Opposing Roles for TrkA and p75 Neurotrophin Receptors in the Regulation of Sympathetic Neuron Growth and Target Innervation

Judith Kohn

Centre For Neuronal Survival
Department of Neurology and Neurosurgery
McGill University, Montreal
August 31, 2001

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of
Doctor of Philosophy

ABSTRACT

Neurotrophins are key regulators of neuronal survival and growth. In sympathetic neurons, NGF generates positive biological signals through TrkA, while the p75NTR transduces negative responses. Activating the p75NTR with BDNF while NGF/TrkA signals are robust does not inhibit survival *in vitro,* but p75NTR activation causes apoptosis if TrkA activation is suboptimal. These findings support opposing roles for these receptors with respect to survival, but there is less data supporting their opposing roles in the regulation of neuronal growth and target innervation. The experimental work presented in this thesis has explored this theme in sympathetic neurons.

First, I showed that a balance between the activation of TrkA and p75NTRs is necessary for achieving appropriate levels of growth and target innervation, so they act in a functionally antagonistic manner to regulate biological responses that lie beyond survival. I found that activating the p75NTR with BDNF when TrkA activation was robust, inhibited growth responses *in vitro* without affecting survival. Such regulation may occur through an inhibitory BDNF: p75NTR autocrine loop, since sympathetic neurons and their targets produce BDNF. I addressed the physiological relevance of this finding by examining innervation of a sympathetic target organ, the pineal gland, in BDNF-deficient mice. When BDNF expression is reduced or absent, this target is hyperinnervated by sympathetic fibres. Since the inhibitory effect of p75NTR is decreased when BDNF levels are low or absent, this indicates that BDNF can act through a p75NTR autocrine loop to regulate NGF-mediated growth and target innervation. These findings support the hypothesis that target innervation is regulated by a balance of positively and negatively acting neurotrophins produced by sympathetic neurons and/or their targets.

I also investigated how altering the balance of TrkA/p75NTR signals would affect NGF-mediated sympathetic neuron growth. Since the balance of signaling may vary spatially, depending on the ratio of p75NTR to TrkA expression, I determined the spatial localization of TrkA and p75NTRs by immunocytochemistry. Since I found a differential distribution, I used confocal microscopy to measure relative receptor density, and showed that TrkA was expressed at much higher levels on the cell body

relative to neurites, while the p75NTR exhibited a more even distribution. This differential expression means that the receptor ratio differs on cell bodies versus neurites; if this ratio determines which signals predominate, the receptor ratio along a neuron may influence how it responds to different spatial sources of neurotrophins in its environment.

Since TrkA and p75NTRs exhibit a functional antagonism, I used a receptor co-patching technique to address whether or not this antagonism might occur though a physical interaction. If two receptor classes form patches, this implies that they interact physically. I found that some, but not all TrkA and p75NTRs on neurites were co-patched, suggesting that some physical interaction may occur in processes only.

Finally, I overexpressed TrkA in these neurons using adenoviral approaches to test whether altering receptor ratio would increase sensitivity to NGF, and lead to enhanced biological responsiveness. Levels of NGF that were previously insufficient for survival became permissive, and also led to enhanced neurite outgrowth. This supports an inhibitory role for the p75NTR in sympathetic neuron growth, as increasing TrkA expression appears to override the p75NTRs inhibitory effect, sensitizing these neurons to NGF. The data presented here support the hypothesis of a balance between positive and negative signals being necessary to achieve appropriate levels of growth and target innervation.

RÉSUMÉ

Les neurotrophines sont des régulateurs-clés de la survie et de la croissance neuronale. Dans les neurones sympathiques, le NGF génère des signaux biologiques positifs par le biais de TrkA, tandis que p75NTR transmet des réponses négatives. Le fait d'activer p75NTR par le BDNF alors que les signaux NGF/TrkA sont robustes n'inhibe pas la survie in vitro, mais l'activation de p75NTR provoque l'apoptose si l'activation de TrkA est sub-optimale. Ces découvertes supportent des rôles opposés pour ces récepteurs en regard de la survie. Cependant, il y a moins de données supportant leur rôle opposé quant à la régulation de la croissance neuronale et l'innervation ciblée. Les travaux expérimentaux présentés dans cette thèse ont exploré ce thème au niveau des neurones sympathiques.

Premièrement, j'ai montré qu'une balance entre l'activation de TrkA et p75NTR est nécessaire pour atteindre des niveaux appropriés de croissance et d'innervation ciblée afin qu'ils agissent d'une façon antagoniste mais fonctionnelle pour réguler les réponses biologiques qui transcendent la survie. J'ai trouvé qu'en activant p75NTR avec le BDNF lorsque l'activation de TrkA était robuste, les réponses au niveau de la croissance in vitro étaient inhibées sans toutefois affecter la survie. De telles régulations peuvent survenir par le biais d'un BDNF inhibiteur: la boucle autocrine p75, puisque les neurones sympathiques et leurs cibles produisent du BDNF. J'ai abordé la signification physiologique de cette découverte en examinant l'innervation d'un organe-cible sympathique, la glande pinéale, chez des souris déficientes en BDNF. Lorsque l'expression de BDNF est réduite ou nulle, cette cible est hyper-innervée par des fibres sympathiques. Puisque l'effet inhibiteur de p75NTR est diminué lorsque les niveaux de BDNF sont bas ou nuls, ceci indique que le BDNF peut agir par le biais d'une boucle autocrine p75NTR afin de réguler la croissance médiée par le NGF ainsi que l'innervation ciblée. Ces découvertes supportent l'hypothèse voulant que l'innervation ciblée est régulée par un équilibre entre les neurotrophines agissant de façon positive et négative et qui sont produites par les neurones sympathiques et/ou leurs cibles.

J'ai aussi examiné l'effet de l'altération de l'équilibre entre les signaux TrkA/p75NTR sur la croissance neuronale sympathique médiée par le NGF. Puisque la balance de la signalisation peut varier de façon spatiale, selon le ratio de l'expression de

p75NTR et de TrkA, j'ai déterminé la localisation spatiale de TrkA et de p75NTR par immunocytochimie. Étant donné que j'ai trouvé une distribution différentielle, j'ai utilisé la microscopie confocale pour mesurer la densité relative des récepteurs et j'ai montré que TrkA était exprimé à de plus forts niveaux dans le corps cellulaire comparativement aux neurites, alors que p75NTR était distribué de façon plus égale. Cette expression différentielle signifie que le ratio des récepteurs varie au niveau du corps cellulaire comparativement aux neurites; si ce ratio détermine quel signal prédomine, le ratio des récepteurs le long d'un neurone pourrait influer sur la façon dont le neurone répond à des sources de neurotrophines provenant de différentes régions environnantes.

Puisque TrkA et p75NTR font preuve d'un antagonisme fonctionnel, j'ai utilisé une technique de "plaques" afin de déterminer si cet antagonisme pourrait survenir par le biais d'une interaction physique. Si les deux classes de récepteurs forment des "plaques", cela implique qu'ils interagissent physiquement. J'ai trouvé que certains, mais pas tous les TrkA et p75NTR sur les neurites, forment des "plaques" ensemble, ce qui suggère qu'une certaine interaction physique peut survenir uniquement pendant les processus.

Finalement, j'ai surexprimé TrkA dans ces neurones en utilisant une approche à l'aide d'adénovirus afin de tester si le fait d'altérer le ratio des récepteurs augmenterait la sensibilité au NGF et amènerait une réponse biologique rehaussée. Les niveaux de NGF qui étaient auparavant insuffisants pour la survie sont devenus permissifs et ont mené à une croissance rehaussée des neurites. Ceci supporte la notion d'un rôle inhibiteur pour p75NTR au niveau de la croissance neuronale sympathique puisque l'augmentation de l'expression de TrkA semble surmonter l'effet inhibiteur de p75NTR, sensibilisant ces neurones au NGF. Les données présentées ici supportent l'hypothèse qu'un équilibre entre les signaux positifs et négatifs est nécessaire afin d'arriver à des niveaux appropriés de croissance et d'innervation ciblée.

ACKNOWLEDGMENTS

This thesis would not have been possible without the help of many people. First and foremost, I would like to thank Dr. Freda Miller for giving me the opportunity to work in a dynamic, interesting environment that is full of great ideas, and great ideas mean great science. In your laboratory, neuroscience is definitely the focus, but at the same time, a sense of perspective is maintained so that it is also an enjoyable place to work. This is a rare combination, and I have learned a lot about neuroscience and about the logistics of running a successful laboratory. Your knowledge, creativity, energy, and enthusiasm really make a difference.

I also owe a thank-you to the members of my graduate advisory committee, Drs. David Kaplan and Tim Kennedy, for their advice, suggestions and insight regarding my PhD research. You each had a slightly different perspective on my work, so I learned much from both of you.

I would like to acknowledge the help of Dr. George Kuchel for his contributions to the early work on the pineal gland. Thanks as well to Dr. Nick Marsh for his help with the finer points of western blotting.

All the many members of the Miller lab whom I have had the pleasure of working with deserve thanks for their input as well: Bernie, Audrey, Jean, Carrie, Rahul, Ruth, Dan, Irene, Shernaz, Dave, Marta, Doron, Christine, Jasi, Greg, Hiba, Andrew G, Andrew V, Fanie, Raquel, Catherine, Josée, Jim, Mathieu, Kirmo, Mahnaz, Paul, Aviva, and Xiu Ming.

Finally, but as importantly, I wish to thank my husband Howard for his marvelous sense of humour, unending support and patience throughout my time as a graduate student. Thanks for understanding exactly what I need.

Financial assistance was provided by the Jeanne Timmins Costello and FCAR (Fonds Pour La Formation De Chercheurs Et L'Aide A La Recherche) studentships.

TABLE OF CONTENTS

CHAPTER 3 - TrkA OVEREXPRESSION ENHANCES THE ABILITY OF SYMPATHETIC NEURONS TO RESPOND TO NGF

CHAPTER 4 - GENERAL DISCUSSION

ABBREVIATIONS

BDNF	Brain-derived neurotrophic factor
ChAT	Choline acetyl transferase
CNS	Central nervous system
DBH	Dopamine beta hydroxylase
DRG	Dorsal root ganglia
EGF	Epidermal growth factor
ERK	Extracellular signal-related kinase (also referred to as MAPK)
FRAP	Fluorescence recovery after photobleaching
GFAP	Glial fibrillary acidic protein
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
JNK	c-Jun amino-terminal kinase
KCl	Potassium Chloride
kDa	Kilodalton
MAPK	Mitogen activated protein kinase (see also ERK)
MEK	MAPK kinase
mRNA	Messenger RNA
NGF	Nerve growth factor
PC12 nnr	Non-neurotrophin responsive PC12 cell
NTR	Neurotrophin
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NT-6	Neurotrophin-6
NT-7	Neurotrophin-7
NTR	Neurotrophin receptor
P75NTR	p75 neurotrophin receptor
PC12	Pheochromocytoma-12
PDGF	Platelet-derived growth factor
PDGFR-β	Platelet-derived growth factor receptor beta
PI3-K	Phosphatidylinositol 3-kinase
PNS	Peripheral nervous system
PTB	Protein tyrosine binding
SH2	Src homology 2
SCG	Superior cervical ganglion
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor1
Trk	Tropomyosin-related kinase

LIST OF FIGURES

CONTRIBUTIONS OF AUTHORS AND STATEMENT OF ORIGINALITY

Chapter 2

Kohn J, Aloyz RS, Toma JG, Haak-Frendscho M, Miller FD (1999). Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. Published in the Journal of Neuroscience 19: 5393-5408.

My contribution to this paper includes all of the work carried out for Figures 2.1 through 2.6. Dr. Mary Haak-Frendscho provided the anti-BDNF antibody used in Figures 2.2c and 2.4a, b, and c. For the experiments in Figure 2.7, I harvested the pineal glands used to generate this figure with the help of Dr. Jean Toma, while the Western blot analysis was performed by Dr. Raquel Aloyz. For Figure 2.8, Dr. Jean Toma again helped with the harvesting of the pineal glands, but I performed the experiments that were used to generate the Figure. For Figure 2.9, I harvested the pineal glands and Dr. Jean Toma and I together harvested the carotid arteries; Dr Raquel Aloyz performed the Western blot analysis. I processed all of the data and helped with the writing of this paper under the guidance of Dr. Freda Miller.

Chapter 3

Kohn J, LeSauteur L, Kaplan DR, Miller FD (2001). TrkA overexpression enhances the ability of sympathetic neurons to respond to NGF. Manuscript is being prepared for submission.

My contribution to this paper includes performing all of the experiments presented in the paper, and processing all the data obtained. Dr. Lynne LeSauteur provided the 5C3 ascites monoclonal antibody used in Figure 3.3, and Dr. David Kaplan provided anti-phosphotyrosine antibodies for some of the data that was not shown. I wrote this paper with suggestions and comments from Drs. Freda Miller and David Kaplan.

The work described in **Chapters 2** and **3** constitute **"original scholarship and an advancement of knowledge**" in the field of **neuroscience**.

RATIONALE

The neurotrophin family of growth factors and their receptors play key roles in the survival, growth, and differentiation of sympathetic neurons. In this population of neurons, biological responses are mediated through two different classes of receptors that are co-expressed, the TrkA and the p75NTR. Numerous studies support opposing roles for these receptors in sympathetic neuron survival, but there is less data supporting a functional antagonism between TrkA and p75NTRs with respect to growth and the achievement of appropriate target innervation. Since a balance between p75NTR and TrkA signals has previously been shown to be critical for neuronal survival, the question was whether or not such a signaling balance was also relevant to growth and innervation. The studies presented in this thesis addressed how functional interactions between these receptors determine levels of sympathetic neuron growth and target innervation in response to NGF, and how the ratio of receptor expression, and therefore predominance of signaling, can influence levels of sympathetic neuron survival and growth. This work also addressed how appropriate sympathetic target innervation is achieved, which from a more global perspective, may be extrapolated to the rest of the nervous system, since potentially similar mechanisms may play an essential role in the matching of neurons with their physiological targets.

OBJECTIVES

This thesis had 5 main objectives. The **first** of these was to determine whether activation of the p75NTR in cultured neonatal sympathetic neurons under conditions permissive for survival could negatively regulate NGF-mediated neurite outgrowth, as it does survival. The focus was on how a balance between p75NTR activation by BDNF and TrkA receptor activation by NGF could influence sympathetic neuron outgrowth *in vitro*. The **second** objective was to determine the physiological relevance of these *in vitro* findings to target innervation using a sympathetic target organ, the pineal gland, as the model system. Sympathetic neurons attempting to innervate a target will encounter target-derived BDNF, and they themselves synthesize it, but since they do not express the TrkB neurotrophin receptor, BDNF, in this context, is a specific ligand for p75NTR. This suggests that a BDNF/p75NTR autocrine loop may be one

mechanism whereby levels of target innervation are negatively regulated, or more globally, how axons approaching inappropriate targets may be repelled or inhibited from innervating that target. The **third** objective was to examine the spatial distribution of p75NTR and TrkA receptors on neonatal sympathetic neurons. I wanted to determine whether the spatial distribution differed on cell bodies versus neurites, as such a difference would mean that the p75NTR to TrkA receptor ratio also differs spatially. Since the predominance of either TrkA or p75NTR signals may depend on the ratio of these receptors, a differential ratio could affect signaling events downstream of receptor activation. This has relevance to how a sympathetic neuron might respond to different spatial sources of neurotrophins that it encounters as it grows. Since these receptors have been shown to have opposing roles, the **fourth** objective was to determine whether interactions between TrkA and p75 might be physical as well as functional. The **fifth** and final objective was to determine how altering the ratio of p75NTR to TrkA (using adenoviral approaches to overexpress TrkA) could influence the survival and growth of sympathetic neurons *in vitro*. Since these receptors have opposing roles and the ratio of receptor expression may affect the balance between p75NTR and TrkA signaling, experimentally altering the ratio in favour of TrkA might lead to enhanced NGF/TrkA-mediated biological events through an increased sensitivity to NGF. This would support the notion that a balance between positive and negative signals is required to achieve proper levels of survival, growth, and target innervation.

CHAPTER 1 - LITERATURE REVIEW

LITERATURE REVIEW

The role of this chapter is to review the literature and survey the data that relates to the relationship between TrkA and p75NTRs on sympathetic neurons, emphasizing how they interact to ensure that growth, and target innervation occur at physiologically appropriate levels. Although regulation of neuronal survival by TrkA and the p75NTR was not a main focus of the work presented in this thesis, it is also reviewed briefly, for the sake of completeness.

This chapter will first introduce the neurotrophin family of proteins and their receptors, with the aim of illustrating their roles in the survival, outgrowth, and target innervation of sympathetic neurons. Mechanisms used by neurons developmentally (and potentially, later on) to regulate growth will be reviewed. Since some of the studies presented in the first manuscript of this thesis focus on sympathetic innervation of the pineal gland, a sympathetic target organ, its structure will be reviewed. As this thesis explores the theme of interactions between TrkA and p75NTRs in the regulation of growth and survival, and they may physically interact to do so, studies of their physical interactions are presented. A receptor co-patching technique used to study physical interactions in some experiments in Chapter 3 might not be familiar to the reader, so this method of studying receptor interactions is briefly addressed. Finally, a brief review of TrkA signaling pathways in sympathetic neurons will be presented. The topics selected for the Literature Review were chosen to provide a clear introduction to the body of work carried out for this thesis, which will follow in subsequent chapters.

1) The neurotrophin family of proteins

a) Overview

During development, many cell populations require specific stimuli to maintain survival. These include growth factors, cell-cell contact, hormones or cytokines, or electrical activity. In the peripheral nervous system, survival is mediated by the neurotrophins (Barde, 1989), a group of well-conserved polypeptide growth factors which are expressed primarily in the nervous system. Although the number of identified neurotrophin molecules is limited, the actions of these structurally and functionally

similar proteins are complex, and they act in many ways, on many cell populations of the nervous system, both centrally and peripherally.

b) **Identification and characterization of NGF**

The prototypical member of the neurotrophin family of growth factors, nerve growth factor (NGF), was first discovered over 50 years ago. It was observed that sensory and sympathetic ganglia in the developing chick nervous system exhibited hypertrophic growth responses after receiving mouse sarcoma tumour transplants (Bueker, 1948; Levi Montalcini and Hamburger 1951, 1953; Levi-Montalcini et al., 1954; Levi-Montalcini, 1987). The soluble factor secreted by the tumour was later identified by Rita Levi-Montalcini and Viktor Hamburger and called NGF, due to its ability to promote nerve growth, and it was purified and characterized a decade later (Cohen, 1960; Bocchini and Angeletti, 1969). NGF exists as a 26 kDa homodimer of 13 kDa polypeptides, referred to as βNGF, and the interaction is stable, even at physiologically low concentrations of NGF (Bothwell and Shooter, 1977). Since its discovery in the 1950s, much has been learned about its biological roles.

c) **Identification and characterization of BDNF**

The second neurotrophin to be identified was brain-derived neurotrophic factor (BDNF). This neurotrophin was isolated and purified from porcine brain where it is present in very small amounts (Barde et al., 1982), and was found to stimulate growth of sensory neurons (Barde et al., 1982; Lindsay et al., 1985). Sequence analysis of BDNF indicated that it shared a high degree of structural homology with NGF (Leibrock et al., 1989), with 50% of the amino acid sequence of these neurotrophins being identical.

d) **Identification of other members of the neurotrophin family**

Since the neurotrophins are a highly conserved group of molecules, the discovery of amino acid sequence homologies between NGF and BDNF led to the isolation, purification, and characterization of the other members of the neurotrophin family. These include the mammalian neurotrophin-3 (NT-3; Hohn et al., 1990; Jones

and Reichardt, 1990, Maisonpierre et al., 1990, Rosenthal et al., 1990;), and neurotrophin 4/5 (NT-4/5; Berkmeier et al., 1991; Halböök et al., 1991;Ip et al., 1992), as well neurotrophin 6 (NT-6; Götz et al., 1994), and neurotrophin 7 from fish (NT-7; Lai et al., 1998; Nilsson et al., 1998).

2) The neurotrophin receptors

a) Overview

It is now known that the biological activities of the neurotrophins are transduced by two distinct classes of receptors: the p75NTR (reviewed in Barker, 1998; Majdan and Miller, 1999; Barrett, 2000) and the Trk receptor tyrosine kinases (reviewed in Kaplan and Stephens, 1994; Greene and Kaplan, 1995). Such biological actions include apoptosis, survival, growth, development, maintenance, and repair. The realization that two separate receptor classes mediate these biological events, however, is a relatively recent finding. Initial studies aimed at characterizing mammalian neurotrophin receptors indicated that there were two distinct NGF receptor populations on neuronal cells and on NGF responsive cell lines, based on dissociation kinetics and equilibria (Sutter et al., 1979). These findings were based on differences in both binding affinity and kinetics, and the different receptor populations were then labeled low or high affinity, or called kinetically fast or slow (Frazier et al., 1974; Sutter et al., 1979; Schecter and Bothwell, 1981; Godfrey and Shooter, 1986). Purification studies and cross-linking experiments with ^{125}I-NGF suggested that two receptor proteins of 75-80 and 130-140 kDa existed (Massagué et al, 1981; Buxser et al., 1983; Grob et al., 1983; Puma et al., 1983; Kouchalakos and Bradshaw, 1986; Marano et al., 1987). Eventually, it was determined that there were two distinct families of mammalian neurotrophin receptors, the Trk tyrosine kinase family of receptors (TrkA, TrkB and TrkC), and the p75NTR.

b) Identification and characterization of the p75NTR

The first of the neurotrophin receptor proteins to be cloned was the 75-80 kDa protein (Johnson et al., 1986, Radeke et al., 1987), which is presently referred to as the p75NTR. Based on the kinetic and affinity studies, and since its cytoplasmic domain

contained no apparent signaling motifs, p75NTR was originally believed to function as a positive regulator of TrkA activity in studies with neuronal cell lines (Benedetti et al., 1993; Ip et al., 1993b; Barker and Shooter, 1994; Verdi et al., 1994). This was based upon observations of an increase in both the number of high-affinity NGF binding sites (Hempstead et al., 1991; Mahadeo et al., 1994) and NGF-mediated TrkA activation (Barker and Shooter, 1994; Mahadeo et al., 1994) when TrkA and p75NTRs were co-expressed in transformed cells, relative to when each receptor was expressed individually. Initially, data obtained in studies using mice with targeted deletions of the p75NTR supported these findings (Lee et al., 1992), but other defects observed in these mice are not consistent with the original findings. For example, more recent evidence, both *in vivo* and *in vitro* indicates that the p75NTR can signal on its own, and depending on cellular context, the associated signaling cascades can lead to apoptosis (reviewed in Miller and Kaplan, 1998; Kaplan and Miller, 2000). The p75NTR appears to signal apoptosis in a Trk-independent manner, since its activation can induce apoptosis in sympathetic neurons whose survival was maintained by the depolarizing agent KCl (Aloyz et al., 1998; Vaillant et al., 1999). Re-examination of the p75NTR knockout mouse supports the idea that the receptor is associated with cell death in some populations of developing or injured neurons. For example, in $p75^{-/-}$ mice, the number of basal forebrain cholinergic neurons is increased (Yeo et al., 1997), programmed cell death of sympathetic neurons is significantly decreased (Bamji et al., 1998), and in motor neurons, apoptosis is decreased after axonal injury (Ferri et al., 1998).

Structural analysis of the p75NTR indicates homologies with the fas and TNFR1 receptor family. The p75NTR is classified as a member of this family despite its limited sequence similarity, due to the presence of conserved cysteine residues in a forty amino acid motif within the extracellular domains of all these proteins. These conserved cysteine residues, which are repeated two to six times, are the defining characteristic of the TNF receptor family (Banner et al., 1993). These residues, which occur in the extracellular domain of the p75NTR, are involved in neurotrophin binding as shown by deletion analysis and mutagenesis studies (Welcher et al., 1991; Yan and Chao, 1991; Baldwin et al., 1992). Several members of this family of transmembrane

proteins also contain a region of weak homology in the intracellular domain which has been designated as the "death domain" (Casaccia-Bonnnefil et al., 1998).

The p75NTR interacts with all of the mammalian members of the neurotrophin family with similar affinities (Ernfors et al., 1990; Rodriguez-Tebar et al., 1990; Hallbook et al., 1991; Squinto et al., 1991; Rodriguez-Tebar et al., 1992), as indicated by the generation of similar equilibrium binding constants in binding studies (Chao et al, 1986; Radeke et al., 1987; Rodriguez-Tebar et al., 1990, 1992). Although the neurotrophins all bind to the p75NTR, they do so at residues found at different spatial regions of the p75NTR molecule (Ryden et al., 1995), which suggests that their actions on the p75NTR differ, and may have evolved to serve specific functions (Bartlett, 2000), which are not as yet clearly defined.

c) **Expression of the p75NTR in the peripheral neurons**

The peripheral nervous system originates from a distinct group of precursor cells called the neural crest, and the p75NTR is expressed in ganglia derived from the neural crest, such as the SCG (Shecterson and Bothwell, 1992; Wetmore and Olson, 1995). Neural crest cells have been reported to express p75NTR from the beginning of their segregation from the dorsal neural tube (Stemple and Anderson, 1992). In the SCG, p75NTR mRNA is first detectable prenatally, and its expression increases postnatally as the animal matures, continuing into adulthood (Shecterson and Bothwell, 1992; Wetmore and Olson, 1995). More specifically, levels of p75 mRNA increase 5 to 10-fold between the time of birth and adulthood (Buck et al., 1987). All populations of sympathetic neurons express the p75NTR (Shecterson and Bothwell, 1992; Wetmore and Olson, 1995). In sympathetic neurons, the expression level of the p75NTR is probably regulated by the amount of NGF secreted by its target tissues. This is supported by studies that demonstrated an increase in p75NTR mRNA expression when postnatal animals and cultured sympathetic neurons were treated with increasing amounts of NGF (Miller et al., 1991; Ma et al., 1992).

In the developing nervous system, p75NTR is expressed in neurons that project their axons over long distances (Buck et al., 1987; Ernfors et al, 1988; Yan and Johnson, 1988; Large et al., 1989; von Bartheld et al., 1991). During the period of

axonal outgrowth or dendritic arborization, high levels of p75NTR are expressed by many populations of sympathetic and sensory peripheral neurons; once their neuronal processes reach their target tissues, p75NTR expression is greatly downregulated (Yan and Johnson, 1988).

It is thought that signaling by the p75NTR plays a physiological role after injury or stress, since many different neuronal populations upregulate its expression at this time (Ernfors et al., 1989; Armstrong et al., 1991; Dusart et al., 1994; Kokaia et al., 1998; Martinez-Murillo et al., 1998; Roux et al., 1999). For example, cortical neurons which do not normally express the p75NTR have been shown to express it after seizure activity, and many of these same neurons were also found to be undergoing apoptosis (Roux et al., 1999). Also, neuron-specific expression of the intracellular domain of the p75NTR led to the death of injured facial motor neurons in transgenic mice (Majdan et al., 1997).

d) **Identification and characterization of TrkA**

TrkA is a member of the Trk tyrosine kinase family of receptors. The Trk tyrosine kinase family of receptors are transmembrane proteins which contain an extracellular neurotrophin binding domain, a transmembrane portion, and an intracellular tyrosine kinase domain with intrinsic catalytic activity (Berkmeier et al., 1991; Klein et al., 1991a). Structurally, they resemble the EGF and PDGF receptor tyrosine kinases (Ullrich and Schlessinger, 1990). When NGF binds to Trk, receptor dimerization follows, which leads to autophosphorylation of specific phosphotyrosine resides within the catalytic kinase domain (Kaplan et al., 1991a, b). The activated subunits then serve as scaffold for the signal transduction events that follow (Lemmon and Schlesinger, 1994). The subsequent intracellular signaling cascades that are initiated lead to neuronal survival and growth (reviewed in Kaplan and Miller, 2000).

It is now well established that the Trk family of tyrosine kinase receptors mediates the survival of sympathetic neurons. The Trk oncogene is a protein that was first discovered in colon cancer cells (Martin-Zanca et al., 1986), and the corresponding proto-oncogene product, p140trk (TrkA) contains a tropomyosin domain linked to a receptor tyrosine kinase with intrinsic catalytic activity. TrkA was the first member of

the Trk family to be cloned (Martin-Zanca et al., 1989), and was also the first identified neurotrophin receptor that was able to mediate the biological effects of NGF (Kaplan et al., 1991a,b). Using PC12 cells, it was established that NGF was a TrkA ligand by demonstrating that NGF could phosphorylate tyrosine residues, leading to tyrosine kinase activity in these cells (Kaplan et al., 1991a, b). Furthermore, PC12 cells overexpressing TrkA displayed enhanced levels of TrkA activation and accelerated differentiation as indicated by a faster induction of neurite outgrowth (Hempstead et al., 1992). Using $TrkA^{-/-}$ mice, it was clearly demonstrated that the TrkA receptor is critical for the trophic effects of NGF. Mice with a targeted disruption of the TrkA gene exhibit a massive loss of sympathetic neurons of the SCG, as well as a decrease in the number of forebrain cholinergic neurons that project to the hippocampus and cortex (Smeyne et al., 1994). Animals carrying a deletion of either the TrkA or NGF genes basically lack a peripheral nervous system (Crowley et al., 1994; Smeyne et al., 1994). TrkA has been demonstrated to be essential for the survival of sympathetic neurons after E15.5, and for proper innervation of their distal targets (Fagan et al., 1996). In $TrkA^{-/-}$ mice, a significant deficit is apparent at E17.5, and becomes progressively worse after birth (Fagan et al 1996). Recent studies using mice with targeted deletions of both the gene coding for the proapoptotic protein BAX, and TrkA (or NGF) also support a role for TrkA (and NGF) in sensory axonal growth (Patel et al., 2000). This mouse is the ideal model to use for such studies, as TrkA (and NGF) signals are not required for survival when BAX is not expressed, so their role in growth and development could be addressed.

e) **Identification of other members of the Trk family**

Subsequent to the discovery of TrkA, the other members of the Trk family were also cloned. These include TrkB (Klein et al., 1989; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991) and TrkC (Lamballe et al., 1991). Although the different Trk receptors do have their preferred ligands, there is some cross-reactivity with the other neurotrophins, and the Trks bind these molecules with affinities similar to those observed for the p75NTR. More specifically, TrkA preferentially binds NGF, but also binds NT-3 and NT-4. It does not, however, bind BDNF (Cordon-Cardo et al., 1991;

Hempstead et al., 1991; Klein et al., 1991a). TrkB preferentially binds BDNF and also recognizes NT-3 and NT-4/5, but does not recognize NGF (Squinto et al., 1991; Soppet et al., 1991; Klein et al., 1991b, 1992; Ip et al., 1992, 1993a). Finally, TrkC's ligand of choice is NT-3 (Lamballe et al., 1991; Ip et al., 1993a).

Splice variants of the Trk receptors also exist as truncated isoforms. Truncated receptor proteins have been observed for TrkA (Barker et al., 1993), TrkB (Klein et al., 1990; Middlemas et al., 1991) and TrkC (Tsouflas et al., 1993; Valenzuela et al., 1993). These receptors lack the catalytic tyrosine kinase domain, and their exact function is unclear, but it has been suggested that they may act as dominant inhibitory proteins that prevent productive receptor dimerization in order to downregulate signaling by the intact receptor protein (Eide et al., 1996; Huang and Reichardt, 2001). Thus, they might potentially function as a "sink" by sequestering neurotrophins in order to regulate signaling of the full length receptor protein (Fryer et al., 1997). Ligand binding to truncated TrkB and TrkC isoforms can also regulate intracellular signaling pathways (Baxter et al., 1997; Hapner et al., 1998; Reichardt et al., 2001). This type of modulation is dependent upon intracellular sequences that are specific to the truncated isoforms of TrkB (Baxter et al, 1997). The activity of truncated TrkC is not necessarily eliminated, but its substrate specificity becomes altered (Guiton et al., 1995; Tsouflas et al., 1996; Meakin et al., 1997).

It has also been suggested that differential splicing of the Trk receptor mRNAs leads to the generation of proteins with differences in their extracellular domains. Such variations can affect ligand interactions, which, in turn, can affect the ability of some of the neurotrophins to activate these receptors. (Meakin et al., 1992; Clary & Reichardt, 1994; Shelton et al., 1995; Garner et al., 1996; Strohmaier et al., 1996). This affects the responsiveness of neurons to neurotrophins. For example, a TrkB isoform lacking a short amino acid sequence in its juxtamembrane domain can only be activated by BDNF, and not NT-3 or NT-4, which can also normally bind to TrkB (Strohmaier et al., 1996). Such isoforms are expressed in embryonic DRG neurons, and are thought to restrict the neuronal responsiveness of TrkB expressing neurons (Boeshore et al., 1999).

f) **Expression of Trk receptors in the sympathetic nervous system**

Trk receptor mRNA expression has been reported in both the mature and developing sympathetic ganglia. Embryonically, TrkC mRNA is detectable at E13, while TrkA mRNA is expressed a bit later, at E16-18 (Ernfors et al., 1992; Schecterson and Bothwell, 1992). Temporally, expression patterns of TrkA and TrkC mRNAs overlap with each other, with TrkC expression observed at E14.5, but not TrkA (Birren et al., 1993). At E15.5 or later, TrkA mRNA is easily detected while the expression of TrkC is decreased. Late in embryonic development, around E19.5, TrkC is no longer detectable, while TrkA expression is upregulated (Birren et al., 1993). This reciprocal pattern of expression suggested that survival of sympathetic neurons during development was supported sequentially, first, early on, by NT-3 (via TrkC), and then by NGF (via TrkA) at later stages. This was supported by initial studies of NT-3 deficient mice, in which proliferating neuroblasts underwent increased apoptotic death very early on during their formation, resulting in a loss of approximately 50% of sympathetic neurons (ElShamy et al., 1996). Unlike in mice with a targeted deletion of the NT-3 gene, mice deficient in TrkC did not show a similar defect, either embryonically or postnatally (Fagan et al., 1996). This implies that NT-3 acts through TrkA, not TrkC, to promote survival.

TrkB has not been reported to be present on sympathetic neurons (Ernfors et al., 1992; Fagan et al., 1996; Garcia-Suarez et al., 1996; Belliveau et al., 1997).

3) The role of the neurotrophins and their receptors during development of the sympathetic nervous system

a) The neurotrophic hypothesis

During the development of the sympathetic nervous system, many more neurons are generated than are actually required for appropriate levels of connectivity, thus intrinsic mechanisms are invoked that limit neuronal survival and process outgrowth. This is illustrated by the neurotrophic hypothesis, which was based upon studies of sympathetic neurons that are absolutely dependent upon NGF during the period of target competition and programmed cell death *in vivo* (Levi-Montalcini and

Booker, 1960a, b). This period begins neonatally, and extends into the first few weeks of postnatal life.

The underlying premise of this hypothesis, in its original form, is that interactions between a developing peripheral neuron and its target(s) play a vital role in neuronal survival, and by extension, growth, and differentiation (reviewed in Barde, 1989). These parameters are regulated by target-derived neurotrophic factors such as NGF (Thoenen and Barde, 1980; Levi-Montalcini, 1987). Ultimately, competition for limiting amounts of trophic factors match the number of innervating neurons to target cells (Oppenheim, 1991), since the amount of NGF synthesized by the target is limiting and is enough to maintain the survival of only a fraction of the innervating neurons (Levi-Montalcini, 1987; Thoenen and Barde, 1980). Those neurons sequestering enough neurotrophin will survive and go on to innervate the target, and those that do not compete successfully for trophic support will be rapidly eliminated by naturally-occurring cell death, through an apoptotic cascade. Thus the number of neurons are matched to the size of their corresponding targets. Mechanisms that match neurons to their appropriate targets, however, are less well understood.

Apoptosis, or naturally-occurring cell death, is a hallmark of vertebrate nervous system development. It was previously believed that neurons that did not successfully compete for trophic support underwent apoptosis only because of the absence of an active survival signal. However, it is becoming increasingly apparent that apoptotic cell death, at least in sympathetic neurons, is probably due to the presence of active death signals that are suppressed by Trk activation, and is not simply due to the lack of a survival signal (Majdan et al., 2000). This is supported by recent studies that indicate that the survival of developing sympathetic neurons is regulated by opposing signals whose activities are normally balanced. In this fashion, both survival signals that emanate from targets and activate TrkA, and apoptotic factors that act on the p75NTR will determine if a sympathetic neurons lives or dies (Majdan and Miller, 1999). As will be discussed in the first paper in this thesis, such a balance between TrkA and p75NTR signals also regulates the levels of sympathetic neuron growth and target innervation.

b) **Trophic interactions between sympathetic neurons and their targets**

"Trophic interactions are defined operationally as the long-term interdependent relationships of neurons and the cells they innervate, which may be either non-neuronal cells or other neurons. Long-term denotes effects that take place over days, weeks, or months" (Purves et al., 1988).

Early studies published by Viktor Hamburger (1977) indicated that trophic interactions were responsible only for regulating neuronal number during development. However, it is presently accepted that trophic interactions are also responsible for synaptic connections that are made, and such interactions persist throughout the life of the organism. Trophic signals reaching neurons after the time of programmed cell death are important for maintenance of neuron-target-interactions, and are used to adjust the pattern and degree of innervation to any changes in the size or architecture of the targets (Purves et al., 1988). Once a neuron reaches its target, the density of innervation that is achieved will be determined, at least partially, by growth factors produced by the target cells (Campenot, 1982 a, b; Edwards et al., 1989; Causing et al., 1997).Thus interactions between developing peripheral neurons and their targets play a role in determining neuronal phenotype, and it has been demonstrated that NGF regulates gene expression in both developing and mature sympathetic neurons (reviewed in Miller, 1994). NGF specifically upregulates the expression of a subset of growth associated genes, as well as those involved in the differentiation of neonatal sympathetic neurons. These include genes encoding the p75NTR (Miller et al., 1991; Ma et al., 1992), Tα1 α-tubulin (Mathew and Miller, 1990; Ma et al., 1992), and tyrosine hydroxylase (Miller et al., 1991; Ma et al., 1992). This occurs in a concentration-dependent fashion, and demonstrates that trophic interactions between neuron and target can affect the morphology of a neuron itself. Further support for this comes from studies in which the transcription of the Tα1 α-tubulin promoter was repressed by target contact in both developing and mature neurons. This suggests that retrograde, target-derived "stop-growth" signals can regulate the transcription of genes that are necessary for axonal growth to achieve proper levels of target innervation (Wu et al., 1997).

c) **NGF and the sympathetic nervous system**

NGF is the primary survival factor for neonatal sympathetic neurons, and it has been demonstrated that many peripheral tissues that are innervated by sympathetic neurons, such as the skin, the pinna of the ear, and the iris express NGF (Korsching and Thoenen, 1983a; Heumann et al., 1984; Shelton and Reichardt, 1984), even though the SCG itself does not (Heumann et al., 1984). The level of NGF expression seems to be correlated with innervation density (Korsching and Thoenen, 1983a; 1988; Heumann et al., 1984; Shelton and Reichardt, 1984). Early studies indicated that NGF protein was found in the SCG itself, but later studies found that this was atttributable to retrograde transport of the protein from innervated target tissues (Korsching and Thoenen, 1983b, 1985, 1988), since the SCG does not express NGF mRNA (Heumann et al., 1984).

The necessity of NGF for sympathetic neuron survival was originally demonstrated using antibodies to NGF, which induced the death of sympathetic neurons (Levi-Montalcini and Booker, 1960b), and has been confirmed in studies using mice with a targeted disruption of the NGF gene. In these mice, sensory as well as sympathetic ganglia exhibit a profound cell loss, and by postnatal day 3, the volume of the SCG was decreased by 90% (Crowley et al., 1994).

Besides maintaining survival, responses of sympathetic neurons to NGF include growth, neurotransmitter metabolism and the expression of neuron-specific genes (Chun and Patterson, 1977; Mathew and Miller, 1990; Ma et al., 1992; Miller, 1994; Belliveau et al., 1997). Once enough NGF is sequestered to maintain neuronal survival, all of these responses increase in a graded fashion, over a broad concentration range, from 10 to 100 ng/ml (Belliveau et al., 1997). Concentrations of NGF below 10 ng/ml are not sufficient for maintaining neuronal survival and the level of biological responsiveness observed parallels the levels of TrkA activation that occurs (Belliveau et al., 1997). The degree of neurite outgrowth and gene expression that occurs is therefore dependent on the levels of NGF that sympathetic neurons are exposed to. Such a broad response curve is biologically relevant, as these neurons need to be responsive to limiting concentration of NGF during the time of target competition, but also need to be able to respond more strongly to increasing concentrations of NGF when the size of the target area increases as an animal grows (Majdan and Miller, 1999).

d) **Expression of BDNF in the sympathetic nervous system**

Although BDNF is the neurotrophin most widely expressed in the adult central nervous system (Katoh-Semba et al., 1997), there is evidence that BDNF is also expressed by mature and developing sympathetic neurons (Shecterson and Bothwell, 1992; Causing et al., 1997). Using *in situ* hybridization as the detection system, sympathetic ganglia from embryonic day 14.5 (E14.5) to postnatal day 1 (P1) mouse were shown to express BDNF mRNA (Shecterson and Bothwell, 1992). The expression of BDNF mRNA in P1 SCG has been confirmed by northern blot and RNAse protection assays (Causing et al., 1997), both in vivo and in cultured neonatal sympathetic neurons. However, although BDNF is present in the P1 SCG, BDNF does not induce Trk receptor activation in neonatal sympathetic neurons, as these neurons only express TrkA and low levels of TrkC (Belliveau et al., 1997), which do not bind BDNF.

Finally, Causing et al (1997) also used Western blot analysis to show that biologically-active BDNF protein is synthesized in the adult SCG.

e) **Expression of NT-3 in the sympathetic nervous system**

NT-3 (and its preferred receptor, TrkC) are expressed very early during development, well before the programmed cell death and the period of target innervation (Lewin and Barde, 1996). Many NGF-dependent neurons in the PNS including neurons of the SCG are initially dependent on NT-3 during the very early stages of development. At developmental stage E14-E15, sympathetic neuron precursors are dependent on NT-3 for survival (Birren et al., 1993; DiCicco-bloom et al., 1993; reviewed in Chalazonitis, 1996). Around the time of birth, SCG neurons become dependent upon NGF for survival, a dependency that is correlated with high levels of TrkA mRNA expression and a downregulation of TrkC mRNA (Birren et al., 1993; DiCicco-Bloom et al., 1993).

Although NT-3 mRNA has been detected in the sympathetic nervous system during development, NT-3 protein does not seem to be expressed in the adult SCG (Zhou and Rush, 1993). NT-3 plays an important role in sympathetic neuron

development, since neuron number is decreased by up to 50% in the SCG of postnatal NT-3 deficient transgenic mice (Ernfors et al., 1994; Farinas et al., 1994; Wyatt et al., 1997).

f) Beyond the neurotrophic hypothesis - neurotrophins and growth mechanisms

i) Neurite outgrowth and axonal guidance

During the development of the nervous system, neurons undergo commitment to a neuronal fate, which is followed by terminal mitosis. After this stage, they begin to differentiate and put forth neurites. As process out growth occurs, developing neurites become polarized, with some becoming axons, and some dendrites. Axons extend and pathfind their way to appropriate targets that they will proceed to innervate, but normal development of the nervous system is dependent upon the ability of axonal growth cones to correctly pathfind and recognize their biological targets.

The question of how a system as complex as the nervous system is able to establish appropriate neuronal circuitry during development is still very much an ongoing issue, and up until recently, it was thought that sympathetic axons were guided to their targets by gradients of target-derived NGF. This was supported by studies showing that intracranial injection of NGF led to extensive growth of sympathetic axons into the brain (Menesini-Chen et al., 1978). However, since NGF is not synthesized in the targets of SCG neurons until their earliest axons begin to arrive (Korsching and Thoenen, 1988), and since these neurons do not express Trk receptors until they reach their targets, this suggested that target-derived NGF did not play a role in long-range axonal guidance for sympathetic neurons (Davies et al., 1987). The interpretation was that NGF instead acted locally and not over longer distances to promote neuronal outgrowth. The local growth promoting effect of NGF has been illustrated both *in vivo* (Edwards et al., 1989; Miller et al., 1994) and *in vitro* (Campenot 1977, 1982a, 1987). *In vivo*, transgenic mice with NGF expression directed to pancreatic beta-cells exhibited an increased density of sympathetic axons around the islets (Edwards et al., 1989), and local injections of NGF into the irides of adult rats induced spouting of sympathetic fibres from the SCG (Miller et al., 1994). Experiments *in vitro* used compartmented cultures to demonstrate that the ability of NGF to promote

neurite growth involves local mechanisms. However, there are also recent studies that have showed that neurotrophins do act as guidance factors *in vitro* in spinal neurons (Ming et al., 1997, 1999) and DRG neurons (Gallo et al., 1997; Lentz et al., 1999), and *in vivo* in trigeminal sensory neurons (O'conner and Tessier-Lavigne). The question of whether neurotrophins act as guidance factors or are required for axonal initiation/ elongation may finally be answered in future studies by using a line of transgenic mice with targeted deletions in both the propapoptotic protein BAX and TrkA or NGF (Patel et al., 2000). This model "uncouples" survival and growth pathways, so that the roles of NGF/TrkA in neuronal growth can be studied without the requirement of NGF for survival. Data from these studies indicates that a lack of NGF signals does not affect the ability of sensory neurons to respond to guidance and branching cues, since central DRG axonal projections were observed in the spinal cord. Whether or not NGF is required for sensory initial axon elongation was not completely clear. Although neurons of these double knockout mice were not found in major nerve trunks and axon numbers within cutaneous nerves were decreased, it is possible that these neurons may have extended short projections which were subsequently retracted (Patel et al., 2000; Kaplan et al., 2000). For elongation of sensory and motor axons in mixed nerves, however, all the neurotrophins are required, since a combination of function-blocking antibodies to NGF, NT-3, and BDNF was able to dramatically inhibit their extension into developing limb buds of mice, at age E10.5. Neurite outgrowth, at this developmental stage, occurs well before axons have reached their final target (Tucker et al., 2001).

As a neurite begins pathfinding, and grows towards its presumptive target, it must traverse a relatively large area, and will encounter many different cell types that express a variety of molecular guidance cues. Such cues must be either ignored, or appropriately read and integrated, so that the proper level and type of connectivity can be achieved (reviewed in Mueller et al., 1999). The machinery for neurite outgrowth and guidance is found in growth cones located in nerve terminals (Mitchison and Kirshner, 1988; Mueller, 1999). Growth cones sample their environment by means of filopodial and lamellar protrusions, and react to positive or negative cues by moving towards or away from such guidance molecules (Oakley and Tosney, 1993; Gomez and

Letourneau; 1994; Fan and Raper; 1995; Kuhn et al., 1995; Goodman, 1996). Cytoskeletal rearrangements in response to such molecular interactions are the means by which growth cones are redirected towards their appropriate targets during their migration (Bentley and O'Connor, 1994; Lin et al., 1994; Tanaka and Sabry, 1995; Challacombe et al., 1996).

After arriving in their target fields, neuronal processes arborize in characteristic and specific patterns. In the CNS, axons from a single subpopulation of neurons need to distribute themselves throughout a large target field and form topographic maps. In the PNS, arriving nerves generally contain mixed populations of neurons, frequently containing a mix of autonomic, sensory/and or motor axons which must be sorted into the appropriate areas. For the nervous system to achieve proper connectivity and function correctly, there are a number of cues that are used. These growth/guidance cues can be either attractive or repulsive, with both types acting together to elicit appropriate connectivity. Since the neurotrophins (and their receptors) can influence neuronal growth, positive and negative neurotrophic cues will be discussed below, both generally, and also with respect to growth of the sympathetic nervous system.

ii) **Target Innervation**

Developing sympathetic neurons that have sequestered enough NGF to ensure their survival will grow and mature morphologically, as represented by their ability to innervate their targets. As discussed above, the role of NGF goes beyond just being a permissive survival factor. Once neurons have reached their physiological targets, neuronal processes must receive biological signals that tell them to grow into and innervate these targets. They exhibit a great deal of plasticity, in that frequent remodeling of neuronal connectivity occurs until the degree and pattern of innervation is appropriate for a particular target. Such mechanisms ensure that transiently overproduced connections are eliminated. In the case of sympathetic neurons, the principal biological signal for both neuronal growth and target innervation is provided by NGF (Levi-Montalcini, 1987).

NGF may or may not be involved in long distance axon guidance, as discussed above, but it has been shown to influence the growth of fibres that have already reached

their targets. Thus, the neurotrophins and their receptors are important not only for achieving appropriate target innervation, but also for ensuring proper innervation patterns. In sympathetic and sensory neurons, the local availability of target-derived NGF will influence the degree of terminal axonal branching and dendritic complexity *in vivo* (Purves et al., 1988), and neurite growth *in vitro* (Campenot, 1982a, b). It follows that the degree of morphological complexity varies according to the size of the target, resulting in a match between the size of the target, and the number of innervating neurons. Upon reaching their targets, axons will arborize in specific patterns. The axonal and dendritic arborizations of sympathetic neurons are less complex in neonatal animals, and this pattern becomes more complex, and the axonal arbor increases in size as long as the target field, and the animal, continue to grow (Purves et al., 1988). Thus even after neuronal number has been established after the period of naturally occurring cell death, this plasticity ensures that neuronal morphology continues to change in accordance with the growth of the body, through ongoing rearrangement/and or growth of axonal and dendritic branches.

Mice deficient in both BAX and TrkA or NGF have served as a model for studying the roles of TrkA/NGF in axon growth and differentiation, as introduced in the section above (Patel et al., 2000). Without expression of the BAX protein, many neuronal populations do not undergo naturally-occurring cell death (White et al., 1998), and cultured sympathetic and DRG neurons from $BAX^{-/-}$ mice survive indefinitely in the absence of NGF (Deckworth et al., 1996; Lentz et al., 1999). Thus this model allowed the role of NGF/TrkA signaling in axon growth and differentiation to be studied without the involvement of apoptosis. With respect to target innervation, sensory axons of $BAX^{-/-}/TrkA^{-/-}$ transgenic mice did not arborize in peripheral cutaneous target fields, suggesting that NGF/TrkA signaling is required for target field innervation and for maintenance of axonal projections (Patel et al., 2000; Kaplan et al., 2000).

iii) **Sympathetic innervation of the pineal gland**

The pineal gland, believed by Descartes to be the "seat of the soul," is named for its pine cone-like (Latin: *pinea*), shape. Because it is an experimentally accessible

sympathetic target organ, it a convenient structure for the study of sympathetic neuron growth and target innervation. For this reason, and also because it does not receive any other peripheral innervation from motor or sensory neurons (Stanley et al., 1987), it is the model that was selected to study growth and innervation in the work presented in this thesis.

The pineal gland, which arises from the roof of the third ventricle, is a midline sympathetic target organ that receives bilateral and overlapping sympathetic innervation (Kapers, 1960; Owman, 1964). The innervating neurons originate in the left and right SCG (Bowers et al., 1984; Stanley et al., 1987), and their axons project to the pineal by way of the right and left internal carotid nerves (Zigmond et al., 1981; Bowers et al., 1982, 1984). They ultimately reach the pineal gland via the two nervi conarii, nerve bundles at the posterior end of the gland. HRP tracing and bilateral superior ganglionectomy studies in the rat indicate that all of the sympathetic neurons innervating the pineal gland originate in the SCG (Kapers, 1960; Owman, 1964; Bowers et al., 1984). It has been reported that some axons enter the anterior portion of the pineal gland from the pineal stalk (Kappers, 1960). There is also some evidence of minor synaptic inputs from the CNS to the pineal gland in the rat and the guinea pig, possibly from the habenular region (Ronnekleiv and Moller, 1979; Dafney, 1980; Korf and Wagner, 1980; Semm et al., 1981). However, other studies using retrograde tracing methods find no evidence of any innervation originating from the habenular nuclei, or any other diencephalic, midbrain, pontine or medullary structure (Stanley et al., 1987).

Levels of NGF are reported to be high in the pineal gland of developing animals (Weskamp and Otten, 1987; Wright et al., 1987), as in other sympathetic target organs, such as the iris and the submaxillary gland (Korsching and Thoenen, 1983a). Since the pineal gland is primarily innervated by sympathetic fibres from the SCG, high levels of NGF in this target organ is consistent with its established role in the differentiation and maintenance of sympathetic neurons (Levi-Montalcini and Angeletti, 1968; Ruit et al., 1990).

There is histochemical evidence that innervating sympathetic neurons establish functional connections with pinealocytes shortly after birth in the rat (Hakanson et al.,

1967; Machado et al., 1968b; Wiklund, 1974; van Veen et al., 1978), a finding which has been confirmed by electron microscopy (Machado, 1971). However, these innervating fibres reside mainly at the surface of the gland as indicated by monoaminergic immunofluorescence studies (Hakanson et al., 1967; Machado, 1971; Machado et al., 1968a, b; van Veen et al., 1978). By postnatal day 2, fibres invade the gland (van Veen et al., 1978), projecting varicose terminals around blood vessels and parenchyma (Machado et al., 1968b; Wiklund, 1974), and by the fifth postnatal day, fibres are visible throughout the pineal gland, although not in all areas, and not to the same degree that is observed in the adult (van Veen et al.,1978; Wiklund,1974). Staining intensity continues to increase during the second postnatal week, and by postnatal day 16 is similar to that observed in the adult, but the number of varicose terminals is lower (Hakanson et al., 1967). By the third week of postnatal life, the intensity of norepinephrine fluorescence, and the distribution and number of varicose terminals closely resemble those of the adult animal (Hakanson et al., 1967; Machado, 1971; Machado et al., 1968b; van Veen et al., 1987; Wiklund; 1974). By the time of weaning, the number of varicose terminals within pinealocytes and around blood vessels is equal to that measured in the adult (Machado et al., 1968a, b).

g) **Growth regulation**

i) **Positive and negative cues**

Both positive and negative cues are required to ensure that neuronal growth proceeds appropriately. Positive guidance cues range from being merely permissive, such as a permissive substrate in an otherwise non-permissive environment, to true attractive signals. Although positive cues are obviously required for neurons to grow, inhibitory or repulsive cues are equally important. Such cues ensure that patterns and levels of target innervation achieved are appropriate for the size of the target, and that the target is a physiologically relevant match for incoming neuronal processes. By inhibiting axonal growth that is misdirected, and to prevent inappropriate innervation, repulsive cues can repel growing neuronal processes, and help them to instead reach their physiological targets (Pini. 1993; Fan and Raper, 1995; Messersmith et al., 1995; Püschel et al., 1995; Raper et al., 2000).

In the case of sympathetic neurons, the neurotrophins can either help or hinder neurite outgrowth, depending on cellular context, such as receptor and neurotrophin cohorts present in the cellular environment. In these neurons, TrkA mediates positive events, while activation of the p75NTR is associated with negative signals (Bamji et al., 1998; Chapter 2 of this thesis). In cases where neurotrophins or their receptors are either overexpressed or absent, there are reports of perturbations in the both the levels and the patterns of growth and innervation. Such events will be reviewed below.

ii) **Neurotrophins and growth regulation**

NGF is normally produced by sympathetic target tissues, but not by sympathetic neurons themselves (Ernfors et al., 1992). Ectopic expression of NGF under the control of the DBH promoter in sympathetic neurons caused enhanced neurite outgrowth from the SCG and within the sympathetic trunk, and there are also some reported instances of inappropriate axon growth, but not inappropriate target innervation. Although these neurons were able to find their physiological targets, these targets had fewer innervating terminal sympathetic fibres (Hoyle et al., 1993). NGF overexpression therefore prevented axons from establishing a normal innervation pattern and density.

NGF overexpression under the control of the GFAP promoter induced robust ingrowth of sympathetic fibres to inappropriate neural tissues such as the cerebellar deep white matter and inferior cerebellar peduncles, and aberrant fibres were also observed in regions of the hippocampal formation (Kawaja and Crutcher, 1997). Sensory neurons have also been reported to invade the cerebellum in this line of transgenic mice (Kawaja et al., 1997). These findings are unusual because regions that contain white matter are not normally permissive for axonal growth due to the presence of factors associated with CNS myelin and oligodendrocytes that are inhibitory to growth. As will be discussed in the subsequent section on neurotrophin receptors and growth regulation, when the p75NTR is functionally ablated, the level of growth becomes enhanced even further. This supports the findings that I will present in subsequent chapters, of p75NTR's ability to negatively regulate neuronal growth.

Overexpression of NGF in skin under the control of a keratin promoter has been demonstrated to cause hypertrophy of sympathetic and sensory nerves, as well as

enhanced sympathetic innervation (Albers et al., 1997; Davis et al., 1997), and some aberrant sympathetic target innervation was observed in the region of the mystacial pads and vibrissae (Davis et al., 1997). Sympathetic targets such as footpads, sweat glands, and blood vessels also exhibited disruptions in their innervation patterns. Innervation of these targets was decreased, and instead, sympathetic neurons formed a dense aberrant plexus just below the epidermis, where they were found intermingled with sensory fibres (Guidry et al., 1998). Overexpression of NGF in the skin also increases sensory innervation density, selectively promoting innervation by NGF-dependent nociceptive neurons (Stucky et al., 1999).

These findings together demonstrate that neurotrophin overexpression, specifically NGF, especially when directed to a particular area leads to increased innervation density, and sometimes, aberrant innervation. Studies using other systems in which other neurotrophins are overexpressed or absent yield similar results, implying that this is a more global phenomenon in the nervous system. Mice overexpressing NT-3 in the skin show increased density of sensory nerve endings (Albers et al., 1996), and in mice with targeted deletions of the NT-3 gene sympathetic fibers failed to invade the pineal gland and external ear, although other targets were normally innervated. Sympathetic fibers of mice carrying one functional copy of the NT-3 gene did reach the pineal gland, but failed to branch and form a normal plexus (ElShamy et al., 1996). The application of exogenous NT-3 was able to rescue the sympathetic target deficit of NT-3-deficient mice.

Overexpression of BDNF under the control of the keratin promoter has been shown to alter the sensory innervation to the skin (LeMaster et al., 1999), and transgenic mice overexpressing BDNF under the control of the nestin promoter exhibit a significant decrease in the levels of sensory innervation of the gustatory papillae (structures containing taste buds) (Ringstedt et al., 1999). In these mice, gustatory nerves entering the tongue exhibited abnormal innervation patterns, with tangles and disorganization of fibres. Parts of the tongue were not innervated, with the nerve fibres projecting there but terminating at sites of ectopic BDNF expression, where they formed abnormal branches and sprouts, rather than continuing to project to the papillae (Ringstedt et al., 1999). Since the nerve fibres were able to project to the tongue, the

outgrowth of gustatory neurons was not inhibited by BDNF overexpression, but target innervation was.

Similar results were obtained in a study in which BDNF and NT-4 were overexpressed in the epithelium of the tongue (Krimm et al., 2001). Fungiform papillae were poorly innervated by gustatory fibres in BDNF and NT-4 overexpressing animals, with some fibres approaching, but not penetrating the lingual epithelium. Aberrant innervation was also observed. In BDNF overexpressors, some gustatory fibres innervated inappropriate targets, connecting with nongustatory filiform papillae, rather than fungiform papillae. Although both lines of mice exhibited decreased target innervation as well as aberrant target innervation, the pattern differed slightly in BDNF versus NT-4 overexpressors, suggesting that the spatial distribution of BDNF and NT4 within lingual epithelia is important for appropriate target selection (Krimm et al., 2001).

Studies using neuronal cultures show that neurotrophins also have the ability to regulate growth *in vitro*. Neurotrophins can elicit turning responses of growth cones toward a source of neurotrophin and can modulate responses of the axonal growth cone (Gundersen and Barrett, 1980; Gallo et al., 1997; Ming et al., 1997; Paves and Saarma, 1997; Tuttle and O'Leary, 1998). Growth cones of DRG neurons have been shown to turn towards a local source of NGF, while BDNF can cause the growth cones of these neurons to collapse (Paves and Saarma, 1997). The collapsing effect of BDNF on growth cones, with subsequent neurite retraction has also been shown in embryonic *Xenopus* spinal neurons (Wang and Zheng, 1998). In experiments in which chick DRG neurons were cultured in the presence of NGF-bound beads, the turning response was shown to be a TrkA-mediated event because it could be inhibited by: (1) antibodies directed against the NGF extracellular domain, (2) elevating the concentration of NGF in the medium to saturate TrkA receptors, and (3) the addition of K252a to the medium (Gallo et al., 1997). When BDNF was added to the culture medium, the turning response of the sensory neurons was greatly reduced. This supports the ability of the p75NTR to modulate TrkA activity.

Similar results were obtained in mass cultures of NGF-dependent cutaneous DRG neurons from chick embryos. When these neurons were exposed to BDNF in the

medium, growth cone collapse was observed, and in separate experiments, neurite outgrowth towards BDNF-secreting fibroblasts was also inhibited (Cahoon-Metzger et al., 2001). The inhibitory effect of BDNF was mediated through the p75NTR because: (1) function blocking anti-p75NTR antibodies completely prevented BDNF-induced growth cone collapse, (2) K252a did not block the collapse. These cutaneous neurons express BDNF message and protein much more strongly in the epidermis than in the dermis. Since the normal region of termination for cutaneous neurites is the dermis, BDNF in the epidermis is likely to play a role in feather patterning by restricting axons from entry into the epidermis (Cahoon-Metzger et al., 2001).

In compartmented cultures of adult rat DRG neurons, NGF, but not BDNF or NT-3, was able to elicit outgrowth into a side compartment containing one of these neurotrophins. Antibodies to NGF placed in this compartment caused a cessation of neurite outgrowth (Kimpinski et al., 1997) The addition of BDNF to a side compartment containing NGF was able to inhibit neurite extension in a dose-related manner. This is similar to what has been observed in cultured SCG neurons (Chapter 2, this thesis). BDNF was able to inhibit the growth of sympathetic neurons in a dose-related manner, independent of its effects on survival, supporting a role for the p75NTR as a mediator of inhibitory growth signals. Although the adult DRG neurons used in this study do not require NGF for their survival, they do require it to stimulate their growth, and the addition of BDNF was able to inhibit outgrowth in a dose-related manner. Evidence for the ability of the p75NTR to inhibit neuronal growth and TrkA to promote it, is presented in the section below.

iii) **The neurotrophin receptors and growth regulation**

When neurotrophin receptors are overexpressed, normal growth becomes perturbed. *In vitro*, overexpression of TrkA receptors in PC12 cells has been demonstrated to induce a more rapid NGF-induced differentiation, as illustrated by a significantly decreased time for these cells to elaborate neurites (Hempstead et al., 1992). In studies of $TrkA^{-/-}$ mice, sympathetic neurons were able to innervate their surrounding vasculature at developmental age E15.5, a time during which sympathetic neurons have started to reach more distal targets. The vasculature, however, is one of

the earliest targets to receive sympathetic innervation, and the E15.5 TrkA$^{-/-}$ submaxillary gland, a target that normally becomes innervated later on during development failed to receive the normal complement of incoming sympathetic fibres (Fagan et al., 1996). Thus a lack of TrkA does not impair the ability of these neurons to extend processes during the early stages of development, with the deficit becoming apparent later on during development.

The expression of the p75NTR can also negatively regulate the growth and target innervation of a number of neuronal populations. It has been demonstrated that transgenic mice with targeted deletions of the p75NTR have alterations in sympathetic and sensory innervation. Sympathetic deficits include significantly reduced innervation of the lateral footpads and pineal gland (Lee et al., 1994a,b), and reduced sensory cutaneous innervation (Lee et al., 1992, 1994b). However, the absence of the p75NTR can also lead to enhanced sprouting and growth of sympathetic axons when NGF is overexpressed (Hannila and Kawaja, 1999; Walsh et al., 1999a), as well as alterations in their directional growth (Walsh et al., 1999b). Sympathetic neurons of transgenic mice that are deficient in functional p75NTR expression and which overexpress NGF in astrocytes under the control of the GFAP promoter (NGF/p75$^{-/-}$mice) were able to invade the white matter of the optic tract (Hannila and Kawaja, 1999), forming fibre bundles. Sympathetic fibres from this line of p75NTR-deficient mice also robustly and aberrantly innervate the cerebellar deep white matter, inferior cerebellar peduncles and cerebellar folia as do mice that overexpress NGF in astrocytes (Kawaja and Crutcher, 1997), but do so even more robustly (Walsh et al., 199a).

Finally, mice that lack functional p75NTR expression exhibit an increase in hippocampal innervation originating from TrkA-positive basal forebrain cholinergic neurons (Yeo et al., 1997). Besides altering the degree of target innervation, the absence of the p75NTR also disrupted the pattern of innervation, since ChAT-positive fibres were decreased in the hippocampal layer that is normal highly innervated, and increased in other regions.

How does the p75NTR modulate neuronal growth? The mechanism by which the p75NTR modulates neuronal growth is still unclear. Recently, Barde and colleagues (Yamashita et al., 1999) have shown, using a yeast two-hybrid system, that the

p75NTR interacts with and can modulate the activity of Rho, a GTPase that is associated with signaling to the growth cone cytoskeleton (MacKay et al., 1996; van Leeuwen et al., 1997). Such an interaction supports a link between the p75NTR, neurotrophins and the cytoskeleton. RhoA, when activated, has been previously shown to mediate negative signals in neurons, leading to growth cone collapse, and PC12 cell neurite retraction (Jalink et al., 1994; Tigyi et al., 1996; Katoh et al., 1998). When the intracellular domain of the p75NTR was expressed in retinal ganglion cells that do not express Trk receptors and respond to p75NTR activation by elaborating neurites, Rho was robustly activated, in a constitutive fashion. However, upon neurotrophin binding to the p75NTR, this p75-dependent Rho activation was suppressed (Yamashita et al., 1999). Since Rho activation is associated with the inhibition of neuronal growth (Lehmann et al., 1999), the p75NTR could regulate growth in either a positive or negative manner, depending on the proportion of unliganded to liganded p75NTR in the local microenvironment (Kaplan and Miller, 2000).

The study by Yamashita et al (1999) supports a positive role for p75NTR in modulating neuronal growth, but it was carried out in retinal ganglion cells that do not express Trk receptors. It remains to be seen how co-expression of Trk receptors might affect the interaction between p75NTR and Rho. Cellular context is an important factor since the p75NTR, in some instances, has been reported to positively affect Trk receptors (Davies et al., 1993; Verdi et al., 1994; Lee et al., 1994b), and in others, to inhibit them (Bamji et al., 1998; Yoon et al., 1998; Kohn et al, 1999; Walsh et al., 1999). Since the p75NTR can associate with TrkA, B, and C (Bibel et al., 1999), signaling differences among Trk receptors must also be considered, since, for example, in sympathetic neurons that express the p75NTR, TrkA and TrkB can signal differently, even within the same cellular context (Atwal et al., 2000).

h) **Autocrine survival and growth mechanisms**

By definition, an autocrine/paracrine growth factor, in the context of the nervous system, is a molecule that is produced by a neuron itself, and can act to influence that same neuron, or neighboring neurons, in some fashion. This differs slightly from the terminology used in other biological systems, in which the term

"autocrine" has come to mean acting on a cell itself, while "paracrine" refers to a local action on neighboring cells.

It has been suggested that local autocrine mechanisms may be involved in mediating the biological roles of the neurotrophins, in certain contexts. The notion of autocrine mechanisms playing a role in neuronal survival was based on studies in which both BDNF and its receptor, TrkB, were demonstrated to be localized to the same population of DRG neurons (Acheson et al., 1995). Such colocalization has also been reported in CNS neurons (Yan et al., 1997), and autocrine growth factors have been demonstrated to be important for neuronal survival (Acheson et al., 1995; Davies, 1996; Lindholm et al., 1996). During development, DRG neurons are dependent upon target-derived neurotrophins for survival, but this is lost in adulthood, possibly due to an autocrine loop, in which these neurons synthesize and release BDNF (Acheson et al., 1995). Experiments using antisense strategies to significantly decrease BDNF expression resulted in a significant decrease in neuronal survival, supporting the notion that adult DRG neurons lose their trophic dependence and maintain survival by an autocrine mechanism (Acheson et al., 1995).

It has been shown that in cultured neonatal sympathetic neurons, exogenous NT-3 can synergize with NGF to maintain survival *in vitro*, acting as an "accessory" survival factor that activates TrkA receptors, but can not maintain survival as well as NGF (Belliveau et al., 1997; Orike et al., 2001). Since NT-3 is produced in vivo during development in a subpopulation of sympathetic neurons from E14.5 to P1 (Schecterson and Bothwell, 1992), and in target fields of sympathetic neurons (Ernfors et al., 1992; Schecterson and Bothwell, 1992), this suggests a potential autocrine role for NT-3 during the development of sympathetic neurons.

With respect to autocrine mechanisms being involved in biological responses of sympathetic neurons independent of survival, there is evidence for autocrine growth regulation. In the same study by Belliveau et al (1997) cited above, and in a study by Orike et al. (2001), exogenous NT-3 was demonstrated to be as efficient as NGF at promoting the outgrowth of neonatal sympathetic neurons *in vitro*; since sympathetic neurons at this stage express NT-3 (; Schecterson and Bothwell, 1992) as do some of their target fields neurons (Ernfors et al., 1992; Schecterson and Bothwell, 1992), this

suggests that NT-3 may potentially act as an autocrine growth factor, as well as a survival factor.

Sympathetic neurons express bioactive hepatocyte growth factor (HGF) during development (Maina et al., 1998) and postmitotically (Yang et al., 1998), as well as its receptor, the Met tyrosine kinase (Yang et al., 1998). This suggests an autocrine function for this factor. HGF can selectively promote optimal growth but not survival in these neurons, with similar efficacy as that observed with NGF itself (Maina et al., 1998; Yang et al., 1998). It is possible that such a mechanism could positively influence axonal growth during periods when extrinsic sources of growth factors are limiting, such as during axonal outgrowth at the time of development, or perhaps after injury.

Another example of an autocrine mechanism involving growth of sympathetic neurons is provided by studies using human keratinocytes. In the skin, a major target of sympathetic neurons, NGF has been shown to be synthesized and released by normal human keratinocytes (Di Marco et al., 1991; Yaar et al., 1991; Pincelli et al.,1994) which express both TrkA and p75 NTRs (Di Marco et al., 1993), as well as by murine keratinocytes (Tron et al., 1990). Since NGF is synthesized and released by keratinocytes that express TrkA, it has been suggested that that autocrine mechanisms are responsible for keratinocyte proliferation (Pincelli et al., 2000). This is supported by the observations TrkA is constitutively activated in the absence of exogenous NGF (Zhai et al., 1995) and that K252, also in the absence of exogenous NGF, was able to inhibit DNA synthesis in keratincocytes (Di Marco et al., 1993). Moreover, medium from keratinocytes has been shown to promote outgrowth of sensory neurons (Di Marco et al, 1991) and of PC12 cells (Pincelli et al., 2000), and exogenous NGF upregulates NGF mRNA in keratinocytes (Pincelli et al., 2000). Further support for a TrkA:NGF autocrine loop comes from experiments in which normal human keratinocytes were transfected with NGF cDNA, and these cells showed a higher rate of proliferation, relative to controls, suggesting that a TrkA:NGF autocrine loop exists in human skin cells (Pincelli et al., 2000).

In sympathetic neurons a BDNF: p75NTR autocrine loop has been suggested as a negative regulatory mechanism involved in neuronal apoptosis (Aloyz et al., 1998; Bamji et al, 1998). These neurons synthesize BDNF (Causing et al., 1997) that they

process and secrete (Causing CG, Aloyz RA and FD Miller, unpublished observation), and which, in these cells, acts as a specific p75NTR ligand, since sympathetic neurons do not express the TrkB neurotrophin receptor (Ernfors et al., 1992; Belliveau et al., 1997). Bamji et al (1998) have shown during the period of naturally occurring cell death, this may be representative of an ongoing autocrine BDNF: p75NTR apoptotic mechanism.

In Chapter 2 of this thesis, I have taken this model one step further and addressed the possibility of BDNF as part of this same autocrine loop, but as one that regulates neuronal growth, as well as survival. There is evidence that neurotrophic factors such as BDNF can act in a negative fashion to influence neuronal growth in the peripheral nervous system (Kimpinski et al., 1997; Paves and Saarma, 1997; Wang and Zheng, 1998; Chapter 2 of this thesis). As discussed above, an autocrine BDNF:75NTR loop may be recruited in sympathetic neurons to limit neuronal survival during the time of naturally-occurring cell death, but evidence will be presented in the first paper in this thesis, for just such a mechanism as a negative regulator of sympathetic neuron growth, as well as survival. I have shown that the pineal gland of $BDNF^{-/-}$ neurons is hyperinnervated with sympathetic fibres, suggesting that a BDNF:p75NTR autocrine loop can also antagonize NGF-mediated target innervation.

4) Interactions between TrkA and p75NTRs

a) Overview

Many NGF-responsive neuronal populations including sympathetic neurons co-express both TrkA and p75NTRs. The original idea for a functional interaction between these receptors came from studies in which the p75NTR, when coexpressed with TrkA in cell lines that express neither of these receptors, generated greatly increased levels of high affinity neurotrophin binding sites (Hempstead et al., 1991; Kaplan et al., 1991; Mahadeo et al., 1994). Since the concentration of NGF in target tissues is extremely limiting, being in the subpicomolar range (Meakin and Shooter, 1992; Barde, 1989), a high-affinity binding site is necessary to ensure that enough can be sequestered to ensure the neuronal survival during development. Such a high-affinity site was postulated to involve both TrkA and and p75NTRs, because the spatial conformation of

binding sites on a neurotrophin molecule was thought to be facilitory for such a physical interaction; TrkA and p75NTR binding sites on neurotrophins are are believed to overlap to a significant degree (Bothwell et al., 1995). With respect to the composition of the high-affinity binding site, TrkA and p75NTR heterodimers or homodimers were both proposed (Hempstead et al., 1991; Jing et al., 1992; Ibáñez et al., 1993). The number of high affinity sites has recently shown to be regulated by the ratio of TrkA and p75NTRs, as well as by transmembrane and cytoplasmic domains within these molecules (Esposito et al., 2001).

Exactly how the p75NTR interacts with the Trk receptor family is still unclear. What has become clear is that these receptors do interact. The biophysical and biochemical studies that have been used to address this question will be reviewed below.

b) **Do the p75NTR and TrkA interact biophysically?**

Ever since TrkA and p75NTRs were shown to both be capable of binding neurotrophins, the possibility of an association between them has existed, since they both can signal on their own, can modulate each other's activities, and often mediate opposing events. The question remains as to how TrkA and p75NTRs interact to regulate survival, growth, and target innervation. A number of studies have suggested or demonstrated that TrkA and p75NTRs form a physical association (Hempstead et al., 1991; Wolf et al.,1995; Ross et al., 1996; Bibel et al., 1999; Mischel et al., 2001). Based on biophysical data, it has been reported that p75 and TrkA are in close physical proximity, and may form a heteromolecular complex in vivo (Ross et al., 1996).

It has been difficult to document any direct physical association between these NTRs. The question remains as to whether the interaction between TrkA and p75NTRs in sympathetic neurons is functional or physical, or is perhaps dependent upon both types of interactions. How they interact may depend upon cellular context, especially since different cellular systems have different cellular ratios of these receptors. A number of methods have been used to address whether p75 and TrkA interact physically to exert their effects. Two methods that have been used are receptor coimmunoprecipitaion and receptor co-patching.

i) **Evidence from biochemical studies**

Demonstrating direct interactions between TrkA and p75NTRs has been difficult to do reliably. Co-immunoprecipitation of chemically crosslinked ^{125}I-radiolabeled NGF with antibodies directed against TrkA and p75NTR suggested but did not demonstrate a complex between the receptors (Huber and Chao, 1995), despite the fact that the embryonic neural tissues used were enriched with p75NTR and Trk receptors. Reversibly crosslinking Trk and p75NTR receptors expressed in Sf9 cell lines was also unsuccessful (Gargano et al., 1997).

Barde and colleagues (Bibel et al., 1999) were recently able to demonstrate a direct interaction between TrkA and p75NTRs by co-immunoprecipitation after overexpressing them in A293 kidney cells; an interaction between p75NTR and Trks B and C was also demonstrated. The TrkB-p75NTR interaction was mediated by intra- and extracellular domains. The tagging procedure that they used may explain why they were successful while previous groups were not. They observed a physical interaction between p75NTR and all Trk receptors having a hemagglutinin epitope at their N-terminal end (Vesa et al., 2000), while others did not. They suggested cellular context as another possibilty, with cytoplasmic proteins within A293 cells potentially stabilizing the interaction (Bibel et al., 1999). Some other technical reasons have been suggested for the lack of success in previous cross-linking studies. If reactive amino acid side chains are not suitably juxtaposed in protein complexes, or if protein-protein interactions that occur in plasma membranes are not strong enough to withstand detergent and immunoprecipitation procedures and maintain their association, the experimental procedures may fail, and so are not necessarily conclusive (Bothwell, 1995). In the studies by Gargano et al (1997), antibodies directed against the p75NTR were able to efficiently co-immunoprecipitate p75NTR-TrkA cross-linked complexes, but antibodies to TrkA could not.

ii) **Evidence from biophysical studies**

Biophysical methods to study receptor interactions were implemented because of the difficulties in demonstrating protein complexes using crosslinking or

immunoprecipitation. Unlike biochemical methodologies, these methods do not use detergents to solubilize membranes, and may therefore detect more interactions.Biophysical methods used to study protein-protein interactions include FRAP or (**F**luorescence **R**ecovery **A**fter **P**hotobleaching) and receptor co-patching. FRAP has been used to assess the lateral mobility of neurotrophin receptors expressed in PC12 nnr cell lines and Sf9 insect cells by baculoviral vectors (Wolf et al., 1995). Receptors can move laterally within the plane of the plasma membrane. In cells that do not express TrkA and are not responsive to NGF, the p75NTR has been shown to be highly mobile, but becomes immobilized upon the expression of TrkA within these cells (Venkatakrishnan et al., 1990). FRAP studies indicated that although the p75NTR was freely mobile when expressed alone, co-expression of TrkA with the p75NTR was able to limit the diffusion of the p75NTR within the cytoplasmic membrane (Wolf et al., 1995). This implies a physical association, but does not prove that it occurs through a specific intermolecular complex. The interaction required intact p75NTR cytoplasmic and TrkA kinase domains, although the interaction could still occur in the absence of ligand.

The ability of receptors to move laterally within the cell membrane underlies the design of the co-patching experiments that have previously been used to detect a p75NTR-TrkA intermolecular complex. This technique was also used in some studies presented in Chapter 3 of this thesis. The co-patching technique referred to in Chapter 3 was adapted from similar studies (Ross et al., 1996) that used Sf9 insect cell lines which overexpress TrkA and the p75NTR through a baculoviral expression system. The co-patching technique is designed to detect whether TrkA and p75NTRs interact physically. In this immunocytochemical technique, receptors are induced to "form patches" by the addition of an antibody directed against either one of the proteins of interest. The cells are are then fixed to prevent any further receptor-induced aggregation. The second protein of interest is then antibody-tagged, and two different fluorochromes are used to visualize the cells. If the 2 receptors, in this case p75NTR and TrkA physically interact, they should both be found within the patches, and the TrkA-p75NTR complex is referred to as a "copatch."

In the copatching experiments reported by Ross et al (1996), a specific physical interaction was demonstrated between the p75NTR and TrkA, but not between the p75NTR and TrkB or any other receptor tyrosine kinases such as drosophila Torso, or PDGFR-β (Ross et al., 1996). NGF was not required for this interaction. Domain mapping experiments indicated that although the extracellular domains of TrkA and p75NTRs were sufficient for complex formation, the intracellular domains could regulate high-affinity binding by through conformational changes within the complex (Ross et al., 1996).

Many of the experiments described in this section have been able to suggest a close relationship between the p75NTR and TrkA, but reliable methods for readily isolating the TrkA and 75NTRs from neurons while they remain in close physical association with each other, remain elusive. The biophysical experiments descrobed have been carried out in transfected cell lines in which the expression of receptor proteins is artificially high, and the p75NTR:TrkA stoichiometry may not be representative of that which occurs in *vivo*. In neonatal sympathetic neurons, the ratio of p75:TrkA (mRNA) is at least 10:1 (Mahadeo et al., 1994; Verdi et al., 1994; Chao and Hempstead, 1995).

5) **TrkA signal transduction in sympathetic neurons**

a) **TrkA signaling pathways**

Neurotrophins exert a number of different biological effects on their target neurons, which has led to a number of investigations into the intracellular pathways that ultimately relay the resultant molecular signals. During the period of naturally occurring cell death, and *in vitro*, sympathetic neurons have an absolute requirement for NGF to prevent them from undergoing apoptosis, and the survival signals that are generated originate from the TrkA receptor. Signaling pathways downstream of TrkA maintain neuronal survival, and in sympathetic and sensory neurons, this occurs through the Ras/PI3-kinase/Akt signal transduction pathway. TrkA phosphorylates specific residues found within its intracellular catalytic domain that serve as docking sites for downstream signaling molecules (Kaplan and Miller, 2000).

The TrkA signal transduction pathway was originally described and characterized using the PC12 cell model. These cells exhibit a neuronal phenotype upon treatment with NGF, differentiating into cells which resemble sympathetic neurons, and which share similar characteristics (reviewed in Kaplan and Miller, 1997). Using PC12 cell mutants which lack TrkA and are therefore non-neurotrophin responsive PC12 nnr), it was demonstrated that TrkA was the transducer of NGF's biological actions, since when exposed to NGF, these mutants do not differentiate, and NGF-responsiveness could be restored upon expression of TrkA (Loeb et al., 1994). In support of TrkA as the mediator of NGF's biological functions, it was shown that TrkA overexpression in PC12 cells increased the rate of neurite outgrowth in response to NGF (Hempstead et al., 1992).

Since NGF is a target-derived factor for sympathetic neurons, it is believed that its mode of action for promoting neuronal survival is retrograde signaling. Both NGF itself and tyrosine phosphorylated Trk receptors are retrogradely transported in sympathetic neurons (Hendry et al., 1974; Johnson et al., 1978; Korsching and Thoenen, 1983; Palmatier et al., 1984; Nagata et al., 1987; Tsui-Pierchala and Ginty, 1999), but the retrograde signal carrier that mediates NGF-dependent survival of sympathetic neurons is not clear.

Upon NGF binding to TrkA, receptor dimerization occurs and kinase activity is induced, leading to autophosphorylation of tyrosine residues (Kaplan et al., 1991a, b; Klein et al., 1991). These phosphorylated tyrosine residues act as docking sites for a number of adapter proteins, which contain PTB or SH2 domains. This allows downstream substrates to associate and interact with TrkA (Koch et al., 1991; van der Geer and Pawson, 1995), thus coupling Trk to a number of intracellular signaling pathways, such as the Ras-MEK-MAPK and PI-3 kinase-Akt pathways (reviewed in Stephens and Kaplan, 1994; Kaplan and Miller, 1997, 2000).

In this fashion, NGF initiates a series of downstream signaling events in sympathetic neurons, such as the activation of the small GTP-binding protein p21RAS (Segal and Greenberg,1996; reviewed in Kaplan and Miller, 1997, 2000). Ras, was the first neurotrophin-activated signaling protein that was shown to mediate the survival of sympathetic neurons. It activates several downstream effectors, including Raf and PI3-

K (Rodriguez-Viciana et al., 1994; Vojtek and Der, 1998). Ras has been shown to be necessary and sufficient for mediating NGF-induced survival of both PC12 cells and cultured rat sympathetic neurons, since antibodies directed against Ras, or functional inhibition of endogenous Ras by dominant-inhibitory Ras will inhibit the survival of sympathetic neurons (Nobes and Tolkovsky, 1995; Mazzoni et al.,1999). Alternatively, constitutively-active Ras was able to rescue cultured sympathetic neurons from NGF withdrawal (Mazzoni et al., 1999).

Since constitutively-active Ras and dominant-inhibitory Ras were able to rescue or inhibit survival by approximately 50 percent (Mazzoni et al., 1999), this implies that other signaling pathways exist which might act with Ras to regulate sympathetic neuron survival. In fact, Ras does not act directly to promote survival. Instead, it acts as a molecular "switch," directing NGF-generated signals into a number of signaling pathways. Two of these pathways, PI3-K and its downstream substrate, the serine/threonine kinase Akt and the MEK/MAPK pathway are the major downstream effectors of neurotrophin and Ras-activated survival (Kaplan and Miller, 2000). The first piece of evidence supporting a direct interaction between Ras and PI3-K was reported in PC12 cells (Rodriguez-Viciana et al., 1994) where it was shown that inhibition of Ras suppressed NGF-mediated PI 3-K activity, and more recently, it was shown that Ras-mediated survival could be blocked pharmacologically by inhibiting PI3-K (Mazzoni et al., 1999).

PI3-K activity has been shown to be responsible for the NGF-mediated survival of cultured rat sympathetic neurons (Crowder and Freeman, 1998), since constitutively active PI 3-K or Akt were able to rescue rat sympathetic neurons from NGF withdrawal-induced cell death (Crowder and Freeman, 1998), and pharmacological inhibition of PI3-K is able to inhibit both Ras- and NGF-dependent survival (Crowder and Freeman, 1998; Mazzoni et al., 1999). Since both Ras and PI3-K are involved in NGF-mediated sympathetic neuron survival (Nobes and Tolkovsky, 1995; Crowder and Freeman, 1998), and PI 3-K has been shown to be a downstream effector of Ras, this suggested that Ras and PI3-K share a comon survival pathway.

A recent study of PI3-K signaling in sympathetic neurons has shown that this NGF/TrkA effector can relay different functional signals in spatially distinct parts of

the same neuron during long-range retrograde signaling. Ginty and colleagues (Kuruvilla et al., 2000), using compartmented cultures, found that PI3-K signaling in distal axons, but not cell bodies, was required for the initiation (but not propagation) of retrograde NGF transport and signaling. Since PI3-K signaling was required to maintain survival of sympathetic neurons receiving only distally-applied NGF, and PI3-K signaling in the cell body contributes to the survival of sympathetic neurons, PI3-K signaling in distal axons appears to control PI3-K signaling in the cell body; PI3-K activity within cell bodies is required to generate activated Akt (a target of PI3-K acvtivity; see below) in cell bodies, since activated Akt generated by PI3-K signaling in distal axons will not translocate to the cell body. Thus PI3-K signaling in both axons and cell bodies contributes to the survival of sympathetic neurons supported only by distally-applied NGF. PI3-K signaling within the cell body is thought to be critical for neuronal survival as it promotes Akt activation, as well as the activation of other downstream effectors of survival. The authors of this study suggested that PI3-K signaling in distal axons was an indirect contributor to neuronal survival, as it is necessary for the initiation of retrograde transport and signaling.

The serine/threonine kinase Akt is a major target of PI3-K activity (Andjelkovic et al., 1998; Ashcroft et al., 1999), but it is likely that there are other targets as well. Akt generates its survival-promoting activity by inhibiting the activity of apoptotic proteins. Studies utilizing dominant-inhibitory Akt in sympathetic neurons have shown that Akt is necessary for about 80% of NGF-mediated survival (Crowder and Freeman, 1998; Vaillant et al., 1999; Virdee et al., 1999; Kaplan and Miller, 2000). It has recently been demonstrated that NGF plus neuronal activity synergize to increase neuronal survival through PI 3-K and Akt. Sympathetic neurons treated with suboptimal concentrations of NGF plus KCl were able to maintain survival through a synergistic mechanism, whereby concentration of these agents that were not previously permissive for survival, now acted together to maintain neuronal survival (Vaillant et al., 1999). Thus, in this context, depolarization and neurotrophic factors seem to act together to optimize survival, an effect that could be reversed by inhibition of PI 3-K.This synergy stimulated maximal Akt activity and neuronal survival, which suggests that Akt is potentially a convergence point for different types of survival signals (Kaplan and

Miller, 2000). What this suggests is that during development, at stages where survival signals are suboptimal, neurons which are active or those which have received many inputs from other neuronal cells may have a competitive edge over those that are inactive, due to increased Akt activity in these active cells. This could potentially have some relevance during the time of sympathetic target innervation, when later-arriving neurons that may already have multiple inputs from other neurons, could out-compete those later-arriving neurons that do not. This might be particularly relevant to sympathetic neurons, since in these cells, dendritic growth occurs later on during development (Voyvodic, 1987), when target-derived NGF might be limiting.

The Ras-MEK-MAPK signaling pathway may also be used to promote neuronal survival, but unlike the PI 3-K pathway, may also help to protect neurons from injury or toxicity-induced death, rather than from the absence of trophic factors. This pathway seems to have a more prominent role in TrkB than in TrkA-mediated survival. Sympathetic neurons engineered to express TrkB have been shown to use both the Ras-MEK-MAPK pathway and the PI3-K pathways to signal survival, whereas TrkA uses only the latter (Atwal et al., 2000). With respect to axonal growth of sympathetic neurons, both PI3-K and MEK proteins work together to regulate local axonal growth (Atwal et al., 2000). The MEK-ERK pathway has been shown to phosphorylate microtubule-associated proteins, such as tau (Roder et al., 1993; Garcia Rocha and Avila, 1995) that regulate the stability of microtubules and regulate axonal elongation, and MAPK/ERKs can also phosphorylate neurofilament proteins (Veeranna et al., 1998). In like fashion, PI3-K also associates with or regulates a number of cytoskeletal proteins such as actin (Rodriguez-Viciana et al., 1997), tubulin (Kapeller et al., 1995), and actin-regulating protein (Toker and Cantely, 1997).

There is evidence from PC12 cells that Trk receptors signal growth versus survival differently, depending on whether they are internalized, or whether they are in the plasma membrane. NGF-mediated survival is optimal when Trk receptors remain in the plane of the membrane, where they mediate prolonged activation the PI3-K/Akt survival pathway. Alternatively, neurite outgrowth requires receptor internalization (Zhang et al., 2000). The authors proposed that after ligand binding, Trk activation, and and receptor endocytosis, Trk receptors within coated vesicles are protected from

deactivation by membrane-embedded phosphatases (Tisi et al., 2000). They then remain phosphorylated and catalytically active. This enables them to optimally active substrates that are required for differentiative responses, such as as neurite outgrowth, but also prevents them from supporting efficient survival because they cannot efficiently activate Akt. A similar separation of trophic and neuritogenic signaling was reported based on the rate of TrkA receptor internalization in PC12 cells, with rapidly internalizing NGF-TrkA complexes mediating survival, while slowly internalizing ligands were associated with neuritogenesis (Saragovi et al., 1998). This suggest that the location of activated Trk receptors regulates signaling and ultimately, biological responses.

b) **Trk signaling and the p75NTR**

Recent studies suggest that the ability of the p75NTR to mediate negative signals is dependent upon the level of Trk activation (Bamji et al., 1998; Davey and Davies, 1998). More specifically, the ability of the p75NTR to signal is decreased by concomitant activation of the Trk receptor, and neurotrophin-induced apoptosis through the p75NTR occurs much more effectively when Trk receptors are not activated (Davey and Davies, 1998; Yoon et al 1998) or suboptimally activated (Bamji et al., 1998). In cell lines that do not express Trk but do express the p75NTR, Trk expression can suppress the ability of NGF to induce the hydrolysis of sphingomyelin which generates ceramide, an apoptotic mediator (Dobrowsky et al., 1995).

Trk receptor activation is able to silence the p75NTR-mediated JNK-p53-BAX cell death pathway in oligodendrocytes (Yoon et al., 1998) and sympathetic neurons (Aloyz et al., 19998. In sympathetic neurons, the JNK cascade can also be suppressed by Ras activation (Mazzoni et al., 1999). The JNK-p53-BAX apoptotic pathway is activated by p75NTR activation and after NGF withdrawal (Aloyz et al., 1998).

Although Trk activation modulates p75NTR signaling, there is bi-directional cross talk between Trk and p75NTRs, with the p75NTR also able to modulate some Trk signaling pathways (Kaplan and Miller, 2000). The finding in sympathetic neurons, that the p75NTR is only able to signal apoptosis when Trk is not activated, or when Trk activation is suboptimal (Bamji et al., 1998), suggests that Trk signaling silences

apoptotic signals generated through the p75NTR. Further evidence for this is provided by observations of robust TrkA activation blocking the p75NTR-mediated death of trigeminal sensory neurons (Davey and Davies, 1998), and inhibition of NGF-induced apoptosis in oligodendrocytes ectopically expressing TrkA (Yoon et al., 1998). Thus p75NTR-signaling events in response to neurotrophin activation are a function of Trk receptor repertoire or activation status.

REFERENCES

Acheson A, Barde YA, Thoenen H (1987). High K^+-mediated survival of spinal sensory neurons depends on developmental stage. Exp Cell Res 170: 56-63.

Acheson A, Barker PA, Alderson RF, Miller FD, Murphy RA (1991). Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to nerve growth factor. Neuron 7: 265-275.

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

Albers KM, Perrone TN, Goodness TP, Jones ME, Green MA, Davis BM (1996). Cutaneous overexpression of NT-3 increases sensory and sympathetic neuron number and enhances touch dome and hair follicle innervation. J Cell Biol 134: 487-497.

Albers KM, Wright DE, Davis BM (1997). Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. J Neurosci 14: 1422-1432.

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD (1998) P53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol 143: 1691-1703.

Andjelkovic M, Suidan HS, Meier R, Frech M, Alessi DR, Hemmings BA (1998). Nerve growth factor promotes activation of the alpha, beta, and gamma isoforms of protein kinase B in PC12 pheochromocytoma cells. Eur J Biochem 251: 195-200.

Armstrong DM, Brady R, Hersh LB, Hayes RC, Wiley RG (1991). Expression of choline acetyltransferase and nerve growth factor receptor within hypoglossal motoneurons following nerve injury. J Comp Neurol 304: 596 –607.

Ashcroft M, Stephens RM, Hallberg B, Downward J, Kaplan DR (1999). The selective and inducible expression of endogenous PI 3-kinase in PC12 cells results in NGF-mediated survival, but defective neurite outgrowth. Oncogene18: 4586-4597.

Atwal JK, Massie B, Miller FD, Kaplan DR (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and PI3-Kinase. Neuron 27: 265-277.

Baldwin AN, Bitler CM, Welcher AA, Shooter EM (1992). Studies on the structure and binding properties of the cysteine rich domain of rat low affinity nerve growth factor receptor (p75NGFR). J Biol Chem 267: 8352-8359.

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.

Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, Loetscher H, Lesslauer W (1993). Crystal structure of the soluble human 55kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. Cell 73: 431-445.

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

Barde YA (1989). Trophic factors and neuronal survival. Neuron 2:1525-1534.

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 PC12cells. Neuron 13:203-215.

Barker PA (1998). P75NTR: A study in contrasts. Cell Death Differ 5: 346-356.

Bartlett GL (2000). The p75 neurotrophin receptor and neuronal apoptosis. Prog Neurobiol 61: 205-229.

Baxter GT, Radeke MJ, Kuo RC, Makrides V, Hinkle B, et al. (1997). Signal transduction mediated by the truncated TrkB receptor iso-forms, TrkB.T1 and TrkB.T2. J Neurosci 17: 2683–90.

Belliveau, DJ, Krivko I, Kohn J, Lachance C, Pozniak C, Rusakov D, Kaplan D, Miller FD (1997). NGF and NT-3 both activate TrkA on sympathetic neurons, but differentially regulate survival and neuritogenesis. J Cell Biol 136: 374-388.

Benedetti M, Levi A, Chao MV (1993). Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci USA 90: 7859-7863.

Bentley D, O'Connor T (1994). Cytoskeletal events in growth cone steering. Curr Opin Neurobiol 4: 43-48.

Berkmeier LR, Winslow JW, Kalan DR, Nikolics K, Goeddel DV, Rosenthal A (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7: 857-866.

Bibel M, Hoppe E, Barde Y-A (1999). Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18: 616-622.

Birren SJ, Lo L, Anderson DJ (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development 119: 597-610.

Bocchini V and Angeletti PU (1969). The nerve growth factor: Purification as a 30,000-molecular-weight-protein. Proc Natl Acad Sci USA 64: 787-794.

Boeshore KL, Luckey CN, Zigmond RE, Large TH (1999). TrkB isoforms with distinct neurotrophin specificities are expressed in predominantly nonoverlapping populations of avian dorsal root ganglion neurons. J Neurosci 19: 4739-4747.

Bothwell M and Shooter EM (1977). Dissociation equilibrium constant of β-nerve growth factor. J Biol Chem 252: 8532-8536.

Bothwell M (1995). Functional interactions of neurotrophins and their receptors/ Annu Rev Neurosci 18: 223-253.

Bowers CW, Zigmond RE (1982). The influence of the frequency and pattern of sypathetic nerve activity on serotonin N-acetyltransferase in the rat pineal. J Physiol Lond 330: 279-296.

Bowers CW, Dahm LM, Zigmond RE (1984). The number and distribution of of sympathetic neurons that innervate the rat pineal gland. Neuroscience 13: 87-96.

Boyd JG, Posse de Chaves E, Gordon T (2000). Inhibitory effect of high dose brain-derived neurotrophic factor on motor axonal regeneration may be mediated bya ceramide-dependent mechanism. Soc Neurosci Abstr 26: 844.

Bredesen DE, Rabizadeh S (1997). P75 NTR and apoptosis: Trk-dependent and Trk-independent effects. Trends Neurosci 20: 287-290.

Buck CR, Martinez HJ, Black IB, Chao MV (1987). Developmentally regulated expression of the nerve growth receptor gene in the periphery and brain. Proc Natl Acad Sci USA 84: 3060-3063.

Bueker ED (1948). Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbosacral nervous system. Anat Rec 102: 369-390.

Buxser SE, Kelleher DJ, Watson L, Puma P, Johnson GL (1983). Change in state of nerve growth factor receptor. Modulation of receptor affinity by wheat germ agglutinin. J Biol Chem 258: 3741-3749.

Cahoon-Metzger S, Wang G, Scott SA (2001). Contribution of BDNF-mediated inhibition in patterning avian skin innervation. Dev Biol 232: 246-254.

Campenot RB (1977). Local control of neurite development by nerve growth factor. Proc Natl Acad Sci USA 74: 4516-4519.

Campenot R (1982a). Development of sympathetic neurons in compartmentalized cultures: I. Local control of neurite growth by nerve growth factor. Dev Biol 93: 1-12

Campenot R (1982b). Development of sympathetic neurons in compartmentalized cultures: II. Local control of neurite survival by nerve growth factor. Dev Biol 13-21.

Camepnot RB (1987). Local promotion of neurite sprouting in cultured sympathetic neurons by nerve growth factor. Dev Brain Res 37: 293-301.

Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Kong H, Chao MV (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383: 716-719.

Casaccia-Bonnefil P, Kong H, Chao MV (1998). Neurotrophins: the biological paradox of survival factors eliciting apoptosis. Cell Death Diff 5: 357-364.

Causing CG, Gloster A, Aloyz R, Bamji SX, Chang E, Fawcett J, kuchel G, Miller FD (1997). Symaptic innervation density is regulated by neuron-derived BDNF. Neuron 18: 257-267.

Challacombe JF, Snow D, Letourneau PC (1996). Role of the cytoskeleton in growth cone motility and axonal elongation. Semin Neurosci 8: 67-80.

Chalazonitis A (1996). Neurotrophin-3 as an essential signal for the developing nervous system. Molecular Neurobiology 12: 39-53.

Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, Sehgal A (1986). Gene transfer and molecular cloning of the human NGF receptor. Science 232: 518-521.

Chao, MV, Hempstead BL (1995). P75 and Trk: a two receptor system. Trends Neurosci 18: 321-326.

Chun LLY, Patterson PH (1977). Role of nerve growth factor in the development of rat sympathetic neurons in vitro. I. Survival, growth, and differentiation of catecholamine production. J Cell Biol 75: 694-704.

Clary DO, Reichardt LF (1994). An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc Natl Acad Sci USA

91: 11133–37.

Cohen S (1960). Purification of a nerve-growth promoting protein from mouse salivary gland and its neurocytotoxic antiserum. Proc Natl Acad Sci 46: 302-311.

Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF et al. (1991). The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66: 173-183.

Crowder RJ, Freeman RS (1998). Phosphatidyl inositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18: 2933-2943.

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.

Dafney N (1980). Photic input to rat pineal gland conveyed by both sympathetic and central afferents. J Neural Transm 48: 203-208.

Davey F, Davies AM (1998). TrkB signaling inhibits p75-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. Curr Biol 8: 915-918.

Davies AM, Bandtlow C, Heumann R, Korsching S, Rohrer H, Thoenen H (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature 326: 353-358.

Davies AM (1996). Paracrine and autocrine actions of neurotrophic factors. Neurochem Res 21: 749-753.

Davies AM, LeeKF, Jaenisch R (1993). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron 11: 565-574.

Davis BM, Fundin BT, Albers KM, Goodness TP, Cronk KM, Rice FL (1997). Overexpression of nerve growth factor in skin causes preferential increases among innervation to specific sensory targets. J Comp Neurol 387: 489-506.

Deckwerth TL, Elliott JL, Knudson CM, Johnson EM, Snider WD, Korsmeyer, SJ (1996). Bax is required for neuronal death in adult sensory neurons after trophic factor deprivation and during development. Neuron 17: 401–411.

Dobrowsky RT, Jenkins GM, HannunYA (1995). Neurotrophins Induce Sphingomyelin Hydrolysis. Modulation by co-expression of p75NTR with trk receptors. J Biol Chem 270: 22135-22142.

DiCiccio-Bloom E, Friedman WJ, Black IB (1993). NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. Neuron 11:1101-1111.

DiMarco, E Marchisio PC, Bondanza S, Franzi AT, Cancedda R, De Luca M (1991). Growth-regulated synthesis and secretion of biologically active nerve growth factor by human keratinocytes. J Biol Chem 266: 21718-21722.

DiMarco E, Mathor M, Bondanza S, Cutuli N, Marchisio PC, Cancedda R (1993). 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 268: 22838-22846.

Dusart I, Morel MP, Sotelo C (1994). Parasagittal compartmentation of adult rat Purkinje cells expressing the low-affinity nerve growth factor receptor: changes of pattern expression after a traumatic lesion. Neuroscience 63:351–356.

Edwards RM, Rutter WJ, Hanahan D (1989). Directed expression of NGF to pancreatic ß cells in transgenic mice leads to selective hyperinnervation of the islets. Cell 58: 161-170.

Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF (1996). Naturally occurring truncated TrkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 16: 3123–29.

Elshamy WM, Linnarsson S, Lee KF, Jaenisch R, Ernfors P (1996). Prenatal and postnatal requirements of NT-3 for sympathetic neuroblast survival and innervation of specific targets. Development 122: 491-500.

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

Ernfors P, Henschen A, Olson L, Persson H (1989). Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2:1605–1613.

Ernfors P, Ibañez CF, Ebendal T, Olson L, Persson H (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographic expression in the brai. Proc Natl Acad Sci (USA) 87: 5454-5458.

Ernfors P, Merlio JP, Persson H (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. Eur J Neurosci 4: 1140-1158.

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

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

Esposito D, Patel P, Stephens RM , Perez P, Chao MV, Kaplan DR, Hempstead BL (2001). The cytoplasmic and transmembrane domains of the p75 and TrkA receptors regulate high affinity binding to NGF. J Biol Chem (in press).

Fagan AM, Zhang H, Landis S, Smeyne RJ, Silos-Santiago I, Barbacid M (1996). TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. J Neurosci 16: 6208-18.

Fan J, Raper JA (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron 14: 263-274.

Fariñas I, Jones K, Backus C, Wang X, Reichardt L (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369: 658-661.

Ferri CC, Moore FA, Bixby MA (1998). Effects of facial nerve injury on mouse motoneurons lacking the p75 low-affinity neurotrophin receptor. J Neurobiol 34:1-9.

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

Frazier WA, Boyd LF, Bradshaw RA (1974). Properties of the specific binding of ^{125}I-nerve growth factor to responsive peripheral neurons. J Biol Chem 249: 5513-5519.

FryerRH, Kaplan DR, Kromer LF (1997). Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite out growth in vitro. Exp Neurol 148: 616-627.

Gallo G, Lefcort FB, Letourneau PC (1997). The TrkA receptor mediates growth cone turning toward a localized source of nerve growth factor. J Neurosci 17: 5445–5454.

Garcia Rocha M, Avila J (1995). Characterization of microtubule-associated protein phosphoisoforms present in isolated growth cones. Brain Res. Dev Brain Res 89: 47–55.

Garcia-Suarez O, Naves FJ, Del Valle ME, Esteban I, Bronzetti E, Vasquez E, Vega JA (1996). Distribution of p75 and trl-neurotrophin receptor proteins in adult human sympathetic ganglia. Anat Embryol (Berl) 193: 577-593.

Gargano N, Levi,A. and Alema,S (1997). Modulation of nerve growth factor internalization by direct interaction between p75 and TrkA receptors. J Neurosci Res 50:1–12.

Garner AS, Menegay HJ, Boeshore KL, Xie XY, Voci JM, et al (1996). Expression of TrkB receptor isoforms in the developing avian visual system. J. Neurosci. 16:1740–52.

Godfrey EW and Shooter EM (1986). Nerve growth factor receptors on chick embryo sympathetic ganglion cells: binding characteristics and development. J Neurosci 6: 2543-2550.

Gomez TM, Letourneau PC (1994). Filopodia initiate choices made by sensory neuron growth cones at laminin/fibronectin borders *in vitro*. J Neurosci 14: 5959-5972.

Goodman CS (1996). Mechanisms and molecules that control growth cone guidance. Annu Rev Neurosci 19:341-377.

Götz R, Koster R, Winkier 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.

Greene LA and Kaplan DR (1995). Early events in neurotrophin signalling via TrkA and p75 receptors. Curr Opin Neurobiol 5: 579-587.

Grob PM, Berlot CH, Bothwell MA (1983). Affinity labeling and partial purification of nerve growth factor receptors from rat pheochromocytoma and human melanoma cells. Proc Natl Acad Sci USA 80: 6819-6823.

Guidry G, Landis SC, Davis BM, Albers KN (1998). Overexpression of nerve growth factor in epidermis disrupts the distribution and properties of sympathetic innervation in footpads. J Comp Neurol 393: 231-243.

Guiton M, Gunn-Moore FJ, Glass DJ, Geis DR, Yancopoulos GD, Tavare JM (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: 20384–90.

Gunderson RW, Barrett JN (1980). Characterization of the turning response of dorsal root neurites toward nerve growth factor. J Cell Biol 87: 546-554.

Hakanson R, Lombard des Gouttes M-N, Owman C (1967). Activities of tryptophan hydroxylase, dopa decarboxylase, and monoamine oxidase as correlated with the appearance of monoamines in developing rat pineal gland. Life Sci 6: 2577-2585.

Halböök F, Ibañez 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.

Hamburger V(1977). The developmental history of the motor neuron. Neurosci Res Prog Bull 15 (suppl III): 1-37.

Hannila SS, Kawaja MD (1999). Nerve growth factor-induced growth of sympathetic axons into the optic tract of mature mice is enhanced by an absence of p75NTR expression. J Neurobiol 39: 51-66.

Hapner SJ, Boeshore KL, Large TH, Lefcort F (1998). Neural differentiation promoted by
truncated TrkC receptors in collaboration with p75(NTR). Dev Biol 201: 90–100.

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.

Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, Kaplan DR (1992). Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron 9: 883-896.

Hendry IA, Stockel K, Thoenen H, Iversen LL (1974). The retrograde axonal transport of nerve growth factor. Brain Res 68: 103-121.

Heumann R, Korsching S, Scott J, Thoenen H (1984). Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. EMBO J 3: 3183-3189.

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

Heumann R, Lindholm D, Bandtlow C, Meyer M, Radeke MJ, Misko TP, Shooter E, Thoenen H (1987b). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerves during development, degeneration and regeneration. Proc Natl Acad Sci USA 84: 8735-8739.

Higgins GA, Koh S, Chen KS, Gage FH (1989). NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. Neuron 3: 247-256.

Hohn ALJ, Bailey K, Barde YA (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 334: 339-341.

Hoyle GW, Mercer EH, Palmiter RD, Brinster RL (1993). Expression of NGF in sympathetic neurons leads to excessive axon outgrowth from ganglia but decreased terminal innervation within tissues. Neuron 10: 1019-1034.

Huang EJ, Reichardt LF (2001). Neurotrophins: roles in neuronal development and function. Ann Rev Neurosci 24:677–736.

Huber LJ, Chao MV (1995). A potential interaction of p75 and TrkA receptors revealed by affinity cross-linking and immunoprecipitation. J Neurosci Res 40: 557-563.

Ibáñez CF, Ilag LL, Murray-Rust J, Persson H (1993). An extended surface of binding of Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. EMBO J: 2281-2283.

Ip, NY, Ibañez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, LeBeau MM, Espinosa R, Squinto SP, Persson H, Yancopoulos GD (1992). Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution and receptor specificity. Proc Natl Acad Sci USA 89:3060-3064.

Ip NY, Li Y, Yancopoulos GD, , Lindsay RM (1993a). Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J Neurosci 13: 3394-3405.

Ip NY, Stitt Tn, Ttaapley P, Klein R, Glass DJ, Fandl J, Green LA, Barbacid M, Yancopoulos GD (1993b). Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. Neuron 10: 137-149.

Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 126: 801-810.

Jing S, Tapley P, Barbacid M (1992). Nerve growth factor mediates signal transduction through Trk homodimer receptors. Neuron 9: 1067-1079.

Johnson EM, Andres RY, Bradshaw RA (1978). Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I] NGF. Brain Res 150: 319-331.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao MV (1986). Expression and structure of the human NGF receptor. Cell 47: 545-554.

Jones KJ, Reichardt LF (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.

Kapeller R, Toker A, Cantley LC, Carpenter CL (1995). Phosphoinositide 3-kinase binds constitutively to alpha/beta- tubulin and binds to gamma-tubulin in response to insulin. J Biol Chem 270: 25985–25991.

Kaplan DR, Martin-Zanca D, Parada LF (1991a). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350: 158-160.

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

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

Kaplan DR, Miller FD (1997). Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 9: 213-221.

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

Kaplan D, Zirrgiebel U, Atwal J (2000). Center stage for NGF on peripheral (but not central) sensory neuron outgrowth. Neuron 25: 253-259.

Kappers JA (1960). The development, topographical relations and innervation of the *epiphysis cerebri* in the albino rat. Z Zellforsch mikrosk Anat 52: 163-215.

Katoh H, Aoki J, Ichikawa A, Negishi M (1998). P160 RhoA-binding kinase ROKα induces neurite retraction. J Biol Chem 273:2489-2492.

Katoh-Semba R, Takeuchi IK, Semba R, Katoh K (1997). Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. J Neurochem 69:34-42.

Kawaja MD, Crutcher KA (1997). Sympathetic axons invade the brains of mice overexpressing nerve growth factor. J Comp Neurol 383: 60-72.

Kawaja MD, Walsh GS, Petrucelli K, Coome GE (1997). Sensory nociceptive axons invade the cerebellum of transgenic mice overexpressing nerve growth factor. Brain Res 774: 77-86.

Kimpinski K, Campenot RB, Mearow K (1997). Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. J Neurbiol 33: 395-410.

Klein R, Parada LF, Coulier F, Barbacid M (1989). trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J 8: 3701-3709.

Klein R, Conway D, Parada LF, Barbacid M (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic domain. Cell 61: 647-656.

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

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991b). The trkB tyrosin protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66: 395-403.

Klein R, Lamballe F, Bryant S, Barbacid M (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. Neuron 8: 947--56.

Koch CA, Anderson D, Moran MF, Ellis C, Pawson T (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252: 668-674.

Kohn J, Aloyz RS, Toma JG, Haak-Frendscho M, Miller FD (1999). Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. J Neurosci 19: 5393-5408.

Kokaia Z, Andsberg G, Martinez-Serrano A, Lindvall O (1998). Focal cerebral ischemia in rats induces expression of p75 neurotrophin receptor in resistant striatal cholinergic neurons. Neuroscience 84:1113–1125.

Korf HW, Wagner U (1980). Evidence for a nervous connection between the brain and pineal organ in the guinea pig. Cell Tissue Res 209: 505-510.

Korsching S, Thoenen H (1983a). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. Proc Natl Acad Sci USA 80: 3513-3516.

Korsching S, Thoenen H (1983b). Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. Neurosci Lett 39: 1-4.

Korsching S, Thoenen H (1985). Treatment with 6-OH-dopamine and colchicine decreases nerve growth factor levels in sympathetic ganglia and increases them in the corresponding target tissues. J Neurosci 5: 1058-1061.

Korsching S, Thoenen H (1988). Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev Biol 126: 40-46.

Kouchalakos RN and Bradshaw RA (1986). Nerve growth factor receptor from rabbit sympathetic ganglia membranes. Relationship between subforms. J Biol Chem 261: 16054-16059.

Krimm RK, Miller KK, Kitzman PH, Davis BM, Albers KM (2001). Epithelial overexpression of BDNF or NT4 Disrupts targeting of taste neurons that innervate the anterior tongue. Dev Biol 232: 508-521.

Kuhn TB, Schmidt MF, Kater SB (1995). Laminin and fibronectin guidepost signal sustained but opposite effects to passing growth cones. Neuron 14: 275-285.

Kuruvilla R, Ye H, Ginty DD (2000). Spatially and functionally distinct roles of the PI3-kinase effector pathway during NGF signaling in sympathetic neurons. Neuron 27: 499-512.

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 (1991). trkC, a new member of the trk family of tyrosine protein kinases, si a receptor for neurotrophin-3. Cell 66: 967-979.

Large TH, Weskamp G, Helder JC, Radeke MJ, Misko TP, Shooter EM, Reichardt LF (1989). Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. Neuron 2: 1123-1134.

Lee K-F, 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 K-F, Bachman K, Landis S, Jaenisch(1994a). Dependence on p75 for innervation of some sympathetic targets. Science 263: 1447-1449.

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

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999). Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19: 7535-7547.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde YA (1989). Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341: 149-152.

LeMaster AM, Krimm RF, Davis BM, Noel T, Forbes ME, Johnson JE, Albers KM (1999). Overexpression of brain-derived neurotrophic factor enhances sensory innervation and selectively increases neuron number. J Neurosci 19: 5919-5931.

Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD (1999). Neurotrophins support the development of diverse sensory axon morphologies. J Neurosci 19: 1038–1048.

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

Levi-Montalcini R, Angeletti PU (1968). The nerve growth factor. Physiol Rev 48: 534-569.

Levi-Montalcini R, Booker B (1960a). Excessive growth of the sympathetic ganglia evoked by a protein isolated from mouse salivary glands. Proc Natl Acad Sci USA 46: 373-381.

Levi-Montalcini R, Booker B (1960b). Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve growth protein. Proc Natl Acad Sci USA 46: 381-390.

Levi Montalcini R, 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, Hamburger V (1953). A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. J Exp Zool 123: 233-287.

Levi-Montalcini R, Meyer H, Hamburger V (1954). In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Cancer Research 14: 49-57.

Lin C, Thompson CA, Forscher P (1994). Cytoskeletal reorganization underlying growth cone motility. Curr Opin Neurobiol 4: 640-647.

Lindholm D, Carroll P, Tzimagiogis G, Thoenen H (1996). Autocrine-paracrine regulation of hippocampal neuron survival by IGF-1 and the neurotrophins BDNF, NT-4 and NT-3. Eur J Neurosci 8: 1452-1460.

Lindsay RM, Thoenen H, Barde YA (1985). Placode and sensory neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. Dev Biol 112:319-328.

Loeb DM, Stephens RM, Copeland T, Kaplan DR, Greene LA (1994). A Trk nerve growth (NGF) factor point mutation affecting interaction with phospholipase C-gamma 1 abolishes NGF-promoted peripherin induction but not neurite outgrowth. J Biol Chem 269: 8901-8910.

Ma Y, Campenot RB, Miller FD (1992). Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117:135-141.

Machado AB, Machado CR, Wragg LE (1968a). Catecholamines and granular vesicles in adrenergic axons of the developing pineal body of the rat. Experienta 24: 464-465.

Machado CRS, Wragg LE, Machado ABM (1968b). A histichemical study of sympathetic innervation and 5-hydroxytryptamine in the developing pineal body of the rat. Brain Res 8: 310-318.

Machado ABM (1971). Electron microscopy of developing sympathetic fibers in the rat pineal body. The formulation of granular vesicles. Prog Brain Res 34: 171-185.

Mackay DJG, Nobes CD, Hall A (1996). The Rho's progress: a potential role during neuritogenesis for the Rho family GTPases. Trends Neurosci 18: 496-501.

Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994). High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. J Biol Chem 269: 6884-6891.

Maina F, Hilton MC, Andres R, Wyatt S, Klein R, Davies AM(1998). Multiple roles for hepatocyte growth factor in sympathetic neuron development.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990). Neurotrophon-3: a new neurotrophic factor related to NGF and BDNF. Science 247: 1446-1451.

Majdan M, Lachance C, Gloster A, Aloyz R, Zeindler C, Bamji S, Bhakar A, Belliveau D, Fawcett J, Miller FD, Barker PA (1997). Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. J Neurosci 17:6988-6998.

Majdan M, Miller FD (1999). Neuronal life and death decisions: functional antagonism between the Trk and p75 neurotrophin receptors. Int J Dev Neurosci 17: 153-161.

Majdan M, Aloyz R, Miller FD (2001). TrkA is not required to maintain sympathetic neuron survival in the absence of p75. In revision for publication in J Cell Biol.

Marano N, Dietzschold B, Earley JJ Jr, Schatteman G, Thompson S, Grob P, Ross AH, Bothwell M, Atkinson BF, Koprowski H (1987). Purification and amino terminal sequencing of human melanoma nerve growth factor receptor. J Neurochem 48: 225-232.

Martinez-Murillo R, Fernandez AP, Bentura ML, Rodrigo J (1998) Subcellular localization of low-affinity nerve growth factor receptor-immunoreactive protein in adult rat purkinje cells following traumatic injury. Exp Brain Res 119: 47–57.

Martin-Zanca D, Hughes SH, Barbacid M (1986). A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature 319: 743-748.

Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M (1989). Molecular and biochemical characterization of the human trk proto-oncogene. Mol Cell Biol 9: 24-33.

Massagué J, Guillette BJ, Czech MP, Morgan CJ, Bradshaw RA (1981). Identification of a nerve growth factor receptor protein in sympathetic ganglia membranes by affinity labeling. J Biol Chem 256: 9419-9424.

Mathew TC, Miller FD (1990). Increased expression of Tα1 α-tubulin mRNA during collateral and NGF-induced sprouting of sympathetic neurons. Dev Biol 141: 84-92.

Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D (1999). Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J Neurosci 19: 9716-9727.

Meakin SO, Shooter (1992). The nerve growth factor family of receptors. Trends Neurosci. 15: 323-331.

Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM (1992). The rat Trk proto-oncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc. Natl. Acad. Sci. USA 89:2374–78.

Meakin SO, Gryz EA, MacDonald JI (1997). A kinase insert isoform of rat TrkA supports
nerve growth factor-dependent cell survival but not neurite outgrowth. J Neurochem 69: 954–67.

Menesini-Chen MG, Chen JS, Levi-Montalcini R (1978). Sympathetic nerve fibers ingrowth in the central nervous system of neonatal rodent upon intracerebral NGF injections. Arch Ital Biol 116: 53-84.

Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14: 949-959.

Miller FD (1994). Nerve growth factor and neuronal gene expression. Prog Brain Res 103: 23-33.

Miller FD, Mathew TC, Toma JG (1991). Regulation of nerve growth factor receptor gene expression by NGF in the developing peripheral nervous system. J Cell Biol 112: 303-312.

Miller FD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG (1994). Nerve growth factor derived from terminals selectively increases the ratio of p75 to TrkA NGF receptors on mature sympathetic neurons. Dev Biol 161: 206-217.

Miller FD, Kaplan FM (1998). Life and Death decisions: a biological role for the p75 neurotrophin receptor. Cell Death Diff 5: 343-345.

Ming GL, Lohof AM, Zheng JQ (1997). Acute morphogenic and chemotrophic effects of neurotrophins on cultured embryonic Xenopus spinal neurons. J Neurosci 17: 7860-7871.

Mischel PS, Smith SG, Vining ER, Valletta JS, Mobley WC, Reichardt LF (2001). The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. J Biol Chem 276: 11294-11301.

Mitchison T, Kirschner M (1998). Cytoskeletal dynamics and nerve growth. Neuron 1: 761-772.

Mueller BK (1999). Growth cone guidance: first steps towards a deeper understanding. Annu Rev Neurosci 22: 351-388.

Nagata Y, Ando M, Takahama K, Iwata M, Hori S, and Kato K (1987). Retrograde transport of endogenous nerve growth factor in superior cervical ganglion of adult rats. J Neurochem 49: 296-302.

Nobes CD, Tolkovsky AM (1995). Neutralizing anti-p21Ras Fabs suppress rat sympathetic neuron survival induced by NGF, LIF, CNTF and cAMP. Eur J Neurosci 7: 344-350.

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

Oakley RA, Tosney KW (1993). Contact-mediated mechanisms of motor axons segmentation. J Neurosci 13: 3773-3792.

O'Connor R, Tessier-Lavigne M (1999). Identification of maxillary factor, a maxillary process-derived chemoattractant for developing trigeminal sensory axons. Neuron 24: 165-178.

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

Orike N, Thrasivoulou C, Wrigley A, Cowan T (2001). Differential regulation of survival and growth in adult sympathetic neurons: an *in vitro* study of neurotrophin responsiveness. J Neurobiol 47: 295-305.

Owman C (1964). Sympathetic nerves probably storing two types of monoamines in the rat pineal gland. Int J Neuropharmacol 2:105-112.

Palmatier MA, Hartman BK, Johnson EM Jr (1984). Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. J Neurosci 4: 751-756.

Paves H, Saarma M (1997). Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons. Cell Tissue Res 290: 285–297.

Pincelli C, Sevignani C, Manfredini R, Grande A, Fantini F, Bracci-Laudiero L (1994). Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes. J Invest Dermatol 1994:103:13-18.

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

Pini A (1993). Chemorepulsion of axons in the developing mammalian CNS. Science 261: 96-98.

Puma P, Buxser SE, Watson L, Kelleher DJ, Johnson GL (1983). Purification of the receptor for nerve growth factor from A875 melanoma cells by affinity chromatography. J Biol Chem 258: 3370-3375.

Purves D, Snider WD, Voyvodic JT (1988). Trophic regulation of nerve cell morphology and innervation on the autonomic nervous system. Nature 336: 123-128.

Püschel AW, Adams RH, Betz H (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron 14: 941-948.

Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LI, Bredesen DE (1993). Induction of apoptosis by the low-affinity NGF receptor. Science 261: 345-348.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325: 593-597.

Raper JA (2000). Semaphorins and their receptors in vertebrates and invertebrates. Curr Opin Neurobiol 10: 88-94.

Ringstedt T, Ibanez CF, Nosrat CA (1999). Role of Brain-Derived Neurotrophic Factor in Target Invasion in the Gustatory System. J Neurosci 19:3507–3518.

Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR, Nikolics K, Winslow JW (1990). Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4: 767-773.

Roder HM, Eden PA, Ingram VM (1993). Brain protein-kinase PK40erk converts TAU into a PHF-like form as found in Alzheimer's disease. Biochem Biophys Res Commun 193: 639–647.

Rodriguez-Tebar A, Dechant G, Barde Y-A (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4: 487-492.

Rodriguez-Tebar A, Dechant G, Gotz R, 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.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370: 527-532.

Rodriguez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A, Downward J (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell 89: 457–467.

Ronnekleiv OK, Moller M (1979). Brain-pineal nervous connections in the rat: an ultrastructure study following habenular lesion. Expl Brain Res 37: 551-562.

Ross A, Daou M-C, McKinnon CA, Condon PJ, Lachyankar MB, Stephens RM, Kaplan DR, Wolf DE (1996). The neurotrophin receptor, gp75m forms a complex with the receptor tyrosine kinase TrkA. J Cell Biol 132: 945-953.

Roux PP, Colicos MA, Barker PA, Kennedy TE (1999). p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure. J Neurosci 19: 6887–6896.

Ruit KG, Osborne PA, Schmidt RE, Johnson EM, Snider WD (1990). Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. J. Neurosci 10: 2414-2419.

Ryden M, Murray Rust J, Glass D, Ilag LL, Trupp M, Yancopoulos GD, McDonald NQ, Ibañez CF (1995). Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75LNGFR in NT-4 signaling. EMBO J 14: 1979-1990.

Saragovi HU, Zheng WH, Maliartchouk S, DiGugliemoi BM, Mawal YR, Kamen A, Woo SB, Cuello AC, Debeir T, Neet KE (1998). A TrkA-selective, fast internalizing nerve growth factor-antibody complex induces trophic but not neuritogenic signals. J Biol Chem 52:34933-34940.

Schecter AL, Bothwell MA (1981). Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. Cell 24:

867-874.

Schecterson LC, Bothwell M (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9: 449-463.

Segal RA, Greenberg ME (1996). Intracellular signaling pathways activated by neurotrophic factors. Annu Rev Neurosci 19: 463-489.

Semm P, Schneider T, Vollrath L (1981). Morphological and electrophysiological evidence for habenular influence on the guinea-pig pineal gland. J Neural Transm 50: 247-266.

Shelton DL, Reichardt LF (1984). Expression of the β nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc Natl Acad Sci USA 81: 7951-7955.

Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, et al. (1995). Human Trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci 15: 477–91.

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.

Soppet D, Escandon E, Maragos J, Middlemaas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplna DR, Hunter T, et al (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the TrkB tyrosine kinase receptor. Cell 65: 895-903.

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

Stanley LC, Horikawa K, Powell EW (1987). Innervation of the superficial pineal of the rat using retrograde tracing methods. Am J Anat 180: 249-254.

Stemple DL, Anderson DJ, (1992). Isolation of a stem cell for neurons and glia form the mammalian neural crest. Cell 71: 973-985.

Strohmaier C, Carter B, Urfer R, Barde YA, Dechant G (1996). A splice variant of the neurotrophin receptor TrkB with increased specificity for brain-derived neurotrophic factor. EMBO J 15: 3332–37.

Stucky CL, Koltzenburg M, Schneider M, Engle MG, Albers KM, Davis BM (1999). Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. J Neurosci 19: 8509–16.

Sutter A, Riopelle RJ, Harris-Warwick RM, Shooter EM (1979). Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J Biol Chem 254: 5972-5982.

Tanaka E, Sabry E (1995). Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell 83: 171-176.

Thoenen H, Barde YA (1980). Physiology of nerve growth factor. Physiol Rev 60: 1284-1335.

Tigyi G, Fischer DJ, Sebok A, Yang C, Dyer DL, Miledi R (1996). Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca^{2+} signalling and Rho. J Neurochem 66: 537-548.

Tisi M, Xie Y, Yeo T, Longo F (2000). Downregulation of LAR tyrosine phsophatase prevents apoptosis and augments NGF-induced neurite outgrowth. J Neurobiol 42:477-486.

Toker A, Cantley LC (1997). Signaling through the lipid products of phosphoinositide-3-OH kinase. Nature 387: 673–676.

Toma JG, Rogers D, Senger DL, Campenot RB, Miller FD (1997). Spatial regulation of neuronal gene expression in response to nerve growth factor. Dev Biol 184: 1-9.

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

Tsoulfas P, Stephens RM, Kaplan DR, Parada LF (1996). TrkC isoforms with inserts in the
kinase domain show impaired signaling responses. J Biol Chem 271: 5691–97.

Tsouflas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez JL, Rosenthal A, Nikolics K, Parada LF (1993). The rat trkC locus encodes multiple neurogenic receptors that exhibit differential responses to neurotrophin-3 in PC12 cells. Neuron 10:975-990.

Tsui-Pierchala BA, Ginty DD(1999). Characterization of an NGF-P-TrkA retrograde signaling complex and age-dependent regulation of TrkA phosphorylation in sympathetic neurons. J Neurosci 19: 8207-8218.

Tucker KL, Meyer M. Barde Y-A (2001). Neurotrophins are required for nerve growth during development. Nat Neurosci 4: 29-37.

Tuttle R, O'Leary DD (1998). Neurotrophins rapidly modulate growth cone response to the axon guidance molecule, collapsin-1. Mol Cell Neurosci. 11: 1–8.

Ullrich A and Schlessinger J (1990). Signal transduction by receptors with tyrosine kinase activity. Cell 61: 203-212.

Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD (1999). Depolarization and neurotrophins converge on the phosphatidylinositol-3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 146: 955-966.

Valenzuela DM, Maisonpierre PC, Glass DJ, Rojas E, Nunez L, Kong Y, Gies DR, Stitt TN, Ip NY, Yancopoulos GD (1993). Alternative forms of rat trkC with different functional capabilities. Neuron 10: 963-974.

van der Geer P, Pawson T (1995). The PTB domain: a new protein module implicated in signal transduction. Trends Biochem Sci 20: 277-280.

van Leeuwen FN, Kain HET, van der Kammen RA, Michiels F, Kranenburg OW, Collard JG (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. J Cell Biol 139: 797-807.

van Veen T, Brackmann M, Moghimzadeh E (1978). Postnatal development of the pineal organ in the hamster's *phodopus sungorus* and *mesocricetus auratus*. Cell Tiss Res 189: 241-250.

Veeranna AND, Ahn NG, Jaffe H, Winters CA, Grant P, Pant HC. (1998). Mitogen-activated protein kinases (Erk1,2) phosphorylate Lys-Ser-Pro (KSP) repeats in neurofilament proteins NF-H and NF-M. J Neurosci 18: 4008–4021.

Virdee K, Xue L, Hemmings BA, Goemans C, Heumann R, Tolkovsky AM (1999). Nerve growth factor-induced PKB/Akt activity is sustained by phoshoinositide 3-kinase dependent and independent signals in sympathetic neurons. Brain Res 837: 127-142.

Venkatakrishnan G, McKinnon CA, Pilapil CG, Wolf DE, Ross AH (1990). Nerve growth factor receptors are preaggregated and immobile on responsive cells. Biochemistry 30: 2748-2753.

Verdi JM, Birren SJ, Ibañez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994). p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron 12: 733-745.

Vesa J, Krüttgen A, Shooter EM (2000). p75 reduces TrkB tyrosine autophosphorylation in response to brain-derived neurotrophic factor and neurotrophin 4/5. J Biol Chem 275: 22414-22420.

Vojtek AB, Der CJ (1998). Increasing complexity of the Ras signaling pathway. J Biol Chem 273: 19925-19928.

Von Bartheld CS, Heuer JG, Bothwell M (1991). Expression of nerve growth factor receptors in the brain and retina of chick embryos: comparison with cholinergic development. J Comp Neurol 310: 103-129.

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

Voyvodic JT (1987). Development and regulation of dendrites in rat superior cervical ganglion. J Neurosci 7: 904-912.

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 Q, Zheng JQ (1998). CAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. J Neurosci 18: 4973-4984.

Welcher AA, Bitler CM, Radeke MJ, Shooter EM (1991). Nerve growth factor binding domain of the nerve growth factor receptor. Proc Natl Acad Sci USA 88: 159-163.

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. J Neurochem 48: 1779-1786.

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

White FA,Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD (1998). Widespread elimination of naturally occurring neuronal death in BAX-deficient mice. J Neurosci 18: 1428–1439.

Wilklund L (1974). Development of serotonin-containing cells and the sympathetic innervation of the habenular region in the rat brain. Cell Tiss Res 155: 231-243.

Wolf DE, McKinnon CA, Daou M-C, Stephens RM, Kaplan DR, Ross AH (1995). Interactions with TrkA immobilizes gp75 in the high affinity growth factor receptor complex. J Biol Chem 270: 2133-2138.

Wright LL, Beck C, Perez-Polo JR (1987). Sex differences in nerve growth factor levels superior cervical ganglia and pineals. Int J Dev Neurosci 5: 383-390.

Wu W, Gloster A, Miller FD (1997). Transcriptional repression of the growth-associated T alpha1 alpha-tubulin gene by target contact. J Neurosci Res 48: 477-487.

Wyatt S, Pinon LGP, Ernfors P, Davies AM (1997). Sympathetic neuron survival and TrkA expression in NT3-deficient mouse embryos. EMBO J 16: 3115–31 23.

Yaar M. Grossman K, Eller M, Gilchrist B (1991). Evidence for nerve growth factor-mediated paracrine effects in human epidermis. J Cell Biol 115: 821-828.

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

Yan Q, Johnson EM, Jr (1988). An immunohistochemical study of the nerve growth factor receptor in developing rats. J Neurosci 8: 3481-3498.

Yan H and Chao MV (1991). Disruption of cysteine-rich repeats o f the p75 nerve growth factor receptor leads to loss of ligand binding. J Biol Chem 266: 12099-12104.

Yan Q, Rosenfeld RD, Matheson CR, Hawkins N, Lopez OT, Bennett L, Welcher AA (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience 78: 431-48.

Yang, X-M, Toma JG, Bamji SX, Belliveau DJ, Kohn J, Park M, Miller FD (1998). Autocrine hepatocyte growth factor provides a local mechanism for promoting axonal growth. J Neurosci 18: 8369-8381.

Yeo TT, Chua-Couzens J, Butcher LL, Bredesen DE, Cooper JD, Valletta JS, Mobley WC, Longo FM (1997). Absence of p75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. J Neurosci 17: 7594-7605.

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.

Yoshida K, Gage FH (1992). Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factot and other cytokines. Brain Res 569: 14-25.

Zhai S, Pincelli C, Yaar M, Gonsalves J, Gilchrist B (1995). The role of nerve growth factor in preventing keratinocyte apoptosis. J Invest Dermatol 104: 572.

Zhang Y-Z, Moheban DB, Conway BR, Bhattacharyya A, Segal RA (2000). Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. J Neurosci 20: 5671-5678.

Zhou XF, Rush RA (1993). Localization of neurotrophin-3-like immunoreactivity in peripheral tissues of the rat. Brain Res 621: 189-199.

Zigmond RE, Baldwin C, Bowers CW (1981). Rapid recovery of function after partial denervation of the rat pineal gland suggests a novel mechanism for neural plasticity. Proc Natl Acad Sci USA 78: 3959-3963.

HYPOTHESIS

The survival of sympathetic neurons is dependent upon a physiological balance between activation of TrkA and p75NTRs, with TrkA mediating positive and the p75NTR transducing negative biological signals. Our laboratory has previously shown that activating the p75NTR with its specific ligand BDNF in the presence of robust NGF-induced TrkA does not suppress survival *in vitro*, but if TrkA activation is weak, coincident p75NTR activation is able to inhibit survival. Since these receptors have opposing roles, and the proapoptotic effect of the p75NTR occurs only when NGF survival signal are suboptimal, we hypothesized that the p75NTR might inhibit other TrkA-mediated responses such as neuronal growth when NGF signals are robust. Another prediction, based on opposing roles for these receptors, is that increasing TrkA levels while maintaining a constant rate of p75NTR expression will allow sympathetic neurons to become more sensitive to TrkA ligands through more efficient Trk-mediated suppression of inhibitory signals. Therefore, a balance between positive and negative signals is required to achieve appropriate levels of growth and target innervation.

CHAPTER 2

CHAPTER 2

Preface

Previous work addressing the opposing roles of the TrkA and p75NTRs has focused mainly on neuronal survival. There is evidence that sympathetic neuron survival is not simply a function of TrkA activation, but can also be influenced by neurotrophins such as BDNF. In the context of sympathetic neurons that do not express TrkB, BDNF is a specific ligand for the p75 neurotrophin receptor, and it signals via p75NTR to mediate apoptosis (Aloyz et al., 1998; Bamji et al., 1998).

Although much has been learned about how TrkA and the p75NTR function in an antagonistic fashion to regulate neuronal survival, there has been less emphasis on studying how these receptors functionally interact to regulate neuronal growth and target innervation. In the first paper that follows, I addressed this issue of p75NTR as a mediator of inhibitory signals for growth by measuring NGF-induced neuronal growth and showing that activation of the p75NTR with BDNF *in vitro* could inhibit neonatal sympathetic neuron outgrowth. To show that this finding was physiologically relevant, I then used immunocytochemical techniques to determine whether reduced or no p75NTR activation would influence target innervation *in vivo.* The pineal glands from BDNF-deficient transgenic mice were utilized, as in the absence of BDNF, activation of the p75NTR on sympathetic neurons would be affected.

This paper was published in the Journal of Neuroscience as:
Kohn J, Aloyz RS, Toma JG, Haak-Frendscho M, Miller FD (1999). Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. J Neurosci 19: 5395-5408.

SECTION:Developmental Neurobiology

SENIOR EDITOR: Dr. Dennis D.M. O'Leary

FUNCTIONALLY ANTAGONISTIC INTERACTIONS BETWEEN THE TrkA AND p75 NEUROTROPHIN RECEPTORS REGULATE SYMPATHETIC NEURON GROWTH AND TARGET INNERVATION

Judi Kohn, Raquel S. Aloyz, Jean G.Toma, Mary Haak-Frendscho+, and Freda D. Miller#

Centre for Neuronal Survival, Montreal Neurological Institute, McGill University, Montréal, Québec, Canada, H3A 2B4.

+Department of Immunology, R&D, Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin 53711, U.S.A.

#To whom correspondence should be addressed

Running Title: Antagonistic interactions between p75 and TrkA

Corresponding Author: Dr. Freda D. Miller, Centre for Neuronal Survival, Montreal Neurological Institute, 3801 rue University, Montréal, Québec, Canada, H3A 2B4. Telephone: 514-398-4261. FAX: 514-398-1319. E-mail: mdfm@musica.mcgill.ca

Number of text pages: 45 **Figures**: 9

Abstract: 212 words. **Introduction**: 498 words.

Acknowledgements: We thank Christine Pozniak and Marta Majdan (both of McGill University) for their help with the $BDNF^{+/-}$ and $p75NTR^{-/-}$ colonies, and other members of the Miller laboratory for their advice and input throughout the course of this work. This study was supported by grants from the Canadian Medical Research Council and NeuroSciences Network (to F.D. Miller). F.D. Miller is a Killam Scholar and, during the course of this work, R. Aloyz was a NeuroSciences Network fellow.

ABSTRACT

In this report, we provide evidence that NGF and BDNF have functionally antagonistic actions on sympathetic neuron growth and target innervation, with NGF acting via TrkA to promote growth and BDNF via p75NTR to inhibit growth. Specifically, in cultured sympathetic neurons that themselves synthesize BDNF, exogenous BDNF inhibits and function-blocking BDNF antibodies enhance process outgrowth. Both exogenous and autocrine BDNF mediate this effect via p75NTR since, i) BDNF does not inhibit growth of neurons lacking p75NTR, ii) function-blocking p75NTR antibodies enhance NGF-mediated growth, and, iii) $p75NTR^{-/-}$ sympathetic neurons grow more robustly in response to NGF than do their wildtype counterparts. To determine the physiological relevance of this functional antagonism, we examined the pineal gland, a well-defined sympathetic target organ. BDNF is present in the pineal gland during target innervation, and incoming sympathetic axons are p75NTR-positive. Moreover, the pineal glands of $BDNF^{+/-}$ and $BDNF^{-/-}$ mice are hyperinnervated with sympathetic fibres, and tyrosine hydroxylase levels are elevated. Increased tyrosine hydroxylase is also observed in the $BDNF^{+/-}$ carotid artery, another sympathetic neuron target. Thus, BDNF, made by sympathetic neurons and/or their target organs, acts via p75NTR to antagonize NGF-mediated growth and target innervation, suggesting that sympathetic target innervation is determined by the balance of positively- and negatively-acting neurotrophins present in developing, and potentially, mature targets.

Key words: nerve growth factor, brain-derived neurotrophic factor, sympathetic neurons, target innervation, neurotrophin receptor, TrkA, p75NTR, pineal gland.

The neurotrophic factor hypothesis states that neuronal growth and survival are regulated by target-derived neurotrophic factors, such as nerve growth factor (NGF) (reviewed in Thoenen and Barde, 1980; Levi-Montalcini, 1987), so that competition for limiting amounts of trophic factors match the number of innervating neurons to target cells (Oppenheim, 1991). This hypothesis is largely based upon peripheral sympathetic neurons, which are absolutely dependent upon NGF during the period of target competition (Levi-Montalcini, 1987). During this developmental window, target-derived NGF is thought to regulate the density of target innervation by stimulating terminal growth (Miller et al., 1994), and by serving as a discriminator that allows elimination of neurons that fail to sequester adequate target territory.

Target-derived NGF binds to two different cell surface receptors on sympathetic neurons to elicit these responses: the tyrosine kinase receptor, TrkA (Kaplan et al., 1991a; 1991b; Cordon-Cardo et al., 1991; Klein et al., 1991), and the p75 neurotrophin receptor (p75NTR) (Johnson et al., 1986; Radeke et al., 1987). In addition to these two receptors, postmitotic sympathetic neurons express low levels of another Trk family member, TrkC (Belliveau et al., 1997). Two lines of evidence indicate that NGF binding to TrkA alone is sufficient to mediate sympathetic neuron survival and growth. First, ligand-mediated activation of TrkA, but not p75NTR, supports sympathetic neuron growth and survival (Weskamp and Reichardt, 1991; Ibáñez et al., 1992; Clary et al., 1994; Belliveau et al., 1997; Bamji et al., 1998). Second, all sympathetic neurons are lost in $TrkA^{-/-}$ mice (Smeyne et al., 1994) as they are in $NGF^{-/-}$ mice (Crowley et al., 1994).

Implicit to the neurotrophic factor hypothesis is the assumption that positive

signals, such as those elicited by target-derived NGF binding to TrkA, are sufficient to determine both the life or death of a developing neuron and the appropriate level of target innervation. However, we have recently demonstrated that sympathetic neuron survival is not only determined by TrkA, but is also regulated by negatively-acting neurotrophins like BDNF, which signal though p75NTR to mediate neuronal apoptosis (Aloyz et al., 1998; Bamji et al., 1998). Specifically, when survival signals are suboptimal, BDNF-mediated activation of p75NTR causes sympathetic neuron apoptosis. Moreover, in $BDNF^{-/-}$ mice, sympathetic neuron number is increased, and in $p75NTR^{-/-}$ mice, the normal period of sympathetic neuron death is greatly delayed. This latter deficit in apoptosis is intrinsic to sympathetic neurons, since cultured $p75NTR^{-/-}$ neurons die much more slowly than their wild-type counterparts in the absence of NGF. Thus, naturally-occurring sympathetic neuron death is regulated by positively- and negatively-acting neurotrophins that signal through TrkA versus p75NTR.

Since the apoptotic effect of p75NTR signalling occurs only under suboptimal survival conditions, we hypothesized that p75NTR might also inhibit other TrkA-mediated responses when survival conditions are optimal. In this report, we test this hypothesis, and demonstrate that BDNF acts via p75NTR to inhibit NGF-mediated growth and target innervation. Thus, the balance of signalling mediated by the TrkA versus p75 neurotrophin receptors ultimately determines both the survival and growth of developing sympathetic neurons.

MATERIALS AND METHODS

Mass Cultures of Sympathetic Neurons

Mass cultures of pure sympathetic neurons from the superior cervical ganglion (SCG) of postnatal day 1 Sprague Dawley rats (Charles River Breeding Laboratories, St. Constant, QUE) were prepared as previously described (Ma et al., 1992). Neurons were plated at low density (approximately 1 ganglion/well) in Nunclon 4-well culture dishes (Gibco BRL, Burlington, ONT), coated with either rat tail collagen or poly-D-lysine and laminin (both from Collaborative Biomedical Products, Bedford, MA). Culture medium was UltraCulture (BioWhittaker, Walkersville, MD), supplemented with 3% rat serum (Harlan Bioproducts, Madison, WI), 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from BioWhittaker), and for days 2 and 3, 7 μM cytosine arabinoside (Sigma-Aldrich Canada Ltd., Oakville, ONT).

CD-1 mouse sympathetic neurons were cultured by a modification of the method used to prepare rat neurons. Mouse cultures were essentially prepared the same way, but were dissociated in UltraCulture medium rather than in Hanks' balanced saline solution. 3% fetal bovine serum (Gibco) was used instead of rat serum, and 3.5 μM cytosine arabinoside was added to the culture medium on Day 1 post-plating.

NGF used in these experiments was purified from mouse salivary glands and supplied by Cedarlane Laboratories Ltd. (Hornby, ONT). The sources of recombinant human BDNF were PeproTech Inc. (Rocky Hill, NJ), for the neuritogenesis assays, and Promega Corporation, (Madison, WI), for the rhBDNF neutralization experiments. The p75NTR function-blocking antibody REX (Weskamp and Reichardt, 1991) was the kind gift of Dr. L. Reichardt (University of California, San Francisco, CA). REX is

directed against the extracellular domain of p75NTR, and was used as an antiserum at a dilution of 1:100 (Weskamp and Reichardt, 1991). Rabbit serum (Gibco) of the same concentration was used as the negative control for REX. Anti-Human BDNF (Promega), was used at 10 μg/ml. As a negative control for anti-BDNF, nonimmune chicken IgY (Promega) was used at up to 40 μg/ml in BDNF neutralization experiments, and 10 μg/ml in neuritogenesis experiments.

BDNF Neutralization

To test the capacity of anti-BDNF to neutralize BDNF, TrkB-expressing NIH-3T3 cells (the kind gift of Dr. D. Kaplan, McGill University) were cultured in Dulbecco's modified Eagle medium. Briefly, cells were washed twice and incubated for 1 hour at 37°C in buffer, followed by a 30 minute wash at 37°C in a phosphate-free buffer. Treatment consisted of incubating cells for 5 minutes with either 50 ng/ml BDNF (Promega) or with BDNF preadsorbed for 4 hours at 4°C with increasing concentrations of the BDNF antibody (5-40μg/ml). In addition, TrkB-3T3 cells were treated with medium only, or medium plus 40 μg/ml nonimmune chicken IgY. Following these treatments, cells were lysed, immunoprecipitated with anti-pan Trk (Hempstead et al., 1992), and the immunopreciptates analyzed for TrkB activation by Western blot analysis with phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), as we have previously described (Belliveau et al., 1997; Bamji et al., 1998).

Survival Assays

NGF-dependent neurons were selected by culturing sympathetic neurons for 5 days in the presence of 50 ng/ml NGF, as previously described (Ma et al., 1992; Belliveau et al., 1997; Bamji et al., 1998). Neurons were washed three times for 1 hour each in neurotrophin-free media, and were then fed with media containing 10 ng/ml NGF with or without 100 ng/ml BDNF, 10 μg/ml αBDNF, or a 1:100 dilution of antiserum containing the p75NTR antibody, REX (Weskamp and Reichardt, 1991). Each condition was repeated in triplicate, and analysis of survival was performed 48 hours later by using nonradioactive MTT survival assays that measure mitochondrial function (Celltitre 96; Promega, Madison, WI; Belliveau et al., 1997). Specifically, 50 μl of the MTT reagent was added to 500μl media in each well, and incubated for 2 hours at 37°C. After aspiration of the MTT containing media, 100 μl of a 0.065N HCL/isopropanol mixture was added to each well to lyse the cells, and colorimetric analysis was performed using an ELISA reader. For rat sympathetic neurons, 10 ng/ml NGF represents 100% survival (Bamji et al., 1998), therefore all other values were considered to be relative to 10 ng/ml NGF. For mouse neurons, 7.5 ng/ml NGF represents 100% survival, and was thus considered to be the 100% survival threshold for neuritogenesis experiments.

Analysis of Transgenic Animals

Mice heterozygous for a targeted mutation in the BDNF gene (Ernfors et al., 1994) or homozygous for a targeted mutation in the p75NTR gene (Lee et al., 1992) were obtained from Jackson Labs (Bar Harbor, ME). The $BDNF^{+/-}$ mice were

maintained in a C129/BalbC background. The p75NTR$^{-/-}$ mice were originally generated in a C129 background (Lee et al., 1992), and were crossed back into a C129 background before purchase from Jackson Labs and then maintained as homozygotes. Progeny from BDNF heterozygote crosses were screened for the mutant allele(s) using PCR, as we have previously described (Bamji et al., 1998).

Analysis and Quantification of Process Outgrowth

p75NTR/BDNF regulation of neuronal growth was analyzed using two different types of neuritogenesis assays. Similar results were obtained with both approaches. The first assay, which measures the number of process intersections/neuron, is described in detail in Belliveau et al. (1997) and Yang et al. (1998). Briefly, postnatal day 1 rat sympathetic neurons were cultured in 50 ng/ml NGF for 2 to 3 days in order to upregulate p75NTR, whose increased expression in response to NGF occurs independently of neuronal survival (Miller et al., 1991; Ma et al., 1992). Following a 1 hour washout in neurotrophin-free medium, cultures were maintained for an additional two days in 10 ng/ml NGF plus or minus 100 ng/ml BDNF. Fields in low-density sister cultures were randomly selected and photographed; 6-8 sampling windows were utilized per culture. We then determined, in each field, i) the number of visible neurite intersections, and, ii) the number of neuronal cell bodies. We expressed these data as the number of intersections/number of cell bodies to give us a measure of intersections/neuron. We then determined the mean number of intersections/neuron for all of the photographed fields in a given culture, and used the Student's *t* test to determine the statistical significance of density differences between experimental

groups. Results were expressed as the mean process network density per neuron plus or minus the standard error of the mean.

The second approach allowed us to quantitate, in any given field, i) number of neuronal cell bodies, ii) apoptotic cells, and iii) amount of area covered by neuritic processes. Specifically sympathetic neurons were TUNEL-labelled to visualize apoptotic cells, followed by immunolabelling with anti-α-tubulin to visualize neurites, and then staining with Hoechst 33250 to visualize nuclei. To perform these experiments, P1 SCG neurons were plated onto 8-well Nunc-Nalgene plastic Lab Tek chamber slides (Gibco) that were coated twice, first with a polylysine-collagen mixture, followed by a second collagen coating. Following the experimental treatments, cultures were washed twice with PEM (PIPES-EGTA-$MgCl_2$) buffer, and fixed for 15 minutes in 4% paraformaldehyde in PEM buffer containing 0.25% glutaraldehyde and 0.2% Triton X-100. After three 10 minute washes in PBS, TUNEL-labelling was carried out as previously described (Slack et al., 1996; reagents from Promega) with a Streptavidin-CY3 conjugate (1/2000 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA). Cultures were then washed 3 times for 10 minutes each in PBS, and incubated for two hours with anti-α-tubulin (1/500 in PBS, Clone DM1A; Sigma-Aldrich Canada, Oakville). The tubulin immunolabel was visualized using FITC-conjugated goat anti-mouse IgG (1/800 in PBS, Jackson ImmunoResearch). Finally, cultures were incubated with Hoechst 33250 (2 μg/ml in PBS, ICN Biomedicals, Costa Mesa, CA) for one minute to label cell nuclei, and washed an additional three times for 10 minutes each in PBS. Slides were then coverslipped using Sigma Mounting Medium (Sigma Diagnostics, St. Louis, MO), and viewed by epifluorescence microscopy.

To analyze these cultures, images were captured using a Sony XC-75CE CCD video camera module attached to an Axioskop microscope (Carl Zeiss Canada) and a 16X plan-neofluar lens. The Northern Eclipse image analysis system (Empix Imaging Inc., Mississauga, ONT) was used to analyze these images as follows: images of low density neuronal fields were captured, and the area labelled with tubulin was measured and expressed as a percentage of the total field area. The numbers of Hoechst-positive and TUNEL-positive cell bodies were quantitated by viewing the same field with the appropriate filters, and analyzed using the same software package. For each culture, 7 to 8 independent, randomly-chosen fields were analyzed. These data were expressed as average percentage tubulin immunoreactive area/live cell, and Student's *t*-test was used to determine the statistical significance of differences between experimental groups.

Immunocytochemistry and Analysis of Sympathetic Innervation Density

For quantitative analysis of sympathetic innervation density, P13 to P15 $BDNF^{+/+}$, $^{+/-}$ and $^{-/-}$ mice were deeply anaesthetized with isoflurane, and killed by decapitation. Pineal glands were removed immediately, immersion fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) overnight, and subsequently cryoprotected in graded sucrose solutions. Pineal glands taken from mice of each genotype were cut on a cryostat (12 μm sections), and the entire pineal gland was serially thaw-mounted onto three Superfrost slides (Fisher Scientific, Houston, TX). Thus, each pineal gland was completely represented on each of three slides, and an entire pineal gland of each genotype could be analyzed, after immunostaining, to obtain a quantitative measure of innervation density. These sections were then postfixed in 4%

paraformaldehyde for 10 minutes at room temperature, and washed for 10 minutes in phosphate buffered saline (PBS, pH 7.4). Following non-specific blocking with 4% goat serum and 4% rat serum (both from Jackson ImmunoResearch) plus 0.2% Triton X-100 in PBS (pH 7.4), the sections were incubated overnight at 4°C with a commercially available polyclonal antibody directed against tyrosine hydroxylase (TH, 1:400; Chemicon International, Temecula, CA) in blocking solution. Slides were then washed three times for 10 minutes each in PBS, and incubated for two hours in blocking solution containing a CY3-conjugated secondary antibody (goat anti-rabbit IgG, 1:2000, Jackson ImmunoResearch). Following 3 x 10 minute washes in PBS, slides were coverslipped using Sigma Mounting Medium and viewed by epifluorescence microscopy.

To quantitatively analyze sympathetic innervation density, images were captured and analyzed using the Northern Eclipse Imaging System. For a given slide, the percentage area covered by TH-immunolabelling was measured for every section on that slide and the mean percentage area covered by TH-immunolabelling was determined from all of these sections, thereby avoiding errors due to potential differences in distribution of sympathetic fibers in pineal glands of different genotypes. Comparisons were only made between slides which were processed together.

To directly compare the pattern of p75NTR-immunoreactivity with that of TH-immunoreactivity, two slides containing serial sections obtained from the same P13 $BDNF^{+/-}$ pineal gland were immunostained as described above with either anti-TH (Chemicon) or anti-human p75NTR (Promega, 1/500) antibodies. The secondary antibody used was a CY3-conjugated goat anti-rabbit IgG (1:2000 in blocking solution;

Jackson ImmunoResearch).

Western Blot Analysis

For biochemistry, groups of P13 to P15 pineal glands from $^{+/+}$, $^{+/-}$, or $^{-/-}$ BDNF mice, or portions of $^{+/+}$ or $^{+/-}$ common carotid artery (dissected at the point of bifurcation into the internal and external branches), or adult rat pineal glands or cortex were homogenized in Tris buffered saline (TBS) containing 137mM NaCl, 20 mM Tris (pH 8.0), 1% v/v NP-40, 0.1% SDS, 10% glycerol and the protease inhibitors phenylmethyl sulfonyl fluoride (PMSF, 1 mM), aprotinin (10 μg/ml), leupeptin (0.2 μg/ml), and sodium vanadate (1.5 mM). The tissue was rocked for 10 minutes at 4°C, and following a 10 minute centrifugation at 4°C, the supernatant was collected and lysates normalized for protein concentration using a BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL). For Western blot analysis, equal amounts of pineal, carotid, or cortical protein were boiled in sample buffer for 5 minutes, and separated by 7.5% or 15% SDS-PAGE (7.5% gel for TH, p75NTR, α-tubulin and ERK1, and 15% gel for BDNF). 20 ng of rhBDNF (Amgen, Thousand Oaks, CA) was also run on a 15% SDS-PAGE gel. After electrophoresis, proteins were transferred to 0.2μm nitrocellulose membranes for 1.5 hours at 0.6 amps, and washed three times for 10 minutes each with either PBS (for BDNF) or TBS (for all other proteins). Following a 1.5 hour block in blotto (3% non-fat milk in PBST for BDNF, or TBST for all other proteins) at room temperature, membranes were incubated overnight at 4°C in blocking solution containing either anti-BDNF (1:3000; SantaCruz Biotechnology Inc., Santa Cruz, CA), anti-recombinant human p75NTR (1:10,000; Promega), anti-TH (1:5000; Chemicon), anti-α-tubulin

(1:5000; Calbiochem/Oncogene Research Products, Cambridge, MA), or anti-ERK1 (1:10,000; SantaCruz Biotechnology). The membranes were washed four times for 10 minutes each with either PBST (for BDNF) or TBST (for all other proteins), and then incubated with secondary antibody (1:10,000 goat anti-rabbit HRP [Boehringer-Mannheim, Laval, QUE] for anti-BDNF, anti-p75NTR, anti-ERK1, and anti-TH, or 1:10,000 goat anti-mouse HRP [also from Boehringer] for anti-α-tubulin) for 1.5 hours at room temperature. After 3 x 10 minute washes in PBST (for BDNF protein) or TBST (for all other proteins), detection was carried out using enhanced chemiluminescence (ECL Western blotting detection reagent; Amersham Canada Ltd., Oakville, ONT) and XAR x-ray film (Eastman Kodak Co., Rochester, NY).

RESULTS

BDNF-Mediated Activation of p75NTR Inhibits NGF-Induced Growth of Cultured Sympathetic Neurons

BDNF-mediated activation of p75NTR antagonizes TrkA-mediated sympathetic neuron survival when NGF levels are suboptimal, but has no effect on survival at higher levels of NGF (Bamji et al., 1998; Aloyz et al., 1998). To determine whether p75NTR activation also antagonized other TrkA-mediated biological responses, we focused on sympathetic neuron growth. Specifically, we cultured sympathetic neurons in NGF to maintain their survival, and then activated p75NTR using BDNF. For rat sympathetic neurons, 10 ng/ml NGF mediates 100% sympathetic neuron survival but elicits limited morphological growth and TrkA activation relative to higher concentrations of NGF (Ma et al., 1992; Belliveau et al., 1997), while 100 ng/ml BDNF is sufficient to activate p75NTR in apoptosis experiments (Bamji et al., 1998), but does not bind to the two Trk receptors present on sympathetic neurons, TrkA and TrkC (Belliveau et al., 1997).

Initially, we confirmed, as we have previously reported (Bamji et al., 1998), that the addition of 100 ng/ml BDNF in the presence of 10 ng/ml NGF had no negative effects on sympathetic neuron survival (Fig. 1A); sympathetic neurons were cultured for 5 days in 50 ng/ml NGF, were washed free of neurotrophin, and were then switched into 10 ng/ml NGF plus or minus 100 ng/ml BDNF. Two days later, neuronal survival was measured using MTT assays, which measure mitochondrial function (Belliveau et al., 1997; Bamji et al., 1998). As previously shown, (Bamji et al., 1998), the addition of 100 ng/ml BDNF had no effect on sympathetic neuron survival in 10 ng/ml NGF (Fig. 1A).

We next determined whether p75NTR activation by BDNF affected neuronal growth by measuring the level of neurite extension that occurs in response to 10 ng/ml NGF with or without BDNF. For these experiments, neurons were cultured for 2 to 3 days in 50 ng/ml NGF, were switched to 10 ng/ml NGF plus or minus 100 ng/ml BDNF, and the density of neuritic processes was determined two days later by quantitating the number of neurite intersections per neuron (Fig. 1B,C; 2A,B). Results from 6 separate experiments indicated that BDNF reduced the process network density from 22 to 52%, for an average decrease of 40% (Fig. 1B,C; Fig. 2A,B). Having determined that BDNF reduced neurite density, we then performed a dose-response curve with 50, 100 and 200 ng/ml BDNF, using the same experimental approach. This analysis revealed that 50 ng/ml BDNF produced a small decrease in process density that was not statistically significant, while 100 and 200 ng/ml BDNF both produced similar, significant decreases (Fig. 1D); this dose-response is similar to that observed for the apoptotic effects of BDNF on the same neurons (Bamji et al., 1998).

To confirm that the observed decrease in neurite intersections per neuron reflected a decrease in the total amount of growth in these cultures, we utilized a second approach. As in the previous experiments, neurons were first grown for 2 to 3 days in 50 ng/ml NGF, and then were switched to 10 ng/ml NGF plus or minus 100 ng/ml BDNF for 2 additional days. As a control, neurons were withdrawn from NGF for these final two days. We triple-labelled these neurons (Fig. 3) by, i) TUNEL-labelling to assess the number of apoptotic neurons (pink/red nuclei in Fig. 3), ii) using anti-tubulin to visualize the neuritic network (green in Fig. 3), and iii) using Hoechst 33250 to visualize neuronal nuclei (blue in Fig. 3). We then selected random fields in each sister

culture, and used image analysis to quantitate the amount of area covered by tubulin-immunoreactive processes per Hoechst-labelled neuron, and to determine the number of apoptotic neurons per field. This analysis confirmed that, as indicated by the MTT assay, the number of dying cells was similar in cultures maintained in 10 ng/ml NGF alone versus those in 10 ng/ml NGF plus 100 ng/ml BDNF (Fig. 1E). Moreover, the relative amount of tubulin-immunoreactive processes per neuron was decreased approximately 40% in the neurons treated with BDNF (Fig. 1F,G), a decrease similar to that seen when the number of neuritic intersections per neuron was determined (Fig. 1B,C). Thus, these two approaches indicate that, under these conditions, BDNF was able to antagonize NGF-promoted sympathetic neuron growth with no perturbation of neuronal survival.

Autocrine BDNF, Acting Through $p75^{NTR}$, Decreases Growth of Cultured Sympathetic Neurons

These data suggested that exogenous BDNF is able to activate p75NTR and negatively-influence TrkA-mediated neuritogenesis. However, since sympathetic neurons themselves synthesize BDNF (Causing et al., 1997) that can be detected in conditioned medium obtained from cultured SCG neurons (C.G. Causing, R. Aloyz, and F.D. Miller, unpublished observations), we hypothesized that autocrine BDNF might play a role in negatively regulating levels of sympathetic neuron growth through a BDNF:p75NTR autocrine loop. To test this hypothesis, we used a function-blocking BDNF antibody.

Initially, to ensure that this anti-BDNF was capable of neutralizing BDNF, we

incubated TrkB-expressing NIH-3T3 cells for 5 minutes with 50 ng/ml BDNF plus or minus 5-40 μg/ml of anti-BDNF. TrkB protein was then immunoprecipitated using anti-panTrk, and the immunoprecipitates analyzed by Western blot analysis with anti-phosphotyrosine. As controls, cells were incubated either with culture medium, or medium with BDNF plus 40 μg/ml nonimmune IgY. This analysis revealed that BDNF caused a robust increase in tyrosine phosphorylation of TrkB, and that anti-BDNF inhibited BDNF-stimulated TrkB phosphorylation at concentrations of 10 μg/ml or higher (data not shown). In contrast, the control IgY had no effect on BDNF-mediated TrkB activation (data not shown).

We then used this function blocking anti-BDNF to test the role of autocrine BDNF in sympathetic neuron growth. Initially, we determined whether anti-BDNF had any effect on sympathetic neuron survival under the conditions of our growth experiments; neurons were cultured for 5 days in 50 ng/ml NGF, and then were switched to 10 ng/ml NGF with or without 10 μg/ml anti-BDNF. Measurement of neuronal survival using MTT assays two days later revealed that anti-BDNF had no effect on sympathetic neuron survival (Fig. 4A). We then determined whether anti-BDNF affected neuronal growth under these same conditions; neurons were grown in 50 ng/ml NGF for 2 to 3 days, and then switched into 10 ng/ml NGF plus or minus 10 μg/ml anti-BDNF. Measurement of neurite process density revealed that in 3 separate experiments, cultures exposed to anti-BDNF exhibited an average increase in neuritogenesis of 80% relative to 10 ng/ml NGF alone (Fig. 4B,C; Fig. 2A,C). Nonimmune IgY had no effect on NGF-mediated growth (Fig. 4B), demonstrating the specificity of the effect. Thus, autocrine BDNF inhibits TrkA-mediated neuritogenesis,

at least *in vitro*.

If autocrine BDNF is mediating these effects via p75NTR, then we would predict a similar increase in TrkA-mediated neuritogenesis if we blocked p75NTR. To test this prediction, we performed neuritogenesis experiments using the function-blocking p75NTR antibody, REX. As before, cultures were initally grown for 2 days in 50 ng/ml NGF and were then incubated for 2 additional days with 10 ng/ml NGF with or without REX (Fig. 4D,E; Fig. 2A,D). As a control, sister cultures were incubated with rabbit serum at the same volume as the REX antiserum. These experiments revealed that when p75NTR was blocked by REX, neuritogenesis was enhanced almost 2-fold relative to NGF alone (Fig. 4D,E; Fig. 2A,D), an effect that was not observed with rabbit nonimmune serum (Fig. 4D), and that was similar to the response elicited by anti-BDNF (Fig. 4B,C). The increased neuritogenesis observed with both REX and anti-BDNF is similar to the 2 to 2.5-fold increase that occurs when NGF is increased from 10 to 40 ng/ml NGF (Fig. 4F; Belliveau et al., 1997), a treatment that causes increased TrkA activation (Belliveau et al., 1997), supporting the idea that a BDNF:p75NTR autocrine loop antagonizes TrkA-mediated sympathetic neuron growth.

In a final experiment, we tested whether exogenous BDNF could reverse the effect of REX on neuritogenesis, as it should if REX is acting by disrupting a BDNF:p75NTR loop. Neurons were cultured for 2 to 3 days in 50 ng/ml NGF, and then switched to 10 ng/ml NGF with REX plus or minus 100 ng/ml BDNF for an additional 2 days; in experiments with REX and BDNF, cultures were preincubated with REX for 2 hours prior to the addition of BDNF. These experiments revealed that exogenous BDNF blocked the ability of REX to increase sympathetic neuron growth (Fig. 4G,H).

This antagonism between REX and exogenous BDNF is similar to results we have previously obtained when examining BDNF-induced apoptosis of sympathetic neurons (Bamji et al., 1998), and supports the idea of an inhibitory BDNF:p75NTR growth loop.

p75NTR$^{-/-}$ Sympathetic Neurons Show Enhanced Neuritogenesis in Response to NGF, and Do Not Respond to Exogenous BDNF

Although our data strongly suggest that BDNF acts through p75NTR to antagonize TrkA, they do not conclusively demonstrate the necessity of p75NTR for BDNF's effects. To address this issue more directly, we cultured neurons from both p75NTR$^{-/-}$ and wild type control mice and repeated the neuritogenesis assays. Since mouse sympathetic neurons have been reported to be more sensitive to NGF than rat sympathetic neurons, we initially performed survival assays to determine an appropriate NGF concentration; neurons were maintained for 5 days in 50 ng/ml NGF, were switched to concentrations of NGF ranging from 0.1 to 10 ng/ml NGF for 3 days, and survival was then measured using MTT assays. These experiments revealed that 5, 7.5, and 10 ng/ml NGF were all able to mediate maximal mouse sympathetic neuron survival (Fig. 5A). To ensure that BDNF had no apoptotic effect under these survival conditions, we performed similar experiments with 7.5 or 10 ng/ml NGF plus or minus 100 ng/ml BDNF. MTT assays revealed that, as for rat neurons (Fig 1A), BDNF did not affect mouse sympathetic neuron survival in the presence of optimal concentration of NGF (Fig. 5B). On the basis of these data, we selected 7.5 ng/ml NGF for our experiments, a concentration that was optimal for survival and where BDNF had no

significant effect on survival.

We then examined sympathetic neurons from p75NTR$^{-/-}$ mice to determine first, whether the lack of p75NTR imparted to p75NTR$^{-/-}$ neurons an intrinsic ability to extend more neuritic processes, and second, whether BDNF was acting through p75NTR to inhibit TrkA-mediated growth. To perform these experiments, sympathetic neurons were cultured from p75NTR$^{-/-}$ versus control mice on the same day, were maintained for 3 days in 50 ng/ml NGF, and were subsequently switched to 7.5 ng/ml NGF, plus or minus 100 ng/ml BDNF. Measurement of neuritic process density revealed that p75NTR$^{-/-}$ neurons exhibited an almost 2-fold increase in neurite outgrowth relative to their wild type counterparts (Fig. 5C,D; Fig. 6A,C), a result similar to that observed with the REX and anti-BDNF antibodies in experiments using rat sympathetic neuron cultures (Fig. 4). Moreover, while BDNF decreased the degree of neuritogenesis in wild type mouse cultures by an average of 35% (Fig. 5C,D; Fig. 6A,B), a result similar to that observed with rat neurons (Fig. 1B,C), exogenous BDNF had no effect on growth of p75NTR$^{-/-}$ neurons (Fig. 5C,D; 6C,D). Thus p75NTR is required for BDNF to inhibit NGF-mediated sympathetic neuron growth, and NGF is more effective at eliciting growth in the absence of p75NTR.

BDNF is Present in Sympathetic Target Organs and p75NTR in Sympathetic Neuron Axons During the Period of Target Innervation

If these culture results are relevant to the process of target innervation *in vivo*, then we would predict that BDNF (derived either from incoming sympathetic fibers and/or from target tissue) would be present in sympathetic neuron targets during the

developmental period of target competition. To test this prediction, we focused on the pineal gland, a sympathetic target organ that, i) is bilaterally innervated by neurons from the SCG (Kappers, 1960; Owman, 1964), ii) does not receive any other peripheral innervation from sensory or motor neurons (Stanley et al., 1987), and, iii) is innervated postnatally. Ingrowth of sympathetic fibres to the pineal gland begins during the first week of postnatal life, reaching adult levels after 3-4 weeks (Hakanson et al., 1967). To perform this experiment, lysates of pineal glands from adult rats were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed for the presence of BDNF using a BDNF antibody that we have previously characterized extensively for specificity (Causing et al., 1997; Fawcett et al., 1997; Fawcett et al., 1998). This analysis revealed a BDNF-immunoreactive band in the pineal gland that is the same size as BDNF in the rat cortex, and human recombinant BDNF (Fig. 7A,B). To confirm the identity of this band, we analyzed the pineal gland from mice in which the BDNF gene was mutated by homologous recombination (Ernfors et al., 1994). Western blot analysis revealed that the BDNF-immunoreactive band was completely lost in the pineal glands of $BDNF^{-/-}$ mice (Fig. 7C), as we have previously observed for this BDNF band in other $BDNF^{-/-}$ tissues (Causing et al., 1997; Fawcett et al, 1998).

We next determined whether incoming sympathetic axons were positive for p75NTR over this same timeframe. Immunocytochemical analysis of the rat pineal gland using the anti-p75NTR antibody MC192 revealed the presence of numerous p75NTR-positive fibres throughout the pineal gland at P6 (data not shown), in a pattern similar to that previously observed for tyrosine hydroxylase-positive sympathetic fibres (Kuchel, 1993). To confirm that this immunostaining corresponded to bona fide

p75NTR, we also performed Western blot analysis, which demonstrated that p75NTR was present in both the rat (data not shown) and mouse (Fig. 9) pineal gland during the first few postnatal weeks. Thus, both BDNF and p75NTR are present in the pineal gland at the time of sympathetic target competition.

The Pineal Gland, a Sympathetic Neuron Target Organ, is Hyperinnervated in BDNF$^{+/-}$ and $^{-/-}$ Mice

Together, our *in vivo* and *in vitro* data predict that, when BDNF levels are lowered, sympathetic neuron target innervation should increase. To test this prediction, we examined the level of sympathetic innervation to the pineal gland of BDNF$^{+/-}$ and BDNF$^{-/-}$ mice at P13 to P15. Initially, we analyzed the density of sympathetic fibres immunocytochemically, using an antibody against tyrosine hydroxylase (TH), a protein that is a marker for sympathetic axons. To perform this analysis, we serially sectioned the pineal gland from BDNF$^{+/+}$, $^{+/-}$ and $^{-/-}$ littermates, and performed TH-immunostaining on every third serial section from pineal glands of each genotype. We then used an image analysis system to quantitate the percentage area covered by TH-positive fibers on each of these sections, and averaged the percentage area obtained from sections throughout each pineal gland, thereby ensuring that sampling errors were not incurred due to differences in the pattern of sympathetic innervation to the pineal gland in animals of different genotypes. This analysis revealed that thick, TH-positive fibres were interspersed throughout the entire pineal gland of BDNF$^{+/-}$ and BDNF$^{-/-}$ mice, whereas fibres in the pineal gland of BDNF$^{+/+}$ littermates were less abundant and appeared qualitatively thinner (Fig. 8A-C). This qualitative difference was reflected in

the numbers obtained using image analysis; in each of 4 sets of littermates, the TH-positive innervation density was increased approximately 2 to 3-fold in the pineal glands of $BDNF^{+/-}$ versus $BDNF^{+/+}$ animals, and the level of innervation was similar in the $BDNF^{+/-}$ and $BDNF^{-/-}$ pineal glands (Fig. 8F).

To confirm the sympathetic hyperinnervation in the $BDNF^{+/-}$ and $^{-/-}$ pineal glands, we also measured the level of TH biochemically. Western blot analysis of equal amounts of protein from P13-P15 pineal glands revealed that TH levels were increased in $BDNF^{+/-}$ and $BDNF^{-/-}$ tissue relative to controls (Fig. 9), consistent with the immunocytochemical results (Fig. 8). We also used the same approach to quantitate the levels of p75NTR and α-tubulin, the former of which is present in incoming sympathetic afferents (Fig. 8D,E), and the latter of which is enriched, but not specific to all axons. Western blot analysis revealed that, like TH, levels of both of these proteins were increased in quantity in the pineal glands of $BDNF^{+/-}$ and $^{-/-}$ mice relative to their wildtype littermates (Fig. 9). In contrast, levels of ERK1, a signaling protein that is present in all cells, were similar in all of the samples (Fig. 9).

To determine whether this increase in sympathetic innervation was limited to the pineal gland, or whether it generalized to other sympathetic targets such as blood vessels, we also analyzed the common carotid artery of $BDNF^{+/-}$ versus $BDNF^{+/+}$ littermates. Western blot analysis of equal amounts of protein revealed that, as observed for the pineal gland, levels of TH, p75NTR and tubulin were all increased in the $^{+/-}$ carotid artery (Fig. 9). In contrast, levels of ERK1 were similar (Fig. 9). Thus, when BDNF levels are reduced as they are in the $BDNF^{+/-}$ mice (Fawcett et al., 1998), sympathetic innervation to at least two peripheral targets is increased.

DISCUSSION

Data presented in this paper demonstrate that BDNF-mediated activation of p75NTR antagonizes NGF-mediated growth of sympathetic neurons, thereby playing an essential role in the establishment of appropriate target innervation *in vivo*. Specifically, these experiments indicate that, when sympathetic neurons are cultured under optimal survival conditions, exogenous BDNF and autocrine BDNF made by sympathetic neurons themselves inhibit NGF-promoted neuronal growth. BDNF mediates this inhibition via p75NTR since, i) function-blocking p75NTR antibodies enhance NGF-promoted growth, ii) BDNF cannot inhibit the growth of $p75NTR^{-/-}$ neurons, and, iii) $p75NTR^{-/-}$ neurons grow more robustly in response to NGF than do their wildtype counterparts. The physiological relevance of these culture findings is demonstrated by the pineal gland studies presented in this paper. Specifically, BDNF is present in the pineal gland, and p75NTR is localized to sympathetic neuron fibres at the time of developmental target innervation. When BDNF is reduced or absent, as in $BDNF^{+/-}$ or $BDNF^{-/-}$ mice, the pineal gland is hyperinnervated with sympathetic fibres, and tyrosine hydroxylase levels are increased in two sympathetic targets, the pineal gland and the carotid artery. Together, these data indicate that BDNF, made by sympathetic neurons and/or their target organs, acts via p75NTR to antagonize NGF-mediated growth and target innervation, suggesting that sympathetic target innervation is determined by the balance of positively- and negatively-acting neurotrophins present in developing target organs.

What is the biological rationale for having two neurotrophin receptors, one of which, TrkA, mediates survival and growth, and one of which, p75NTR, acts

antagonistically to cause apoptosis and inhibit growth? With regard to neuronal survival, we have previously proposed that p75NTR provides a molecular mechanism for ensuring rapid and active apoptosis when a neuron is unsuccessful in competing for adequate amounts of the appropriate neurotrophin (Aloyz et al., 1998; Bamji et al., 1998; Miller and Kaplan, 1998). We propose that the antagonistic effects of TrkA and p75NTR on growth, as described here, are part of the same biological mechanism, as exemplified in the following three situations. First, if a sympathetic neuron is late-arriving and/or reaches an inappropriate target, then TrkA would be only weakly induced as a consequence of the lack of NGF, and p75NTR would be robustly activated by neurotrophins such as BDNF, leading to the rapid apoptotic elimination of that neuron. Second, if a sympathetic neuron extends only one main axon collateral, and that collateral reaches an appropriate target and sequesters NGF, then TrkA would be robustly activated, allowing for survival of that neuron. The third possibility would occur when a sympathetic neuron extends several axon collaterals or terminal branches. If one of those collaterals reaches an appropriate target and sequesters NGF, then the subsequent TrkA activation would retrogradely mediate survival of the neuron and would locally promote terminal growth of that axon. If the second collateral arrives at a target that is already innervated or that is inappropriate, then the low level of available NGF would cause only weak TrkA activation, and p75NTR would be robustly activated by neurotrophins such as BDNF. In this final case, p75NTR activation would not affect neuronal survival, since survival would be maintained by TrkA activation from the other collateral. Instead, the major effect would be on target innervation itself, with p75NTR acting locally to attenuate the growth of that specific axon branch. The net result would

be selection of one collateral over the other. In this way, p75NTR would act as a "fine-tuning" mechanism that would allow both the selection of those neurons that reach appropriate targets at an appropriate time, and the selection and maintenance of axon collaterals or terminal branches that meet the same criteria. Disruption of such a mechanism could well explain the perturbations in sympathetic neuron number (Bamji et al., 1998) and sympathetic innervation (Lee et al., 1994) observed previously in the $p75NTR^{-/-}$ mice. In the absence of p75NTR, sympathetic target organs that are innervated late, such as the pineal gland, are never appropriately innervated (Lee et al., 1994), suggesting that the disruption of appropriate competition for innervation of early sympathetic targets completely disrupts the later period of sympathetic target innervation.

Data presented here also indicate that BDNF is one p75NTR ligand that is responsible for regulating sympathetic neuron growth. Specifically, we have demonstrated that when BDNF in the pineal gland is reduced or absent, both the density of sympathetic innervation and the levels of tyrosine hydroxylase are increased. We believe that this increased pineal innervation is directly due to the loss of BDNF for the following reasons. First, the pineal gland does not receive peripheral sensory or motor innervation, making it unlikely that the observed effects are due to a BDNF-dependent loss of, for example, sensory innervation. Second, although sympathetic neuron number is increased approximately 30% in $BDNF^{-/-}$ mice (Bamji et al., 1998), the increases in innervation level and tyrosine hydroxylase are two to three-fold in the pineal gland, indicating that the degree of hyperinnervation is greater than the increase in cell number. Third, and perhaps most importantly, increased innervation to the pineal gland is also

seen in $BDNF^{+/-}$ mice, which are not delayed developmentally, and which have normal sympathetic and sensory neuron numbers. There are several precedents for such a BDNF gene dosage effect where the absence of one BDNF allele is sufficient to significantly perturb nervous system structure and/or function (Korte et al., 1995; Carroll et al., 1998; Fawcett et al., 1998), strongly suggesting that even minor alterations in the ratios of the neurotrophins are of major physiological importance. In this regard, we believe that the most likely explanation for our findings is that the BDNF present in the pineal gland inhibits NGF-mediated target innervation, and that this inhibition is lost when BDNF is reduced or absent.

What is the source of BDNF in vivo? Although the central nervous system is the most abundant postnatal source of BDNF (Ernfors et al., 1990; Hofer et al., 1990; Phillips et al., 1990), it is clear that many peripheral targets, including dermal mesenchyme, mandible, whisker pad (Schecterson and Bothwell, 1992), as well as muscle, heart and lung (Maisonpierre et al., 1990) synthesize BDNF. Moreover, these same targets synthesize NGF (Levi-Montalcini, 1987; Schecterson and Bothwell, 1992), suggesting that targets themselves determine the precise neurotrophin cohort encountered by arriving axons. However, sympathetic neurons also synthesize BDNF (Schecterson and Bothwell, 1992; Causing et al., 1997), and cultured neonatal sympathetic neurons secrete processed BDNF into the media (C.G. Causing, R. Aloyz, and F.D. Miller, unpublished observations). Moreover, our data indicate that this autocrine BDNF is sufficient to inhibit NGF-mediated growth, at least in culture. However, it is not possible, on the basis of the data presented here, to assess the relative contribution of target-derived versus sympathetic neuron-derived BDNF in vivo.

Nonetheless, it is tempting to speculate that if BDNF is anterogradely trafficked into sympathetic axons as it is into sensory (Zhou and Rush, 1996) and central (von Bartheld et al., 1996; Altar et al., 1997; Conner et al., 1997; Fawcett et al., 1998) axons, then secretion from sympathetic terminals may well provide a cellular mechanism whereby "successful" terminals could eliminate and/or make the environment unfavourable for later-arriving axon collaterals. Precedent for a related mechanism derives from the neuromuscular junction, where active synaptic sites apparently destabilize inactive synapses in their vicinity (Colman et al., 1997).

How does p75NTR inhibit TrkA-mediated neuronal growth? One potential mechanism involves p75NTR-mediated generation of intracellular ceramide (Dobrowsky et al., 1994; 1995). Recently, Posse de Chaves et al. (1997) demonstrated that elevation of intracellular ceramide in distal sympathetic neurites locally inhibited NGF-promoted sympathetic axon growth. Thus, as proposed by the authors, activation of p75NTR could well attenuate neurite growth via increased ceramide. A similar, and potentially additive attenuation would occur if activated p75NTR directly downregulated TrkA-mediated growth signals by serine-threonine phosphorylation of the TrkA receptor via ceramide-activated kinases (McPhee and Barker, 1997). The net outcome of these ceramide-driven mechanisms would be inhibition of growth, and potential axonal retraction. This receptor cross-talk is also likely to be bidirectional; robust TrkA activation would likely silence a p75NTR-mediated ceramide flux, as previously demonstrated in PC12 cells (Dobrowsky et al., 1995), thereby further favouring axonal growth. Such negative feedback between these two receptors provides a mechanism for biasing the cell to one of two outcomes, growth or no growth, thereby

ensuring that axon collaterals that are only minimally successful in terms of sequestering target territory do not remain to compete during this critical developmental period.

Does this functional antagonism between TrkA and p75NTR generalize to neurons other than sympathetic neurons? The most compelling case that it does, to at least some degree, derives from studies on basal forebrain cholinergic neurons. In $p75NTR^{-/-}$ mice, the number of basal forebrain cholinergic neurons increases (van der Zee et al., 1996) and the hippocampus is hyperinnervated (Yeo et al., 1997), two phenotypes reminiscent of sympathetic neurons in $p75NTR^{-/-}$ and $BDNF^{-/-}$ mice (Lee et al., 1994; Bamji et al., 1998; data presented here). Such functional antagonism may also occur, in at least some situations, for TrkA-positive sensory neurons. For example, the local NGF-promoted growth of adult sensory neurons is inhibited by BDNF (Kimpinski et al., 1997), cultured dorsal root ganglia neurons showed a decrease in growth cone turning towards NGF-coated beads in the presence of BDNF (Gallo et al., 1997), and functional ablation of p75NTR using antisense oligonucleotides enhanced the survival of cultured postnatal sensory neurons (Barrett and Bartlett, 1994). Moreover, one recent study indicates that this functional antagonism between p75NTR and TrkA may not only regulate the innervation of appropriate targets, but may also allow axons to distinguish between permissive and nonpermissive growth substrates. In particular, when NGF is overexpressed in astrocytes of transgenic mice, absence of p75NTR leads to robust sympathetic axon growth on myelinated tracts in the mature CNS, indicating that p75NTR plays a significant role in making CNS myelin an inhibitory growth environment, at least for sympathetic axons (Walsh et al., 1999). However, it is

also clear that, like other members of the p75NTR family, the outcome of p75NTR-mediated signal transduction is a function of cellular context. For example, depending upon the cellular environment, p75NTR regulates cell migration (Anton et al., 1994), gene expression (Itoh et al., 1995), and can positively modulate TrkA signalling (Barker and Shooter, 1994; Verdi et al., 1994).

In summary, our studies provide evidence for a mechanism whereby two receptors coexpressed in sympathetic neurons, TrkA and p75NTR, can functionally interact to regulate process outgrowth during the time of target innervation, thereby ensuring appropriate matching of neurons and their target territory. Thus, not only do neurotrophins regulate neuronal survival and growth, depending on the particular receptors that they activate, but they may also provide a mechanism whereby neurons can recognize whether they are exposed to the "right" versus the "wrong" targets. Such functional antagonism, mediated by TrkA and p75NTR, appears to be essential for appropriate sympathetic neuron target innervation, and similar mechanisms may well turn out to play an essential role in the matching of neurons with their targets throughout the nervous system.

REFERENCES

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J. Cell Biol. 143, 1691-1703.

Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay R, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 389:856-860.

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.

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.

Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. Neuron 13:203-2215.

Barrett GL, 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.

Belliveau DJ, Krivko I, Kohn, Lachance C, Pozniak C, Rusakov D, Kaplan D, Miller FD (1997) NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. J Cell Biol 136: 375-388.

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

Causing CG, Gloster A, Aloyz R, Bamji SX, Chang E, Fawcett J, Kuchel G, Miller FD (1997) Synaptic innervation density is regulated by neuron-derived BDNF. Neuron 18: 257-267.

Chandler C, Parsons L, Hosang M, Shooter E (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 259:6882-6889.

Clary DO, Weskamp G, Austin LR, Reichart LF (1994) TrkA cross-linking mimics neuronal responses to nerve growth factor. Mol Biol Cell. 5:549-563.

Colman H, Nabekura J, Lichtman JW (1997) Alterations in synaptic strength preceding axon withdrawal. Science 275:356-361.

Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S (1997) Distribution of brain-

derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci 17:2295-2313.

Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF, Barbacid M (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173-183.

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 76:1-20.

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.

Dobrowsky RT, Jenkins GM, Hannun YA (1995) Neurotrophins induce sphingomyelin hydrolysis: modulation by co-expression of p75 with Trk receptors. J Biol Chem:22135-22142.

Ernfors P, Wetmore C, Olson L, Persson L (1990) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family.

Neuron 5:511-526.

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

Fawcett JP, Aloyz R, McLean JH, Pareek S, Miller FD, McPherson PS, Murphy RA (1997) Detection of brain-derived neurotrophic factor in a vesicular fraction of brain synaptosomes. J. Biol. Chem. 272, 8837-8840.

Fawcett JP, Bamji SX, Causing CG, Aloyz R, Ase AR, Reader TA, McLean JH, Miller FD (1998) Functional evidence that BDNF is an anterograde neuronal trophic factor in the CNS. J Neurosci 18:2808-2821.

Gallo G, Lefcort FB, Letourneau PC (1997) The TrkA receptor mediates growth cone turning toward a localized source of nerve growth factor. J Neurosci 17:5445-5454.

Hakanson R, Lombard Des Gouttes M-N, Owman C (1967) Activities of tryptophan hydroxylase, dopa decarboxylase and monoamine oxidases correlated with the appearance of monoamines in the developing rat pineal gland. Life Sci 6:2577-2585.

Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, Kaplan DR (1992) Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron 9:883-896.

Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde Y-A (1990) Regional distribution of brain-derived neurotrophic factor mRNA in adult mouse brain. EMBO J 9:2459-2464.

Ibáñez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H (1992) Disruption of the low affinity receptor binding site in NGF allows for neuronal survival and differentiation by binding to the trk gene product. Cell 69:329-341.

Itoh K, Brackenbury R, Akeson RA (1995) Induction of L1 mRNA in PC12 cells by NGF is modulated by cell-cell contact and does not require the high-affinity NGF receptor. J Neurosci 15:2504-2512.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao MV (1986) Expression and structure of the human NGF receptor. Cell: 47:545-554.

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

Kaplan DR, Martin-Zanca D, Parada LF (1991b) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350:158-160.

Kappers JA (1960) The development, topographical relations and innervation of the

epiphysis cerebri in the albino rat. Z Zellforsch mikrosk Anat 52:163-215.

Kimpinski K, Campenot RB, Mearow K (1997) Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. J Neurobiol 33: 395-410.

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.

Korte M, Carroll P, Wolf E, brem G, Thoenen H, Bonhoeffer T (1995) Hipppocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc. Natl. Acad. Sci. USA 92, 8856-8860.

Kuchel GA (1993) Alterations in target innervtion and collateral sprouting in the aging sympathetic nervous system. Exp Neurol 124: 381-386.

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, Bachman K, Landis S, Jaenisch R (1994) Dependence on p75 for innervation of some sympathetic targets. Science 263:1447-1449.

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

Ma Y, Campenot RB, Miller FD (1992) Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117: 135-141.

MacPhee IJ, Barker PA (1997) Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduced TrkA signalling while increasing serine phosphorylation in the TrkA intracellular domain. J Biol Chem 272:23547-23551.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Firth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a new neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Miller FD, Kaplan DR (1998) Life and Death decisions: a biological role for the p75 neurotrophin receptor. Cell Death Diff 5:343-345.

Miller FD, Mathew TC, Toma JG (1991) Regulation of nerve growth factor receptor gene expression by NGF in the developing peripheral nervous system. J Cell Biol 112: 303-312.

Miller FD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG (1994)

Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. Dev Biol 161:206-217.

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

Owman C (1964) Sympathetic nerves probably storing two types of monoamines in the rat pineal gland. Int J Neuropharmac 2:105-1112.

Phillips HS, Hains JM, Laramee GR, Rosenthal A, Winslow JW (1990) Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science 250:290-294.

Posse de Chaves EI, Bussière M, Vance DE, Campenot RB, Vance JE (1997) Elevation of ceramide within distal neurites inhibits neurite growth in cultured rat sympathetic neurons. J Biol Chem 272:3028-3035.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-596.

Slack RS, Belliveau DJ, Rosenberg M, Atwal J, Lochmuller H, Aloyz R, Haghighi A, Lach B, Seth P, Cooper E, Miller FD (1996) Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. J. Cell Biol. 135, 1-12.

Stanley LC, Horikawa K, Powell EW (1987) Innervation of the superficial pineal of the rat using retrograde tracing methods. Am J Anat 180:249-254.

Schecterson LC, Bothwell M (1992) Nevel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9:449-463.

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

Thoenen H, Barde Y-A (1980) Physiology of nerve growth factor. Physiol Rev 60:1284-1335.

Van der Zee CEEM, Ross GM, Riopelle RJ. Hagg T (1996) Survival of cholinergic forebrain neurons in developing $p75^{NFGR}$-deficient mice. Science 274:1729-1732.

Verdi JM, Birren SJ, Ibáñez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994) p75(NGFR) regulates trk signal transduction and NGF-induced differentiation in MAH cells. Neuron 12:733-745.

von Bartheld CS, Byers MR, Williams R, Bothwell M (1996) Anterograde transport of neurotrophins and axodendritic transfer in the developing visual system. Nature 379:830-833.

Walsh GS, Krol KM, Crutcher KA, Kawaja MD (1999) Enhanced neurotrophin-induced axon growth in myelinated portions of the central nervous system in mice lacking the p75 neurotrophin receptor. J. Neurosci (in press).

Weskamp G, Reichardt LF (1991) Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. Neuron 6:649-663.

Yang X-M, Toma JG, Bamji SX, Belliveau DJ, Kohn J, Park M, Miller FD (1998) Autocrine hepatocyte growth factor provides a local mechanism for promoting axonal growth. J. Neurosci. 18, 8369-8381.

Yeo TT, Chua-Couzens J, Butcher LL, Bredesen DE, Cooper JD, Valletta JS, Mobley WC, Longo FM (1997). Absence ofp75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. J Neurosci 17:7594-7605.

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

FIGURE LEGENDS

Figure 1: **BDNF decreases the growth of sympathetic neurons *in vitro* without affecting their survival.** (A) Results of colorimetric MTT assays to measure mitochondrial function and cell survival. Neonatal sympathetic neurons were cultured in 50 ng/ml NGF for 5 days, washed free of neurotrophin-containing medium, and then switched for 2 days to NGF or NGF plus BDNF. The data derive from a representative survival assay which was performed in triplicate. In these assays, absolute values are normalized so that the value obtained with 0 neurotrophin is 0% survival, while that obtained with 10 ng/ml NGF is considered 100% survival. Error bars represent SEM. The values obtained for NGF versus NGF plus BDNF were not significantly different ($p>0.05$). (B,C,D) Quantitative analysis of neurite process density in sympathetic neuron cultures grown in the presence of NGF or NGF plus BDNF. (B) Six separate experiments were performed to determine the effect of BDNF on NGF-mediated process density in sympathetic neurons. Sympathetic neurons were plated at low density on collagen or poly-D-lysine and laminin in 50 ng/ml NGF for 2 to 3 days, and were then switched to 10 ng/ml NGF plus or minus 100 ng/ml BDNF for 2 days. In all six experiments, significantly fewer neurite intersections were observed after exposure to NGF+BDNF versus NGF alone ($*P<0.05$). (C) The experiments shown in (B) were normalized so that the neurite density at 10 ng/ml NGF is 100, and then averaged to provide an index of the relative neurite density. (D) Dose-response curve to determine the effect of different concentrations of BDNF on NGF-mediated process density. Experiments were performed as in (B). No significant different in process density was observed when 50 ng/ml BDNF was added, but statistically significant differences were

seen with both 100 and 200 ng/ml BDNF (*P<0.05). The data derive from one neurite outgrowth assay, where each condition was sampled 4-5 times. (E) Results of TUNEL-labelling to measure apoptotic neurons. Neurons were treated as in (A), but were TUNEL-labelled to measure apoptotic cells, and then stained with Hoechst 33250 to quantitate total neuronal nuclei. 7-8 fields of cells were analyzed per treatment in each experiment, and results are expressed as the percentage of TUNEL-labelled nuclei/total neuronal nuclei. Results represent the mean ± SEM. Note that BDNF had no effect on the number of TUNEL-labelled neurons in these experiments. (F,G) Quantitative analysis of the area covered by tubulin immunoreactive neurites in sympathetic neuron cultures grown in 10 ng/ml NGF with or without BDNF. The same fields of cells shown in (E) were analyzed for the percentage of area within a given field that was covered by tubulin-immunoreactive processes. These data were normalized for the total number of neuronal cell bodies in the same field (as indicated by Hoechst-staining), and then used to determine the relative tubulin immunolabelled area/neuron. Results represent the mean ± SEM; in both experiments, BDNF caused a statistically-significant decrease in tubulin-immunoreactive processes (*P<0.05), with no effect on neuronal apoptosis (panel E). (G) The experiments shown in (F) were normalized so that the tubulin immunolabelled area at 10 ng/ml NGF was 100, and then averaged to provide an index of the mean relative tubulin immunolabel.

Figure 2: **Exogenous BDNF inhibits and anti-BDNF and anti-p75NTR enhance NGF-promoted growth of sympathetic neurons in vitro.** Phase contrast micrographs of cultured neonatal rat sympathetic neurons maintained in 50 ng/ml NGF for 2 to 3

days, and then switched to (A) 10 ng/ml NGF, (B) 10 ng/ml NGF plus 100 ng/ml BDNF, (C) 10 ng/ml NGF plus 10 μg/ml anti-BDNF, or (D) 10 ng/ml NGF plus anti-p75NTR (REX). Exogenous BDNF inhibited and BDNF or p75NTR antibodies enhanced process outgrowth. Scale bar = 65 μM.

Figure 3: **BDNF decreases neurite outgrowth in cultured neonatal rat sympathetic neurons without decreasing their survival.** Digitized micrographs of postnatal day 1 sympathetic neuron cultures triple-labelled to visualize neurite outgrowth (α-tubulin, GREEN), apoptotic cells (TUNEL-labelled, RED/PINK), and total number of cells in the culture (Hoechst nuclear stain, BLUE). Cultures were grown in 50 ng/ml NGF for 2 days, and then switched to either (A) 10 ng/ml NGF, (B) 10 ng/ml NGF plus 100 ng/ml BDNF, or (C) withdrawn from NGF. After 2 additional days, the cultures were labelled as indicated above. Arrows point to nuclei of apoptotic cells. Note that the addition of BDNF to the cultures did not increase the degree of TUNEL-labelling over that observed in cultures treated with NGF alone, whereas cultures in which NGF was withdrawn exhibit a high number of TUNEL-labelled neurons and a complete disintegration of neurites. Magnification = 160X.

Figure 4: **Function-blocking antibodies directed against BDNF or p75NTR enhance growth of sympathetic neurons without affecting their survival.** (A) Results of colorimetric MTT assays to meaure mitochondrial function and cell survival. Neonatal sympathetic neurons were cultured in 50 ng/ml NGF for 5 days, washed free of neurotrophin-containing medium, and then switched for 2 days to 10 ng/ml NGF or 10

ng/ml NGF plus 10 μg/ml anti-BDNF (α-BDNF). These data represent the values obtained in a representative survival assay which was performed in triplicate. In these assays, absolute values are normalized so that the value obtained with 0 neurotrophin is 0% survival, while that obtained with 10 ng/ml NGF is considered 100% survival. Error bars represent SEM. These two values were not significantly different (P=0.3710). **(B-H) Quantitative analysis of neuritic process density in sympathetic neuron cultures grown in the presence of NGF, NGF plus anti-BDNF (B,C), NGF plus REX (D,E), increased NGF (F), or (G,H) NGF and REX plus or minus BDNF.** (B) Three separate experiments were performed to determine the effect of anti-BDNF on NGF-promoted neurite process growth in sympathetic neurons. Sympathetic neurons were plated at low density in 50 ng/ml NGF on collagen, or poly-D-lysine and laminin for 2 to 3 days, and then switched for an additional two days to 10 ng/ml NGF plus or minus 10 μg/ml anti-BDNF (α-BDNF) or, as a control, 10 μg/ml nonimmune IgY. In all three experiments, significantly more neurite intersections were observed after exposure to anti-BDNF relative to NGF alone (*P<0.05). (C) The experiments shown in (B) were normalized so that the neuritic density at 10 ng/ml NGF is 100, and then averaged to provide an index of the relative neurite density. (D) Three separate experiments were performed to determine the effect of anti-p75NTR on NGF-promoted neurite process growth in sympathetic neurons. Sympathetic neurons were treated as in (B), except that they were treated with a 1:100 dilution of p75NTR antiserum (REX) or, as a control, with the same dilution of nonimmune rabbit serum. In all three experiments, significantly more neurite intersections were observed after exposure to anti-p75NTR than after NGF alone (*P<0.05). Serum itself had no effect (P>0.05). (E) The

experiments shown in (D) were normalized so that the neuritic density at 10 ng/ml NGF is 100, and then averaged to provide an index of the relative neurite density. (F) The increase in relative neurite process density in 40 ng/ml NGF relative to 10 ng/ml NGF. These data represent the results from one experiment, and are shown here for comparison only. We have previously documented the reproducibility and significance of this increase in Belliveau et al. (1997). (G) Three separate experiments were performed to determine whether BDNF could antagonize the p75NTR-mediated increase in sympathetic neuron growth. Sympathetic neurons were treated as in (B), except that they were treated with a 1:100 dilution of p75NTR antiserum (REX) plus or minus 100 ng/ml BDNF. In all three experiments, exogenous BDNF significantly inhibited the REX-induced increase in process density (*$P<0.05$). (H) The experiments shown in (G) were normalized so that the neuritic density at 10 ng/ml NGF is 100, and then averaged to provide an index of the relative neurite density.

Figure 5: **Analysis of neurite outgrowth in response to NGF or NGF plus BDNF in p75NTR$^{-/-}$ versus p75NTR$^{+/+}$ sympathetic neurons.** (A,B) Results of colorimetric MTT assays to meaure mitochondrial function and survival of murine sympathetic neurons in response to NGF or NGF plus BDNF. (A) Neonatal murine sympathetic neurons were cultured in 50 ng/ml NGF for 5 days, washed free of neurotrophin-containing medium, and switched for 2 days to various concentrations of NGF as indicated on the x-axis. Results from one representative experiment performed in triplicate are shown. In these assays, absolute values are normalized so that the value obtained with 0 neurotrophin is 0% survival, while that obtained with 10 ng/ml NGF is

considered 100% survival. Error bars represent SEM. Note that 5 ng/ml NGF is capable of eliciting maximal survival of murine sympathetic neurons. (B) Neonatal murine sympathetic neurons were cultured as in (A), and were then switched into various concentrations of NGF plus 100 ng/ml BDNF, as denoted on the x-axis. These data represent values from one representative experiment performed in tripicate. Values are normalized as in (A), and error bars represent SEM. As with rat sympathetic neurons, BDNF does not affect survival in optimal concentrations of NGF. (C,D) Quantitative analysis of neuritic process density in p75NTR$^{-/-}$ versus wildtype murine sympathetic neurons in response to NGF or NGF plus BDNF. In the two separate experiments shown here, p75NTR$^{-/-}$ versus wildtype neonatal sympathetic neurons were cultured as sister cultures for 3 days in 50 ng/ml NGF, and were then switched for an additional 2 days to 7.5 ng/ml NGF plus or minus 100 ng/ml BDNF. One asterisk denotes values that were significantly different in the comparison between NGF versus NGF plus BDNF (*$P<0.05$) while two asterisks denote those values that were significantly different in the comparison between p75NTR$^{-/-}$ versus wildtype (WT) neurons in response to 7.5 ng/ml NGF (**$P<0.05$). Note that p75NTR$^{-/-}$ neurons grow more robustly than wildtype neurons in response to the same concentration of NGF. Note also that BDNF significantly reduces the NGF-mediated growth of wildtype ($P<0.05$), but not p75NTR$^{-/-}$ (in both experiments, $P>0.38$) neurons. (D) The experiments shown in (C) were normalized so that the neuritic density of wildtype neurons at 10 ng/ml NGF was 100, and then averaged to provide an index of the relative neurite density.

Figure 6: **p75NTR$^{-/-}$ sympathetic neurons show enhanced neuritogenesis in response to NGF, and do not respond to exogenous BDNF.** Phase contrast micrographs of cultured Coomassie Blue-stained (A,B) wildtype and (C,D) p75NTR$^{-/-}$ murine sympathetic neurons maintained in 50 ng/ml NGF for 2 days, and then switched to (A,C) 7.5 ng/ml NGF, or (B,D) 7.5 ng/ml NGF plus 100 ng/ml BDNF. As observed in cultured rat sympathetic neurons (Fig. 2B), exogenous BDNF inhibited NGF-promoted process outgrowth in wildtype but not p75NTR$^{-/-}$ murine sympathetic neurons. Note that the degree of process outgrowth is greatly enhanced in p75NTR$^{-/-}$ neurons, relative to their wildtype counterparts. Scale bar = 65 μM.

Figure 7: **BDNF is present in the pineal gland during the period of sympathetic target innervation, as detected by Western blot analysis.** (A) Western blot analyis for BDNF in the adult rat pineal gland. Tissue lysates were separated on polyacrylamide gels, transferred to nitrocellulose, and probed with an antibody to BDNF that we have previously extensively characterized (Causing et al., 1997; Fawcett et al., 1997; Fawcett et al., 1998). (B) Pineal gland BDNF is the same size as recombinant human BDNF. Western blot analysis of tissue lysates revealed that the band seen in the adult pineal gland (Pineal) is similar in size to recombinant human BDNF (rhBDNF) and to BDNF in the adult rat cortex (Cortex). Lysates from the brain and pineal contain equal amounts of protein. (C) Western blot analysis of BDNF in the pineal gland of BDNF$^{-/-}$, and BDNF$^{+/+}$ littermates at P13 to P15. Note that the BDNF-immunoreactive band is not present in the BDNF$^{-/-}$ pineal gland.

Figure 8. **The pineal gland is hyperinnervated with sympathetic fibres in BDNF$^{+/-}$ and BDNF$^{-/-}$ mice at P13.** (A-C) Immunocytochemical analysis of tyrosine hydroxylase, a specific marker for sympathetic axons, in sections of the pineal gland from (A) BDNF$^{+/+}$, (B) BDNF$^{+/-}$, and (C) BDNF$^{-/-}$ littermates. Note that the density of TH-positive fibers is increased in both the BDNF$^{+/-}$ and $^{-/-}$ sections relative to the section from the control littermate. (D,E) Immunocytochemical analysis of TH (D) and p75NTR (E) in sections from the same P13 BDNF$^{+/-}$ pineal gland. Note that, although the p75NTR-immunoreactivity is somewhat fainter, the pattern of immunoreactivity is similar to that seen with anti-TH. (F) Quantitative analysis of the relative amount of pineal gland area covered by TH-immunoreactive fibers in BDNF$^{+/+}$, $^{+/-}$ and $^{-/-}$ animals, obtained using sections similar to those shown in panels A-C. For details of the analysis, see the Results and Materials and Methods sections. Each experiment represents the results obtained from the pineal glands of one set of littermates of different genotypes. Note that, in all four experiments, the amount of TH-positive innervation in the BDNF$^{+/+}$ pineal gland was significantly lower than that seen in either the BDNF$^{+/-}$ or BDNF$^{-/-}$ pineal glands (*$P<0.05$). Magnification is 160X in panels A-E.

Figure 9. **Levels of sympathetic axon markers are increased in sympathetic targets in BDNF$^{+/-}$ and BDNF$^{-/-}$ mice.** Western blot analysis of tyrosine hydroxylase (TH), p75NTR (P75), α-tubulin (Tubulin) and ERK1 (Erk1) in equal amounts of protein from the pineal glands and carotid arteries of BDNF$^{+/+}$, BDNF$^{+/-}$, and BDNF$^{-/-}$ littermates at P13 to P15. Note that the blots shown for ERK1 are reprobes of the same blots shown for p75NTR, in the case of the pineal gland, and for α-tubulin, in the case of the carotid

artery.

Figure 2.1

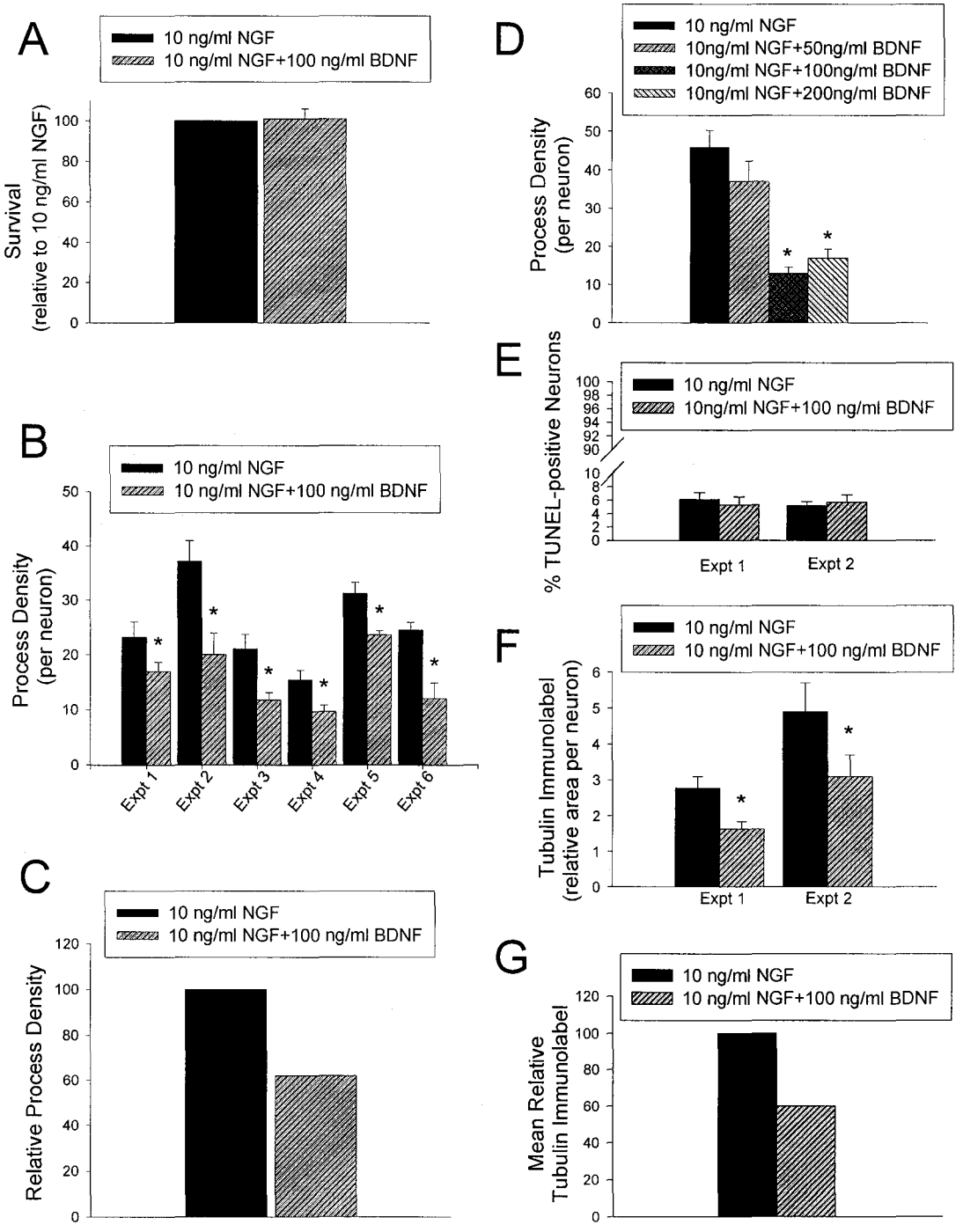

A
10 ng/ml NGF
10 ng/ml NGF+100 ng/ml BDNF
Survival
(relative to 10 ng/ml NGF)
B
10 ng/ml NGF
10 ng/ml NGF+100 ng/ml BDNF
Process Density
(per neuron)
Expt 1
Expt 2
Expt 3
Expt 4
Expt 5
Expt 6
C
10 ng/ml NGF
10 ng/ml NGF+100 ng/ml BDNF
Relative Process Density
D
10 ng/ml NGF
10ng/ml NGF+50ng/ml BDNF
10ng/ml NGF+100ng/ml BDNF
10ng/ml NGF+200ng/ml BDNF
Process Density
(per neuron)
E
10 ng/ml NGF
10ng/ml NGF+100 ng/ml BDNF
% TUNEL-positive Neurons
Expt 1
Expt 2
F
10 ng/ml NGF
10 ng/ml NGF+100 ng/ml BDNF
Tubulin Immunolabel
(relative area per neuron)
Expt 1
Expt 2
G
10 ng/ml NGF
10 ng/ml NGF+100 ng/ml BDNF
Mean Relative
Tubulin Immunolabel

Figure 2.2

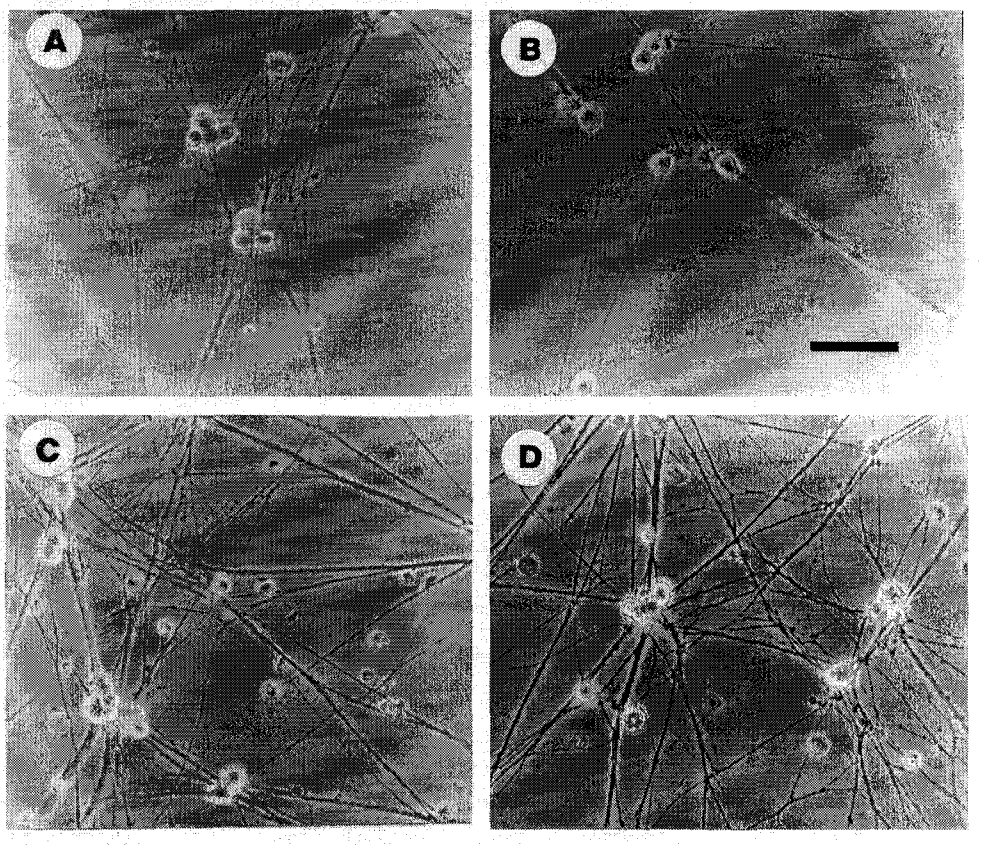


Figure 2.3

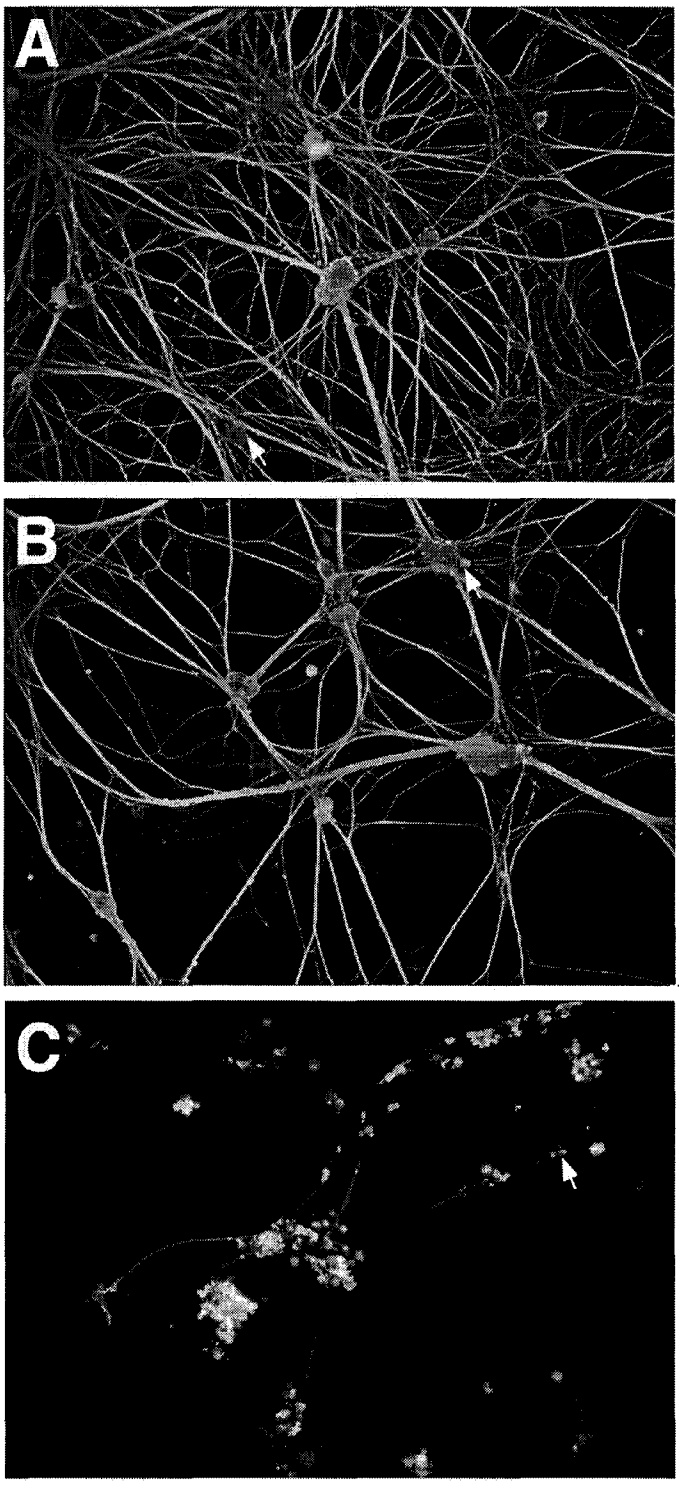


Figure 2.4

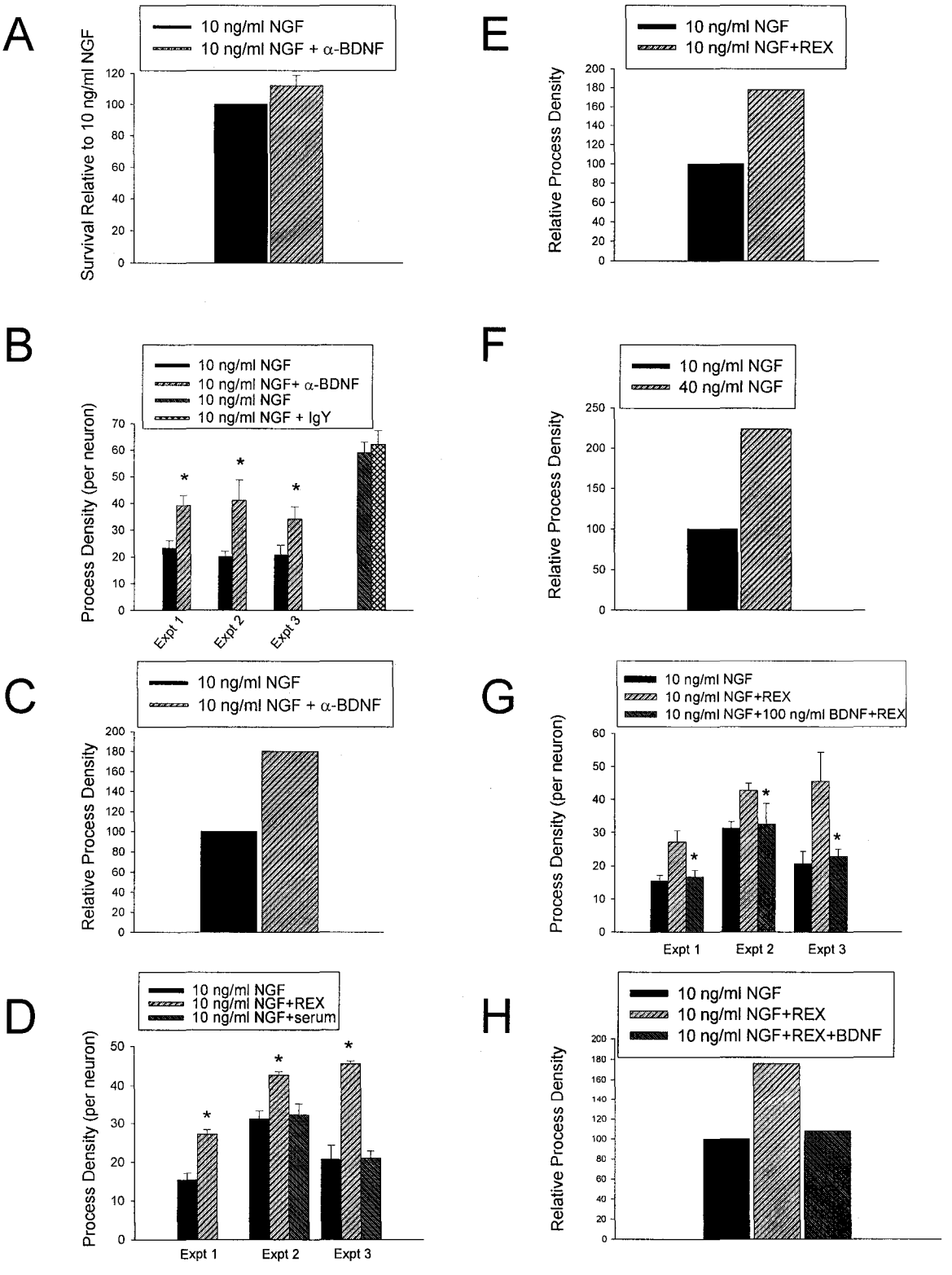

A
10 ng/ml NGF
10 ng/ml NGF + α-BDNF
Survival Relative to 10 ng/ml NGF
B
10 ng/ml NGF
10 ng/ml NGF+ α-BDNF
10 ng/ml NGF
10 ng/ml NGF + IgY
Process Density (per neuron)
Expt 1
Expt 2
Expt 3
C
10 ng/ml NGF
10 ng/ml NGF + α-BDNF
Relative Process Density
D
10 ng/ml NGF
10 ng/ml NGF+REX
10 ng/ml NGF+serum
Process Density (per neuron)
Expt 1
Expt 2
Expt 3
E
10 ng/ml NGF
10 ng/ml NGF+REX
Relative Process Density
F
10 ng/ml NGF
40 ng/ml NGF
Relative Process Density
G
10 ng/ml NGF
10 ng/ml NGF+REX
10 ng/ml NGF+100 ng/ml BDNF+REX
Process Density (per neuron)
Expt 1
Expt 2
Expt 3
H
10 ng/ml NGF
10 ng/ml NGF+REX
10 ng/ml NGF+REX+BDNF
Relative Process Density

Figure 2.5

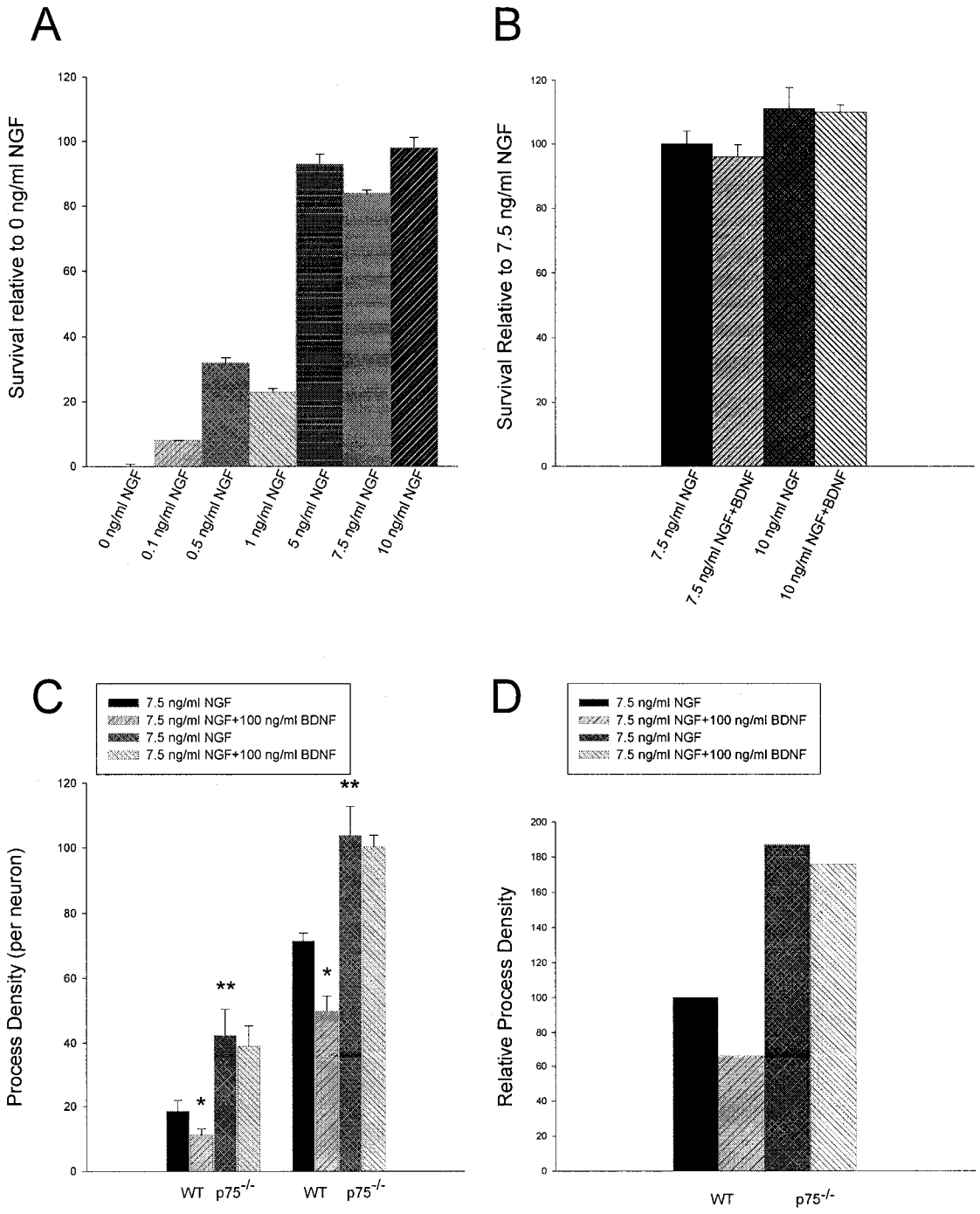

A
Survival relative to 0 ng/ml NGF
120
100
80
60
40
20
0
0 ng/ml NGF
0.1 ng/ml NGF
0.5 ng/ml NGF
1 ng/ml NGF
5 ng/ml NGF
7.5 ng/ml NGF
10 ng/ml NGF
B
Survival Relative to 7.5 ng/ml NGF
120
100
80
60
40
20
0
7.5 ng/ml NGF
7.5 ng/ml NGF+BDNF
10 ng/ml NGF
10 ng/ml NGF+BDNF
C
7.5 ng/ml NGF
7.5 ng/ml NGF+100 ng/ml BDNF
7.5 ng/ml NGF
7.5 ng/ml NGF+100 ng/ml BDNF
Process Density (per neuron)
120
100
80
60
40
20
0
*
**
*
**
WT
p75-/-
WT
p75-/-
D
7.5 ng/ml NGF
7.5 ng/ml NGF+100 ng/ml BDNF
7.5 ng/ml NGF
7.5 ng/ml NGF+100 ng/ml BDNF
Relative Process Density
200
180
160
140
120
100
80
60
40
20
0
WT
p75-/-

Figure 2.6

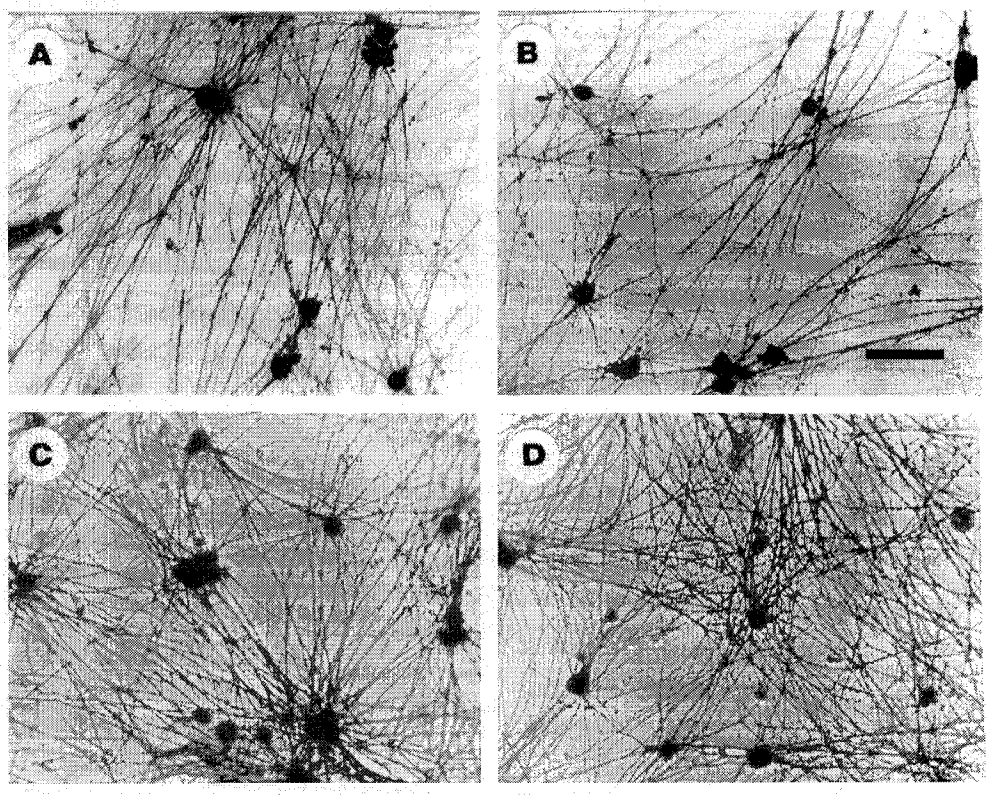


Figure 2.7

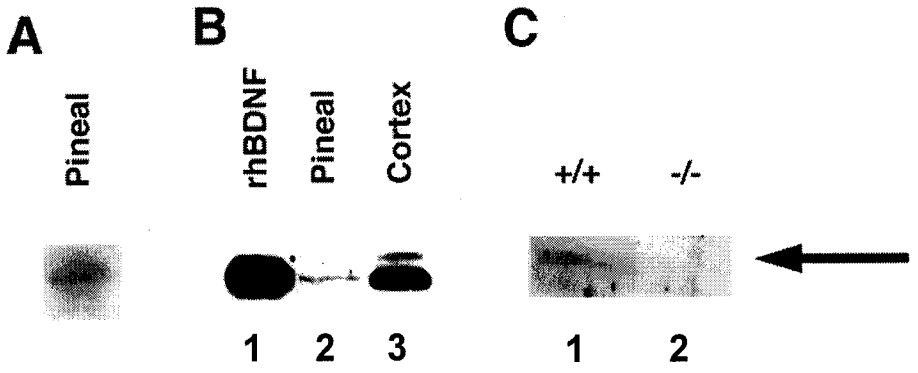
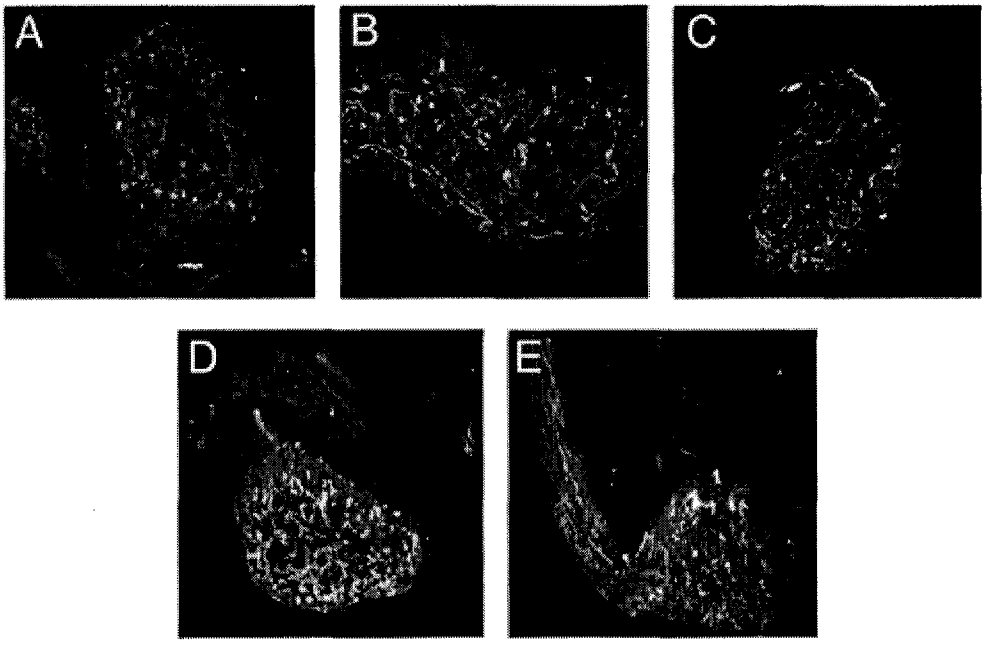


Figure 2.8


A
B
C
D
E

F

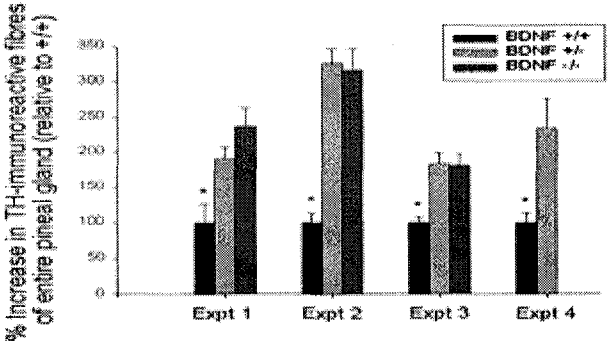
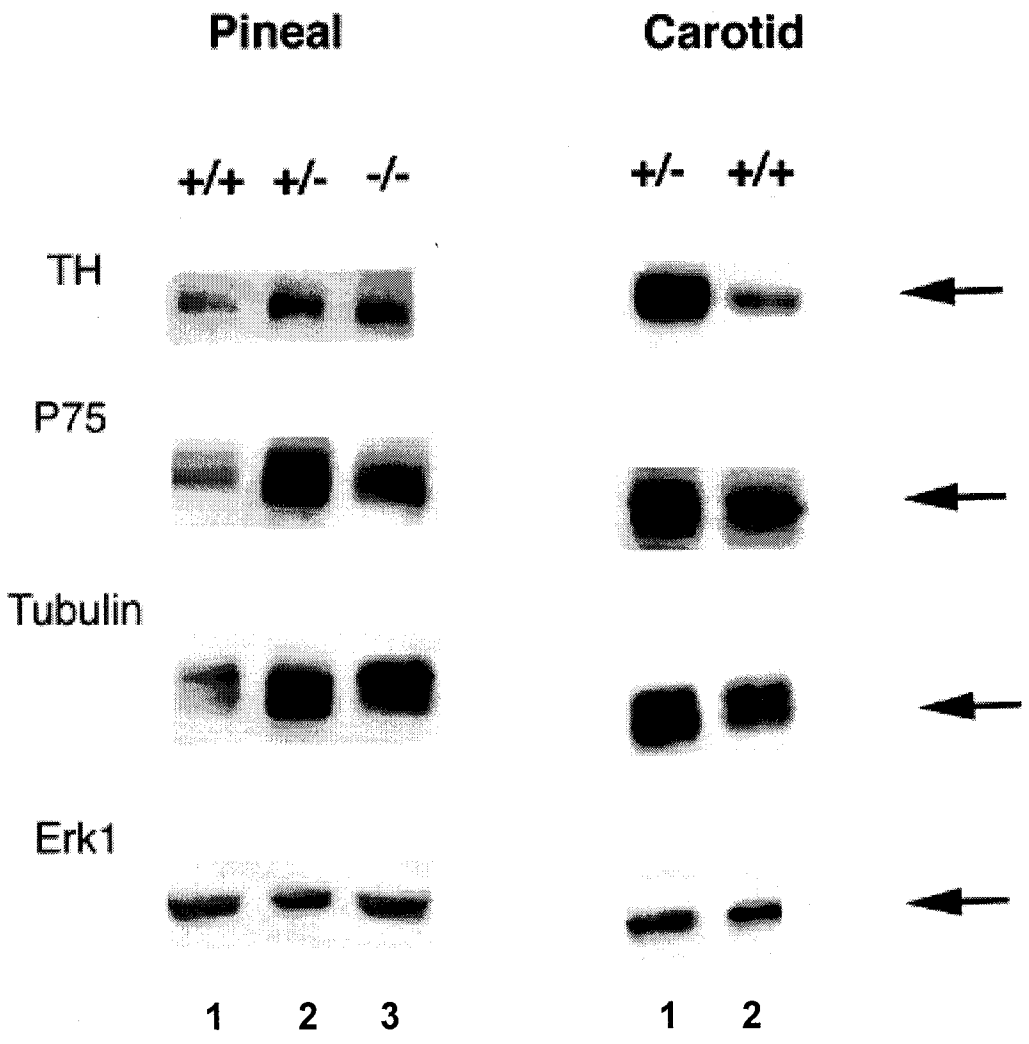

% Increase in TH-immunoreactive fibres of entire pineal gland (relative to +/+)
BDNF +/+
BDNF +/-
BDNF -/-
Expt 1
Expt 2
Expt 3
Expt 4

Figure 2.9



CHAPTER 3

CHAPTER 3

Preface

The previous paper focused on how functional interactions between TrkA and p75NTRs could regulate the growth and target innervation of sympathetic neurons. In this paper, this theme is explored further. I addressed how increasing the levels of TrkA can influence biological responsiveness of sympathetic neurons to NGF, potentially allowing more efficient inhibition of the p75NTR's inhibitory signals.

I used adenoviral approaches to overexpress TrkA, and demonstrated that levels of this receptor are a key determinant of biological responsiveness to NGF. Since such responsiveness may be regulated spatially, I characterized the distribution of TrkA and p75NTRs on sympathetic neurons. I found that although TrkA and p75NTRs are both expressed all over these neurons, they are differentially distributed with respect to their spatial localization. I showed, immunocytochemically, that TrkA is enriched in the cell body, while the p75NTR is more evenly distributed. This means that the ratio of these receptors varies spatially, depending upon which compartment of a neuron (cell body versus processes) is being considered. I used confocal microscopy to measure the relative density of these receptors on the neuronal surface, and found, as the immunocytochemical analysis suggested, that TrkA is expressed at much higher levels in the cell body than in neurites, while the expression of the p75NTR was much less variable between compartments. Since TrkA and p75NTRs are differential distributed with respect to spatial compartments, their ratio also varies spatially. This might regulate signaling events that may be generated in the different compartments.

Finally, since TrkA and p75NTRs interact to regulate biological responsiveness, are coexpressed, and can regulate each other's signaling pathways, the potential exists for direct physical interactions between them. I addressed whether or not they form a complex by attempting to co-patch these receptors on the surface of sympathetic neurons. I found that some, but not all TrkA and p75NTRs are co-patched on neurites,

but not cell bodies, implying that interactions between them are more likely to be functional than physical.

This paper is being prepared for submission.

TrkA OVEREXPRESSION ENHANCES THE ABILITY OF SYMPATHETIC NEURONS TO RESPOND TO NGF

Judi Kohn, Lynne LeSauteur, David R. Kaplan and Freda D. Miller

Brain Tumour Research Centre
Montreal Neurological Institute
McGill University
Montreal, Quebec
Canada
H3A 2B4

Abstract

Previous studies from our laboratory have demonstrated that a balance between positive-NGF/TrkA and negative p75NTR signaling regulates the biological responsiveness of sympathetic neurons, and determines how well they survive, grow, and innervate their physiological targets. In this report, we used adenoviral approaches to examine how increased levels of TrkA expression might shift this balance to alter the TrkA:p75NTR receptor ratio and increase the sensitivity of neonatal sympathetic neurons to NGF. Since TrkA and p75NTRs have functionally opposing roles in sympathetic neurons with respect to both survival and growth, their cellular ratio is an important determinant of biological responsiveness in this neuronal population. We first used immunocytochemical approaches to characterize the spatial localization of these receptors. We determined that TrkA is preferentially expressed in cell bodies, while the p75NTR is more ubiquitously expressed along the neuronal surface. Using confocal microscopy, we determined that the relative difference in p75NTR density between cell bodies and neurites is less than 2:1, while for TrkA, the relative density is higher on the cell body than on neurites (a difference of almost 4:1). This indicates that the ratio of TrkA: p75NTR is higher on cell bodies than on neurites and implies that signaling events may differ spatially. Since TrkA and p75NTRs have opposing roles, the question of whether they interact biophysically to modulate each other's activities was investigated. We used a receptor co-patching technique to determine whether they form complexes in culture, and whether any such complexes were localized to specific neuronal compartments. Our analysis indicated that some, but not all TrkA and p75NTRs on neurites (but not cell bodies) were co-patched, indicating that most interactions between these receptors, at least in our model system, are more likely to be functional than physical. To test whether increased TrkA expression could alter the sensitivity of these neurons to NGF, we overexpressed TrkA using an adenoviral expression system and tested whether previously suboptimal NGF signals would become permissive for survival. MTT survival assays indicated that concentrations of NGF that could not previously support survival (<10 ng/ml NGF) were able to do so in a dose related manner, and 10 ng/ml NGF was able to stimulate more neurite outgrowth in TrkA-infected cultures relative to control. This demonstrates that levels of TrkA in

sympathetic neurons are a key determinant of biological responsiveness, and suggests that altering the TrkA-p75NTR signaling balance in favor of positive-TrkA signals enhances responsiveness. Together, these studies indicate that a balance between TrkA and p75 activation in sympathetic neurons is essential for achieving physiologically appropriate levels of neuronal survival and growth.

Introduction

For most peripheral nervous system neurons, survival growth, and cell maintenance is dependent upon a family of neurotrophic factors known as the neurotrophins (Barde, 1989). This family is comprised of nerve growth factor (NGF; Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF; Barde et al., 1982), and neurotrophins 3 (Ernfors et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990), 4/5 (Berkmeier et al., 1991; Ip et al., 1992), 6 (Gotz et al., 1994) and 7 (Lai et al., 1998; Nilsson et al., 1998). These factors mediate neuronal survival, differentiation, and growth by interacting with two distinct classes of cell-surface receptors, the Trk family of tyrosine kinases (Barbacid, 1994) and the p75NTR (Johnson et al., 1986; Radeke et al., 1987). The Trk receptors preferentially bind specific neurotrophins, with NGF binding to TrkA (Cordon-Cardo et al., 1991; Kaplan DR et al., 1991a,b; Klein et al., 1991), BDNF and NT-4/5 binding to TrkB (Soppet et al., 1991), and NT-3 specifically binding TrkC, but also binding Trks A and B, albeit with lower affinity (Lamballe et al., 1991; Tsouflas et al., 1993). This is in contrast to the p75NTR, which binds all the neurotrophins (Rodriguez-Tébar et al., 1990; 1992).

There is a large body of evidence which indicates that the neurotrophins act through Trk and p75NTRs to regulate the survival and growth of developing neurons of the peripheral nervous system (reviewed in Snider, 1994; Majdan and Miller, 1999; Kaplan and Miller, 2000). We have previously shown that naturally-occurring cell death of sympathetic neurons is the result of both suboptimal TrkA receptor activation, and coincident activation of the p75NTR (Bamji et al., 1998). These receptors act in an antagonistic fashion, with TrkA mediating positive-survival signals and the p75NTR transducing negative or apoptotic responses. They have also been shown to have opposing roles in the regulation of sympathetic neuron growth and target innervation (Kohn et al., 1999). Exactly how they regulate each other's activities to achieve a physiological balance between life and death is not completely clear, and is an ongoing area of investigation in the field of neurotrophin signal transduction (reviewed in Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Nor is it understood exactly how they interact to regulate growth and achieve appropriate levels of target innervation. Since they are coexpressed in sympathetic neurons and can act

antagonistically to modulate each other's signals (Bamji et al., 1998; Kohn et al., 1999), the question remains as to how they interact to achieve a physiological balance in the sympathetic nervous system. A p75NTR-TrkA heteromolecular complex has been suggested, based on biochemical/molecular data (Hempstead et al., 1991; Bibel et al., 1999; Esposito et al., 2001; Mischel et al., 2001), biophysical data from co-patching studies with Sf9 cell lines (Ross et al., 1996), and FRAP experiments with Sf9 and PC12 cell lines (Wolf et al., 1995). Although data obtained from these studies suggested that there was a physical interaction between TrkA and p75NTRs, results obtained from high levels of receptor expression in cell lines may not be representative of the situation in primary neurons or *in vivo*.

Whether or not TrkA and p75NTRs interact physically, the biological responses of sympathetic neurons to NGF might be partially regulated by the spatial distribution of TrkA and p75NTRs on the neuronal surface. Since they have opposing roles, the cellular ratio of these receptors may determine the signaling events that occur downstream of their activation. Target-derived NGF, which upregulates the expression of the P75NTR, but not TrkA, has been demonstrated to increase the ratio of p75: TrkA on nerve terminals (Miller et al., 1994).

Using compartmented cultures of sympathetic neurons, Toma et al. (1997) found that biological responses to NGF varied with the spatial source of NGF, with NGF application to the cell body always generating more robust gene expression. This may occur if there is a differential distribution of TrkA and p75NTRs. A spatial difference in receptor expression could mean that the ratio of TrkA to p75NTR also varies spatially. This, in turn could mean that signaling events could also vary, depending on the cellular compartment. Thus a neuron might respond differently to neurotrophins that it might encounter developmentally, depending on the spatial location of its source.

Since TrkA and p75NTRs have been demonstrated to have opposing roles which together determine the outcome of biological events such as survival (Bamji et al., 1998) and growth (Kohn et al., 1999), a prediction from these data is that increasing levels of TrkA expression might also increase the sensitivity of sympathetic neurons to TrkA ligands such as NGF. In this paper we test this hypothesis, and show that the

levels of TrkA are a key determinant of biological responsiveness to NGF, since increasing TrkA expression levels allowed sympathetic neurons to become more responsive to NGF. Although we do not formally demonstrate that this occurs because the ratio of TrkA: p75NTR is altered, our demonstration of spatial differences in TrkA-p75NTR receptor ratio taken together with the published data from Toma et al (1997) discussed above suggests that it is a possibility.

Materials and Methods

Mass cultures of sympathetic neurons

Mass cultures of pure sympathetic neurons from the superior cervical ganglion (SCG) of postnatal day (P)1 Sprague Dawley rats (Charles River Breeding Laboratories, St. Constant, Quebec, Canada) were prepared as described previously (Ma et al., 1992). For biochemistry, neurons were plated at medium density (100 x 10^3 cells per well) in 6-well culture dishes (Falcon Labware/Beckton Dickinson, Lincoln Park, NJ) coated with rat tail collagen (Harlan Bioproducts, Madison, WI). For co-patching and immunocytochemical analyses, cultures were plated at low density (approximately 1 ganglion per well) in Nunclon 4-well culture dishes (Life Technologies, Burlington, Ontario, Canada) containing 13 mm coverslips (Fisher Scientific, Nepean, Ontario, Canada) coated with poly-d-lysine and laminin (Collaborative Biomedical Products, Bedford, MA), and for survival assays, neurons were plated in 96-well plates (Falcon) coated with rat tail collagen at a density of 3 x 10^3 cells per well. Culture medium was UltraCulture (BioWhittaker, Walkersville, MD) , supplemented with 3% rat serum (Harlan Bioproducts, Madison, WI), 2mM glutamine, 100 U/ml penicillin,100 µg/ml streptomycin (all from BioWhittaker), and for days 2 and 3, 7µM cytosine arabinoside (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). NGF used in these experiments was purified from mouse salivary glands and supplied by Cedarlane Laboratories (Hornby, Ontario, Canada), and was used at 50 ng/ml during routine maintenance, and between 1 and 50 ng/ml during experimental manipulations. For some experiments, anti-human NT-3 (Promega Corporation, Madison, WI) was used in the medium at a concentration of 10 µg/ml.

Immunocytochemistry

To determine the relative densities of TrkA and p75NTRs on cell bodies and neurites of P1 rat sympathetic neurons, cultures were maintained for 5 days in 50 ng/ml NGF, and characterization of TrkA and p75NTRs was carried out using standard immunocytochemical methodologies. Sympathetic neuron cultures were washed twice in PBS, and then fixed for 10 minutes with 4% paraformaldehyde in the presence of 0.05% Triton-X 100. Following a 5-minute wash in PBS, non-specific binding was

blocked by incubation for 1 hour in PBS containing 4% goat serum and 4% rat serum in PBS. Primary antibodies were prepared in blocking solution. The p75 NTR antibody Mc192, which is directed against the extracellular domain of the receptor, was the kind gift of Dr. R.A. Murphy (McGill University, Montreal, Quebec, Canada) and was used at a concentration of 3 μg/ml. The TrkA antibody RTA , which recognizes the extracellular domain of TrkA, was generously provided by Dr. L. Reichardt (University of California, San Francisco, CA), and used at a concentration of 1:500. After a two hour incubation at room temperature, cultures were washed three times in PBS (10 minutes each), and the p75 immunolabel was visualized using FITC-conjugated goat anti-mouse IgG (1:800 in blocking solution; Jackson ImmunoResearch Laboratories, West Grove, PA), while the TrkA was visualized using Cy3-conjugated goat anti-rabbit IgG (1:2000 in blocking solution; Jackson ImmunoResearch), all by epifluorescence microscopy.

Receptor Co-patching Assay and Measurements of Relative Receptor Density

For semiquantitative determination of relative receptor density, TrkA and p75 immunolabeled neurons were scanned with a Confocal Laser Scanning Microscope (Wild Leitz, Heidelberg, Germany), and its densitometry function was used to determine the relative density of TrkA and p75NTRs on neuronal cell bodies versus neurites (separately for each receptor) by measuring the intensity of the fluorescent label. Three separate experiments were performed, and for each experiment, 10 to 15 random samplings were obtained for either cell bodies or processes, and an average measurement was determined for each. The difference in fluorescence intensity between cell body and neurites was then determined by comparing the measurements obtained for each cellular compartment.

To determine if interactions between TrkA and p75NTRs were biophysical, cultures of neonatal rat sympathetic neurons were maintained for 5 days in 50 ng/ml NGF, induced to co-patch as described below, and processed for double-label co-patching immunocytochemically, also as described below. The technique used for receptor co-patching in sympathetic neurons was adapted from the methods used by Ross et al. (1996) in Sf9 cells. Before undergoing fixation, neuronal cultures were

incubated for 30 minutes at room temperature with the anti-p75 antibody Mc192 (5 to 10 μg/ml) in order to induce aggregation of p75 receptors, and to visualize the p75 aggregates, an FITC-conjugated goat anti-mouse IgG was added for 1 hour. Following this incubation period, cultures were fixed for 10 minutes in 4% paraformaldehyde to prevent any further antibody-induced redistribution of receptors, and the RTA antibody was added for another hour. Finally, a Cy3-conjugated goat anti-rabbit IgG was added to visualize TrkA. Each channel was scanned separately, and optically sectioned with the confocal microscope in order to view the receptors at different levels of the cell, and in this way, it was determined whether TrkA receptors were included in the receptor aggregates and hence co-patched in an intermolecular complex. As a control, sister cultures were processed by standard double-label immunocytochemical methods, using the same antibodies. Neuronal cultures were washed twice with PBS, fixed for 10 minutes with 4% paraformaldehyde, and blocked for 1 hour in PBS containing 4% normal rat serum and 4% normal goat serum (both from Jackson ImmunoResearch Laboratories, West Grove, PA). Neurons were then incubated with both RTA (1:400) and Mc192 (3 μg/ml) for 2 hours at room temperature, in blocking solution. Following three 10-minute washes in PBS, cells were incubated for an additional 2 hours in blocking solution containing both FITC-conjugated goat anti-mouse and CY3-conjugated goat anti-rabbit IgGs (1:800 and 1:1000 respectively, both from Jackson). After three 10-minute washes in PBS, coverslips were removed, mounted on glass microscope slides using Sigma mounting medium, and viewed by confocal microscopy, as described above.

Adenoviral Infection and TrkA overexpression

To increase the ratio of TrkA to p75 in sympathetic neurons, cultures prepared from P1 rats were maintained for 2 days in Ultraculture containing 50 ng/ml NGF for growth assays or 5 days in 50 ng/ml NGF for survival assays and biochemistry. Cultures were then infected for 24 hours with different MOIs (multiplicity of infection, or pfu/cell) of recombinant adenovirus expressing either the human TrkA receptor or a GFP-expressing control virus. During the viral infection, cultures were maintained in ultraculture supplemented with 10% fetal bovine serum and 50 ng/ml NGF. After 24

hours, cultures were returned to their normal maintenance medium, and allowed to express the protein of interest for 48 hours before undergoing further treatments. To generate the viral dose-response survival curve, cultures were infected with either 10, 25, or 50 MOI TrkA adenovirus, or 50 MOI GFP adenovirus as described above, and then treated with various concentrations of NGF, as described below.

Survival Assays

NGF-dependent neurons were selected by culturing sympathetic neurons for 3 days in the presence of 50 ng/ml NGF, as described previously (Ma et al., 1992; Belliveau et al., 1997; Bamji et al., 1998; Kohn et al., 1999). After 3 days, neurons were infected as described above, and 2 days post-infection, neurons were washed free of neurotrophin using three successive 1 hour washes in neurotrophin-free Ultraculture, and then fed with medium containing NGF, ranging from 1 to 50 ng/ml NGF. 48 hours after treatment, cell viability was assayed by the addition of MTT reagent (3(4,5-dimethylthio-zol-2-yl)2,5-diphenyltetrazolium bromide; Sigma) for three hours at 37°C. The reaction product was solubilized by the addition of 100 µl of a 0.65N HCL/isopropanol mixture, and measured on a spectrophotometer (570 nm/690 nm).

Analysis of process outgrowth

To determine whether overexpression of the TrkA receptor would enhance the growth of sympathetic neurons grown in 10ng/ml NGF, cultures were maintained for 3 days, and infected with TrkA adenovirus or control GFP adenovirus as described above. 48 hours post-infection, cultures were washed free of neurotrophin for three hours, and fed with Ultraculture containing 10 ng/ml NGF. After 2 additional days *in vitro*, random phase images comparing the degree of outgrowth were captured with a Sony XCCE CCD video camera module, and digitized with the Northern Eclipse image analysis system (Empix Imaging, Mississauga, Ontario, Canada). As a biochemical correlate of this morphological data, western blot analysis (as described below) to detect α-tubulin-expression levels was performed.

Western blot analysis

For biochemistry, cells were washed twice with ice-cold PBS and lysed in ice-cold TBS lysis buffer (20 mM Tris, pH 8.0, 137.5 mM NaCl, 10% glycerol, 1% NP-40, o.5mM sodium vanadate, 1 mg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF) for 20 minutes at 4°C while rocking. Neurons used for detecting human TrkA expression were lysed under non-reducing conditions, which involved the addition of 20mM iodoacetamide (pH 7.8) to the lysis buffer immediately before lysis. Lysates were scraped to remove them from the plate, and microfuged for 10 minutes at 10,000 rpm. Protein concentration was determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). Lysates (50μg) were either boiled with sample buffer, or 150-250 μg of protein was first immunoprecipitated overnight with anti-panTrk (203B; Hempstead et al., 1992). Immunoprecipitated protein was incubated and rocked for two hours at 4°C with Protein A Sepharose beads, and the beads were then isolated, washed, resuspended in sample buffer, and boiled for 5 minutes. Proteins lysed under non-reducing conditions were resuspended in DTT-free sample buffer. Samples were electophoresed on 7.5% SDS-PAGE gels, transferred to 0.2μm nitrocellulose membranes, blocked in 3% blotto (5% blotto for DO-7 antibody), and probed with the appropriate antibodies overnight at 4°C: anti-p53 (D0-7, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-recombinant human p75NTR (1:5000; Promega), anti-α-tubulin (1:5000; Calbiochem/Oncogene Research Products, Cambridge, MA), anti-pan Trk (203B; 1:2000), anti-human TrkA (5C3, 1:1000, LeSauteur et al., 1996; anti-rat TrkA (RTA; 1:5000), or anti-ERK (c-16 Santa Cruz;1:5000). Blots were incubated with secondary antibodies (Boehringer-Mannheim, Laval, Quebec, Canada) for 1 hour, and then detection performed using ECL (Amersham Canada Ltd., Oakville, Ontario) and RAR x-ray file (Eastman-Kodak, Rochester, NY).

RESULTS

The p75 to TrkA ratio differs on cell bodies versus neurites of sympathetic neurons

Immunostaining

The first step in studying the distribution of TrkA and p75NTRs was to characterize their expression and spatial distribution on sympathetic neurons. Neonatal rat sympathetic neurons express both the TrkA and the p75 NTRs and are neurotrophin-responsive. However, previous studies utilizing compartment cultures demonstrated that biological responsiveness to NGF, at the level of neuronal gene expression differed, depending on whether NGF was applied to the central cell body compartment, or to the distal chambers containing neurites (Toma et al., 1997). This suggested that the spatial distribution of these receptors (and/or signalling components), and therefore the ratio of TrkA:p75 receptors varies due to a differential distribution of these receptors. To determine whether TrkA and p75NTRs were differentially distributed along the surface of sympathetic neurons, neonatal rat sympathetic neurons were cultured and immunolabeled with antibodies directed against the p75NTR (MC192, which recognizes the ECD of p75NTR) and TrkA (using RTA, which also recognizes the ECD, but that of TrkA).

After immunostaining, p75NTR immunoreactivity was relatively evenly distributed (Figure 3.1A), whereas the TrkA immunolabel appeared to be enriched in neuronal cell bodies relative to neurites (Figure 3.1A). Although increasing NGF levels *in vitro* has been demonstrated to upregulate the levels of p75, but not TrkA, in neonatal sympathetic neurons (Toma et al., 1997), increasing the NGF concentration from 20 to 50 ng/ml NGF had no observable effect on the pattern of immunostaining, either for TrkA or for the p75NTR (Figure 3.1A).

Confocal fluorescence intensity measurements

To semi-quantitatively determine relative differences in levels of TrkA and p75NTRs on neuronal cell bodies and neurites, we double labeled sympathetic neuron cultures with RTA and MC192 as described above, and used confocal microscopy to measure the relative densities of TrkA and p75NTRs by determining fluorescence

immunolabel intensity. Using conventional epifluorescence microscopy, we observed that TrkA is preferentially expressed in cell bodies while the p75NTR is more evenly distributed along the cell as described above. We also determined by confocal microscopy that there is a 3.5- to 4-fold difference in the intensity of TrkA immunostaining between cell bodies and neurites (Figure 3.1B). For the p75NTR, the relative difference in staining intensity as determined by confocal microscopy was approximately 1.5- to 2-fold (Figure 3.1B).

Receptor Co-patching

Since TrkA and p75NTRs are co-expressed on sympathetic neurons, can signal autonomously to modulate each other's activities, and exhibit functional antagonism (Bamji et al, 1998; Kohn et al., 1999), there is potential for direct interactions between these cell surface proteins. Their potential for direct interaction was addressed by co-patching TrkA and p75NTRs on cultured sympathetic neurons to look for the formation of receptor complexes or "patches," and the presence of co-patching was determined by confocal microscopy. This technique was adapted from the methods used by Ross et al (1996), and takes advantage of receptor mobility within the plane of the plasma membrane. Before fixation, cultures were incubated with MC192, which induces the aggregation of p75NTRs, followed by an IgG-conjugated fluorochrome. After allowing for receptor patches to form in this manner, neurons were fixed to prevent further redistribution, and labeled with RTA and another fluorochrome. The fluorescence was then visualized to determine whether or not TrkA receptors are included with the p75NTR patches, or co-patched with the p75NTR, which is suggestive of an intermolecular complex.

Figure 3.2 shows the results of a typical co-patching experiment. Panels A-F illustrates neurons that have undergone co-patching immunocytochemistry, while panels G-L represent control neurons that have been processed using standard immunocytochemical techniques. TrkA-immunolabeled cell bodies are shown in panels A (patched) and G (unpatched), while TrkA-immunolabeled neurites are indicated in panels D (patched) and J (unpatched); p75-immunolabeled cell bodies are shown in panels B (patched) and H (unpatched), while p75-immunolabeled neurites are indicated

in panels E (patched) and K (unpatched). The individual TrkA and p75 images are digitally combined in the overlay to observe any co-patch formation (for example, the images in panels A and B were overlaid to generate panel C). As indicated in panel F, some co-patching was observed, mainly along sympathetic neurites (refer to the arrow for an example of a co-patch), but much less so in the cell body compartment (panel C). This indicates that only small population of TrkA and p75NTRs form co-patches or complexes. The implication of this is that although there may be some biophysical interactions between them, TrkA and p75NTRs receptors are more likely to interact functionally than physically, at least in sympathetic neurons.

To illustrate that it is the co-patching technique itself that induces the receptor redistribution observed on patched neurons, cultures were double-labeled by standard double-label immunocytochemical techniques, using the same TrkA and p75NTR antibodies and fluorochromes used for the co-patching experiments. In cultures processed using standard immunocytochemistry, no co-patches were observed (panels I and L), indicating that the ability of the receptors to co-patch was the result of the experimental manipulation and was not simply artefactual.

Increasing TrkA levels enhances NGF-mediated survival

Survival and growth of sympathetic neurons is dependent upon a physiological balance between activation of p75 and TrkA neurotrophin receptors, with TrkA mediating positive, and the p75NTR transducing negative biological responses. We have previously shown that activating the p75NTR with it specific ligand BDNF in the presence of robust NGF-induced survival signals through TrkA does not suppress survival *in vitro* (Bamji et al. 1998). However, if TrkA activation is weak, concomitant p75NTR activation now inhibits survival. One prediction of this data is that increasing TrkA levels at a constant level of the p75NTR will allow sympathetic neurons to become more sensitive to TrkA ligands through more efficient TrkA-mediated suppression of inhibitory signals. To test this hypothesis, we have increased the level of TrkA in sympathetic neurons using recombinant adenovirus expressing human TrkA. We investigated whether TrkA overexpression would correlate increased survival in a

dose-related manner, and using biochemical approaches, we asked whether increased expression of TrkA was correlated with increased TrkA activation.

Expression Levels

To demonstrate that the human TrkA was being expressed by sympathetic neurons in vitro after adenoviral infection, TrkA was immunoprecipitated, western blot analysis was performed, and the membrane probed with an antibody specific to human TrkA (5C3 ascites) in order to differentiate between exogenous human and endogenous rat TrkA. As indicated in Figure 3.3A, the 5C3 antibody recognizes only human TrkA (lane 2, top). When reprobed with the RTA antibody which recognizes the endogenous rat, but not human TrkA receptors, expression levels are similar (Figure3.3A, bottom), indicating that endogenous TrkA is not altered in any way by the infection and expression of exogenous human TrkA. Furthermore, overexpression of TrkA had no observable effect on the levels of p75NTR expression (Figure 3.3C, lanes 1 and 2). The exogenously expressed TrkA protein was functional, as it could be activated by NGF stimulation. This was detected using a 4G10 antibody, which recognizes phosphotyrosine residues (data not shown).

A pan-Trk antibody (203B) was used to determine total Trk protein levels. As indicated in Figure 3.3B (lanes 1-4, top), the degree of exogenous TrkA expression was related to the MOI of TrkA adenovirus used to transduce the cells. Endogenous TrkA levels are also shown after reprobing the blot with RTA antibody (lanes 1-4, bottom).

Survival assays

TrkA adenovirus was used to increase the levels of TrkA receptors. Two days post-infection, neurons were washed free of neurotrophin, and induced for 48 hours with NGF, at concentrations ranging from 0 to 50 ng/ml. Neuronal survival was measured using MTT assays, which measure mitochondrial function (Belliveau et al., 1997; Bamji et al., 1998; Kohn et al., 1999). TrkA infected neurons required less NGF for their survival relative to uninfected or GFP-infected neurons (Figure 3.4A). This effect was particularly pronounced at lower (limiting) concentrations of NGF. For example, 1 ng/ml NGF, which has been previously show to maintain only 20% survival

of sympathetic neurons (Belliveau et al., 1997), was able to maintain approximately 3 times as much survival in TrkA-infected neurons relative to uninfected-controls, and almost twice as much as in GFP-infected controls. (Figure 3.4A). A similar enhancement of survival has been observed in cultured neurons obtained from p75 -/- mice when compared to neurons cultured from wild type littermates (Kohn et al., 1999). TrkA-infected cultures not receiving any NGF (0 ng/ml NGF) were able to maintain the same degree of survival as those receiving 1 ng/ml of NGF (Figure 3.4A). This suggests that overexpression of TrkA seems to allow these neurons to survive independently of NGF, at least in the first few days in culture. This also suggests that TrkA or a downstream effector of TrkA such as Ras (a signaling molecule that regulates sympathetic neuron survival), may be constitutively activated when TrkA is overexpressed. Previous work from our lab has demonstrated that constitutively activated Ras was able to rescue sympathetic neurons from NGF withdrawal (Mazzoni et al., 1999). Also sympathetic neurons that lack functional p75NTR are able to survive for a longer period after NGF withdrawal, before undergoing apoptosis (Bamji et al., 1998). Thus either overexpression of TrkA and functional ablation of the p75NTR both impart enhanced biological responsiveness to sympathetic neurons, supporting opposing roles for them in this neuronal population.

As a morphological correlate, neurons were examined by phase microscopy for signs of stress. Figure 3.4B illustrates that at 1 ng/ml NGF, TrkA-infected neurons show no morphological indications of apoptosis. This is in contrast to the GFP-infected culture, which shows typical morphological signs of NGF-withdrawal-induced cell death normally observed in control neurons, such as cell body crenation and process degeneration (Figure 3.4B). The ability of TrkA-infected neurons to maintain their survival when NGF concentrations are limiting is consistent with increased sensitivity to NGF. As well as examining morphological indicators of apoptosis, a biochemical correlate of apoptosis was also necessary. Cultures were infected with either 50 MOI of TrkA or GFP adenovirus, and western blot analysis for p53 protein was carried out, as p53 has been previously shown to be an indicator of apoptosis when it is upregulated (Aloyz et al., 1997). Two days post-infection, GFP- and TrkA adenovirus-infected cultures were incubated for an additional 48 hours with either 1 or 10 ng/ml of NGF,

lysed, and probed for p53 protein (Figure 3.4C, lanes 1-3). Only the GFP-infected control culture maintained in 1 ng/ml NGF (Figure 3.4C, lane 1), showed an upregulation of p53, which is consistent with both the survival data, and our morphological observations. Thus, the survival, morphological and biochemical data, when taken together, indicate that increasing the levels of TrkA in sympathetic neurons has a positive effect on neuronal survival.

Increasing TrkA levels enhances NGF-mediated growth

Since the level of overexpression achieved with 50 MOI of TrkA adenovirus was sufficient to significantly increase the survival of sympathetic neurons over controls, we wanted to determine whether increased TrkA expression would also have a positive effect on another NGF/TrkA mediated biological response, sympathetic neuron outgrowth. We have previously shown that blocking p75NTR activation does significantly enhance sympathetic neuron outgrowth (Kohn et al., 1999), so we hypothesized that a similar mechanism might be involved when TrkA levels were increased, since these receptors have opposing biological roles. Two days post-infection with either 50 MOI GFP or 50 MOI TrkA adenovirus, cultured sympathetic neurons were maintained for a further 48 hours in 10 ng/ml NGF, and examined by phase microscopy to see whether neurons overexpressing TrkA showed enhanced growth responses, relative to GFP-infected controls. Neurons overexpressing TrkA exhibited more robust outgrowth relative to GFP controls (Figure 3.5A). Interestingly, the enhanced growth observed in TrkA-infected neurons is similar to what we have shown previously, when the p75NTR was antagonized by function blocking antibodies or its expression was absent (Kohn et al., 1999). This supports our finding that increasing TrkA levels positively affects biological responsiveness of sympathetic neurons, potentially by overriding negative p75NTR signals. Western blot analysis of α-tubulin (Figure 3.5b), a growth-associated marker, also shows that neurite outgrowth is enhanced in TrkA-infected neurons maintained in 10 ng/ml NGF (lane 2), relative to their GFP-infected counterparts (lane 1). ERK expression levels indicate that similar

amounts of protein were loaded in each lane. Together, these data indicate that increasing the levels of TrkA enhances neuronal growth as well as survival, observations that are consistent with increased sensitivity to NGF.

Discussion

Data presented in this paper demonstrate that increasing the expression of TrkA enhances the sensitivity of neonatal sympathetic neurons to NGF, thereby enhancing biological responsiveness *in vitro*. More specifically, these experiments showed that increasing the levels of TrkA allowed sympathetic neurons to respond strongly to concentrations of NGF that were previously non-permissive for survival, and also allowed them to grow more robustly. Overexpression of TrkA increased the sensitivity of sympathetic neurons to NGF because (1) a high degree of neuronal survival was achieved in suboptimal concentrations of NGF, (2) TrkA-infected neurons maintained in low NGF did not upregulate p53 protein, a molecule whose expression is upregulated in sympathetic neurons when NGF is withdrawn or when p75 is activated (Aloyz et al., 1998), and (3) growth responses to NGF were enhanced. Taken together, these data indicate that levels of TrkA are a key determinant of biological responsiveness in sympathetic neurons.

Although we do not formally demonstrate that responsiveness is enhanced by TrkA overexpression because positive signals become more efficient at overriding p75NTR's inhibitory signals, the idea is supported by the following studies from our laboratory. (1) Cultured sympathetic neurons from p75NTR$^{-/-}$ mice survive longer after NGF withdrawal than do control neurons (Bamji et al., 1998). (2) Sympathetic neurons from p75NTR$^{-/-}$ animals have an intrinsic ability to grow more robustly in culture, relative to their wild type counterparts (Kohn et al., 1999). (3) In sympathetic neurons from p75NTR$^{-/-}$ mice, the normal period of programmed cell death does not occur. (4) TrkA activation is enhanced in PC12 cells overexpressing this receptor; these cells exhibit accelerated differentiation as indicated by a rapid induction of neurite outgrowth (Hempstead et al., 1992). (5) Sympathetic neurons overexpressing a constitutively active form of Ras survive after NGF withdrawal and do not upregulate p53 (Mazzoni et al, 1999) which is downstream of the p75NTR (Aloyz et al., 1998). In all these instances, biological responsiveness is increased because TrkA signals are either unopposed by those of the p75NTR because of its absence, or are strengthened when it,

or one of its downstream effectors are more active. This supports the idea of increased sensitivity to NGF being due to a decrease in inhibitory signals from the p75NTR.

Since TrkA and p75 have opposing roles in sympathetic neurons (Bamji et al., 1998; Kohn et al., 1999), their biological responses to NGF might be partially regulated by the spatial distribution of TrkA and p75NTRs on the neuronal surface. Since they have opposing roles, the cellular ratio of these receptors may determine the signaling events that occur downstream of their activation. The neuronal ratio of p75NTR to TrkA which has previously been shown to vary as a function of NGF concentration (Miller et al., 1994), is important in modulating TrkA activity and subsequent biological responses. Using compartmented cultures of sympathetic neurons, Toma et al (1997) observed that NGF-induced gene expression was always more robust when NGF was applied to cell bodies, than when it was applied to distal axons. This spatial difference in biological responsiveness may be attributed to a differential distribution of TrkA and p75NTRs on cell bodies versus neurites of sympathetic neurons, and a differential distribution leads to a TrkA: p75NTR ratio that varies spatially. Our spatial localization and confocal data show just such a differential distribution of TrkA and p75NTRs, so the receptor ratio does differ spatially in sympathetic neurons. Since the ratio differs spatially, the outcome of signaling events might also differ spatially, leading to different biological responses in different cellular compartments. PI3-K, a signaling molecule that is associated with both NGF/Trk-mediated growth and NGF/Trk-mediated survival pathways in sympathetic neurons (Atwal et al., 2000), has been shown to mediate biological events differently, depending on the cellular compartment that it is activated in (Kuruvilla et al., 2000). Since receptor ratio may regulate TrkA signaling and downstream activation of PI3-K, this suggests that biological responsiveness can be regulated by receptor ratio.

The ratio of TrkA and p75NTRs has recently been shown to regulate the number of high affinity binding sites that occur (Esposito et al., 2001). A higher percentage of receptors were demonstrated to exhibit high-affinity binding when they were expressed in near equimolar ratios, at least in cell lines. High affinity binding,

which is particularly important developmentally when neurons compete for limiting quantities of target-derived growth factors, could also regulate signaling events in different cellular compartments, and ultimately biological responsiveness. If the receptor ratio determines if binding is of high-affinity, it follows that biological responsiveness would also be regulated by this ratio. During the time of naturally-occurring cell death, the NGF concentration in sympathetic target is in the subpicomolar range (Barde Y-A, 1989; Meakin and Shooter, 1992). It therefore follows that neurotrophin binding to its receptor site must, by necessity, be of high-affinity if NGF is to support the survival of an adequate number sympathetic neurons. Each receptor, on its own, binds NGF with low affinity ($K_d = 10^{-9}$ M; Kaplan et al., 1991; Hempstead et al., 1991; Mahadeo et al., 1994; Chao and Hempstead, 1995). When they are coexpressed, binding affinity increases to 10^{-11}M (Hempstead et al., 1991; Esposito et al., 2001), a condition that is necessary since target-derived NGF is limiting. There is evidence for high affinity binding sites being regulated not only by the ratio of p75NTR and TrkA receptors, but also by specific domains within these molecules. The transmembrane and cytoplasmic domains of p75NTR and TrkA have both been shown to be responsible for high affinity site formation (Esposito et al., 2001).

Since TrkA and p75NTRs are both necessary for high affinity binding, have the ability to modulate each other's signals, and are co-expressed in many neuronal populations, this suggests that they might interact by forming a physical complex. It has also been proposed that the conformation of binding sites on a neurotrophin might be conducive to the formation of p75NTR-TrkA heterodimers, since the TrkA and p75NTR binding sites in NGF are thought to overlap (Hempstead et al., 1991). Previous chemical cross-linking studies did not successfully demonstrate a physical association between the TrkA and p75NTRs (Huber and Chao, 1995; Gargano et al., 1997), but recently, a physical interaction between p75NTR and TrkA, TrkB and TrkC has been demonstrated (Bibel et al., 1999). These receptors were co-immunoprecipitated in A293 cells transfected with both receptor types, and their interaction was found to be mediated by the intracellular and extracellular receptor domains (Bibel et al., 1999). Co-immunoprecipitation of TrkA and p75NTRs that were

co-expressed in *Xenopus* oocytes has also been demonstrated (Mischel et al., 2001). FRAP, or photobleaching recovery experiments, have also demonstrated physical interactions between TrkA and the p75NTR, through both the intra- and extracellular domain of the receptors (Wolf et al., 1995). Previous studies in which p75NTR and TrkA were co-expressed in Sf9 cells used a receptor co-patching technique (Ross et al., 1996) to demonstrate a heteromolecular complex between them. They demonstrated that all TrkA and p75NTRs in these cells were co-patched. This interaction was specific for TrkA, since the p75NTR did not co-patch with other receptor tyrosine kinases, and this study also showed that both the extracellular and intracellular domains were important for this interaction (Ross et al., 1996). Our data showed that in primary cultures of sympathetic neurons, some, but not all TrkA-p75NTR receptors formed co-patches on neurites, and this was not as apparent in cell bodies. The limited degree of patching we observed might be related to expression levels of these receptors in primary neurons versus cell lines. The previously published report by Ross et al (1996) used Sf9 cell lines in which TrkA and p75NTRs were expressed at high levels through a baculovirus vector expression system (Ross et al., 1996). In PC12 cells and in neonatal sympathetic neurons, the ratio of p75:TrkA (mRNA) is at least 10:1 (Mahadeo et al, 1994; Verdi et al., 1994; Chao and Hempstead, 1995), and the p75:TrkA stoichiometry is likely to be different in Sf9 cells. Moreover, in a sympathetic neuron, the receptor stoichiometry will vary with the spatial locale, since, as our immunocytochemical and confocal data shows, there is a differential distribution of TrkA and p75NTRs on cell bodies and neurites. Why was co-patching observed only in the neurites of sympathetic neurons? One could speculate that if stoichiometry affects receptor conformation, and conformation determines whether or not receptors can physically interact, the stoichiometry that is permissive for a physical interaction might exist in neurites, but not in cell bodies, at least in sympathetic neurons. Whatever the reason, our data suggests that in sympathetic neurons, interactions between TrkA and p75NTRs are more likely to be functional than physical.

The data presented here indicate that the level of TrkA expression is a key determinant of biological responsiveness of sympathetic neurons, since increasing the expression levels of TrkA allowed sympathetic neurons to become more sensitive to

NGF. Potentially, this occurs by a more efficient suppression of the p75NTR's inhibitory signals since the balance of signaling may be shifted toward TrkA. Since TrkA and p75NTRs have opposing roles in sympathetic neurons, a balance between positive and negative signaling can determine whether a neuron lives or dies, or how well it can grow and innervate its target. If the cellular ratio of TrkA and p75NTR influences neuronal biology by regulating the balance of signaling, this ratio may play a key role in determining the absolute levels of TrkA substrate activation in response to NGF, and ultimately, biological responsiveness. Since sources of neurotrophins are more widespread that just target cells, the cohort of neurotrophins that a neurons might encounter in its microenvironment can influence its biology. Glial cells produce neurotrophins, as do peripheral neurons themselves, making autocrine interactions between neurons possible. Consequently, neurons are exposed to neurotrophins from a number of different cellular sources that are often spatially segregated with respect to complex neuronal morphology. Spatial differences in the TrkA:p75NTR ratio makes sense biologically, especially during development, as these differences could allow neurons to respond differently to different cohorts of neurotrophins that it might "see" in its different spatial regions. Based on spatial differences in receptor ratio, the following scenario is a possibility. If a sympathetic neurite that is attempting to innervate its target encounters a physiologically inappropriate neurotrophin such as BDNF in its local microenvironment, the higher ratio of p75NTR:TrkA in the neurite could generate inhibitory signals through p75NTR that are more robust than those generated via TrkA. The neurite would be repelled, and would not grow towards a non-permissive target. At the same time, retrograde TrkA survival signals would emanate either from another collateral that has grown towards a permissive environment, or from the permissive microenvironment surrounding the cell body, a cellular compartment which carries a higher ratio of TrkA: p75NTR. Such local mechanisms could enable misdirected neurites to retract without the neuron itself undergoing apoptosis, while appropriately directed processes would continue to grow and innervate the target. In such a scenario, appropriate levels of growth and target innervation could be achieved, and it is the local receptor ratio that would ultimately determine just which

signals predominate spatially to regulate the biology of the sympathetic neurons in this fashion.

References

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan, DR, Miller FD (1998). P53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J. Cell Biol 143:1691-1703.

Atwal JK, Massie B, Miller FD, Kaplan DR (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and PI3-Kinase. Neuron 27: 265-277.

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 (1994). The Trk family of neurotrophin receptors. J Neurobiol 25:1386-1403.

Barde Y-A (1989). Trophic factors and neuronal survival. Neuron 2:1525-1534.

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

Belliveau DJ, Krivko I, Kohn J, Lachance C, Pozniak C, Rusakov D, Kaplan DR, Miller FD (1997). Ngf and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. J Cell Biol 136: 375-388.

Berkmeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7: 857-866.

Bibel M, Hoppe E, Barde Y-A (1999). Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18: 616-622.

Chao MV, Hempstead BL (1995). P75 and Trk: a two receptor system. Trends Neurosci 18: 321-326.

Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF, Barbacid M (1991). The trk tyrosine protein kinase mediates the mitigenic properties of nerve growth factor and neurotrophin-3. Cell 66: 173-183.

Ernfors P, Ibanez CF, Ebendal T, Olson L, 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. Proc Natl Acad Sci USA 87: 5454-5458.

Esposito D, Patel P, Stephens RM , Perez P, Chao MV, Kaplan DR, Hempstead BL (2001). The cytoplasmic and transmembrane domains of the p75 and TrkA receptors regulate high affinity binding to NGF. J Biol Chem (in press).

Gargano N, Levi,A. and Alema,S (1997). Modulation of nerve growth factor internalization by direct interaction between p75 and TrkA receptors. J Neurosci Res 50:1–12.

Gotz KR, 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.

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.

Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, Kaplan DR (1992). Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron 9: 883-896.

Hohn ALJ, Bailey K, Barde YA (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 334:339-341.

Huber LJ, Chao MV (1995). A potential interaction of p75 and TrkA receptors revealed by affinity cross-linking and immunoprecipitation. J Neurosci Res 40: 557-563.

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

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

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991a). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252: 554-559.

Kaplan DR, Martin-Zanca D, Parada LF (1991b). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350: 158-160.

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

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.

Kohn J, Aloyz RS, Toma JG, Haak-Frendscho M, Miller FD (1999). Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. J Neurosci 19: 5395-5408.

Kuruvilla R, Ye H, Ginty DD (2000). Spatially and functionally distinct roles of the PI3-kinase effector pathway during NGF signaling in sympathetic neurons. Neuron 27: 499-512.

Lai KO, Fu WY, Ip FC, 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 (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66: 967-979.

LeSauteur L, Maliartchouk S, Le Jeune H, Quirion R, Saragovi HU (1996). Potent human p140-TrkA agonists derived from an anti-receptor monoclonal antibody. J Neurosci 16: 1308-1316.

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

Ma Y, Campenot RB, Miller FD (1992). Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117:135-141.

Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994). High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. J Biol Chem 269: 6884-6891.

Majdan M and Miller FD (1999). Neuronal life and death decisions: functional antagonism between the Trk and p75 neurotrophin receptors. Int J Dev Neurosci 17: 153-161.

Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D (1999). Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J Neurosci 19: 9716-9727.

Meakin SO, Shooter (1992). The nerve growth factor family of receptors. Trends Neurosci. 15: 323-331.

Miller FD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG (1994). Nerve growth factor derived from terminals selectively increases the ratio of p75 to TrkA NGF receptors on mature sympathetic neurons. Dev Biol 161: 206-217.

Mischel PS, Smith SG, Vining ER, Valletta JS, Mobley WC, Reichardt LF (2001). The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. J Biol Chem 276: 11294-11301.

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.

Patapoutian A, Reichardt LF (2001). Trk receptors: mediators of neurotrophin action. Curr Opin Cell Biol 11: 272-280.

Radeke MJ, Misko TP, Hsu C, Hertzenberg LA, Shooter EM (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325: 593-596.

Rodriguez-Tébar A, Dechant G, Barde YA (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4: 487-492.

Rodriguez-Tébar A, Dechant G, Gotz R, Barde YA (1992). Binding of neurotrophin-3 to its neuronal receptor and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J 11: 917-922.

Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR, Nikolics K, Winslow JW (1990). Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4: 767-773.

Ross AH, Daou, M-C, McKinnon CA, Condon PJ, Lachyankar MB, Stephens RM, Kaplan DR and Wolf DE (1996). The neurotrophin receptor gp75, forms a complex with the receptor tyrosine kinase TrkA. J Cell Biol 132:945-953.

Snider WD (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 7:627-638.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, Nikolocs K, Parada LF (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell 65: 895-903.

Toma JG, Rogers D, Senger DL, Campenot RB, Miller FD (1997). Spatial regulation of neuronal gene expression in response to nerve growth factor. Dev Biol 184: 1-9.

Tsouflas P, Soppet D, Escandon E, Tessarollo L, Remendoza-Ramirez JL, Rosenthal A, Nikolics K, Parada LF (1993). The rat trkC locus encodes multiple neurogenic receptors that exhibit differential responses to NT-3 in PC12 cells. Neuron 10: 975-990.

Verdi JM, Birren SJ, Ibañez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994). p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron 12: 733-745.

Wolf DE, McKinnon CA, Daou M-C, Stephens RM, Kaplan DR, Ross AH (1995). Interactions with TrkA immobilizes gp75 in the high affinity growth factor receptor complex. J Biol Chem 270: 2133-2138.

Figure Legends

Figure 3.1. TrkA and p75NTRs are differentially expressed on sympathetic neurons thus the ratio of TrkA to p75NTR differs spatially.

(A) Immunocytochemical analysis of the expression of TrkA and p75NTRs in cultured neonatal sympathetic neurons maintained in either 20 or 50 ng/ml NGF. Note that TrkA is preferentially expressed in cell bodies while p75NTR expression is more even along both cell bodies and neurites. (B) Densitometric analysis of relative receptor density on cell bodies versus neurites of cultured sympathetic neurons, by confocal microscopy. Each experiment was repeated three times, and 10 to 15 determinations of receptor density (fluorescence intensity) were made, per experiment, for randomly selected area in each spatial compartment (cell bodies and neurites), and then compared. Note that this is a semiquantitative measure; the relative density of TrkA is higher on cell bodies than on neurites, while p75NTRs are more evenly distributed. Maintaining cultures in 50 ng/ml of NGF instead of 20 ng/ml did not alter the relative density of p75NTRs between cellular compartments.

Figure 3.2. Some, but not all TrkA and p75NTR receptors form co-patches on neurites of cultured sympathetic neurons.

(A-F) Double-label co-patching immunocytochemistry, indicating that some TrkA and p75NTRs on neurites but not cell bodies may physically interact. Immunolabeled cell bodies (A-C) and neurites (D-F) after co-patching immunocytochemistry. Digitized micrographs of TrkA-labeled (A,D) and p75-labeled (B,E) neurons are presented individually to show the formation of TrkA or p75NTR patches; the individual images from the double-labeling were digitally combined (overlay: C,F) to visualize any TrkA-75NTR co-patches that have formed. The arrow in (F) is pointing to a TrkA-p75NTR co-patch. (G-L) Standard double-label immunocytochemistry, indicating that no patching or co-patching occurs when neurons are processed using typical immunocytochemical methodologies. Immunolabeled cell bodies (G-I) and neurites (J-L) after undergoing regular double-label immunocytochemical processing. Digitized micrographs of TrkA-labeled (G,J) and p75-labeled (H,K) neurons and the digitally combined double-label images (overlay: I,L) are shown.

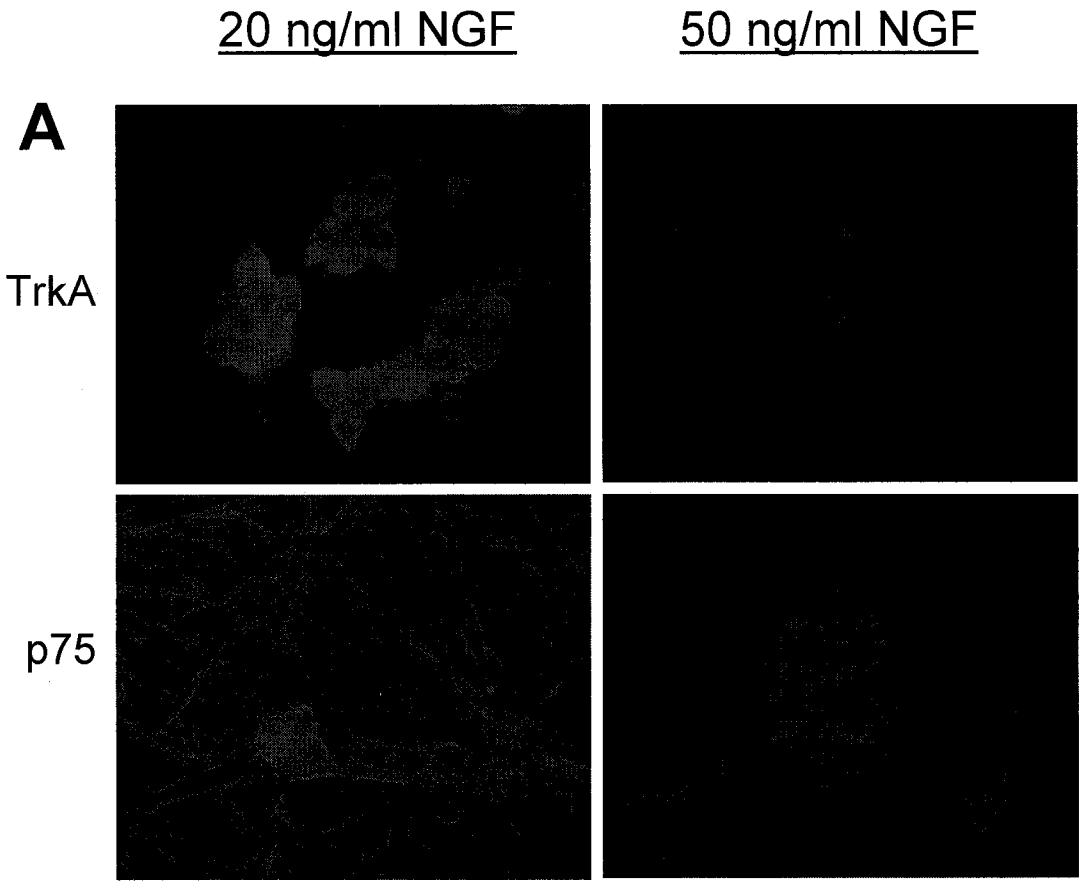
Figure 3.3. Detection of recombinant TrkA adenovirus in sympathetic neurons. Neonatal SCG neurons were cultured for 2 days and infected overnight with either 50 MOI recombinant adenovirus expressing either the human TrkA receptor or a GFP-expressing control virus, and assessed for expression after 48 hours. (A) Expression of exogenous recombinant human Trk protein after total Trk immunoprecipitation with a pan-Trk antibody, detected using anti-human TrkA (5C3 antibody). (B) Reprobe with the RTA antibody, which recognizes only endogenous rat TrkA, indicating that levels of endogenous TrkA are similar in both lanes. (C) Total Trk protein probed with 203B antibody after pan-Trk immunoprecipitation. Cultures were infected with increasing MOIs of TrkA adenovirus or GFP adenovirus. Levels of exogenous TrkA expression were increased with increasing MOI. Reprobe with RTA antibody indicates that endogenous TrkA levels were similar in control and infected cultures. (D) Expression of the p75NTR in control and TrkA-infected cultures. Note that overexpression of TrkA did not alter levels of the p75NTR.

Figure 3.4. Increasing TrkA expression increases sensitivity to NGF, enhancing neuronal survival in response to limiting amounts.
(A) Result of colorimetric MTT survival assay to measure mitochondrial function and cell survival. Neonatal sympathetic neurons were maintained for 3 days in 50 ng/ml NGF before being infected with either TrkA or GFP-adenovirus, or left uninfected. After 48 hours, cultures were washed free of neurotrophin and treated with concentrations of NGF ranging from 1 to 50 ng/ml for 2 days. The data are from a representative survival assay that was performed in triplicate. Note that in TrkA-infected cultures, extremely limiting amounts of NGF (and even no NGF) were extremely efficient at maintaining neuronal survival. (B) Morphological indicator of enhanced survival when NGF is limiting. Phase contrast micrographs of neurons maintained in 1 ng/ml NGF post-infection. The TrkA-infected neurons show no loss of viability when maintained in 1 ng/ml NGF, unlike neurons in the GFP-infected control culture. Note the crenated cell bodies and process degeneration in the control. (C) p53 protein levels are not upregulated in TrkA-infected cultures maintained in low NGF. As

a biochemical correlate of apoptosis, western blot analysis for p53 protein was performed on cultures transduced for 48 hours with either TrkA or GFP-adenovirus, and then maintained for an additional 2 days in 1 ng/ml NGF. As a negative control, a GFP-infected culture was maintained in 10 ng/ml NGF. Note that only the GFP-infected culture maintained in 1 ng/ml NGF showed an upregulation of p53 protein. ERK reprobe indicates that equal amounts of protein were loaded in each lane.

Figure 3.5. Increasing TrkA expression enhances neuronal growth.
(A) Morphological indicator of enhanced growth. Phase contrast micrograph of GFP- and TrkA adenovirus-infected neurons maintained in 10 ng/ml NGF for 2 days post-infection. Growth is more robust in TrkA-infected culture. (B) Biochemical correlate of enhanced growth after infection with TrkA adenovirus. Western blot analysis of α-tubulin expression also indicates enhanced growth after TrkA-adenoviral infection, relative to GFP-infected control. ERK reprobe indicates that similar amounts of protein were loaded.

Figure 3.1



B

Receptor	[NGF] ng/ml	N	* Average relative intensity difference
TrkA	20	3	3.5
TrkA	50	3	3.8
p75	20	3	1.8
p75	50	3	1.6

* cell bodies versus neurites (10-15 determinations per N)

Figure 3.2

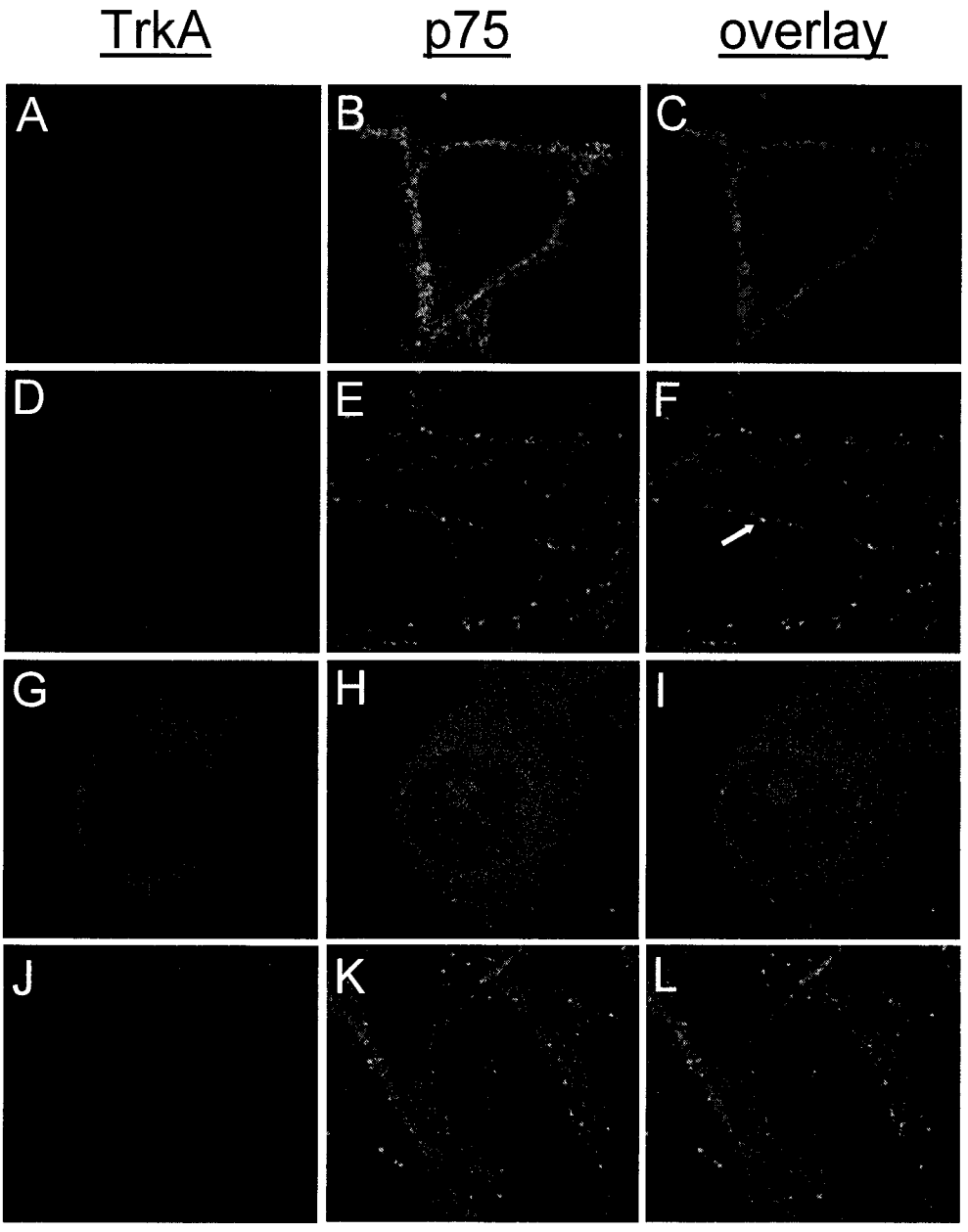


Figure 3.3

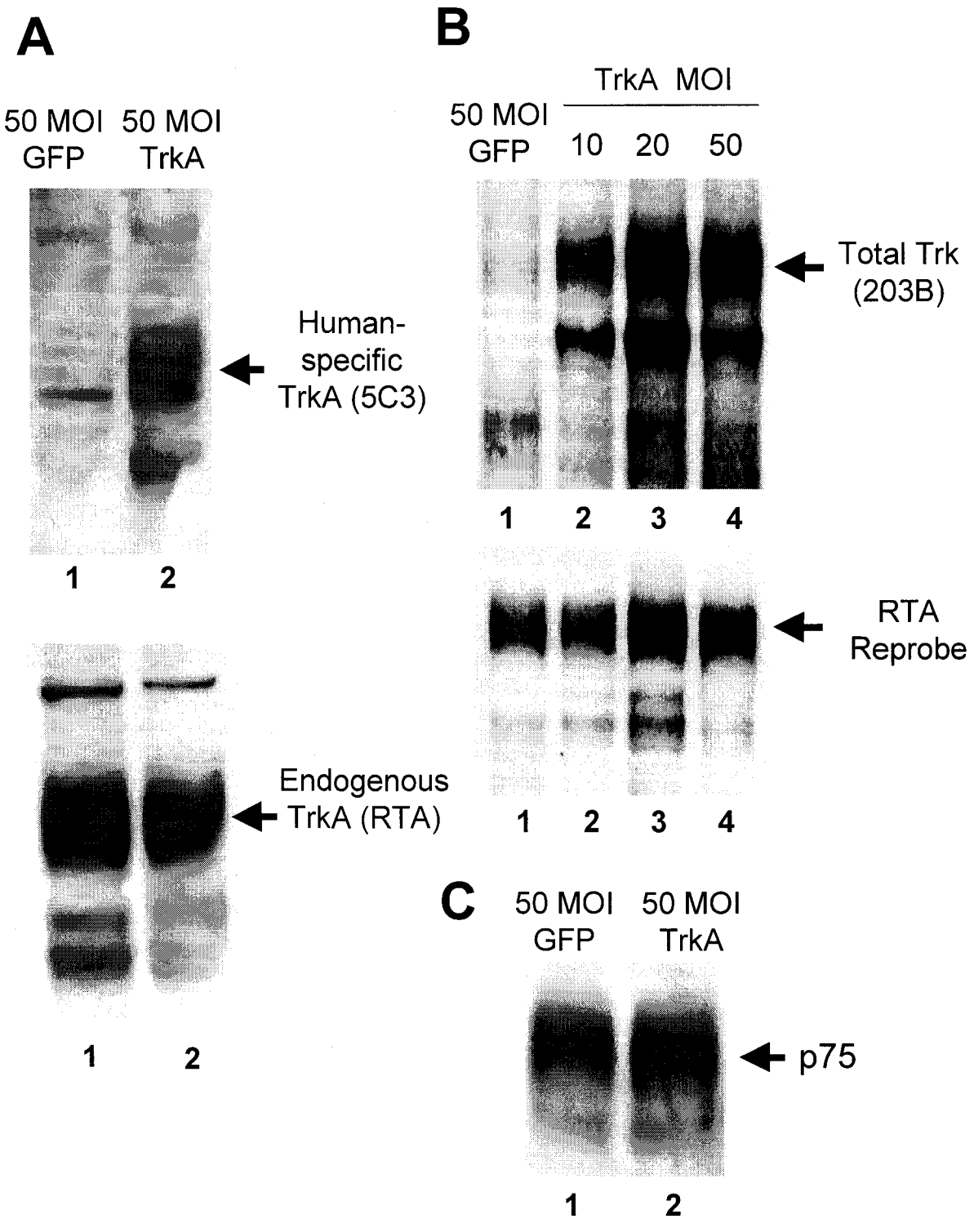
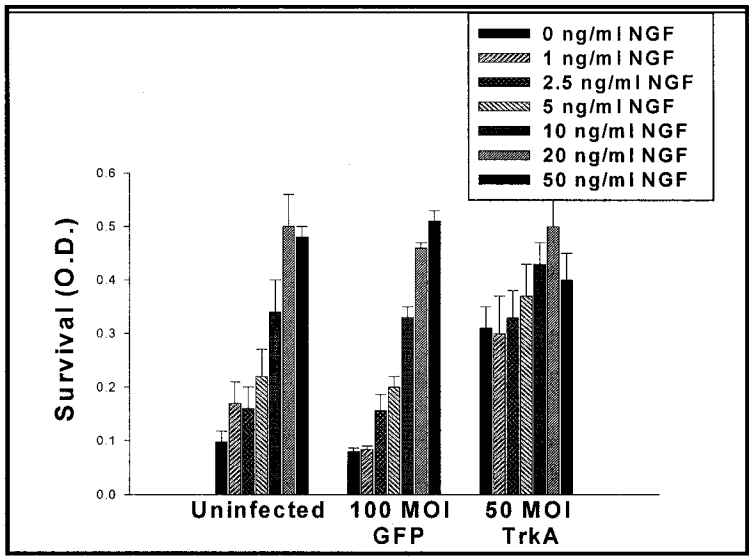
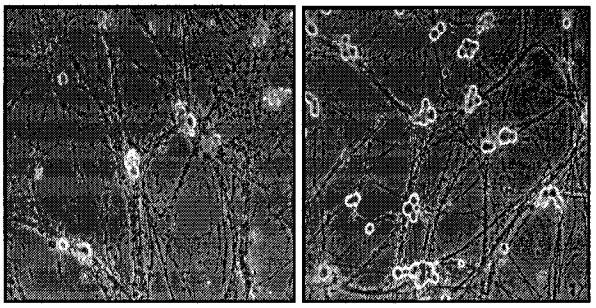

A
50 MOI GFP
50 MOI TrkA
Human-specific TrkA (5C3)
1
2
Endogenous TrkA (RTA)
1
2
B
TrkA MOI
50 MOI GFP
10
20
50
Total Trk (203B)
1
2
3
4
RTA Reprobe
1
2
3
4
C
50 MOI GFP
50 MOI TrkA
p75
1
2

Figure 3.4

A



B



1 ng/ml NGF

50 MOI GFP

50 MOI TrkA

C

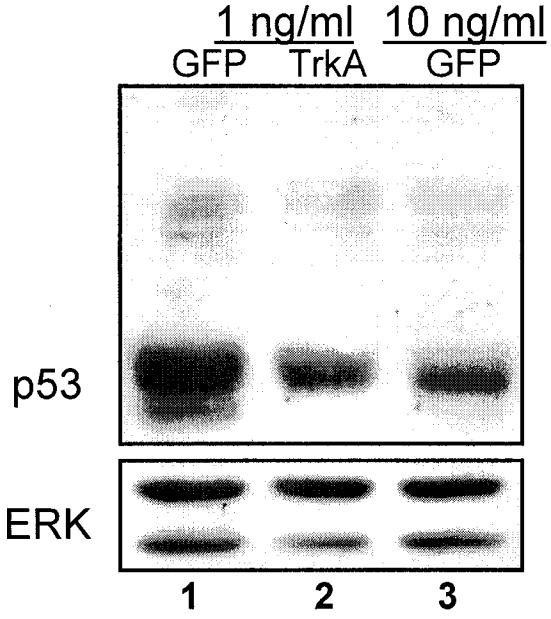
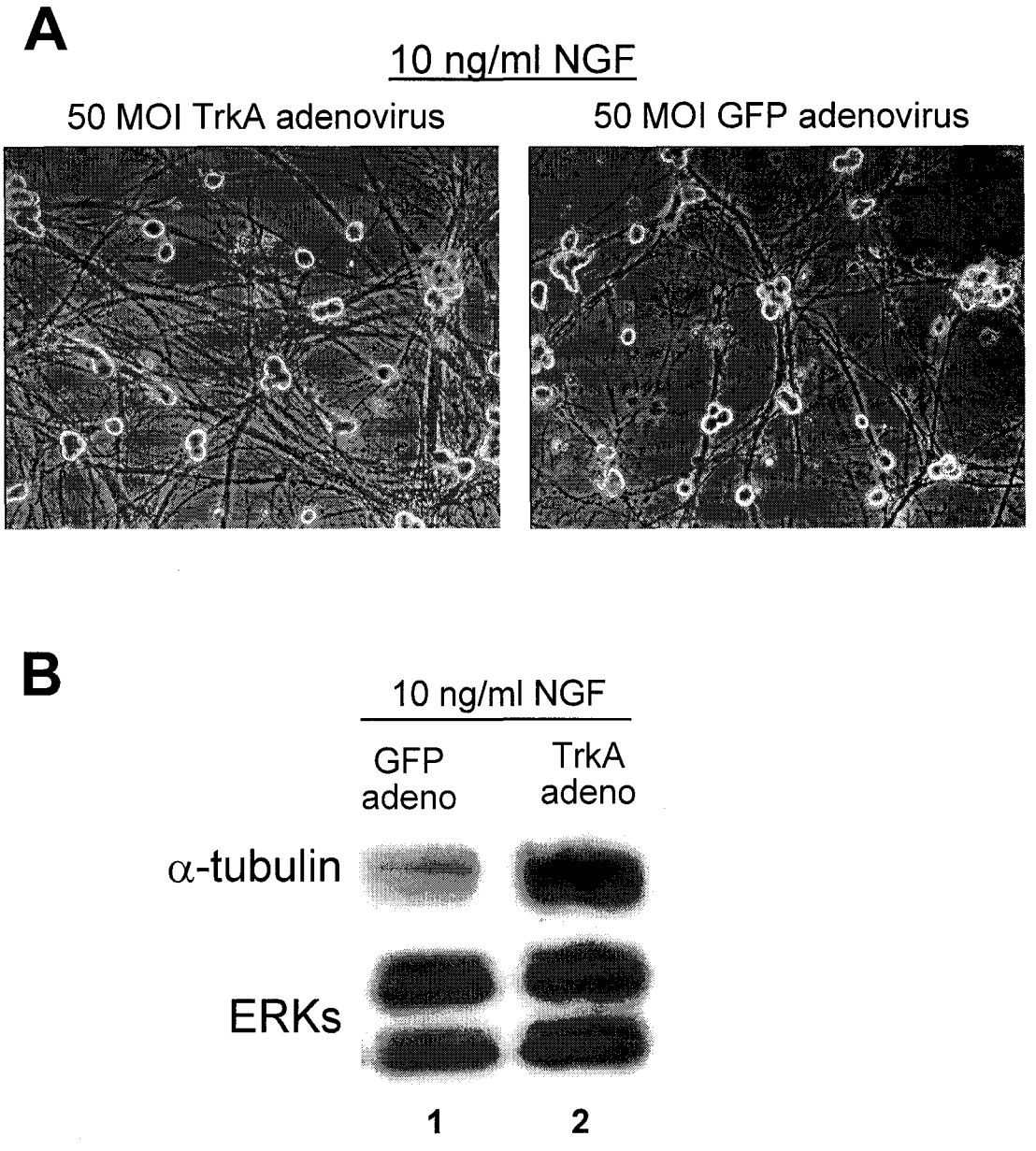


Figure 3.5


A
10 ng/ml NGF
50 MOI TrkA adenovirus
50 MOI GFP adenovirus
B
10 ng/ml NGF
GFP adeno
TrkA adeno
α-tubulin
ERKs
1
2

CHAPTER 4 - GENERAL DISCUSSION

GENERAL DISCUSSION

a) **Summary**

The data presented in this thesis have examined how the TrkA and p75NTRs on sympathetic neurons can act antagonistically to ensure that levels of growth and target innervation are physiologically appropriate, and how the ratio of these receptors can influence survival and growth responses of these neurons. Whether or not they physically interact to achieve a balance between positive and negative signals was also addressed.

Experiments in the first paper used morphological, biochemical, and transgenic approaches to show that in the presence of optimal NGF/TrkA survival signals, activation of the p75NTR with its specific ligand BDNF can inhibit TrkA-mediated sympathetic neuron outgrowth *in vitro* without negatively affecting survival. To determine the physiological or *in vivo* relevance of this p75NTR-mediated growth inhibition, I examined the innervation of a sympathetic target organ, the pineal gland. Using a BDNF-deficient transgenic mouse model, I demonstrated that when p75NTR activation is reduced or absent, target innervation is enhanced, presumably due to decreased inhibitory growth regulation. This suggests that p75NTR activation is necessary *in vivo* to ensure that appropriate connectivity is achieved. I concluded that NGF and BDNF have functionally antagonistic actions with respect to sympathetic neuron growth and target innervation, with NGF acting via TrkA to promote growth, and BDNF acting via p75NTR to inhibit growth. Furthermore, the expression of BDNF was spatially and temporally relevant to such a hypothesis since BDNF is expressed in the pineal gland or its vicinity during the period of target innervation. Since the p75NTR is present in the right place and at the right time, these data are also consistent with the possibility of a BDNF: p75NTR autocrine loop that may be recruited as a mechanism for regulating neurite outgrowth. Such a mechanism could ensure that target innervation is appropriate with respect to both levels of innervation and appropriate targeting, in that it could prevent a neuron from innervating an aberrant target that it may encounter. Thus physiologically appropriate levels of innervation

likely require a balance between positively- and negatively-acting signals from neurotrophins in the local microenvironment of potential targets.

Since the first paper indicated that TrkA and p75NTRs play opposing roles with respect to signaling neuronal growth, the experiments in the second paper were designed to test whether increasing TrkA levels in sympathetic neurons, using adenoviral approaches would affect biological responsiveness by enhancing their sensitivity to NGF. Presumably, elevated TrkA levels could alter the balance of TrkA-p75NTR signaling, and enable NGF-mediated TrkA activation to suppress inhibitory signals more efficiently, leading to enhanced survival and growth. Moreover, since these receptors have opposing roles, the cellular ratio of p75NTR: TrkA in different spatial compartments of a neuron might also determine the outcome of local signaling events in response to neurotrophins encountered in the local microenvironment. I demonstrated that TrkA levels in sympathetic neurons are a key determinant of biological responsiveness, since both survival and growth responses to NGF were enhanced after TrkA overexpression. I then demonstrated that since TrkA and p75NTRs were differentially expressed with respect to their spatial localization, the ratio of TrkA: p75 also differs spatially, being higher on the cell body than in neurites. Thus, signaling events and biological responses are likely to differ spatially as well. This question was addressed immunocytochemically and by confocal microscopy. Finally, the fact that TrkA and p75NTRs are are co-expressed on sympathetic neurons and have opposing roles with respect to neuronal biology suggests that there is potential for a physical interaction between them. I addressed this possibility by attempting to co-patch TrkA and p75NTRs on sympathetic neurons, and found that some, but not all TrkA and p75NTRs formed co-patches on sympathetic neurites but not cell bodies. Based on these data, I concluded that interactions between these receptors are more likely to be functional than physical.

Globally, the experiments presented in this thesis, when taken together, indicate that TrkA and p75NTRs have opposing roles in sympathetic neurons, with TrkA mediating positive and p75NTR mediating inhibitory biological signals. Since the

distribution of these receptors varies spatially, so does the receptor ratio. A differential ratio implies that there are likely to be differences between the two cellular compartments with respect to the signaling of biological responses. Interactions between TrkA and the p75NTR although functional, are less likely to also be physical. Finally, a balance between TrkA and p75NTR activation is essential for achieving physiologically appropriate levels of growth, and ultimately target innervation.

More specific issues related to this body of work are discussed below.

b) **The p75NTR is inhibitory to sympathetic neuron growth**

The ability of the neurotrophins to regulate axonal growth is supported by their abilities to promote neurite outgrowth in neurotrophin-sensitive neuronal populations (Snider and Johnson, 1989). Opposing signals from the TrkA and p75NTRs, at least in sympathetic neurons, provides a mechanism for regulating NGF-mediated growth and target innervation. However, such an inhibitory role for the p75NTR is only now becoming more apparent. Earlier studies of p75 found that its most prominent inhibitory activity was the regulation of cell death (Rabizadeh et al., 1993; Casaccia-Bonefil et al., 1996; Frade et al., 1996; Bredesen and Rabizadeh, 1997; Majdan et al., 1997; Bamji et al., 1998). However, since the p75NTR is also expressed when neurons are not undergoing apoptosis, this suggested that the p75NTR has other physiological functions during development. One of these is the ability to negatively regulate neuronal growth and innervation patterns, as has been demonstrated in a number of neuronal populations (Kimpinski et al., 1997; Yeo et al., 1997; Walsh et al., 1999a, b; Cahoon et al., 2001).

Support for p75NTR as a negative-mediator of biological activity in sympathetic neurons comes from experiments with p75- and BDNF-deficient mice. In $p75^{-/-}$ mice, the normal period of naturally occurring cell death is delayed (Bamji et al., 1998), and sympathetic neuron growth is enhanced *in vitro* (Chapter 2). Furthermore, our finding of pineal gland hyperinnervation when BDNF is absent, and elevated levels of sympathetic neuron survival in BDNF -/- mice (Bamji et al., 1998) also support an inhibitory role for the p75NTR.

To attain appropriate levels of target innervation, signals put forth by neurons must, by design, be both stimulatory and inhibitory, and must be balanced to match the specific needs of the target with respect to levels of innervation. All of these functions are related to the ability of NGF to bind to and activate TrkA. It is been demonstrated many times that TrkA mediates positive biological signals, but the role of the p75NTR is less clear, since in some contexts it plays a positive role, and in others, a negative or inhibitory role. The following scenario can be proposed in sympathetic neurons, based on the data in Chapters 2 and 3. Developmentally, the role of the p75NTR may be to signal in an inhibitory fashion to ensure that the levels of target innervation attained do not exceed what a target organ might require at a particular developmental stage. The level of innervation required is based on the size of the target at that time of an animal's life. As an animal grows, it will be faced with increasingly larger target areas that will require innervation, and mechanisms need to be available to oppose TrkA's stimulatory signals if necessary. Alternatively, if an axon is misdirected and p75NTR becomes activated by a neurotrophin such as BDNF from a non-permissive environment (or perhaps from a competing neuron since symapthetic neurons make BDNF), this will ensure that the axon will not continue to grow along an aberrant pathway. Thus the p75NTR, is, in a sense, a "fine-tuning" mechanism to ensure that innervation proceeds appropriately, with respect to both the degree of innervation, and its patterning.

There are many studies in which the p75NTR was demonstrated to inhibit the growth of sympathetic neurons. One of the more compelling of these was the report from Walsh et al (1999a) who found that the absence of functional p75NTR coupled to NGF overexpression allowed adult sympathetic neurons to vigorously sprout into CNS myelin of the cerebellum, a notoriously non-permissive environment. This was clear evidence for an inhibitory role for the p75NTR in sympathetic neurons. Although the approach used to examine role of the p75NTR in sympathetic neuron growth differed from ours, the conclusions that can be reached are similar. Both studies indicate that when the p75NTR is absent, or its activation is blocked, its normal inhibitory function with respect to growth and target innervation is attenuated.

A role for p75NTR in the regulation of target innervation was originally postulated based on observation of perturbed growth patterns in $p75^{-/-}$ mice (Lee et al.,

1994). In this case, the deficiency of p75NTR was shown to inhibit innervation of some, but not all sympathetic targets. Lack of sympathetic innervation was reported for the pineal gland and specific lateral footpads. These targets are further away from the sympathetic ganglia containing their innervating neurons, and so will be innervated at a later time than more proximal targets. It is possible that in the absence of p75NTR, unopposed TrkA signals may lead to enhanced innervation of targets that are innervated earlier during development, somehow preventing the growth of collaterals to targets that are normally innervated later on, such as the pineal gland. This is supported by the TrkA overexpression data presented in Chapter 3. Overexpression of TrkA, which might be likened to the unopposed TrkA signals suggested above when p75NTR is absent, led to enhanced neurite growth, even in the absence of NGF. The hyperinnervation I observed in mice lacking BDNF might be attributable to a similar mechanism, since the activation of the p75NTR is reduced in the absence of this neurotrophin, potentially leading to stronger TrkA signaling. The studies by Guidry et al (1998) support such a scenario. Mice overexpressing NGF under the control of the keratin promoter showed decreased innervation of sweat glands in footpads and blood vessels, and dense plexus of sympathetic fibres was directed towards the dermis. Thus TrkA's unopposed signals may be able disrupt innervation patterns in this fashion, especially if the p75NTR is absent, as in the studies by Lee et al. Also, if a sympathetic neuron is late in arriving at its target as in the above scenario, it will not be exposed to adequate levels of trophic support it needs once it arrives. It may locally "see" inhibitory neurotrophins such as autocrine BDNF, made either by other sympathetic neurons or their targets, which will activate the p75NTR, and the process may be "told" to retract.

Another question that can be asked relates to the differences in levels of pineal gland innervation when the defiency was in p75NTR versus the BDNF deficiency. In the pineal gland, the absence of p75 leads to a virtual absence of incoming sympathetic fibres (Lee et al., 1994). This is in contrast to observations presented here, in which the absence of BDNF protein leads to hyperinnervation of the same sympathetic target, the pineal gland. If both molecules play inhibitory roles in sympathetic neurons, why does one deficiency cause hyperinnervation, and the other,

complete disruption? At first glance, this appears contradictory, as one might have predicted that an absence of BDNF or the absence of p75 itself, would lead to the same phenotype. The exact opposite turns out to be true. The function of the p75NTR and of BDNF, although both inhibitory, will by design, be different, since one of these molecules is a receptor and the other a neurotrophin. If one looks at the function of the receptor and ligand as opposite sides of the same coin, the absence of receptor blocks the ability of the neuron to reach its target, while the absence of ligand allows it to innervate its target too well.

In the case of BDNF-deficient neurons, sympathetic neurons are known to synthesize NT-3, and NT-3 can bind to and activate the p75NTR. In this manner, inhibitory signaling via the p75NTR is not completely disrupted, even though the observed hyperinnervation suggests that it is probably not expressed in the pineal at high enough levels to limit innervation. At E14.5, 10 to 15% of neurons in sympathetic ganglia express NT-3 mRNA, while 40 to 50% express BDNF mRNA (Schecterson and Bothwell, 1992). NT-3 protein is not expressed in the adult SCG (Zhou and Rush, 1993). Alternatively, in the case of the receptor, the p75NTR may play a role in regulating directional growth, so its absence will lead to disruptions in innervation, potentially due to the lack of inhibitory signaling. If axons that normally innervate the pineal are misdirected early on during development, they may never reach their target. A potential scenario is as follows:

Axons use positive and negative cues to guide them towards their presumptive targets. If these cues are developemntally regulated, and if a neuron arrives at a guidepost too early or too late, that cue may not be integrated, and the axon will not find its way to its target. Neurotrophins are expressed in regions that are crossed by axons in their trajectory towards these targets. These neurotrophins are there to provide them with trophic support, or sometimes guidance cues, as they are guided along their route. Many neurotrophins are developmentally regulated, so that if an axon expressing the p75NTR is is too late or too early in reaching the guideposts it needs to properly direct its growth, it may see the "wrong neurotrophin." If the p75NTR is strongly activated by an inappropriate neurotrophin, the axon might be inhibited from continuing along its route. It may also not "see" and therefore not integrate critical

directional cues. One could also imagine that if the p75NTR is *not* present in an axon, a similar mechanism might be in place, but instead of being inhibited or guided by neurotrophins, or potentially by other molecular cues, such a p75NTR-deficient axon would ignore them, and in this way, be misdirected. The end result, in both thse cases might be that the axon would never reach its target. In support of this idea, Walsh et al (1999a) show that mice lacking functional p75NTR expression show a disorganization in innervation pattern, and innervate myelinated portions of the cerebellum, a structure that is not normally innervated by sympathetic neurons, and moreover, is non-permissive for growth.

The p75NTR has been reported to inhibit neuronal regeneration post-axotomy. A regenerative response could be induced in injured TrkB-expressing motor neurons by the application of low-doses of BDNF, but the response was inhibited by higher doses of this neurotrophin (Boyd et al., 2000). Such a biphasic dose-response may seem paradoxical at first. Why is BDNF able to inhibit growth of a neuron expressing its preferred receptor? This may be consistent with a paradigm in which BDNF preferentially binds to and activates its principal receptor (TrkB) when BDNF is present in lower doses. However, as the dose of BDNF is increasesd, TrkB receptor saturation may be achieved, and BDNF would now also bind more p75NTR molecules, generating the inhibitory response reported. Survival would be maintained through BDNF/TrkB-related survival signals, but the co-activation of p75 by BDNF although not robust enough to induce an apoptotic response, might be sufficient to inhibit the regenerative response of these neurons. In support of axonal growth inhibition being a p75-mediated response, Boyd et al. (2000) reported that BDNF/TrkB-mediated axonal regeneration could proceed in the presence of the function blocking anti-p75 antibody (REX IgG). Thus when p75NTR signaling was inhibited, BDNF could now only bind to its preferred recceptor TrkB, which was able to signal a positive response.

The question remains as to exactly how the p75NTR inhibits TrkA-mediated neuronal growth. I have shown that activation of the p75NTR with BDNF is inhibitory to TrkA/NGF-mediated sympathetic neuron growth. Recently, it has been shown that activation of the p75NTR can downregulate the TrkA-dependent raf-MEK-MAPK pathway (R Aloyz, FD Miller, DR Kaplan, unpublished data), which, in sympathetic

neurons, is a major pathway that is coupled to their outgrowth (Atwal et al., 2000; Kaplan and Miller, 2000).

Another possible mechanism is the p75NTR-mediated generation of ceramide. Posse de Chaves (1997) reported that local elevation of ceramide in distal neurites, but not in cell bodies of sympathetic neurons is able to inhibit axonal growth. Since the p75NTR can activate sphingomyelin hydrolysis, leading to the generation of ceramide, this is a potential mechanism by which it mediates the inhibition of growth. This inhibition was not attributed to apoptosis (Posse de Chaves, 2001). Thus activation of the p75NTR might increase ceramide flux, and inhibit growth in this fashion. Since p75NTR and TrkA can modulate each other's activities, and ceramide-dependent kinases can activate serine-threonine phosphorylation of the Trk receptor to inhibit its activity (MacPhee and Barker, 1997), this is a mechanism that was proposed to explain how p75NTR-mediated ceramide flux in distal neurites might occur. However, more recently, this same group reported that treating distal axons with ceramide was able to inhibit the uptake of NGF by distal axons by approximately 70%, suggesting that the inhibition of axonal growth by ceramide might be partially due to impaired endocytosis of NGF. This inhibition of endocytosis was not attribute to decreased TrkA phosphorylation (Posse de Chaves, 2001).

c) **Levels of TrkA are a key deteminant of biological responsiveness**

The work presented in Chapter 2 indicated that inhibitory signals carried by the p75NTR could be suppressed by robust TrkA activation. Based on these findings, we hypothesized that increasing the levels of TrkA while leaving p75 expression unaltered might increase the sensitivity of sympathetic neurons to TrkA ligands by enhancing the ability of Trk to suppress inhibitory p75NTR signals. This has been examined in another neuronal model. Basal forebrain cholinergic neurons normally express both TrkA and p75NTRs. This neuronal population in p75NTR-deficient mice exhibit neuronal hypertrophy, the magnitude of which was reported to be similar to that seen after NGF infusion (Yeo et al., 1997). This implies that the absence of the p75NTR imparted increased NGF responsiveness to these neurons, or potentially, increased

responsiveness to TrkA signaling pathways. Another possibility is that the p75NTR could independently suppress the responsiveness of these neurons (Yeo et al., 1997).

We asked a similar question, but approached it by altering the levels of TrkA, rather than ablating the p75NTR. We tested whether increasing the levels of TrkA would impart an increased sensitivity to NGF in sympathetic neurons, which implies, but does not demonstrate that the ratio of TrkA to p75 has been altered. Although we cannot formally conclude that we altered the TrkA to p75NTR ratio, our data suggest that by increasing levels of TrkA, we did so, since we were able to increase the sensitivity of sympathetic neurons to NGF. After overexpression of TrkA, they required much less NGF to survive, and also grew more robustly in response to 10 ng/ml NGF, a concentration previously shown to maintain robust survival of cultured sympathetic neurons (Bamji et al, 1998). We have also observed that sympathetic neurons of $p75NTR^{-/-}$ mice grow more robustly in reponse to NGF. Overexpression of TrkA in PC12 cells has also been demonstrated to enhance growth responses to NGF, as these cells differentiated and elaborated neuronal processes much more rapidly than did control cells (Hempstead et al., 1992).

Like alterations in p75NTR expression, alteration in TrkA expression, or alterations in its level of activation lead to changes in levels of growth and target innervation. Hoyle et al (1993) reported that overxpression of NGF within sympathetic neurons under the control of the DBH promoter allowed neurons to reach their targets, but innervation density was lower than normal. This may be a situation whereby overexpression of NGF and therefore robust TrkA signals may prevent a neuron from growing away from a richer environment, in this case, themselves, toward a poorer one, in this case their physiological targets. The net result was aberrant target innervation. Thus proper levels of target innervation require inhibitory signals, as well as stimulatory ones.

These studies together indicate that levels of TrkA are a key determinant of biological responsiveness. Increasing the levels of TrkA enhances the sensitivity of sympathetic neurons to NGF for both growth and survival, which implies that the balance of signaling is shifed towards TrkA. Sensitivity to NGF is important

biologically, as it allows a sympathetic neuron to be responsive to limiting concentrations of NGF, a situation that occurs developmentally.

d) **A balance between TrkA and p75NTR signals regulates levels of sympathetic neuron growth and target innervation**

Our data shows that for biological functions to proceed normally, a balance between TrkA and p75NTR signaling must be achieved. The data indicates that these receptors have opposing roles in sympathetic neurons, and more evidence has recently been provided from work with transgenic mice deficient in both TrkA and p75NTRs. Neurons that express only TrkA, but not the p75NTR show increased numbers of SCG neurons postnatally, while neurons that express only the p75NTR and not TrkA are deficient in sympathetic neurons, and die early in postnatal life (Bamji et al., 1998; Majdan et al., 2000). Majdan et al (2001) have recently demonstrated that in transgenic mice lacking both TrkA and p75NTRs, neurons do not undergo apoptosis. Since neurons lacking TrkA but expressing p75NTR do not survive, this demonstrates that one of the prinicpal functions of TrkA signaling appears to be the silencing of a p75NTR-mediated apoptotic signaling cascade. This also further supports opposing roles for TrkA and p75NTRs. These findings are consistent with a shift in biological responsiveness when levels of either TrkA or p75NTR are altered. The p75NTR, when present without TrkA induces apoptosis of sympathetic neurons, while the expression of TrkA without the p75NTR, leads to abnormally increased survival, at least developmentally (Majdan et al., 2001; Bamji et al., 1998). Obviously such increases or decreases in survival are not desirable biologically, but they do illustrate that what is important biologically is a balance between negative and positive signals.

The recurring theme in the experiments presented in this thesis is that the p75 NTR and TrkA have opposing biological roles, with TrkA mediating positive events such as survival, and the p75NTR mediating negative events such as apoptosis. We have shown that opposing roles for TrkA and p75NTRs extend to other biological events in sympathetic neurons, namely growth and target innervation. The way that TrkA and p75NTRs functionally interact to ensure that levels of target innervation are appropriate (described in Chapter 2) are an extension of the mechanism explaining how

this balance regulates neuronal survival (Majdan and Miller, 1999). In both cases, only those neurons that are "fit" will go on to survive and grow, and only "correct" targets become, and remain innervated. Unsuccessful neurons are eliminated by apoptosis, or aberrant collaterals are retracted. The biological rationale for having two neurotrophin receptors that function antagonistically with respect to survival (and growth) is illustrated by the following scenario:

If a sympathetic neuron is unsuccessful at competing for limiting amounts of target-derived NGF during the period of naturally-occurring cell death, the proapoptotic activity of the p75NTR may be one mechanism whereby such unsuccessful neurons are eliminated. Successful competitors that sequester enough NGF will have their TrkA receptors robustly activated, thus any concomitant activation of the p75NTR by ligands such as BDNF will not generate an apoptotic signaling cascade. Unsuccessful neurons may include those that are late arriving at the target, or those reaching inappropriate targets. In such cases, NGF may be limiting, and TrkA receptors will be weakly activated, or not activated at all, whereas p75NTRs, which binds all mammalian neurotrophins, will be more strongly activated. The net outcome is the elimination of neurons that have not successfully competed for trophic support.

Another determinant of biological responsiveness of sympathetic neurons is receptor ratio, which is determined by the spatial localization of these receptors. TrkA is expressed preferentially in the cell body, while the p75NTR is expressed quite ubiquitously along the neuronal surface. This means that the ratio of TrkA to p75NTR is higher in the cell body than it is in neurites, so in the area of the cell body, TrkA positive signals are likely to predominate. Alternatively, TrkA signaling will likely be lower on neurites, where p75NTR signaling may be more robust. This has biological relevance, if one considers that each of these receptors signals antagonistically. Since p75NTR signals are inhibitory, and the TrkA:p75NTR ratio is lower in neurites, it follows that the role of local p75NTR signals during development may be to locally limit growth responses when they are overly robust, or when a neurite is attempting to innervate an aberrant target. On the other hand, since TrkA signals mediate positive

events, and the cell body is enriched in trkA, a higher ratio here makes sense, with respect to signaling survival.

Spatial regulation of biological responsiveness might be important during migration, axonal pathfinding, or when competing for target territory, when a neuron must respond to spatially segregated cues, so it can appropriately innervate its target (Toma et al., 1997). Spatial regulation of responsivness will affect how a neuron may respond to different spatial sources of neurotrophins or cohorts of neurotrophins that it may encounter in its microenvironment as it grows or remodels itself. If a sympathetic neuron growing towards its target is dependent upon NGF for survival, and survival is maintained through retrograde signals that are transported back to the cell body, it makes sense for TrkA signalling pathways to predominate in the cell body of a neuron. Alternatively, cues for remodeling or for axonal growth should be local, and it makes sense that TrkA growth signals should be less predominant there, allowing p75NTR signals to predominate.

Although neurotrophins have been classically considered to be mainly target-derived, there are indications that neurotrophins are made by glial cells (Heumann et al.,1987a,b; Acheson et al., 1991; Yoshida and Gage, 1992), and as our data shows, peripheral neurons themselves synthesize neurotrophins (Ernfors et al., 1992; Schecterson and Bothwell, 1992; Acheson et al., 1995). This means that neurons are likely to "see" neurotrophins from several sources that are spatially segregated with respect to neuronal morphology.

TrkA and p75NTRs are independently regulated. In sympathetic as well as sensory and basal forebrain cholinergic neurons, p75NTR levels are greatly upregulated by exposure to NGF (Higgins et al., 1989; Miller et al., 1991) while TrkA levels remain unchanged (Belliveau et al., 1997), at least in sympathetic neurons. Since TrkA and p75NTRs are differentially distributed, NGF-mediated signaling and biological responsiveness will not only differ in axons versus cell bodies, but will also differ as a function of long-term exposure to NGF itself, since NGF exposure upregulates the p75NTR.

e) **Conclusions**

Although much has been learned about the neurotrophins and their receptors, especially within the last decade, more questions than answers remain regarding their interactions. It is not entirely clear how TrkA and the p75NTR act together to achieve proper levels of growth and target innervation, especially since the contribution of each receptor in regulating growth at different developmental growth stages is unclear. They may play different roles depending on the biological event, such as axon initiation and neurite elaboration, guidance, target innervation, terminal arborization, and even remodeling in the adult animal. Their roles after injury and during regeneration are also unclear. Also, neurotrophin expression is also developmentally regulated, so that the cohort of receptors expressed on a particular neuron or type of neuron may signal differently, depending on the stage of development that it is in.

Finally, since in sympathetic and cutaneous sensory neurons, p75 transduces inhibitory growth signals yet in certain other neurons it cooperates with TrkA, one of the most important determinants of biological responsiveness is likely to be cellular context. Cellular context is a key differentiating factor for how a neurotrophin or neurotrophin receptor acts, and there are many examples in which the same receptor type, or neurotrophin can have very different functions. For example, the studies by Atwal et al (2000) indicate that in sympathetic neurons, TrkA and TrkB signal differently, so how they each interact with p75NTR is also likely to be different. Their signaling pathways are becoming better understood, and characterizing them further will help to address many as-yet-unanswered questions about TrkA and p75NTR. What can be concluded from the data presented here is that in the context of neonatal sympathetic neurons, the TrkA and p75NTRs have functionally opposing roles and together can regulate levels and patterns of growth and target innervation.

Appendix

a) **Evidence for the production and release of BDNF from sympathetic neurons**:

One of the hypotheses presented in this thesis is that autocrine BDNF might potentially play a role in the negative regulation of sympathetic neuron growth through a BDNF: p75NTR autocrine loop. Although BDNF is more commonly found in the CNS and in peripheral sensory neurons, there is also evidence for it being present in sympathetic neurons.

In vitro, BDNF has been detected in conditioned medium obtained from cultured neonatal rat SCG neurons after several days in culture (CG Causing, R Aloyz, and FD Miller, unpublished observations). Since it is detectable in these cultures which have undergone treatment with an anti-mitotic agent to eliminate non-neuronal cells, the BDNF is derived from sympathetic neurons. Further evidence for sympathetic neurons being a source of BDNF has also been obtained from studies *in vivo.*

In vivo, BDNF mRNA and protein have been detected using both Northern and Western blot analysis as well as by immunocytochemistry in postnatal mouse sympathetic neurons (Causing et al., 1997). In situ hybridization has also been used to detect BDNF mRNA in mouse sympathetic neurons, from embryonic day 14.5 (E14.5) to postnatal day 1 (P1) (Schecterson and Bothwell, 1992).

b) **Numbers of experimental animals used**:

In **Chapter 2**, for the generation of the growth data in Figures 1-6, experiments were all repeated at least in triplicate (three separate cultures, prepared on different days). There are approximately twelve rat or mouse pups, on average, per litter, therefore thirty-six animals were used for each of these experiments. For the generation of Figure 7 (Western blot analysis), approximately ten pineal glands were required for each BDNF genotype (BDNF $^{+/+}$, $^{+/-}$, and $^{-/-}$), representing ten animals per genotype. For Figure 8, three pineals glands were used to generate the quantitative hyperinnervation data for each of the three BDNF genotypes, thus the averaged data is representative of three animals per genotype. For the Western blot analysis shown in Figure 9, approximately ten pineal glands or sets of carotid arteries were required for each BDNF genotype (BDNF $^{+/+}$, $^{+/-}$, and $^{-/-}$), representing 10 animals per genotype.

In **Chapter 3**, for the immunocytochemical analyses shown in Figures 1 and 2, experiments were repeated in triplicate (three separate cultures, prepared on different days). For the Western blot analyses (Figure 3), survival date (Figure 4), and growth data (Figure 5), again, experiments were repeated in triplicate (three separate cultures, prepared on different days).

References

Acheson A, Barker PA, Alderson RF, Miller FD, Murphy RA (1991). Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to nerve growth factor. Neuron 7: 265-275.

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

Atwal JK, Massie B, Miller FD, Kaplan DR (2000). The TrkB-Shc signals neuronal survival and local axon growth via MEK and PI3-kinase.

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.

Belliveau, DJ, Krivko I, Kohn J, Lachance C, Pozniak C, Rusakov D, Kaplan D, Miller FD (1997). NGF and NT-3 both activate TrkA on sympathetic neurons, but differentially regulate survival and neuritogenesis. J Cell Biol 136: 374-388.

Boyd JG, Posse de Chaves E, Gordon T (2000). Inhibitory effect of high dose brain-derived neurotrophic factor on motor axonal regeneration may be mediated bya ceramide-dependent mechanism. Soc Neurosci Abstr 26: 844.

Bredesen DE, Rabizadeh S (1997). P75 NTR and apoptosis: Trk-dependent and Trk-independent effects. Trends Neurosci 20: 287-290.

Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Kong H, Chao MV (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383: 716-719.

Cahoon-Metzger S, Wang G, Scott SA (2001). Contribution of BDNF-mediated inhibition in patterning avian skin innervation. Dev Biol 232: 246-254.

Ernfors P, Merlio JP, Persson H (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. Eur J Neurosci 4: 1140-1158.

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

Guidry G, Landis SC, Davis BM, Albers KN (1998). Overexpression of nerve growth factor in epidermis disrupts the distribution and properties of sympathetic innervation in footpads. J Comp Neurol 393: 231-243.

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

Heumann R, Lindholm D, Bandtlow C, Meyer M, Radeke MJ, Misko TP, Shooter E, Thoenen H (1987b). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerves during development, degeneration and regeneration. Proc Natl Acad Sci USA 84: 8735-8739.

Higgins GA, Koh S, Chen KS, Gage FH (1989). NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. Neuron 3: 247-256.

Hoyle GW, Mercer EH, Palmiter RD, Brinster RL (1993). Expression of NGF in sympathetic neurons leads to excessive axon outgrowth from ganglia but decreased terminal innervation within tissues. Neuron 19: 1019-1034.

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

Kimpinski K, Campenot RB, Mearow K (1997). Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. J Neurbiol 33: 395-410.

Lee KF, Bachman K, Landis S, Jaenisch R (1994). Dependence on p75 for innervation of some sympathetic targets. Science 263: 1447- 1449.

MacPhee IJ, Barker PA (1997). Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduced TrkA signaling while increasing serine phosphorylation in the TrkA intracellular domain. J Biol Chem 272: 23547-23551.

Majdan M, Lachance C, Gloster A, Aloyz R, Zeindler C, Bamji S, Bhakar A, Belliveau D, Fawcett J, Miller FD, Barker PA (1997). Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. J Neurosci 17:6988-6998.

Majdan M, Miller FD (1999). Neuronal life and death decisions: functional antagonism between the Trk and p75 neurotrophin receptors. Int J Devl Neurosci 17: 153-161.

Majdan M, Aloyz R, Miller FD (2001). TrkA is not required to maintain sympathetic neuron survival in the absence of p75. In revision for publication in J Cell Biol.

Miller FD, Mathew TC, Toma JG (1991). Regulation of nerve growth factor receptor gene expression by NGF in the developing peripheral nervous system. J Cell Biol 112: 303-312.

Posse de Chaves E, Bussiere M, Vance DE, Campenot RB, Vance JE (1997). Elevation of ceramide within distal neurites inhibits neurite growth in cultured rat sympathetic neurons. J Biol Chem 272: 3028-3035.

Posse de Chaves E, Bussiere M, MacInnis B, Vance DE, Campenot RB, Vance JE (2001). Ceramide inhibits axonal growth and nerve growth factor uptake without compromising the viability of sympathetic neurons. J Biol Chem (in press).

Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LI, Bredesen DE (1993). Induction of apoptosis by the low-affinity NGF receptor. Science 261: 345-348.

Schecterson LC, Bothwell M (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9: 449-463.

Snider WD, Johnson Jr EM(1989). Neurotrophic molecules. Ann Neurol 26: 489-506.

Toma JG, Rogers D, Senger DL, Campenot RB, Miller FD (1997). Spatial regulation of neuronal gene expression in response to nerve growth factor. Dev Biol 184: 1-9.

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.

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

Yeo TT, Chua-Couzens J, Butcher LL, Bredesen DE, Cooper JD, Valletta JS, Mobley WC, Longo FM (1997). Absence of p75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. J Neurosci 17: 7594-7605.

Yoshida K, Gage FH (1992). Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factot and other cytokines. Brain Res 569: 14-25.

Zhou XF, Rush RA (1993). Localization of neurotrophin-3-like immunoreactivity in peripheral tissues of the rat. Brain Res 621: 189-199.