

THE EFFECTS OF NEUROTROPHINS ON NEURITE  
GROWTH IN CULTURED ADULT SENSORY NEURONS

CENTRE FOR NEWFOUNDLAND STUDIES

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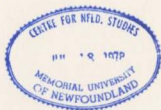
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0-612-73554-0

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# **The effects of neurotrophins on neurite growth in cultured adult sensory neurons**

By

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A dissertation submitted to the School of Graduate Studies in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy

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September 2001

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## **Acknowledgments**

First and foremost I would like to thank my parents Jean and Dr. Joe Kimpinski for their support during all these years. I would like to thank my supervisor Dr. Karen Mearow for her help and contributions during my time at Memorial. I would also extend thanks to my committee members Drs. Penny Moody-Corbett and John MacLean. I would like to recognize the members of the Division of Neuroscience for their help. A special thanks to Dr. Detlef Bieger for his knowledge of honey and bridges. I thought it was a sure thing.

## **List of Publications**

Work from this thesis has been previously been published as noted below:

Kimpinski, K., Campenot, R.B. and K.M. Mearow (1997) Effects of the neurotrophins NGF, NT-3 and BDNF on neurite growth from adult sensory neurons in compartmented cultures. *J. Neurobiol.* 33:395-410.

Kimpinski, K., Jelinski, S. and K.M. Mearow (1998) Normal Trk-p75 interactions are required for optimal neurite growth in adult DRG neurons. *Neurosci.* 93:253-263.

Kimpinski, K. and K.M. Mearow (2001) Neurite growth promotion by nerve growth factor and insulin-like growth factor-1 in cultured adult sensory neurons: role of phosphoinositide 3-kinase and mitogen activated protein kinase. *J. Neurosci. Res.* 63:486-499.

## Abstract

The compartmented culture method was used to study the regulation of adult sensory neurite growth by neurotrophins. We examined the effects of the neurotrophins nerve growth factor (NGF), neurotrophin-3 (NT3), and brain derived neurotrophic factor (BDNF) on neurite elongation from adult rat dorsal root ganglion (DRG) neurons. Initial proximal neurite growth (within center compartments) did not require neurotrophins. Subsequent elongation into distal compartments resulted from NGF but not NT3 or BDNF treatment. After neurites had extended into distal compartments, treatment with anti-NGF resulted in the cessation of growth with no significant neurite retraction. After axotomy of growing neurites in distal compartments, NGF was necessary to support regrowth. In the same paradigm, both NT3 and BDNF did not result regrowth. The results demonstrated that unlike *in vivo* nerve regeneration, *in vitro* regrowth does require NGF.

Given the above results, the next focus of study was to determine the individual contributions of the NGF receptors to the growth response. The requirement of both TrkA and the p75 neurotrophin receptor in neurite growth was examined using several experimental interventions. As expected, inhibition of TrkA activation using K252a totally blocked distal neurite extension into NGF containing compartments. Results showed using BDNF to interfere with NGF binding to p75, found that the addition of BDNF to NGF containing distal compartments reduced distal neurite extension. In contrast MC192 which alters the interaction of NGF with p75, completely inhibited NGF dependent neurite growth. Both compounds were found to partially attenuate NGF induced TrkA phosphorylation. However only MC192 appeared to activate p75 based on immunocytochemical evidence

showing nuclear localization of NF $\kappa$ -B.

These results demonstrate that both TrkA and p75 play a role in neurite growth response to NGF. Furthermore our evidence suggests that any alteration in optimal TrkA-p75 interactions, or direct activation of p75 at the expense of TrkA, results in an inhibition of NGF-dependent neurite growth.

NGF/TrkA signaling has a number of common pathways in which to elicit various physiological responses. Many of the same pathways are used by other growth factors that also are capable of producing neurite growth in adult sensory neurons. To investigate the influence of other growth factors in addition to NGF, the effects of IGF-1 EGF and FGF on neurite growth from adult rat dorsal root ganglion (DRG) neurons were examined. As expected, NGF elicited robust neuritic growth in both the dissociated and compartmented cultures. The growth response to IGF-1 was similar. There was minimal neurite growth in response to EGF or FGF. In addition, IGF-1 (but neither FGF nor EGF), when applied to cell bodies in compartmented cultures, potentiated the distal neurite growth into NGF-containing side compartments.

In order to determine the contribution of signaling intermediates downstream of receptor activation, we used pharmacological inhibitors and western blotting. The PI 3-kinase inhibitor, LY294002 attenuated neurite growth evoked by NGF, IGF and EGF in dissociated cultures, while the MEK inhibitor PD98059 only diminished growth in IGF treated cultures. Immunoprecipitation and western blotting results demonstrated differential activation of MAPK, PI 3-kinase, PLC $\gamma$ 1 and SNT by the different factors. Activation of PI 3-kinase and SNT by both NGF and IGF-1 correlated with their effects on

neurite growth. These results support the hypothesis that the PI 3-kinase pathway, and the SNT protein play an important role in neuritogenesis.

In conclusion neurite growth of adult DRG neurons is mediated by NGF *in vitro* and required TrkA activation. In comparison these results indicate that inhibition of NGF binding to p75 partially inhibits growth while activation of the receptor abolishes the response. Further assessment of NGF-TrkA signaling pathways, using other growth factors for comparison (i.e. IGF-1 and EGF) indicate that PI 3-kinase and SNT signaling intermediates are important contributors to the growth process. These experiments are a part of a larger focus on NGF and its role in neurite growth in neurons (adult DRG neurons) that do not require this factor for survival. Furthermore these studies will hopefully contribute to elucidating the processes involved in neurite growth.



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## List of Abbreviations

Akt/PKB	Protein Kinase B
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumen
CGRP	Calcitonin Gene Related Peptide
CNS	Central Nervous System
DRG	Dorsal Root Ganglion
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
ERK	Extracellular-signal Related Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FRS2	Fibroblast Growth Factor Related Substrate 2
Gab	Grb2 Associated Binder 2
GDNF	Glial cell Derived Growth Factor
Grb2	Growth Factor Related Bound Protein 2
IB4	Isolectin-B4
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IP	Immunoprecipitation
IRS	Insulin Receptor Substrate
K-252a	8R*,9S*,11S*(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cycloocta(c,d,e)trindene-1-one
K-252b	9-carboxylic acid derivative of K-252a
L15	Leibowitz 15 medium
LY292004	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
NF $\kappa$ B	Nuclear Factor kappa B
NGF	Nerve Growth Factor
NT3	Neurotrophin 3
NT4/5	Neurotrophin 4/5
PBS	Phosphate Buffered Saline
PC12 cells	pheochromocytoma-12 cells
PD98059	2'-amino-3'-methoxyflavone
PI-3K	Phosphoinositol-3 Kinase
PKC	Protein Kinase C
PLC $\gamma$ 1	Phospholipase C $\gamma$ 1
PMSF	Phenylmethyl Sulphonyl Fluoride
PNS	Peripheral Nervous System
p75	p75 neurotrophin receptor



p75NTR	p75 neurotrophin receptor
Ret	Glial cell Derived Growth Factor Receptor
RTK	Receptor Tyrosine Kinase
ROD	Relative Optical Density
SBTI	Soyabean Trypsin Inhibitor
SCG	Superior Cervical Ganglion
SDS	Sodium Dodecyl Sulfate
SHP-2	SH2 containing Protein Tyrosine Phosphatase
SMC	Sphingomyelinase-ceramide
SNT	suc-associated neurotrophic factor induced tyrosine phosphorylated target
SOS	Son of Sevenless intermediate protein
SP	Substance P
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline and Tween 20
Trk	High Affinity Neurotrophin Receptor
Trk-IR	High Affinity Neurotrophin Receptor-Immunoreactivity

## **1.0 CHAPTER I: INTRODUCTION**

### **1.1 The Dorsal Root Ganglia**

The Dorsal Root Ganglion (DRG) contains the cells that act as first order relay neurons for the majority of sensory modalities from the trunk and appendages. The heterogeneity of the neurons that make up the DRG reflects the wide range of sensory impulses that they convey. Neurons within the ganglia produce a single afferent which bifurcates (pseudo unipolar distribution) with one projection to dorsal horn cells in the spinal cord and the second to specific sensory receptors in the periphery. The peripheral projections are classified according to fibre diameter, degree of myelination and the sensory modality conveyed. Large diameter, heavily myelinated A $\alpha$ /A $\beta$  fibres innervate sensory receptors responsible for mechanoreception (touch) and proprioception (position sense). Small diameter, lightly myelinated A $\delta$  fibres and small diameter, unmyelinated C fibres convey painful stimulation (nociception) and crude touch (Gardner et al., 2000). This description is an obvious simplification of a complex subject in order to provide the reader with a background of the physiological processes that occur within the DRG. This is necessary as neurotrophins have specific actions on the separate cell types within the ganglia that appears to correlate with physiological function.

The development and physiologic functioning of the DRG is significantly impacted upon by neurotrophins and their consequent receptors. This thesis investigates the response of adult DRG neurons to neurotrophins *in vitro*.

### **1.1.1 The role of neurotrophins and neurotrophin receptors**

#### **1.1.1.1 Background**

The family of neurotrophin molecules are based on the prototypic neurotrophin Nerve Growth Factor (NGF; Levi-Montalcini and Booker 1960; Barde et al., 1982; Leibrock et al., 1989; Mobley et al., 1976). The cloning of the NGF molecule has allowed for the discovery of related molecules of the same family including Brain Derived Neurotrophic Factor (BDNF: Hohn et al., 1990), Neurotrophin-3 (NT-3: Maisonpierre et al., 1990; HallBrook et al., 1991) and Neurotrophin 4/5 (NT-4/5: Berkemeier et al., 1991; Ip et al., 1991).

To date two classes of receptors have been identified through which these neurotrophins produce their effects. These two classes consist of the Low Affinity Neurotrophin Receptor, referred to as p75 (for review see Chao 1994, Chao and Hempstead 1995; Barker 1998; Casaccia-Bonnel et al., 1998) and the Trk family of tyrosine kinase receptors (for review see Barbacid 1994; Kaplan and Miller 1997). Presently three Trk receptors have been identified. NGF binds solely to TrkA (Kaplan et al., 1991a; Klein et al., 1991a). BDNF and NT-4/5 bind to TrkB (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991; Berkemeier et al., 1991; Ip et al., 1991; Klein et al., 1992 ref). NT-3 binds primarily to TrkC (Klein et al., 1991b Lamballe et al., 1991). In addition, NT-3 also has a diminished affinity for Trk A and TrkB compared to the TrkC receptor (Cordon-Cardo et al., 1991; Klein et al., 1991b Klein et al., 1991b; Lamballe et al., 1991). All neurotrophins bind to the p75 low affinity neurotrophin receptor (p75) as well as their high affinity Trk receptors (Rodriguez-Tebar et al., 1990 and 1992; Ernfors et al., 1990;

Hallbook et al., 1991; Squinto et al., 1991).

In addition to the full length Trk receptors (receptors composed of an extracellular binding domain, a transmembrane portion and an intracellular tyrosine kinase domain) several truncated forms have been discovered. Two distinct TrkB isoforms and several TrkC isoforms lacking the intracellular kinase domain have been reported (Middlemas et al., 1991; Valenzuela et al., 1993). In DRG neurons, truncated TrkB isoforms are expressed in distinct neuronal populations and are hypothesised to regulate neurotrophin responsiveness (Boeshore et al., 1999).

#### **1.1.1.2 Expression of neurotrophin receptors within the DRG**

There are a number sub-populations of neurons within the DRG responsive to NGF, BDNF, NT-3 and NT-4/5. While there is variation among studies, approximately 5-20% and 10-20% of neurons in the adult DRG express TrkB and TrkC, respectively. TrkA expressing neurons in the DRG are estimated to be in the range of 40-50% (Mu et al., 1993; McMahon et al., 1995; Molliver et al., 1995; Kashiba et al., 1996). The range of values reflect the experimental differences between studies due to the age of the animals used and the spinal levels from which the DRGs were obtained. These receptors appear to be expressed in non overlapping patterns with the exception of 5-15% of neurons that co-express both TrkA and TrkC (Wright and Snider, 1995). The p75 receptor is co-expressed in 90% of TrkA and TrkB expressing neurons but only 50% of TrkC expressing neurons (Wright and Snider, 1995). The p75 receptor does not appear to be expressed in neurons that do not express Trk receptors (Wright and Snider, 1995).

Developmentally neurotrophin responsiveness, primarily survival effects, have been shown using knockout mice that are deficient in either NGF, BDNF or NT-3 or their corresponding receptors. Animal knockouts have provided further evidence indicating the importance of these molecules during DRG development. As a result, both NGF or TrkA knockout mice display 70-80% loss of neurons in the DRG, with the affected population being the smaller peptidergic neurons (Smeyne et al., 1994; Crowley et al., 1994). Cell death resulting from either the lack of NGF or TrkA is thought to involve preferential activation of c-jun (see discussion on p75 signalling) and consequent upregulation of the pro-apoptotic protein BAX (Vogelbaum et al., 1998; Patel et al., 2000). Down regulation of BAX in BAX-/- plus TrkA/NGF -/- mice prevents the developmental loss of DRG neurons (Patel et al., 2000).

NT-3 or TrkC knockouts appear to influence the larger proprioceptive neurons in the DRGs, with loss of about 20% of neurons in the TrkC -/- mice and up to 55% loss in the NT-3-/- mice (Ernfors et al., 1994; Klein et al., 1994). Both the BDNF -/- and TrkB-/- animals also display a 30-50% loss of neurons in the DRGs (Klein et al., 1993). Along the same lines NT4 -/- mice do not develop TrkB expressing neurons in the DRG (Liebl et al., 2000).

#### **1.1.1.3 Physiological significance of neurotrophins *in vivo***

The response toward a given neurotrophin in a subset of neurons within the ganglia generally correlates with Trk expression. For example small to medium diameter neurons express TrkA and are responsive to NGF (Crowley et al., 1994; Smeyne et al., 1994).

There is a subset of small diameter neurons unresponsive to NGF in the adult. However these neurons are lost during development in TrkA  $-/-$  mice, strongly indicating that TrkA is expressed in these cells during development (Silos-Santiago et al., 1995). The large diameter neurons appear to express TrkC and are NT-3 responsive accordingly (Zhou and Rush 1995; Kucera et al., 1995; Oakley et al., 1997).

While there are some exceptions, neuronal subpopulations responsive to NGF and NT-3 appear to correlate with specific sensory modalities (as reviewed in Mendell 1999). Small to medium sized NGF responsive neurons give rise to small myelinated and unmyelinated fibres subserving pain and crude touch ( $A_\delta$  and C fibres) while larger NT-3 responsive neurons give rise to larger myelinated afferents subserving proprioception ( $A\beta$  fibres; reviewed in Wright and Snider 1996).

Immunocytochemical studies of TrkA and TrkC expression and associated markers in the DRG have revealed that these neurotrophin receptors are distinctly located within NGF and NT-3 responsive neuronal populations, respectively (as reviewed in Mendell 1999). This has provided further evidence for the hypothesis that the neurotrophins NGF and NT-3 regulate the functions of distinct neurons in the ganglia (Wright and Snider 1996). Evidence from TrkA/NGF knockout mice show severe deficits in pain sensation and are consistent with the idea that these neurons subserve nociception (Crowley et al., 1994; Smeyne et al., 1994). Knockout mice for p75  $-/-$  (the low affinity neurotrophin receptor) also show a loss of nociceptive neurons and aberrant nociceptive functioning with impaired pain sensation (Stucky and Koltzenburg 1997). NGF has a wide range of effects on nociceptive afferents including regulation of afferent sprouting, modulating response to

stimuli and inducing pathological responses such as hyperalgesia (Diamond et al., 1991a,b; Shu and Mendell 1999).

Immunocytochemistry shows that several targets of peripheral DRG projections including muscles and associated tendons express NT-3 (Zhou and Rush 1995). In fact proprioceptive Ia afferents and muscle spindles are dependent on NT-3 for normal development (Kucera et al., 1995). In the absence of peripheral targets, exogenous NT-3 rescues muscle spindles and muscle spindle afferent formation (Oakley et al., 1997; Wright et al., 1997). Such evidence supports the concept of NT-3 responsive neurons subserving proprioception.

Experiments using NT-3  $\pm$  heterozygous mutant mice (these mice survive past birth in contrast to their homozygous littermates but still have a significant decrease in NT-3) reveal a decrease in both small diameter and large diameter neurons (Airaksinen et al., 1996). An explanation for this finding is the reliance of TrkA expressing neurons (in the adult) on NT-3 during development. At embryonic day 11.5, all neurons are expressing TrkC and require NT-3 for survival. By embryonic day 13.5, TrkC mRNA is down regulated and a subpopulation of these neurons (approximately 80%) express TrkA and are reliant on NGF for survival (White et al., 1996). Cells within the DRGs of NT-3  $\pm$  mice undergo premature differentiation and cell death of neuronal precursors. A number of these precursors are destined to become TrkA expressing/NGF responsive. The result is a depletion in the number of neuronal precursors during a critical period in the production of TrkA expressing neurons and a decrease in the overall number of TrkA neurons (Farinas et al., 1996; Eishamy and Ernfors 1996; Lefcort et al., 1996).

The hypothesis that proprioception and nociception are regulated by TrkC/NT-3 responsive and TrkA/NGF responsive neurons, respectively, have lead researchers to postulate a more specific role for BDNF responsive/TrkB neurons. BDNF  $-/-$  mice show a 34% reduction in DRG neurons with no effect on normal proprioception (Jones et al., 1994). Transgenic mice over expressing BDNF affect specific mechanoreceptors producing larger Meissner corpuscles and increased densities of Merckel cells (LeMaster et al., 1999). Along similar lines, several experiments have shown that BDNF  $-/-$  (homozygous) and BDNF  $-/+$  (heterozygous) mice have impaired functioning of slow adapting mechanoreceptors (Carroll et al., 1998). These deficits are reversed with BDNF application (Carroll et al., 1998).

However, determining the physiological influence of BDNF on specific sensory modalities may be difficult. The effects of BDNF may be redundant with other neurotrophins, specifically NT-3 and NT-4/5. Both ligands bind to TrkB and are expressed in peripheral tissues (Lindsay 1996). Therefore the use of transgenic mice to study the physiological significance of BDNF in the DRG may be complicated by compensation by other neurotrophins during development. For instance, NT-3  $-/+$  mice show deficits in D-hair and slow adapting mechanoreceptors. Merkel cells and their afferent projections are also lost (Airaksinen et al., 1996). In addition p75  $-/-$  knockout mice show a decrease in sensitivity of D-Hair cells to mechanical stimulation with sparing of low threshold mechanoreceptors (Stucky and Koltzenburg 1997).

The majority of studies have focussed on the effects of neurotrophins produced by peripheral targets and their consequent effects on DRG neurons. However it has become



apparent that the DRG can produce neurotrophins that are transported in an anterograde fashion to target tissues. In the peripheral nervous system BDNF is transported into targets in the periphery and spinal cord by adult DRG neurons. The forward transport of BDNF in the DRG is hypothesised to be important in synaptic modelling (Zhou and Rush 1996). Anterograde transport of BDNF by NGF responsive neurons may act as a mechanism where dorsal horn neurons within the spinal cord can be modulated by neurons in the periphery (Michael et al., 1997; as reviewed in Altar and DiStefano 1998).

### **1.1.2 *In vitro***

#### **1.1.2.1 Sensory neurons: Factors affecting adult versus neonatal survival**

NGF is required for the survival of embryonic and neonatal, but not adult sensory neurons *in vitro* (Lindsay 1988). Studies examining the effects of NGF withdrawal on survival have primarily focussed on sympathetic neurons. In such cases growth factor withdrawal results in the initiation of apoptotic cell death characterised by DNA fragmentation and increased protein synthesis (Deckworth and Johnson 1993; Edwards and Tolkovsky 1994). Along the same line, inhibitors of protein synthesis prevent apoptosis induced by NGF withdrawal (Martin et al., 1988). The prevention of apoptosis by inhibition of protein synthesis is a strong indication that the death process in sympathetic neurons (after NGF withdrawal) is an inherently active process rather than a passive response. The requirement of NGF for survival in neonatal DRG neurons suggests a similar apoptotic response to NGF withdrawal (Kleese and Parada 1998).

In this regard the expression of a number of proteins involved in apoptosis (Bax and

p53) are essential for cell death *in vivo* (Deckworth et al., 1996; Easton et al., 1997). *In vitro* p53 expression is sufficient to induce death of neonatal sympathetic neurons (Slack et al., 1996). Activation of p53 has been reported to occur through the MEKK/Jun kinase pathway (Derijard et al., 1994; Yan et al., 1994). Jun kinase can be activated by p75 and NGF withdrawal (Bamji et al., 1998; Estus et al., 1994). Recently work by Aloyz and colleagues has correlated p75 activation/NGF withdrawal to Jun kinase activation of p53 to induce cell death in sympathetic neurons (Aloyz et al., 1998).

Survival effects can be mimicked by the related neurotrophin NT-3 which also binds to TrkA but with a lesser affinity compared to NGF (Cordon-Cardo et al., 1991). Consequently higher concentrations of NT-3 are required to produce the equivalent survival effects seen with NGF at lower concentration in sympathetic neurons (Belliveau et al., 1997). However, in the same study such increases in NT-3 concentrations were not required for equivalent neuritogenesis produced by NGF. Therefore differential activation of TrkA may be important in mediating different biological responses such as growth and survival.

In the adult animal, reliance on NGF for survival is lost and adult DRG neurons can survive in culture in the absence of exogenous trophic support (Lindsay 1988). Critical examination of the data from these culture experiments does not exclude early cell death of specific populations since such experiments are usually done 24-48 hours after initial plating of the cells (Lindsay 1988). More recent data have implicated autocrine production of BDNF by adult DRG neurons as a requirement for survival. Anti-sense oligonucleotides to BDNF resulted in death of approximately 30% of the neurons in culture (Acheson et al.,

1995). The effects were reversed by the addition of BDNF and to a lesser extent by NT-3 (Acheson et al., 1995).

In addition to neurotrophins, Glial Cell Derived Growth Factor (GDNF) influences the survival and growth of a subset of neurons. Small diameter, lectin IB4 binding neurons rely on GDNF for survival. This requirement begins on embryonic day 15 and continues into adulthood (Molliver et al., 1997a). GDNF, however does not affect TrkA/CGRP expressing neurons as IB4 binding and TrkA expressing subpopulations do not significantly overlap within the ganglia (Molliver et al., 1997a). The same population of neurons is protected by GDNF after axotomy (Bennett et al., 1998). GDNF counters the decrease in TMP (thiamine monophosphatase; a marker for the neuronal population), down regulation of IB4 binding and somatostatin expression seen after sciatic nerve axotomy (Bennett et al., 1998). Similar results using explant cultures, show increased neurite growth and decreased apoptotic cell death of adult DRG neurons (Leclerc et al., 1997).

#### **1.1.2.2 Effects of growth factors on neurite growth in adult sensory neurons**

Although adult DRG neurons do not require neurotrophins for survival, neurite growth can be elicited from adult DRG neurons by a wide array of growth factors. In low density cultures, NGF and BDNF enhance neurite growth and neurite arborisation (Lindsay 1988; Yasuda et al., 1990). NT-3 also has the ability to produce neurite growth but effects equivalent to that seen with NGF require a ten fold concentration increase (Mohiuddin et al., 1995).

In addition to the neurotrophins there are also a number of other factors that

influence growth. These growth factors include Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF), Fibroblast Growth Factor (FGF) and GDNF (Recio-Pinto et al., 1986; Matheson et al., 1996). The effect of aFGF has been shown to enhance but not initiate neurite growth. aFGF increased NGF induced neurite length in DRG neuronal cultures 2 fold over NGF controls (Mohiuddin et al., 1996). In contrast bFGF has no apparent effect on the initiation of neurite growth (Kimpinski and Mearow unpublished observations). In culture GDNF has comparable effects to NGF on the percentage of neurite bearing neurons and neurite extension (Gavazi et al., 1999). This effect was more prominent in IB4/ret expressing neurons (Gavazi et al., 1999). Further discussion on the growth effects of other growth factors are presented elsewhere in this introduction (EGF, see section 3.2; IGF, see section 3.3; see also discussion concerning compartmented cultures, section 4).

### **1.1.3 Other roles for NGF in adult sensory neurons**

While NGF is not required for survival of adult sensory neurons, it does play a role in maintaining phenotype and regulating gene expression. For example, NGF has the ability to upregulate the expression of both TrkA and p75 in PC12 cultures (Zhou et al., 1995). The same upregulation of Trk and p75 reported in PC12 cells has been observed in adult DRG neuronal cultures (Lindsay et al., 1990). Upregulation of NGF receptors may provide a mechanism for NGF to regulate the intensity of its own signalling pathways. NGF is capable of regulating neuropeptide expression of CGRP and substance P (SP) in DRG neurons (Lindsay and Harmar 1989; Mulderry 1994). CGRP and SP may be

involved in pain transmission and provide a link between NGF and nociception. In addition to those already mentioned, there are a wide variety of genes upon which NGF exerts an influence. These topics are out of the scope of this overview.

#### **1.1.4 Summary**

Neurons of the DRG are responsive to a wide variety of growth factors and neurotrophins. In particular, neurotrophins appear to influence select cell populations within the ganglia. These influences include survival, axonal growth, sensory function and gene expression. The underlying mechanisms for the actions of neurotrophins (and growth factors) occur at the molecular level. Interactions of neurotrophins with their receptors and the resulting signalling pathways are reviewed in the following sections.

#### **1.2 Neurotrophin signal transduction**

Since the classification of the Trk proto-oncogene as the high affinity receptor for NGF there has been a significant increase in our understanding of the process of signal transduction by this class of growth factors (Kaplan et al., 1991a,b). However, it has become apparent that data concerning pathways responsible for biological outcomes in cell lines (particularly PC12 cells) have not necessarily translated well to primary neurons (Kaplan and Stephens 1994; Kaplan and Miller 1997). The p75 receptor itself has also emerged to have a greater contribution to these processes. The following sections attempt to present the reader with a background knowledge of Trk/p75 signal transduction taken primarily from work with PC12 cells and highlight the differences that occur with primary

neurons.

### 1.2.1 Trk-p75 interactions

Both Trk and p75 receptors play an important role in the interaction with neurotrophins and each influence the other receptor's association with potential ligands. Cell lines expressing either p75 or Trk have been shown to have dissociation constants of approximately  $10^{-9}$ M, corresponding to low affinity binding sites (Hempstead et al., 1991). However when Trk and p75 are coexpressed approximately 10% of the binding exhibited by NGF corresponds to high affinity sites ( $K_d$   $10^{-11}$  M). The same study indicated that p75 is necessary for this high affinity binding of neurotrophins (Hempstead et al., 1991). In addition, p75 alters the binding kinetics of neurotrophins to their receptors. For example, cell lines expressing p75 or Trk alone show rates of dissociation of a  $t_{1/2}$  equal to that of 1 minute for p75 compared to 70 minutes exhibited by Trk receptors. However when p75 and Trk receptors are coexpressed, a 25 fold increase in the rate of dissociation occurs compared to cells solely expressing Trk, which appears to coincide with high affinity binding (Mahadeo et al., 1994). Therefore the interaction of either p75 or Trk with a neurotrophin ligand is greatly influenced by whether one or both receptors are present.

Several roles for p75 in neurotrophin signalling have been suggested. To date the majority of studies have focussed on the modulation of neurotrophin binding. How p75 alters neurotrophin binding to Trk receptors still remains a topic of debate. Two models have been proposed. The first model or the conformational model involves the dimerization of both p75 and Trk to form a high affinity binding site (Chao and Hempstead

1995; Barker 1998; Casaccia-Bonnel et al., 1998). The second model or the confrontational model suggests p75 acts as a sink for the neurotrophin and presents the molecule to the Trk receptor by either forming a heteromeric receptor complex or providing a micro environment where local concentrations of the neurotrophin are increased (Chao and Hempstead 1995). While there are various arguments for either model, conclusive evidence for the formation of a heteromeric receptor combination involving both p75 and Trk is lacking. Such a lack of convincing evidence comes from cross-linking studies using brain tissue where Trk and p75 could not be coimmunoprecipitated (Huber and Chao 1995). In contrast, copatching studies in baculovirus-insect cells over expressing TrkA and p75 show formation of heteromeric receptor complexes in the presence or absence of NGF (Ross et al., 1996). The same mechanism for p75 interaction with both TrkB and TrkC has been reported (Bibel et al., 1999).

A greater picture of Trk and p75 has emerged, indicating a survival versus death process depending on which receptor signalling cascades predominate (Kaplan and Miller 1997). For example, p75 signalling in the absence of Trk activation results in cell death most likely acting through c-jun kinase (Yoon et al., 1998). Similarly, p75 activation decreases neuronal growth and innervation of target tissues (Kohn et al., 1999). In the situation where Trk is activated in combination with p75, cell survival, neuronal growth and increased target innervation predominate (Yoon et al., 1998; Kohn et al., 1999; Kaplan and Miller 1997). Chapter 3 of this thesis focuses on these concepts.

### **1.2.2 Trk signal transduction (see Figure 1.1)**

NGF and its Trk receptor signal transduction pathways have been the most widely studied of all the neurotrophins. For this reason the majority of the evidence concerning neurotrophin signalling is derived from using NGF as a model. Potential signalling pathways will be discussed in the following section.

The Trk receptor consists of an extracellular portion, which interacts with its neurotrophin ligand, a transmembrane domain, and an intracellular region containing a tyrosine kinase domain (Reichardt and Farinas 1997). Through phosphorylation of the intracellular tyrosine kinase domain, Trk receptors initiate a variety of intracellular actions responsible for various cell functions such as proliferation, differentiation, survival and growth (Reichardt and Farinas 1997). Currently much of the research involving signal transduction pathways of the Trk receptor are concerned with receptor autophosphorylation, the subsequent phosphorylation of intracellular substrates, determining how this phosphorylation is regulated and its relation to the wide variety of cellular responses ascribed to neurotrophins (see figure 1.1).

The first step in Trk signalling is the phosphorylation of the intracellular tyrosine kinase upon ligand binding (Kaplan et al., 1991b, see figure 1.1). It has been shown in culture experiments in a wide variety of cell lines and neurons that treatment with NGF results in rapid autophosphorylation of the intracellular tyrosine kinase occurring within 5 minutes (Kaplan et al., 1991b). As a result, various intracellular substrates become phosphorylated through a number of steps involving serine/threonine and tyrosine kinases. Substrates phosphorylated by Trk include phosphatidylinositol-3 kinase (PI3-kinase), suc-



associated neurotrophic factor induced tyrosine phosphorylated target (SNT), phospholipase  $C_\gamma$  ( $PLC_\gamma$ ), and Shc. The Trk autophosphorylation site pY674/675 correlates with Trk catalytic activity (Segal et al., 1996). Trk autophosphorylation sites pY490 and pY785 activate Shc and  $PLC_\gamma$  respectively (Segal et al., 1996). The autophosphorylation site pY490 is also responsible for activation of PI3-kinase (Hallberg et al., 1998). The phosphorylation site for SNT remains an area of debate (for review see Friedman and Greene 1999).

The ras pathway has been implicated in neurite growth, PC12 cell differentiation and sympathetic neuron survival (Nobes et al., 1996; Pang et al., 1995; Stephens et al., 1994). Activation of the pathway by Trk initially involves phosphorylation of Shc (Obermeier et al., 1993). Shc in turn binds to growth factor related bound protein 2 (Grb2) which acts as a link between Shc and the Son of Sevenless (SOS) intermediate protein (Rozakis-Adcock et al., 1992). SOS acts to increase phosphorylation of ras resulting in an increase in the ratio of bound GTP to GDP (Maruta and Burgess 1994). The active form of ras, T-Ras, binds b-raf and this protein through phosphorylation activates MEK (MAP kinase kinase; Wood et al., 1992; Vaillancourt et al., 1994). The next step in the cascade involves phosphorylation of Mitogen Activated Protein Kinase (MAPK) by MEK (Gomez and Cohen 1991). MAPK likely regulates nuclear translocation and transcription of NGF-inducible genes (see figure 1.1).

Reports have shown that sustained p21ras activation is sufficient for survival of sympathetic neurons in the absence of NGF (Nobes et al., 1996). There is conflicting evidence, where activation of ras resulted in survival and neurite growth of embryonic DRG

neurons but not sympathetic neurons (Borasio et al., 1989, 1993). However Kleese and Parada (1998) have provided evidence that while ras activation is necessary for survival, these effects are not mediated through MAPK. Rather PI3-kinase in addition to ras serves redundant functions in maintaining survival (Kleese and Parada 1998). These results suggest an interaction between ras and PI3-kinase signalling pathways in sensory neurons (see below).

MAPK is activated by NGF in both sympathetic and sensory neurons. However activation has been reported to be transient in sympathetic neurons but sustained in adult sensory neurons (Klinz and Heumann 1995). The use of the MEK inhibitor PD 98059 has helped the understanding of the role MAPK plays in PC12 cells (Dudley et al., 1995). PD 98059 blocks differentiation and associated neurite outgrowth in PC12 cells (Pang et al., 1995). In sympathetic neurons MAPK promotes survival and process outgrowth (Frodin et al., 1994). While in adult sensory neurons, MAPK does not appear to be involved in either growth or survival (Klinz et al., 1996; but see Ganju et al., 1998).

PLC $\gamma$  is transiently and rapidly phosphorylated by activated Trk upon NGF binding. Along with the ras pathway PLC $\gamma$  has been shown to be important in neurite outgrowth in PC12 cells (Loeb et al., 1991; Kim et al., 1991; Vetter et al., 1991; Zhou et al., 1995). Both PLC $\gamma$  and MAPK appear to serve redundant functions in PC12 cells (Stephens et al., 1994). The phosphorylation of PLC $\gamma$  has also been reported in adult DRG neurons (Ganju et al., 1998).

SNT is a specific target of Trk activation independent of other signalling pathways

and plays a number of roles in neuronal functioning. SNT is rapidly phosphorylated by NGF binding to TrkA (Rabin et al., 1993). Consequentially, the phosphorylated form of SNT localizes to the nucleus where it likely exerts its effect on gene transcription. Furthermore, deletion of a juxta-membrane sequence of TrkA in PC12 cells inhibited phosphorylation of SNT but not PLC $\gamma$ , SHC or PI-3 Kinase (Peng et al., 1995). SNT appears to play a role in neuritogenesis since a lack of SNT activation corresponded to a severe deficit in neurite promotion (Peng et al., 1995).

Recent evidence has shown that the SNT molecule is identical to the Fibroblast Growth Factor Receptor Substrate-2 (FRS2), a phosphorylation target that is able to bind Grb2/SOS complexes (Kouhara et al., 1997). FRS2 is phosphorylated by TrkA and can elicit activation of a number of molecules including Crk, SH-PTP-2 and src (Meakin et al., 1999; Ong et al., 2000). The identification of SNT as FRS2 has provided tremendous insight into the effects of Trk signalling on cell cycle progression and gene transcription that were otherwise difficult to explain by other major signalling pathways. In addition to TrkA, TrkB activation of SNT has been reported and the same pathway may be possible for TrkC (Easton et al., 1999).

Several studies have reported that PI3-kinase can be directly phosphorylated by Trk (Carter and Downs 1992; Soltoff et al., 1992; Obermeier et al., 1993). Ohmichi and colleagues failed to replicate Trk association with the enzymatic 85 kD portion of PI3-kinase but did see a 110 kD band with immunoblotting that likely represents the catalytic portion of the PI3-kinase enzyme (Ohmichi et al., 1992). However, results in PC12 cells indicate that predominant activation of PI3-kinase by Trk involves the signalling

intermediate Gab1 (Grb2 associated binder-1) versus direct interaction (Holgado-Madruga et al., 1997).

PI3- kinase is suggested to be important in neurite outgrowth in PC12 cells (Kobayashi et al., 1997). Studies using wortmannin, an inhibitor of PI-3 kinase, blocked neurite outgrowth during the late phase of PC12 cell differentiation (Kimura et al., 1997). In addition to wortmannin, another inhibitor of PI-3 Kinase, LY294002, has been described (Vlahos et al., 1994). There are conflicting reports on whether this compound results in death of sympathetic neurons but there is agreement that PI3-kinase is involved in survival (Crowder and Freeman 1998; Philpott et al., 1997). In contrast, inhibition of PI3-kinase has been reported to not affect survival of adult sensory neurons (Bartlett et al., 1997). In sympathetic neurons, survival effects of PI-3 kinase were correlated with activation of the signalling intermediate Akt and inhibition of this pathway decreased survival (Crowder and Freeman 1998; Philpott et al., 1997).

In summary it has been suggested that two basic arms of Trk signalling exist. Using PC12 cells as a model, there is strong evidence that signalling through PI3-kinase/Akt results in survival effects whereas downstream activation of ras (i.e. MAPK), PLC $\gamma$  and SNT/FRS2 induce other biological effects such as differentiation and neurite growth (Kleese et al., 1999; Ashcroft et al., 1999; see also previous discussion within this section).

The exception to this rule is that both ras and PI3-kinase can produce survival effects (see above). Inhibition of either ras or PI3-kinase results in death of embryonic sensory neurons. Where the convergence of both the ras and PI 3-kinase pathways

occurs is still to be completely determined. However it has been reported that ras is capable of activating PI 3-kinase in PC12 cells (Rodriguez-Viciano et al., 1994). Activation of PI 3-kinase may be the mechanism by which ras signalling can diverge from acting through MAPK and initiate survival responses (Kleese and Parada 1998; Hallberg et al., 1998).

### **1.2.3 p75 signal transduction (see Figure 1.2)**

In addition to the Trks, neurotrophins bind to a second receptor, the p75 receptor. p75 has been reported to bind all neurotrophins with equal affinity but differing kinetics and is generally associated with low affinity binding (Rodriguez-Tebar et al., 1990, 1992). Unlike the Trks, p75 has a less clearly defined role but has been implicated in apoptosis and cell survival (Rabizadeh et al., 1993; reviewed in Carter et al., 1996b; Carter and Lewin 1997; Bredesen and Rahbizadeh 1997; Kaplan and Miller 1997; Barker 1998; Casaccia-Bonnel et al., 1998).

While p75 appears to be neither necessary nor sufficient for many aspects of neurotrophin signalling, it does play a role in regulating responses to the neurotrophins. Evidence suggests that p75 modulates the cellular response to NGF by acting to increase the cellular sensitivity or responsiveness to NGF (Chao and Hempstead 1995; Ryden et al., 1997). Receptor co-expression studies have indicated that co-expression of p75 with Trk results in increased high-affinity binding sites (Hempstead et al., 1991; Battleman et al., 1993; Mahedee et al., 1994). NGF binding to p75 has also been shown to enhance Trk activity and responsiveness to NGF (Barker and Shooter 1994; Berg et al., 1991;

Hantzopoulos et al., 1994; Verdi et al., 1994). Inhibition of NGF binding to p75 using either blocking antibodies or BDNF, which binds p75 but not TrkA, resulted in decreased numbers of high-affinity binding sites; as a consequence, biological responses were not fully blocked but required higher concentrations of NGF to be elicited (Barker and Shooter 1994; Westkamp and Reichardt 1991). Similarly neurons from p75<sup>-/-</sup> mice require increased amounts of NGF for survival compared to wild-type animals (Davies et al., 1993). A mutant NGF molecule incapable of binding p75, still elicits neurite outgrowth and survival but increased concentrations are required compared to the normal NGF (Ryden et al., 1997; Ibanez et al., 1992).

In addition to modulatory influences on Trk activity, p75 also possesses intrinsic signalling capabilities. First, NF $\kappa$ -B activation and translocation to the nucleus is induced by NGF acting through p75 in Schwann cells (Carter et al., 1996b). The same results could not be replicated using BDNF or NT-3 (Carter et al., 1996b). Second, the p75 receptor also results in phosphorylation of Jun kinase, which has been implicated in cell death processes (Cassacia-Bonnet et al., 1996). Third, the sphingomyelinase pathway can be activated by p75, resulting in the production of ceramide which has been implicated in apoptosis (Dobrowsky et al., 1994; Hannun and Obeid 1995) and the regulation of neurite outgrowth (Furuya et al., 1995; Posse de Chaves et al., 1997). Furthermore, activation of Trk antagonizes sphingomyelin hydrolysis produced by p75 (Dobrowsky et al., 1995). As a result it has been postulated that Trk activation acts to inhibit pathways signalling through p75.

#### **1.2.4 Summary**

The present view of neurotrophin signalling encompasses three basic ideas. First both Trk and p75 interact with neurotrophins in a way that influences the other receptor. The majority of evidence suggests that p75 acts to enhance the activity of the Trk receptor when coexpressed. Second, both Trk and p75 activate specific signal transduction cascades. Third, predominant activation of either receptor results in different biological effects. For example, p75 signalling (in the absence of Trk activation) results in cell death while Trk signalling apparently can over ride p75 and produce effects such as survival and neurite growth.

### **1.3 Other Tyrosine Kinase Receptors/ligands and their effects on DRG neurons**

In addition to neurotrophins, DRG neurons are responsive to a number of other growth factors. The following is a short summary of the growth factors and their receptors pertinent to this study.

#### **1.3.1 Generalized concepts of Tyrosine Kinase Receptor signalling**

Of the numerous groups and classifications of receptor tyrosine kinases it has become apparent that even though these receptors are structurally different (with the exception of an intracellular tyrosine kinase) they activate similar intracellular targets. This is interesting because RTKs can have widely varying cellular effects. The reasons for the differing biological results could be a result of subtle differences in the cascade of intracellular proteins activated and/or the duration and intensity of this activation (Marshall

1995). Such issues have been addressed to a large degree in the current literature but the emerging idea that a single receptor can have different effects in varying cell types through the same pathways still clouds the issue. It is possible that different biological effects are important in differing cell types and that the function of a receptor needs to be characterized for each particular cell type. Such is the case for primary sensory neurons where RTKs like the Trk receptors play such an important biological role both *in vitro* and *in vivo*.

### **1.3.2 Epidermal Growth Factor, its signalling pathways and its effects on DRG neurons (see Figure 1.3)**

EGF enhances the growth of various cell types *in vitro*. EGF binds to the EGF receptor (EGFR), through which it exerts its biological effects. EGFR is a kinase receptor with an intracellular tyrosine kinase domain (as reviewed in Yamada et al., 1997). It is the effects of EGF on DRG neurons that is focussed on in chapter 4 of this thesis.

EGF binding the EGFR receptor results in dimerization and autophosphorylation on intracellular tyrosine residues (Schlessinger and Ullrich 1992; Van der Geer et al., 1994). These activated tyrosine residues act as high affinity sites for a number of proteins containing SH2 (src homology 2) domains (Anderson et al., 1990; Cohen et al., 1995; Pawson 1995). These SH2 containing proteins include PLC $\gamma$ , Ras-GTPase activating protein, Ras-GAP, Grb2, Nck, SHC and SHP-2 (Van der Geer et al., 1994). Activation of Grb2 links EGFR to the ras signalling pathway and eventual activation of MAPK (Buday and Downward 1993).



SHP-2 (a SH2 containing protein tyrosine phosphatase) and ras-GAP are two signalling intermediates activated by EGFR that may negatively regulate EGFR activity (Feng and Pawson 1994; Moran et al., 1990). SHP-2 is a protein tyrosine phosphatase that results in the dephosphorylation of activated proteins. Ras-GAP acts to stimulate the GTPase activity of ras resulting in conversion to the GDP bound inactive state. In doing so it is likely that Ras-GAP negatively regulates ras activation by EGF (Boguski and McCormack 1993).

EGFR stimulates PI3-kinase but does not directly bind to it (Cochet et al., 1991; Hu et al., 1992; Rafioni and Bradshaw 1992). This is contrast to other RTKs such as Platelet Derived Growth Factor Receptor that can bind directly to PI3-kinase (Klippel et al., 1992; Copper and Kashishian 1993; Obemeier et al., 1993). PI3-kinase can also be activated by other EGF receptors, including ErbB-3, that have been shown to mediate its activity (Soltoff et al., 1994; Soltoff and Cantley 1996; Holgado-Madruga et al., 1996). This activation is through a heteromeric configuration with the EGFR receptor through consensus sequences on ErbB-3. Cbl has been shown to associate with PI-3 Kinase in response to EGF as well as Gab (Soltoff and Cantley 1996). Gab is a docking protein similar to the IRS proteins that interact with the insulin and insulin like growth factor receptors. Gab is can act as a docking protein for a number of SH-2 containing proteins including PI3-kinase, PLC $\gamma$ , Grb-2, and SHP-2 and capable of activating PI3-kinase (Holgado-Madruga et al., 1996).

The EGFR receptor is capable of activating the non-receptor tyrosine kinase JAK and the latent cytoplasmic transcription factor STAT (Signal Transducer and Activation of

Transcription). STAT1 and STAT3 are activated through the EGFR receptor (Ruff-Jamison et al., 1993; Sandowski et al., 1993; Zhong et al., 1994). JAK 1 is also activated (Shuai et al., 1993) and is the most likely candidate for activating STAT1 and STAT3.

Within the DRG approximately 70% of the neurons express EGFR (Huerta et al., 1996). In embryonic DRG neurons, EGF does not play a role in cell survival when compared to TGF (Chalazonitis et al., 1992). Comparatively EGF has been found to induce moderate neurite outgrowth in adult sensory neurons (see chapter 4).

### **1.3.3 Insulin-like Growth Factors, its signalling pathways and its effects on DRG neurons (see Figure 1.4)**

There are two members of the Insulin-like Growth Factor Family; Insulin-like Growth Factor-1 (IGF-1) and Insulin-like Growth Factor-2 (IGF-2). IGF-1 and IGF-2 act as growth factors and promote insulin like metabolic activities in a wide range of cell and tissues. Actions of IGF-1 and IGF-2 are mediated through the receptor tyrosine kinase Insulin-like Growth Factor Receptor-1 (IGFR-1). A second IGF receptor exists (IGFR-2) but lacks the intracellular tyrosine kinase responsible for initiating the effects of the IGFR-1 receptor upon IGF binding (as reviewed in Folli et al., 1996; Feldman et al., 1997). The function of the IGFR-2 is unknown but is thought to act as a scavenger receptor to sequester IGF (Folli et al., 1996; Feldman et al., 1997).

Upon IGF binding to the  $\alpha$ -subunit of IGFR-1, activation of the tyrosine kinase located on the  $\beta$ -subunit occurs (Petruszelli et al., 1982). The intracellular domain contains an ATP binding site and seven potential autophosphorylation sites (Tonquist and Auruch

1988; White et al., 1988, Feener et al., 1993). Both IGF-1 and IGF-2 bind to IGFR-2 but to date the signalling pathways for this receptor have not been elucidated. The binding of IGF to both the IGFR-1 and IGFR-2 receptors are mediated by six Insulin-like Growth Factor Binding Proteins (IGFBPs 1-6; reviewed in Folli et al., 1996). IGFBPs are thought to play a role in mediating the biological activity of IGF (Folli et al 1996).

The IGFR-1 receptor activates the Insulin Receptor Substrate protein (IRS1 and IRS-2). The IRS proteins are particularly good substrates for IGFR-1 and contribute significantly to IGF signalling (Sun et al., 1993; Hubbard et al., 1994). The IRS proteins are cytoplasmic proteins with 20 potential tyrosine phosphorylation sites and 40 possible serine phosphorylation sites. The tyrosine phosphorylation of IRS proteins enables activation of the PI-3 Kinase complex, fyn tyrosine kinase Grb2, Nck and SHPTP2 (Backer et al., 1992; Folli et al., 1992; Kuhne et al., 1993; Sun et al., 1993). It has been reported that through PI3-kinase, IGF-1 can activate p70 S6 Kinase and GLUT4 mediated glucose transport (Cheatham et al., 1994; Chung et al., 1994; Myers et al., 1994).

In cerebellar granule neurons, IGF-1 activates PI3-kinase resulting in neuronal survival (D'Mello et al., 1997). Similar reports using PC12 cells suggests IGF produces survival effects through activation of both MAPK and PI3-kinase. Activation of both pathways is reported to have a synergistic effect on survival indicating that each molecule may use a different mechanism to produce the same outcome (Parrizas et al., 1997).

IGF has been reported to activate the ras pathway and consequently MAPK activity by two independent mechanisms. The first involves the direct activation of SHC by the NPXY (tyrosine 960) consensus sequence of the juxta membrane region of IGFR-1 (Wolf

et al., 1995). In addition to direct activation by the IGFR-1 receptor, activation of IRS leads to ras pathway activation by phosphorylation of Grb2 (Lowenstein et al., 1992). Novel activation of IRS-1 and IRS-2 by BDNF through TrkB in cortical neurons has been reported in the literature (Yamada et al., 1997). However the study did not show direct activation of IRS by TrkB.

Both IGF-1 and IGF-2 are expressed in the DRG and are upregulated during peripheral nerve injury (Glazner et al., 1994). IGF-1 has also been shown to increase the rate of sciatic nerve regeneration in vivo and enhance neurite outgrowth from adult sensory neurons (Glazner et al., 1993; see chapter 4). In culture IGF-1 is capable of maintaining survival of embryonic DRG neurons in the absence of NGF (Russel et al., 1998). This effect was reversed with the PI3-kinase inhibitor LY294002 indicating a primary role for this signalling pathway in IGF mediated survival of sensory neurons (Russell et al., 1998). In adult cultures IGF-1 promotes regeneration of DRG neurons (FERNYHOUGH et al., 1993; Akahori and Horie 1997). These growth effects of IGF-1 correspond to the expression of IGFR-1 in culture (see chapter 4).

### **1.3.4 Summary**

EGF and the IGF signal through receptor tyrosine kinases. Both RTKs activate a number of common pathways including ras/MAPK and PI-3 kinase. Pathways activated by EGF and IGF share similarities with that of the signalling cascades of neurotrophins. The questions of why growth factors that activate similar substrates have different effects in the same cell type remains an area of debate.

#### 1.4 Compartmented cultures (see Figure 1.5)

The development of the compartment culture has given the experimenter an extremely adaptable culture method to study numerous questions relating to regulation of neuronal function, growth and survival. The basic premise of the compartment culture is to isolate different regions of the neuron in separate compartments. The simplest example of this method are cultures where sympathetic/sensory neuronal cell bodies and proximal axons are housed in a separate compartment from their corresponding distal processes and terminals. This culture method has been exploited to increase the knowledge of how growth factors produce localized growth of axonal terminals, retrograde transport of NGF and localized effects of pharmacological agents and growth factors (Campenot 1994).

In mass cultures it is obvious that culture conditions, drug treatments and the inclusion of growth factors are equivalent along the entire structure of an individual neuron. Given the fact that some neurons extend axons through the body measuring up to a metre or more it is likely that global conditions seen in culture are not the case *in vivo*. Different regions of a neuron are obviously acted upon by different physiological factors. Most notably, this is occurring when neurons are acted on by target derived growth factors/neurotrophins at distal portions of axons/terminals (Korsching 1993).

The basic premise of the neurotrophic theory is that neurons during development innervating their intended targets are maintained by trophic influences exerted by the target tissue. Those neurons that miss their targets or innervate inappropriate targets do not receive the same trophic support and eventually die. NGF is a prototypical trophic factor that is required by sympathetic neurons to survive and is supplied by targets including the

salivary glands and iris *in vivo* (Levi-Montalcini and Booker 1960; Johnson et al., 1972). In such situations, exposure to NGF is primarily localised to the distal portions of growing axons. The same localized action occurs in adult DRG neurons where nerve terminals undergo sprouting in the skin in response to NGF (Diamond et al., 1976 and 1991a,b).

The compartment culture system allows the experimenter to plate neurons in one compartment and stimulate neurite growth into an adjacent compartment. This results in the cell bodies (including more proximal portions of neurites) of the neurons and their distal neurites (including the growth cones/terminals) being housed in two separate compartments (Campenot 1977, 1992). Isolation is the main advantage of the compartment culture and allows for the manipulation of one region of a neuron without applying the same treatment to other areas of the cell. It is the isolation of distal neurites which more closely resembles the environment encountered by nerve terminals *in vivo*. In this way compartmented cultures have the advantage over mass cultures. In this thesis it was our objective to use these advantages to study neurite growth in adult sensory neurons.

#### **1.4.1 Localized effects of neurotrophins on neurons in compartmented cultures**

It was originally discovered that NGF was necessary in distal compartments in order to elicit the growth of distal neurites into that compartment (Campenot 1977). NGF is required for the neuronal survival of sympathetic neurons (Levi-Montalcini and Booker 1960). It is interesting that NGF supplied to distal neurites is sufficient to maintain survival when the neurotrophin is removed from compartments containing cell bodies (Campenot

1982b).

The removal of NGF from distal compartments results in retraction of distal neurites back to compartments containing cell bodies. This effect is not reversed by application of NGF to cell bodies (Campenot 1982a). In adult DRG neurons that are not reliant on NGF for survival (Lindsay 1988) retraction of distal neurites was not observed but growth was halted (see chapter 2). The differences in local control of distal neurites by NGF in each case is likely a result of the different survival effects of the neurotrophin in each cell type (chapter 2; see discussion).

#### **1.4.2 Contributions of cell bodies/proximal axons versus distal neurite/terminals to the growth process**

A major finding of studies using the compartment culture to study neurite growth in sympathetic neurons is that such growth appears to be a local effect, heavily regulated within the distal neurite itself. Cell body effects seem to be important in more long term processes such as upregulation of growth genes such as  $T\alpha 1$   $\alpha$ -tubulin (Mathew and Miller 1990) but do not have an important role in more immediate growth responses. In fact local activation of TrkA receptors by NGF (and consequent signal transduction) is responsible for distal neurite growth and retrograde transport, which is important for other mechanisms such as survival, is not necessary in this regard (for review see Campenot 1994).

The essential components for neurite growth have been shown to be produced locally in distal neurites. All membrane lipids with the exception of cholesterol are produced locally in distal neurites and the rate of synthesis within the cell body does not

have a major influence on growth (Vance et al., 1991). In addition tubulin transport from the cell body is not a limiting factor for neurite growth and local turnover of  $\beta$ -tubulin within distal neurites does occur (Campenot et al., 1996). To add to the hypothesis of local control of growth within the neurite, structural proteins such as tubulin and actin are produced at neurite terminals (Eng et al., 1999). Although production of tubulin and actin only represent 1% of the total synthesis within the neuron, this process may play a physiological role in neurite growth (Eng et al., 1999). Given the above finding it appears that more rapid growth responses are controlled locally within distal neurites.

Factors affecting neurite growth have also been found to be important only when localized to distal regions of the neuron (distal neurites/terminals). For example, Protein Kinase C (PKC) is a necessary component of neurite growth but inhibitors of the enzyme are only effective when added to compartments containing distal neurites. The application of the PKC inhibitors staurosporine, sphingosine, calphostin C and chelerythrine only inhibited growth when applied to distal neurites but had no effect at cell bodies (Campenot et al., 1991,1994). The same localized effects are seen with cell permeable analogues of ceramide (Posse de Chaves et al., 1997). This evidence is compelling support for the idea of local control of nerve terminal growth but specific inhibitors for specific targets of TrkA signal transduction have yet to be studied on distal neurites (see above). Data pertaining to neurite regulation by ceramide is still in question given data that ceramide inhibits phosphorylation of Trk (MacPhee and Barker 1997). It remains to be seen if ceramide produces its effects through a specific pathway (likely those involved in programmed cell death), through a direct inhibition/alteration of the Trk receptor or both (but see also



MacPhee and Barker 1999).

### **1.4.3 The contributions of retrograde transport of neurotrophins, p75 and Trk to the processes involved in neurotrophin signalling**

#### **1.4.3.1 Background**

The process of retrograde transport of neurotrophins includes the binding of the molecules to receptors expressed on the cell surface, internalization of the receptor-ligand complex and transport to the cell body (Ure and Campenot 1997). It has been shown that NGF is transported in a retrograde manner sympathetic neurons (Henry et al., 1974a,b; Stockel et al., 1974, Johnson et al., 1978). NGF transported back to cell bodies remains relatively intact until it is degraded at the cell body (Henry et al., 1974a; Stockel et al., 1974, Johnson et al., 1978; Grimes et al., 1996). Intact NGF appears to be transported as a complex with its tyrosine kinase receptor (Ehlers et al., 1995). Observations of the lack of effect when NGF alone is injected into responsive cell types suggests that such a complex is necessary for signalling during retrograde transport (Rohrer et al., 1982; Seeley et al., 1983).

In addition to NGF, the neurotrophins BDNF and NT-3 and NT-4 undergo retrograde transport in both the peripheral and central nervous systems (Distefano et al., 1992; Sobreviela et al., 1996). It is interesting that normal p75 functioning is necessary for the transport of BDNF and NT-3 but does not significantly affect NGF. The disruption of p75 function by antibodies blocking neurotrophin binding has its greatest effects on BDNF and to a slightly less degree on NT-3 with no real effect on NGF transport (von Bartheld

et al., 1996b; Curtis et al., 1995). While all three neurotrophins have similar affinities for p75 the order of effect correlates very well with the rate constants for each molecule. The rate constant (a measure of the time a ligand remains bound to the receptor) is slowest for BDNF ( $5.8 \times 10^{-4}/s$ ; i.e. bound to p75 for the longest period of time) slightly faster for NT-3 ( $1.4 \times 10^{-2}/s$ ) and fastest for NGF (too fast to be measured accurately). Such differences in rate constants have been postulated as a method for the discrimination between different binding sites for neurotrophins (Rodriguez-Tebar et al., 1990 and 1992). The above receptor binding data could explain the necessity of normal p75 expression for maximum transport of BDNF and NT-3. Longer periods of p75 binding are necessary to stabilize p75-TrkB or p75-TrkC complexes on the cell surface. Consequently, these complexes undergo retrograde transport. The second possibility is that the increased time of binding of NT-3 and BDNF to p75 results in greater amounts of the neurotrophin being transported along with the receptor. This speculation is complicated by other studies suggesting a very slow rate of dissociation of NGF from its receptor (Mahadeo et al., 1994). The reasons for such discrepancies may be dependent upon procedural differences, culture differences or the cell type used.

#### **1.4.3.2 Retrograde transport of NGF in compartmented cultures**

The contributions that retrograde transport of neurotrophins make toward the physiological effects of these molecules is still a matter of some controversy. Studies by Ure and Campenot (1997) have shown that NGF supplied to distal neurites in compartment cultures is not transported until one hour after application. To add to this, the same study

also shows that only 2-25% of the NGF associated with distal axons was transported each hour. The large majority of NGF therefore appears to be associated with TrkA and p75 receptors on the surface of distal neurites (Ure and Campenot 1997). Given the now well characterized Trk signalling pathways that are activated by the initial NGF-TrkA interaction at the cell surface, the role of retrograde transport of NGF is becoming less certain.

Increased evidence for a lesser contribution of retrograde transport in NGF signalling than once theorized is derived from a number of important findings. The first is that there are very specific and well characterized pathways, which upon binding of NGF to its receptor, can produce a large number of its biological effects (Kaplan and Stephens 1994). The second is that second messenger signalling initiated through TrkA can reach the cell body up to one minute after activation of the receptor (Senger and Campenot 1997). A rapid mechanism for communication between cell surface receptors and the nucleus brings into question the function of retrograde transport of NGF-Trk complexes. Why would retrograde transport (of NGF-Trk complexes) be necessary for transport of signals to the nucleus to regulate gene transcription if other rapid means of nuclear signalling exist. Third, many of the biological responses such as neurite growth seen in compartment cultures are regulated at the neurite terminals not at cell bodies (see chapter 2). The evidence appears to exist for a greater role of signal transduction rather than retrograde transport of NGF-Trk complexes in such processes as neurite growth. Therefore this thesis focussed specifically on Trk signalling pathways and their contributions to neurite growth.

#### **1.4.4 Summary**

The majority of research in the past few years has focussed more towards signal transduction rather than receptor transport. There is substantial evidence to argue that most of the biological effects of Trk result from receptor signalling. In contrast, recent evidence has emerged that argues strongly that Trk receptor uptake and transport to the nucleus is necessary for pCREB activation (Riccio et al., 1997). The pCREB protein is an activator of gene transcription and is an important mediator of neurotrophin induced gene expression and cell survival (Finkbeiner et al., 1997; Riccio et al., 1997). Retrograde transport of neurotrophins/Trk receptors may therefore play a more prominent part in long term changes associated with these factors such as changes in gene expression (Campanot 1994). More studies employing the use of the compartment culture method, should provide a clearer picture as to what the actual contribution of retrograde transport is to the entire NGF-TrkA signalling process.

#### **1.5 Overall objectives and hypothesis**

The overall objective of this work was to examine neurotrophin regulation of neurite growth in adult DRG neurons. The first step taken toward this goal was to establish an *in vitro* model where NGF and other neurotrophins could be applied to adult sensory neurons in a manner analogous to that in animal models. To accomplish this objective, the compartmented culture system was modified to be used with adult sensory neurons. It was our aim to characterize this system to determine which neurotrophins could produce a working model of neurite growth. To do this, each neurotrophin was supplied to various

compartments housing either cell bodies or distal neurites to test what combinations could produce neurite outgrowth into distal compartments (see chapter 2). The author's main hypothesis are as follows:

1. DRG neurons express all three types of Trk receptors and therefore NGF, BDNF and NT-3 should be able to elicit growth of distal neurites in compartment cultures. Neurite growth should occur in a dose dependent form.
2. Neurites growing in side/distal compartments after axotomy (*in vitro*) will require the presence of the same neurotrophin (initially used to produce elongation).
3. Neurites growing into distal compartments should express the Trk receptor specific to the neurotrophin producing the neurite growth.

Once a working compartmented culture model was developed the next objective was to ascertain the individual contribution of the neurotrophin receptors to the growth response of distal neurites (results are provided in chapter 3). The receptors of interest were p75 and TrkA (see chapter 2 for specific results). To address this question the drug K-252a was used to block TrkA phosphorylation. K-252a is a selective inhibitor of Trk phosphorylation and blocks activation of intracellular target substrates for Trk receptors. MC192 was used to disrupt p75-NGF binding and BDNF to compete with NGF to bind p75 to ascertain the receptors functioning in the growth process. The author's main hypothesis are as follows:

1. The activation of TrkA via NGF would be necessary for neurite growth. Consequently the use of K-252a should completely inhibit growth.
2. Alteration of optimal p75-Trk interaction would result in sub-optimal growth of distal neurites when compared to controls. Competitive inhibition of NGF binding to p75 using BDNF or the use of MC192 to disrupt NGF binding to p75 should result in measurable decreases in neurite growth.
3. Activation of p75 dependent pathways using either BDNF or MC192 if present should result in a greater decrease in neurite growth compared to experimental conditions that only alter p75-TrkA interaction with NGF. Therefore activation of p75 dependent pathways are expected to be detrimental to neurite growth.

To assess the contributions of Trk signalling pathways towards neurite growth two methods were utilized. The first method was to look at components of the Trk signalling cascade (PLC $\gamma$ , MAPK, PI3-kinase and SNT) which are also activated by other trophic factors (EGF and IGF-1). We hypothesised that other growth factors that produce neurite growth in adult DRG neurons may do so through the same pathways as activated by NGF. Therefore a correlation between neurite growth elicited by a number of growth factors and their effects on PLC $\gamma$ , MAPK, PI3-kinase and SNT/FRS2 might be drawn. The second method used was to employ the use of specific inhibitors of these pathways to determine their effects on growth (PD 98059: MEK/MAPK; LY 294002: PI3-kinase). The inhibitors

were assessed as to their ability to block neurite outgrowth initiated by NGF, EGF and IGF. The results are presented in chapter 4. The author's specific hypothesis are as follows:

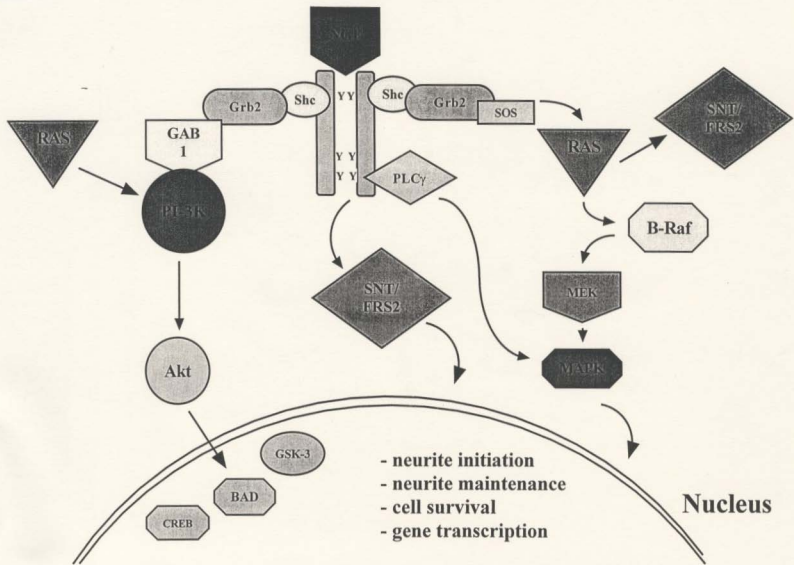
1. Growth factors (NGF, IGF-1, EGF and FGF) that produce neurite growth should activate similar intracellular pathways. A correlation between neurite growth and specific signalling pathways should be able to be made between growth factors.
2. The use of inhibitors to specific signalling intermediates should result in inhibition of neurite growth if these pathways are involved.
3. A correlation between data from hypothesis 1 and 2 (immediately above) should reveal common specific signalling pathways that initiate or maintain neurite growth. Therefore data from experiments using growth factors should correlate with data using chemical inhibitors.

Results and discussion for each set of hypothesis' listed above correspond to chapters 2,3, and 4 respectively. The overall objective of the thesis is three fold: 1. To determine which neurotrophins produce distal neurite growth in compartmented cultures. 2. To examine the contribution of the individual neurotrophin receptors (Trk and p75) to this growth process. 3. To further determine which neurotrophin receptor signalling processes contribute to neurite growth.

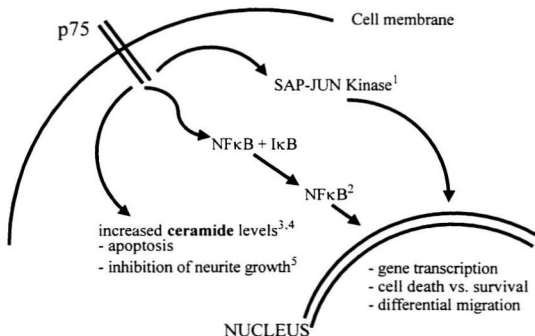
It is anticipated that these studies will contribute to the understanding of the

mechanisms by which NGF and its receptors regulate neurite growth in adult sensory neurons. These studies may also provide a basis for *in vivo* experiments which are not as easily done as in culture.

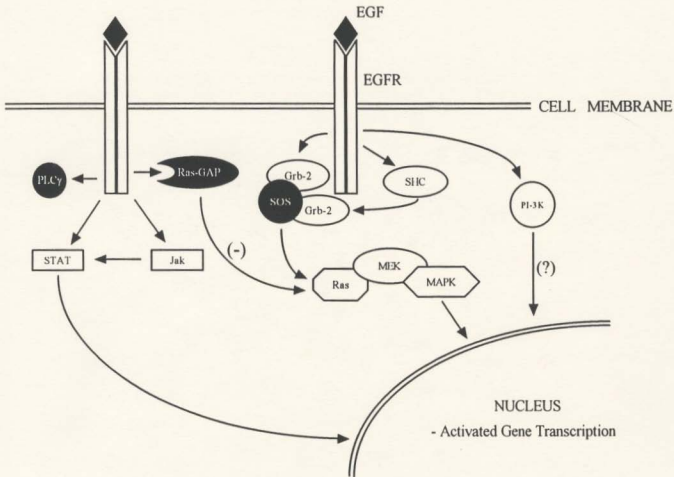




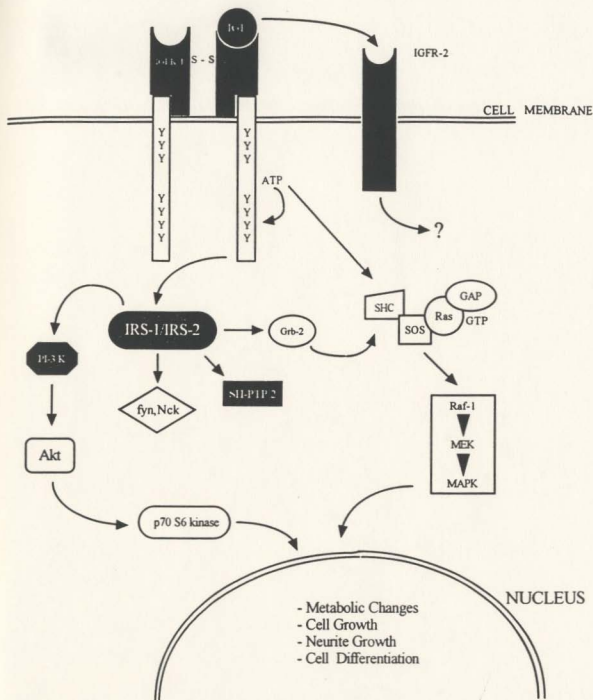
**Figure 1.1** Proposed signal transduction pathways for TrkA



**Figure 1.2** Suggested signal transduction pathways activated by p75.  
 (1. Cassacia-Bonnefil et al., 1996; 2. Carter et al., 1996b; 3. Dobrowsky et al., 1994; 4. Dobrowsky et al., 1995; 5. Posse de Chaves et al., 1997).

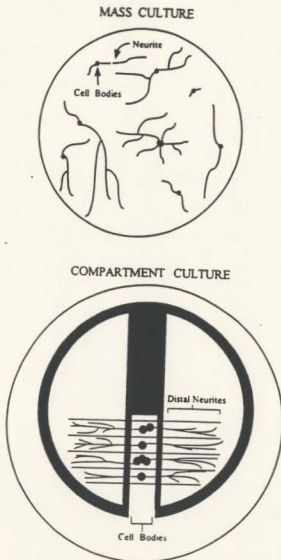


**Figure 1.3** The proposed current view of EGF Receptor signalling in neuronal cells.  
Adapted from Yamada et al. Progress in Neurobiology, 51:19-37, 1997



**Figure 1.4** Proposed model of IGF Receptor intracellular signalling.  
Adapted from Folli et al. *Molecular Neurobiology* 13(2):155-183. 1996

**Figure 1.5** Diagram of the two culture methods used in this study. Top: An illustration of a conventional mass culture. In the mass culture, neurites and cell bodies are contained in the same compartment/media. Bottom: An illustration of a compartment culture. The cell bodies are housed in the center compartment. The distal neurites are housed in distal/side compartments. The cell bodies and neurites are divided by a Teflon barrier (in black). Different media and growth factors can be supplied to these separate compartments.



## **2.0 CHAPTER II: Effects of the neurotrophins Nerve Growth Factor, Neurotrophin-3, and Brain Derived Neurotrophic Factor on neurite growth from adult sensory neurons in compartment cultures.**

### **2.1 Introduction**

Nerve growth factor (NGF), BDNF, and neurotrophin-3 (NT3) are members of the neurotrophin (NT) family of molecules. These factors have been shown to influence the differentiation, development, and survival of a wide variety of neurons in both the central nervous system (CNS) and the peripheral nervous system (PNS). Many studies have provided information pointing to the importance of the NTs in the differentiation, early development and early postnatal survival of various classes of sensory neurons (reviewed in Davies, 1996; Kalchauer, 1996; Ockel et al., 1996). While postnatal and adult sensory neurons do not require NTs for their continued survival, the continued maintenance of particular mature phenotypic characteristics (including peptide content and physiologic responsiveness) seems to at least be responsive to, if not dependent upon, NTs such as NGF and NT3 (Mulder, 1994; Wright and Snider, 1996; reviewed in Lindsay, 1992).

Neurotrophins also play a major role in the regulation of neuronal growth, and particularly in growth or regeneration as a response to nerve injury. In the mature PNS, many types of neurons possess the capability both to sprout new axon collaterals when presented with the appropriate stimulus and to regenerate axons that have been severed. In adult dorsal root ganglion (DRG) neurons, NGF is required for collateral sprouting of intact neurons, yet has no observable effects on the regeneration of those neurons (Diamond et al., 1992a,b; Mearow et al., 1994; Mearow and Kril, 1995). Although these studies have provided evidence concerning the role of NGF in the regulation of axonal

growth, the *in vivo* system cannot be easily manipulated to investigate the underlying mechanisms involved in collateral sprouting as compared to regeneration. The compartment culture model (Campenot, 1997, 1992) provides an attractive system with which to further investigate the role of NTs in regulating adult neuronal outgrowth. In this model, the neuronal cell bodies and the proximal portions or their neurites remain in a center compartment, while the distal portions of the axons extend into side compartments that are effectively isolated from the center chamber. In this way, local effects of NTs or other agents on either the distal neurite terminals or the cell bodies can be examined. This, unlike usual culture systems, is perhaps a closer approximation of the *in vivo* situation, especially in the case of the DRG neurons whose cell bodies and terminals are separated by substantial distances and are thus not likely exposed to similar environments.

Studies on the influence of NTs *in vitro* have generally used either embryonic or neonatal tissues; while these neurons tend to be easier to establish and maintain in culture, one complicating factor is that neuronal survival is often dependent on the presence of a particular NT. In this regard, the use of adult DRG neurons presents a useful model for differentiating between the survival and growth-influencing effects of the NTs, since these neurons survive independently of added NTs (Lindsay, 1988; but see Acheson et al., 1994).

In the present study, our objectives were to evaluate the feasibility of using adult DRG neurons in the compartment culture system and to examine the influence of NGF, NT3, and BDNF on the growth of neurites from both neonatal and adult neurons in such cultures. The results of these experiments have indicated that adult DRG neurons adapt

readily to the compartment system and provide a useful model with which to examine both local and retrograde effects of NTs. In these cultures (in which the neuronal cell bodies were plated in the absence of NTs), NGF alone was able to elicit distal neurite elongation from the DRG neurons. Once neurites had extended into the distal compartments, further extension could be stopped by anti-NGF treatment. Furthermore, following axotomy at a time when the neuritic growth had stabilized, NGF was also required for neurite regeneration. Neither BDNF nor NT3 alone was able to elicit growth of neurites out of the central compartments. The combination of BDNF plus NGF resulted in inhibition of neuritic extension, while the combination of NT3 plus NGF had little effect over and above that of NGF alone. The results are discussed with respect to the role of the NTs and the similarities and differences between the growth processes exhibited by sensory neurons *in vivo* and *in vitro*.

## **2.2 Materials and Methods**

### **2.2.1 Culture procedures**

Dorsal root ganglia were dissected from postnatal day 1 or adult (100-200g) Sprague-Dawley rats (supplied by Memorial University Vivarium). Ganglia were removed from all spinal levels and the roots were trimmed and then chemically dissociated by separate incubations in 0.25% collagenase (GIBCO-BRL, Burlington, Ontario, Canada), 0.25% trypsin (GIBCO-BRL), and finally, 0.52 mg/mL soybean trypsin inhibitor (SBTI), 0.04 mg/mL DNase, and 3 mg/mL bovine serum albumin (BSA) (Sigma, St. Louis, MO). Adult ganglia were treated with collagenase for 1.5 h at 37°C,



followed by trypsin for 30 min, and finally, with the SBTI-DNAse for 5 min. Neonatal ganglia were dissociated with 30 min collagenase, 15 min trypsin, and SBTI-DNase 5 min. The ganglia were then mechanically dissociated by trituration with a flame-narrowed Pasteur pipette in L15 medium with 2% fetal calf serum (FCS) (GIBCO-BRL). The resulting cell suspension in L15 medium supplemented with 2% FCS, 100 U/mL penicillin/streptomycin (GIBCO-BRL), and 3 g/100mL glucose (Sigma) was centrifuged (1000 rpm) through a cushion of 15% BSA (Fraction V; Sigma) in L15 medium; this procedure eliminates much of the cellular debris and results in a neuronally enriched pellet. The resulting cellular material was suspended in complete L15-air medium supplemented with 1.5% methylcellulose and neurotrophins when required.

Compartment cultures were constructed as described by Campenot (1992), with a modification in the culture substrate: 35-mm culture dishes were coated sequentially with polyornithine (10 ug/mL; Sigma) for one hour and laminin (5 ug/mL; Sigma) for one hour. Excess substrate was removed with a quick rinse in sterile water. Parallel tracks in the substrate were made by scratching the culture dish surface with a pin-rake (Tyler Research Instruments, Edmonton, Alberta, Canada). Subsequently, a three-chambered Teflon divider (Tyler Research Instruments) was then seated on top of the tracks as described elsewhere (Campenot, 1992).

Dissociated neurons were plated into the center compartments and maintained in L15-air medium containing 2% FCS, penicillin/streptomycin plus 1.5% methylcellulose, and 20  $\mu$ m cytosine arabinoside (Sigma). For neonatal cultures, 2.5S NGF (Cedarlane Labs, Hornby, Ontario, Canada) was added to a final concentration of

10 ng/mL. Adult neurons did not require NGF in the center compartments for survival. Cultures had neurites emerging into the side compartments after 3 days, and after 5 days neurites were well established; at this point, NGF was removed from the center compartments. Compartments containing only neurites received varying concentrations of neurotrophins (NTs), ranging from 0 to 100 ng/mL of either NGF, BDNF, or NT3 (Cedarlane Labs; Amgen, Thousand Oaks, CA). In some situations anti-NGF immunoglobulin Gs (IgGs) (Cedarlane Labs, Hornby, Ontario, Canada) were added at a concentration of 100-200 ng/mL to neutralize NGF. Medium was changed every 3-5 days and cultures were maintained in an air atmosphere at 37°C.

The anti-NGF antibody has been previously characterized (Coughlin and Collins, 1985; Diamond et al., 1992a,b; van der Zee et al., 1995). It is cross-reactive with NGF and NT3 on enzyme-linked immunosorbent assays (J. Diamond, personal communication) and appears to inhibit neurite production by neonatal mouse DRG neurons in response to both NGF and NT3 (van der Zee et al., 1995). However, it should be noted that the concentrations of the anti-NGF IgG preparation employed by van der Zee et al., (1995) are two orders of magnitude higher than those we used in our experiments (10 µg/mL vs. 100-200 ng/mL), and higher than those required to completely block NGF's activity. This anti-NGF antibody has been previously shown to neutralize the activity of purified mouse NGF (200 ng/mL) half-maximally at 100 ng/mL and completely at 500 ng/mL (Coughlin and Collins, 1985).

### **2.2.2 Treatments**

Experimental manipulations included either the cell bodies or the neurites being exposed to differing concentrations of NGF, BDNF, or NT3 (0-100 ng/mL of complete medium). Axotomies were performed on day-7 cultures after measurements of neurite extension had been recorded. A jet of sterile water was projected at the most proximal portions of neurites growing into the side compartments using a syringe and a 23-gauge needle; this procedure was repeated four times in total and resulted in compartments totally devoid of neurites.

### **2.2.3 Measurements**

Cultures were examined using the x16 objective of a Zeiss inverted microscope equipped with an ocular micrometer. Growth measurements were taken as neurite extension from the proximal edge of the grease barrier of the side compartment to the most distal neurite processes in a given track. Of the total 20 tracks in a side compartment, only 14 were measured because the cultures consistently showed little or no neurite growth in the top or bottom three lanes. Data from the 14 lanes of each culture were pooled with equivalent cultures at the same time point. The total number of cultures examined at each time point ranged from 10 to 15. Data were analyzed using two-way analysis of variance with MINITAB statistical software. Error bars in all figures indicate standard errors of the mean (SEM); where error bars are not seen, they do not exceed the width of the symbol. In all cases, significance was considered to be  $p < 0.05$  and is indicated with an asterisk. Neuritic density measurements were

performed as described below.

#### **2.2.4 Immunocytochemistry**

The medium was carefully aspirated from the cultures, which were then washed with phosphate-buffered saline (PBS) and fixed for 10 min in 4% formaldehyde in PBS. After washing in PBS, the cultures were incubated in a blocking solution (10% normal goat serum, 0.1% Triton X in PBS) for 1 h. Subsequently the cultures were exposed to the primary antibodies for overnight incubation at 4°C.

The primary antibodies employed are as follows: for p75, the monoclonal MC192 (Oncogene Sciences, Cambridge, MA; 1:100 dilution); TrkA, polyclonal SC-118, non-reactive with TrkB or TrkC (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution); TrkB, polyclonal SC-12, non-reactive with TrkA or TrkC, or truncated TrkB (Santa Cruz Biotechnology; 1-500 dilution); TrkC, polyclonal SC-117, non-reactive with TrkA or TrkB (Santa Cruz Biotechnology; 1-500 dilution). The cultures were washed and exposed to the biotinylated-secondary antibody for 1 h at room temperature; visualization of staining was completed using a Vectastain Kit (Vector Labs, Burlingame, CA) and diaminobenzidine (DAB). Alternatively, fluorescein isothiocyanate (FITC)-tagged secondary antibodies were used. Cultures were then cover slipped under glycerol, examined, and photographed either with a Leitz photomicroscopic system or a Hund Wilovert-S inverted microscope. Using a computer-based imaging system (Northern Exposure; EMPIX Imaging, Mississauga, Ontario, Canada), density measurements were made on cultures where neurites had extended into NGF, subsequent to

immunocytochemical staining for p75 and visualization using horseradish peroxidase-avidin and DAB.

## **2.3 Results**

### **2.3.1 Culture characteristics**

In these experiments, we examined the influence of the presence or absence of various neurotrophins on the survival and neurite growth of both neonatal and adult DRG neurons. Dissociated neurons were always plated in the center compartments of the three-chambered Teflon dividers, essentially as previously described (Campenot, 1992); in our cultures, the medium in the center or side compartments was identical except for the addition of NT(s). The measurements of neurite extension were taken from the point at which the neurites emerged from under the grease barrier to the most distal point in the lanes being examined; it should be noted that at this point the neurites had already extended in excess of 0.5 mm, since the width of the barrier itself was 0.5 mm.

Within 24 h of plating, neuritic growth was observed in the center compartments. However, there was a lag phase before neurites were observed extending into the side compartments. Generally, in most of the cultures examined, it was possible to view neuritic processes just about to emerge into the distal compartments, and in certain experimental conditions (see below) where elongation into the distal compartments did not occur, one could still observe neurites at the edge of the compartment barrier. This suggests that under the differing conditions where elongation did not proceed, the

neurons or neurites still had an equivalent opportunity to extend into the distal compartments given the appropriate stimulus.

We also observed that in the case of the adult neurons, none of the NTs were required for survival, as expected. With respect to the neonatal neurons, NGF was required for their survival; while we did not carry out extensive analyses, BDNF and NT3 did not appear to have significant effects on the neonatal survival and could not replace NGF in this regard.

### **2.3.2 Influence of NGF on DRG neurite growth in compartment cultures**

In initial experiments, neurons were plated in the center compartment in the presence of 10 ng/mL NGF. In these cultures, there was no neurite extension of adult or neonatal neurons into side compartments that contained equivalent (10 ng/mL) or less NGF. However, if the distal compartments contained 100 ng/mL NGF, then there was substantial extension of the neonatal neurites but only minimal growth of the adult neurites (Fig. 2.1 A-B, see below). Subsequently, all cultures were plated in the absence of NGF in the center compartments. In such cultures, there was extensive growth of both the neonatal and adult neurites into distal NGF-containing chambers (Fig. 2.1 C-D). While the adult neurons did extend neurites in the absence of NGF in the center compartment, there was never any significant elongation into distal compartments that did not contain NGF.

The total extensions that both the neonatal and adult neurites reached at the end of the experiments (9 days *in vitro*) were similar for all concentrations of NGF. The rate

of neurite extension over the 9-day period ranged from 106-136  $\mu\text{m}/\text{day}$  for the adults and 105-157  $\mu\text{m}/\text{day}$  for the neonatal neurons. A significant dose-dependent effect of the NGF was observed on the density of the neurites, with greatly increased neuritic density in the cultures containing the higher concentrations of NGF (Fig. 2.2; see Table 2.1). Thus, while the overall distance that the neurites grew was not that different between conditions, the effect of increasing NGF concentrations was either to increase the branching of the neurites resulting in an increased density or to increase the number of neurons that extended neurites into the distal compartments. Back labeling experiments using fluorescent markers did not provide consistent results with respect to this issue; that is, there was no clear evidence of differences in the number of neurons labeled with the differing concentrations of NGF.

A significant observation was that while the adult neurons survived in the absence of NGF, the neonatal neurons were able to survive plating in the absence of NGF only if there was a distal compartment with NGF available. When the neonatal neurons were plated in the absence of NGF and not provided with an NGF-containing distal compartment, by day 5 of culture, the percentage of surviving neurons was <20% (data not shown). The neonatal cultures were employed as a way of assessing optimal conditions for the adult neurons. Because of the low survival in the absence of NGF and our primary interest in adult neuronal growth, subsequent experiments focused on adult cultures.

### **2.3.3 Influence of BDNF and NT3 on DRG neurite growth in compartment cultures**

The influence of BDNF and NT3 on eliciting neurite growth was also investigated. Cultures were prepared as above, plating the neurons in the absence of any NT in the center compartment. The distal compartments contained 0, 1, 10, or 100 ng/mL of BDNF, or 1, 10, or 100 ng/mL of NT3. With respect to the adult cultures, neither BDNF nor NT3 in any of the concentrations used in these experiments was able to support neurite growth into the distal compartments (Figs. 2.3 and 2.4).

To ascertain whether these cultures were healthy and capable of extending processes into the side compartments, the following manipulation was carried out. At day 7 of culture, the medium in the distal compartments (originally containing BDNF or NT3) was replaced with medium containing 25 ng/mL NGF. Following a 1-day lag, all the cultures displayed relatively rapid neurite growth into the NGF-containing compartments (Fig. 2.3 A-B), indicating that there was nothing intrinsically impaired in these cultures. It is also important to note that in these cultures, in the absence of any NT in the side compartment for 7 days (i.e., the 0 ng/mL BDNF and NT3 conditions), NGF was still able to elicit neurite elongation at essentially the same rate of growth (250-300  $\mu\text{m}/\text{day}$ ).

To rule out the possibility that lack of NTs was deleterious to any specific neuronal population, cultures were plated with various combinations of NTs in the center and distal compartments. Thus, neurons were plated with 1 ng/mL BDNF in the center compartment and provided with distal compartments containing 100 ng/mL BDNF or NGF, or 10 ng/mL NT3; 1 ng/mL NT3 in the center and 100 ng/mL BDNF or



NGF, or 10 ng/mL NT3 in the sides, and finally, 1 ng/mL NGF in the center and 100 ng/mL BDNF or NGF, or 10 ng/mL NT3 in the sides. The results of this experiment confirmed the previous observations that neuronal growth occurred only into distal compartments containing NGF; there was no significant outgrowth into any of the compartments containing NT3 or BDNF or no NT (Table 2.2).

#### **2.3.4 BDNF Plus NGF in distal compartments results in an inhibition of extension**

To assess the possibility that the combination of BDNF plus NGF in the distal compartments might have an additive influence on neurite extension, cultures were set up with 100 ng/mL BDNF with either 10 ng/mL NGF or 100 ng/mL NGF. As suggested by the studies of Barker and Shooter (1994), our expectation was that BDNF would interfere with the action of NGF, likely by competing for p75 low-affinity receptor-binding sites. The data from these experiments are presented in Figure 2.4 (A), where it is apparent that the effect of adding BDNF to NGF was to inhibit neurite extension. With higher concentrations of BDNF (1  $\mu$ m/mL; see chapter 3), the extension was almost completely inhibited. These results suggest the importance of the appropriate interactions of both p75 and TrkA in mediating the actions of NGF.

#### **2.3.5 NT3 plus NGF in distal compartments is not additive**

In the case of NT3, we were interested in determining whether the addition of NT3 to NGF would potentiate the neuritic growth response. Our initial experiments were carried out with concentrations of NT3 that should activate TrkC receptors

primarily, although at higher concentrations NT3 can also act via the TrkA receptor (Rodríguez-Tebar et al., 1992; Belliveau et al., 1997). We carried out further experiments using 100 ng/mL NT3 and found that even at this higher concentration, there was no distal neurite extension (Fig. 2.4 B). To investigate the possibility that NT3 might potentiate the effects of NGF, neurons were provided with compartments containing 100 ng/mL NT3 plus either 10 ng/mL or 100 ng/mL NGF, and the amount of the distal extension was quantitated. The results are presented in Figure 2.4(B), where it is apparent that NT3 does not act to increase the distal neurite extension; there were no obvious increases in neuritic density in these cultures, either. Thus, while NT3 can activate TrkA, it also competes for p75, with the end result conceivably being that the possible positive influences of NT3 are offset by an effective decrease in the ratio of p75:TrkA available for NGF.

### **2.3.6 Neurons plated in the presence of anti-NGF exhibit normal neurite extension into NGF-containing distal compartments**

Our observations indicated that there was significant neuritic growth in the center compartments in the absence of added NGF. To examine whether there might be some endogenous NGF being produced by the small number of non-neuronal cells that were often found in the cultures, the effect of plating neurons in the presence of anti-NGF was assessed.

Neurons were exposed to 200 ng/mL anti-NGF IgGs at the time of plating and for the course of the experiment. The presence of anti-NGF in the central compartments

had little effect on initial proximal neurite growth. There was no obvious difference in the amount of initial proximal neurite outgrowth, although we could not quantitate this initial outgrowth because the density of the plated neurons made it difficult to determine the identified neuronal source of given neuritic processes. Furthermore, the addition of anti-NGF to the central compartment did not significantly influence distal extension into NGF-containing side compartments, as shown in Figure 2.5 (A).

### **2.3.7 Influence of NGF removal in established cultures**

As the neurons required NGF to extend into the distal compartments, we then examined whether a continued supply of NGF was necessary for further extension. In these experiments, the adult cultures were allowed to grow neurites into the distal compartments containing the various concentrations of NGF. At 7 days *in vitro*, the medium containing NGF was removed and replaced with medium containing anti-NGF (200 ng/mL affinity-purified IgGs), and the cultures were assessed for neurite growth or regression over the next 5 days. The effect of the NGF removal (i.e., the anti-NGF) was to block any further neurite growth (Fig. 2.5 B). There was a small amount of neurite regression, primarily of very fine processes that had extended out from neurite bundles (Fig. 2.2 d). In contrast, the sister cultures which were maintained in NGF displayed continual outgrowth over the same period of time (Fig. 2.5 B), thus demonstrating the requirement of a continual source of NGF for neuritic growth.

### **2.3.8 Effects of NGF on neurite regeneration following axotomy in compartment cultures**

One of our interests in pursuing these experiments was to determine whether this *in vitro* system would provide an adequate model with which to investigate the differential role of NGF observed in the regulation of collateral sprouting and regeneration of adult sensory neurons *in vivo*. While neurite growth from dissociated cells in culture is necessarily a regenerative response initially, it was possible that once the neurites extended into the distal NGF-containing compartments, the growth would stabilize and could be used to approximate the *in vivo* model. One of the advantages of using the compartment cultures is that the distal growth can be axotomized in a relatively precise fashion, and the regrowth or regeneration of those neurites further examined. In these experiments, cultures of both neonatal and adult DRG neurons were plated in the center compartments in the absence of NGF and allowed to grow for 7 days into distal compartments containing NGF. On day 7, the neurites in the distal chambers were axotomized using a stream of distilled water, and the regrowth of the neurites was evaluated under differing conditions. Axotomized neurites rapidly regrew into compartments containing NGF (Fig. 2.6 A-B). However, in the presence of anti-NGF in the distal compartments, there was no regrowth of the axotomized neurons, thus indicating a requirement of NGF for this regeneration (Fig. 2.6 C). Neither BDNF nor NT3 (100 ng/mL) was able to support the regeneration of the axotomized neurons (Fig. 2.6 D). There was little difference between the neonatal and adult neurons in terms of the requirement of NGF for regeneration; however, the axotomized neonatal

neurons were able to regenerate processes more quickly than the adults, such that at the day-1 examination time point, neurites were already established in the distal compartments containing NGF (Fig. 2.6 A). These results indicating a requirement for NGF for the regeneration are different from the *in vivo* situation, where NGF has been shown to have little influence (Diamond et al., 1992b).

### **2.3.9 Immunocytochemical assessment of the compartment cultures**

Immunocytochemistry using antibodies directed against p75 and TrkA, TrkB and TrkC was performed to provide some assessment of the neuronal composition of the cultures. As expected, the staining for TrkA-IR and p75-IR was found in a majority (>50%) of the neurons in the cultures; fewer of the cultured neurons displayed TrkC-IR or TrkB-IR. Figure 2.7 presents representative photomicrographs of the respective immunoreactive neurons in the center compartments paired with the corresponding distal neurite compartments. The neurites in the distal compartments were positively stained for p75 and TrkA (Fig. 2.7); no staining was observed with either the TrkB or TrkC antibodies.

## **2.4 Discussion**

The aim of the present study was to investigate the regulation of neurite growth from adult sensory neurons by the NTs, NGF, BDNF, and NT3. Our findings demonstrate that NGF was the only NT able to elicit elongation of distal neurites in compartment cultures of both neonatal and adult neurons. There was no neuritic

elongation into compartments containing either BDNF or NT3. NGF produced maximal neurite growth when applied solely to the distal neurite compartment, and subsequent growth was halted when the NGF was effectively removed or neutralized using anti-NGF. In addition, neurite growth was affected by NGF concentrations with the highest concentration of NGF resulting in a much greater density of neuritic growth, which was most likely due to both increased numbers and increased branching of neurites. Our results show that NGF was the only NT of those tested which produced distal neurite growth in compartment cultures, and thus provide a model for further study of NGF-dependent neuronal responses.

The neonatal neuron cultures were initially employed as a way of assessing optimal conditions for the adult neurons, and also because of the differences in requirements of NGF for survival, it was conceivable that neonatal neurons might exhibit some differences compared to adult neurons. Findings of the present study indicated that neonatal neurons had the same characteristic growth responses as adult neurons in compartment cultures; growth rates, neurite elongation, and responses after axotomy of the neonatal neurons were comparable to those of the adult DRG neurons. The major difference between these cultures was the fact that to survive *in vitro*, the neonatal neurons required access to NGF. NGF could be removed from the cell body compartment and the neurons would survive only if the distal neurite compartment was provided with NGF. Because of our primary interest in the response of the adult PNS to injury and NTs, we subsequently focused primarily on the adult DRG cultures.

### 2.4.1 Control of axon growth by NGF

Adult DRG neurons do not require NGF or other NTs for neurite elaboration in culture, although the presence of these factor results in increased neuritic growth (Lindsay, 1988; Jiang et al., 1995; Mohiuddin et al., 1995; Smith and Skene, 1997).

In our cultures of adult DRG neurons, the neuronal cell bodies did not require exposure to NGF for either initial neurite growth or subsequent extension into the distal NGF-containing compartments. This is demonstrated by the findings that the neurons were routinely plated in the absence of NGF, and further, if the neurons were plated in the presence of anti-NGF, there was little obvious alteration in the amount of proximal neurite growth in the center compartment or subsequent distal extension into NGF-containing compartments (Fig. 2.5 A). Interestingly, if NGF was provided to the cell body compartment, there was little growth of the adult neurites into the distal NGF-containing compartments (Fig. 2.1). The ability of the neonatal neurons to extend into the higher concentration of NGF may reflect differences in the NGF sensitivity of developing neurons.

Nerve growth factor appeared to influence the growth of DRG neurites in a local manner, such that exposure of the cell bodies to NGF, while eliciting increased growth locally, did not in itself result in growth into the distal chambers. The fact that the NGF in the center compartment also inhibited the distal extension into NGF-containing compartments may indicate that the neurons growing in the presence of NGF have exhausted their potential to grow into the side compartments. Alternatively, this may be a result of altering the neuronal response to injury. A recent *in vivo* study suggests that

provision of exogenous NGF via intrathecal infusion impairs peripheral axonal regeneration, perhaps by reducing the cell body response to injury (Gold, 1997). In that model, provision of NGF to the neurons results in a significant delay in both the onset and maximal distance of axonal extension subsequent to a peripheral nerve crush. It is conceivable that in the present experiments, NGF may have a similar influence.

Dorsal root ganglion neurons appear to have an intrinsic capability for two distinct forms of neurite growth *in vitro*: an initial relatively short arborizing form of growth followed by a switch to neurite elongation (Smith and Skene, 1997). These authors suggest that most DRG neurons are constitutively competent to undergo arborizing growth, but very limited longer neurite extension; this arborization occurs in the absence of NGF or other factors, but can be influenced by NGF or density of plating. The subsequent switch to elongation requires a transcription-dependent transition, which may be triggered by interruption of a signal from the periphery, as would occur after nerve injury or explantation into culture (Smith and Skene, 1997). Their results support the idea that many adult DRG neurons *in vivo* are essentially maintained in an arborization-competent state, which would be exemplified by the ability of certain neurons to maintain or increase their terminal fields in response to alterations in NGF [i.e. to undergo collateral sprouting in response to increased NGF levels (Diamond et al., 1992a; Mearow et al., 1994)]. On the other hand, Smith and Skene (1997) suggested that competence for elongation is normally suppressed in intact neurons by signals conveyed from the peripheral targets, and can be induced by disruption of ongoing signals, such as occurs with nerve injury.



The nature of the signal(s) is unclear, but is not likely to be simply a loss of transport of NGF, for example, since prior studies have demonstrated that neither NGF nor anti-NGF treatment *in vivo* affects the regeneration of damaged adult DRG neurons (Diamond et al., 1992b; Mearow et al., 1994; Mearow and Kril, 1995). Our results (discussed above) also point to the possibility that exposure of the neuronal cell bodies to NGF suppresses the elongation competence and places the neurons in the arborization state.

#### **2.4.2 BDNF and NT3 do not support distal neurite extension**

Our results demonstrate that neither BDNF nor NT3 was effective in promoting distal neurite extension. While it is clear that the DRGs are composed of a heterogeneous population of neurons, including those that express receptors for NGF, BDNF, and NT3, only NGF elicited distal neurite growth or elongation, presumably from the TrkA-expressing neurons. In the adult DRGs, TrkA-expressing neurons comprise approximately 40-50% of the total, while TrkB and TrkC-expressing cells account for anywhere from 5 to 30 and 10 to 20%, depending upon the spinal level (McMahon et al., 1994; Wright and Snider, 1995; Molliver et al., 1995). In line with these studies, our observations indicated that a majority (>50%) of the neurons in the cultures were p75-IR and TrkA-IR. One possibility to account for the fact that neither BDNF nor NT3 supported neurite extension into the distal compartments is that there were insufficient numbers of neurons expressing TrkB or TrkC in our cultures for BDNF or NT3 to elicit significant neurite elongation. However, we carried out immunocytochemistry using the

commercially available antibodies for TrkC and TrkB, and our preliminary observations indicate that <10-20% of the neurons were TrkC or TrkB-IR. Similar results were observed with DRG explants in culture (Edstrom et al., 1996). Explants of adult mouse lumbar DRGs with attached segment of nerve or root cultured in gels of extracellular matrix material displayed small amounts of neurite growth in the absence of NTs. However, the addition of NGF substantially increased both the amount and length of such neurites. While a much smaller effect of NT3 was observed, BDNF had no apparent influence upon the neurite growth from such explants; the results were suggested to be attributable to the relatively low percentage of TrkB-expressing neurons in the lumbar DRGs (Edstrom et al., 1996).

While we have evidence that the cultures do contain BDNF- and NT3-responsive neurons, and that exposure of the cell bodies and proximal axons to these NTs results in enhanced growth (data not shown), it may be that the neurons do not extend sufficiently long neurites to reach the distal compartments, or that the NTs must act on cell body receptors to get a response. For example, sympathetic neurons are capable of different responses depending upon whether the cell bodies, distal neurites, or both are exposed to trophic factors (Toma et al., 1997). Miller et al., (1994) showed that exogenous NGF applied to the terminal fields can up regulate TrkA or p75 mRNAs independently and also promote differential expression of the receptors on the terminals versus cell bodies such that there is an increase in the ratio of p75:Trk on the terminal neurites.

We were also interested in determining whether BDNF or NT3 could affect the

response of the neurons to NGF, and thus carried out experiments in which neurons plated in the absence of the NTs were provided with distal compartments containing BDNF plus NGF, or NT3 plus NGF. The biological activities of the NTs are mediated by binding to their cognate high-affinity Trk receptors: NGF to TrkA; BDNF/NT4 to TrkB; NT3 to TrkC and, at higher concentrations, also to TrkA (reviewed in Barbacid, 1994). All the NTs also bind with lower affinity to the p75 receptor, and while the function of p75 is less clear, proposed roles include enhancing the binding of the NTs to the Trks and modulating Trk signaling (Barker and Shooter, 1994; Hantzopoulos et al., 1994; reviewed in Chao, 1994, and Carter and Lewin, 1997). p75 has been postulated to be involved in NGF-mediated signaling by being able to increase the binding of NGF to TrkA by increasing the local availability of NGF (Barker and Shooter, 1994). Using excess BDNF to block binding of NGF to p75 (since BDNF will compete with NGF for p75, but not TrkA), the co-treatment of PC12 cells with BDNF and NGF resulted in a decreased activation of TrkA. This diminished activation was indicated by cross linking experiments to be due to a reduction in binding of NGF to TrkA (Barker and Shooter, 1994). In our experiments, the combination of BDNF with NGF resulted in the inhibition of neurite extension, and in similar experiments using a monoclonal antibody against p75, MC 192, our results have also shown a decrease in neurite extension (see chapter 3). The results are consistent with a significant role of the p75 receptor in the regulation of this neurite growth response by NGF. The ratio of p75:Trk receptor has been postulated as being an important factor in the response of cells to NGF, and by altering the ability of NGF to bind to p75, one is effectively altering this ratio, and thus the

response of the neurons to NGF (e.g., Benedetti et al., 1993; Miller et al., 1994).

We also examined whether NT3 would have similar effects to BDNF or would potentiate the response of NGF, perhaps through its actions on TrkA. In our initial experiments, 1 or 10 ng/mL of NT3 was employed to more specifically activate TrkC receptors; at higher concentrations NT3 has been shown to also activate the TrkA receptors in sympathetic neurons (e.g., Belliveau et al., 1997), although it does not do the same in PC12 cells (Ip et al., 1993). However, distal neurite extension did not occur in any concentration of NT3. Our results (Fig. 2.4 B) demonstrate that the combination of NT3 plus NGF did not effect an increased amount of neurite extension, nor were there any noticeable alterations in neuritic density. While NT3 can activate TrkA, it also interacts more efficiently with p75 than NGF (Rodriguez-Tebar et al., 1992) and could thus interfere with the binding of NGF in a similar fashion as BDNF. To account for the lack of effect of the combination of NT3 plus NGF, it is conceivable that the effects of NT3 on TrkA are offset by potential inhibitory influences via binding to p75.

#### **2.4.3 Differences between *in vivo* and *in vitro* growth responses**

As discussed above, adult sensory neurons *in vitro* have been shown to exhibit two forms of axonal growth that in some respects could be considered similar to *in vivo* growth responses. Thus, the arborizing form of growth, which is suggested as the default state of many adult DRG neurons (Smith and Skene, 1997), may be similar to the *in vivo* sprouting of the NGF-responsive population of nociceptive neurons, and elongation may correspond to regeneration.

Collateral sprouting of intact adult DRG neurons requires NGF; the only DRG neurons that have been shown to sprout in the adult mammal have been the nociceptive, NGF-responsive neurons (Diamond et al., 1987, 1992a). Anti-NGF treatment halts the sprouting, while systemic NGF results in a hyper innervation of the terminal fields (Diamond et al., 1992a,b). In contrast, there is no observable sprouting from non-nociceptive afferents—that is, the non-NGF responsive neurons, including those that express TrkB and TrkC receptors (Diamond et al., 1987 1992a,b; Doucette and Diamond, 1988). However, corresponding *in vivo* experiments testing the efficacy of BDNF or NT3 on influencing either sprouting or regeneration have been difficult to perform because of the lack of sufficient quantities of either blocking antisera or the NTs themselves.

A similar sprouting response can be approximated in the compartment culture system once the neurites have extended into the distal compartments (e.g. Campenot, 1987). It is possible that some of the mechanisms through which NGF elicits growth of distal neurites in compartment cultures are analogous to those exerted by the NT during collateral sprouting *in vivo* (but see below). NGF is supplied only in the distal compartments in our cultures, a manner which mimics the *in vivo* situation to a certain extent, and the action of NGF in both cases primarily influences the growth response, as opposed to a survival effect. Each response is reliant on NGF and is halted or abolished by the removal of the neurotrophin (Diamond et al., 1992a; Mearow and Kril, 1995), indicating that both collateral sprouting and growth of distal neurites in the compartment cultures require a constant supply of NGF.

In contrast, the regeneration of peripheral sensory afferents *in vivo* occurs in the absence of NGF (Rich et al., 1984; Diamond et al., 1992b), although NGF has been shown to locally promote growth of the central processes of DRG neurons (Oudega and Hagg, 1996; Tuszinsky et al., 1994). In other experiments, however, intrathecal infusion of NGF at the time of peripheral nerve injury has been demonstrated to delay the onset and rate of regeneration (Gold, 1997).

The *in vivo* model predicts that the regeneration is NGF independent and that it continues to be independent of NGF even when regenerating within the target tissue (Diamond et al., 1992b). In the present experiments, regeneration *in vitro* seems to have both NGF-independent and NGF-dependent components. Thus, the initial growth of proximal neurites in the adult cultures (i.e., within center compartments) which is necessarily a regenerative response occurred in the absence of any added neurotrophic factors and was unaffected by treatment with anti-NGF. The subsequent and significant elongation and increases in the complexity of terminal branching patterns, however, required the presence of NGF. The regrowth after *in vitro* axotomy also required the presence of NGF, a result which is at variance with the predictions of the *in vivo* model. The reasons for the difference between the *in vivo* and *in vitro* results are not clear, but could involve differential interactions with the growth substratum and NGF or other NTs. In addition to its trophic effects, NGF has long been suggested to exhibit trophic influences on growing neurites, particularly with respect to extension of growth cones, for example (Campanot, 1995). Our observations *in vitro* suggest that this may be a possibility. Some limited regrowth was observed in the

absence of NGF in the culture medium, perhaps owing to the interaction of neurites with residual substratum-bound NGF. However, when anti-NGF was added to the medium in these distal compartments, no regeneration of neurites was observed. In *in vivo* experiments, while neither NGF nor anti-NGF affects the regeneration of the sensory fibers (Diamond et al., 1992b), the regrowing fibers were able to grow along a favorable substratum, i.e., the degenerating nerve sheaths rich in various extracellular matrix molecules that can influence nerve growth (Reichardt, 1993; Gavazzi and Cowen, 1996). Furthermore, this regrowth could very well have been influenced by factors other than NGF, such as the insulin-like growth factors (Glazner et al., 1993; Zhuang et al., 1996) or fibroblast growth factors (Danielson et al., 1988; Eckenstein et al., 1991).

In conclusion, we have shown that NGF alone was able to sustain growth or elongation of distal neurites in compartment cultures of both adult and neonatal DRG neurons. The neurons exhibited both NGF-independent and -dependent forms of growth. BDNF and NT3 did not elicit such neurite growth, nor did they appear to be able to positively influence NGF-dependent growth. Use of the compartment system affords a unique model to study aspects of the regulation of adult neurite growth that are often difficult to pursue *in vivo*, as well as providing a paradigm for specific investigation of nociceptive afferents.

**Table 2.1 Neurite Densities from Neurons Cultured with 0, 1, 10 and 100 ng/mL NGF in Distal Compartments**

Distal Compartment (ng/mL NGF)	Density per Lane ( $\mu\text{m}^2 \times 10^3$ )
Control	0
1	$38.47 \pm 14.6 \mu\text{m}^2$ *
10	$94.18 \pm 10.1 \mu\text{m}^2$ *
100	$140.50 \pm 14.4 \mu\text{m}^2$ *

Neuritic densities are taken as the average area covered by the neurites growing across lanes in distal compartments. Values are expressed as the average density for single lanes. Control = no NGF added to side compartments. Density was measured on day 7 *in vitro* and represents the mean  $\pm$  S.E.M. of 10 to 15 lanes from two to three cultures. Differences are significant ( $p < 0.001$ ).



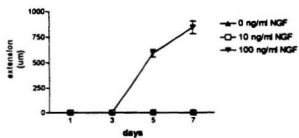
**Table 2.2 Neurite Extension from Neurons Cultured with Neurotrophins in the Center Compartment**

Center Compartment (10 ng/mL)	Side Compartment (100 ng/mL)		
	NT3	BDNF	NGF
control	—	—	+(969 ± 210 μm)
NT3	—	—	+(701 ± 139 μm)
BDNF	—	—	+(725 ± 28 μm)
NGF	—	—	+(537 ± 29 μm)

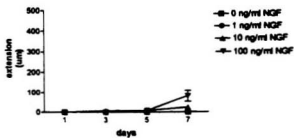
The addition of neurotrophins (NGF, BDNF, and NT3) to the neuronal cell body compartment did not result in increased growth into distal compartments, nor did it induce any neurite growth into distal compartments containing neurotrophins other than NGF. Control = no neurotrophin. Extension was measured as described in Methods on day 7 *in vitro* and represents the mean ± S.E.M. of four to six compartments.

**Figure 2.1 Nerve growth factor (NGF) is required for distal neurite growth of sensory neurons in compartment cultures.** Dissociated sensory neurons were plated in the center compartments of three-chambered Teflon dividers in either the presence of NGF[(A) neonatal neurons or (B) adult neurons,] or the absence of NGF [(C) neonatal neurons or (D) adult neurons]; the distal compartments contained medium with no NGF or varying concentrations of NGF. Measurements of growth were taken as total neurite extension as described in Methods. (A) Neonatal sensory neurons plated in compartments with NGF (10 ng/mL) displayed no growth into distal compartments containing 10 ng/mL NGF, but did extend into distal compartments with 100 ng/mL NGF. (B) Adult sensory neurons plated in compartments with NGF (10 ng/mL) displayed little growth into the NGF-containing distal compartments over the 7-day culture period. In contrast, neurons plated in the absence of NGF [(C) neonatal, (D) adult] showed extensive distal neurite growth into the side chambers containing NGF; no growth was seen in the absence of NGF. Each point represents the mean of 7-10 cultures  $\pm$  S.E.M.

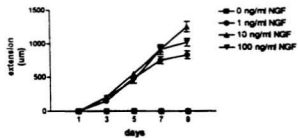
A



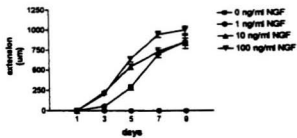
B



C



D

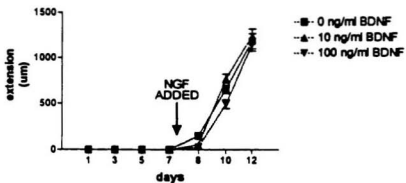
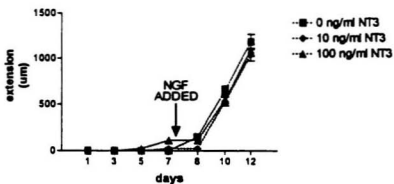


**Figure 2.2 Density of neuritic growth increases with increasing NGF**

**concentrations in the side compartments.** Distal neurites were labeled with Fluorogold (2% in sucrose/HBBS) for 2-4 h; the cultures were then washed with HBSS and the medium was replenished and incubated for a further 24h. The cultures were subsequently fixed and labeled neurites were visualized with ultraviolet illumination. The photomicrographs are representative examples of neurites growing into distal compartments containing (A) 100 ng/mL, (B) 10 ng/mL, or (C) 1 ng/mL NGF. In some cultures at day 7, anti-NGF was added to the medium; (D) and (E) are representative examples of cultures treated with anti-NGF for 5 days and labeled with Fluorogold. Calibration bar = 100  $\mu$ m.

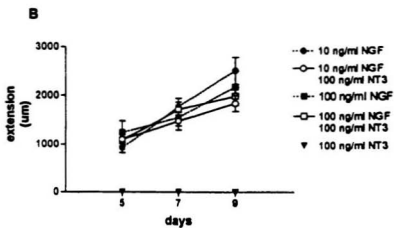
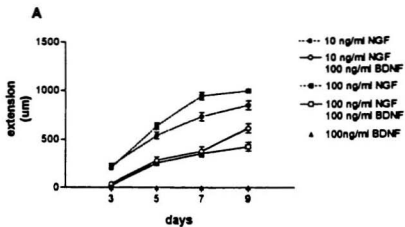


**Figure 2.3 Neither BDNF nor NT3 supported neuritic growth into the side compartments.** Adult neurons were center-plated in the absence of added NTs and provided with distal compartments containing BDNF (A) or NT3 (B). There was no neurite growth into the side chambers containing BDNF or NT3. On day 7 *in vitro*, the medium in the side chambers was replaced with medium containing 25 ng/mL NGF. Replacing BDNF or NT3 with NGF resulted in rapid growth of neurites into the side chambers. Each point represents the mean  $\pm$  S.E.M. of seven cultures.

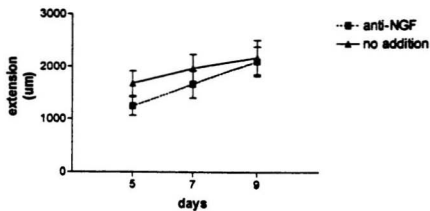
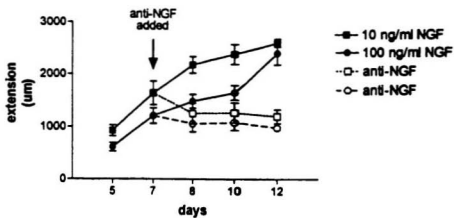
**A****B**

**Figure 2.4 The effects of combining NGF with BDNF or NT3 in the distal compartments.** (A) Neurons were plated in the absence of neurotrophin and provided with distal compartments containing either NGF (10 or 100 ng/mL), BDNF (100 ng/mL), or a combination of BDNF (100 ng/mL) with 10 or 100 ng/mL NGF. There was no extension into compartments containing BDNF alone (solid triangles); extension proceeded normally into the NGF-containing compartments. The combination of BDNF with NGF resulted in an inhibition of the distal extension, although it was not blocked totally (open circles and squares). (B) Neurons were plated in the absence of neurotrophins and provided with distal compartments containing either NGF (10 or 100 ng/mL), NT3 (100 ng/mL), or a combination of NT3 (100 ng/mL) or 10 or 100 ng/mL NGF. There was no extension into compartments containing NT3 alone (solid triangles). There was no further increase in extension in compartments with both NT3 and NGF compared with the NGF-only compartments. Each point represents the mean  $\pm$  S.E.M. of four cultures.

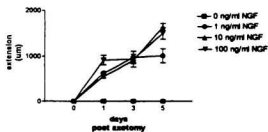
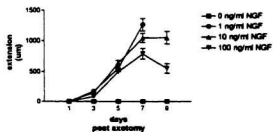
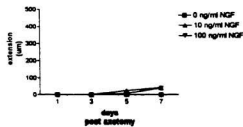
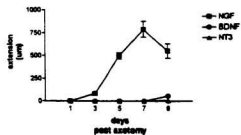




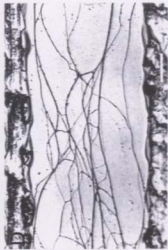
**Figure 2.5 Anti-NGF applied to cell bodies did not block distal neurite extension, but did if applied to the distal neurites.** (A) Neurons were plated in the center compartments in the presence of 200 ng/mL anti-NGF (affinity-purified IgGs); sister cultures were plated in the usual medium in presence of control IgGs. Distal neurite extension was measured from days 5 to 9 after plating; there was no significant difference in the amount of neurite extension. (B) Removal of NGF with anti-NGF treatment halted neurite extension but did not result in neurite retraction over a period of 5 days. Adult neurons were allowed to send neurites into side compartments with NGF (10 or 100 ng/mL) for 7 days. On day 7, the NGF was removed from half the cultures and replaced with anti-NGF (200 ng/mL affinity-purified IgG); the remaining cultures were maintained with their respective concentrations of NGF. Growth measurements were then recorded for 5 more days. There is no further neurite extension in the anti-NGF treated cultures; the open squares are the anti-NGF treated cultures that were initially grown with 10 ng/mL NGF, and the open circles represent the anti-NGF treated cultures originally exposed to 100 ng/mL NGF. Sister cultures maintained with NGF for the same 5 days show the normal rate of neurite extension (solid squares and circles). Each time point represents the mean  $\pm$  S.E.M. for five cultures.

**A****B**

**Figure 2.6 Nerve growth factor was required for regrowth following in vitro axotomy of both neonatal and adult neurons.** Neonatal (A) and adult (B) neurons were grown in compartment cultures for 7 days. On day 7, distal neurites in side compartments containing NGF (1, 10, or 100 ng/mL) were axotomized with a jet of distilled water as described in Methods, and the NGF-containing medium was replenished. As can be seen in (A) and (B), neurons rapidly regrew neurites into these compartments, provided NGF was present. (C) Anti-NGF blocked the regrowth of neurites. As above, after day 7 of culture, the neurites of adult neurons were axotomized, but the NGF-containing medium was replaced with anti-NGF (200 ng/mL IgG). There was no significant regrowth into the compartments in the absence of NGF. (D) Neither BDNF nor NT3 could substitute for NGF in promoting regrowth of neurites after axotomy. As above, after 7 days of culture and neurite extension into NGF-containing medium, axotomy was carried out; the NGF-containing medium was replaced with that containing either BDNF (plus anti-NGF) or NT3 (plus anti-NGF). There was no significant regrowth into either BDNF- or NT3-containing (100 ng/mL) compartments. Each point represents the mean  $\pm$  S.E.M. for six to ten cultures.

**A****B****C****D**

**Figure 2.7 Immunocytochemical staining for p75 and TrkA.** Cultures were fixed and processed for immunocytochemistry as described in methods. (A, B) p75 immunoreactivity was visualized using an FITC-tagged secondary antibody and photographed with fluorescence optics. (A) Neurons in the cell body compartment; (B) neurites in the distal compartment. (C,D) TrkA immunoreactivity was visualized using an HRP-tagged secondary antibody and diaminobenzidine, and photographed with transmitted light optics. (C) Neurons in the center compartment; (D) neurites in the distal compartment.



### **3.0 CHAPTER III: The anti-P75 antibody, MC192, and Brain Derived Neurotrophic Factor inhibit Nerve Growth Factor dependent neurite growth from adult sensory neurons.**

#### **3.1 Introduction**

The neurotrophins have been shown to regulate neuronal differentiation, development and survival, and for many classes of neurons the dependence on these factors for continued survival extends through adulthood. In contrast, as sensory neurons mature nerve growth factor (NGF) is no longer required for their survival, but is important in maintaining phenotypic specializations, as well as being involved in physiological functioning and innervation patterns of nociceptive afferents (Diamond et al., 1992; Lindsay 1988; McMahon et al., 1995; Mulderry 1994; Rueff and Mendell 1996).

The neurotrophins exert their effects through binding to the Trk family of receptors and the p75 neurotrophin receptor (p75NTR). The biological effects of NGF are primarily mediated by high affinity binding to the receptor tyrosine kinase, TrkA (Kaplan et al., 1991; Kaplan and Miller 1997; Segal and Greenberg 1996). In addition NGF, like the other neurotrophins, binds p75NTR. p75NTR has been reported to bind all neurotrophins with equal affinity but differing kinetics (Rodriguez-Tebar et al., 1990, 1992). Unlike the Trks, p75NTR has a less clearly defined role but has been implicated in apoptosis and cell survival Bamji et al., 1998; Carter et al., 1996; Casaccia-Bonnet et al., 1996; Rabizadeh et al., 1993; Van der Zee et al., 1996). While p75NTR appears to be neither necessary nor sufficient for many aspects of neurotrophin signaling, it is reported to play a role in regulating responses to the



neurotrophins. Evidence suggests that p75NTR acts to increase the cellular sensitivity or responsiveness to NGF (Ryden et al., 1997). Receptor co-expression studies have indicated that co-expression of p75NTR with Trk results in increased high-affinity binding sites (Hempstead et al., 1991; Mahadeo et al., 1994), and NGF binding to p75NTR has also been shown to enhance Trk activity and responsiveness to NGF (Barker and Shooter 1994; Hantzopoulos et al., 1994; Verdi et al., 1994). Inhibition of NGF binding to p75NTR using brain-derived neurotrophic factor (BDNF), which binds p75NTR but not TrkA, or anti-p75 blocking antibodies resulted in decreased numbers of high-affinity binding sites; as a consequence, biological responses were not fully blocked but required higher concentrations of NGF to be elicited (Barker and Shooter 1994; Weskamp and Reichardt 1991). A mutant NGF molecule that can no longer bind p75, still elicits neurite outgrowth and survival but at increased concentrations compared to the normal NGF (Ryden et al., 1997). Similarly, neurons from p75<sup>-/-</sup> mice require increased amounts of NGF for survival compared to wild-type animals (Davies et al., 1993). In addition to modulatory influences on Trk activity, p75NTR also possesses intrinsic signaling capabilities (Carter et al., 1996; Casaccia-Bonnel et al., 1996; Dobrowsky et al., 1994). For example, the sphingomyelinase pathway can be activated via p75NTR, resulting in the production of ceramide which has been implicated in apoptosis (Hannun and Obeid 1995) and the regulation of neurite outgrowth (Posse de Chaves et al., 1997; Schwartz and Futerma 1997).

In order to further investigate the contribution of p75NTR to the NGF-induced growth response in adult primary sensory neurons, we have adapted the compartment

culture model for use with adult dorsal root ganglion (DRG) neurons (Campenot 1977; Kimpinski et al., 1997). This model allows for a distinction to be made between the effects of NGF on axon terminals as opposed to its effects on neuronal cell bodies. We have previously shown that NGF alone elicits growth of distal neurites from DRG neurons; BDNF and NT-3 are without effect when provided alone. However, we also observed that the combination of BDNF with NGF resulted in a decrease in neurite extension (Kimpinski et al., 1997). BDNF and the monoclonal antibody against p75 (MC192) have previously been used to interfere with p75NTR binding of NGF, resulting in decreased response to NGF (Chandler et al., 1984; Barker and Shooter 1994; MacPhee and Barker 1997). Thus, we have used a similar approach with adult DRG neurons to study the contribution of p75NTR to NGF-induced neurite growth. Our results indicate that these treatments result in an inhibition of neurite outgrowth likely acting primarily through their effects on p75NTR.

## **3.2 Materials and methods**

### **3.2.1 Cell preparation**

Dorsal root ganglia (DRGs) were dissected from young adult (100-150g) Sprague-Dawley rats (Memorial University of Newfoundland Vivarium). Animals were anaesthetized and decapitated prior to DRG removal. Neuronal dissociation and resuspension were carried out as previously described (Kimpinski et al., 1997). Neurons were plated in compartment cultures at a density of 5-6 ganglia per compartment culture, or in 24- or 96-well plates at a density of 5000-7000 and 20-50

neurons per well respectively.

### **3.2.2 Culture conditions**

Compartment cultures were constructed as previously described (Campenot 1977; Kimpinski et al., 1997). In all cultures, the neurons were plated in the middle compartment (M) in the absence of added NGF and allowed to grow into adjacent side compartments (S) containing NGF, or NGF+BDNF or NGF+MC192. Cultures generally had neurites emerging into the side compartments after 3 days and the neurites were fully established after 5 days. Medium provided to the neurons (middle compartment) consisted of L15 with fetal bovine serum (FBS, 2%) and penicillin/streptomycin (100 units) plus 1% methylcellulose. Medium supplied to distal neurites (side compartments) contained N2 supplement (Gibco, BRL) rather than FBS. Cytosine arabinoside (20 mM) was added at the time of plating of the neurons for 24 hours, and added again on days 3-7 if required. Medium changes occurred one day after plating and every 2-3 days thereafter. In the mass cultures (24- and 96-well plates) the medium was the same as that supplied to distal compartments with the exclusion of methylcellulose. Medium included either NGF (Cedarlane Labs, Hornby Ont.), BDNF (Peprotech, Rocky Hill, NJ) or the monoclonal anti-p75 antibody MC192 (Oncogene Science, Cambridge, MA) where appropriate.

### **3.2.3 Immunoprecipitation and western blotting.**

Protein analysis was undertaken using the following methods. Samples were lysed on ice with 50-100 ml of lysis buffer per compartment or well. The lysis buffer consisted

of tris buffered saline (TBS) with 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium orthovanadate (all chemicals were obtained from Sigma, St. Louis, MO). Lysates were centrifuged at 10,000 rpm for 10 min to pellet debris, and the supernatants used immediately or frozen for future use. Protein concentrations were determined using the BioRad DC assay system (BioRad Corp.).

Extracts from 3 middle and 6 side compartments, or 3-4 wells from 24-well mass cultures were pooled for immunoprecipitation with antibodies to TrkA (Trk 763, Santa Cruz Biotech, Santa Cruz, CA), TrkB or TrkC (generous gifts of Dr. D. Kaplan) or p75 (MC192, Oncogene Science). In some experiments pan-Trk antibodies were employed (TrkC14, Santa Cruz; a203, generously provided by Dr. D. Kaplan). The lysates were precleared in order to prevent any non-specific binding by IgGs using 50% protein-A-sepharose at 4°C for 2 hours. These lysates (50-100 mg protein) were then immunoprecipitated with the above antibodies (1mg per reaction) overnight at 4°C. Immunoprecipitates were captured with protein-A-agarose and re-suspended in sample buffer containing 10% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT), and 0.005% bromophenol blue. For western analysis of p75, 20-25 mg total protein in lysis buffer was combined with 5x sample buffer with the exclusion of DTT. Total cell lysates were also analysed with an anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Saranac Lake, NY). The samples were then electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECL, Amersham, Buckinghamshire, England). The membranes were blocked in 3% BSA in TBST (tris-buffered saline with

0.2% (v/v) Tween-20) and subsequently blotted with primary antibodies (TrkA 763, 1:1000; MC192 1:500; 4G10, 1:2000). Enhanced chemiluminescence (ECL, Amersham Corp.) was used to visualize specific bands using X-ray film.

#### **3.2.4 Tyrosine phosphorylation assays**

TrkA phosphorylation was assayed after treatment of the neurons with NGF, NGF plus BDNF, or NGF plus MC192 for 30 min and 24 hrs, using western blotting of total cell lysates, as well as immunoprecipitated TrkA. Anti-phosphotyrosine (PY, clone 4G10, UBI) was used as the assay antibody; western blots were sequentially probed with anti-TrkA to confirm the PY localization. Bands in the autoradiographic films were quantitated by densitometry (Northern Exposure Imaging system, EMPIX Imaging, Mississauga, Ont.), and the intensities expressed relative to the 0 NGF condition. Statistical analyses of 6 independent experiments were carried out using paired t-tests and repeated measures Anova with Bonferroni multiple comparison post-tests.

#### **3.2.5 Immunocytochemistry.**

Culture medium was carefully aspirated from the cultures which were then washed with phosphate-buffered saline (PBS) and fixed for 15 min in 4% formaldehyde in PBS. After washing in PBS, the cultures were incubated in blocking solution (10% normal goat serum, 0.1% TritonX in PBS) for 1 hr. Incubation with anti-p75 Ab MC192 (1:500 dilution) or anti-NF $\kappa$ B (1:500, Santa Cruz Biotech) was carried out overnight at 4°C. The cultures were washed and exposed to the biotinylated secondary antibody for 1 hr at RT;

visualization of staining was completed using a Vectastain Kit (Vector Labs, Burlingame, CA) and diaminobenzidine (DAB). Cultures were then cover-slipped under glycerol, examined and photographed either with a Leitz photomicroscopic system or a Hund Wilovert-S inverted microscope.

### **3.2.6 Measurements of neurite growth**

For measurements of neurite extension, 10-16 lanes per side compartment per culture were counted as previously described (Kimpinski et al., 1997). Extension data was expressed as the average of the total length a group of neurites extended across each lane from the point of emergence under the barrier. Cultures were measured using a Hund Wilovert-S inverted microscope and optical micrometer. Measurements of neurite density were performed using cultures immunostained with anti-p75 (see above). The monoclonal antibody MC192 was used as it was found to be the most effective for visualizing these neurites and provided the best representation of fine neurites, which are not always visible under phase microscopy. Density of neurites was quantified using digitized images and the Northern Exposure Imaging System (EMPIX Imaging, Mississauga, Ont.). The data were plotted as the mean and standard error ( $\pm$  SEM). Statistical analyses were carried out using analysis of variance (one way ANOVA) or Students t- test.

## **3.3 Results**

### **3.3.1 Characterization of TrkA as the only Trk receptor found on distal neurites of adult DRG neurons in compartment cultures.**

In compartment cultures of adult DRG neurons, only NGF produces any measurable growth of neurites into the distal or side compartments; neither BDNF nor NT-3 produced significant or permanent growth into distal compartments, as we have previously reported (Kimpinski et al., 1997). In these cultures, only the distal or side (S) compartments contained any trophic factors; the neurons were always plated in the central or middle (M) in medium containing no trophic factors. We examined neurites harvested from the distal compartments for the expression of the Trk receptors. Immunoprecipitation (IP) of equivalent amounts of protein from neurite samples was carried out with antibodies specific for TrkA, B or C as described in the Experimental Procedures. Immunoblot analysis of the IP samples using the same antibodies revealed that these neurites expressed only TrkA (Fig. 3.1A). Attempts to localize TrkB (using antibodies recognizing both full length and truncated TrkB) and TrkC on distal neurites were unsuccessful. In addition, we also used a pan-Trk antibody (a203) to carry out the immunoprecipitations, followed by immunoblotting with TrkB-in and TrkC-in antibodies (kindly provided by Dr. D. Kaplan), and obtained similar results. This evidence indicates that the distal neurites in the compartment cultures express only TrkA, and thus the use of BDNF to compete with NGF for binding to p75NTR at distal neurites is not complicated by the presence of TrkB receptors.

### **3.3.2 Expression of TrkA and p75NTR on cell bodies/proximal neurites in comparison to distal neurites.**

We also examined the expression of p75NTR (and TrkA) on cell bodies plus

proximal neurites and distal neurites. Neuronal samples were taken from the middle (M) compartments and neurite samples from the distal or side (S) compartments. p75 and TrkA were immunoprecipitated from lysates as described in Experimental Procedures. Blots were then probed with antibodies to either TrkA or p75; representative blots are presented in Fig 1B. Both p75NTR and TrkA were robustly expressed by cell bodies (M) and distal neurites (S) in cultures sampled at day 5 and day 7 in vitro (Fig. 3.1B).

### **3.3.3 Trk A is necessary for neurite outgrowth in compartment cultures.**

In order to establish that TrkA is necessary for neurite growth in compartment cultures we employed the protein kinase inhibitor K-252a, which also inhibits Trk autophosphorylation and subsequent signaling in response to NGF (Berg et al., 1991; Tapley et al., 1992). Concentrations of 200 nM - 2 mM have been shown to result in complete inhibition of NGF-induced Trk phosphorylation in PC12 cells (Dobrowsky et al., 1995; Maliartchouk and Saragovi 1997). K-252b, a related isomer which requires higher concentrations than K252a to inhibit the actions of NGF (Knusel et al., 1992), was also used. Distal neurite extension was blocked in cultures treated with 1 mM K-252a in the distal compartments, but neurites in control and 1 mM K-252b-containing cultures grew normally (Fig. 3.2). These results indicate a requirement for TrkA activity during NGF-dependent neurite growth in adult sensory neurons.

### **3.3.4 NGF-dependent neurite growth is decreased by BDNF.**

We had previously observed that when distal neurites are exposed to NGF plus



BDNF there was a diminution of neurite extension over time. We were interested in further pursuing this observation to test the possibility that BDNF was having this effect by interfering with TrkA and p75 activation by NGF. We thus examined the effects of BDNF in combination with NGF on distal neurite extension, based on the assumption that BDNF would displace NGF from p75NTR receptors (Barker and Shooter 1994; MacPhee and Barker 1997). Measurements were taken of neurite extension into distal compartments containing 1-100 ng/ml NGF alone, or in combination with varying concentrations of BDNF (1-100 ng/ml) and the results plotted in Figure 3.3 A-C. In general, with 1-100 ng/ml NGF in distal compartments, the inclusion of BDNF (at the time of plating) resulted in decreased neurite elongation. However, even at BDNF concentrations in excess of 100-fold greater than that of NGF, some growth remained (e.g., compartments containing 1 and 10 ng/ml NGF, Fig. 3.3A and B). In all cultures, some neurite elongation was present even at the highest concentration of BDNF (Fig. 3.3A-C) and this remaining growth was similar at all concentrations, ranging from 316 to 403 mm extension. This suggests that either BDNF was not totally inhibiting NGF binding to p75NTR or that NGF, acting through TrkA alone, is capable of stimulation of moderate neurite growth.

Since there was still significant neuritic growth in the presence of BDNF and NGF, we used a second method to quantitate the growth, in which we measured neurite density (i.e., total area covered by distal neurites) as described in the Materials and Methods section. Table 3.1 presents the quantitation of neurite density at d7 in the culture conditions shown in Fig 3A-C (100 ng/ml BDNF with varying concentrations of NGF), while Fig 3.4 provides representative photomicrographs of neuritic growth patterns in these

cultures. The density measurements demonstrated two points, first, that while the linear extension measurements are useful in most contexts they do not illustrate the full extent of neuritic growth in these particular experiments. For example, the neurite extension values in 1 ng/ml and 10 ng/ml NGF are quite similar, yet the overall density of growth is approximately double in the 10 ng/ml NGF treatment. Second, the effect of BDNF on inhibiting NGF-induced neurite growth is much more apparent when the total neuritic growth or density is evaluated, particularly in the 10ng/ml and 100 ng/ml NGF conditions (Table 3.1).

### **3.3.5 The monoclonal anti-p75 antibody MC192 also decreases NGF-dependent neurite growth.**

MC192, a monoclonal antibody to p75NTR, alters the normal binding parameters of NGF to p75 resulting in slightly increased affinity of p75 for NGF (Chandler et al., 1984), although it has also been suggested that it may act as an agonist or at least mimic ligand binding to the receptor (Casaccia-Bonnel et al., 1998) or an antagonist (Maliarchouk and Saragovi 1997). However it has been shown that, at least for PC12 cells, this interference with normal binding can affect the binding of NGF to TrkA and result in a concomitant decrease in Trk activity (Barker and Shooter 1994; Hantzopoulos et al., 1994; Lachance et al., 1997). We thus used MC192 in experiments to further examine the role of p75NTR in neurite extension in both compartment and mass cultures.

In initial experiments to test the effectiveness and concentration of MC192 required, we found that when cultures were exposed to 10 ng/ml of NGF with varying amounts of

MC192, there was a dose dependent effect where distal neurite elongation was almost completely inhibited at 2 mg/ml (Fig. 3.5A). For further experiments we chose to use 1 mg/ml, and as observed in Figure 3.5B, when MC192 was added to distal compartments along with varying concentrations of NGF (1-100 ng/ml) at the time of plating, there was a significant decrease in neurite elongation into those compartments in comparison to NGF alone. In contrast to the results obtained with NGF-BDNF co-treatment, this inhibition was almost complete. In these experiments, neuritic density was not quantitated, since it was obvious that there was little or no neurite growth when MC192 was present in the medium.

### **3.3.6 The effects on Trk A phosphorylation following NGF stimulation with co-application of MC192 or BDNF.**

The possibility that the effects of BDNF and MC192 on neurite growth were being exerted via an influence on Trk signaling was assessed by examining the tyrosine phosphorylation of TrkA using both Western blotting of total cell lysates and immunoprecipitation with anti-TrkA followed by immunoblotting with anti-phosphotyrosine. In these experiments, the neurons were grown in mass cultures rather than compartment cultures in order to obtain sufficient material for analyses and were maintained for two days in medium containing no NGF. On the third day, the cultures were stimulated with fresh medium containing NGF (10 ng/ml) alone or in combination with MC192 (1 mg/ml) or BDNF(100 ng/ml); prior to the experimental treatments, the cultures were pretreated for 30 min with MC192 or BDNF where appropriate. Protein samples were taken at 30 min and 24 hours after stimulation; equivalent amounts of protein were electrophoresed on

SDS-acrylamide gels, followed by western blotting with anti-phosphotyrosine antibody. In one set of experiments, equivalent amounts of protein were immunoprecipitated with anti-TrkA, and the blots sequentially probed with anti-phosphotyrosine and TrkA antibodies. A representative blot of one such experiment is presented in Figure 3.6, where it can be observed that there is activation of TrkA by NGF above the basal levels, and that this activation is decreased by the inclusion of BDNF. Densitometric quantitation of western blots of total lysates sampled at 30 min or 24 hours post-treatment and statistical analyses were performed as described in the Materials and Methods. The relative optical density (R.O.D.) values for the treated samples are expressed relative to the basal R.O.D. values for each individual experiment. The data from these experiments are presented in Table 3.2 as the mean  $\pm$  SEM (n=6). In the 30 min stimulation experiments, all treatments were significantly different from the values obtained in the absence of stimulation (basal levels, R.O.D. value = 1.0), while in the longer term experiments the NGF and NGF+MC192 groups were still different from the basal level. The data indicate that the inclusion of MC192 or BDNF resulted in a diminution of Trk activation as assessed using this tyrosine phosphorylation assay. In addition, the BDNF treatment appeared more effective at inhibiting the Trk activation than the MC192 treatment. Our data also demonstrate that there is a prolonged activation of Trk after NGF stimulation, although basal levels are also relatively higher than what is observed in cultures of embryonic or neonatal DRG neurons (K. Mearow, unpublished observations).

### **3.3.7 MC192 treatment results in nuclear translocation of NF $\kappa$ B in adult sensory**

## neurons.

While the p75NTR receptor plays a role in NGF-mediated responses that are a result of Trk activation, it is also clear that p75NTR can act autonomously to trigger signaling events, including activation of the sphingomyelinase cascade (Dobrowsky et al., 1994 and 1995), stimulation of Jun kinase (Casaccia-Bonnel et al., 1996) and activation of the transcription factor NF $\kappa$ B (Carter et al., 1996).

Since one consequence of p75 signaling has been shown to be activation of the transcription factor NF $\kappa$ B and translocation to the nucleus (Carter et al., 1996), and since DRG neurons do express NF $\kappa$ B under various circumstances (Doyle and Hunt 1997), we asked whether the manipulations that were resulting in decreased growth could also activate NF $\kappa$ B, and thus provide some indication of whether p75 was being directly activated. Thus, immunocytochemistry for NF $\kappa$ B was carried out on cultures treated with no growth factors (control), NGF, NGF plus BDNF or NGF plus MC192 for varying times after plating (Fig. 3.7A-F). Three independent culture experiments were carried out, and for each time point and condition in each experiment the number of neurons counted ranged from 400-500. The number of cells displaying nuclear localization for NF $\kappa$ B were quantitated and expressed as a percentage of total neurons counted and the data are presented in Figure 3.8. Initially, about 10% of the plated neurons displayed nuclear staining under all conditions. However, by 1 day post-plating the cultures treated with MC192 alone or in combination with BDNF and NGF had significantly increased numbers of cells with nuclear staining for NF $\kappa$ B compared to the controls, which was not restricted

to any particular cell size. Since neither NGF nor BDNF alone or in combination resulted in the increased nuclear staining, it is likely that the effect is due primarily to the influence of MC192. Indeed, it appears that the co-treatment of MC192 with NGF or BDNF resulted in a decrease in the effectiveness of MC192; there were no significant differences between the MC192+NGF or MC192+BDNF data for d1, d3 and d5 (Fig. 3.8).

Based on reports that p75 activation can result in NF $\kappa$ B activation, these results suggest that treatment of sensory neurons with the anti-p75 antibody MC192 is activating p75NTR which could have important implications in the regulation of the growth process.

### **3.4 Discussion**

The compartment culture system provides a unique opportunity to study neurotrophin regulation of the neurite growth from adult sensory neurons. Using this model, NGF-responsive neurons can be isolated from neuronal populations expressing other Trk receptors. We have previously shown that NGF, but neither BDNF nor NT-3, elicits growth of distal neurites (Kimpinski et al., 1997). In the present study we show that this NGF-elicited growth requires TrkA activation, and also points to the importance of appropriate interactions between TrkA and p75 in mediating the effects of NGF on neuritic growth. In particular we show that: i) the distal neurites growing into NGF-containing compartments express only TrkA but not TrkB nor TrkC, as well as p75; ii) K252a, a tyrosine kinase inhibitor displaying specificity for TrkA, totally blocks distal neurite growth into NGF; iii) BDNF inhibits the NGF-dependent neurite outgrowth, both in terms of neurite extension and total neuritic density; iv) MC192, a monoclonal antibody against p75 which

does not block p75-NGF binding, more completely inhibits neurite outgrowth, both in compartment and mass cultures; v) NGF-stimulated activation of TrkA phosphorylation is decreased by co-treatment with either BDNF nor MC192; and finally vi) that MC192 results in a prolonged activation of NF $\kappa$ B in a sub-population of these adult sensory neurons. Our rationale for using BDNF and MC192 was that they both bind p75, and in a number of other studies have been assumed to modify or interfere with the binding of NGF to TrkA and thus its activation (Barker and Shooter 1994; Carter et al., 1996; Lachance et al., 1997; MacPhee and Barker 1997). Our results demonstrate that both TrkA and p75 play a role in neurite growth response to NGF, and further suggest that any alteration in optimal TrkA-p75 interactions, or possibly direct activation of p75 at the expense of TrkA, results in an inhibition of NGF-dependent neurite growth in adult sensory neurons.

There has been much discussion of the proposed function(s) of p75 in various cell types and the consensus is that it depends upon the cellular context in which p75 is expressed (reviewed in Barker 1998, Bredesen et al., 1998; Casaccia-Bonnel et al., 1998; Kaplan and Miller 1997; Miller and Kaplan 1998). However, there are two general scenarios that can be envisaged. The first is that the optimal effect of NGF requires interactions between both p75 and TrkA. NGF binding to the p75 receptor enhances Trk phosphorylation in a variety of cell types (Barker and Shooter 1994; Berg et al., 1991; Hantzopoulos et al., 1994; Verdi et al., 1994). If the normal NGF activation or interaction of p75 and TrkA is somehow altered, then the resulting response will be less than if there were no perturbations. In situations where there is no p75 available to be activated by NGF, then one might expect that neuronal survival or growth might be decreased or

perturbed in some manner. For example, embryonic sensory neurons from p75-deficient mice exhibit a decreased survival response and require higher NGF concentrations to reproduce the effects of NGF seen in wild type animals (Davies et al., 1993; Lee et al., 1994). The second is the situation where other ligands, such as BDNF or MC192, are present that compete with NGF for p75 and prevent or interfere with activation by NGF. Here, the p75-ligands can alter the responsiveness of TrkA to NGF, such as the attenuation of TrkA activation seen when BDNF, MC192, or p75 blocking antibodies are used (Barker and Shooter 1994; Lachance et al., 1997; MacPhee and Barker 1997). However, a different interpretation of the same scenario is that these p75 ligands are able to activate p75-dependent or autonomous signaling which might either act to directly suppress Trk activation (MacPhee and Barker 1997) or be sufficiently antagonistic to the Trk signaling to effect an inhibition of the NGF effect. There are a number of examples of the interplay between TrkA and p75 signaling dependent upon the ratio of expression and activation of the 2 receptors, where suppression of TrkA by p75 or suppression of p75 by TrkA have been observed (Twiss et al., 1998; Yoon et al., 1998). The concept of functional antagonism has been put forward to explain how neurotrophic factors can effect very different biological responses depending on the context of expression and activation (Casaccia-Bonnel et al., 1998, Kaplan and Miller 1997; Miller and Kaplan 1998).

One interpretation of our results is that p75 contributed to, but was not necessary for, the NGF-dependent growth of distal neurites. Thus in the situation where p75 was not available to NGF, such as in the presence of BDNF or MC192, NGF-dependent neurite growth was inhibited, due perhaps to events downstream of the decreased Trk activation.



There are a number of recent reports that support a role for p75 in neurite growth. For example, DRG neuronal growth cone responses to localized sources of NGF also have been shown to require both TrkA and p75 (Gallo et al., 1997). Sympathetic neuron sprouting in NGF over-expressing, p75-deficient (NGF+/+p75<sup>-/-</sup>) hybrid transgenic mice occurs similar to that observed in the NGF+/+ mice, but the overall pattern and extent of growth is perturbed (Walsh et al., 1999).

However, another possibility is that activation of p75 using BDNF and MC192 results in activation of a signaling cascade that is antagonistic to Trk signaling and that may override the growth-promoting effects of NGF-Trk signaling. Recent studies by Barker and colleagues have confirmed earlier results that BDNF (Barker and Shooter 1994; MacPhee and Barker 1997) and that blocking NGF binding to p75 with a p75-blocking antibody (rather than BDNF) also results in reduced Trk activation in both PC12 cells and neonatal sympathetic neurons (Lachance et al., 1997). Additionally, it was suggested that BDNF may be directly activating p75, and that some downstream signaling event may be responsible for the modulation of Trk activation, since the addition of ceramide to cultures of PC12 cells also resulted in decreased Trk activation via serine-threonine phosphorylation of the Trk receptor (MacPhee and Barker 1997). Our own investigations of Trk signaling in the presence of NGF, NGF+BDNF or NGF+MC192 have suggested that there are differences between PC12 cells, sympathetic neurons and DRG neurons (Mearow and Kimpinski, unpublished observations). Adult DRG neurons appear to express relatively less TrkA than either the sympathetic neurons or PC12 cells, although we are able to detect basal and induced levels of phosphorylated Trk. In our experiments,

using tyrosine phosphorylation of TrkA as an assay, we observed alterations in the activation of TrkA when the neurons were exposed to NGF plus either MC192 or BDNF, similar to what has been reported with PC12 cells and neonatal SCG neurons (Barker and Shooter 1994; Lachance et al., 1997; MacPhee and Barker 1997). The modest decrease in Trk activation coupled with the possibility of autonomous p75 signaling could culminate in the decreased response to NGF observed, namely the reduction in neurite growth. Why BDNF, which appeared more effective in inhibiting Trk activation by NGF than MC192, has less of an effect on neurite growth is not clear, although it seems likely that the two ligands are acting via different pathways (see below). BDNF activation of p75 has also been reported to decrease the density of neuritic growth in cultured SCG neurons grown in low NGF. Both anti-BDNF and a p75-blocking antibody, REX, resulted in increased neurite density, a result which was attributed to the inhibition of p75 activation by autocrine BDNF released by the cultured neurons (Kohn et al., 1998). In p75<sup>-/-</sup> mice, increased numbers of basal forebrain neurons are observed compared to wild-type controls (Van der Zee et al., 1996) as well as neuronal hypertrophy and increases in the amount of target innervation from these neurons (Yeo et al., 1998). In these studies, it would appear that the presence of p75 (coupled with low or absent Trk signaling) provides a negative influence on neuronal survival and growth.

We have observed that MC192 had more of an effect on inhibiting growth compared to BDNF. While it seems clear that BDNF binds p75 and can compete out NGF, the manner in which MC192 is acting in our cultures is not as clear. MC192 increases the affinity of p75 for NGF (Chandler et al., 1984) and this has been suggested to effectively

sequester NGF from TrkA, consequently inhibiting/interfering with binding (Barker and Shooter 1994). MC192 may be acting as an agonist or mimicking ligand binding to p75. While it has been proposed that MC192 can act synergistically with NGF and TrkA (Maliarchouk and Saragovi 1997), this does not appear to be the case in our experiments. There is also evidence that MC192 can inhibit the effects of NGF acting via p75. MC192 was shown to block NGF-induced release of dopamine from mesencephalic neurons that do not express TrkA, although it had little influence on BDNF-mediated dopamine release in the same neurons (Blochl and Sirrenberg 1996). In other studies, in contrast to the effects of MC192, use of a p75-blocking antibody did not have any significant influences on the NGF-induced neurite growth from PC12 cells and DRG neurons (Weskamp and Reichardt 1991). This suggests that simply blocking the binding of the neurotrophins to p75 does not result in the inhibition of growth, but rather that an activation of p75 and subsequent signaling may be required.

We have found that nuclear localization of NF $\kappa$ B occurs with MC192 treatment of neurons. Such translocation to the nucleus is indicative of NF $\kappa$ B activation by the p75 receptor (Carter et al., 1996). The fact that MC192 alone appears to activate NF $\kappa$ B suggests that the MC192 is acting somewhat differently from the neurotrophins, perhaps triggering signal transduction cascades that are distinct from those activated by NGF or BDNF. This possibility is currently being addressed. NF $\kappa$ B is associated with an anti-apoptotic effect in a number of cell types, and has also been suggested to play a role in neuronal plasticity and the resistance of adult DRG to apoptosis after neurotrophin

withdrawal (Doyle and Hunt 1997; O'Neill and Kaltschmidt 1997).

Further, signaling via p75 has been shown to activate the sphingomyelinase-ceramide (SMC) pathway resulting in increased ceramide levels (Dobrowsky et al., 1994). A recent study of sympathetic neurons in compartment cultures has shown that increased ceramide concentrations in the distal neurite compartments inhibits growth (Posse de Chaves et al., 1997). Ceramide analogues have also been shown to reduce NGF-stimulated Trk activation (MacPhee and Barker 1997). It is possible that in our cultures, that MC192 or BDNF, besides simply altering NGF binding to p75, may be activating p75 signaling via the SMC pathway. With respect to BDNF, normal binding to p75 may not be sufficient for activation and consequently may be antagonized by remaining levels of Trk activity (Dobrowsky et al., 1995).

Our results demonstrate that both TrkA and p75 play a role in neurite growth response to NGF, and further suggest that any alteration in optimal TrkA-p75 interactions, or direct activation of p75 at the expense of TrkA, results in an inhibition of NGF-dependent neurite growth in adult sensory neurons. Our evidence supports the hypothesis that p75 signaling may inhibit certain aspects of Trk signaling required for neurite growth, and add to the growing body of evidence indicating that this interplay between receptors is widespread throughout the nervous system, although the actual response is dependent upon the cellular context. During development such a functional interaction between Trk and p75 receptors is postulated to regulate process outgrowth during the time of target innervation to ensure appropriate matching of neurons and their target territory (Kaplan and Miller 1997). It seems very likely that similar mechanisms exist to provide for the

extensive plasticity of the mature peripheral nervous system.

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**Table 3.1 Total neuritic density (mean  $\times 10^3 \mu\text{m}^2$ ,  $\pm$  SEM) of neurites growing into NGF alone or NGF + BDNF (100 ng/ml).**

Treatment	1 ng/ml NGF	10 ng/ml NGF	100 ng/ml NGF
NGF alone	51 $\mu\text{m}^2$ ( $\pm$ 15)	92 $\mu\text{m}^2$ ( $\pm$ 10.5)	145 $\mu\text{m}^2$ ( $\pm$ 7.7)
NGF + BDNF (100 ng/ml)	24 $\mu\text{m}^2$ ( $\pm$ 3.5)	50 $\mu\text{m}^2$ ( $\pm$ 4.2)	58.5 $\mu\text{m}^2$ ( $\pm$ 4.5)

Total neurite density (in  $\mu\text{m}^2$ ) was determined as described in Experimental Procedures.

n=15 lanes from 3 independent cultures for each treatment condition.

**Table 3.2 Quantitation of relative Trk phosphorylation following experimental treatments.**

Treatment	NGF	NGF + MC192	NGF + BDNF
30 min stimulation	1.88 ± 0.10 <sup>a</sup>	1.56 ± 0.11 <sup>a, b</sup>	1.38 ± 0.11 <sup>a, b, c</sup>
24 hr stimulation	1.72 ± 0.16 <sup>a</sup>	1.47 ± 0.08 <sup>a, b</sup>	1.24 ± 0.04 <sup>b, c</sup>

Data (mean relative optical density ± SEM, n=6 independent experiments) are expressed relative to the basal level (no NGF stimulation) for each independent experiment. Data were analysed using paired t-tests, as well as with repeated measures ANOVA with Bonferroni's multiple comparison post-test. **a** - sig. different from basal values,  $p < 0.001$  (NGF, NGF +MC192- 30 min) and  $p < 0.01$  (NGF+BDNF- 30 min; NGF+MC192 - 24 hr); **b** - sig. different from NGF treatment,  $p < 0.05$  (NGF+MC192),  $p < 0.01$  (NGF+BDNF - 24 hr) and  $p < 0.001$  (NGF+BDNF - 30 min); **c** - sig. different from NGF+MC192 treatment,  $p < 0.05$ .

**Figure 3.1 A. Distal neurites express TrkA, but not TrkB or TrkC.** Protein lysates were prepared from neurites growing into the side or distal compartments of compartment cultures containing 100 ng/ml NGF. Equivalent amounts of protein were immunoprecipitated (I.P.) with specific anti-Trk antibodies and probed with the same anti-Trk A (A), TrkB (B) or TrkC (C) antibodies (1° ab). Immunoblot analysis reveals that TrkA, but neither TrkB nor TrkC, is expressed on the distal neurites that have extended into the NGF-containing side compartments. **B. p75NTR and TrkA are expressed on both cell bodies and neurites.** The p75 receptor is expressed on both cell bodies (M, middle compartment) and distal neurites (S, side compartments) as shown by immunoprecipitation with anti-p75 antibody MC192 and probing with the same antibody (top panel, B). Immunoprecipitation for TrkA and probing with the same antibody (bottom panel, B) shows that TrkA is also expressed on both cell bodies (M) and distal neurites (S) in compartment cultures. d5, d7 - lysates were sampled at either 5 d or 7 d in vitro. Each blot is representative of a minimum of three experiments showing similar results.

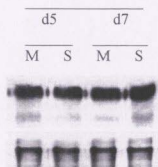


**A**

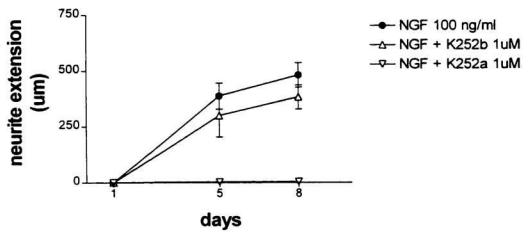
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A	B	C	1°ab



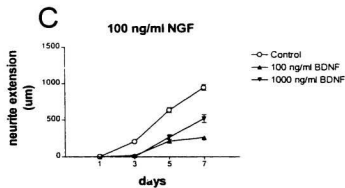
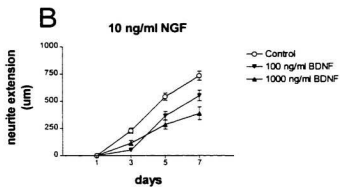
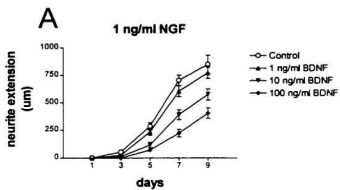
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**B****p75****Trk**

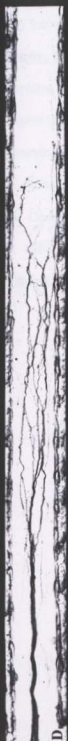
**Figure 3.2 TrkA activation is required for neurite growth.** Adult DRG neurons were plated in centre compartments and allowed to grow into distal or side compartments containing NGF (100 ng/ml) alone or with the inclusion of either K-252a (1 mM) or K252b (1 mM). K-252a, but not K-252b, inhibited the growth of distal neurites in compartment cultures. Each point represents lanes with growing neurites from 9-11 cultures  $\pm$  SEM.



**Figure 3.3 BDNF inhibits NGF-mediated neurite growth.** Neurite elongation of distal neurites extending into side compartments containing BDNF (0-1000 ng/ml) and NGF at concentrations of 1 ng/ml (A), 10 ng/ml (B) and 100 ng/ml (C). Linear growth (extension) was measured as described in the Experimental Procedures. A decrease in neurite elongation was observed in cultures containing NGF plus BDNF in comparison to NGF containing controls. Each point represents 96 lanes from 3 cultures  $\pm$  SEM.



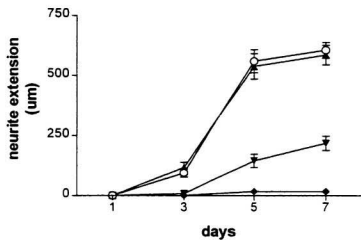
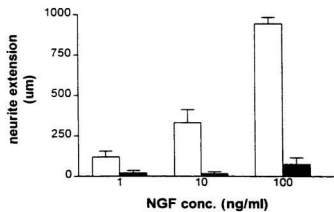
**Figure 3.4 Photomicrographs of distal neurite growth into NGF or NGF + BDNF containing compartments.** Representative examples of distal neurites growing into side compartments containing either NGF alone (1 ng/ml, A; 10 ng/ml, C; 100 ng/ml, E), or the corresponding cultures with NGF plus 100 ng/ml BDNF (B, D, F). Scale bar represents 100  $\mu$ m.



**Figure 3.5 The anti-p75 antibody MC192 inhibits NGF-mediated neurite growth. (A)**

Neurite extension of distal neurites extending into distal compartments containing 100 ng/ml NGF plus MC192 at concentrations ranging from 0.02-2 mg/ml was measured as described in Experimental Procedures. -○- control, 10 ng/ml NGF only; -▲- +0.02 mg/ml MC192; -▼- + 0.2 mg/ml MC192; -◆- + 2.0 mg/ml MC192. (B) The effects of MC 192 (1 mg/ml) on distal neurite growth cultured in varying NGF concentrations (1, 10 or 100 ng/ml). Neurite growth into compartments containing both NGF and MC192 was decreased compared to growth in NGF alone. Open bar - NGF alone; filled bar - NGF + 1mg/ml MC192. Each point in (A) represents 128-160 lanes from 4-5 cultures  $\pm$  S.E.M.; each bar in (B) represents 75 lanes from 3 cultures  $\pm$  S.E.M.



**A****B**

**Figure 3.6 NGF induced TrkA phosphorylation is decreased by co-treatment with BDNF and MC192.** Adult neurons were cultured in mass cultures for 2 days in the absence of NGF and subsequently treated for an additional 24 hours with no growth factors, NGF (10 ng/ml), NGF (10 ng/ml) + MC192 (1 mg/ml) or NGF (10 ng/ml) + BDNF (100 ng/ml). Cultures were then harvested for protein and immunoprecipitation with anti-TrkA was performed as described in Experimental Procedures. Blots were then sequentially probed with primary antibodies to phosphotyrosine (PY, ab4G10) and TrkA. This representative example shows an increase in the phosphorylation with all treatments compared to control; a decreased phosphotyrosine signal is observed when BDNF is co-applied with NGF. Lane 1 - no growth factor; lane 2 - NGF; lane 3 - NGF+MC192; lane 4 - NGF+BDNF.

1 2 3 4

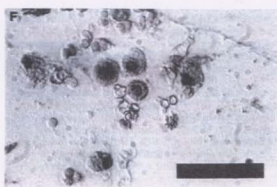
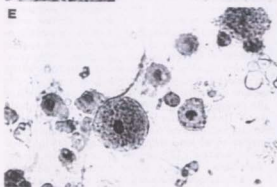
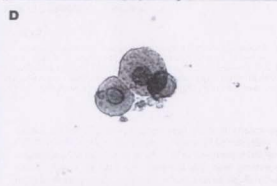
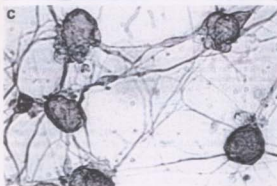
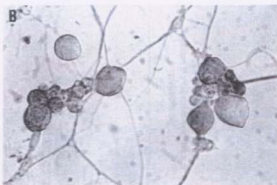
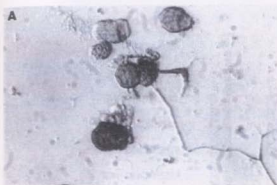


Trk

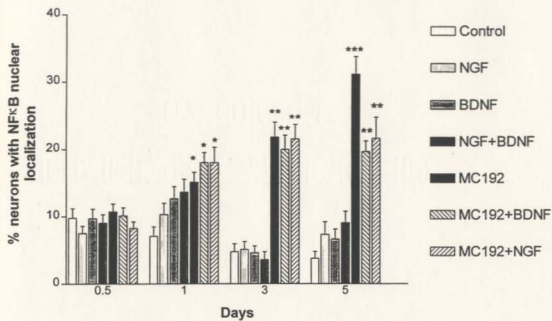


PY

**Figure 3.7 MC192 treatment results in activation of NF $\kappa$ B as assayed immunocytochemically.** Sensory neurons were cultured for 3 days with the following treatments: (A) no growth factors, (B) 10 ng/ml NGF, (C) 10 ng/ml NGF + 100 ng/ml BDNF, (D) MC192 (1 mg/ml), (E) 10 ng/ml NGF + 1 mg/ml MC192, (F) 100 ng/ml BDNF + 1 mg/ml MC192. Immunocytochemistry for NF $\kappa$ B was carried out as described in the Experimental Procedures. Increased numbers of cells displaying nuclear localization of NF $\kappa$ B were observed with the MC192 treatment. Scale bar represents 12  $\mu$ m.



**Figure 3.8 Quantitation of NF $\kappa$ B immunostaining.** The percentage of neurons displaying nuclear localization of NF $\kappa$ B was quantitated for each condition and the data presented in the histogram (mean  $\pm$  SEM). For each condition and each time point 400-500 neurons were evaluated. In all conditions containing MC192, a prolonged increase in the number of positive cells was observed, which is likely due primarily to the influence of MC192, since neither NGF nor BDNF alone effected a similar increase. Data were analyzed using ANOVA followed by Bonferroni multiple comparison post-testing. \* - significantly different from control,  $p < 0.05$  ; \*\* - significantly different from control, NGF, BDNF and NGF+BDNF,  $p < 0.001$ ; \*\*\* -significantly different from all other groups,  $p < 0.01$ .



#### **4.0 CHAPTER IV: Neurite growth promotion by Nerve Growth Factor and Insulin-like Growth Factor-1 in cultured adult sensory neurons. The role of Phosphoinositide 3-Kinase and Mitogen Activated Protein Kinase.**

##### **4. 1 Introduction**

The role of nerve growth factor (NGF) in the regulation of neurite growth has been the subject of numerous investigations. *In vivo*, NGF has been shown to be required for the collateral sprouting of undamaged nociceptive neurons into adjacent denervated target areas, although it has no observable effects on the regeneration of these neurons in the periphery (Diamond et al., 1992a,b; Mearow et al., 1994). *In vitro*, NGF induces neurite growth from adult sensory neurons in a variety of culture models (Lindsay 1988; Edstrom et al., 1996; Kimpinski et al., 1997). While peripheral sensory axon regeneration can occur in the absence of NGF, other growth factors, in particular the insulin-like growth factors (IGFs), and fibroblast growth factors (FGF) have been implicated in regeneration *in vivo* (eg., Danielson et al., 1988; Kanje et al., 1989; Glazner et al., 1993; Zhuang et al., 1996). These factors also promote neurite growth from sensory neurons *in vitro*. Insulin, IGF-I and IGF-II increase survival and neurite outgrowth in cultured embryonic sympathetic and sensory neurons (Recio-Pinto et al 1986), and IGF-I, but not IGF-II, also enhances neurite growth from cultured adult DRG neurons (Fernyhough et al., 1993; Akahori and Horie, 1996). Both FGF-2 (basic FGF, Malgrange et al., 1994) and FGF-1 (acidic FGF, Mohiuddin et al., 1996) promote adult DRG neurite growth *in vitro*. Although DRG neurons are reported to express EGF receptors (Huerta et al., 1996) there is little evidence for EGF influencing neurite growth.

These growth factors signal through specific receptor tyrosine kinases, and ligand



binding to the receptors results in the activation of a number of common intracellular signaling intermediates including Ras, mitogen activated protein kinase kinase (MEK), mitogen activated protein kinase (MAPK or ERK, extracellular-signal related kinase), phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) and phosphoinositide 3-kinase (PI 3-kinase) (reviewed in Greene and Kaplan, 1995; Folli et al., 1996; Yamada et al., 1997; Kaplan and Miller, 2000).

Information on signaling pathways important in or required for neuritogenesis come primarily from studies on the NGF-induced differentiation of PC12 cells, although emerging studies using primary neurons suggest that there may be differences in the contributions of the various components to either neurite growth or neuronal survival. Both the Ras-MAPK and PI 3-kinase pathways appear to be required for NGF-induced differentiation and neuritogenesis in PC12 cells with the Ras-MAPK pathway reported to be essential for differentiation and neuritogenesis in PC12 cells (Pang et al., 1995; Robinson et al., 1998; Korhonen et al., 1999). In primary neurons, inhibition of MAPK activation has been shown either have no effect (Klinz et al., 1996; Ganju et al., 1997) or to result in decreased neuritic growth (Creedon et al., 1996). PI 3-kinase activated pathways are suggested to play a primary role in the promotion of cell survival (Dudek et al., 1996; Philpott et al., 1997; Crowder and Freeman, 1998; Klesse and Parada, 1998), although PI 3-kinase has also been shown to be sufficient for neurite outgrowth (Kimura et al., 1994; Jackson et al 1996; Kita et al., 1996; Ashcroft et al., 1999; Korhonen et al., 1999). Since PI 3-kinase clearly plays a large role in the survival promoting effects of neurotrophins and other growth factors, its role in neuritogenesis may be overshadowed in experimental systems where the cells or neurons exhibit neurotrophin or growth factor-dependent survival. Another

signaling component reported to be uniquely associated with differentiation and neuritogenesis in PC12 cells is the SNT protein (Rabin et al , 1993; Peng et al., 1995). The FRS-2 protein recently isolated appears to be homologous to SNT, and has been shown to be important in PC12 cell neuritogenesis evoked by a number of growth factors (Wang et al 1995; Kouhara et al., 1997; Meakin et al., 1999).

It was the objective of this study to investigate the activities and possible interactions of NGF, IGF-1, FGF and EGF in eliciting neurite growth from adult sensory neurons, as well as investigating potential signaling pathways required for neurite growth. Our results indicate that NGF and IGF-I both promoted robust neurite growth in compartment and dissociated cultures, and that IGF-I acted to potentiate the effects of NGF. In addition, activation of PI 3-kinase and SNT were correlated with this growth response, while inhibition of PI 3-kinase was more effective in attenuating neurite growth from both NGF and IGF than was inhibition of MAP kinase kinase.

## **4.2 Materials and methods**

### **4.2.1 Dorsal Root Ganglion dissociation and culture methods.**

DRGs were dissected from young adult (100-120g) Sprague-Dawley rats (Memorial University of Newfoundland vivarium) and dissociated as previously described (Kimpinski et al., 1997; 1999). Briefly, ganglia were taken from all spinal cord levels and sequentially incubated with 0.25% collagenase (Gibco BRL, Life Technologies, Burlington, Ontario) for one hour and 0.25% trypsin (Gibco BRL) for 30 minutes. The ganglia were then mechanically dissociated by trituration through a series of flame-narrowed Pasteur

pipettes. The resulting cell suspension in L15 AIR medium (Gibco BRL) supplemented with 2.0% FBS, 100 units penicillin/streptomycin (P/S; Gibco BRL), and subsequently plated and maintained in serum-free L15 media with N2 medium supplement (Gibco BRL). Neurons were plated in the centre chambers of compartment cultures at a density of 5-6 ganglia per compartment culture. For dissociated cultures, 24- and 96-well plates were plated at a density of 5000-7000 and 20-50 neurons per well respectively for protein analysis and growth measurements. Cells were plated at a density of approximately 1500 cells per well in 96-well plates for survival assays. Media was supplemented with NGF (Cedarlane Labs, Hornby, Ontario), IGF-I (Sigma), bFGF (Sigma) or EGF (Sigma) where appropriate.

#### **4.2.2 Compartment cultures.**

Compartment cultures were constructed as previously described (Kimpinski et al., 1997, 1999). In all cultures the neurons were plated in the middle compartment in the absence of added growth factors and allowed to extend neurites into adjacent side (distal) compartments which contained medium with the added growth factors. Media supplied to the neurons and neurites was L15 AIR with the same supplements described above.

#### **4.2.3 Culture treatments**

Stock concentrations of all inhibitors were made in DMSO. During growth experiments and survival assays growth factors and inhibitors were added upon plating and maintained throughout the experiment. During experiments assessing the effects of

inhibitors on protein phosphorylation, cultures were grown in L15 media with N2 supplement for 3 days and then were washed with L15 media without additives several times. Cultures were then incubated with the inhibitor diluted in L15 media without additives for two hours, and subsequently stimulated using the same L15 media plus inhibitors and growth factors (100 ng/ml) for specified periods of time.

#### **4.2.4 Measurements of neurite growth**

Neurite growth was quantitated as previously described (Kimpinski et al., 1997; 1999). For measurements of neurite extension in compartment cultures, 12-16 lanes per side compartment per culture were counted. Extension data is expressed as the average of the total length a group of neurites extended across each lane from the point of emergence under the barrier into the distal compartment. Neurite outgrowth in mass or dissociated cultures was quantified by counting the number of neurons in a culture that produced processes twice the diameter of the cell body. The percentage of neurons with neurites versus the total number of neurons per culture for each treatment was determined. The data was plotted as the mean and standard error for each treatment at a particular time point assuming each culture as n=1.

#### **4.2.5 Neuronal survival/MTT assays**

Cultures were maintained for 2-3 days with the indicated growth factors and inhibitors. Cell survival was measured using a standard MTT cell proliferation/death assay (Promega Corp., Madison WI). Stock solutions of MTT in phosphate buffered saline (PBS,

5ug/ml) were diluted 1:10 with PBS, 200 ul added per well, and the plates incubated at 37°C for 2.5 hours. Media and dye solution were removed and the cells lysed with 250 ul stop solution (isopropanol/concentrated hydrochloric acid; 500:1) per well; 100 ul of lysate was transferred to a clean 96 well plate and the absorbency was read at 570 nm on an ELISA plate reader, with background subtraction at 630 nm.

#### **4.2.6 Immunoprecipitation and western blotting.**

Protein was extracted from cultures using lysis buffer containing TBS with 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 ug/ml aprotinin, 1 ug/ml leupeptin, and 0.5 mM sodium orthovanadate. Samples were taken by washing cells with Hanks Balanced Salt Solution with 0.5 mM Orthovanadate and lysed with 50-100 ul of buffer per compartment or well. Extracts from 3-4 wells in mass cultures were pooled for immunoprecipitation.

Lysates were pre-cleared in order to prevent non-specific binding by IgGs using 50% protein-A-sepharose (Santa Cruz Biotechnology, Santa Cruz CA)) at 4°C for 2 hours. The lysates were then immunoprecipitated with 1-2 ug specific antibody per reaction overnight at 4°C. The product was precipitated out with protein-A/Gplus-agarose (Santa Cruz). The precipitate was suspended in 25 ul sample buffer containing 10% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT; Gibco BRL), and 0.005% bromophenol blue (Sigma). For Western analyses, 30 ug of total protein in lysis buffer was combined with 5x sample buffer (as above).

Samples were electrophoresed on 10% SDS polyacrylamide gels and transferred

electrophoretically onto nitrocellulose membranes. The blots were blocked in 3% BSA/TBST (Tris-Buffered Saline and Tween-20 at 0.2% v/v), and subsequently incubated with primary antibodies (1-500-1:1000) overnight at 4°C. The protein bands were then visualized using secondary antibodies conjugated to horseradish peroxidase (anti-rabbit or anti-mouse IgG- HRP; Boehringer Mannheim, 1:10,000) and enhanced chemiluminescence (ECL; Amersham). The blots were then exposed to X-ray film and analysed. Antibodies used included anti- ERK1/MAPK, PLC $\gamma$ 1, PI 3-kinase, EGFR, TrkA, IRS-1 (Santa Cruz), anti-phosphotyrosine (4G10), IGFR1 (Upstate Biotechnology, Lake Placid, NY), anti-phosphospecific MAP kinase (New England Biolabs). p13<sup>wc</sup> agarose beads (Upstate Biotechnology) were used to precipitate SNT.

#### **4.2.7 Statistical analyses**

Data were analyzed for significance using ANOVA followed by Bonferroni correction for multiple comparisons.

### **4.3 Results**

#### **4.3.1 Growth effects of NGF, EGF and IGF on adult DRG neurons in dissociated cultures.**

Neurons plated in 96-well plates (mass cultures) were exposed to the various growth factors and number of neurons expressing neurites was evaluated over 60 hrs post-plating. The data, expressed as the mean %  $\pm$  SEM, are presented in Figure 4.1. The percentage of neurons producing neurites in response to NGF and IGF-1 was similar,

approximately 70% at 60 hours (Fig. 4.1C). EGF had a lesser effect but was still able to promote neurite growth in 45% of the neurons compared to the 20% of the neurons that exhibited neurites in control cultures (Fig. 4.1B), while the number of neurons with neurites in response to FGF was not significantly different from the control condition (Fig. 4.1A). Neither EGF nor IGF significantly increased the percentage of responding neurons when neurons were exposed to co-incubation with NGF (Fig. 4.1D).

#### **4.3.2 Growth effects of NGF, EGF and IGF on adult DRG neurons in compartment cultures**

Compartment cultures were used to further investigate the abilities of NGF, IGF, EGF and FGF to promote neurite growth when applied to specific neuronal regions (cell bodies versus distal neurites). Neurons were plated in the central compartments in the absence of added growth factors. NGF, IGF-I, EGF or bFGF were added to the distal compartments and neurite extension into those compartments quantified as described in the Materials and Methods. As shown in Fig 2, neither FGF (Fig. 4.2A) nor EGF (Fig. 4.2B) were able to promote much neurite extension into the distal compartments. In contrast, both NGF and IGF-1 supported substantial neurite extension (Fig. 4.2C-D). The length of distal neurites extension into the IGF-containing distal compartments was comparable to that in NGF, but the appearance of these neurites was somewhat different. Neurites growing into NGF exhibited more fasciculation, while IGF-1-induced neurites grew in a more random pattern. Representative examples of the neurite growth into EGF-, IGF-1- and NGF-containing distal compartments are presented in Figure 4.3.

We also tested whether combinations of the growth factors with NGF in the distal compartments would have any additive effects. Neurites growing into NGF alone or in combination with EGF or IGF-1 showed no differences in elongation (NGF  $1231 \pm 67$   $\mu$ m; NGF + EGF  $1058 \pm 78.5$   $\mu$ m; NGF + IGF  $1259 \pm 60.9$   $\mu$ m; 7 days in culture, n=96 lanes from 4 cultures per treatment). This data corresponds to those from mass cultures where neither EGF nor IGF potentiated the growth promoting effects of NGF. We then asked whether differential exposure of the neurons (i.e., cell bodies in the central compartments vs distal neurites in the side compartments) to the factors would have any effect. Figure 4.4 is a summary of the data obtained from this experimental series. (n= 5-6 cultures per treatment; the mean neurite extension measured at d7 is presented for each condition). Provision to the cell body compartment of NGF or any of the other factors alone or in combination did not promote anterograde growth of the neurites into distal compartments not containing any growth factors. Of the factors tested, only IGF-I when applied to the cell body compartment had any effect on neurite growth into NGF-containing side compartments. Application of IGF to the cell bodies resulted in an almost 2-fold enhancement of neurite growth into NGF compared to the NGF-induced neurite growth in the absence of the IGF treatment (Fig. 4.4C). This effect appeared to be due primarily to the potentiation of the NGF-induced growth since IGF-1 does not promote anterograde growth of distal neurites into compartments containing no growth factors.

#### **4.3.3 Effects of the inhibition of MEK and PI 3-kinase on the survival of adult DRG neurons.**



Prior to assessing the effects of MEK and PI 3-kinase inhibition on neurite growth, we examined whether the inhibitors would influence the survival of these adult neurons as they do for embryonic or neonatal DRG neurons (Mearow, unpublished observations). The results of the MTT assays are presented in Figure 4.5 and show that inhibition of MEK (PD 98059) or PI 3-kinase (LY29004) had no significant effects on the survival in the presence or absence of EGF, IGF-1 or NGF. The lack of significant survival effects indicates that further experiments to measure neurite outgrowth using these compounds are not biased by cell death in our cultures.

#### **4.3.4 Effects of the inhibition of MEK and PI 3-kinase on neurite outgrowth**

Inhibition of the activation of MAP kinase by PD98059 had no significant effects on the percentage of neurons exhibiting neurite outgrowth in mass cultures in response to NGF or EGF (Fig. 4.6A-C). PD98059 did produce a slight inhibition of growth in IGF-1 at concentrations of 10 and 100  $\mu$ M (Fig. 4.6B).

In contrast to MAPK inhibition, the inhibition of PI 3-kinase by LY29004 had a significant effect on neurite-producing neurons in response to NGF and IGF-1 (Fig. 4.7A-B), but his effect was not as pronounced with respect to EGF (Fig. 4.7C). It should be noted that while 20  $\mu$ M LY29004 inhibited growth significantly, a high concentration of the inhibitor was also employed for comparison and to determine if growth could be blocked completely. This high concentration was effective in essentially abolishing the growth in IGF but not in the presence of NGF, suggesting the contribution of other pathways.

#### **4.3.5 Biochemical confirmation of the effects of the inhibitors**

Western blotting of lysates obtained from control and NGF stimulated cultures treated with PD98059 or LY29004 was used to assess whether the inhibitors were acting as expected. Lysates were sequentially probed with phospho-specific Akt or MAPK antibodies (Fig. 4.8, pAkt or pMAPK) and then reprobed with anti-Akt or MAPK (Fig 8, Akt or MAPK). As shown in the figure, in the presence of NGF there is phosphorylation of MAPK and Akt (a downstream target of PI 3-kinase) compared to the control, non-stimulated cells. The presence of 50 mM PD98059 during the NGF stimulation results in an inhibition of the MAPK activation. Similarly, the presence of the LY29004 during the NGF stimulation inhibits the Akt activation. These results demonstrate that the inhibitors are acting to inhibit MEK and PI 3-kinase actions as they were assumed to do.

#### **4.3.6 Biochemical analysis of the effects of NGF, IGF-1 and EGF on signaling intermediates**

To extend the observations obtained with the inhibitors on neurite growth, we investigated the potential signaling intermediates activated by NGF, IGF or EGF, that might underlie the biological responses (i.e., neurite growth). Stimulation experiments were carried out as described in the Materials and Methods. Equivalent amounts of protein from the cell lysates were immunoprecipitated with anti-PI 3-kinase, anti-ERK1, anti-PLC $\gamma$ 1 (Santa Cruz) or incubated with p13<sup>Suc1</sup>-conjugated agarose beads. Following electrophoresis and transfer of the IP samples to nitrocellulose, the blots were sequentially probed with anti-phosphotyrosine (anti-pY, clone 4G10), followed by the primary antibody

used for the immunoprecipitation (eg., samples immunoprecipitated with anti-ERK1 were probed with anti-pY and anti-ERK1). Representative examples of these experiments are presented in Figure 4.9.

As seen in Figure 4.9A, all the factors tested activated MAPK/ERK1 in these neurons as expected. Although there was some basal activation in these experiments, the growth factor stimulation was able to increase MAPK/ERK phosphorylation above the basal levels. The effects of NGF were more pronounced than either EGF or IGF-1. Our results indicated that while there is a detectable activation at 15 min of stimulation, the MAPK activation is maintained for the full 60 min.

PLC $\gamma$ 1 is another signaling intermediate that is recruited to the TrkA receptor after NGF binding. We examined the activation of PLC $\gamma$ 1 by NGF and IGF; because of limited amounts of samples, only these two factors which promoted the most neurite growth were assessed. NGF stimulation of the neurons for 15-60 min resulted in phosphorylation of PLC $\gamma$ 1, with the peak of the activation appearing at 30 min and declining thereafter. In contrast, IGF-1 treatment did not result in any detectable PLC $\gamma$ 1 phosphorylation.

Phosphorylation of PI 3-kinase was greater than basal levels following stimulation for 60 min with both IGF-1 and NGF, while EGF stimulation did not result in increased phosphorylation above the basal levels (Fig. 4.9C).

SNT/FRS2 is also a target of Trk phosphorylation and is activated independently of other signal transduction pathways during RTK signaling (Rabin et al 1993). Stimulation (60 min) with NGF, IGF-1 and FGF activated SNT/FRS2, while EGF did not activate SNT above control levels (Fig. 4.9D). A time course of the activation of SNT was carried out for

NGF and IGF and showed a very strong phosphorylation signal at 45 min of NGF stimulation and at 60 min for the IGF.

#### **4.4 Discussion**

In this study we have examined the activity and interactions of NGF, IGF-1, EGF and bFGF in promoting neuritic growth from adult rat sensory neurons. In dissociated cultures, neurite growth was assayed by quantitating the percentage of plated neurons that elaborated neurites over the time course of the experiments. In these experiments, NGF and IGF-1 resulted in the highest levels of neurite growth; the effect of bFGF and EGF were indistinguishable from the control cultures (neurons grown in the absence of added growth factors). There were no significant additive effects observed when combinations of the factors were tested in the dissociated cultures. However, in these cultures the presence or absence of neurites, but not the amount of growth was the assayed variable. In order to quantitate the extent of growth and to examine potential interactions we employed the compartment culture system Kimpinski et al., 1997).

In the compartment system we measured the distal neurite extension into side compartments containing different growth factors initially. The results here again showed that both NGF and IGF-1 elicited robust distal neurite growth, but there was minimal growth into either bFGF or EGF. We then examined the effect of various combinations of growth factors – application to the cell body compartment alone, to the distal compartment alone or in both compartments. Co-application of NGF +EGF or +bFGF in any combination had no added effect compared to the results seen with NGF alone. When NGF +IGF-1 were

both provided in the distal compartments, there was a slight increase above that seen with either factor alone. However, the largest effect was observed when IGF-1 was provided to the cell bodies and the NGF to the distal neurites; in these experiments, the growth into the NGF-containing compartments was almost double that in the control situation (where there was no growth factor in the central compartment).

Immunocytochemistry for TrkA, EGFR, and IGFR1 indicated that all three receptors are expressed in our cultures of adult DRG neurons (data not shown), and while we did not assess whether individual neurons were expressing more than one of the receptors, our estimates of the percentages of neurons expressing TrkA ( $64.4 \pm 2.5\%$ ), EGFR ( $71.3 \pm 4.5\%$ ) or IGFR ( $70.9 \pm 3.9\%$ ) would suggest that there is overlap in the expression profiles. Previous work has demonstrated that DRG neurons express a variety of growth factor receptors including the EGF receptor (Huerta et al., 1996), FGF 1 receptor (Grothe et al., 1997), insulin and IGF 1 receptors (Karagiannis et al., 1997); mRNA for an insulin-related receptor has also been shown to be co-expressed with TrkA mRNA in DRG neurons (Reinhardt et al., 1997).

We were also interested in investigating what potential signalling pathways were involved in promoting neurite growth. Adult DRG neurons do not require exogenous neurotrophins for their continued survival in vitro (Lindsay et al., 1988; Kimpinski et al., 1997, 1999), and so it is potentially possible to distinguish neurite-promoting influences of the neurotrophins from those required for survival. The signal transduction pathways activated by NGF binding to TrkA have been the subject of numerous investigations and are similar in many respects to those activated by other tyrosine kinase growth factor

receptors (i.e., EGFR and IGFR) (Folli et al 1997; Kaplan and Miller, 2000). While much of this work has been carried out on cell lines there are more and more studies using primary neurons that suggest that although the general concepts are similar, the activation and contribution of the different signalling components depends on the cell types and context of the experiment. Following binding of NGF to Trk, a number of proteins are recruited to the activated receptor, including PLC $\gamma$ 1, Shc, FRS2/SNT. This recruitment serves to link the receptor to intracellular signalling pathways such as the Ras-MAPK and PI 3-K cascades, shown to be required for NGF-induced neuritogenesis and survival in a variety of cell types. While it seems clear that signalling via PI 3-K and its downstream targets plays an important and primary role in cell survival, the evidence for the requirement of the Ras-MAPK pathway alone for neurite growth in primary neurons is less convincing.

Our data indicates that inhibition of MAPK activation had little effect on NGF-evoked neuritogenesis, although it did decrease the percentage of cells with neurites exposed to IGF-1. However, it should be noted that this particular inhibitor may not result in complete inhibition of MAPK activation. PD 98059 is a selective inhibitor of MEK1 activation, which should lead to inhibition of activation of its downstream target, MAPK. However, it does not inhibit activated MEK and it has also been noted that in response to strong activators such as NGF or EGF, residual MEK activity may be sufficient to activate MAPK (Alessi et al., 1995). Our results would further suggest that PI 3-K contributes to neurite growth in these cells, since inhibition of PI 3-K resulted in significant decreases in the percentage of neurons with neurites in both the NGF and IGF-1-treated cultures. Our Western blotting

results show that all the factors induce activation of MAPK, although there is a more pronounced increase over the control condition with NGF. PI 3-K is more strongly phosphorylated by IGF than NGF or EGF, and all factors except EGF result in the phosphorylation of SNT/FRS2. Interestingly, IGF does not phosphorylate PLC $\gamma$ 1, while NGF does so as expected; this result has been observed following IGF treatment of other cell types (Seely et al., 1995; Choi et al., 1995).

Previous reports have provided evidence that MAPK activation is a requirement for NGF-dependent neuritogenesis in PC12 cells (Cowley et al., 1994; Pang et al 1995), while PLC $\gamma$ 1 is required for FGF-induced neuritogenesis, although it may act via downstream activation of MAPK (Hall et al.,1996). FRS2/SNT also links the FGF-R to the MAPK pathway (Kouhara et al., 1997;Wang et al., 1996), and has been further implicated as necessary for NGF-induced neuritogenesis in PC12 cells (Meakin et al., 1998). Over expression of activated MEK promotes neuritogenesis (Robinson et al., 1997), but Over expression of dominant inhibitory MEK does not inhibit outgrowth (Sarner et al., 2000). In contrast to the results of experiments with cell lines, inhibition of MAPK activation has been reported to have little or no effect on neurite growth from primary neurons (Creedon et al., 1997; Klinz et al., 1996; Ganju et al., 1998).

The effect of the MEK inhibitor on IGF-induced growth compared to NGF-induced growth may relate to the fact that IGF apparently does not activate PLC $\gamma$ 1. In a situation where both PLC $\gamma$  and MAPK can be activated (i.e., with NGF), the inhibitory effect of the PD98059 on MEK may be somewhat attenuated by concomitant activation of the pathway via PLC $\gamma$ 1 (Stephens et al 1994) and one might not expect to see a large effect on

neuritogenesis. In the case where PLC $\gamma$  is not activated (i.e., with IGF), then inhibition of MEK may have more influence on the growth response.

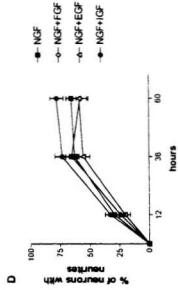
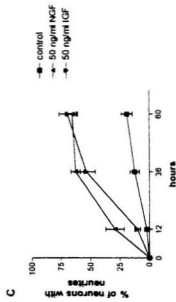
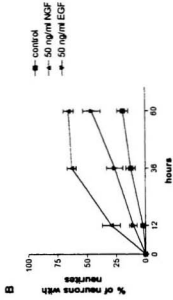
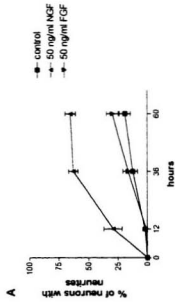
The PI 3-K pathway also plays a role in neuritogenesis, and our results would suggest that for these primary neurons it is a major contributor to the growth response. PI 3-K has been shown to be required for growth factor-induced changes in cell cytoskeletal elements (eg., Feldman et al., 1997; Jackson et al., 1996) and IGF -1 has been shown to enhance growth cone motility via the PI 3-K pathway in neuroblastoma cells (Feldman et al., 1997). Recently it has been shown that Over expression of the Gab1-docking protein, which binds to Grb2 and PI 3-K and can link TrkA to both PI 3-K and MAPK signalling pathways, induces neurite growth and survival in PC12 cells by activating both pathways, although the MAPK pathway appears to be indispensable for the neuritogenesis response (Korhonen et al., 1999). Over expression of activated PI 3-K also promotes neurite growth in neuroblastoma and PC12 cells (Kita et al., 1998; Jackson et al., 1996). In PC12 cells, PI 3-K may be more important for neurite extension rather than for the initiation of neurites (Ashcroft et al., 1999), suggesting that a sustained activation of PI 3-K may be required for neuritogenesis

While our results would support a primary role of PI 3-K and a lesser role for MAPK in neurite outgrowth induced by NGF and IGF-1, it is much more likely that several converging or overlapping signaling pathways are interacting at various points in the cellular response to result in neuritogenesis. For example, while we have focused our investigations on PI 3-K and MAPK, others have shown that PI 3-K acting via Jun N-terminal kinase (JNK) also contributes to neuritic growth in PC12 cells (Kobayashi et al.,

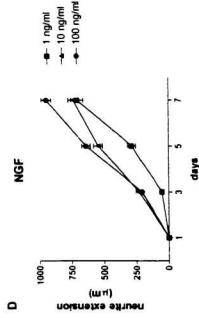
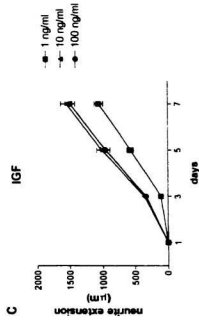
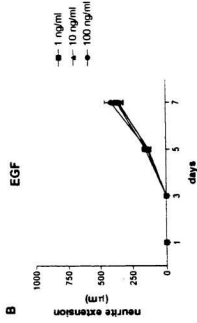
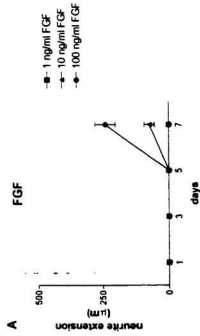


1997). Ras is known to be required for neurite growth and survival in a variety of cells, and while the most commonly cited pathway is Ras-MAPK, Ras can also activate PI 3-K (eg., Downward, 1997). The potentiating effect of IGF on NGF-evoked neurite growth could be explained by the suggestion that neuritogenesis requires a threshold level of signaling strength and duration, and that the rate of responsiveness can be accelerated as the signal is enhanced (Greene and Kaplan, 1995). Why other combinations of growth factors do not elicit such a response is not clear, although it could be that NGF stimulates the signaling pathways sufficiently and that further enhancement by other growth factors is not detectable in the experimental system used in these studies. In this regard, in ongoing experiments, we have observed that although the percentage of neurite-bearing neurons is not different, the extent and complexity of neuritic growth is substantially increased in the presence of dual growth factor combinations and this can be differentially influenced by inhibition of the various signaling pathway components (Jones, Rahimtula and Mearow, in preparation).

**Figure 4.1 Neurite Growth in mass or dissociated cultures.** Neurons were plated at low densities in 96 well cultures and measured for the percentage of neurons extending neurites versus total neurons per culture. Both application of EGF (B) and IGF-1 (C) increased the percentage of neurite bearing neurons per culture above control (no growth factor), while FGF has little effect on neurite growth. However neither IGF-1 nor EGF increased the percentage of responding neurons when co-applied with NGF in comparison to NGF alone (D). Each point represents n=12-16 cultures ( $\pm$  SEM) per time point.

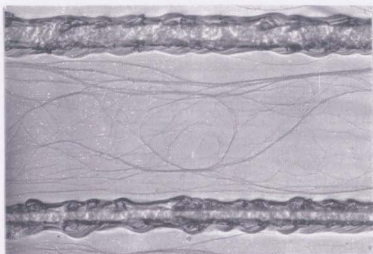


**Figure 4.2 Neurite growth in compartment cultures.** Neurons were plated in middle compartments of three well compartment cultures containing no growth factors; side compartments contained the indicated growth factors . Measurements were made of neurite growth into distal compartments containing 1-100 ng/ml of the indicated growth factors over 7 days. FGF (A) and EGF (B) elicited only minimal neurite growth, while both IGF (C) and NGF (D) evoked more substantial growth. Each point represents n=96 lanes ( $\pm$  SEM) from four separate cultures.

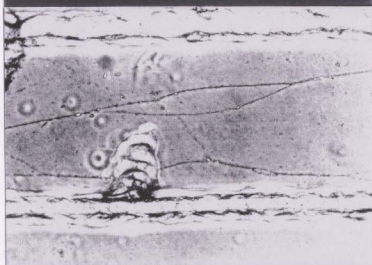


**Figure 4.3 Representative examples of distal neurites growth.** Phase contrast photographs were taken at 7 days *in vitro* of neurite growth into side compartments containing NGF (A), EGF (B) or IGF-1 (C) at 100 ng/ml. Scale bar represents 100  $\mu$ m.

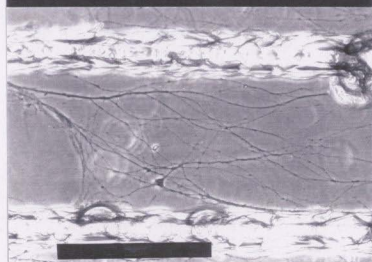
A



B

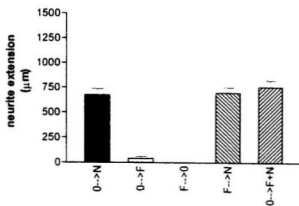
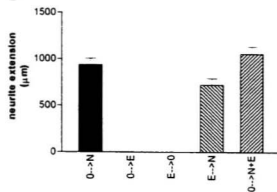
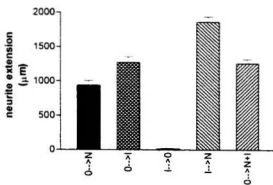


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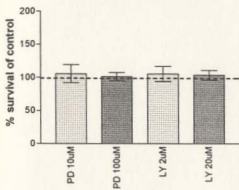
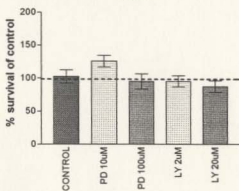
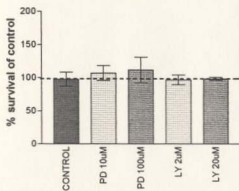
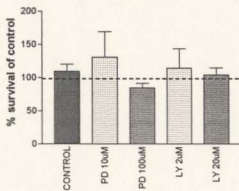


**Figure 4.4 Schematic summary of growth data in compartment cultures.** Neurite extension results (mean + SEM, n= 5-6 cultures from 3 separate culture experiments; 7 div) from combinations of growth factor manipulations are presented for FGF (A), EGF (B) and IGF (C) compared in each case to the NGF control condition (0->N). 0->N, I, E or F - indicates 0 growth factor in the center compartment and the indicated factor in the distal compartment. E, I, F, or N->0 - the indicated growth factor is present in the center compartment, but absent from the distal compartment. E, I or F->N - the indicated factors are present in the centre compartment with NGF in the distal compartment.

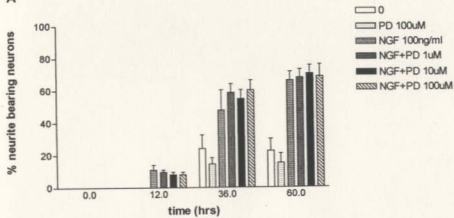
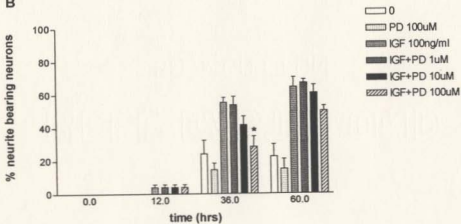
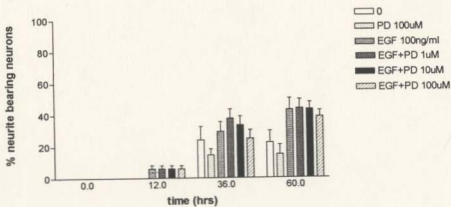


**A****B****C**

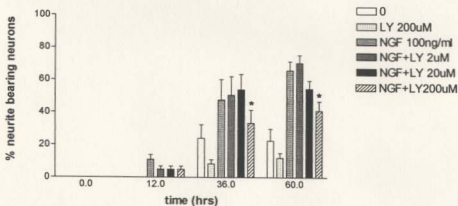
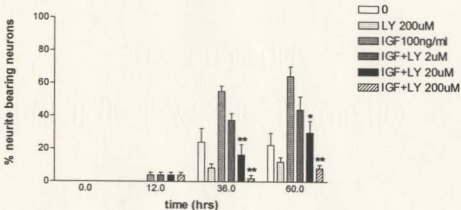
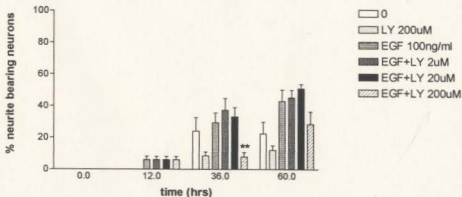
**Figure 4.5 Survival effects of inhibitors of MAP kinase kinase and PI 3-kinase on adult DRG neurons.** Neurons were cultured for three days with the inhibitors PD (PD98059) or LY (LY294002) and subsequently assayed for cell survival using an MTT-based colorimetric assay. None of the inhibitors tested resulted in significant decrease in cell survival in the absence of growth factors (A), or in the presence of NGF (B), IGF (C) or EGF (D). Data are presented as % of survival compared to the control (the absence of the inhibitor in the absence of presence of the appropriate growth factor). Each bar is representative of n=3 independent experiments  $\pm$  SEM.

**A****NO GROWTH FACTORS****B****+ NGF****C****+ IGF****D****+ EGF**

**Figure 4.6 Effect of MEK inhibition on neurite growth.** Neurons were plated at low densities in 96 well cultures and measured for the percentage of neurons extending neurites compared to total neurons per culture. The MEK inhibitor PD 98059 (10-100  $\mu$ M) did not have significant effects on neurite promotion by NGF (A) or EGF (C); the highest concentration of PD 98059 (100  $\mu$ M) resulted in a significant decrease in the percentage of neurite-bearing neurons in the IGF cultures. Each point represents  $n=12-16$  cultures  $\pm$  SEM per time point \* - significantly different from growth factor control ( $p < 0.01$ ).

**A****B****C**

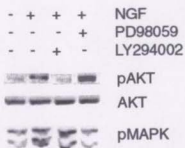
**Figure 4.7 Effect of PI 3-kinase inhibition of neurite growth.** Neurons were plated at low densities in 96 well cultures and measured for the percentage of neurons extending neurites versus total neurons per culture. The PI 3-kinase inhibitor LY294002 significantly decreased the percentage of neurite bearing neurons in IGF-containing cultures at 36 and 60 hrs (B). In the NGF-containing cultures, significant decrease in neurite-bearing neurons was observed at 60 hrs (A), while in the EGF cultures, only the highest concentration of LY294002 had a significant effect (C). Each point represents  $n=12-16$  cultures  $\pm$  SEM per time point. \* - significantly different from growth factor control ( $p < 0.01$ ), \*\* -  $p < 0.001$ .

**A****B****C**

**Figure 4.8 Biochemical assessment of the effects of the MEK and PI 3-kinase inhibitors.** Neurons were cultured as described and treated with 100  $\mu$ M LY294002 or PD98059 for 2 hrs and then stimulated with 50 ng/ml of NGF for 10 min. Protein lysates were prepared and equivalent amounts run on a 10 % SDS-acrylamide gel. Western blotting using the indicated antibodies was carried out as described in the Materials and Methods. NGF induces phosphorylation of Akt (pAKT) and MAPK (pMAPK) that is inhibited by LY29004 or PD98059, respectively. The blots were sequentially probed with the non-phosphospecific antibodies to indicate the amount of specific protein present (Akt or MAPK).



A

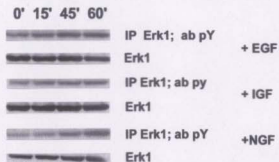


B

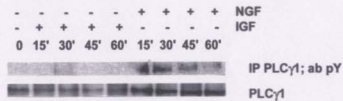


**Figure 4.9 Biochemical analyses of signaling components activated by NGF, IGF or EGF.** Neurons were stimulated for the indicated times with EGF, IGF or NGF. Subsequently, protein lysates were prepared as described in the Materials and Methods. 50-100 ug of protein were then subjected to immunoprecipitation with the indicated antibodies; immunoprecipitates were electrophoresed and the blots were sequentially probed with the 4G10 anti-phosphotyrosine antibody (pY) and then the antibody used for immunoprecipitation (IP). **Panel A:** Lysates were immunoprecipitated with anti-ERK1 at the indicated time points and probed with anti-phosphotyrosine (pY) to determine MAPK activation. Activation was increased (compared to the 0 time point) with growth factor treatment by NGF and to a lesser extent by IGF-1 and EGF. **Panel B:** Lysates from cultures treated with NGF or IGF for the indicated times were immunoprecipitated with anti-PLC $\gamma$ 1 and probed with anti-phosphotyrosine (pY), followed by anti-PLC $\gamma$ 1. Only NGF was effective in PLC $\gamma$  activation as assessed by the anti-pY probe. **Panel C:** Lysates from cultures treated with growth factors for 1 hr were immunoprecipitated with anti-PI 3-kinase (p85) and probed with anti-pY, followed by anti-PI 3-kinase. All the factors resulted in phosphorylation of PI-3 K compared to the control, although both IGF and NGF appeared more effective than EGF. **Panel D and E:** Lysates from cultures treated with the indicated growth factors for 1 hr were incubated with p13suc1-conjugated agarose beads to precipitate SNT. The IPs were then probed with anti-phosphotyrosine (pY). Note that EGF is ineffective in phosphorylation of SNT. Blots are representative examples of triplicate experiments.

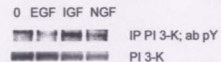
A



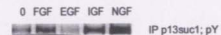
B



C



D



## **5.0 CHAPTER V: DISCUSSION and CONCLUSIONS**

### **5.1 Analysis of neurite growth in compartment cultures.**

The basic theme of this thesis was to determine the mechanisms regulating growth in adult DRG neurons. Adult neurons were selected for their clinical significance in neurological disease. Many neurological diseases involve older neurons. These neurons have a diminished capacity for growth compared to their neonatal counterparts. In order to address such problems as neuropathy, studies such as this involving adult neurons are required.

As mentioned previously, mature DRG neurons survive independent of exogenous trophic factors, eliminating confounding variables related to survival responses. This advantage of mature DRG neurons has facilitated the study in conditions where cells exhibit naive *in vitro* responses to exogenously applied neurotrophins and growth factors (Lindsay 1988). This is an important point in regards to compartment culture studies because adult DRG neurons can be plated in compartments without added trophic support. Distal neurites can grow into adjacent side compartments without previous interaction with other neurotrophins in direct contrast to sympathetic neurons which require NGF (Campenot 1977). Therefore it was possible to study the ability of neurotrophins to elicit distal neurite growth free of external complicating factors. We found that of the three neurotrophins studied (NGF, BDNF and NT-3) only NGF was able to produce significant neurite growth (Kimpinski et al., 1997; see chapter 2).

This was an important study based on the fact that other work had only dealt with neurotrophins in mass cultures (Mohiuddin et al., 1995; Yasuda et al., 1990; etc). DRG

neurons *in vivo* extend their processes over long distances (up to one metre) producing a situation where neurite terminals and cell bodies are subjected to different environmental conditions. Unlike mass culture systems, the compartment culture better replicates the *in vivo* situation. Few studies had classified the responses of neurites to direct neurotrophin application by eliminating complicating interactions at the cell body. To the author's knowledge this is the first reported use of adult DRG neurons in compartment cultures.

Our results did differ somewhat using the compartment culture in comparison to mass cultures. For example, NT-3 did not have the same neurite promoting effects when placed solely at neurite terminals compared to global application (Kimpinski et al., 1997; Mohiuddin et al., 1995; see chapter 2). Several reasons could account for this finding. First, NGF has a higher affinity for TrkA than NT-3 and this higher affinity for TrkA may be necessary to produce sufficient activation to elicit distal neurite growth (Rodriguez-Tebar et al., 1992). In neonatal sympathetic neurons, increased concentrations of NT-3 are required to produce equivalent neuritogenesis compared to that produced by lesser amounts of NGF (Belliveau et al., 1997). These results may point to specific differences in the actions of NGF and NT-3 on distal neurites *in vivo*. Perhaps these differences may be important during innervation of specific end targets during embryogenesis or after axotomy.

Second, the percentage of neurons responsive to NT-3 may not be adequate to produce distal neurite growth in compartment cultures. Depending on the age of the DRG and the study quoted, neurons expressing TrkC only constitute 10-20% of the cell in the

ganglia (Mu et al., 1993; McMahon et al., 1995; Kashiba et al., 1996). Lastly, NT-3 may play distinct roles in adult neurons by regulating function within the DRG rather than inducing neurite growth (Airakensinen et al., 1996; Mendell 1999).

The reason why distal neurite growth occurs with NGF but not NT-3 may provide a better understanding of neurotrophin functioning. The lack of an effect for NT-3 could be a result of different binding kinetics exhibited by NT-3 versus NGF toward the p75 receptor (Rodriguez-Tebar et al., 1990,1992 but see Mahadeo et al., 1994). NGF remains bound to p75 for longer periods in comparison to NT-3 (Rodriguez-Tebar et al., 1990,1992). It has been postulated that p75 interactions may allow for the discrimination among the various neurotrophins in different cell types (Rodriguez-Tebar et al., 1990,1992; Carter et al., 1996). We have observed that distal neurites express p75 at significantly lower levels than found on cell bodies (Kimpinski and Mearow unpublished data). Perhaps p75 levels on distal neurites optimize TrkA-NGF interactions given the nature of NGF-p75 binding. In comparison, the shorter periods of p75 binding by NT-3 may be insufficient to produce neurite growth. Where p75 is highly expressed on cell bodies, in mass cultures this may result in sufficient activation by NT-3 to produce neurite growth.

It was noted that the rate of regeneration after axotomy was significantly increased in neonatal neurons. However the rate of elongation for both neonatal and adult neurons were comparable. Several factors could account for these findings. First, it was obvious that growth of distal neurite was more robust in neonatal neurons. It is likely that more neurons grew under the silicon barrier and as a result there was a greater response after axotomy. Second, neonatal neurons at this time point (post natal day 1) are innervating

peripheral targets and may be more able to adapt to processes such as axotomy when compared to adult neurons. Third, as mentioned previously it is a widely held tenet in neuroscience that older neurons do not regenerate (if at all) as well as younger neurons. Further study is needed to fully address this question. Such studies were out of the context of this thesis due to our intention to focus on adult neurons.

## **5.2 Contributions of the NGF receptors TrkA and p75 to the growth response.**

With initial experiments indicating a prominent role for NGF in compartment cultures of adult DRG neurons, our next objective was to determine the individual contributions of the NGF receptors. We used a number of experimental manipulations to determine the roles of TrkA and p75 in distal neurite growth. As expected growth was absolutely dependent on TrkA. However p75 functioning appeared to have two influences on neurite growth depending on the experimental circumstance.

Inhibition of NGF binding to p75 by excess BDNF (via competition for the p75 receptor) reduced, but did not completely inhibit, distal neurite growth. Using the monoclonal antibody MC192 to alter NGF binding to p75 suppressed practically all neurite growth. MC192 acts to increase binding of the neurotrophins to the p75 receptor (Chandler et al., 1984). In this case, based on NF $\kappa$ B immunocytochemistry, MC192 but not BDNF (alone) treatment resulted in p75 activation. The outcome of simply blocking interaction of p75 and NGF may act to decrease optimum activation of TrkA receptors resulting in a decrease in neurite growth (Barker and Shooter 1994). However, it is also possible that activation of p75 by MC192/NGF may generate intracellular signals inhibitory

to neurite growth (Dobrowsky et al 1994; Posse de Chaves et al., 1997; MacPhee and Barker 1997). In certain circumstances, p75 activation independent of Trk activity is important for regulating neurite growth. Such possibilities could exist in development during competition for target innervation and in the adult during growth responses such as regeneration and collateral sprouting (Diamond et al., 1976 and 1991a,b). The Mearow lab is particularly interested in these phenomena and data from compartment cultures could provide a clue as to how these receptors are functioning *in vivo* (Mearow et al., 1994). It may be that while TrkA is required for this process, p75 is also necessary to produce an optimal response (Mearow and Kril 1995; Diamond et al., 1995).

Other studies indicate that p75 activation is capable of generating ceramide (Dobrowsky et al., 1994) and that intracellular ceramide is inhibitory to neurite growth (Posse de Chaves et al., 1997). Our work also correlates p75 activation with inhibition of neurite growth (Kimpinski et al., 1999; see chapter 3). Further study using DRGs in compartment cultures could provide the basis to consolidate the above information with work done by Barker and colleagues (LaChance et al., 1997; MacPhee and Barker 1997).

### **5.3 Growth effects in DRG neurons versus other cell types: Response to NGF.**

Experiments with DRG neurons in compartment cultures reveal several important differences from previous experiments using neonatal sympathetic neurons. Upon NGF withdrawal, distal neurites of sympathetic but not adult DRG neurons retract back to compartments housing cell bodies (Campenot 1982a; Kimpinski et al., 1997; see chapter 2). Compartments containing sympathetic cell bodies require NGF, because of this fact



the experimental paradigm is different in the case for DRG neurons. However this finding does illustrate the different growth effects produced by NGF on different cell types (Campenot 1982b). The different responses of sensory and sympathetic neurons in this case are probably due in part to the differences in survival requirements.

Overlapping effects of survival due to NGF within the same cell type complicate conclusions drawn about growth effects. This is particularly relevant in PC12 cells where experimental interventions that block neurite growth may in fact be impinging on mechanisms responsible for survival and differentiation. Inhibition of processes involved in NGF induced differentiation consequently block neurite growth. This could be a contributing explanation for why inhibition of NGF signalling pathways are not generally applicable from PC12 cells to neurons. For example blocking MAPK activity resulting from NGF treatment in PC12 cells inhibits neurite formation (Pang et al., 1995). However similar experiments in adult DRG neurons have no effects on growth (Klinz et al., 1996).

The fact that the effects of NGF on PC12 cells are not always reproducible in primary neurons is an interesting concept in itself. Treatment of PC12 cells with NGF induces differentiation into cells resembling (morphologically and biochemically) sympathetic neurons. The differentiation process has been reported to be biphasic in nature where the early phase is characterized by sharpened cellular morphology and the later stage by neurite growth (Kimura et al., 1994). Late phase neurite growth can be inhibited by blocking PI3-kinase with wortmannin, while earlier phases may be dependent on other signals including the ras/MAPK pathway (Kimura et al., 1994). However more recent studies have suggested that PI3-kinase may have limited effects on neurite growth and that PI3-kinase expression alone cannot maintain neurites over extended periods of

time (Hallberg et al., 1998; Ashcroft et al., 1999). In contrast our findings indicate that PI3-kinase is a major signalling pathway in eliciting neurite growth in adult DRG neurons (see chapter 4).

In sympathetic neurons there appears to be an overlapping of survival effects resulting from NGF signalling pathways. Both PI3-kinase and ras pathway activation have been reported to be sufficient to maintain the survival of sympathetic neurons (Crowder and Freeman 1998; Philpott et al., 1997; Nobes et al., 1996; see also introduction section 2.2, Trk signal transduction). This raises two questions regarding adult DRG neurons. Can specific pathways be ascribed only to a single function or do pathways that regulate neuronal survival serve redundant functions such as neurite outgrowth? In fact there is evidence that NGF signalling pathways do serve redundant functions. In PC12 cells, PLC $\gamma$ 1 and MAPK have overlapping effects (Stephens et al., 1994). Given the vast number of the cellular changes that must occur to result in a process as complex as survival or neurite growth it would be assumed that there would be an inherent redundancy. This may be especially relevant when it is applied to measuring neurite growth in general rather than examining further end point measurements (up regulation of genes involved in growth, synthesis of structural proteins, etc.)

Perhaps neurite growth is reliant on several pathways, each responsible for different aspects of the process. It may be that pathways that are rate limiting are those that have been described as "required". These processes are likely to be quite different in primary neurons versus PC12 cells. In adult DRG neurons it has been proposed that two separate phases occur during growth. The first phase is "arborization" where neurite growth displays

more branching but less permanent neurites. The second phase "elongation" consists of more permanent neurites growing at length. The second process is differentiated by the necessity of transcription of various growth genes (Smith and Skene 1997). How signalling by growth factors fits into this picture is still a matter under study.

#### **5.4 Effects of growth factors on DRG neurons.**

In an attempt to determine what signalling pathways are responsible for neurite growth we used an array of growth factors and assessed their ability to produce neurite outgrowth. Our experiments employed EGF, IGF-1 and NGF. These growth factors signal through tyrosine kinase receptors which activate many of the same intracellular signalling pathways ascribed to TrkA (see section 3, introduction). We hypothesised that growth factors which elicit growth are using similar pathways to produce this effect. In mass culture NGF, IGF-1 and EGF to a lesser extent produced neurite growth. In compartment cultures the same profile of growth responses was observed.

Next, several of the major pathways that are activated by the Trk receptor including SNT, MAPK, PI3-kinase and PLC $\gamma$ 1 were selected for study (Kaplan and Stephens 1994). All factors appeared to activate MAPK in adult DRG neurons. SNT was activated by both IGF and NGF, but not by EGF. PI3-kinase appeared to be constitutively activated in these neurons regardless of growth factor treatment but was most sensitive to IGF-1. PLC $\gamma$ 1 is not activated by the IGFR-1 receptor compared to TrkA (for TrkA activation of PLC $\gamma$ 1: Loeb et al., 1991; Kim et al., 1991; no reported evidence as such exists for IGFR-1). Studies using inhibitors showed that PD 98059 (an inhibitor of MEK) had no effect on neurite

growth in adult DRGs (Klinz et al., 1996). This result was expected based on the fact that EGF activates MAPK but does not produce substantial neurite growth (see chapter 4). In contrast, IGF which does not significantly activate PLC $\gamma$ 1 produces neurite growth comparable to NGF (see chapter 4).

Based on data that both IGF and NGF but not EGF activate SNT/FRS2 and the fact that LY 294002, an inhibitor of PI3-kinase, decreases neurite growth, it is very likely that these pathways are involved in neurite extension (Vlahos et al., 1994). These data are supported by deletions in the juxtamembrane sequence of TrkA resulting in lack of SNT activation. The same PC12 cell line showed no neurite propagation in response to NGF application (Peng et al., 1995). The effects of PI3-kinase activity on neurite growth in adult DRG neurons has not been previously reported.

PI3-kinase is an important second messenger in lipid metabolism and activation of survival molecules such as Akt (Fisher et al., 1992; Chung et al., 1994). Much of the evidence regarding PI3-kinase indicates this molecule is involved in survival of various cell types through the indirect activation of Akt (see introduction, section 2.2). There is limited evidence for a role in the initiation of neurite growth (Ashcroft et al., 1999). However all the studies dealing with the subject used cell types reliant on NGF for survival (see introduction, section 2.2). Further study with adult sensory neurons may help to better define the role of PI3-kinase outside of this molecule's effect on survival.

### **5.5 Further studies directly applicable to this thesis.**

Further experiments directly applicable to this thesis but not performed due to time

and supply constraints include two specific cases. The first would have been to use a mutant NGF molecule (NGF3T) that binds to TrkA but not p75 (Ibanez et al., 1992). Growth experiments using BDNF and MC192 in combination with NGF3T would provide decisive evidence as to whether inhibition of neurite growth was due to Trk-p75 interactions with NGF or as a result of direct p75 receptor activation. For instance, in the case of application of BDNF in combination with NGF3T, there should be no effect on neurite growth compared to NGF3T alone. This would indicate that the effect of BDNF is due to inhibition of optimal Trk-p75 interactions by displacing NGF at the p75 receptor. If MC192 is activating p75, neurite growth should be inhibited/decreased when added in combination with NGF3T compared to NGF3T treatment alone. This prediction is based on data that MC192 irrespective of neurotrophins seem to activate p75 as measured by nuclear localization of NF $\kappa$ -B (Kimpinski et al., 1998; see section 2, discussion and conclusions).

A limiting factor for these studies was the amount of material (cultures) available. Ideally a complete study of Trk signal transduction in DRG neurons would be important to evaluating the results reported in this thesis. However, such studies are out of the scope of this work and are in the most practical sense a thesis in their own right. These experiments are now being performed in Dr. Mearow's laboratory (Mearow K.M. unpublished data). Experiments using viral vectors to transfect neurons with over expressing active or dominant inhibitory genes to various Trk signalling molecules could maximize the use of a limited number of cultures and strengthen data derived from the use of chemical inhibitors. These experiments would provide better evidence for the hypothesis put forth in this thesis that SNT and PI3-kinase are the major mediators of

neurite growth in adult DRG neurons.

## 5.6 Future directions

Continued study of the ideas and data presented here should proceed in two directions. The first aspect is to undergo a more rigorous study of the contribution of p75 to the growth process. More work looking at Trk independent signalling pathways could provide a direct link between p75 activation and neurite growth. It is possible that p75 activation is inhibiting neurite growth by inducing ceramide production (Posse de Chaves et al., 1997). Conversely p75 activation may be inhibiting TrkA by phosphorylation of serine/threonine residues directly on Trk receptors (MacPhee and Barker 1997; see section 2, conclusions).

The second avenue of study should consist of expanding our knowledge of Trk signalling in these cells. Compared to work done in PC12 cells and sympathetic neurons less is known about the influences of neurotrophins in adult sensory neurons. A complete study comparing Trk signalling in DRG neurons to studies in PC12 cells will provide a strong basis to study Trk-NGF effects specific to the DRG. For example, TrkA activation in DRG neurons induces SP and CGRP production (Lindsay and Hammar 1989; Mulderry 1994). How NGF is influencing peptide transmitter synthesis in these nociceptive neurons has helped the understanding of how NGF is involved in pain sensation. In comparison, there is little evidence for NGF/TrkA signalling and neurite growth in adult sensory neurons *in vitro* other than the correlation between activation and growth. A model for the study of DRG neurons can be derived from work done by Campenot and colleagues using

sympathetic neurons in compartment cultures. This work has provided a greater understanding of the process of neurite growth in response to NGF, including the synthesis of structural proteins (i.e. tubulin), the production of cell membrane constituents and the effects of ceramide in growing neurites (see introduction, section 4). A similar study of the processes that occur between TrkA activation and neurite growth are necessary in the adult DRG *in vitro*.

### **5.7 Relevance to the current understanding of regeneration of the PNS**

Damage to the PNS either through trauma or metabolic processes such as diabetes currently have limited potential for recovery. Few if any specific treatments are available. Understanding of the mechanisms of NGF induced growth could eventually lead to treatment of neuropathy. The use of neurotrophins could promote innervation of specific end organ targets after traumatic axotomy (see Mendell 1999).

The understanding of processes that inhibit growth such as p75 activation (presented in chapter 3) can also be used to induce selected innervation of specific targets. Innervation of inappropriate targets can play a role in a number of pathological processes such as abnormal pain states. Prevention of such problems include reflex sympathetic dystrophy (RSD). Altering or inhibiting pathological sprouting of sympathetic neurons could result in significant pain resolution. Data implicating p75 in the process of growth inhibition could lead to the production of pharmacological agents which activate the receptor.

Along further lines, pharmacological agents that activate specific signalling pathways, could be developed to induce axonal growth. Our studies indicate that

molecules such as SNT and PI-3 kinase are potential targets. A strong understanding of the processes that underlie neurite growth are necessary to develop further avenues of clinical study.

## 5.8 Summary

Described in this thesis a number of components regulating neurite growth in adult DRG neurons. Growth factors that produce substantial neurite growth in adult DRG neurons include IGF-1 and NGF. These factors are capable of producing growth applied globally (cell bodies + neurites) or selectively to specific regions (cell bodies or neurites). These growth effects appear to be reliant on PI3-kinase activation and SNT activity to induce neurite growth (see figure 5.1).

A closer examination of the NGF receptors TrkA and p75 show that TrkA is absolutely required for any growth and p75 is necessary for optimum growth. However activation of p75 signalling pathways independent of trk may have an inhibitory effect. It is hoped that these experiments in culture can provide a basis for the examination of similar processes, difficult to study *in vivo*.



**Table 5.1 Summary of the aspects regulating neurite growth by neurotrophins in adult DRG neurons.** This table summarizes the data presented in this thesis from the level of the growth factor, to the receptor level, to the molecular signaling level.

	<b>Effect on distal neurite growth</b>
<b>Growth Factor Level</b>	NGF - significant growth effects BDNF - no growth effects NT-3 - no growth effects
<b>Receptor Level</b>	TrkA - required p75 competitive inhibition - inhibitory p75 activation - significantly inhibitory
<b>Molecular Pathway Level</b>	SNT - promotes optimal growth PI-3 kinase - promotes growth MAPK - no determinable effect PLC $\gamma$ 1 - no determinable effect

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