphorylated epitopes of Raf-1, Mek1/2 and Erk1/2 to measure activation of these proteins under stimulation with NGF or epithelial growth factor (EGF) (Santos et al., 2007).

In vitro kinase activity assay allows direct measurements of the activation state of protein kinases. A promising high-throughput kinase assay was developed by Janes and co-authors to measure activity of several kinases in parallel (Janes et al., 2003). Using this assay, the authors were able to identify the kinetic component of the network activation which mediated anti-apoptotic action of insulin on colon epithelial cells.

Flow cytometry is one of the most potent methods for parallel measurements of the activation of multiple network components (Irish et al., 2004; Krutzik et al., 2008). Importantly, flow cytometry provides measurements on single cells and therefore it allows quantification of signaling heterogeneity in the studied cell population. This allows the identification of cells of different types or in different physiological states within a heterogeneous population (Irish et al., 2006).

Mass spectrometry approach for the quantification of protein phosphorylation has developed rapidly over the last few years (Kratchmarova et al., 2005; Pflieger et al., 2008). The method allows the measurement of phosphorylation of hundreds of specific sites in the same experiment and identification of novel phosphorylation sites (Wolf-Yadlin et al., 2007).

The above listed experimental methods are difficult to apply to small cell populations, for instance to a limited neuronal population cultured from a specific region of the nervous system. Reduction of the amount of cell material sufficient for measurements will be probably one of the most important directions for the future development of these techniques.

Data Integration Into a Whole-Cell Network

Parallel monitoring of activities of multiple molecules in a signaling network allows to reveal connectivity and details of signal propagation in intracellular signaling networks (Janes et al., 2003; Sachs et al., 2005; Santos et al., 2007; Wolf-Yadlin et al., 2007). In the future the number of measured molecules and time points will increase to get a more full and accurate description of signals that govern physiological functions.

The problem of uniting all signaling molecules of a cell into a single network reminds to some extent of Laplacian determinism. Let us imagine that we could know: 1) positions of all molecules within a cell, 2) activation states of signaling proteins, and 3) how signaling inputs are turned into the activity output at each type of a signaling protein. Then we would be able to predict the cell behaviour at high accuracy. This ideal view of future cell biology rises the question of cognitive ability of human science to handle this level of complexity. How much information is a modern researcher able to process on causes and consequences in a whole-cell network? Are modern mathematics and informatics ready to interpret and integrate experimental measurements on hundreds of signaling components into a network model for prediction of cell behaviour? The tools for data analysis and human ability to address complex regulation of physiological functions are probably the most important research components for filling the gap between current studies on simple pathways and the desired understanding of integral cell function (Figure 1).

Convergence of Signals

Modern experimental cell biology mostly deals with finding activatory and inhibitory connections from one signaling molecule to another or from a signaling molecule to a cell physiologic output. The problem of these results is that they often turn out to have a very limited, unreliable predictive value. In many cases an outcome of a signaling cascade is highly dependent on a cell type (Miller-Jensen et al., 2007). Even within a certain, well defined experimental system some signaling connections are highly affected by culturing conditions like cell density, substrate coating, etc. One explanation for signaling variability is that the activity of a signaling molecule is usually regulated not by a single input from an upstream level of a pathway, i.e. signaling pathways are not linear. Instead, two or more different signaling inputs usually affect the same downstream signaling molecule by means of covalent modifications and/or complex formation. For instance different types of receptor inputs converge in intracellular signaling networks so that a cellular response to activation of one receptor type can be strongly affected by activation/inhibition of another receptor type. In this way a signaling connection between two molecules can be switched from inhibition to

activation and *vice versa* by a "side input" from another pathway. An example of this kind is provided by pharmacological remodelling of the attraction/repulsion response of axonal growth cones to receptor activation. Growth cone repulsion induced by polarized activation of a G-protein coupled receptor CXCR4 can be switched to attraction by inhibiting protein kinase C (PKC) or by application of a cyclic GMP analog 8-Br-cGMP (Xiang et al., 2002).

Boolean Networks and NGF Signaling

Cell culture techniques allow to easily probe multiple ligand combinations for their effects on living cells. A range of cell events can be monitored in response to ligand stimulation including cell survival, morphological responses and molecular events like protein or RNA expression, protein modification, targeting, etc. Understanding connectivity and crosstalk of signaling pathways upstream of the monitored event reveals the mechanism of the ligand – response causal connection. Thereby the cell response can be predicted and manipulated, for instance by pharmacological tools. Boolean logics provides a simple and informative way to reveal and describe the input-output relation in those cases when the activity (output) of a signaling molecule is regulated by two or more regulatory inputs from other signaling molecules. It assumes that the causal connections between network inputs and outputs are determined by logical operations performed at the nodes of the network (Arkin and Ross, 1994; Bray, 1995; Sun and Zhao, 2004).

Boolean interpretation of biochemical reactions was proposed in 1960-s after the discovery of the operon control of gene transcription and allosteric enzyme regulation, for references please see (Arkin and Ross, 1994). Some recent applications of Boolean formalism include analysis of the cell cycle progression in endothelial cells (Huang and Ingber, 2000) and description of synthetic genetic networks in *E.coli* (Guet et al., 2002). Dueber and co-authors used domain recombination to construct signaling proteins which perform desired Boolean operations in eucaryotic cells (Dueber et al., 2003; Dueber et al., 2004).

We previously implemented a Boolean network formalism to retrieve signaling connections between cell surface receptors, intracellular protein kinases and axonal growth in dorsal root ganglion (DRG) neurons (Paveliev et al., 2007). We demonstrated that signaling inputs from laminin and neurotrophic factors are integrated with each other by a conjunctive operator (AND) which is associated with Src family kinases (SFKs). In addition to that laminin also activated a different SFK-independent pathway that involved the cyclin-dependent kinase 5 (Cdk5). The two signaling pathways converge at a disjunctive operator (OR). Later in this chapter we discuss the focal adhesion kinase (FAK) as a possible molecular substrate for the Boolean functions in this signaling network.

A signaling network that integrates three types of receptor inputs was studied by Zhou and co-authors (Zhou et al., 2006). It was demonstrated that cultured sensory neurons grow axons when stimulated simultaneously with NGF and laminin. The third input to the signaling network was aggrecan – a member of Chondroitin Sulphate ProteoGlycan (CSPG) family. Aggrecan efficiently inhibited axonal growth induced by NGF+laminin. Here we propose a Boolean model to describe convergence of receptor inputs in the signaling network based on the experimental results by Zou and co-authors (Zhou et al., 2006). In Figure 2A

signaling inputs from NGF and laminin are integrated by a conjunctive operator (AND). Inhibition of the laminin-induced signaling by CSPG is expressed through a negative Boolean operator (NOT). The two operators enclosed in the dotted frame in Figure 2 may physically represent together a signaling protein which is activated by laminin and inhibited by CSPG. Alternatively the operator NOT may be a protein (for instance, a phosphatase) which is activated by CSPG and inhibits the laminin-activated signaling pathway.

The experimental data indicate that the signaling network was substantially remodelled when mice were subjected to a preconditioning lesion (PCL) of sciatic nerves (Zhou et al., 2006). PCL becomes the fourth input to the network (Figure 2B). Under this posttraumatic condition synergism between laminin and NGF was no longer required for axonal growth. Therefore in the case of PCL signaling inputs from NGF and laminin converge on the disjunctive operator (OR) (Figure 2B). Inhibitory analysis indicates that Src and the integrin-linked kinase (ILK) are downstream of both laminin and NGF (Zhou et al., 2006). Therefore similarly to the study by Paveliev and co-authors (Paveliev et al., 2007), Src is likely to be the integration node for signals coming from NGF and laminin. The molecular mechanism of the network remodelling remains unclear. Janus kinase (JAK) and a transcription factor STAT3 were previously suggested to mediate effects of PCL on axonal growth (Liu and Snider, 2001). PCL may affect expression of a protein or a group of proteins which control the type of signal integration between laminin and NGF. Otherwise PCL may affect covalent modification or allosteric regulation of Src, ILK or another signaling protein that controls the type of the Boolean function that switches NGF and laminin signaling inputs to the axonal growth output.

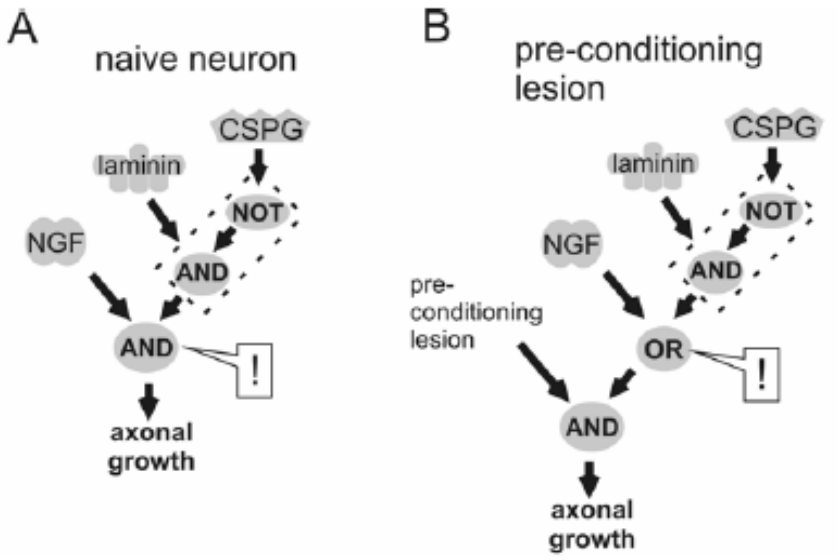

Figure 2. A Boolean model of the NGF-activated axonal growth based on the experimental results by (Zhou et al., 2006). A. In naïve neurons conjunction (AND) of NGF and laminin induces axonal growth. B. After a pre-conditioning lesion (PCL) either NGF or laminin alone is enough to activate axonal growth (input disjunction, OR). The change of the Boolean operator between laminin and NGF which happens after PCL is marked with the exclamation mark. CSPG – chondroitin sulphate proteoglycan.

A physical substrate of the Boolean operators presented in Figure 2 may be a single protein which has regulatory inputs from NGF, laminin and CSPG. Alternatively each of the Boolean functions in Figure 2 may be performed by a separate signaling protein and those proteins are connected with each other into a pathway.

We have discussed now Boolean models that we constructed based on the results of inhibitory analysis performed by Paveliev and co-authors (Paveliev et al., 2007) and Zhou and co-authors (Zhou et al., 2006). The essential result of Boolean modelling in these cases is explicit formal definition of the type of the input-output relation at the signal convergence points in the studied signaling pathways. This formalization is necessary to describe a system which includes both synergistic and mutually independent signaling elements. A wide range of processes that transform a sum of receptor inputs into a cell physiological response can be clarified and reduced to the level of a Boolean model. Most importantly, this allows prediction and manipulation of physiological responses to pharmacological treatments, gene perturbation, etc. (Huang, 1999; McAdams and Shapiro, 1995; Paveliev et al., 2007).

FAK as a Hypothetical Integrator of NGF- and ECM-Induced Signals

Attributing Boolean operations to a signaling network that integrates two or more receptor inputs rises the question of a physical substrate – which particular signaling molecules or molecular complexes integrate signals in the Boolean-type fashion.

The identity of a signaling molecule or a multimolecular complex that receives signaling inputs from the SFK-dependent and –independent pathways and converts these inputs into axonal growth remains unclear (Paveliev et al., 2007). Here we hypothesize that Focal Adhesion Kinase (FAK) is the molecular substrate that integrates the two pathways and performs the disjunctive Boolean operation (OR) leading to axonal growth.

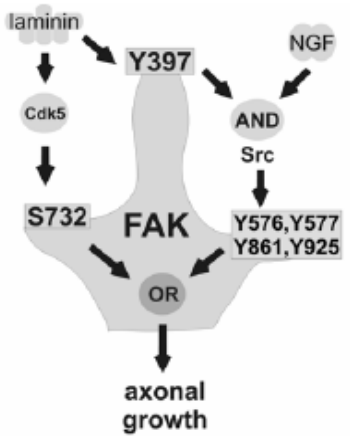

Figure 3. FAK as a possible physical substrate for the Boolean network described in (Paveliev et al., 2007). FAK-Src signaling complex is capable of integrating signaling inputs from laminin and NGF. FAK phosphorylation on Ser732 (S732) by Cdk5 is suggested to mediate the effect of laminin on axonal growth in the absence of NGF. Y – tyrosine.

SFK-FAK co-signaling downstream of NGF and laminin. Src and FAK form a dual signaling complex in which the two kinases activate each other (Mitra et al., 2005). Integrin activation by laminin leads to FAK phosphorylation on tyrosine 397 (Tyr397) (Tucker et al., 2005). It is known from experiments in non-neuronal cells that Src docking to FAK pTyr397 leads to activation of Src (Mitra et al., 2005). Our experimental results suggest that Src integrates the laminin/integrin/FAK pTyr397 signaling input with the NGF/TrkA signaling input, and thereby performs conjunctive (AND) Boolean operation (Figure 3) (Paveliev et al., 2007). Activated Src, in turn, phosphorylates FAK on Tyr576 and Tyr577 inducing FAK kinase activity. In addition, Src phosphorylates FAK on Tyr861 and Tyr925 and these phosphorylated residues become docking sites for several signaling proteins including paxillin and p130Cas (Mitra et al., 2005). Activation of p130Cas, in turn, leads to activation of Rac and Cdc42 and promotes axonal growth (Liu et al., 2007).

A recent work highlighted the role of Src-FAK co-signaling in DRG axonal growth induced by simultaneous treatment with NGF and laminin (Tucker et al., 2008). As distinct from our study (Paveliev et al., 2007), the authors applied laminin after cell culture plating and observed early onset of axonal growth (already at 6 hours after ligand application) in rat DRG neurons treated with NGF or laminin or both. Despite these differences of the experimental setup, some observations made by Tucker and co-authors support our current hypothesis on the mechanism of laminin/NGF-induced axonal growth (Figure 3). Neurons were transfected with the FAK-related non-kinase (Frnk) which served as FAK inhibitor. This treatment resulted in reduced phosphorylation of both FAK Y397 and Src. Moreover, suppression of FAK with Frnk blocked the NGF/laminin-activated axonal growth (Tucker et al., 2008). FAK and its homolog Pyk2 were also suggested to integrate signaling inputs from growth factors (EGF, IGF-1) and integrin ligands (lamini, collagen) in a pathway leading to neurite outgrowth in phaeochromocytoma and neuroblastoma cells (Ivankovic-Dikic et al., 2000).

Cdk5 signaling downstream of laminin. In the absence of NGF laminin is able to promote axonal growth through a different, SFK-independent mechanism. We have demonstrated that the NGF-independent axonal growth on a laminin-coated substrate was not affected by the SFK inhibitors (Paveliev et al., 2007). By contrast, this type of growth was blocked by the Cdk5 inhibitor roscovitine. Moreover, we observed laminin-dependent induction of Cdk5 activity at the time of axonal growth onset.

Laminin is also known to activate Cdk5 in cerebellar macroneurons and in a differentiated neuroblastoma cell line and in both cases this signaling pathway leads to the activation of axonal growth (Li et al., 2000; Paglini et al., 1998). FAK turns out to be a likely candidate to mediate the effect of laminin/Cdk5 signaling on axonal growth (Figure 3). The supporting evidence comes from the study by Xie and co-authors (Xie et al., 2003; Xie and Tsai, 2004). The authors demonstrate that Cdk5 phosphorylates FAK on Ser732 and this signaling event is required for neuronal migration.

These data together suggest that FAK can mediate both the NGF-dependent and –independent signaling pathways that we described previously (Paveliev et al., 2007). In this case FAK may be the convergence point for the two pathways and the physical substrate for the disjunctive Boolean operator (OR) (Figure 3).

Quantitative Studies of NGF Signaling

As any scientific description Boolean modelling is an approximation of a process which allows to predict certain (but not all) features of the studied process. The level of accuracy and detail depends on the amount of experimental data that describe various features of the studied process. In this sense Boolean description of diverging and converging signaling pathways provides a transition step between the empirical description – "this activates that" - and detailed mathematical models of signaling networks. By doing so Boolean modelling contributes to filling the gap between the present unsatisfactory understanding of signal networking and the desired integrated knowledge on cell signaling (Figure 1). The limitation of Boolean formalism is that it is difficult to apply to the analysis of partial, quantitative changes in the measured output parameter. Indeed, Boolean modelling relies on the "all-or-none" approximation of cell responses so that the measured parameter is binarized into the values of 0 or 1. Different mathematical tools are used for the analysis of partial changes in the activation state of intracellular signaling networks. Among those are differential equations, modular response analysis and Bayesian network formalism.

Differential equations have long been used in biochemistry to model signaling and metabolic pathways (Smith et al., 2002; Vilar et al., 2003; Wolkenhauer et al., 2005). Two studies on NGF-stimulated pheochromocytoma cells have combined the ordinary differential equation models with experimental measurements of signaling events activated by NGF (Aoki et al., 2007; Sasagawa et al., 2005). The study by Sasagawa and co-authors implicated differential activation of Ras and Rap1 by EGF and NGF as a mechanism of the difference in the Erk1/2 activation kinetics in response to these growth factors (Sasagawa et al., 2005; Stork, 2005). The work of Aoki and co-authors highlighted the role of the phosphatidilinositol-5-phosphatase SHIP2 in the NGF-activated neurite growth in PC12 cells (Aoki et al., 2007). This study takes advantage of fluorescence resonance energy transfer (FRET) which gives an exclusive chance to trace activation of signaling molecules in real time inside living cells.

Modular response analysis (MRA) is a highly promising mathematical method developed by Kholodenko and co-authors for discovery and quantitative study of causal connections in signaling and gene networks (Bruggeman et al., 2002; Kholodenko et al., 2002). The method was designed to analyse experimental measurements on a set of network components. A single component of the network is perturbed (for instance by a chemical inhibitor, siRNA, etc.) and changes in the states of all component within the set (global response coefficients) are measured in response to this perturbation. Global response coefficients are measured for perturbations of all components within the set and subjected to mathematical analysis. The outcome is a map of connections and values of connection strength between signaling molecules within the studied set. The method reveals both direct connections between the measured molecules and indirect connections mediated by network components which were not included into the measured set (Kholodenko et al., 2002).

MRA was recently applied to reveal signaling connections between Raf (Raf-1 and B-Raf), Mek1/2 and Erk1/2 in the pathway activated by NGF or EGF in PC12 cells (Santos et al., 2007). Santos and co-authors demonstrate the existence of a negative feedback loop from Erk to Raf under EGF stimulation. By contrast, the Erk-Raf feedback loop is positive in the

case of NGF stimulation (Kholodenko, 2007; Santos et al., 2007). Furthermore MRA revealed temporal changes in the strength of signaling connections between Raf, Mek and Erk, i.e. this method of analysis provides a new approach for the study of signal dynamics within the network. Based on the computed map of signaling connections the authors predicted a switch-like response of Erk1/2 activation to the change of NGF concentration. This prediction was experimentally verified by means of flow cytometry (Santos et al., 2007).

MRA has a potential to become an extremely important method for experimental cell biology. It provides a unique opportunity for studying connectivity and remodelling of signaling networks under different physiological and pathological conditions. Rapid development of the methods for systematic gene perturbation (Bjorklund et al., 2006) and large-scale monitoring of site-specific protein phosphorylation (Wolf-Yadlin et al., 2007) suggests that MRA may soon be applied to large signaling networks. This would provide enormous amount of novel information on cell function and pathology.

Bayesian network approach uses probabilistic analysis of correlation between activation states of network components to infer causal connections within the network (Pe'er, 2005; Sachs et al., 2002; Sachs et al., 2005). Similarly to MRA Bayesian network analysis can be performed on experimental measurements of activation states of multiple network components subjected to activatory or inhibitory perturbations. Sachs and co-authors performed flow cytometry measurements of 11 phosphorylated signaling proteins and phospholipids in T cells under various conditions including stimulation of 3 types of cell surface receptors and pharmacological perturbation of 7 network components (Sachs et al., 2005). The data sets were analyzed with the Bayesian network structure inference algorithm and the majority of inferred causal connections were in agreement with the currently accepted consensus network topology. Bayesian analysis requires high numbers of individual measurements for accurate retrieval of network connections (Sachs et al., 2005). This may become a limitation for applying Bayesian network algorithms to experimental measurements obtained by Western blot and other methods that use signal averaging over the whole cell population.

Spatial Quantification of Signaling Networks

The majority of cell functions are highly compartmentalized so that concentration and activity of the same signaling molecule may be regulated in very different ways depending on subcellular location. At the same time most of the current techniques for measuring signaling activities provide average values over the whole cell volume or over large cell populations (please see the section “Activity measurements on multiple network components” of this chapter). A much more promising technique for adequate monitoring of signaling networks is quantification of subcellular distribution of activated signaling molecules. Confocal microscopy makes it possible to localize activation sites at sub-micrometer resolution. Therefore local intensity peaks are highly contrasted against background leading to high signal-to-noise ratio. This is a very important advantage as compared to whole-cell averaging of a signal in flow cytometry and averaging over a large number of cells in Western blot analysis. Current advance in technical opportunities for imaging and image analysis is in

sharp contrast with very limited steps that have been taken so far in this direction in cell biology.

Every signaling protein executes its function by communicating to signaling partners on sub-micrometer scales. These small volume events are then orchestrated on 10-50μm scale (a eukaryotic cell size) to compose a whole-cell physiological response. Therefore local measurements of signaling activities should be incorporated into a whole-cell model of signal propagation. An exciting study in this direction was made by Smith and co-authors (Smith et al., 2002) revealing crucial steps of the Ran protein transport between cytoplasm and nucleus. Development of high throughput microscopy techniques and corresponding analysis methods will probably have a major influence on cell biology in the coming decades (Schubert et al., 2006; Starkuviene and Pepperkok, 2007).

This approach is not limited to any narrow field of cell biology (a particular signaling pathway). Instead, it will inevitably expand the current scope of cell biology in general.

Conclusion

NGF-activated signaling networks provide a promising experimental model for solving the problem of monitoring and manipulating complex causal connections within a wider range of intracellular signaling networks. Revealing coordination and information exchange between multiple receptor inputs and physiological outcomes requires “model-experiment coupling” approach reinforced by high throughput experimental measurements and “biologist-friendly” tools of mathematical analysis. Boolean logics provides a reductionist approach to complex signal networking that may serve as a transition step between empirical cell biology and advanced mathematical models. Quantitative analysis of network effects should become a routine concept for cell biology experimentalists. If we want to achieve predictable manipulation of cell functions we need to understand how causal connections between parts determine behaviour of the whole.

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References

Aoki, K., Nakamura, T., Inoue, T., Meyer, T., and Matsuda, M. (2007). An essential role for the SHIP2-dependent negative feedback loop in neuritogenesis of nerve growth factor-stimulated PC12 cells. *J. Cell Biol.*, 177, 817-827.

Arkin, A. and Ross, J. (1994). Computational functions in biochemical reaction networks. *Biophys. J.*, 67, 560-578.

Bhalla, U. S. (2003). Understanding complex signaling networks through models and metaphors. *Prog. Biophys. Mol. Biol.*, 81, 45-65.

Bhalla, U. S., Ram, P. T., and Iyengar, R. (2002). MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science*, 297, 1018-1023.

Bjorklund, M., Taipale, M., Varjosalo, M., Saharinen, J., Lahdenpera, J., and Taipale, J. (2006). Identification of pathways regulating cell size and cell-cycle progression by RNAi. *Nature*, 439, 1009-1013.

Bray, D. (1995). Protein molecules as computational elements in living cells. *Nature*, 376, 307-312.

Bruggeman, F. J., Westerhoff, H. V., Hoek, J. B., and Kholodenko, B. N. (2002). Modular response analysis of cellular regulatory networks. *J. Theor. Biol.*, 218, 507-520.

Dueber, J. E., Yeh, B. J., Bhattacharyya, R. P., and Lim, W. A. (2004). Rewiring cell signaling: the logic and plasticity of eukaryotic protein circuitry. *Curr. Opin. Struct. Biol.*, 14, 690-699.

Dueber, J. E., Yeh, B. J., Chak, K., and Lim, W. A. (2003). Reprogramming control of an allosteric signaling switch through modular recombination. *Science*, 301, 1904-1908.

Guet, C. C., Elowitz, M. B., Hsing, W., and Leibler, S. (2002). Combinatorial synthesis of genetic networks. *Science*, 296, 1466-1470.

Huang, S. (1999). Gene expression profiling, genetic networks, and cellular states: an integrating concept for tumorigenesis and drug discovery. *J. Mol. Med.*, 77, 469-480.

Huang, S. and Ingber, D. E. (2000). Shape-dependent control of cell growth, differentiation, and apoptosis: switching between attractors in cell regulatory networks. *Exp. Cell Res.*, 261, 91-103.

Irish, J. M., Hovland, R., Krutzik, P. O., Perez, O. D., Bruserud, O., Gjertsen, B. T., and Nolan, G. P. (2004). Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell*, 118, 217-228.

Irish, J. M., Kotecha, N., and Nolan, G. P. (2006). Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat. Rev. Cancer*, 6, 146-155.

Ivankovic-Dikic, I., Gronroos, E., Blaukat, A., Barth, B. U., and Dikic, I. (2000). Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat. Cell Biol.*, 2, 574-581.

Janes, K. A., Albeck, J. G., Peng, L. X., Sorger, P. K., Lauffenburger, D. A., and Yaffe, M. B. (2003). A high-throughput quantitative multiplex kinase assay for monitoring information flow in signaling networks: application to sepsis-apoptosis. *Mol. Cell Proteomics.*, 2, 463-473.

Katz, P. S. and Clemens, S. (2001). Biochemical networks in nervous systems: expanding neuronal information capacity beyond voltage signals. *Trends Neurosci.*, 24, 18-25.

Kholodenko, B. N. (2007). Untangling the signalling wires. Nat.Cell Biol., 9, 247-249.

Kholodenko, B. N., Kiyatkin, A., Bruggeman, F. J., Sontag, E., Westerhoff, H. V., and Hoek, J. B. (2002). Untangling the wires: a strategy to trace functional interactions in signaling and gene networks. *Proc. Natl. Acad. Sci. USA*, 99, 12841-12846.

Kratchmarova, I., Blagoev, B., Haack-Sorensen, M., Kassem, M., and Mann, M. (2005). Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. *Science*, 308, 1472-1477.

Krutzik, P. O., Crane, J. M., Clutter, M. R., and Nolan, G. P. (2008). High-content single-cell drug screening with phosphospecific flow cytometry. *Nat. Chem. Biol.,* 4, 132-142.

Li, B. S., Zhang, L., Gu, J., Amin, N. D., and Pant, H. C. (2000). Integrin alpha(1) beta(1)-mediated activation of cyclin-dependent kinase 5 activity is involved in neurite outgrowth and human neurofilament protein H Lys-Ser-Pro tail domain phosphorylation. *J. Neurosci.,* 20, 6055-6062.

Liu, G., Li, W., Gao, X., Li, X., Jurgensen, C., Park, H. T., Shin, N. Y., Yu, J., He, M. L., Hanks, S. K., Wu, J. Y., Guan, K. L., and Rao, Y. (2007). p130CAS is required for netrin signaling and commissural axon guidance. *J. Neurosci*., 27, 957-968.

Liu, R. Y. and Snider, W. D. (2001). Different signaling pathways mediate regenerative versus developmental sensory axon growth. *J. Neurosci.,* 21, RC164.

McAdams, H. H. and Shapiro, L. (1995). Circuit simulation of genetic networks. *Science,* 269, 650-656.

Miller-Jensen, K., Janes, K. A., Brugge, J. S., and Lauffenburger, D. A. (2007). Common effector processing mediates cell-specific responses to stimuli. *Nature,* 448, 604-608.

Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005). Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol*., 6, 56-68.

Paglini, G., Pigino, G., Kunda, P., Morfini, G., Maccioni, R., Quiroga, S., Ferreira, A., and Caceres, A. (1998). Evidence for the participation of the neuron-specific CDK5 activator P35 during laminin-enhanced axonal growth. *J. Neurosci*., 18, 9858-9869.

Papin, J. A., Hunter, T., Palsson, B. O., and Subramaniam, S. (2005). Reconstruction of cellular signalling networks and analysis of their properties. *Nat. Rev. Mol. Cell Biol*., 6, 99-111.

Paveliev, M., Lume, M., Velthut, A., Phillips, M., Arumae, U., and Saarma, M. (2007). Neurotrophic factors switch between two signaling pathways that trigger axonal growth. *J. Cell Sci*., 120, 2507-2516.

Pe'er, D. (2005). Bayesian network analysis of signaling networks: a primer. *Sci. STKE*., 2005, l4.

Pflieger, D., Junger, M. A., Muller, M., Rinner, O., Lee, H., Gehrig, P. M., Gstaiger, M., and Aebersold, R. (2008). Quantitative proteomic analysis of protein complexes: concurrent identification of interactors and their state of phosphorylation. *Mol. Cell Proteomics.,* 7, 326-346.

Sachs, K., Gifford, D., Jaakkola, T., Sorger, P., and Lauffenburger, D. A. (2002). Bayesian network approach to cell signaling pathway modeling. *Sci. STKE*., 2002, E38.

Sachs, K., Perez, O., Pe'er, D., Lauffenburger, D. A., and Nolan, G. P. (2005). Causal protein-signaling networks derived from multiparameter single-cell data. *Science,* 308, 523-529.

Santos, S. D., Verveer, P. J., and Bastiaens, P. I. (2007). Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. *Nat. Cell Biol.,* 9, 324-330.

Sasagawa, S., Ozaki, Y., Fujita, K., and Kuroda, S. (2005). Prediction and validation of the distinct dynamics of transient and sustained ERK activation. *Nat. Cell Biol*., 7, 365-373.

Schilling, M., Maiwald, T., Bohl, S., Kollmann, M., Kreutz, C., Timmer, J., and Klingmuller, U. (2005). Computational processing and error reduction strategies for standardized quantitative data in biological networks. *FEBS J.,* 272, 6400-6411.

Schubert, W., Bonnekoh, B., Pommer, A. J., Philipsen, L., Bockelmann, R., Malykh, Y., Gollnick, H., Friedenberger, M., Bode, M., and Dress, A. W. (2006). Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nat. Biotechnol.,* 24, 1270-1278.

Smith, A. E., Slepchenko, B. M., Schaff, J. C., Loew, L. M., and Macara, I. G. (2002). Systems analysis of Ran transport. *Science*, 295, 488-491.

Starkuviene, V. and Pepperkok, R. (2007). The potential of high-content high-throughput microscopy in drug discovery. *Br. J. Pharmacol.,* 152, 62-71.

Stork, P. J. (2005). Directing NGF's actions: it's a Rap. Nat.Cell Biol., 7, 338-339.

Sun, N. and Zhao, H. (2004). Genomic approaches in dissecting complex biological pathways. *Pharmacogenomics*., 5, 163-179.

Swameye, I., Muller, T. G., Timmer, J., Sandra, O., and Klingmuller, U. (2003). Identification of nucleocytoplasmic cycling as a remote sensor in cellular signaling by databased modeling. *Proc. Natl. Acad. Sci. USA*, 100, 1028-1033.

Tucker, B. A., Rahimtula, M., and Mearow, K. M. (2005). Integrin activation and neurotrophin signaling cooperate to enhance neurite outgrowth in sensory neurons. *J. Comp Neurol.,* 486, 267-280.

Tucker, B. A., Rahimtula, M., and Mearow, K. M. (2008). Src and FAK are key early signalling intermediates required for neurite growth in NGF-responsive adult DRG neurons. *Cell Signal*., 20, 241-257.

Vilar, J. M., Guet, C. C., and Leibler, S. (2003). Modeling network dynamics: the lac operon, a case study. *J. Cell Biol.,* 161, 471-476.

Weng, G., Bhalla, U. S., and Iyengar, R. (1999). Complexity in biological signaling systems. *Science*, 284, 92-96.

Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D. A., and White, F. M. (2007). Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc. Natl. Acad. Sci. USA*, 104, 5860-5865.

Wolkenhauer, O., Ullah, M., Wellstead, P., and Cho, K. H. (2005). The dynamic systems approach to control and regulation of intracellular networks. *FEBS Lett.*, 579, 1846-1853.

Xiang, Y., Li, Y., Zhang, Z., Cui, K., Wang, S., Yuan, X. B., Wu, C. P., Poo, M. M., and Duan, S. (2002). Nerve growth cone guidance mediated by G protein-coupled receptors. *Nat. Neurosci*., 5, 843-848.

Xie, Z., Sanada, K., Samuels, B. A., Shih, H., and Tsai, L. H. (2003). Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell*, 114, 469-482.

Xie, Z. and Tsai, L. H. (2004). Cdk5 phosphorylation of FAK regulates centrosome-associated miocrotubules and neuronal migration. *Cell Cycle*, 3, 108-110.

Zhou, F. Q., Walzer, M., Wu, Y. H., Zhou, J., Dedhar, S., and Snider, W. D. (2006). Neurotrophins support regenerative axon assembly over CSPGs by an ECM-integrin-independent mechanism. *J. Cell Sci*., 119, 2787-2796.

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Chapter 14

Nerve Growth Factor Mediated Effects in the Development of Orbitopathy Associated with Graves' Disease

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Abstract

Nerve growth factor (NGF) is not restricted to cells of neuronal origin but also extends to cells of the immune system, and to cells that neurons innervate such as muscles, glands and adipose tissue. NGF plays a role in inflammatory responses and in tissue repair via its two classes of receptors, p75 and tyrosin kinase (TrkA).

Graves' orbitopathy represents a special link between thyroid and orbital autoimmunity with a complexity of neuronal activity. The clinical symptoms of hyperthyroidism could associate - depending on catecholamine amount - with an increase of myocyte hypertrophy or apoptosis. Production of NGF could be induced by T helper - 2 activation. An aberrant apoptosis has been demonstrated in nodular goitre due to increased proteasome activity contrary to the death receptor mediated form in thyroid autoimmunity. The proper balance between NGF mediated proliferative processes and enhanced apoptosis could be demonstrated during the manifestation of Graves' orbitopathy.

The expression of NGF receptors could locally modulate the inflammatory and reparative responses. Data about the role of NGF in conjunctival and corneal epithelium integrity suggest its importance in orbitopathy. NGF displays stimulatory effects on fibroblasts and does not influence collagen production but promotes their phenotype into myofibroblasts.

NGF via its dual effect on survival and apoptosis, which could manifest in hypertrophy and degenerative damages represents a special neuronal regulatory system in Graves' orbitopathy. NGF mediated events in the development of orbitopathy associated with Graves' disease are independent of direct effects of thyroid hormones. Recombinant

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human NGF may be useful therapy in orbitopathy, which has been applied for corneal ulcers successfully.

Introduction

1. Nerve Growth Factor and Its Importance in Graves' Orbitopathy

Graves' orbitopathy represents a special link between thyroid and orbital autoimmunity and neuronal activity. Neuronal innervation extends not only to cells of immune system but also to cells of glands, muscle and adipose tissues [1]. Nerve growth factor (NGF) is a neurotrophic factor for peripheral sympathetic, sensory and cholinergic neurons although its pleiotropic effects are involved in the modulation of inflammatory and immune processes [2,3,4]. NGF is a key in the balance between survival or tissue repair and degenerative effect or apoptosis.

NGF is a member of the neurotrophin family with a widespread non-neuronal activity. NGF is a pentameric protein complex with a 130 000 molecular weight, where the β-subunit is responsible for all biological activities [5,6]. The action of NGF is mediated by two cell surface receptors: the high-affinity tyrosine kinase receptor A (TrkA) that is specific for NGF, and the low-affinity receptor, p75. The neurotropic activity of NGF is mediated only by TrkA inducing activation of MAPK (mitogen- activated protein kinase) and promoting neuronal cells survival.

NGF is synthesized in different target tissues, where its main function is to regulate the survival and differentiation of sympathetic and sensory neurons [7,8]. Orbital tissue is a highly innervated organ where NGF plays a potent effect on the development of the retina, corneal epithelium, lens, iris, ciliary bodies and optic nerves, as well as on innervation and inflammatory events with the promotion of recovery processes [9,10,11].

NGF is a trophic factor for endocrine organs such as thyroid glands [12]. Thyroid hormones exert preventing effects on neuronal development, remyelination and oligodendrocyte activation [13]. Increased levels of NGF were demonstrated in autoimmune diseases such as diabetes mellitus, Graves' disease, rhematoid arthritis, multiple sclerosis, psoriasis [14,15,16]. Elevated serum levels of NGF could be detected in hyperthyroid Graves' disease, but NGF levels also were modestly high in hypothyroid form [17]. However, Graves' patients with orbitopathy showed lower detectable serum NGF than those who have no eye signs. NGF is present in the mammotroph cells of the anterior pituitary and cosecrets with prolactin via a dopamine-regulated mechanism highlighting their roles in stressful conditions [18].

The main cell sources of NGF levels represent the immune system including mast cells, eosinophils, lymphocytes, monocytes, macrophages as well as structural cells such as fibroblast, epithelial cells, smooth muscle cells, keratinocytes and adipocytes [19,20,21,22,23,24,25,26,27]. These cells are able to produce and secret NGF, which positively acts on its receptor expressions leading to the activation of the cells themselves. NGF is an important activator factor for B and T lymphocytes, mast cells, fibroblast and adipocytes, which play a central role in the development of Graves' orbitopathy with the

cosecretion of different cytokines [28,29,30,31,32,33,34,35]. These cytokines possess inflammatory (IL-1, IL-6, IFN-γ,TNF-α) or inhibitory (IL-4, IL-10) effects on autoimmune processes but the secretion and activating effect of NGF are also modulated by them [20,24,36,37,38,39,40,41].

The sympathetic nervous system plays a central role in the lipolysis and secretion of leptin and inflammatory response proteins in white adipose tissue (WAT) [24]. NGF is expressed not only in white but also in brown adipose tissue showing its implication in the thermogenesis and obesity [42]. The evidence for NGF synthesis in WAT is locally associated with cutaneous wound healing and atherosclerotic lesions [43]. Adipokines are various proteins such as leptin, adiponectin, plasminogen activator inhibitor-1, haptoglobin, metallothionen, and classical cytokines and growth factors (TNF-α, IL-6, transforming growth factor-β) which are secreted from WAT. This date confirms, that adipose tissue is an active endocrine organ. Besides an increase of adipose tissue, obesity nowadays represents a chronic stimulation of WAT via elevated levels of inflammatory cytokines. This chronic systemic WAT activation leads to atherosclerosis, metabolic abnormalities such as diabetes, insulin resistance, dyslipidemia and hypertension [44]. The secretion and neurotropic effect of NGF are associated with the inflammatory responses of WAT which may induce orbitopathy and/or pretibial myxedema in Graves' disease.

1.1. Nerve Growth Factor as a Coordinator for Cell Survival and Apoptosis in the Thyroid Gland and Orbital Tissues

The control of cell survival and death by NGF is mediated by two transmembrane glycoproteins, the TrkA and p75 neurotrophin receptors. p75 is a member of the TNF (tumor necrosis factor) cytokine receptor superfamily. Expression of p75 in sympathoadrenal cells increases the expression of TrkA and enhances NGF regulated differentiation. Both p75 and TrkA receptors could express on the orbital tissues involved in Graves' orbitopathy [9,10,11]. Competitive signaling between TrkA and p75 NGF receptors was demonstrated in the cell survival, but the activation of p75 may initiate the cell death alone [45,46]. The apoptosis of sympathetic neuron death is mediated by the p75 receptor, while the TrkA receptor is necessary for the neuron survival [47]. Antibodies against Trk receptors delay disease progression in neuromuscular degeneration [48]. Vascular smooth muscle cell apoptosis is mediated by p75 activation besides reduced Trk activation [27].

Catecholamines have complex effects on the target cells (myocyte, adipocyte, thyrocyte, fibroblast) including growth, survival and the ability of triggering apoptosis [49,50,51]. Catecholamines have distinct effects on cell survival [Figure 1]. However, low catecholamine concentration may protect the cells against apoptosis through a coupling between cAMP pathway and MAPK activation [52]. The vertebrate inner ear like orbital tissue is characterized by ectoderm originate and expresses NGF receptors. IGF-1 (insulin-like growth factor-1) and NGF are both growth factors which are involved in inner ear development [53]. In this network, IGF-1 and NGF represent a balance between the cell death and the activation of survival where IGF-1 may block the activation of the p75 death receptor.

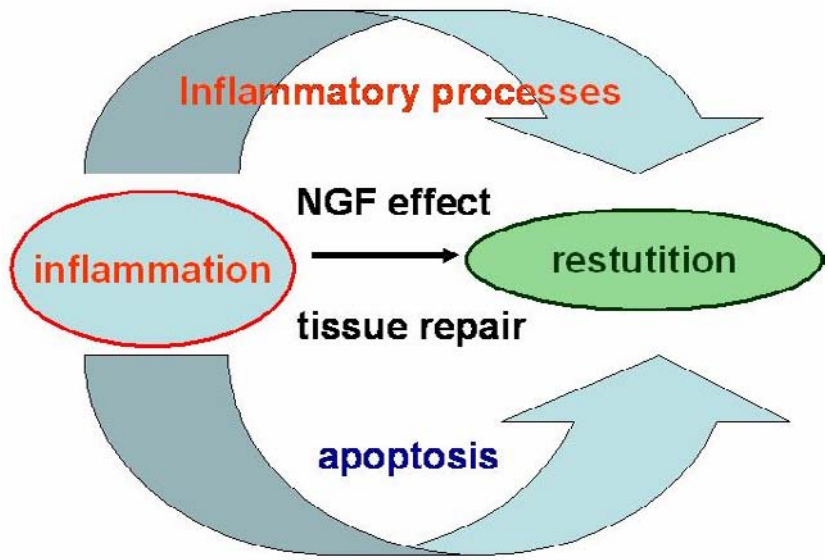

Figure 1. Nerve growth factor (NGF) plays an active role in the inflammation and restutition.

Normal thyroid cells are resistant to Fas-mediated apoptosis, the inhibition of which could be broken by the sensitization of cytokines (IFNγ and IL-1β) [54,55]. MAPK activation was proven which may be responsible for the resistance of normal thyroid cells to Fas-mediated apoptosis [56]. Nongenomic actions of thyroid hormones could exert anti-apoptotic effects via activation of MAPK [57]. NGF also enhances the survival, phagocytosis, and superoxide production of neutrophils [58].

1.2. Nerve Growth Factor Released from Cells that are Involved in Immune Inflammatory Processes of Orbitopathy

NGF is not restricted to cells of neuronal origin: it also extends to cells of immune system. These include mast cells, basophils, eosinophils, monocytes, lymphocytes and fibroblasts, endothels and vascular smooth muslce cells [19,21,25,26,27]. NGF displays stimulatory effects on human skin, lung, and orbital fibroblasts [22,39,41]. Macrophage-derived cytokines IL-1β, TNFα, IL-10 are increased in orbital adipose tissue highlithing the presence of macrophage activation in orbitopathy [34]. NGF receptor bearing mast cells are responsible for the augmentation of allergic reactions that are frequently associated with Graves' orbitopathy. Prostaglandins realease from adipocytes and mast cells lead to the secretion of proinflammatory cytokines and chemoattractants, the amounts of which are modulated by NGF [20,23,24,38]. However, the synthesis of prostaglandins is influenced by T helper type 1 and 2 cytokines production [31]. Cytokine IL-1β and immunoglobulins from Graves' patients are capable to induce hyaluronan synthesis in orbital fibroblasts while IL-1β markedly may contribute to NGF secretion of fibroblasts [39,41,59]. Recent results suggest the pathogenic role of the IGF-1 receptor via anti-IGF-1 antibodies in the orbital fibroblasts activation [30,32]. High-affinity TrkA receptors are present on the human ocular surface suggesting the role of NGF in the ocular inflammation and corneal epithelial proliferation and differentiation [9]. 80 % of T helper lymphocytes (CD4+) express TrkA receptors in the

ocular tissues. Vernal keratoconjunctivitis (VKC) is characterized by the infiltration of lymphocytes (mostly Th2), eosinophils and mast cells. NGF displays a profibrogenic activity during wound-healing and tissue-repair. Increased NGF expression with both p75 and TrkA receptors could be demonstrated in patients with VKC in comparison with those in healthy persons [41]. NGF induces differentiation from conjunctival fibroblast to myofibroblast and modulates collagen production. Experimental data showed in rabbits that an increase in NGF levels contributes to high intraocular pressure [11]. Increased production of NGF via inflammatory, allergic or autoimmune events could link to the senzitisation of sensory neurons eliciting hyperalgesia in the affected region [8].

NGF receptors are present on T and B lymphocytes and are involved in the autoimmune processes. TrkA receptors are expressed after T cell activation mediated by antigens or APC (antigen presenting cells) [46,60]. NGF secretion is supported by T helper 2 cytokines, the dominance of which is present in orbitopathy [59,61].

1.3. Nerve Growth Factor and Sympathicotonia Result in Hypertrophy of the Target Tissues

The clinical symptoms of hyperthyroidism are associated with an increase of catecholamine which is a central mediator of sympathetic innervation. NGF as a trophic factor of sympathetic neurons is synthesized and secreted in the target tissues (e.g. adipose, thyroid, orbital, muscle tissues) influencing the density of their sympathetic innervation, the expression of adrenoreceptors, and the balance between survival and apoptosis [62]. An increase of catecholamine is characterized by physiological stress which may cause damage to muscle cells and adipocytes via inducing insulin resistance. Inflammatory immune responses via elevated levels of IL-1β, IL-6 and TNFα lead to that similar result: an impairment of insulin signal transduction through decreasing glucose uptake [63,64,65]. NGF may induce apoptosis of sympathetic neurons via its p75 receptors but that action may be abolished by the activation of TrkA receptors. Chronic stimulation of β-adrenergic receptors may associate either with myocyte hypertrophy or with an increase of apoptosis through the mitochondrial death pathway [49,50,66]. Myocyte contractility modulates both the catecholamine cytotoxicity as well as the cytoprotective effect of NGF. NGF suppresses β-adrenergic receptor induced apoptosis with proposing hypertrophy [Figure 2]. NGF possesses an angiogenetic effect through the stimulation of vascular endothelial growth factor (VEGF), which is involved in stromal hypertrophy [26,27]. Peripheral nerve injury induces an increase in α-adrenergic receptor expression [67]. Catecholamines through α-adrenergic receptor stimulations, which are directly trophic for vascular wall could contribute to hypertrophy and severity of atherosclerosis [68]. Apoptosis of multinucleated muscle fibers may be different from that seen in mononucleated cells, and depending on the presence of muscle denervation, dystrophin-deficiency [69]. Apoptosis of multinucleated muslce cells is characterized by sarcoplasmic segment degradation, which does not contribute to immediated loss of the whole muscle fibers but results in the long-time process of muscle fiber atrophy (after denervation, in primary myopathies). Cell death mechanism in inflammatory myopathies is likely to be necrosis and not apoptosis. Both pathological pathways of muscle cell death may explain the different outcome of orbitopathy in Graves' disease.

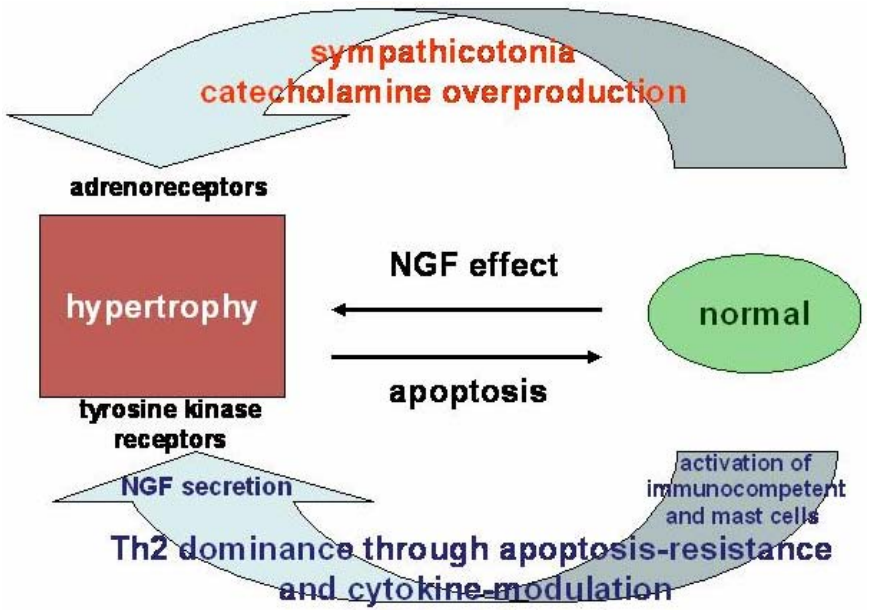

Figure 2. Nerve growth factor (NGF) is involved in hypertrophy.

1.4. Development of Th2 Dominance Stimulates the Chronic Inflammatory Processes in Graves' Orbitopathy

NGF exerts specific effects on immune functions. Trk receptors are present on T and B cells [19,46]. NGF promotes growth and differentiation of T and B lymphocytes similar to those of other myeloid progenitor cells. The antigen stimulation increases the expression of functional Trk receptors where the high affinity receptor alone is sufficient to mediate biologic activity of NGF on T and B lymphocytes [47,70,71]. The ablation of TrkA function in the immune system is associated with elevated serum levels of certain immunoglobulin classes. The predominance of Th2 cells, which is characterized by allergic diseases is promoted by NGF and by the different affection of T helper cells through activation-induced apoptosis [72,73]. Circulating memory/effector Th1 cells are selectively affected in the activation-induced apoptosis which fact promotes the immune balance toward surviving Th2 cells [74]. Th2 dominance is explained not only by Th1 cell apoptosis-susceptibility but also by Th2 cell apotosis-resistance induced through the phosphatidylinositol 3'-kinase (PI3-kinase) signaling pathway [75]. Th2 derived cytokines, IL-4, IL-5 exert inhibiting effects on Fas-mediated apoptosis [76]. IL-10 cytokine increases and IFNγ inhibits the secretion of NGF [59]. A similar switch in the balance between Th1 and Th2 cells toward Th2 cells could be demonstrated by NGF treatment in experimental allergic encephalomyelitis [77]. Immune, inflammatory and mast cells could contribute to Th2 switching directly in a cell receptor mediated manner in stress, catecholamine overproduction and allergic responses [78,79,80].

1.5. Thyroid Hormones and NGF

Thyroid homones play a crucial role in cell and neuronal differentiation, growth and metabolism. Thyroid hormones exert a modulating effect on lymphocyte activity via the

protein kinase C signaling pathway [81]. Higher T and B cell proliferation and activation are present in hyperthyroidism in comparison with hypothyroidism [78,82]. Increased serum NGF levels associated with hyperthyroidism in Graves' disease but the levels of NGF were lower in patients with orbitopathy compared to those who had no eye symptoms [17]. Nongenomic effects of thyroid hormones are regulated by PI3-kinase/Akt pathway similar to those are involved in NGF action [83].

Conclusion

2. Pathomechanism of Graves' Orbitopathy with the Interactions of Nerve Growth Factor Mediated Effects

Graves' disease is an autoimmune thyroid disorder in which the thyroid glands became infiltrated by lymphocytes and activated by autothyroid antibodies directed against the TSH receptor. TSH receptor activation leads to an increase of thyroid hormones and thyroid glands enlargement. The mononuclear cell infiltration of orbital and pretibial connective tissues is characterized by extrathyroideal manifestations of Graves' disease. Immunocompetent cells synthesize inflammatory mediators such as cytokines, chemoattractants, which activate fibroblasts, the critical initiating elements of the inflammatory responses [30,31,34,35]. NGF is a new neuronal factor in the immune inflammatory responses, which synthesizes locally, and exerts its special effect on fibroblasts as well as other immunocompetent cells. Graves' orbitopathy represents a link between thyroid and orbital autoimmunity with a complexity of neuronal activity. There are clinical signs which could not be explained simply by thyroid autoimmunity in Graves' orbitopathy: 1. Thyroid gland enlargement by failed TSH receptor antibodies, 2. Extraocular muscle enlargement, 3. Orbitopathy associating with Hashimoto's thyroiditis, 4. The different or failed manifestation of orbitopathy in Graves' disease. The recent data suggests the following hypothetical explanation of the disease complexity based on autoimmune, hormonal and neuronal interactions that seem to be involved in the pathomechanism of Graves'orbitopathy [Figure 3]. These interactions stress four important processes, which are dominantly present in Graves' orbitopathy: a/ Inflammation and immune responses, b/ hypertrophy, c/ Th2 dominance, d/ apoptosis. Thyroid autoimmunity results in an increase of autoantibodies, such as TSH receptor antibody associated with elevated levels of thyroid hormones and the stimulation of sympathoadrenal activity with elevated catecholamine production. However, other factors - like chronical stress, iodine excess, allergic responses, infections - could also initiate enormous sympathoadrenal activity. Catecholamines stimulate the density of sympathetic innervation, the synthesis of NGF and induce insulin resistance in adipose, skeletal muscle tissues. Chronic stimulation of catecholamine could contribute to the development of hypertrophy through increased NGF production besides of the catecholamines' exerted effect on apoptosis induction. NGF neurotrop factor acts as an inhibitor on β-adrenergic receptor induced apoptosis proposing hypertrophy. Locally increased production of NGF in Graves'orbitopathy is involved in inflammatory events of thyroid glands and orbital tissue - such as in adipose and eye muscle tissues - via fibroblast, adipocyte, myocyte and immunocompetent cell activation. This new

data revealed that NGF may play a concommitant role in the orbital and thyroid inflammatory events. NGF could modulate cytokine effects and act toward Th2 dominance. The importance of Th2 dominance seems to be crucial in Graves' orbitopathy. Th2 dominance exerts modulating effect on cell apoptosis and shifts the balance toward cell survival by suppressing apoptosis. NGF activity on target cells also promotes these processes via Trk receptors contributing to cell survival. Th2 dominance could be respected as stimulating factor for hypertrophy and inflammatory events.

The orbital (or adipose tissue) manifestation in Graves' disease seems to be depending on the expression of NGF receptors. Ocular tissues express both NGF receptors after cytokine stimulation of inflammatory processes. It is a very interesting fact that the tissues of the ocular symptoms based on NOSPECT express both NGF receptors [84]. The low levels of NGF in hyperthyreoid Graves' orbitopathy compared to those who had no eye symptoms may have resulted from increased binding of NGF to their receptors. The presence of the p75 NGF receptor could be respected as the damage inducing factor.

In conclusion, although the pathomechanism of Graves' orbitopathy has not been yet revealed exactly, the recent data suggests the crucial role of neuronal activity via NGF trophic factor in thyroid autoimmunity. The concommitant effect of NGF is confirmed in immune inflammatory events. The interactions of NGF with catecholamines promote better understanding of tissue hypertrophy and contribute to impaired insulin action and suppression of apoptosis. Th2 dominance, which is promoted by catecholamines, cytokines and NGF is responsible for the maintaince of immune inflammatory events.

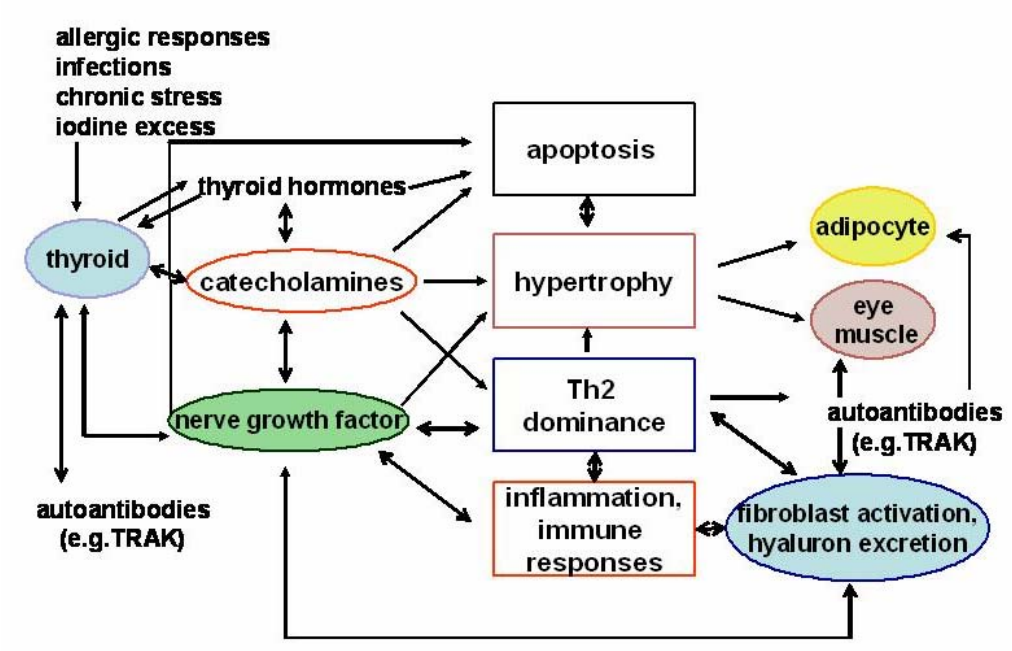

Figure 3. Graves' orbitopathy is characterized by a complexity of immune inflammatory, hormonal, and sympathoadrenal actions.

3. Neuronal Damages in Orbitopathy Give a New Challange in the Therapy

3.1. Pentoxifyllin Efficancy in the Neuro-Immune Inflammatory Processes of Orbitopathy

Pentoxifyllin, a methylxanthine derivate is a nonspecific phosphodiesterase inhibitor that downregulates several proinflammatory cytokines including TNFα, IL-1β. Pentoxifyllin has other functions which were used clinically to improve erythrocyte deformability and capillary circulation and inhibition of platelet adherence [85]. The efficancy of pentoxifyllin has been proven in Graves' orbitopathy in our previous study [86]. We demonstrated its blocking effect on serum TNFα levels and the production of glucosaminoglycan of human retroorbital tissue cultures. This drug was also able to inhibit the expression of HLA-DR molecules of retroorbital tissue. Much data has already established that pentoxifyllin could act as an inhibitor agens in inflammatory processes and as a protectiv agens in endotoxin-induced diseases [87]. Pentoxifyllin via dowregulating TNFα, IL-1β may result in a decrease of NGF levels, which contributes not only to the inhibition of inflammatory events, but also blocks NGF effect on cell hypertrophy, survival and the balance toward Th2 dominance.

3.2. Steroid Effects on the Neuro-Immune Inflammatory Processes of Orbitopathy

Corticosteroids are used as a widespread applied treatment in active orbitopathy. Their antiinflammatory effects are also involved in neural and immune inflammatory processes. Corticosteroids could induce a decrease in both the fibroblasts produced and cytokine-stimulated NGF secretions [39,88]. Corticosteroids inhibit IL-1β and TNFα stimulated NGF secretions but they also inhibit other proinflammatory factors (IL-6, IL-8, IL-11).

3.3. The Possibility for the Local NGF Therapy in Orbitopathy

Recently recombinant human nerve growth factor (rhNGF) are available for clinical treatment of inflammatory and neurodegenerative diseases [89,90]. Systemic therapy of NGF is not recommended because of its hypertrophy inducing effects but its local administration in ocular diseases seems to be effective. Corneal ulcers after impairment of sensory innervation were treated by exogenous NGF [91,92]. The preliminary, uncontrolled study suggested that NGF could improve the corneal ulcers through direct action on the epithelium and induction healing with the restoration of corneal sensitivity. The exogenous applied rhNGF had no local or systemic side effects.

In summary, NGF represents a new factor in the pathomechanism of Graves' orbitopathy. NGF via its dual effect on survival and apoptosis involved in hypertrophy and degenerative damages NGF plays a crucial role in the immune inflammatory responses. This data indicates that the development of Graves' orbitopathy is characterized by a complexity of immune inflammatory, hormonal, and sympathoadrenal actions.

References

[1] Heumann, R: Regulation of the synthesis of nerve growth factor. *J. Exp. Biol*. 1987; 132:133-150.

[2] Päth, G.;Braun, E; Meents, N; Kerzel, S; Quarcoo, D; Raap, U; Hoyle, GW; Nockher, WA; Renz, H. Augmentation of allergic early-phase reaction by nerve growth factor. *Am. J. Respir. Crit. Care Med*, 2002; 166:818-826.

[3] Sanico, AM; Stanisz, AM; Gleeson, TD; Bora, S; Proud, D; Bienenstock, J; Koliatsos, VE; Togias, A. Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. *Am. J. Respir. Crit. Care Med.* 2000; 161:1631-1635.

[4] Kyriakis, J; Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev*. 2001; 81:807-869.

[5] Reichardt, LF. Neurotrophin-regulated signalling pathways. *Phil. Trans R. Soc. B* 2006; 361:1545-1564.

[6] Bibel, M; Barbel, Y. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes and Development* 2000; 14:2919-2937.

[7] Mendell, LM; Albers, KM; Davis, BM. Neurotrophins, nociceptors, and pain. *Microsc. Res. Tech*. 1999; 45:252-261.

[8] Shu, XQ; Mendell, LM. Neurotrophins and hyperalgesia. *Proc. Natl. Acad. Sci. USA* 1999; 96: 7693-7696.

[9] Lambiase, A; Bonini, S; Micera, A; Rama, P; Bonini, S; Aloe, L. Expression of nerve growth factor receptors on the ocular surface in healthy subjects and during manifestation of inlfammatory diseases. *Invest Ophthalmol. Vis. Sci.* 1998; 39:1272-1275.

[10] Lambiase, A; Manni, L; Bonini, S; Rama, P; Micera, A; Aloe, L. Nerve growth factor promotes corneal healing: Structural, biochmical, and molecular analyses of rat and human corneas. *Invest Ophthalmol. Vis. Sci.* 2000; 41:1063-1069.

[11] Lambiase, A; Bonini, S; Manni, L; Gbinelli, E; Tirassa, P; Rama, P; Aloe, L. Intraocular production and release of nerve growth factor after iridectomy. *Invest Ophthalmol. Vis. Sci.* 2002; 43:2334-2340.

[12] Dicou, E; Lee, J; Brachet, P. Synthesis of nerve growth factor mRNA and precursor protein in the thyroid and parathyroid glands of the rat. *Proc. Natl. Acad. Sci. USA* 1986; 83:7084-7088.

[13] Fernandez, M; Guiliani, A; Pirondi, S; D'Intino, G; Giardino, L, Aloe, L, Levi-Montalcini, R; Calza, L. Thyroid hormone administration enhances remyelination in chronic demyelinating inflammatory disease. *Proc. Natl. Acad. Sci. USA* 2004; 101:16363-16368.

[14] Aloe, L; Skaper, SD; Leon, A; Levi-Montalcini, R. Nerve growth factor and autoimmune diseases. *Autoimmunity* 1994; 19:141-150.

[15] Pincelli, C. Nerve growth factor and keratinocytes: a role in psoriasis. *Eur. J. Dermatol*. 2000; 10:85-90.

[16] Calzá, L; Giardino, L; Pozza, M; Micera, A; Aloe, L. Time-course changes of nerve growth factor, corticotropin-releasing hormone, and nitric oxide synthase isoforms and

their possible role in the development of inflammatory response in experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA* 1997; 94:3368-3373.

[17] Molnár, I; Bokk, Á. Decreased nerve growth factor levels in hyperthyroid Graves' ophthalmopathy highlighting the role of neuroprotective factor in autoimmune thyroid diseases. *Cytokine* 2006; 35:109-114.

[18] Missale, C; Boroni, F; Sigala, S; Buriani, A; Fabris, M; Leon, A; Toso, RD; Spano, PF. Nerve growth factor in the anterior pituitary: Localization in mammotroph cells and cosecretion with prolactin by a dopamine-regulated mechanism. *Proc. Natl. Acad. Sci. USA* 1996; 93:4240-4245.

[19] Labouyrie, E; Dubus, P; Groppi, A; Mahon, FX; Ferrer, J; Parrens, M; Reiffers, J; de Mascarel, A; Merlio, JP. Expression of neurotrophins and their receptors in human bone marrow. *Am. J. Pathol.* 1999; 154:405-415.

[20] Marshall, JS; Gomi, K; Blennerhassett, MG; Bienenstock, J. Nerve growth factor modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism. *J. Immunol.* 1999; 162:42714276.

[21] Höglund, CO; de Blay, F; Oster, JP; Duvernelle, C; Kassel, O; Pauli, G; Frossard, N. Nerve growth factor levels and localisation in human asthmatic bronchi. *Eur. Respir. J.* 2002; 20:1110-1116.

[22] Micera, A; Vigneti, E; Pickholtz, D; Reich, R; Pappo, O; Bonini, S; Maquart, FX; Aloe, L; Levi-Schaffer, F. Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts, demonstrating a direct role for this factor in tissue repair. *Proc. Natl. Acad. Sci. USA* 2001; 98:6162-6167.

[23] Bulló, M; Peeraully, MR; Trayhurn, P. Stimulation of NGF expression and secretion in 3T3-L1 adipocytes by prostaglandins PGD_2, PGJ_2, and Δ^{12}-PGJ_2. *Am. J. Physiol. Endocrinol. Metab*. 2005; 289: E62-E67.

[24] Peeraully, MR; Jenkins, JR; Trayhurn, P. NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines. *Am. J. Physiol. Endocrinol. Metab.* 2004; 287:E331-E339.

[25] Kobayashi, H; Gleich, GJ; Butterfield, JH; Kita, H. Human eosinophils produce neurotrophins and secrete nerve growth factor on immunologic stimuli. *Blood* 2002; 99:2214-2220.

[26] Emanueli, C; Salis, MB; Pinna, A; Graiani, G; Manni, L; Madeddu, P. Nerve growth factor promotes angiogenesis and arteriogenesis in ischemic hindlimbs. *Circulation* 2002; 106:2257-2262.

[27] Wang, S; Bray, P; McCaffrey, T; March, K; Hempstead, BL; Kraemer. R. p75(NTR) mediates neurotrophin-induced apoptosis of vascular smooth muscle cells. *Am. J. Pathol.* 2000; 157:1247-1258.

[28] Han, R; Smith, TJ. Induction by IL-1β of tissue inhibitor of metalloproteinase-1 in human orbital fibroblasts: Modulation of gene promoter activity by IL-4 and INF-γ. *J. Immunol.* 2005; 174:3072-3079.

[29] Valyasevi, RW; Harteneck, DA; Dutton, CM; Bahn, RS. Stimulation of adipogenesis, peroxisome proliferator-activated receptor-γ (PPARγ), and thyrotropin receptor by PPARγ agonist in human orbital preadipocyte fibroblasts. *J. Clin. Endocrinol. Metab.* 2002; 87:2352-2358.

[30] Pritchard, J; Han, R; Horst, N; Cruikshank, WW; Smith, TJ. Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor 1 receptor pathway. *J. Immunol.* 2003; 170:6348-6354.

[31] Han R; Smith, TJ. T helper type 1 and type 2 cytokines exert divergent influence on the induction of prostaglandin E_2 and hyaluronan synthesis by interleukin-1β in orbital fibroblasts: Implications for the pathogenesis of thyroid-associated ophthalmopathy. *Endocrinology* 2006; 147:13-19.

[32] Smith, TJ; Hoa, N. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor. *J. Clin. Endocrinol. Metab*. 2004; 89:5076-5080.

[33] Shih, MJ; Liao, SL; Hou, KT; Smith, TJ, Chuang, LM. Molecular pathology of Müller's muscle in Graves' ophthalmopathy. *J. Clin. Endocrinol. Metab.* 2006; 91:1159-1167.

[34] Kumar, S; Bahn, RS. Relative overexpression of macrophage-derived cytokines in orbital adipose tissue from patients with Graves' ophthalmopathy. *J. Clin. Endocrinol. Metab*. 2003; 88:4246-4250.

[35] Pritchard, J; Horst, N, Cruikshank, W; Smith, TJ. Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts. *J. Immunol.* 2002; 168:942-950.

[36] Pagès, G; Lenorman, P; L'Allemain, G; Chambard, JC; Meloche, S; Pouysségur, J. Mitogen-activated protein kinases $p42^{mapk}$ and $p44^{mapk}$ are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA* 1993; 90:8319-8323.

[37] Irie, Y; Mori, F; Keung, WM; Mizushima, Y; Wakabayashi, K. Expression of neuronal growth inhibitory factor (metallothionein-III) in the salivary gland. *Physiol. Res.* 2004; 53:719-723.

[38] Sawada, J; Itakura, A; Tanaka, A; Furusaka, T; Matsuda, H. Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Blood* 2000; 95:2052-2058.

[39] Olgart, C; Frossard, N. Human lung fibroblasts secrete nerve growth factor: effect of inlfammatory cytokines and glucocorticoids. *Eur. Respir. J.* 2001; 18:115-121.

[40] Fuenzalida, KM; Aguilera, MC; Piderit, DG; Ramos, PC; Contador, D; Quiňones, V; Rigotti, A; Bronfman, FC; Bronfman, M. Peroxisome proliferator-activated receptor gamma is a novel target of the nerve growth factor signaling pathway in PC12 cells. *J. Biochem*. 2005; 280:9604-9609.

[41] Micera, A; Lambiase, A; Stampachiacchiere, B; Sgrulletta, R; Normando, EM; Bonini, S; Bonini, S. Nerve growth factor has a modulatory role on human primary fibroblast cultures derived from vernal keratoconjunctivitis-affected conjunctiva. *Mol. Vis.* 2007; 13:981-987.

[42] Nisoli, E; Tonello, C; Benarese, M; Liberini, P; Carruba, MO. Expression of nerve growth factor in brown adipose tissue: Implications for thermogenesis and obesity. *Endocrinology* 1996; 137:495-503.

[43] Calzá, L; Giardino, L; Giuliani, A; Aloe, L; Levi-Montalcini, R. Nerve growth factor control of neuronal expression of angiogenetic and vasoactive factors. *Proc. Natl. Acad. Sci. USA* 2001; 98:4160-4165.

[44] Toni, R; Malaguti, A; Castorina, S; Roti, E; Lechan, RM. New paradigms in neuroendocrinology: Relationships between obesity, systemic inflammation and the neuroendocrine system. *J. Endocrinol. Invest* 2004; 27:182-186.

[45] Lambiase, A; Bonini,S; Micera, A; Rama, P; Bonini, S; Aloe, L. Expression of nerve growth factor receptors on the ocular surface in healthy subjects and during manifestation of inflammatory diseases. *Invest Ophthalmol. Vis. Sci.* 1998; 39:1272-1275.

[46] Ehrhard, PB; Erb, P; Graumann, U; Schmutz, B; Otten, U. Expression of functional trk tyrosine receptors after T cell activation. *J. Immunol*. 1994; 152:2705-2709.

[47] Yoon, SO; Casaccia-Bonnefil, P; Carter, B; Chao, MV. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J. Neurosci.* 1998; 18:3273-3281.

[48] Ruiz, R; Lin, J; Forgie, A; Foletti, D; Shelton, D; Rosenthal, A; Tabares, L. Treatment with trkC agonist antibodies delays disease progression in neuromuscular degeneration (nmd) mice. *Hum. Mol. Gen*. 2005; 14:1825-1837.

[49] Kuramochi, Y; Lim, CC, Guo, X, Colucci, WS; Liao, R; Sawyer, DB. Myocyte contractile activity modulates norepinephrine cytotoxicity and survival effects of neuregulin-1β. *Am. J. Physiol. Cell Physiol.* 2004; 286:C222-C229.

[50] Viguerie, N; Clement, K; Barbe, P; Courtine, M; Benis, A; Larrouy, D; Hanczar, B; Pelloux, V; Poitou, C; Khalfallah, Y; Barsh, GS; Thalamas, C; Zucker, JD; Langin, D. In vivo epinephrine- mediated regulation of gene expression in human skeletal muscle. *J. Clin. Endocrin. Metab.* 2004; 89:2000-2014.

[51] Arner, P. Editorial: Novel target genes for catecholamines in skeletal muscle. *J. Clin. Endocrinol. Metab*. 2004; 89:1998-1999.

[52] Henaff, M; Hatem, SN; Mercadier, JJ. Low catecholamine concentrations protect adult rat ventricular myocytes against apoptosis through cAMP-dependent extracellular signal-regulated kinase activation. *Mol. Pharmacol*. 2000; 58:1546-1553.

[53] Frago, LM; Caňon, S, de la Rosa, EJ; León, Y; Varela-Nieto, I. Programmed cell death in the developing inner ear is balanced by nerve growth factor and insulin-like growth factor I. *J. Cell Sci.* 2002; 116:475-486.

[54] Wang, SH; Bretz, JD, Phelps, E, Mezosi, E; Arscott, PL; Utsugi, S; Baker, JR. A unique combination of inflammatory cytokines enhances apoptosis of thyroid follicular cells and transforms nondestructive to destructive thyroiditis in experimental autoimmune thyroiditis. *J. Immunol*. 2002; 168:2470-2474.

[55] Mezosi, E; Wang, SH; Utsugi, S; Bajnok, L; Bretz, JD; Gauger, PG; Thompson, NW; Baker, JR. Interleukin-1β and tumor necrosis factor (TNF)-α sensitize human thyroid epithelial cells to TNF-related apoptosis-inducing ligand-induced apoptosis through increases in procaspase-7 and Bid, and the down-regulation of p44/42 mitogen-activated protein kinase activity. *J. Clin. Endocrinol. Metab.* 2004; 89:250-257.

[56] Mezosi, E; Wang, SH, Utsugi, S; Bajnok, L; Bretz, JD; Gauger, PG; Thompson, NW, Baker, JR. Induction and regulation of Fas-mediated apoptosis in human thyroid epithelial cells. *Mol. Endocrinol*. 2005; 19:804-811.

[57] Mezosi, E; Szabo, J; Nagy, EV; Borbely, A; Varga, E; Paragh, G; Varga, Z. Nongenomic effect of thyroid hormone on free-radical production in human polymorphonuclear leukocytes. *J. Endocrinol.* 2005; 185:121-129.

[58] Kannan, Y; Ushio, H; Koyama, H; Okada, M; Oikawa, M; Yoshihara, T; Kaneko, M; Matsuda, H. Nerve growth factor enhances survival, phagocytosis, and superoxide production of murine neutrophils. *Blood* 1991; 77:1320-1325.

[59] Brodie, C. Differential effects of Th1 and Th2 derived cytokines on NGF synthesis by mouse astrocytes. *Fed. Eur. Biochem. Soc*. 1996; 394:117-120.

[60] DeSilva, DR; Jones, EA; Favata, MF; Jaffee, BD; Magolda, RL; Trzaskos, JM; Scherle, PA. Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy. *J. Immunol.* 1998; 160:4175-4181.

[61] Villoslada, P; Hauser, SL; Bartke, I; Unger, J; Heald, N; Rosenberg, D; Cheung, SW; Mobley, WC; Fisher, S; Genain, CP. Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper cell type 1 and 2 cytokines within the central nervous system. *J. Exp. Med*. 2000; 191:1799-1806.

[62] Shelton, DL; Reichardt, LF. Expression of the β-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl. Acad. Sci. USA* 1984; 81:7951-7955.

[63] Del Aguila, LF; Claffey, KP; Kirwan, JP. TNF-α impairs insulin signaling and insulin stimulation of glucose uptake in C_2C_{12} muscle cells. *Am. J. Physiol. Endocrinol. Metab.* 1999; 276:E849-E855.

[64] Del Aguila, LF; Krishnan, RK, Ulbrecht, JS; Farrell, PA; Correll, PH; Lang, CH; Zierath, JR; Kirwan, JP. Muscle damage impairs insulin stimulation of IRS-1, PI 3-kinase, and Akt-kinase in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 2000; 279:E206-E212.

[65] Takano, R; Hisahara, S; Namikawa, K; Kiyama, H; Okano, H, Miura, M. Nerve growth factor protects oligodendrocytes from tumor necrosis factor-α-induced injury through Akt-mediated signaling mechanisms. *J. Biol. Chem*. 2000; 275:16360-16365.

[66] Iwata, M; Yoshikawa, T; Baba, A; Anzai, T; Nakamura, I; Wainai, Y; Takahashi, T; Ogawa, S. Autoimmunity against the second extracellular loop of β_1-adrenergic receptors induces β-adrenergic receptor desensitization and myocardial hypertrophy in vivo. *Circ. Res*. 2001; 88:578-586.

[67] Perl, ER. Causalgia, pathological pain, and adrenergic receptors. *Proc. Natl. Acad. Sci. USA* 1999; 96:7664-7667.

[68] Erami, C; Zhang, H; Ho, JG; French, DM; Faber, JE. α_1-adrenoreceptor stimulation directly induces growth of vascular wall in vivo. *Am. J. Physiol. Heart Circ. Physiol.* 2002; 283:H1577-H1587.

[69] Tews, DS. Role of apoptosis in myopathies. *Basic Appl. Myol.* 2003; 13:181-190.

[70] Lee, FS; Chao, MV. Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc. Natl. Acad. Sci. USA* 2001; 98:3555-3560.

[71] Bamji, SX; Majdan, M; Pozniak, CD; Belliveau, DJ; Aloyz, R; Kohn, J; Causing, CG; Miller, FD. The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J. Cell Biol.* 1998; 140:911-923.

[72] Coppola, V; Barrick CA; Southon, EA; Celeste, A; Wang, K; Chen, B; Haddad, EB; Yin, J; Nussenzweig, A; Subramaniam, A; Tessarollo, L. Ablation of TrkA function in the immune system causes B cell abnormalities. *Development* 2004; 131:5185-5195.

[73] Neumann, H; Misgeld, T; Matsumuro, K; Wekerle, H. Neurotrophins inhibit major histocampatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. *Proc. Natl. Acad. Sci. USA* 1998; 95:5779-5784.

[74] Akdis, M; Trautmann, A; Klunker, S; Daigle, I; Küçüksezer, UC; Deglmann, W; Disch, R; Blaser, K; Akdis, CA. T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells. *FASEB J.* 2003; 17:1026-1035.

[75] Varadhachary, AS; Peter, ME; Perdow, SN; Krammer, PH; Salgame, P. Selective up-regulation of phosphatidylinositol 3'-kinase activity in Th2 cells inhibits caspase-8 cleavage at the death-inducing complex: a mechanism for Th2 resistance from Fas-mediated apoptosis. *J. Immunol.* 1999; 163:4772-4779.

[76] Spinozzi, F; Agea, E; Fizzotti, M; Bassotti, G; Russano, A; Droetto, S; Bistoni, O; Grignani, F; Bertotto, A. Role of T-helper type 2 cytokines in down-modulation of Fas mRNA and receptor on the surface of activated CD4+ T cells: molecular basis for the persistence of the allergic immune response. *FASEB J.* 1998, 12:1747-1753.

[77] Calzá, L; Fernandez, M; Giuliani, A; Giardino, L. Thyroid hormone activates oligodendrocyte precursors and increases a myelin-forming protein and NGF content in the spinal cord during experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA* 2002; 99:3258-3263.

[78] Tsatsoulis, A. The role of stress in the clinical expression of thyroid autoimmunity. *Ann. NY Acad. Sci*. 2006; 1088:382-395.

[79] Elenkov, IJ; Chrousos, GP. Stress, hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann. NY Acad. Sci.* 2002; 966:290-303.

[80] Chrousos, GP. The stress response and immune function: Clinical implications. The 1999 Novera H. Spector lecture. *Ann. NY Acad. Sci.* 2000; 917: 38-67.

[81] Klecha, AJ; Genaro, AM; Gorelik, G; Arcos, MLB; Silberman, DM; Schuman, M; Garcia, SI; Pirola, C; Cremaschi, GA. Integrative study of hypothalamus-pituitary-thyroid-immune system interaction: thyroid hormone-mediated modulation of lymphocyte activity through the protein kinase C signaling pathway. *J. Endocrinol.* 2006; 189:45-55.

[82] Salmaso,C; Bagnasco, M; Montagna, PP; Brizzolara, R; Altrinetti, V; Richiusa, P; Galluzzo, A; Giordano, C. Regulation of apoptosis in endocrine autoimmunity. Insights from Hashimoto's thyroiditis and Graves' disease. *Ann. NY Acad. Sci.* 2002; 966: 496-501.

[83] Hiroi, Y; Kim, HH; Ying, H; Furuya, F; Huang, Z; Simoncini, T; Noma, K; Ueki, K; Nguyen, NH; Scanlan, TS; Moskowitz, MA; Cheng, SY; Liao, JK. Rapid nongenomic actions of thyroid hormone. *Proc. Natl. Acad. Sci. USA* 2006; 103:14104-14109.

[84] Werner, SC: Modification of classification of eye changes of Graves' disease: recommendations of the Ad Hoc Committee of American Thyroid Association. *J* Clin Endocrinol Metab 1977; 44: 203-4.

[85] Wang, W; Zolty, E; Falk, S; Basava, V; Reznikov, L; Schrier, R. Pentoxifylline protects against endotoxin-induced acute renal failure in mice. *Am. J. Physiol. Renal. Physiol.* 2006; 291:F1090-F1095.

[86] Balazs, C; Kiss, E; Vamos, A; Molnar, I; Farid, NR. Beneficial effect of pentoxifylline on thyroid associated ophthalmopathy (TAO): a pilot study. *J. Clin. Endocrinol. Metab.* 1997; 82:1999-2002.

[87] Oliveira-Junior, IS; Brunialti, MKC; Junqueira, VBC; Salomão, R. Effect of pen toxifylline on lung inflammation and gas exchange in a sepsis-induced acute lung injury model. *Braz. J. Med. Biol. Res.* 2006; 39:1455-1463.

[88] Shi, B; Rabin, SJ; Brandoli, C; Mocchetti, I. Dexamethasone induces hyertrophy of developing medial septum cholinergic neurons: Potential role of nerve growth factor, *J. Neurosci.* 1998; 18:9326-9334.

[89] Colangelo, AM; Finotti, N; Ceriani, M; Alberghina, L; Martegani, E; Aloe, L; Lenzi, L; Levi-Montalcini, R. Recombinant human nerve growth factor with a marked activity in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* 2005; 102:18658-18663.

[90] Talapatra, S; Thompson, CB. Growth Factor signaling in cell survival: Implications for cacer treatment. *J. Pharmacol. Exp. Ther.* 2001; 298:873-878.

[91] Siliprandi, R; Canella, R; Carmignoto, G. Nerve growth factor promotes functional recovery of retinal ganglion cells after ischemia. *Invest Ophthalmol. Vis. Sci.* 1993; 34:3232-3245.

[92] Lambiase, A; Rama, P; Bonini, S; Caprioglio, G; Aloe, L. Topical treatment with nerve growth factor for corneal neurotrophic ulcers. *New Engl. J. Med.* 1998; 338:1174-1180.

Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z