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Spatial regulation of neuronal survival, axon growth, and axon degeneration in compartmented cultures of sympathetic neurons

by

Bronwyn L. MacInnis 

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

Department of Cell Biology

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Spatial regulation of neuronal survival, axon growth, and axon degeneration in compartmented cultures of sympathetic neurons" submitted by Bronwyn L. MacInnis in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

In developing sympathetic neurons, nerve growth factor (NGF) interacts with its receptor tyrosine kinase, TrkA, on axon terminals to generate local signals that act within the axon terminals and retrograde signals that ultimately regulate gene expression in the nucleus. The mechanisms by which NGF-mediated retrograde signals are communicated over long distances are poorly understood. The prevalent hypothesis predicts that all retrograde signals are carried by the retrograde transport to the cell bodies of endosomes containing NGF in complex with phosphorylated (p)TrkA, thus maintaining the receptor in its active state until it reaches the cell body. Foremost, this thesis investigates predictions of the signaling endosome hypothesis, by examining the requirement of internalization and retrograde transport of NGF, and of retrograde arrival of phosphorylated TrkA in the cell bodies, in mediating two of the major biological functions of NGF in sympathetic neurons: neuronal survival and axonal growth. Data presented in this thesis demonstrate that the survival of sympathetic neurons can be maintained by binding of NGF to receptors on the axon terminals, without retrograde transport of the growth factor. Furthermore, inhibition of TrkA kinase activity in the cell bodies of neurons supported by a distal source of NGF did not compromise neuronal survival, nor did it prevent survival-associated signaling in the cell bodies or inhibit distal axon growth. In contrast, internalization of NGF/pTrkA complexes in axons was found to be required for NGF-induced phosphorylation of Erk1/2 and NGF-mediated local axon growth. Axon growth was attenuated when phosphorylation of Erk1/2 was prevented by inhibiting MEK kinase activity locally in distal axons, whereas inhibition of MEK in cell bodies/proximal axons had no such effect, suggesting that internalization of NGF/pTrkA

complexes is required to activate MEK/Erk signaling locally in axons where it is required to promote axon growth. These data refute the hypothesis that retrograde transport of NGF/pTrkA-containing signaling endosomes is the exclusive mechanism by which retrograde signals are communicated. Instead they suggest that retrograde signals can reach the cell bodies in the absence of the NGF or the pTrkA that initiated them. They also suggest that endocytosis of NGF/pTrkA complexes may be important for aspects of NGF-induced signaling are distinct from retrograde signaling, including promoting local axon growth.

In other experiments, mechanisms that control local axon degeneration were investigated. Wallerian degeneration of transected distal axons and axonal degeneration due to NGF withdrawal were prevented by pharmacological inhibition of proteasome function, preserving their morphology and mitochondrial metabolic activity for at least 24h. Transected axons treated with the proteasome inhibitor MG132 displayed persistent phosphorylation of Erk1/2, and co-application of a MEK inhibitor, U0126, restored the rapid Wallerian degeneration observed in untreated axons. In contrast, inhibition of axonal caspase activity provided no protection from Wallerian degeneration or NGF withdrawal. These results suggest that axon degeneration is an active, proteasome-mediated mechanism of self-destruction that involves suppression of the MEK/Erk pathway. Its existence has broad implications for understanding axonal degeneration during normal development and neurodegenerative disease.

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LIST OF ABBREVIATIONS

Akt	Serine/Threonine kinase
BDNF	Brain-Derived Neurotrophic Factor
CB/PAx	Cell Bodies/Proximal Axons
CNS	Central Nervous System
CPZ	Chlorpromazine
CREB	Cyclic Adenosine Monophosphate Response Element Binding Protein
DAx	Distal Axons
DMSO	Dimethylsulfoxide
DPM	Disintegrations Per Minute
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EGF	Epidermal Growth Factor
Erk	Extracellular-Regulated Kinase
GFP	Green Fluorescent Protein
IB	Immunoblot
IP	Immunoprecipitation
LY	LY290042
MAPK	Mitogen-Activated Protein Kinase
MDC	Monodansylcadaverine
MEK	MAPK and Erk Kinase
NGF	Nerve Growth Factor
NT	Neurotrophin
NTR	Neurotrophin Receptor
PAGE	Polyacrilamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PC12	Pheochromocytoma cell 12
PI3-kinase	Phosphatidylinositide 3-kinase
PLC	Phospholipase-C
PNS	Peripheral Nervous System
PTB	Phosphotyrosine-Binding

PVDF	Polyvinylidene fluoride
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
TBS	Tris-Buffered Saline
Trk	Tropomyosin-Related Kinase
Wld^S	Wallerian Degeneration Slow

CHAPTER 1

BACKGROUND AND INTRODUCTION

BACKGROUND

Nerve Growth Factor

Nerve growth factor (NGF) is the prototypical member of the neurotrophin family of growth factors, critical mediators of various aspects of nervous system development, maintenance, and function. NGF was discovered as a result of a series of elegant embryological experiments which initiated with the observation that grafting of a mouse sarcoma tumor onto a chick embryo induced a significant increase in the size of the dorsal root ganglion that innervated it (Bueker, 1948). It was later found, by Rita Levi-Montacini and Victor Hamburger, that there was an even greater increase in the size of the innervating superior cervical ganglion (Levi-Montalcini, 1951). Similar effects were produced when the tumor was grafted onto the chorioallantoic membrane of the chick embryo, suggesting that these effects were mediated by a diffusable substance, as there was no direct contact between the embryo and the tumor (Levi-Montalcini, 1952; Levi-Montalcini, 1953). This hypothesis was confirmed by *in vitro* experiments in which profuse neurite outgrowth from superior cervical ganglion and dorsal root ganglion explants was induced by culturing the ganglia with either sarcoma tumor tissue (Levi-Montalcini et al., 1954) or emulsified tumor extract (Cohen et al., 1954). The purification and molecular characterization of this diffusible substance, then aptly named nerve growth factor, was facilitated by the serendipitous discovery that it is present at high levels in the snake venom (Cohen, 1960; Levi-Montalcini and Cohen, 1960). NGF was found to be present in the mouse submaxillary salivary gland, which is homologous to the snake venom gland, at higher concentrations than in any other tissue, although its role in this tissue remains undefined.

NGF is synthesized as a 31 kDa glycosylated precursor that is cleaved by enzymatic convertases to produce the 13.2 kDa mature, processed form of the protein in which six conserved cysteine residues form a series of three disulfide bridges giving rise to a cysteine knot (Angeletti et al., 1973a; Angeletti et al., 1973b), a feature conserved amongst the neurotrophins and common in other secreted proteins. Mature NGF exists as a non-covalently bound homodimer with a total apparent molecular weight of 26.5 (Angeletti et al., 1973a; Angeletti et al., 1973b). Other members of this family of

structurally and functionally related proteins include brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990), neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbook et al., 1991), neurotrophin-6 (NT-6) (Gotz et al., 1994), and neurotrophin-7 (NT-7) (Lai et al., 1998; Nilsson et al., 1998). NGF, BDNF, NT-3, and NT-4/5 sequences are highly conserved amongst vertebrates (although NT-4/5 is not detected in avian species), while NT-6 and NT-7 have been identified only in fish and do not appear to have mammalian orthologues. Each member of the neurotrophin family regulates processes in distinct but overlapping populations of neurons. The neurotrophins are well known as target-derived neuronal survival factors, however, the scope of their actions continues to expand and now includes various processes in the development, maintenance, and pathology of the nervous system, including neural crest cell proliferation and migration, neuronal differentiation, axon growth, synaptic function, and induction of apoptosis (Farinas, 1999; Miller and Kaplan, 2001; Markus et al., 2002a; Segal, 2003).

NGF is produced and constitutively secreted by target tissues that are innervated by sympathetic and sensory neurons of the peripheral nervous system (PNS), thereby providing trophic support to these neurons as their axons reach the final target tissue. Sympathetic neurons in the developing mouse embryo become dependent on NGF for survival at embryonic day 16 (Coughlin and Collins, 1985), prior to which these neurons require NT-3. NT-3 knock-out mice possess 50 % fewer sympathetic neurons and exhibit a significant deficit in sympathetic target innervation (Ernfors et al., 1994; Farinas et al., 1994). By late embryogenesis and into early neonatal development, however, these neurons are acutely dependent on NGF for survival, both *in vivo* and *in vitro*. The importance of NGF in the survival and growth of sympathetic and sensory neurons has been confirmed in *in vivo* experiments in which loss of NGF function by antibody-mediated neutralization (Levi-Montalcini and Booker, 1960), or by targeted disruption of the NGF gene (Crowley et al., 1994), led to the complete loss of sympathetic neurons and the loss of a large percentage of dorsal root ganglion sensory neurons. NGF is also important for the development and function of striatal (Fagan et al., 1997) and basal forebrain cholinergic neurons (McAllister, 2001) in the central nervous system (CNS).

Nerve Growth Factor Receptors: TrkA and p75NTR

TrkA

TrkA, the prototypical member of the Trk family of neurotrophin receptors, was initially characterized as a colon cancer-derived transforming oncogene comprised of tropomyosin fused to an unidentified tyrosine kinase (Martin-Zanca et al., 1986a; Martin-Zanca et al., 1986b). The corresponding proto-oncogene of this tropomyosin-related kinase (Trk) proved to be a member of a highly homologous family of transmembrane receptor tyrosine kinases (Martin-Zanca et al., 1989) that are expressed in discrete neuronal populations and are activated by the binding of discrete neurotrophins: TrkA preferentially binds NGF (Klein et al., 1991), TrkB binds BDNF and NT-4/5 (Klein et al., 1989; Soppet et al., 1991; Squinto et al., 1991), and TrkC binds NT-3 (Lamballe et al., 1991). The specificity of these ligand-receptor interactions is derived from the variable amino termini of the neurotrophins. At higher concentrations than required for NGF binding, TrkA can also bind NT-3 and NT-4/5, and at higher concentrations than required for BDNF or NT4/5, TrkB can also bind NT-3 (Berkemeier et al., 1991; Cordon-Cardo et al., 1991; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991). TrkA is highly expressed in NGF-responsive sympathetic and sensory neurons, and targeted disruption of the TrkA gene in mice produces marked abnormalities in the development of these neuronal populations ((Smeyne et al., 1994); reviewed in: (Snider, 1994)).

TrkA is a 140 kDa type I (single pass) transmembrane protein receptor tyrosine kinase. The extracellular domain, which is absent in the Trk oncogene, is required for ligand binding (Perez et al., 1995). It is comprised of two cysteine-rich regions flanking a leucine-rich motif in the amino-terminal region, followed by two immunoglobulin-like domains in the juxtamembrane region. The intracellular domain of TrkA contains a kinase domain flanked by 70 amino acids on its membrane-proximal side and 15 amino acids at its carboxy-terminus, making it much smaller than most receptor tyrosine kinases (Yano and Chao, 2000). Aside from other Trks, the TrkA kinase domain most closely resembles that of the insulin receptor (Ultsch et al., 1999; Wiesmann et al., 1999).

Binding of dimeric NGF to TrkA induces receptor homodimerization and autophosphorylation of seven tyrosine residues in the kinase domain (Cunningham et al., 1997). The activated tyrosine kinase then rapidly phosphorylates its partner at two additional tyrosines outside of the kinase domain: the juxtamembrane NPXY motif, a consensus binding site for Src homology (SH)-2 or phosphotyrosine binding (PTB) domain-containing molecules such as Shc (Obermeier et al., 1993b; Stephens et al., 1994), and the carboxy-terminal YLDIG motif, a consensus binding site for phospholipase C (PLC) (Obermeier et al., 1993a; Loeb et al., 1994). Phosphorylation and activation of TrkA kinase activity by NGF binding induces subsequent activation of multiple intracellular signaling pathways (discussed below) that ultimately exert the biological effects of NGF.

Much of the current understanding of NGF-mediated signaling mechanisms has been achieved using PC12 cells, a pheochromocytoma adrenal tumor cell line which exhibits several 'neuron-like' phenotypes in response to NGF, including differentiation, survival, and neurite outgrowth (Tischler and Greene, 1975; Greene and Tischler, 1976). PC12 cells lacking TrkA are insensitive to NGF, and re-introduction of TrkA into these cells restores their NGF responsiveness (Loeb et al., 1991; Loeb and Greene, 1993). The PC12 cell line is widely used as a convenient model system by which to mimick NGF-responsiveness of primary sympathetic neurons. However, there are many significant differences between PC12 cells and sympathetic neurons with respect to the nature of NGF-induced signaling and its biological effects, and extrapolation of results obtained from PC12 cells must be done with care.

p75NTR

Initial NGF binding studies in PC12 cells and NGF-responsive sensory and sympathetic neurons suggested the presence of two classes of NGF binding sites: one with a higher affinity (< 100 pM) and one with relatively lower affinity (near 1 nM) (Landreth and Shooter, 1980). The two classes of binding sites proved to represent two distinct receptors. p75NTR was the first NGF receptor to be cloned and characterized, and was found to bind NGF at the lower affinity (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987). As the neurotrophin family expanded it became evident that this

receptor bound all neurotrophins with similar affinity in most cell types (Rodriguez-Tebar et al., 1990; Squinto et al., 1991; Rodriguez-Tebar et al., 1992).

p75NTR was the first-identified member of the tumor necrosis factor (TNF) family of receptors, characterized by a single membrane-spanning domain and the presence of four structurally related cysteine-rich regions in the extracellular domain. However, p75NTR is also distinct from other members of this family, due to its ability to act as a receptor tyrosine kinase co-receptor with the Trks, and because its ligands, the neurotrophins, are soluble rather than the characteristic trimeric type II transmembrane ligands of other TNF family receptors (Roux and Barker, 2002).

The precise role of p75NTR in neurotrophin-mediated functions has remained enigmatic. Unlike the Trks, the intracellular domain of p75NTR does not contain a kinase domain and lacks intrinsic enzymatic activity. However, the intracellular domain does contain multiple motifs that are likely to mediate signaling via association with downstream adaptor proteins. Notably, it contains two potential TRAF binding sites, a type II death domain, a potential G protein activation domain, and a PDZ binding domain (Dechant and Barde, 1997). To date there is evidence that the functions of p75NTR include: acting as a co-receptor with the Trks to create high affinity neurotrophin binding sites; signaling to activate apoptosis; acting as a receptor for secreted immature neurotrophins (known as pro-neurotrophins); facilitating the retrograde transport of neurotrophins; and others. A thorough review of the role of p75NTR in neurotrophin-mediated signaling has been published (Roux and Barker, 2002).

Experiments in this thesis do not assess the role of p75NTR in NGF-mediated signaling. However, as it has been suggested that p75NTR acts as a co-receptor to enhance the affinity of NGF binding to TrkA, and since changes in NGF concentration alter p75NTR protein levels *in vitro* (TrkA levels are not altered) (Song and Posse De Chaves, *Submitted*) (personal communication), the results must be interpreted with the awareness that the cellular ratio and localization of p75NTR and TrkA may be critical to NGF-mediated signaling in sympathetic neurons. Importantly, however, TrkA is sufficient to confer NGF-responsiveness to sympathetic neurons and does not require co-expression p75NTR to elicit the survival or growth-promoting effects of NGF (Patapoutian and Reichardt, 2001).

NGF/phosphorylated (p)TrkA-Induced Signal Transduction

Many of the biological effects of NGF are mediated by the activation of multiple second messenger signaling cascades downstream of the phosphorylation and activation of TrkA (Loeb and Greene, 1993) (represented schematically in Figure 1.1). Mutagenesis studies have shown that Y490 is the principal phosphorylation site leading to the activation of the MEK/Erk and PI3-kinase/Akt signaling pathways which have incurred much of the focus of the survival- and growth-promoting effects of NGF (Atwal et al., 2000; Stephens et al., 1994; Xing et al., 1996). The scaffolding protein Shc is recruited to the NPXpY motif at Y490 of TrkA after NGF stimulation (Obermeier et al., 1993b), which in turn recruits the adaptor protein Grb2, and the guanine nucleotide exchange factor Sos (son of sevenless) to the membrane . Sos activity leads to the activation of Ras by converting it from its GDP to its GTP-bound state (Obermeier et al., 1994; Stephens et al., 1994). Ras activity is central to the survival-promoting effect of NGF, since function-blocking Ras antibodies (Nobes and Tolkovsky, 1995) and over-expression of dominant-negative Ras (Markus et al., 1997; Mazzoni et al., 1999) induce apoptosis of sympathetic neurons in the presence of NGF.

The MEK/Erk pathway

In its simplest form, the MEK/Erk pathway is activated when Ras-GTP promotes the activation of the serine/threonine kinases c-Raf and B-Raf, which phosphorylate and activate MEK1 and MEK2, which respectively activates Erk1 and Erk2 (Erk1/2) by phosphorylation at key threonine and tyrosine residues (reviewed in: (Pearson et al., 2001)). This cascade is a ubiquitous mechanism for communicating and amplifying the cellular response to an extracellular stimulus.

Erk1/2 are members of the MAP kinase family of serine/threonine kinases that were identified by their ability to phosphorylate the microtubule-associated protein MAP-2 (Boulton and Cobb, 1991). It was later found that the targets of these kinases were more diverse than initially recognized, and their critical role in the proliferative effects of

growth factors lead to the renaming of the family as the mitogen-activated protein kinases (Rossomando et al., 1991). To date 20 members of the MAPK family are known to exist (Pearson et al., 2001), of which 4 are known to be activated by neurotrophin/Trk signaling (Erk1, 2, 4 and 5), although only Erk1/2 have so far been shown to be activated by NGF in sympathetic neurons (Grewal et al., 1999). Activated Erk1/2 has multiple targets and is involved in diverse neuronal functions including synaptic plasticity, long term potentiation, and axon outgrowth. They function in the cytoplasm, where they phosphorylate proteins including Tau and neurofilaments promoting cytoskeletal stability, and in the nucleus, where p90Rsk, a substrate of Erk1/2, in turn phosphorylates and activates the cyclic AMP response element binding (CREB) protein. CREB-regulated transcriptional activity is critical to many neuronal processes, including the survival of NGF-dependent sympathetic neurons. Disruption of CREB function by targeted gene deletion or by expression of dominant-negative CREB induces apoptosis of virtually all sympathetic neurons in the presence of NGF *in vitro* (Riccio et al., 1999; Lonze et al., 2002). However, several studies have reported that inhibition of MEK kinase activity has little or no effect on NGF-dependent survival of sympathetic neurons (Atwal et al., 2000; Creedon et al., 1996; Klesse and Parada, 1998; Mazzoni et al., 1999; Virdee et al., 1999; Xue et al., 2000), suggesting that CREB activity is induced by a MEK/Erk-independent pathway in these cells.

The PI3-kinase/Akt pathway

There are two known mechanisms by which NGF-induced phosphorylation of TrkA at Y490 is coupled to the activation of PI3-kinase in peripheral neurons: Membrane-associated Ras-GTP directly binds and activates PI3-kinase, and inhibition of Ras suppresses NGF-mediated PI3-kinase activity (Rodriguez-Viciana et al., 1994; Klesse and Parada, 1998; Mazzoni et al., 1999). In a Ras-independent manner, Shc/Grb2 complexes can also activate PI3-kinase by binding Gab-1, which in turn binds PI3-kinase, allowing it to gain access to its substrates at the inner leaflet of the plasma membrane (Holgado-Madruga et al., 1997). It was found that survival of sympathetic neurons was maintained when a Ras effector mutant that selectively activates PI3-kinase but does not activate the MEK/Erk pathway in response to NGF (Ras(V12)Y40C) was expressed. In

contrast, expression of a Ras effector mutant that selectively activates the MEK/Erk pathway but does not activate PI3-kinase in response to NGF (Ras(V12)Y35S) did not promote NGF-induced survival (Mazzoni et al., 1999; Xue et al., 2000). These findings suggest that the pro-survival effect of Ras is exerted primarily by the activation of the PI3-kinase pathway. Similarly, over-expression of PI3-kinase prevented death of NGF-deprived sympathetic neurons, suggesting that this enzyme is sufficient for survival (Philpott et al., 1997; Crowder and Freeman, 1998). However, evidence that PI3-kinase is required to promote survival in NGF-dependent neurons is conflicting. Whereas several studies have reported that pharmacological or dominant-negative inactivation of PI3-kinase inhibits NGF-mediated survival (Crowder and Freeman, 1998; Mazzoni et al., 1999; Vaillant et al., 1999; Kuruvilla et al., 2000), others have reported only a weak apoptotic effect of PI3-kinase inhibition in NGF-dependent neurons in the presence of NGF (Philpott et al., 1997; Virdee et al., 1999; Tsui-Pierchala et al., 2000). The reasons for these discrepancies are not immediately clear. Interestingly, PI3-kinase is required for the survival of NGF-independent adult sympathetic neurons (Orike et al., 2001).

Phosphatidylinositol-3,4,5-triphosphate (PIP3) generated by PI3-kinase activity induces activation of the serine/threonine kinase Akt by two known mechanisms: PIP3 can bind to and activate Akt directly, and it can also bind to and activate phosphatidylinositide-dependent kinase (PDK)-1, which in turn binds and activates Akt. In contrast to PI3-kinase, the evidence that Akt is critical to NGF-induced survival of sympathetic neurons is decisive: over-expression of constitutively active Akt maintains the survival of NGF-deprived sympathetic neurons, and dominant-negative inhibition of Akt induces apoptosis of approximately 80 % of neurons in the presence of NGF (Crowder and Freeman, 1998; Vaillant et al., 1999; Virdee et al., 1999). Akt is currently viewed to be at the hub of several pathways that mediate the survival-promoting effects of NGF (reviewed in: (Brunet et al., 2001)). Briefly, Akt negatively regulates the activity of molecules in several pro-apoptotic pathways, including the Bcl-2 family member Bad (Datta et al., 1997), members of the Forkhead family of transcription factors (which mediate transcription of pro-apoptotic genes) (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999), and glycogen synthase kinase (GSK)-3 (Pap and Cooper, 1998).

In other cellular systems, Akt has also been shown to positively regulate

molecules in pro-survival pathways, including the transcription factors CREB (Brami-Cherrier et al., 2002; Du and Montminy, 1998; Hayakawa et al., 2002; Perkinton et al., 2002; Pugazhenthi et al., 2000) and NF-kappaB (Kane et al., 1999; Madrid et al., 2000; Ozes et al., 1999; Romashkova and Makarov, 1999), both of which are known to promote NGF-induced survival of sympathetic neurons. Furthermore, a recent study suggests that inhibition of CREB-mediated transcription reduces the expression level of Akt and causes apoptosis in adipocytes, suggesting that signaling via this pathway establishes a survival-promoting positive feedback loop (Reusch and Klemm, 2002). The elucidation of whether CREB and/or NF-kappaB are targets of Akt in NGF-dependent sympathetic neurons will be valuable contributions to the understanding of NGF-mediated pro-survival signaling.

PLC-γ1 Pathways

Phosphorylation of Y785 serves as a direct docking site for PLC-γ, leading to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Obermeier et al., 1994; Obermeier et al., 1993a; Stephens et al., 1994). IP_3 induces release of calcium from intracellular stores, and DAG activates protein kinase C, which can induce activation of the MEK/Erk pathway and induce neurite outgrowth in PC12 cells (Corbit et al., 1999). However, mutation of TrkA at Y785 abolishes NGF-induced PLC-γ binding and activation in sympathetic neurons, but does not affect neuronal survival or axon growth, indicating that PLC-γ1-mediated signaling is not required for these processes (Atwal et al., 2000).

Other NGF/pTrkA-Activated Pathways

In addition to Shc, another scaffolding protein has been found to be recruited to the NPXpY motif at Y490 on activated TrkA. Originally called SNT (for suc-associated neurotrophic factor-induced tyrosine phosphorylated target) by Kaplan and colleagues (Rabin et al., 1993), it was later found to be the same protein as Frs-2 (for fibroblast growth factor receptor substrate-2) and the latter designation was adopted (Kouhara et al., 1997). In PC12 cells, binding to pTrkA induces tyrosine phosphorylation of Frs-2 which then provides a docking site for the adaptor proteins Grb2 and Crk, the protein tyrosine

phosphatase SH-PTP-2, and the tyrosine kinase Src (Meakin et al., 1999). Over expression of Frs-2 in PC12 cells has been shown to induce phosphorylation of Erk1/2, suggesting that Frs-2 may also signal through the MEK/Erk pathway, and deletion of a three amino acid sequence in the juxtamembrane region of TrkA that is also required for Frs-2 activation inhibits NGF-induced neurite outgrowth. Thus, Frs-2 and Shc appear to activate distinct but overlapping pathways, and the regulation of Frs-2 and Shc binding to the NPXpY site may be a mechanism by which specificity in NGF-mediated signal transduction is controlled (Peng et al., 1995).

Other molecules have been found to be recruited to activated TrkA, including the non-receptor tyrosine kinase c-Abl (Yano et al., 2000), the adaptor proteins rAPS and SH2-B (Qian et al., 1998), and the tyrosine phosphatase SHP-2 (Goldsmith and Koizumi, 1997). The signaling pathways and biological functions regulated by the recruitment of these pathways are just beginning to be defined.

NGF-Dependence and NGF Withdrawal-Induced Apoptosis of Sympathetic Neurons

Sympathetic neurons from post-natal day 1 superior cervical ganglia of rats were found to be an ideal *in vitro* model system in which to study the cellular mechanisms and effects of NGF. In addition to the delineation of signaling pathways that control neuronal survival and growth, cultured sympathetic neurons have become a prominent model system for the study of cellular apoptotic mechanisms, which have proven to be remarkably conserved between cell types and through evolution. Sympathetic neurons *in vitro* are dependent on NGF for survival until approximately post-natal day 14, and deprivation of NGF during this period causes them to undergo apoptotic cell death (reviewed in: (Deshmukh and Johnson, 1997)) (represented schematically Figure 1.2). Sympathetic neurons undergoing NGF deprivation-induced apoptosis *in vitro* do not exhibit obvious physical changes for the first 12 h after NGF withdrawl. They begin to show signs of death thereafter, as evidenced at the morphological level by blebbing of the plasma membrane, atrophy of the cell body, condensation of the cytoplasm, and degeneration of the axons. At the ultrastructural level, the nucleus and its genomic DNA begin to condense and fragment at 18 – 24 h after NGF deprivation. This is also the

time frame in which NGF-deprived neurons become 'committed to die', defined as the point at which re-addition of NGF will rescue less than 50 % of the population (Martin et al., 1992). In the final stages of death, which typically ensues 24 – 48 h after NGF withdrawl, the cellular organelles degenerate and the neurons lose structural integrity. Similar changes have been described for sympathetic neurons undergoing naturally occurring apoptosis during development and in newborn mice injected with anti-NGF antibodies.

The biochemical and molecular events that regulate the apoptotic process in sympathetic neurons has been investigated in detail (Deshmukh and Johnson, 1997; Miller and Kaplan, 2001). Briefly, removal of NGF results in down-regulation of pro-survival signaling pathways. Cells constitutively express the basic components of the apoptotic machinery, and it is generally hypothesized that growth factors such as NGF promote survival in part by keeping the apoptotic machinery inactivated (Raff, 1992). Thus, loss of pro-survival signaling initiates pro-apoptotic signaling, leading initially to increases in production of reactive oxygen species and c-jun-mediated transcription of pro-apoptotic effectors, and later to loss of mitochondrial activity and activation of the caspase family of cysteine aspartate-specific proteases that cleave a wide variety of cellular substrates, leading to the rapid demise of the cell.

Local Control of Axon Degeneration

Neurons also have the unique cellular feature in which a part of the cell, namely the axon, can die without spreading to kill the entire neuron. Local withdrawl of NGF from axons of sympathetic neurons *in vitro* induces degeneration of those axons, while their cell bodies are not harmed if exposed to NGF (Campenot, 1982b). A similar phenomenon occurs during development *in vivo*, when inappropriate or superfluous axon branches are selectively removed in a process known as 'branch elimination' or 'pruning', without causing death of the cell body or even the parent axon (O'Leary and Koester, 1993). Furthermore, in several neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's disease, degeneration of the axon precedes death of the cell body, and it has recently been hypothesized that loss of axon function

may be the primary effector of clinical progression of disease (reviewed in: (Coleman and Perry, 2002; Raff et al., 2002)).

Perhaps an even more remarkable example of the intrinsic ability of axons to mediate their own self-destruction program comes from recent insight into Wallerian degeneration, the process by which the distal portion of transected axons degenerate. As loss of connection to the cell body prevents the supply of new proteins to the isolated axon, it was long thought that Wallerian degeneration was a passive process of axonal starvation. However, the discovery of the presence of a spontaneous mutation in mice that confers protection against Wallerian degeneration indicates that such degeneration is an active process that is regulated within the axon (Lunn et al., 1989). In mice possessing this mutation, called *Wld*^{S} for *Wallerian degeneration slow*, the distal portion of severed axons remain viable and able to conduct action potentials for up to 3 weeks *in vivo*, whereas severed axons in wild type mice degenerate within 2 days (Lunn et al., 1989; Perry et al., 1991; Ludwin and Bisby, 1992). *In vitro*, transected *Wld*^{S} axons persist for 6 days, while wild type axons degenerate within hours of disconnection from the cell body (Shaw and Bray, 1977). The protective effect of the *Wld*^{S} mutation is widespread, protecting axons of CNS and PNS neurons, and is an intrinsic property of the axon (Deckwerth and Johnson, 1994; Perry et al., 199; Glass, 1993). In addition to Wallerian degeneration, axons of *Wld*^{S} mice are also resistant to degeneration induced by NGF withdrawl and vincristine neuropathy, although death of the cell body in response to these insults proceeds normally (Deckwerth and Johnson, 1994; Wang et al., 2001).

The Compartmented Culture System

The compartmented culture system (represented diagrammatically in Figure 1.3) was used extensively throughout this thesis. In this system, the cell bodies and proximal axons of neurons are located in a separate fluid environment from their distal axons and growth cones, thus approximating the *in vivo* situation in which cell bodies and their axon terminals are in distinct microenvironments, and permitting independent experimental manipulation and analysis of these cellular compartments. To achieve this segregation, superior cervical ganglia from post-natal day 0 – 2 rats are dissected, enzymatically dissociated, and plated in the center compartment of a 3-compartment Teflon divider

sealed to a collagen-coated tissue culture dish. Axons extend along tracks made in the collagen substrate, passing under the divider, and into distal compartments located on either side of the center compartment. Diffusion between the center compartments and the distal compartments is negligible, thus the fluid environment of each compartment is effectively separated (Campenot and Martin, 2001; Karten et al., 2002). NGF was initially provided to the neurons at 10 – 20 ng/ml in the center compartment, and at 50 – 100 ng/ml in the distal compartments. This NGF regime produces cultures in which, after approximately 5 – 7 days *in vitro*, an extensive axonal network is established in the distal compartments, whereas the center compartment contains the neuronal cell bodies and relatively little axon material.

Compartmented cultures provide the unique opportunity to study NGF-mediated retrograde signaling *in vitro*, and several fundamental concepts of retrograde signaling have been established using this system. Foremost, provision of NGF only to distal axons is sufficient to maintain the survival of sympathetic neurons in a retrograde manner (Campenot, 1982b). In contrast, NGF does not support survival in an anterograde manner, as application of NGF only to cell bodies/proximal axons maintains the viability of the neuronal cell bodies and produces profuse axon growth within that compartment, while the axons in the distal compartments degenerate. Additionally, using compartmented cultures it was found that local application of NGF to distal axons promoted local axon growth, while axons supported by NGF provided in the center compartment do not enter distal compartments that do not contain NGF (Campenot, 1982a). In addition to the local and retrograde effects of NGF-mediated signaling, compartmented cultures provide a unique opportunity to study aspects of neuronal cell biology in which influences of the cell bodies and the distal axons must be controlled independently.

INTRODUCTION

The Neurotrophic Factor Hypothesis

During development, the survival of sympathetic neurons is dependent upon the release of neurotrophins from the target tissues they innervate. However, depending on the population, 20 – 80 % of neurons produced during embryogenesis will die before the organism reaches adulthood (Deshmukh and Johnson, 1997). The majority of this programmed cell death occurs during the developmental period, as the neurons reach their target tissue of innervation (Deshmukh and Johnson, 1997). What determines which neurons live and which die? The neurotrophic factor hypothesis predicts that neurotrophins are released by cells in the target field in limiting quantities, and that those neurons that obtain sufficient neurotrophin support will survive, whereas those that do not will succumb to the default apoptotic program and die (reviewed in: (Oppenheim, 1991)). The formation of this hypothesis was based on the observation that experimentally reducing the size of a target tissue resulted in a corresponding decrease in the number of neurons that survived and innervated it (Purves et al., 1988). Making the survival of neurons dependent on their target would provide a mechanism by which the neuron and target cell population sizes could be coordinated.

Significant experimental support exists for many predictions of the neurotrophic factor hypothesis, especially for neurons of the peripheral nervous system, such as NGF-dependent sympathetic neurons. NGF is produced in limiting quantities by target tissues, its presence is detected by the NGF-specific receptor TrkA at the terminal region of the innervating axons, and ablation of target tissue leads to death of the innervating neurons (Oppenheim, 1991; Johnson and Oppenheim, 1994). Thus, target-derived NGF is critical for the survival of appropriately connected neurons during development. Because of this fundamental role, the mechanism by which NGF promotes survival of sympathetic neurons continues to be an active area of investigation. An important question is how survival signals generated by binding of target-derived NGF to TrkA at axon terminals is communicated to the nucleus in the cell body. Furthermore, this question is of even broader significance and scope, as retrograde signals can also influence neuronal morphology, phenotype and metabolic function, not only during development but also

in the adult organism. A major focus of this thesis is to investigate mechanisms by which local and retrograde signaling by NGF regulates neuronal survival and axon growth.

Retrograde Signaling by Neurotrophins

The mechanisms by which NGF-induced signals are communicated along axons are not well understood. The prevalent hypothesis is that NGF-mediated retrograde signals are carried by the retrograde transport of plasma membrane-derived endosomes containing NGF in complex with active, phosphorylated (p)TrkA (reviewed in: (Ginty and Segal, 2002)). This is the current adaptation of the hypothesis that began with the observation that NGF is retrogradely transported from the target tissue to the cell body *in vivo*, almost 30 years ago. Retrograde NGF transport was originally demonstrated by experiments in which [^{125}I] NGF, injected into the anterior chamber of the adult rat iris, was internalized and retrogradely transport by axons of the sympathetic neurons innervating the eye to the corresponding cell bodies in the superior cervical ganglia (Hendry et al., 1974). Subsequent experiments demonstrated retrograde transport of endogenous NGF in sympathetic and sensory neurons (Palmatier et al., 1984).

It was first thought that retrograde transport of NGF itself elicited the retrograde signal. However, this hypothesis was rejected by experiments in which microinjection of NGF into the cytoplasm or nucleoplasm of PC12 cells did not induce neurotrophic effects, and likewise, the effects of NGF were not inhibited by microinjection of neutralizing NGF antibodies (Heumann et al., 1981; Seeley et al., 1983). These experiments established the requirement for NGF binding to its receptor at the plasma membrane to exert its biological effects, and the model of NGF-mediated retrograde signaling thus evolved to include the co-retrograde transport of TrkA. This model is now commonly referred to as the *signaling endosome hypothesis* of neurotrophin-mediated retrograde signaling (Beattie et al., 1996). It is generally thought that transport of NGF/pTrkA complexes in transport endosomes maintains the receptor in its activated form until it reaches the cell body, where it would then be in close enough proximity to the nucleus to generate signals that influence gene expression to exert the survival-promoting effects of NGF. Thus, NGF would be able to generate signals locally at axon

terminals where it binds to TrkA, along the axon as the endosome is retrogradely transported, and continue to signal once the endosome reaches the cell body.

Signaling endosomes are formed by endocytosis of regions of plasma membrane in which ligand-bound transmembrane receptors, such as NGF-bound TrkA, are concentrated in 'coated pits', formed by the assembly of cytosolic coat proteins of which the major component is clathrin. Clathrin-coated pits are released from the plasma membrane by dynamin, a GTPase that self-assembles into a 'collar' at the neck of the pit and drives the vesiculation of the invaginated membrane. This process, commonly refered to as clathrin-mediated endocytosis, generates clathrin-coated vesicles which carry ligand-receptor complexes into the cell (reviewed in: (Grimes and Miettinen, 2003)). Signaling endosomes have been purified from NGF-stimulated PC12 cells (Grimes et al., 1996; Grimes et al., 1997; Howe et al., 2001; Wu et al., 2001). These vesicles contained NGF in complex with pTrkA, they were associated with clathrin heavy chain, and their formation was blocked by inhibitors of clathrin-mediated endocytosis. Furthermore, the signaling ability of the endosomes was demonstrated, as several members of the MEK/Erk signaling pathway were found to be enriched in their activated form in the vesicle fraction, and the purified vesicles could be used to phosphorylate Elk, a target of Erk1/2, *in vitro*. Association of PI3-kinase and PLC-γ1 was also found, but notably no Akt or pAkt could be detected in the vesicle fraction (Howe et al., 2001).

Importantly, the signaling endosome concept is not limited to neurotrophin-mediated retrograde signaling. Traditionally, receptor-mediated endocytosis was believed to be a mechanism for attenuation of growth factor-mediated signaling, inducing ligand dissociation in acidifying endosomes and receptor degradation in lysosomes and/or recycling to the plasma membrane. However, substantial evidence now indicates that endosomes are platforms for signaling by most ligand-activated receptor tyrosine kinases and G-protein coupled receptors (Grimes and Miettinen, 2003). While it is clear that active receptors signal from the plasma membrane, under many circumstances they have been found to remain bound to ligand and catalytically active after internalization. Several recent studies have used pharmacological inhibitors and dominant-negative constructs of critical effectors of the endocytic process to investigate the role of internalization in growth factor-mediated signaling (Ceresa and Schmid, 2000; Di Fiore

and De Camilli, 2001). Many have reported altered activation profiles of signaling pathways when the receptors are confined to the cell surface. Intriguingly, however, in cases where biological outcomes have been measured, few reports have demonstrated that inhibition of endosomal signaling caused alteration of biological effects of signaling, suggesting that plasma membrane-derived signals were sufficient (Di Fiore and De Camilli, 2001).

The role of endocytosis in NGF-mediated signaling in PC12 cells was investigated by expression of a temperature-sensitive mutant of dynamin, that when expressed, prevents the release of clathrin-coated pits from the plasma membrane (Zhang et al., 2000). This study found that NGF-dependent survival of PC12 cells was not reduced when endocytosis of NGF/pTrkA complexes was inhibited, whereas neuronal differentiation required endocytosis. It was also shown that NGF-induced activation of Akt was increased in magnitude and duration, and activation of Erk1/2 was initially reduced in magnitude but persisted for longer, when NGF/pTrkA signaling was confined to the plasma membrane. Expression of dominant-negative dynamin or treatment with pharmacological inhibitors of endocytosis have also been reported to disrupt NGF-mediated activation of Erk1/2, without reducing TrkA phosphorylation, in PC12 cells and dorsal root ganglion neurons (Howe et al., 2001; Rakhit et al., 2001).

Considerable experimental support for a signaling endosome-mediated mechanism for retrograde signaling by NGF has been established: Retrograde transport of TrkA has been detected *in vivo*, where it was found that TrkA accumulated at the distal side of a ligature in the rat sciatic nerve in an NGF-dependent manner, and that the phosphorylation state of the receptor in the axons was dependent on the availability of NGF at the target field (Ehlers et al., 1995). Using a similar model it has recently been shown that pTrkA in the ligated sciatic nerve accumulates in vesicles that are associated with the retrograde motor protein, dynein (Bhattacharyya et al., 2002). Using compartmented cultures of sympathetic neurons it has been demonstrated that TrkA derived from the axonal plasma membrane is retrogradely transported to the cell bodies/proximal axons, and that at least a fraction of the transported receptor remains associated with NGF and in its phosphorylated form (Tsui-Pierchala and Ginty, 1999). Other experiments using this system have shown that signaling events in the cell

bodies/proximal axons induced by distal NGF are dependent on the arrival of catalytically active TrkA in the cell bodies, as retrograde phosphorylation of CREB, Akt, and several tyrosine phosphorylated proteins was reduced when retrograde TrkA kinase activity was inhibited using K252a (Kuruvilla et al., 2000; Riccio et al., 1997). Similarly, in compartmented cultures of dorsal root ganglion sensory neurons, application of BDNF to distal axons induced accumulation of pTrkB in the cell bodies/proximal axons, a fraction of which could be immunoprecipitated with antibodies to BDNF, suggesting BDNF and activated TrkB had been transported from the axons as a complex (Watson et al., 1999). Also, retrograde phosphorylation of Erk5 in these neurons, which is reported to be required for their survival, was inhibited by K252a-induced inhibition of TrkB kinase activity in the cell bodies/proximal axons, and by expression of a temperature-sensitive dominant negative mutant of dynamin, a protein required for clathrin-mediated endocytosis (Watson et al., 2001).

Evidence for a non-endosomal, non-transport based mechanism of retrograde signaling by NGF has also been described. Senger and Campenot (1997) reported the appearance of pTrkA in the cell bodies/proximal axons of sympathetic neurons within one minute of NGF application to distal axons. Several other tyrosine-phosphorylated proteins were found in the cell bodies/proximal axons within 10 –15 min of distal NGF stimulation. These preceded the first detectable appearance of retrogradely transported [^{125}I] NGF in the cell bodies/proximal axons after addition to distal axons, which did not occur for 30 – 60 min. These results are not consistent with the signaling endosome model, as it predicts that the retrograde appearance of pTrkA and other phosphorylated proteins would be accompanied by the retrograde transport of the NGF that initiated the signal. Instead, these data support the hypothesis that binding of NGF at distal axons induces serial propagation of TrkA phosphorylation at the plasma membrane that travels retrogradely to the cell bodies. The existence of such a cellular mechanism in non-neuronal cells was later demonstrated by Verveer *et al.* (2000), who found that focal EGF stimulation of MCF7 cells, applied using EGF coated microspheres, induced rapid and extensive lateral propagation of ErbB1 phosphorylation to unligated receptors at the plasma membrane.

Local Signaling in Axons and the Control of Axon Growth

In addition to retrograde signaling, NGF induces local signaling events at or near the site of receptor binding in the axon or axon terminal. In a detailed study of the kinetics of [^{125}I] NGF retrograde transport in compartmented cultures of sympathetic neurons it was found that 70 – 95 % of the cell-associated NGF at steady state is associated with the distal axons. Furthermore, following the binding of [^{125}I] NGF at distal axons, there is a significant lag period (of approximately one hour) before retrograde transport of NGF is initiated (Ure and Campenot, 1997). Similarly, a subsequent study of NGF trafficking in cultured sympathetic neurons reported rhodamine-labeled NGF not detectable in vesicles at axon terminals for at least one hour after addition to the culture medium (Weible et al., 2001). The relative abundance of axon-associated NGF, and the relatively long time period during which it is retained at this cellular compartment, suggests that significant NGF-mediated signaling occurs locally in axons. Importantly, many of the downstream targets of NGF-mediated signaling have been shown to be present in axons (including MEK1/2, Erk1/2, PI3-kinase, Akt, and PLC-γ1), and to become activated in axons in response to NGF treatment (Tsui-Pierchala and Ginty, 1999; Atwal et al., 2000; Kuruvilla et al., 2000).

The most appreciated biological effect of local NGF signaling in axons is the stimulation of axon growth. Using compartmented cultures of sympathetic neurons, it was demonstrated that axon growth is induced only in the compartment(s) exposed to NGF (Campenot, 1977; Campenot, 1982a). Also, application of a focal source of NGF (using NGF coated beads) to axons of sensory neurons *in vitro* was shown to induce rapid sprouting of collateral axons (Gallo and Letourneau, 1998). The cellular mechanisms that govern local control of axon growth by NGF are not well understood. Recent evidence suggests that local activation of the MEK/Erk pathway and the PI3-kinase/Akt by NGF in axons is required to promote axon growth, as pharmacological inhibition of either of these pathways in distal axons of sympathetic neurons in compartmented cultures blocks axon extension (Atwal et al., 2000).

Local Mechanisms of Axon Degeneration

Local axon degeneration occurs in a variety of physiological and pathological contexts, and its potential clinical relevance has been highlighted (Coleman and Perry, 2002; Raff et al., 2002). Evidence suggests that selective axon degeneration is mediated by an active and regulated program of self-destruction, rather than passive atrophy as previously thought. However, the mechanisms by which such a process is controlled are virtually undefined. The cloning and characterization of the *Wld*^{S} gene, which confers protection to axons that have been transected from their cell body, or that have been deprived of neurotrophic support, may provide insight into such mechanisms (Conforti et al., 2000; Mack et al., 2001). The *Wld*^{S} gene encodes a chimeric protein comprised of the N-terminal 70 amino acids of UbE4b, an enzyme involved in the polyubiquitinylation of proteins destined for proteasomal degradation, and the complete sequence of Nmnat, an enzyme involved in NAD^{+} synthesis. It appears that the UbE4b component of the chimera is primarily responsible for the *Wld*^{S} phenotype, as transgenic expression of Nmnat fused to a truncated UbE4B sequence does not delay Wallerian degeneration. Intriguingly, however, immunolocalization studies revealed that endogenous *Wld*^{S} protein is primarily nuclear, and is reportedly undetectable in motor neuron or sciatic nerve axons, even though Wallerian degeneration was delayed in these axons (Mack et al., 2001). This suggests that the protective effect of the *Wld*^{S} protein is indirect, potenially functioning in the regulation of expression levels of an unidentified protective protein that is itself targeted to axons.

Several studies have investigated the role of caspase-mediated proteolysis, as caspases are critical effectors of global apoptotic neuronal death, in the search for mechanisms that regulate axon degeneration. The results, however, have been inconsistent, and a general consensus of the role of caspase activity to mechanisms of axon degeneration is not established. Finn *et al.* (2000) reported that treatment with broad-spectrum caspase inhibitors had no effect on the Wallerian degeneration of transected axons of the optic and sciatic nerve and dorsal root ganglion neurons, nor did caspase inhibition delay degeneration due to local neurotrophin withdrawl of axons of dorsal root ganglion neurons in compartmented cultures. These findings lead the authors to hypothesize that the process of selective axon degeneration is mechanistically

distinct from the caspase-mediated process of neuronal apoptosis, speculating that a unique mechanism of degeneration that is confined to axons would protect the cell body. In contrast, however, caspase activity was required for the exposure of phosphatidylserine at axonal membranes in response to local exposure of axons to toxic β-amyloid peptide (Ivins et al., 1998). Caspase activity was also found to be responsible for apoptotic-like features, including phosphatidylserine exposure and mitochondrial membrane permeabilization, elicited by treatment of axon terminal-derived synaptosomes with β-amyloid peptide, staurosporine, and Fe^{2+} (Mattson et al., 1998a; Mattson et al., 1998b).

Introductory Synopsis

The data presented in this thesis address several aspects of the spatial organization of signal transduction induced by exposure of distal axons of sympatheic neurons to NGF. Foremost, mechanisms of retrograde survival signaling by NGF were investigated. Previous results obtained in our laboratory and elsewhere are inconsistent with the hypothesis that all retrograde signals generated by distal NGF stimulation are transmitted by the retrograde transport of NGF/pTrkA-containing signaling endosomes. Thus, experiments were designed to directly assess the validity of several predictions of the signaling endosome model. The results obtained indicate that two presumably critical components of the signaling endosome, NGF and pTrkA, are not required to reach the cell body to effect survival-associated signaling, or neuronal survival itself. Instead, the data presented here are consistent with a model in which pro-survival signaling can be generated by NGF/pTrkA signals at the axonal plasma membrane. In contrast, clathrin-mediated endocytosis was required to promote axon growth, presumably to couple NGF/pTrkA signaling to activation of the MEK/Erk pathway, which is required locally in axons to mediate growth.

In other experiments, mechanisms of the local control of axon degeneration were investigated, using the models of Wallerian degeneration of transected axons and NGF withdrawal. The discovery of the *Wld*^{S} mouse, in which axons are protected from degeneration from various insults including transection from their cell body, indicates that axon degeneration is an active and intrinsically-controlled process. Evidence presented here suggests that axonal degeneration induced by transection from the cell

body or by NGF withdrawal is regulated by proteasomal protein degradation, but not by caspase-mediated proteolytic activity. Furthermore, the proteasome appears to promote axon degeneration by a mechanism that induces downregulation of Erk signaling.

FIGURES

Figure 1.1 Schematic diagram of NGF-induced signal transduction mediated by TrkA in sympathetic neurons

NGF binding to TrkA induces homodimerization and *trans*-activation of the intrinsic tyrosine kinase activity of the receptor. TrkA kinase activity induces phosphorylation of its partner at two tyrosine residues outside the kinase domain: tyrosine 490 and 795. Phosphorylation of TrkA at tyrosine 490, the Shc binding site, leads to activation of the PI3-kinase/Akt and MEK/Erk effector pathways, primarily via the activation of Ras. PI3-kinase/Akt is known to be involved in neuronal survival and contributes to axon growth. MEK/Erk is primarily recognized for its role in mediating local axon growth-promoting effects of NGF. Phosphorylation of TrkA at tyrosine 795 induces recruitment and activation of PLC-γ1, and subsequent activation of DAG and IP_3. The role of this pathway is less characterized.

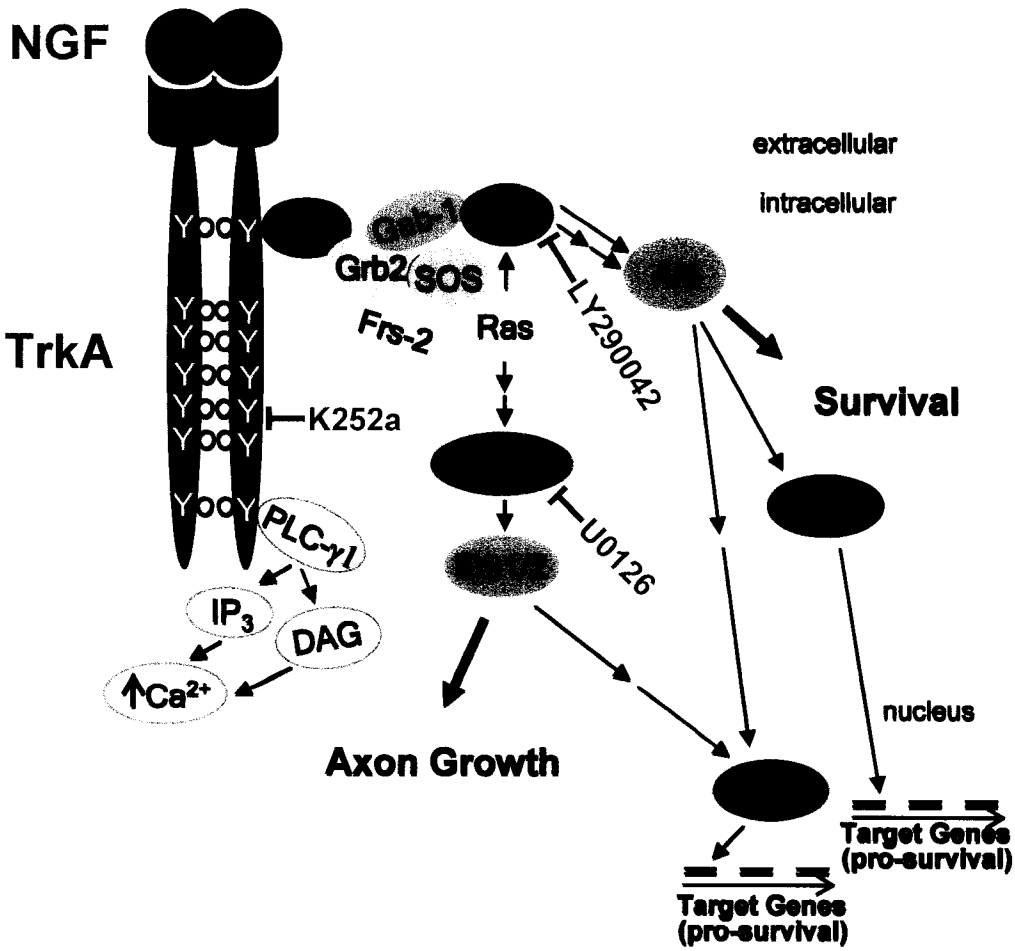


Figure 1.2 NGF-deprivation-induced signal transduction and cellular events leading to apoptosis of NGF-dependent sympathetic neurons

During the period of NGF dependence, withdrawal of NGF from sympathetic neurons results in apoptotic neuronal death. Activation of caspase-mediated proteolytic activity and loss of mitochondrial metabolic activity contribute to the apoptotic process. Activation of the apoptotic program is mediated, at least in part, by the activation of JNK, which induces activation of the transcription factor c-Jun, which regulates the transcription of pro-apoptotic genes including the caspases, and by activation of Bax, which leads to loss of mitochondrial function.

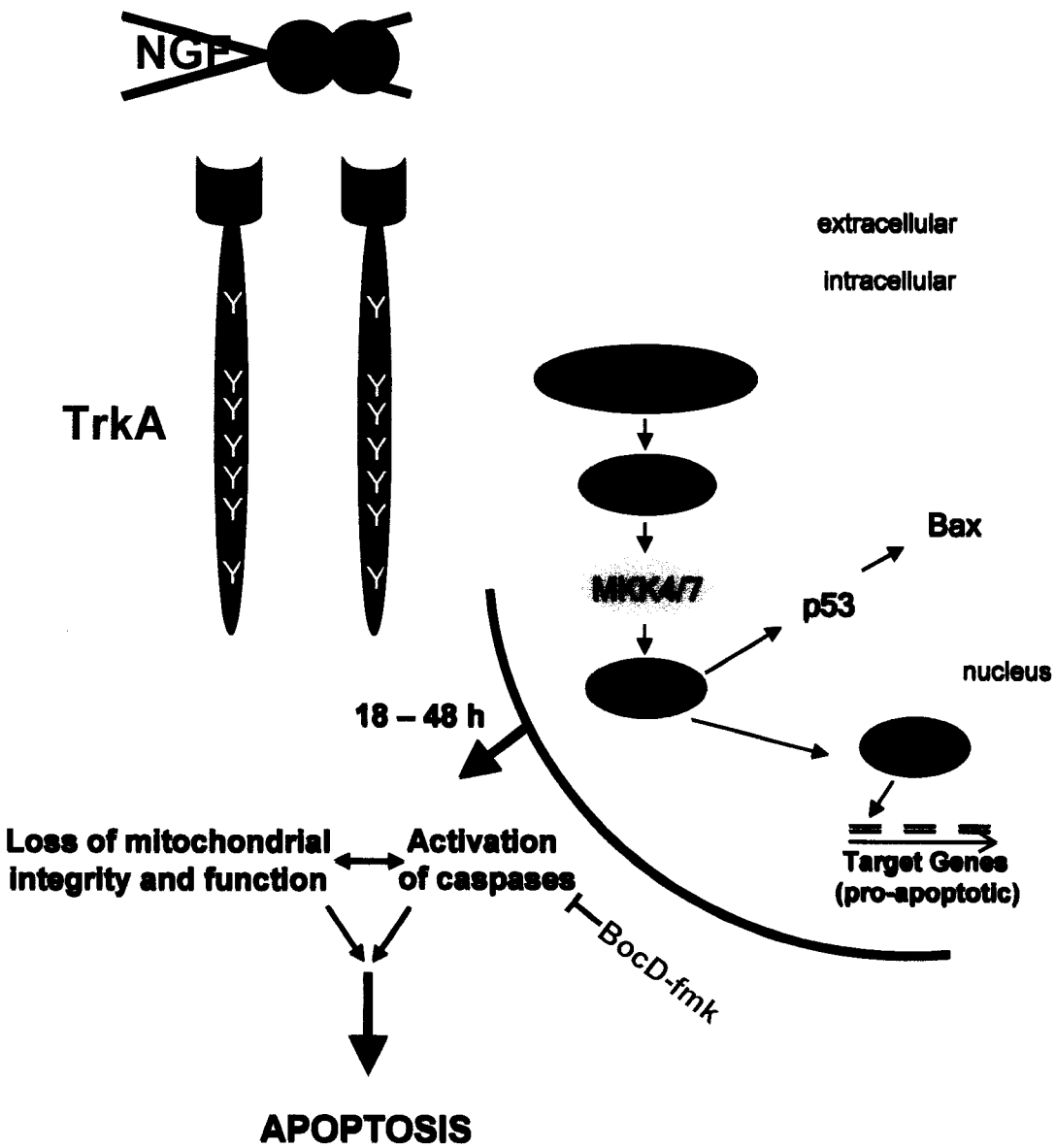
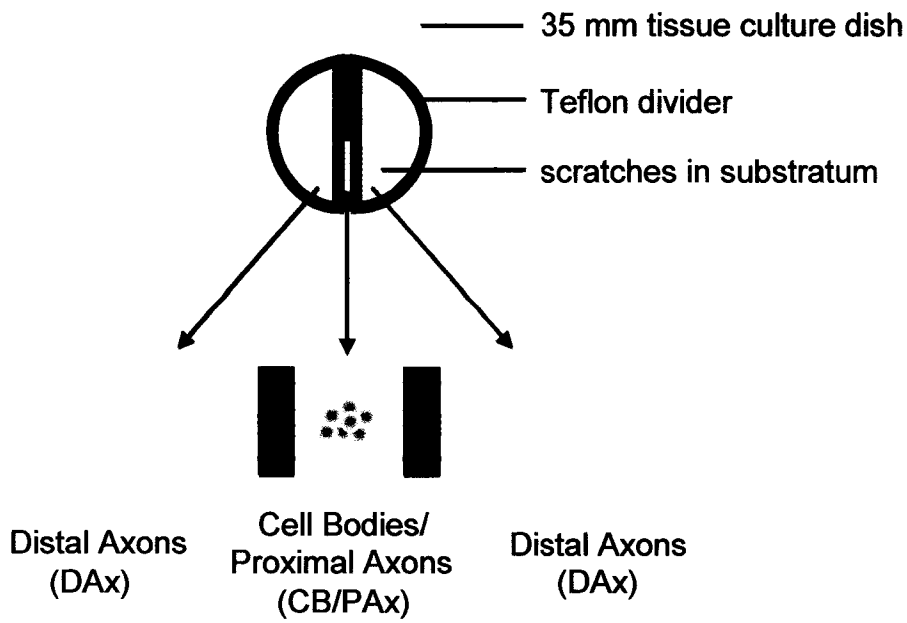


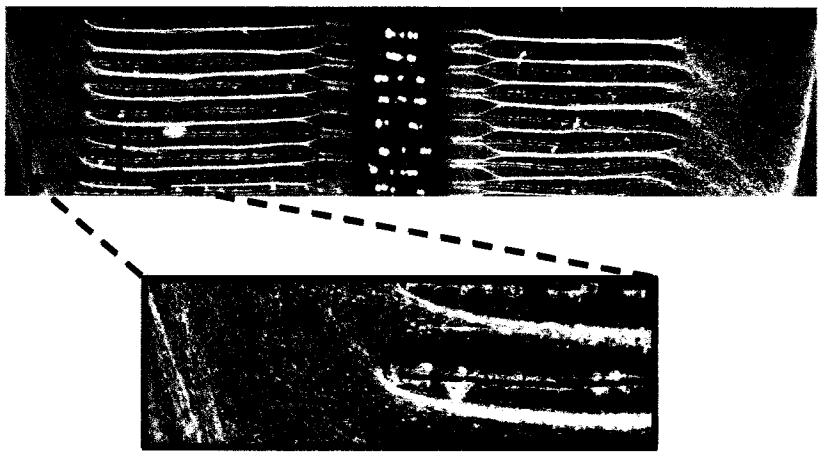
Figure 1.3 The compartmented culture system

A) Schematic diagram of a compartmented culture and an enlargement of one track. The compartmented culture consists of a 35 mm tissue culture dish which has a series of parallel scratches made in the collagen substratum on the floor of the dish, extending under a Teflon divider that has been sealed to the dish with silicone. Tracks between scratches are approximately 0.2 mm wide, the center compartment is approximately 1.5 mm wide, and the barrier between the distal compartments and the center compartment is approximately 1.0 mm wide. Each culture contains up to 20 tracks that can be occupied by neurons. *B)* Fluorescence micrograph of several tracks of neurons that had been maintained in compartmented culture for 7 days and then labeled with the lipophilic dye FM-143, and an enlargement of an area of the distal axons.

A.



B.



CHAPTER 2

Investigation of the Requirements for Internalization and Retrograde Transport of NGF and Retrograde TrkA Kinase Activity in the Support of Retrograde Neuronal Survival and Local Axonal Growth

Data in this chapter pertaining to NGF beads were published in: *MacInnis BL and RB Campenot (2002) Retrograde survival of sympathetic neurons without retrograde transport of Nerve Growth Factor.* Science *296:1536-1539.*

Data in this chapter pertaining to K252a have been accepted for publication in Neuropharmacology as: *MacInnis BL, DL Senger*, and RB Campenot, Spatial Requirements for TrkA Kinase Activity in the Support of Neuronal Survival and Axon Growth in Rat Sympathetic Neurons.* *All data presented in this chapter were obtained by BL MacInnis.

INTRODUCTION

The survival of sympathetic neurons *in vivo* is regulated by nerve growth factor (NGF) provided by the target cells that they innervate (Crowley et al., 1994; Hendry et al., 1974; Levi-Montalcini and Angeletti, 1968). The mechanism by which a distal source of NGF mediates neuronal survival is believed to require retrograde transport to the cell body of signaling endosomes containing NGF/pTrkA complexes, such that the active signaling complexes are in close enough proximity to the nucleus to regulate signaling pathways that lead to transcriptional regulation of survival and other effects on neuronal gene expression. This is believed to be the general mechanism by which all target-derived neurotrophic factors regulate neuronal survival and gene expression.

Until recently, evidence supporting the retrograde transport theory of neurotrophic factor signaling has been largely correlational, most notably that NGF from distal sources is retrogradely transported to the cell bodies in *in vitro* and *in vivo* models (Claude et al., 1982; Hendry et al., 1974; Palmatier et al., 1984; Stoeckel et al., 1976; Ure and Campenot, 1997), and that NGF can be co-transported with pTrkA derived from the axonal plasma membrane in cultured sympathetic neurons (Tsui-Pierchala and Ginty, 1999). However, little is known about the functional significance of the retrograde transport of NGF/pTrkA complexes. Results of functional experiments undertaken with compartmented cultures of rat sympathetic neurons and dorsal root ganglion neurons have suggested that the retrograde transport of neurotrophin complexed with its activated receptor is required for the retrograde activation of Akt and the transcription factor CREB and for other signaling events in the cell body (Bhattacharyya et al., 1997; Kuruvilla et al., 2000; Riccio et al., 1997; Watson et al., 2001; Watson et al., 1999). Also, NGF complexed with pTrkA derived from the surface of distal axons has been detected in cell bodies/proximal axons of sympathetic neurons in compartmented cultures (Tsui-Pierchala and Ginty, 1999).

A direct test of the role of NGF retrograde transport in retrograde signaling was undertaken by supplying sympathetic neurons in compartmented cultures with NGF that had been covalently cross-linked to beads to allow binding and activation of TrkA, but

prevent internalization and retrograde transport of NGF (Riccio et al., 1997). When NGF beads were applied to cell bodies/proximal axons, phosphorylated cyclic-AMP response element binding protein transcription factor (pCREB) accumulated in the nuclei with similar kinetics to the nuclear accumulation of pCREB observed in response to free NGF. This suggests that NGF-induced activation of TrkA at the plasma membrane of the cell bodies could activate the mechanisms producing this response. However, when NGF application was confined to distal axons, only free NGF produced the nuclear accumulation of pCREB. NGF beads did not, suggesting that activation of TrkA by NGF at the plasma membrane of distal axons could not lead to phosphorylation of CREB in the cell bodies.

However, other evidence indicates that internalization of NGF can play a role in signaling that is distinct from providing NGF/pTrkA complexes for retrograde transport. Zhang *et al.* (2000) report that inhibition of NGF internalization in PC12 cells expressing a temperature-sensitive, function-blocking dynamin mutant enhanced the NGF-mediated survival of these cells, but inhibited the NGF-induced neurite outgrowth response. Inhibition of internalization of NGF/pTrkA complexes also increased the magnitude and duration of Akt phosphorylation in response to NGF stimulation, while Erk1/2 phosphorylation was reduced in magnitude but prolonged in duration. In other studies, pharmacological and molecular inhibition of clathrin-mediated endocytosis in cultured dorsal root ganglion neurons and in PC12 cells blocked detectable NGF-induced Erk1/2 activation (Howe et al., 2001; Rakhit et al., 2001; York et al., 2000). There is also evidence for NGF retrograde signaling mechanisms that are independent of retrograde transport of NGF: Senger and Campenot (1997) observed tyrosine phosphorylation of TrkA and several other proteins in the cell bodies/proximal axons of sympathetic neurons in compartmented cultures within 1 to 10 min of NGF application to distal axons, at least 30 min prior to the arrival of the first detectable [^{125}I] NGF transported from the distal axons. As well, basal forebrain cholinergic neurons of p75NTR knock-out mice, in which the retrograde transport of NGF was undetectable (Kramer et al., 1999), nonetheless displayed increased cell body size, increased levels and activity of choline acetyltransferase, and increased target innervation (Kramer et al., 1999; Yeo et al., 1997). The mechanisms for these increases were not defined, but these parameters are

normally maintained by retrograde NGF signals. This suggests that retrograde signaling in these cases may not require the retrograde transport of NGF.

Clearly, the role of internalization and retrograde tranport of NGF/pTrkA complexes in NGF-induced signaling is poorly understood. These results caused us to question the assumption that NGF beads and free NGF activate similar mechanisms except for those that require transport of NGF/pTrkA complexes from distal axons to the cell bodies. Therefore, binding of TrkA to NGF beads could have prevented it from activating retrograde signaling mechanisms that do not directly involve retrograde transport of NGF/pTrkA complexes. In the present study, the role of NGF and pTrkA internalization and retrograde TrkA kinase activity in mediating two of the fundamental biological functions of NGF in sympathetic neurons, survival and axon growth, were investigated. NGF beads were used to determine if preventing internalization of NGF affects the NGF-induced activation of TrkA or NGF-mediated retrograde survival of rat sympathetic neurons in compartmented cultures. We also used pharmacological inhibitors of clathrin-mediated endocytosis to investigate the role of NGF/pTrkA internalization in mediating axonal growth in these neurons. K252a, an inhibitor of TrkA kinase activity, was used to locally block NGF-induced activation of TrkA in only the distal axons or only the cell bodies/proximal axons. We then determined the spatial requirements for TrkA kinase activity for the support of axonal growth, neuronal survival, and survival-associated signaling induced by NGF applied to distal axons. As well,

MATERIALS AND METHODS

Culture Methods

Superior cervical ganglion neurons were obtained from newborn Sprague-Dawley rats (supplied by the University of Alberta Health Sciences Lab Animal Services), enzymatically and mechanically dissociated, and plated into the center compartments of three-compartment dividers as previously described (Campenot, 1992). Compartmented cultures consisted of a Teflon divider (Tyler Research Instruments, Edmonton, AB, Canada) seated with silicone grease (Dow Corning, Midland, MI) onto a collagen-coated 35 mm Falcon tissue culture dish. Cell suspension was plated into the center compartment at a density of 1 ganglion per 4 dishes, and axons extended into the adjacent compartments containing higher concentrations of NGF. For mass cultures, dissociated neurons were plated in 24- or 96-well Linbro tissue culture dishes (ICN Biomedicals, Inc., Aurora, OH), at a density of approximately 1 ganglion per 4 cm^2. Cultures ranged from 7 – 12 days of age at the time they were used in experiments. Age differences in this range made no apparent difference in the results.

Ll5 medium without antibiotics (Gibco Laboratories, Grand Island, NY) was supplemented with the additives prescribed by Hawrot and Patterson (1979) including bicarbonate and methylcellulose. Rat serum (2.5 %), provided by the University of Alberta Laboratory Animal Services, and ascorbic acid (1 mg/ml) were supplied in medium given to the center compartments containing the cell bodies in compartmented cultures, and to mass cultures. Non-neuronal cells were eliminated by supplying 10 μM cytosine arabinoside in the center compartments and in mass cultures during the first 7 days. The 2.5S form of nerve growth factor (NGF) (Alamone Laboratories, Jerusalem, Isreal) was initially supplied in center compartments at 10 – 20 ng/ml to allow cell survival and axon growth. Throughout the culture period and during the experiments the medium in distal compartments and in mass cultures was supplied with 50 ng/ml NGF except where otherwise indicated. Treatment groups within all experiments consisted of cultures from the same plating of neurons maintained under identical conditions until the time of the experiment.

NGF conjugation to beads

NGF was covalently conjugated to 1 μm-diameter beads (FluoSperes amine-modified microspheres, Molecular Probes, Eugene, OR) essentially as previously described (Riccio *et al*, 1997). Briefly, beads were washed 3 times in 0.1 M MES (2-[N-Morpholino] ethanesulfonic acid; pH 6.0), then resuspended to a final concentration of 1 % solids in the same buffer containing NGF (100 μg/ml) and the cross-linking agent EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), hydrochloride, 4 μM; Molecular Probes). The cross-linking reaction was allowed to proceed for 2 h at room temperature with gentle agitation, at which time glycine was added to a final concentration of 0.1 M for 30 min to quench the reaction. The beads were then washed 4 times in a high salt buffer (10 mM sodium phosphate, 1.8 mM potassium phosphate, 1 M sodium chloride, 2.6 mM potassium chloride; pH 4) to remove unbound NGF, incubated overnight in high salt buffer (pH 7.4), and then washed 4 times the following day in high salt buffer (pH 10), to remove adsorbed NGF. All washes were for a minimum of 30 min and were carried out at 4 °C. Beads were pelleted between washes by centrifugation at 10,000 x g at 4 °C for 20 min, and resuspended by gentle tirturation using a glass Pasteur pipette. Control beads underwent the identical procedure except that the NGF (beads alone) or the EDAC (-EDAC) was omitted from the cross-linking step. In experiments in which the effect of free NGF was compared to NGF beads, equivalent vehicle (PBS) was added to the cultures receiving free NGF (50 μl/ml unless otherwise stated).

Radioiodination of NGF

Radioactive iodine-125 ([^{125}I]) was conjugated to NGF by the lactoperoxidase/H_2O_2 method, in our laboratory (Ure and Campenot, 1994, 1997) or at ICN Radiochemicals (Irvine, CA). [^{125}I] NGF was purified from free [^{125}I] in the absence of carrier protein, by gel filtration ([^{125}I] NGF prepared in our laboratory) or by high performance liquid chromatography ([^{125}I] NGF prepared at ICN Radiochemicals). Specific activities averaged 38.2 mCi/mg NGF. [^{125}I] NGF was used within 2 d of iodination for binding to beads, within 5 d of iodination for [^{125}I] NGF-bead experiments, and within 9 days for soluble [^{125}I] NGF internalization and retrograde transport

experiments. In each experiment [^{125}I] NGF from the same preparation was used to compare the effects of soluble [^{125}I] NGF to [^{125}I] NGF beads.

[^{125}I] NGF internalization and retrograde transport analysis

NGF Retrograde transport assays were performed by supplying [^{125}I] NGF at the indicated concentrations to the distal axon compartments of compartmented cultures. Previous results have shown that [^{125}I] NGF is internalized by the distal axons and transported to the cell bodies where it is degraded and the radioactivity is released as a low molecular weight breakdown product into the medium bathing the cell bodies/proximal axons (Ure and Campenot, 1997). In bead experiments transported [^{125}I] was assessed by gamma-counting the medium bathing the cell bodies/proximal axons, which accounts for approximately 90 % of transported radioactivity at steady-state (Ure and Campenot, 1997); in inhibitor experiments the medium bathing the cell bodies/proximal axons and extracts prepared from the cell bodies/proximal axons was counted. To control for non-specific transport, cultures were given medium containing [^{125}I] NGF plus 100-fold excess of unlabeled NGF, and transported radioactivity in this group was subtracted from all other groups given the same concentration of [^{125}I] NGF.

NGF internalization assays were performed by providing 5 ng/ml [^{125}I] NGF to neurons in 96-well plate mass cultures for the indicated times, removing unbound or low affinity bound NGF by rinsing 3 times with ice-cold Krebs-HEPES buffer (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM glucose, and 25 mM HEPES; pH 7.4) (100 µl/well/rinse), then removing surface-associated [^{125}I] NGF by enzymatic cleavage using trypsin (1 µg/ml) in Krebs-HEPES buffer at 4 °C for 30 min. Trypsin-containing buffer was collected and pooled with trypsin-free Krebs-HEPES buffer used to rinse the neurons (2 rinses; 100 µl/well/rinse). Internalized [^{125}I] NGF was then recovered by preparing cell extracts in water. Radioactivity in each fraction was determined by gamma-counting. Non-specific binding/internalization of radioactivity was determined by including a control group given 5 ng/ml [^{125}I] NGF plus 100-fold unlabeled NGF and subtracting the trypsin-sensitive and trypsin-insensitive counts from the respective fractions in all other groups.

Neuronal Survival Assays

For mass cultures: after experimental treatments neurons were incubated for 3 h at 37°C with CellTiter 96 One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI), a derivative of the MTT assay in which cell survival is measured by the conversion of MTT from a yellow to a blue formazan crystal by mitochondrial succinate dehydrogenase in living cells (Mosmann, 1983). The extent of color development was measured quantitatively by plate-reading at a wavelength of 490 nM. Survival of the same neurons was then assessed by staining of neuronal nuclei with Hoechst fluorescent DNA-binding dye. Cultures were fixed with 4 % paraformaldehyde for 20 min and incubated with Hoechst (50 mM in PBS; Molecular Probes, Eugene, OR) for 30 min. Stained nuclei were quantified and images were obtained using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Nikon Digital Camera DX-1200 (Nikon Canada, Toronto, ON). Images were analyzed using Northern Elite V6.0 image capture and analysis software (Empix Imaging, Missisagua, ON). For compartmented cultures: cell survival was first assessed qualitatively by incubation with CellTiter 96 Cell Proliferation Assay for 1 h at 37°C. Neuronal nuclei were then stained with Hoechst 33258 and survival was quantified as described above for mass cultures. For NGF bead experiments: Sympathetic neurons maintained in compartmented cultures for 7-10 d were deprived of NGF using anti-NGF antibodies (24 nM) in all compartments for 3 h, to remove the free NGF in which they were raised. Following this deprivation treatment, anti-NGF antibodies were removed from the distal axon compartments by washing twice over a 30 min period with unsupplemented culture medium. The cultures were then divided into experimental groups of 3 cultures per group. Following experimental treatment, neuronal survival was assessed by Hoechst-staining as described above.

Inhibitor treatments

All reagents were from Sigma (St. Louis, MO) unless otherwise noted. K252a (Calbiochem, San Diego, CA) was prepared as a 2 mM stock in DMSO. LY290042, MDC, CPZ, and U0126 (Promega) were prepared as 1000x concentrated stocks in DMSO. Equivalent DMSO was included in all control culture medium during appropriate experiments (maximum concentration 0.1 %) had no detectable detrimental effects on

the neurons. In experiments using multiple concentrations of K252a, DMSO equivalent to the highest concentration was used in control cultures (0.1 %). Culture medium was routinely changed every 24 h (or every 10-14 h for K252a) to maintain the efficacy of the inhibitors in solution. Anti-NGF antibodies (Cedarlane Biomedicals, Hornby, ON) were used at 24 nM to neutralize NGF.

Immunoprecipitation

Following experimental treatment cultures were rinsed twice with ice-cold Tris-buffered saline (TBS) containing 1 mM sodium orthovanadate and 10 mM sodium fluoride and then lysed in lysis buffer (TBS containing 10 % glycerol, 1 % ipegal, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany) at 4°C for 20 min. Cell lysates were collected, homogenized by repeated passage through a 22 gauge needle, and cleared by centrifugation at 10,000 x g for 5 min. Supernatants were subjected to immunopreciptation with polyclonal anti-Trk (clone 14) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h followed by addition of Protein A/G-plus agarose slurry (50 µl; Santa Cruz Biotechnology) for 2 h with gentle rotation. Immune complexes were recovered by centrifugation, washed 3 times with lysis buffer, boiled in 2 X-strength SDS sample buffer, and resolved by SDS-PAGE.

Immunoblotting

Following experimental treatment, cultures to be analyzed by immunoblotting were rinsed with ice-cold Tris-buffered saline containing 1 mM sodium orthovanadate and 10 mM sodium fluoride (Sigma, Oakville, ON, Canada). Samples were prepared by lysing cells directly into sodium dodecyl sulfate (SDS) sample buffer, boiling for 5 min, and running on 8 % SDS PAGE gels. For immunoblotting, each experimental treatment group contained pooled material from 2 wells of neurons grown in 24 well plates. Proteins were transferred to Immobilon-P PVDF membrane (Millipore Corp., Bedford, MA) using a wet transfer system (Hoefer Scientific Instruments, San Francisco, CA) and immunoblotted using the following antibodies at the indicated dilutions: polyclonal anti-phosphoTrkA (Y490) (1:1000, Cell Signaling Technology, Beverly, MA), monoclonal

anti-phosphotyrosine (clone 4G10) (1:2000, Upstate Biotechnology Inc., Lake Placid, NY) monoclonal anti-phosphoErk1/2 (Thr202/Tyr204) (1:5000, CST), anti-phosphoAkt (Ser473) (1:1000, CST). Equivalent protein loading was confirmed by immunoblotting with polyclonal antibodies to total TrkA protein with anti-TrkA (C-14) (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) and total Erk1/2 protein using anti-Erk1 (C-16) antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), which reacts with both Erk1 and Erk2. Immunoreactivity was determined by enhanced chemiluminescence (SuperSignal West Dura Substrate, Pierce, Rockford, IL). Restore Western Blot Stripping Buffer (Pierce) was used according to manufacturer's directions when membranes were stripped and reprobed with a different primary antibody.

Axonal surface biotinylation and streptavidin biotinylation

Surface proteins at the axonal plasma membrane were biotinylated by briefly rinsing axons with cold PBS and incubating them with sulfo-NHS-LC-biotin (2 mM; Pierce, Rockford, IL) in PBS containing glucose (1 mg/ml) for 30 min at 4 °C. Axons were then washed twice with cold PBS containing glucose to remove excess sulfo-NHS-LC-biotin, and treated according to the experimental design. After treatment, the cell bodies/proximal axons were rinsed twice with cold TBS containing 1 mM sodium orthovanadate and 10 mM sodium fluoride and then lysed for 20 min in non-denaturing lysis buffer (TBS containing 10 % glycerol, 1 % ipegal, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany)) at 4 °C for 20 min. Cell lysates were collected, homogenized by repeated passage through a 22 gauge needle, and cleared by centrifugation at 10,000 x g for 5 min. Supernatants were incubated for 2 h – overnight at 4 °C with 70-100 µl of a 50 % slurry of streptavidin-agarose (Pierce). The biotin-streptavidin-agarose complexes were collected by centrifugation, washed three times in lysis buffer, boiled in 2 X strength SDS sample buffer, and resolved by SDS-PAGE. Immunoblot data were quantified using an Ultrascan XL laser densitometer (Pharmacia LKB Biotecnology Inc., Piscataway, NJ).

Axonal Extension

Distal axons of neurons in compartmented cultures were axotomized with a jet of sterile water delivered via syringe fitted with a 22 gauge needle. Culture medium was then restored to allow axonal regeneration. Axonal extension was measured on individual tracks using an inverted microscope fitted with a stage digitizer (AccuStage, Shoreview, MN) as previously described (Campenot, 1992).

RESULTS

NGF beads induce tyrosine phosphorylation of TrkA

The ability of free NGF and NGF beads to induce tyrosine phosphorylation of TrkA was compared in mass cultures of rat sympathetic neurons. Previous data suggests that a lag period of approximately 1 h exists between the binding of NGF at the plasma membrane and its internalization into vesicular structures (Ure and Campenot, 1997; Weible et al., 2001). Thus, 1 h was chosen as a time point at which to detect potential differences in TrkA phosphorylation in the presence or absence of NGF internalization. Neurons that had been maintained in mass cultures were deprived of NGF for 6 h by exposure to medium lacking NGF but containing 24 nM anti-NGF antibodies (anti-NGF). The cultures were rinsed once to remove the anti-NGF, divided into treatment groups of 2 wells per group, and treated for 1 h with medium containing 0-100 ng/ml free NGF or 0-50 μl/ml NGF beads, as indicated in Figure 2.1.

Cell extracts from cultures in each group were pooled and analyzed by immunoblotting with phosphotyrosine antibodies. The blots were then stripped and reprobed with phosphoTrkA (Y490)-specific antibodies that detect phosphorylation of TrkA at the Shc binding site (Segal et al., 1996). As expected, treatment of neurons with free NGF stimulated tyrosine phosphorylation of TrkA in a concentration-dependent manner (Fig. 2.1A). Treatment with NGF beads also stimulated tyrosine phosphorylation of TrkA detected by both anti-phosphotyrosine (top panel) and anti-phosphoTrkA (Tyr490) (middle panel) antibodies, indicating that NGF beads were able to induce phosphorylation of TrkA including the Shc-binding site. Approximately equivalent phosphorylation of TrkA was obtained with 50 ng/ml NGF and 50 μl/ml of NGF beads. The blot was stripped again and reprobed with antibodies to total TrkA protein (lower panel), which confirmed similar protein loading in all lanes.

NGF beads induce phosphorylation of Akt, but not Erk1/2

In light of the observation that NGF beads promoted similar activation of TrkA as did 50 ng/ml free NGF, we next investigated the ability of NGF beads to activate NGF-

induced signaling pathways downstream of TrkA. Phosphorylation of TrkA at tyrosine 490, which was induced by both NGF and NGF beads, promotes association of Shc and leads to activation of both the MEK/Erk and PI3-kinase/Akt effector pathways (Obermeier et al., 1994; Segal et al., 1996; Stephens et al., 1994). We thus asked whether these pathways were active under conditions where endocytosis of NGF was inhibited. The immunoblot shown in Figure 2.1B is the lower portion (less than 100 kDa) of the blotted membrane from the same experiment as presented in Figure 2.1A. This portion of the membrane was probed with antibodies specific for the phosphorylated forms of Akt (top panel) and Erk1/2 (middle panel). To confirm equivalent protein loading, the blot was stripped and reprobed for total (phosphorylated and unphosphorylated) Erk1/2 protein, which was similar across all lanes (lower panel). As observed for phosphorylation of TrkA, both free NGF and NGF beads induced concentration-dependent phosphorylation of Akt, with 50 ng/ml free NGF and 50 μl/ml NGF beads producing roughly equivalent phosphorylation. This suggests that the PI3-kinase/Akt pathway is activated by TrkA at the plasma membrane, as has previously been reported in PC12 cells (York et al., 2000; Zhang et al., 2000).

In contrast, although free NGF produced robust phosphorylation of Erk1/2, phosphorylation of these molecules was barely detectable in cultures given NGF beads. Thus, whereas 50 μl/ml of NGF beads produced a similar level of tyrosine phosphorylation at the Shc binding site of TrkA as 50 ng/ml free NGF (Fig. 2.1A), downstream signaling to Erk1/2 was strongly inhibited. This suggests that activated TrkA on the neuronal surface must be internalized in order to induce activation of Erk1/2, and that this internalization is blocked by NGF beads. Similar inhibition of growth factor-mediated Erk1/2 phosphorylation induced by inhibition of clathrin-mediated endocytosis has been reported for other receptor tyrosine kinases in multiple cell types (reviewed in: (Ceresa and Schmid, 2000)). Significantly, this phenomenon has recently been reported for TrkA in dorsal root ganglion neurons and PC12 cells, in which pharmacological or molecular inhibition of endocytosis completely blocked NGF-induced Erk1/2 activation (Howe et al., 2001; Rakhit et al., 2001; York et al., 2000).

NGF beads support retrograde survival of sympathetic neurons in compartmented cultures

We next asked whether the phosphorylation and activation of TrkA induced by NGF beads was sufficient to sustain the survival of sympathetic neurons. Neurons in compartmented cultures were given medium lacking NGF and containing anti-NGF in all compartments for 1 h in order to neutralize the NGF in which they were raised. The distal axon compartments were then rinsed twice with NGF-free culture medium to remove the antibodies and cultures were divided into groups according to the treatment applied to the distal axons: one group was given NGF-free culture medium (no NGF), one group was given medium containing 50 ng/ml free NGF, and one group was given medium containing 50 µl/ml NGF beads. Two control groups were included which were given 50 µl/ml of beads that had undergone the same cross-linking protocol without NGF (beads alone), or the same cross-linking protocol with NGF but without the EDAC cross-linking agent (-EDAC). The cell bodies/proximal axons of all groups were maintained in medium containing anti-NGF, such that the neurons were dependent on NGF survival support originating in the distal axons.

After 30 h of treatment, cultures were fixed and labeled with Hoechst dye, then visualized by ultraviolet fluorescence microscopy to identify viable and apoptotic cells. Live, dying, and dead cells are clearly discernible by Hoechst DNA staining: the nuclei of live cells are diffusely stained, whereas dead cells exhibit condensed, fragmented DNA staining characteristic of apoptosis, or lack nuclear staining and appear as "ghosts" (visible cell membrane under phase contrast microscopy, but devoid of nuclear staining). Figure 2.2A shows representative confocal micrographs of Hoechst-stained nuclei of neurons in each treatment group and Figure 2.2B shows the quantification of percent of neurons that survived. As expected, when given 50 ng/ml free NGF in the distal axon compartments, 95 % of neurons displayed live nuclei. Only 22 % of the neurons given no NGF survived, indicating that the initial deprivation treatment had effectively depleted the cultures of NGF. Treatment of cultures with NGF beads on the distal axons for the same time period supported the survival of 81 % of the neurons. This result strongly suggests that internalization and retrograde transport of NGF are not required to

maintain the survival of sympathetic neurons. Control beads did not support neuronal viability significantly above the level maintained in the absence of NGF, indicating that the survival-promoting effect could be attributed specifically to NGF covalently bound to the beads, and not to NGF adsorbed to the beads or the beads themselves.

Retrograde survival could conceivably have been achieved by the release of NGF from the beads into the culture medium, followed by internalization and retrograde transport. However, in experiments similar to that described in Figure 2.2A and B, supernatant media from NGF beads that had supported 30 hours of retrograde survival of one set of cultures (Fig. 2.2C, *solid bars*) failed to support survival of a second set of cultures (Fig. 2.2C, *bars with vertical lines*), whereas free NGF from the first set again supported the survival of a second set. Supernatant media from NGF beads that were preincubated for 30 hours without neurons present also failed to support neuronal survival (Fig. 2.2C, *bars with horizontal lines*), ruling out the possibility that NGF released from the beads may have been depleted from the medium by retrograde transport.

[^{125}I] NGF is not retrogradely transported in biologically significant levels from [^{125}I] NGF beads applied to distal axons

The selective inability of NGF beads to induce Erk1/2 phosphorylation suggests that covalent cross-linking of NGF to beads effectively prevented the internalization of NGF/pTrkA complexes. If internalization was in fact prevented, then the neurons should also be incapable of retrogradely transporting NGF that is presented to the distal axons in bead-bound form. If so, and if retrograde transport of NGF is required for retrograde support of neuronal survival, then NGF beads applied to distal axons should have been unable to support neuronal survival.

To address these issues, NGF was labeled with radioactive iodine ([^{125}I] NGF) and then covalently cross-linked to beads. We first tested whether the [^{125}I] NGF beads, and the soluble [^{125}I] NGF from which the [^{125}I] NGF beads were prepared, produced activation of TrkA. In a similar experiment as described in Figure 1, it was found that 50 µl/ml of the [^{125}I] NGF beads applied to NGF-deprived neurons in mass cultures produced a similar level of TrkA phosphorylation to 50 ng/ml of an aliquot of the soluble

[^{125}I] NGF used to prepare the [^{125}I] NGF beads, which was also comparable to TrkA phosphorylation induced by 50 ng/ml unlabeled free NGF (Fig. 2.3A).

To compare the retrograde transport and survival support of soluble [^{125}I] NGF versus [^{125}I] NGF beads, neurons in compartmented cultures were temporarily deprived of NGF using anti-NGF, as described in the experiment depicted in Figure 2.2. The cultures were then divided into the following five treatment groups: the free NGF transport group given 50 ng/ml soluble [^{125}I] NGF; its unlabeled NGF competition control group given 50 ng/ml soluble [^{125}I] NGF and 5000 ng/ml unlabeled free NGF; the NGF-bead transport group given 50 µl/ml [^{125}I] NGF beads; its unlabeled NGF competition control group given 50 µl/ml [^{125}I] NGF beads and 5000 ng/ml unlabeled free NGF, and a last group given no NGF. Cultures in all groups received anti-NGF in the cell bodies/proximal axons compartments, such that neuronal survival was dependent on trophic support provided by NGF only in the distal axon compartments.

Retrogradely transported [^{125}I] NGF reaches a steady-state concentration in the cell bodies of sympathetic neurons in compartmented cultures within approximately 8 h of application to distal axons. It resides in the cell bodies with a half-life of approximately 3 h, is broken down, and [^{125}I] is released into the medium bathing the cell bodies/proximal axons as a low molecular weight degradation product, presumably [^{125}I] tyrosine (Ure and Campenot, 1994; Ure and Campenot, 1997). After 30 h of incubation the culture medium bathing the cell bodies/proximal axons was collected, which contains approximately 90 % of the retrogradely transported [^{125}I] that is derived from transported [^{125}I] NGF during that time. The nonspecific disintegrations per minute (dpm) from the unlabeled NGF competition control groups were subtracted from the dpm in the transport groups, and specific dpm were then converted to equivalent NGF (shown in pg). While cultures given soluble [^{125}I] NGF in the distal compartments transported an average of 563.6 pg NGF (52026 specific dpm) over the 30 h period, only 0.65 pg (61 specific dpm) were transported by cultures given [^{125}I] NGF beads in distal compartments. Thus, virtually no [^{125}I] NGF originating from [^{125}I] NGF beads given to distal axons was transported to the cell bodies (Fig. 2.3B).

After removal of the culture medium for gamma-counting, these neurons were prepared for Hoechst staining and the percentage of live cells was determined. In

cultures given no NGF during the 30 h incubation, only 26 % of the neurons displayed healthy nuclear staining, indicating that the initial NGF deprivation treatment had effectively depleted the cultures of sufficient NGF to support survival. As expected, 97 % of neurons in cultures given 50 ng/ml soluble [^{125}I] NGF displayed diffusely stained nuclei typical of live cells. Contrary to the prediction of the retrograde transport theory of NGF-mediated neuronal survival, application of [^{125}I] NGF beads to distal axons also supported neuronal survival, with 84 % of the neurons displaying the diffusely stained nuclei of live cells (Fig. 2.3C). These results show that, while NGF in bead-bound form induced phosphorylation of TrkA and produced retrograde survival of rat sympathetic neurons, it did not activate Erk1/2 and was not retrogradely transported.

To explore the possibility that a level of NGF transport that was below detection by our [^{125}I] assay could have supported cell survival of neurons given NGF beads, the relationship between [^{125}I] NGF retrograde transport and neuronal survival in cultures given different concentrations of [^{125}I] NGF at the distal axons was determined. It was then determined where on the transport versus survival curve the [^{125}I] NGF transported from [^{125}I] NGF beads fell. The [^{125}I] NGF used in this experiment was from the same preparation as used for the experiments described in Figure 2.3. Compartmented cultures were deprived of NGF by anti-NGF treatment as described above, so that neuronal survival was dependent upon the [^{125}I] NGF that was subsequently supplied to the distal axons. Cultures were divided into treatment groups, and the distal axon compartments were given concentrations of [^{125}I] NGF ranging from 0.0005 ng/ml to 50 ng/ml, or no NGF, while cell bodies/proximal axons were maintained in anti-NGF antibodies. To control for non-specific transport, a control group was included for each concentration of [^{125}I] NGF tested which received medium containing the appropriate concentration of [^{125}I] NGF plus 100-fold excess unlabeled NGF in the distal axon compartments. The average of the transported radioactivity in these cultures was subtracted from the average transport for the corresponding [^{125}I] NGF concentration. At 50 ng/ml [^{125}I] NGF, non-specific transport accounted for 5 % of the transported counts. The proportion of non-specific transport increased as the concentration of [^{125}I] NGF applied to the distal axons decreased. For [^{125}I] NGF beads, non-specific transport counted for 83 % of the transported counts.

Following 30 h of incubation the culture medium bathing the cell bodies/proximal axons was collected and transported [^{125}I] NGF was determined by gamma-counting. The cells were then fixed and Hoechst stained to assess neuronal survival. The data shown in Figure 2.4 represents the combined results of two independent experiments, performed using cultures from different platings, but using [^{125}I] NGF from the same preparation. Each data point represents the specific transported [^{125}I] NGF and percent survival obtained for an individual compartmented culture. Treatment with [^{125}I] NGF concentrations ranging from 0.0005 to 0.05 ng/ml produced retrograde transport ranging from 0.60 to 3.5 pg NGF, while neuronal survival at these concentrations was approximately 20 %, near the survival maintained in the absence of NGF (21 %). In cultures treated with 0.5 ng/ml [^{125}I] NGF, retrograde transport increased to 12 pg NGF and neuronal survival to 29 %. In contrast, treatment of distal axons with [^{125}I] NGF beads resulted in 84 % survival of the neurons, while only 0.57 pg NGF was transported. A survival rate of 82 % was obtained with 5 ng/ml free NGF, however, this was associated with approximately 150 pg of retrogradely transported [^{125}I] NGF. Thus 5 ng/ml free NGF and NGF beads produced approximately the same level of neuronal survival, while the former associated with retrograde transport of 150 pg and the latter associated with virtually no retrograde transport of NGF at all. These results strongly suggest that retrograde survival by NGF beads is not mediated by the retrograde transport of NGF.

Retrograde transport of TrkA from the axonal plasma membrane is not detectably induced by NGF beads applied to distal axons

The previous experiments establish that retrograde transport of NGF is not essential to communicate the NGF retrograde signal. The signaling endosome model predicts that retrograde transport of NGF is required to maintain TrkA in its ligand-bound, activated form such that it remains catalytically active upon arrival in the cell body. To assess whether retrograde transport of TrkA is induced by application of NGF beads to distal axons, axonal cell surface proteins of neurons in compartmented cultures were labeled with sulfo-NHS-LC-biotin, a membrane-impermeable biotin analogue. Following

the biotinylation procedure, cultures were divided into three treatment groups of 10 cultures/group: one given medium lacking NGF, one given medium containing 50 ng/ml NGF and one given medium containing 50 μl/ml NGF beads at the distal axons, and returned to 37 °C for 8 h. Following this incubation, lysates were prepared from the cell bodies/proximal axons and biotinylated proteins that had been transported during the treatment period were recovered by precipitation with streptavidin-conjugated sepharose beads. Retrogradely transported, biotinylated TrkA was then identified by immunoblotting with antibodies to total Trk protein (Fig. 2.6). Addition of free NGF to distal axons induced an increase of approximately 2.5-fold in the level of retrogradely transported TrkA relative to cultures given no NGF. In contrast, addition of NGF beads did not induce a detectable increase in the transport of TrkA, suggesting that the surrvival-promoting effect of NGF beads can not be attributed to the retrograde transport of TrkA in the absence of NGF.

K252a produces rapid dephosphorylation of pTrkA and prevents accumulation of pTrkA in the cell bodies induced by distal NGF

K252a is a natural alkaloid commonly used to inhibit neurotrophin-induced kinase activity of Trk family receptor tyrosine kinases. It is believed to exert its biological activity by competing with the binding of ATP at the kinase catalytic domain (Kase et al., 1986; Kase et al., 1987). Initially we sought to characterize K252a-mediated inhibition of TrkA kinase activity in sympatheic neurons, both in response to global NGF stimulation in mass cultures, and locally in the cell bodies/proximal axons of neurons in compartmented cultures given NGF only at the distal axons. We first tested the ability of NGF to induce phosphorylation of TrkA and signaling proteins downstream of TrkA in the presence of K252a concentrations commonly used to inhibit Trk kinase activity. Sympathetic neurons in mass cultures were deprived of NGF with 24 nM anti-NGF for 3 h and then pre-treated with medium lacking NGF and anti-NGF and containing 0, 100, 200, or 500 nM K252a for 1 h. TrkA phosphorylation was then induced by addition of 100 ng/ml NGF for 16 h while maintaining the same concentrations of K252a. Cell extracts were analyzed by immunoblotting with antibodies specific to Trk phosphorylated at tyrosines 674/675 (Tyr674/675) in the activation loop of the receptor kinase domain

(Segal et al., 1996). Phosphorylation at these sites is required for the catalytic activity of TrkA and reflects the kinase-active form of the receptor (Coulier et al., 1990; Cunningham et al., 1997; Segal et al., 1996). Under these conditions TrkA phosphorylation was reduced in the presence of all K252a concentrations tested at all time points tested (Fig. 3.1A, lanes 3-5) compared to control cultures not receiving drug treatment (lane 2). Over several experiments, 100 nM K252a had only a partial inhibitory effect on TrkA phosphorylation in response to stimulation with a high concentration of NGF, whereas phosphorylation was reduced to or below the level maintained in the absence of NGF by 200 and 500 nM K252a. These immunoblots were then reprobed with antibodies to phosphorylated Akt and phosphorylated Erk1/2. Similar to its effects of TrkA phosphorylation, K252a effectively inhibited NGF-induced phosphorylation of Akt and Erk1/2, indicating that NGF-induced signaling downstream of TrkA was also inhibited in the presence of K252a.

Previous studies suggest that the effects of K252a are confined to the region of the neuron to which it is applied in compartmented cultures (Riccio et al., 1997; Watson et al., 1999). We confirmed this by testing the compartmentalization of the effect of K252a treatment over the incubation period used in the present experiments. Neurons in compartmented cultures were deprived of NGF using function-blocking anti-NGF antibodies (anti-NGF) in all compartments for 12 h. Concurrently these neurons were treated with K252a (200 nM) applied only to the distal axons or only to the cell bodies/proximal axons, or not treated. While maintaining this K252a distribution the cultures were then stimulated with 200 ng/ml NGF in all compartments for 15 min to induce phosphorylation of TrkA, which was analyzed by immunoblotting with antibodies to pTrkA (Tyr674/675). In neurons not given K252a, pTrkA was detected in the cell bodies/proximal axons and distal axons (Fig. 3.1B, lane 1). Treatment of either distal axons (lane 2) or cell bodies/proximal axons (lane 3) with K252a virtually eliminated TrkA phosphorylation in the region of the neurons to which K252a was applied, without any detectable spread of inhibition to regions of the neurons not directly exposed to K252a. This indicates that biologically effective concentrations of K252a are not achieved in regions of the neurons not directly exposed to K252a, and thus the inhibitor

could be used to block TrkA kinase activity in the cell bodies/proximal axons without directly affecting TrkA activity in the distal axons, and vice versa.

Although the inhibitory effect of K252a on the kinase activity of Trk receptors is well documented, most studies using K252a involve pre-treatment of the cells of interest with the inhibitor before stimulation with neurotrophin. However, to investigate the spatial requirements for TrkA kinase activity in NGF-mediated retrograde signaling it was necessary to determine whether K252a treatment could effectively result in a loss of ongoing TrkA kinase activity. To address this we first determined whether addition of K252a to neurons maintained in NGF produces dephosphorylation of pTrkA. Neurons in mass cultures in the presence of 50 ng/ml NGF were treated with K252a (200 nM) for times ranging from 2.5 to 15 min. The phosphorylation state of TrkA was then assessed by anti-pTrkA (Tyr674/675) immunoblot. Addition of K252a to neurons in the ongoing presence of NGF resulted in the rapid loss of TrkA phosphorylation (Fig. 3.1C). In three replicate experiments, TrkA phosphorylation was virtually undetectable within 5-10 min of K252a addition to the culture medium.

Next we examined neurons in compartmented cultures to determine if addition of K252a to cell bodies/proximal axons reduced the level of TrkA phosphorylation in that compartment when NGF was given only to distal axons. There is evidence in the literature to indicate that it does, although only relatively short time periods have been tested: K252a applied to the cell bodies/proximal axons of sympathetic neurons in compartment cultures inhibited TrkA phosphorylation in the cell bodies in response to distal NGF stimulation for 10 min to 6 h (Kuruvilla et al., 2000; Riccio et al., 1997; Senger and Campenot, 1997), and similar results have been obtained for TrkB in compartmented cultures of dorsal root ganglion neurons (Watson et al., 1999). We asked whether similar effects occur over a 12 h K252a treatment period, as this was the typical incubation period before replenishment for K252a in our experiments. Neurons that had been maintained in compartmented cultures for 6 days were deprived of NGF in all compartments by 12 h treatment with anti-NGF. While maintaining anti-NGF at the cell bodies/proximal axons, cultures were then divided into three groups given medium lacking NGF at the distal axons, medium containing NGF (200 ng/ml) at the distal axons, or given NGF (200 ng/ml) at the distal axons and K252a (200 nM) at the cell

bodies/proximal axons. These treatments were maintained for 12 h, at which time the phosphorylation state of TrkA in the cell bodies/proximal axons of all groups was assessed by Trk immunopreciptation followed by immunoblotting with antibodies to phosphoryated tyrosine. Addition of NGF to the distal axons resulted in an accumulation of pTrkA in the cell bodies/proximal axons (Fig 3.1D, lane 2). In contrast, distal application of NGF did not induce a detectable increase in tyrosine phosphorylation of TrkA in the cell bodies/proximal axons treated with K252a (lane 3).

These results demonstrate that application of K252a results in the rapid dephosphorylation of pTrkA in the ongoing presence of NGF, and that the accumulation of pTrkA in cell bodies/proximal axons that occurs in response to distal NGF is prevented by K252a treatment of the cell bodies/proximal axons. Therefore, these results suggest that K252a in the cell bodies/proximal axons leads to the rapid accumulation of dephosphorylated TrkA, and thus may be unable to relay biologically significant signals locally in the cell body.

Effect of K252a-mediated inhibition of TrkA phosphorylation on neuronal survival

We determined whether NGF-mediated survival of sympathetic neurons was inhibited by K252a. Neurons in mass cultures were treated for 48 h with medium containing NGF (10 ng/ml), NGF plus K252a (100, 200, or 500 nM), or medium lacking NGF and containing anti-NGF. Neuronal survival was assessed in two ways: by determining the fraction of cells with apoptotic nuclei identified by Hoechst staining, and by the MTT assay, in which the yellow MTT tetrazolium dye is reduced to a dark blue formazan precipitate by active mitochondrial succinate dehydrogenase in living cells (Mosmann, 1983). Neuronal viability assayed by MTT staining was reduced in all NGF-treated groups given K252a (Fig. 3.3A), with 200 nM K252a in the presence of 10 ng/ml NGF producing 62 % of the MTT reactivity observed in control cultures not receiving K252a. A similar tendency was obtained when apoptotic and non-apoptotic nuclei were assessed by Hoechst staining (Fig. 3.3B).

These results are consistent with previous reports of decreased neuronal viability in the presence of K252a (Lee and Chao, 2001; Lee et al., 2002; Orike et al., 2001), although K252a-induced neuronal death observed here did not reach the magnitude

induced by NGF deprivation (Fig. 3B, open bar). Approximately 21 % of neurons survived 48h of NGF deprivation, but about 65 % of neurons survived treatment with 200 and 500 nM K252a, assessed by Hoechst staining. The incomplete inhibition of NGF-induced survival by K252a could reflect a survival-promoting effect of K252a itself (Borasio, 1990; Cheng et al., 1994; Glicksman et al., 1995; Roux et al., 2002). To test this we treated neurons for 48 h with anti-NGF and 0, 100, 200, or 500 nM K252a, or with 10 ng/ml NGF. K252a at 100 and 200 nM had no detectable survival promoting effect assayed by MTT, but 500 nM K252a produced a 16 % increase in neuronal survival above the level maintained in control cultures that were deprived of NGF (Fig 3.3C). These results may help explain why K252a does not reduce neuronal survival as effectively as anti-NGF since the inhibition of TrkA phosphorylation at lower concentrations of K252a is less complete, while the more complete inhibition of TrkA phosphorylation by 500 nM K252a may be offset by a relatively small, but significant, survival effect of the K252a itself. Nonetheless, even with incomplete inhibition of TrkA phosphorylation and a small survival promoting effect, the death observed in the presence of K252a was of sufficient magnitude to permit further investigations of the role of TrkA phosphorylation in neuronal survival.

Exposure of cell bodies/proximal axons to K252a does not inhibit retrograde survival mediated by free NGF or NGF beads

We next sought to determine in which cellular compartment(s) of the neuron TrkA kinase activity is required for retrograde survival signaling by NGF. Neurons in compartmented cultures were given anti-NGF in the proximal compartments and 10 ng/ml NGF in distal compartments, while simultaneously the distal axons or the cell bodies/proximal axons of these neurons were treated with 100, 200, or 500 nM K252a. Control cultures were given either 10 ng/ml NGF or anti-NGF in the distal axons compartments with no K252a anywhere. Neuronal viability was assessed after 48 h of treatment. Figure 3.4A shows representative images of MTT-stained neurons, in which live cells are darkly stained and dead cells are not stained, and Hoechst-stained neurons, in which the nuclei of live cells display diffuse labeling and apoptotic cells display condensed, fragmented chromatin or lack of nuclear labeling. These survival assays

suggested that NGF-induced retrograde survival was inhibited by K252a applied to distal axons, but not by K252a applied to cell bodies/proximal axons. This conclusion was supported by quantification of Hoechst-stained nuclei. As expected, NGF provided to the distal axons supported the survival of 95 % of the neurons while 48 h of NGF deprivation in all compartments resulted in only 22 % survival (Fig. 3.4B). K252a applied in distal compartments reduced neuronal survival, with 58 % and 61 % survival occurring in the presence of 200 nM and 500 nM K252a respectively. The magnitude of the inhibition is similar to the magnitude of the inhibition of neuronal survival by K252a in NGF-treated mass cultures (Fig. 3.3B and C) and suggests that the generation of survival signals requires TrkA kinase activity at the site of application of NGF to the neuron. In contrast to the cell death caused by inhibition of TrkA kinase activity in the distal axons, addition of K252a to the cell bodies/proximal axons had a minimal effect on neuronal survival at all of the concentrations tested. Quantification of Hoechst-stained nuclei indicated that survival of at least 87 % of neurons was maintained in the presence of 200 nM K252a applied to cell bodies and proximal axons, not greatly reduced from the 95 % survival observed in NGF control cultures (Fig. 3.4B).

Similar results were obtained when NGF was provided to distal axons in the form of NGF beads. In these experiments, neurons in compartmented cultures were depleted of the NGF in which they were raised (as described in Fig. 2.2), and then given anti-NGF at the cell bodies/proximal axons compartments and medium lacking NGF, or containing 50 ng/ml free NGF or 50 μl/ml NGF beads in distal compartments. Concurrently, the distal axons or the cell bodies/proximal axons of these neurons were treated with or without 500 nM K252a. Neuronal viability was assessed after 30 h of treatment by quantification of Hoechst-stained nuclei (Fig. 3.4C). As expected, distal application of free NGF supported the survival of virtually all of the neurons, whereas in the absence of NGF survival was reduced to 37 %. Application of K252a to distal axons caused a decrease in the survival of neurons supported by either free NGF or NGF beads, reducing survival to approximately 55 % in both groups. In contrast, application of K252a to cell bodies/proximal axons had a virtually no effect on NGF or NGF bead-mediated survival. These results suggest that neuronal survival supported distally by free NGF or NGF beads is not critically dependent on TrkA kinase activity in the cell bodies/proximal axons,

but both treatments require receptor kinase activity at the site of NGF application in the distal axonss to mediate their full survival-promoting effect.

We then tested whether the survival of neurons with K252a applied to cell bodies/proximal axons could be attributed to a survival-promoting effect of K252a that was independent of NGF-mediated trophic support. Neurons in compartmented cultures were deprived of NGF by treatment with anti-NGF in all compartments, while 0, 100, 200, or 500 nM K252a was applied to the cell bodies/proximal axons. Control cultures received 10 ng/ml NGF at their distal axons and no K252a. After 48 h, treatment of cell bodies/proximal axons with 100 nM and 200 nM K252a had no significant effect on survival above the 22 % of neurons maintained in NGF-deprived control cultures given no K252a (Fig. 3.4D). Treatment with 500 nM K252a in the absence of NGF induced an increase to 45 % survival, but this could not account for the 91 % survival observed when 500 nM K252a was applied to the cell bodies/proximal axons of neurons provided with NGF at their distal axons (Fig. 3.4B). This indicated that the survival of neurons treated with K252a on their cell bodies/proximal axons could not be attributed to a survival promoting effect of K252a itself. Thus, K252a treatment of cell bodies/proximal axons did not prevent a retrograde signal generated by NGF at the distal axons from reaching the cell bodies and promoting neuronal survival.

Inhibition of TrkA kinase activity in the cell bodies/proximal axons by K252a does not affect retrograde phosphorylation of Akt or CREB induced by distal NGF

Retrograde transport of pTrkA is believed to be required for the receptor to activate signaling proteins in the cell body that promote neuronal survival. However, K252a treatment of cell bodies/proximal axons of neurons in compartmented cultures blocked the phosphorylation of TrkA in that cellular compartment with little effect on viability. We next asked whether the retrograde activation of two well-characterized pro-survival signaling molecules, Akt and CREB, was maintained in the absence of pTrkA in the cell bodies/proximal axons of cultures treated with K252a in that compartment. Neurons in compartmented cultures were given anti-NGF with or without 200 nM K252a in the center compartment, and anti-NGF or 10 ng/ml NGF in the distal compartments. These treatments were maintained for 24 h at which time the phosphorylation state of

TrkA, Akt and CREB in the cell bodies/proximal axons was assessed by immunoblotting with phospho-specific antibodies (Fig. 3.5A). As expected, pTrkA, as well as pAkt and pCREB, was detected in the cell bodies/proximal axons of cultures treated with NGF at their distal axons (lane 2). The retrograde phosphorylation of TrkA was virtually undetectable in cultures treated with K252a at the cell bodies/proximal axons (lane 3), and was consistently less than the low level of pTrkA that persisted in cultures deprived of NGF during the experiment (lane 1 and 3.5B; see also Fig. 3.1D). In contrast, phosphorylation of Akt and CREB in the cell bodies/proximal axons in response to distal NGF application was not greatly decreased in the presence of K252a at the cell bodies/proximal axons: on average, pAkt decreased by 19 % and there was no signifcant decrease in pCREB (lane 3 and Fig. 3.5B). Importantly, the phosphorylation of Akt and CREB observed under these conditions was dependent on the presence of NGF and not induced by the compound itself, as no increase in pAkt or pCREB above levels maintained in the absence of NGF was detected in cultures treated with K252a in the absence of NGF (lane 4).

K252a weakly promotes neuronal survival at high concentrations, but does not activate Akt or Erk1/2

Our investigations of the survival and axon growth of sympathetic neurons in the presence of K252a and the absence of NGF suggested that 500 nM K252a treatment produced relatively small yet reproducible survival- and growth-promoting effects, whereas 100 and 200 nM K252a did not. To confirm these findings we directly assessed the ability of K252a to support the survival of NGF-dependent sympathetic neurons in the absence of neurotrophin and asked if the survival-promoting effect of K252a increased at concentrations higher than 500 nM. Newly dissected and dissociated neurons were plated in mass culture in medium containing 10 ng/ml NGF, or in medium lacking NGF and containing 0 – 1000 nM K252a. After 24 h (Fig. 3.6A) or 48 h (Fig. 3.6B) of treatment, neuronal survival was assessed by MTT staining. At 24 h, 100 and 200 nM K252a did not promote neuronal survival, while 500 nM produced modest survival and 1000 nM K252a produced substantial survival. Interestingly, this survival-promoting effect at higher concentrations of K252a was greatly diminished by 48 h.

It has recently been reported that K252a induces activation of Akt and Erk1/2 in primary cortical neurons and PC12nnr5 cells, and inhibitors of these pathways block the survival-promoting effect of K252a in these cells (Roux et al., 2002). Significantly, Roux *et al.* also observed K252a-induced phosphorylation of Akt in sympathetic neurons, although the effect was relatively weak (approximately 2-fold) and a survival-promoting effect of the compound was not assessed in these cells. In the present experiments a weak survival-promoting effect was observed, and thus we also directly tested whether activation of Akt and Erk1/2 occurs in sympathetic neurons in response to K252a. Neurons that had been maintained in mass culture in the presence of 50 ng/ml NGF for 6-7 d were deprived of NGF for 6 h and then treated with medium containing 200 ng/ml NGF, or with medium lacking NGF and containing 0 – 1000 nM K252a for 1 h. The activation state of Akt and Erk1/2 were assessed by immunoblotting with phospho-specific antibodies (Fig. 3.6C). As expected, 1h NGF treatment induced phosphorylation of Akt and Erk1/2 (lane 2) however, phosphorylation of these molecules was not induced by treatment with K252a at any of the concentrations tested (lanes 3-6). To rule out the possibility that K252a might induce either a rapid and transient or a delayed activation of Akt or Erk1/2, a similar experiment was performed in which the phosphorylation state of these molecules was assessed at 0.5 h (3.6D, lanes 1-4), 5 h (lanes 5-8), or 15 h (lanes 9-12) after treatment with 200 ng/ml NGF, or with medium lacking NGF and containing 0, 200 or 1000 nM K252a. Again, phosphorylation of Akt or Erk1/2 was not detected in neurons treated with K252a at any of the time points tested. Thus, although higher concentrations of K252a (500 and 1000 nM) promoted survival of NGF-dependent sympathetic neurons in the absence of NGF, the effect is weak, poorly sustained, and does not reflect activation of pro-survival signaling pathways as observed in other neuronal cell types.

Role of local and retrograde PI3-kinase activity in neuronal survival mediated by NGF and NGF beads

Because NGF beads activate Akt, presumably via PI3-kinase, we investigated whether inhibition of PI3-kinase activity with LY294002 (LY) (Vlahos et al., 1994) could block retrograde survival signaling from NGF and NGF beads. It has previously been

shown that LY can be used to locally block NGF-induced PI3-kinase activity in neurons in compartmented cultures, as LY applied to distal axons blocked NGF-induced Akt phosphorylation in the distal axons without effect on Akt phosphorylation in the cell bodies/proximal axons and vice versa (Atwal et al., 2000; Kuruvilla et al., 2000). In the present experiments, neurons in compartmented cultures were depleted of the NGF in which they were raised (as described in the experiments depicted in Fig. 2.2), and then given anti-NGF at the cell bodies/proximal axons and medium lacking NGF, or containing 50 ng/ml free NGF or 50 μl/ml NGF beads at the distal axons. Concurrently, the distal axons or the cell bodies/proximal axons of these neurons were treated with or without 50 μM LY. The use of LY at 50 μM was found to prevent NGF-induced phosphorylation of Akt in neurons in mass cultures (Fig. 2.5A). Neuronal viability was assessed after 30 h of treatment by quantification of Hoechst-stained nuclei, shown in Figure 2.5B. As expected, distal application of free NGF supported the survival of virtually all of the neurons, whereas in the absence of NGF survival was reduced to 30 %. Application of LY to distal axons had little effect on survival promoted by free NGF, only reducing survival to 90 %, while application of LY to cell bodies/proximal axons had a more substantial effect, reducing survival to 55 %. Distal application of NGF beads supported the survival of 83 % of the neurons, and co-application of LY to distal axons reduced the survival to 51 %, while application of LY to cell bodies/proximal axons reduced survival to 63 %. These results suggest that neuronal survival supported distally by free NGF is not critically dependent on PI3-kinase activity in the distal axons, but requires PI3-kinase in the cell bodies/proximal axons to mediate its full survival-promoting effect. Survival maintained by distal application of NGF beads is more critically dependent on the activity of PI3-kinase. This is especially apparent when considered in light of the fact that NGF beads did not induce full survival of the neurons relative to free NGF. The actual NGF bead-induced component of the survival observed in the presence of NGF beads (the survival above that maintained in the absence of NGF) was 53 %. Co-application of LY to distal axons reduced this component to 21 %, a 31 % decrease in the survival maintained by NGF beads when axonal PI3-kinase was inhibited. In contrast, co-application of free NGF and LY at distal axons reduced the free NGF-mediated survival component by only 8 %. This suggests that PI3-kinase activity in the

distal axons may be more important in generating retrograde signals from NGF beads, possibly because this pathway can be activated without internalization, whereas other survival pathways may require internalization. The phenomenon was reversed when PI3-kinase activity was inhibited in the cell bodies/proximal axons: the component of survival mediated by free NGF was reduced by 43 % when cell bodies/proximal axons were treated with LY, whereas LY treatment of the cell bodies/proximal axons reduced the component of survival mediated by NGF beads by only 20 %.

Spatial requirements for TrkA kinase activity in the regulation of axon growth by NGF

The axon growth-promoting effects of NGF involve mechanisms that are localized to the site of extracellular application of NGF (Campenot, 1977; Campenot, 1982a; Campenot, 1987; Campenot, 1994). However, long-term changes associated with continued axonal extension and branching also require the supply of cellular materials synthesized in the cell body, such as Tα1 α-tubulin (Toma et al., 1997) and cholesterol (Posse de Chaves et al., 1997; Vance et al., 1994). This raises the possibility that signals induced by NGF binding at axon terminals instruct the neuron to increase synthesis of proteins and lipids to sustain axon growth. It is unknown whether the signals that promote synthesis and delivery of these materials to the growing axon are communicated by the arrival of activated TrkA in the cell body.

To investigate the role of local and retrograde TrkA kinase activity in mediating axon growth, we determined the effects of different distributions of K252a on axon growth in compartmented cultures of rat sympathetic neurons. K252a has previously been shown to inhibit neurotrophin-induced neurite outgrowth in PC12 cells (Hashimoto, 1988; Hashimoto and Hagino, 1989; Koizumi et al., 1988) and DRG neurons (Doherty and Walsh, 1989; Koizumi et al., 1988). In the present study, distal axons of rat sympathetic neurons in compartmented cultures were axotomized and allowed to regenerate for 24 h with 10 ng/ml NGF supplied in all compartments. Axonal extension was measured, then cultures were divided into treatment groups and axonal extension was monitored for an additional 48 h. All groups were given anti-NGF in the center compartments containing the cell bodies and proximal axons, such that NGF was

available only at the site of axonal growth in the distal axon compartments. Anti-NGF was also given to the distal axons of negative control cultures, which stopped elongating (Fig. 3.2A) and began to deteriorate such that axonal extension was not measurable at 72 h. The axons of control cultures given 10 ng/ml NGF continued to elongate at a rate of about 1.3 mm/day during the treatment period. Three groups of experimental cultures were given 10 ng/ml NGF along with 100, 200, or 500 nM K252a at their distal axons (Fig. 3.2B). Addition of K252a at the site of NGF application inhibited axonal growth and appeared to induce death of the neurons. By 72 h many of the axons in cultures treated with 200 nM and 500 nM K252a were severely degenerated, and it was not possible to measure axonal extension. In contrast, in cultures given 10 ng/ml NGF to the distal axons and 100, 200, or 500 nM K252a to the cell bodies/proximal axons, inhibition of axon growth was not observed (Fig. 3.2C). Rather, the distal axons of these cultures extended at comparable rates to control cultures given NGF and no K252a (compare Fig. 2A). Slight yet detectable axon growth was also observed in additional experiments in which neurons were deprived of NGF in all compartments and exposed to 500 nM K252a at the cell bodies/proximal axons (not shown). This axon growth was associated with a relatively weak survival-promoting affect of 500 nM K252a that was not observed at 100 or 200 nM K252a (see below). At the end of the experiment the relative levels of axonal protein that had accumulated was assessed by harvesting the axonal material in the distal compartments from cultures in each group and immunoblotting with antibodies to β-tubulin, which confirmed the above results: the accumulation of β-tubulin was inhibited by K252a applied to distal axons (Fig. 3.2D, lanes 6-8), but was unaffected by K252a provided to the cell bodies/proximal axons (lanes 3-5). In a similar experiment, in which neuronal survival was supported by 10 ng/ml NGF given to both the distal axons and the cell bodies/proximal axons, the extension of distal axons (Fig. 3.2E) and the accumulation of β-tubulin (Fig. 3.2F) in the distal compartments were again inhibited by distal K252a, but no inhibition was observed when K252a was provided only to the cell bodies/proximal axons. Thus, the inhibition of distal axon growth by distal K252a treatment was not just a consequence of the death of the neurons arising from block of the NGF-induced retrograde survival signal.

Since NGF promotes axonal growth at its site of application to distal axons

(Campenot, 1977; Campenot, 1982a), it is not surprising that K252a applied to distal axons inhibits their growth, presumably by blocking NGF-induced activation of TrkA. However, cultures treated with K252a at the cell bodies/proximal axons remained alive for 48 hr as reflected by the continued growth of their axons. If the retrograde transport to the cell bodies of pTrkA is required for neuronal survival, it would be predicted that K252a treatment at the cell bodies should induce extensive apoptosis. The interesting possibility that phosphorylation of TrkA in the cell bodies may not be required for neuronal survival prompted us to pursue further experiments to investigate the spatial requirements for TrkA phosphorylation in supporting neuronal survival.

Inhibition of clathrin-mediated endocytosis inhibits internalization and prevents retrograde transport of [^{125}I] NGF

To further characterize the role of internalization in the biological functions elicited by NGF in sympathetic neurons, we sought to determine the effect of blocking internalization of NGF on axon growth. NGF beads were not suitable for these studies because the presence of the beads in culture impeded our ability to observe axon growth. Instead, two well-characterized pharmacological inhibitors of clathrin-mediated endocytosis were used to prevent internalization locally at the site of NGF application at the distal axons. Monodansylcadaverine (MDC) and chlorpromazine (CPZ) are mechanistically distinct inhibitors of clathrin-mediated endocytosis: MDC acts by stabilizing clathrin cages thus preventing membrane invagination (Davies et al., 1984), whereas CPZ causes clathrin mis-assembly (Wang et al., 1993). Both of these compounds have been shown to inhibit internalization of [^{125}I] NGF in PC12 cells and dorsal root ganglion neurons (Howe et al., 2001). This study reported that treatment of dorsal root ganglion neurons with either of these inhibitors decreased NGF internalization by approximately 75 %, suggesting that clathrin-mediated endocytosis is the predominant endocytic mechanism for NGF in these cells.

We verified that MDC and CPZ effectively prevent internalization of NGF in sympathetic neurons by two assays: direct analysis of [^{125}I] NGF internalization in the mass cultures, and analysis of [^{125}I] NGF retrograde transport in 3-compartmented cultures. In internalization experiments, neurons in mass cultures were pretreated for 30

min with or without 50 μM MDC or 50 μM CPZ. While maintaining the same drug treatment, cells were then given medium lacking unlabeled NGF but containing 5 ng/ml [^{125}I] NGF for times ranging from 5 min to 6 h. At each time point cells were washed extensively to remove unbound and low affinity-bound [^{125}I] NGF and then subjected to enzymatic cleavage with trypsin at 4 °C to release [^{125}I] NGF bound to surface receptors. Following this treatment, the trypsin bath and the cellular material were collected separately and gamma-counted to determine surface-bound (trypsin-sensitive) and internalized (trypsin-insensitive) [^{125}I] NGF, respectively. The specificity of [^{125}I] NGF binding and internalization was addressed by subtracting the trypsin-sensitive and -insensitive counts obtained from neurons given 5 ng/ml [^{125}I] NGF plus 100-fold excess unlabeled NGF from the counts obtained in the corresponding fraction from each group at each time point. The efficacy of this trypsin treatment in the release of surface bound NGF was confirmed in parallel experiments, in which the neurons were incubated at 4 °C for 30 min prior to, and for 1 h following the addition of [^{125}I] NGF, to allow binding of NGF at the surface but prevent its internalization. Under these conditions it was found that trypsin treatment resulted in the release of 93 % of cell-associated [^{125}I] NGF, indicating that this treatment effectively released surface-associated [^{125}I] NGF. No significant difference was found between the surface-associated [^{125}I] NGF in MDC- or CPZ-treated or control cells at any time point, suggesting that MDC- or CPZ- treatment did not affect the interaction of NGF with its cell surface receptors. In contrast, MDC did cause a substantial reduction in internalization of [^{125}I] NGF. In untreated neurons, significant accumulation of trypsin-insensitive, internalized [^{125}I] NGF was first detectable 1 h after addition of NGF to the culture medium, and continued to accumulate over the course of the experiment (Fig. 2.7A). In contrast, in the presence of MDC or CPZ, internalization of [^{125}I] NGF was reduced by approximately 75 % at both 1 h and 6 h of [^{125}I] NGF treatment, suggesting that internalization of [^{125}I] NGF occurs primarily by clathrin-mediated endocytosis in these neurons, and that inhibitors of this process prevents the accumulation of internalized ligand.

To confirm that MDC and CPZ effectively inhibited internalization of NGF at distal axons, [^{125}I] NGF retrograde transport assays were performed in compartmented cultures. Neurons in compartmented cultures were treated with or without 50 μM MDC

or 50 μM CPZ at the distal axons for 30 min. While maintaining the same drug treatments, axon compartments were then given 50 ng/ml [^{125}I] NGF for 15 h. Following this incubation, the medium and the cellular material were harvested from the cell bodies/proximal axon compartments in each group, and the [^{125}I] content was determined by gamma counting. Specific retrograde transport of [^{125}I] NGF was determined by subtracting counts obtained from cultures given 100-fold excess unlabeled NGF in the presence of 50 ng/ml [^{125}I] NGF. Treatment of distal axons with MDC or CPZ reduced the level of retrograde transport of NGF by 66 % and 61 %, respectively, relative to untreated control cultures (Fig. 2.7B). As these compounds also prevented NGF internalization in mass cultures, and have been reported to inhibit clathrin-mediated endocytosis in several systems including NGF-induced endocytosis of TrkA in primary neurons, it is reasonable to assume that the inhibitory effect of axonal MDC and CPZ treatment on the retrograde transport of [^{125}I] NGF was due to the prevention of NGF (and presumably TrkA) internalization at the plasma membrane, and not due to inhibition of a subsequent step in the trafficking process. These results indicate that the clathrin-dependent endocytic pathway is the predominant mechanism of NGF internalization at distal axons of sympathetic neurons.

Inhibition of clathrin-mediated endocytosis does not affect NGF-induced phosphorylation of TrkA and Akt, but attenuates phosphorylation of Erk1/2

We next examined whether inhibition of clathrin-mediated endocytosis by MDC or CPZ altered the pattern the NGF-induced activation of TrkA, or altered TrkA-mediated signaling to Akt or Erk1/2. Neurons in mass cultures were deprived of NGF for 6 h, pretreated with 50 μM MDC or 50 μM CPZ for 30 min, and then stimulated with or without 50 ng/ml NGF for 1 h while maintaining the same drug treatments. The phosphorylation state of TrkA, Akt and Erk1/2 was assessed by immunoblotting with phospho-specific antibodies as described in the experiment depicted in Figure 2.1. As expected, 1 h NGF stimulation of neurons in the absence of MDC or CPZ induced robust phosphorylation of TrkA, Akt and Erk1/2 (Fig. 2.8). Consistent with the results obtained with NGF beads, treatment with the inhibitors of endocytosis did not reduce NGF-induced phosphorylation of TrkA or Akt, but significantly prevented the

phosphorylation of Erk1/2. Taken with the [^{125}I] NGF internalization and transport data shown in Figure 2.7, these results suggest that inhibition of clathrin-mediated endocytosis and treatment with NGF beads prevents internalization of both NGF and TrkA, as signaling downstream of the receptor is altered in a similar manner with both treatments, and TrkA-induced signaling would be expected to change only if trafficking of the receptor itself was altered.

Inhibition of clathrin-mediated endocytosis at the site of NGF application at distal axons attenuates axon growth

Inhibition of clathrin-mediated endocytosis in PC12 cells expressing dominant-negative dynamin has been shown to prevent NGF-induced neurite outgrowth, suggesting that endosomal NGF/pTrkA signaling is required to mediate this process. We thus asked whether NGF/pTrkA internalization is required to promote axon growth in primary sympathetic neurons. Compartmented cultures were used for these experiments such that the inhibitory effects of MDC and CPZ on clathrin-mediated endocytosis could be confined to site of NGF application at the distal axons, thus preventing NGF internalization without the potentially confounding effects that may arise from inhibiting clathrin-dependent processes in the cell body. Distal axons of neurons in compartmented cultures were axotomized and allowed to regenerate for 24 h in the presence of 50 ng/ml NGF at the distal axons and 10 ng/ml NGF at the cell bodies/proximal axons. Axonal extension was measured, then cultures were divided into treatment groups and axonal extension was monitored for an additional 48 h. The axons of control cultures given 50 ng/ml NGF continued to elongate at a rate of about 1.3 mm/day during the treatment period (Fig. 2.9A), whereas control cultures given medium lacking NGF stopped growing and degenerated extensively (see Fig. 2B). In experimental cultures given 50 μM MDC or 50 μM CPZ in the presence of 50 ng/ml NGF at the distal axons, the rate of axon extension decreased to approximately 50 % of that observed in NGF-treated control cultures (Fig. 2.9A). At the end of the experiment the relative levels of axonal protein that had accumulated was assessed by preparing lysates of the axonal material in the distal compartments from cultures in each group and immunoblotting with antibodies to β-tubulin, an abundant protein of the axonal cytoskeleton. The decreased rate of axon

extension observed in MDC- and CPZ-treated axons was also reflected by a decrease in the relative level of β-tubulin that accumulated in the MDC- and CPZ-treated axon compartments during the experiment indicating that inhibition of clathrin-mediated endocytosis prevent both the rate and the magnitude of axon growth (Fig. 2.9B). Notably, although the growth of axons in the presence of MDC or CPZ was reduced, there was no indication of axonal degeneration, suggesting that these axons were viable. Furthermore, similar results were obtained in experiments in which the NGF initially provided to the cell bodies/proximal axons was replaced with anti-NGF during experimental treatment period, such that the neurons were dependent on NGF provided only to distal axons for axon growth and neuronal survival, suggesting that MDC or CPZ-mediated inhibition of NGF/pTrkA internalization did not cause significant apoptosis of the neurons (not shown).

Local Erk1/2 kinase activity is required to promote local axon growth

It is well established that the axon growth-promoting effect of NGF in sympathetic and dorsal root ganglion neurons and PC12 cells requires Erk1/2 kinase activity. Since clathrin-mediated endocytosis of NGF is required for NGF-induced phosphorylation of Erk1/2 and for NGF-induced axon growth, we asked whether internalized NGF/pTrkA complexes signal to Erk1/2 locally in the axons at the site of NGF uptake, or retrogradely after transport to the cell body. Inhibition of Erk1/2 phosphorylation and activation was achieved using U0126, a specific inhibitor of the respective Erk1/2 kinases, MEK1/2. The optimal concentration for U0126-mediated inhibition of NGF-induced Erk1/2 phosphorylation was determined in neurons in mass cultures by testing drug concentrations in the range typically used to prevent NGF-induced Erk1/2 phosphorylation in primary neurons (25 – 100 μM U0126). It was found that 50 μM U0126 completely blocked detectable phosphorylation of Erk1/2 in response to NGF stimulation (Fig. 2.10A). Compartmented cultures were used to assess the local requirements for Erk1/2 kinase activity in promoting NGF-induced axon growth, in experiments similar to those described above for MDC and CPZ. Distal axons of neurons in compartmented cultures were axotomized and allowed to regenerate for 48 h in the presence of 50 ng/ml NGF at the distal axons and 10 ng/ml NGF at the cell

bodies/proximal axons. Axonal extension was measured, then cultures were divided into treatment groups and axonal extension was evaluated again after an additional 48 h. During the experimental period cultures in all groups were given medium containing anti-NGF at the cell bodies/proximal axons such that they were dependent on NGF provided only to the distal axons. The axons of control cultures given 50 ng/ml NGF continued to elongate at a rate of about 1.1 mm/day during the treatment period (Fig. 2.10B), whereas control cultures given medium lacking NGF stopped growing and degenerated extensively (see Fig. 2.10C). In experimental cultures given 50 μM U0126 in the presence of 50 ng/ml NGF at the distal axons, the rate of axon extension decreased to approximately 20 % of that achieved in NGF-treated control cultures during the treatment period. In contrast, application of U0126 to cell bodies/proximal axons had no effect on the rate of axonal extension (Fig. 2.10B). The inhibitory effect on local axon growth caused by the loss of axonal MEK kinase activity was also reflected by a decrease in the accumulation of axonal β-tubulin in U0126 treated axons (Fig. 2.10C), indicating that both the forward rate of extension and the magnitude of axon growth were substantially reduced in the absence of MEK/Erk signaling. Again, inhibition of MEK activity in cell bodies/proximal axons did not affect the magnitude of axon growth relative to NGF-treated control cultures. These results indicate that MEK kinase activity and subsequent Erk1/2 activation are required locally in axons to promote axon growth. As inhibition of MEK in the cell bodies/proximal axons has virtually no inhibitory effect on distal axon growth, and thus presumably had no inhibitory effect on neuronal survival, these results also suggest that retrograde MEK/Erk signaling is also not required to mediate NGF-induced retrograde survival signals.

DISCUSSION

The mechanisms by which NGF-mediated trophic signals can be communicated from a source of NGF at their axon terminals to the nucleus in the neuronal cell body are poorly understood. The most common theory is that NGF generates signals along axons by the formation of signaling endosomes that transport NGF and pTrkA from the distal site of NGF uptake to the cell body. In this manner it is predicted that retrogradely transported NGF/pTrkA complexes can regulate the activation of signaling proteins in the cell body leading to the transcriptional regulation of gene expression that mediates biological functions of NGF, including neuronal survival and axonal growth. There is substantial evidence supporting many aspects of this theory: It is well documented that NGF is retrogradely transported from the site of uptake at axon terminals to the neuronal cell body, both *in vivo* and *in vitro* (Hendry et al., 1974; Johnson et al., 1978; Korsching and Thoenen, 1983; Palmatier et al., 1984; Ure and Campenot, 1997). It has also been shown that phosphorylated TrkA accumulates distal to a ligature in rat sciatic nerve in an NGF-dependent manner (Ehlers et al., 1995), and more recently that this reflects an accumulation of vesicular pTrk that co-localizes with components of the retrograde motor dynein (Bhattacharyya et al., 2002). As well, NGF complexed with pTrkA derived specifically from the plasma membrane of distal axons has been detected in cell bodies/proximal axons of sympathetic neurons in compartmented cultures (Tsui-Pierchala and Ginty, 1999). Several other studies suggest that retrogradely transported, activated neurotrophin receptors can relay retrograde signals (Bhattacharyya et al., 1997; Kuruvilla et al., 2000; Riccio et al., 1997; Watson et al., 2001; Watson et al., 1999) and that retrograde receptor kinase activity is required for some retrograde signaling responses, especially at shorter time points (Kuruvilla et al., 2000; Riccio et al., 1997; Senger and Campenot, 1997; Watson et al., 2001; Watson et al., 1999). NGF has also been shown to induce the formation of signaling endosomes in PC12 cells, characterized as clathrin-coated vesicles containing NGF and pTrkA, and associated with activated signaling proteins of the MEK/Erk pathway, as well as PI-3 kinase and PLC-γ1 (Griffin and Watson, 1988; Grimes et al., 1997; Howe et al., 2001; Wu et al., 2001). It has also recently been demonstrated that dynamin-dependent endocytosis is required to achieve full activation of Erk5 and CREB in cell bodies/proximal axons of dorsal root ganglion

neurons in compartmented cultures stimulated by distal neurotrophins (Watson et al., 2001).

Previous findings have also been consistent with an alternative mechanism of retrograde signaling in which local activation of TrkA by NGF at axon terminals initiates lateral propagation of phosphorylation to neighboring, unoccupied TrkA receptors, which continues to propagate retrogradely via unoccupied TrkA receptors along the axon to the cell body (Senger and Campenot, 1997). This process has not been directly documented for NGF and TrkA, but has been reported for EGF and ErbB1 in MCF7 cells (Verveer et al., 2000). If present in these neurons, such a mechanism could account for the rapid retrograde phosphorylation of TrkA in cell bodies/proximal axons observed within 1 min of application of NGF to distal axons (Senger and Campenot, 1997). However, the present results are not consistent with this mechanism, since K252a at the cell bodies/proximal axons would be expected to prevent the lateral propagation of TrkA phosphorylation as it reached the cell bodies.

The results presented here clearly demonstrate that the survival of rat sympathetic neurons can be supported without retrograde transport of NGF. Since the survival response requires that an NGF-induced signal reaches the cell body, these results indicate that the retrograde survival signal traveled from the distal axons to the cell bodies unaccompanied by the NGF that generated it. Although several studies support the hypothesis that retrogradely transported, activated Trks carry retrograde signals, there is little evidence that the arrival of pTrk in the cell body is required to mediate neuronal survival evoked by distal neurotrophin. Here we also present evidence that pTrkA is not required to reach the neuronal cell body to communicate NGF-induced signals along axons. These results suggest that retrograde signals promoting survival and axon growth generated by NGF at distal axons reaches cell bodies even when the maintenance and accumulation of NGF-induced TrkA phosphorylation in the cell bodies has been blocked by local application of K252a. Additionally, although retrograde phosphorylation of TrkA was blocked by K252a treatment of cell bodies/proximal axons, the retrograde phosphorylation of Akt and CREB, known pro-survival signaling molecules in sympathetic neurons, was not significantly reduced. These data suggest that the arrival at the cell bodies of signaling endosomes carrying NGF/pTrkA complexes are not the

only mechanism by which NGF at the axon terminals can generate a retrograde survival signal. However, the possibility that signaling endosomes carrying activated TrkA, or lateral propagation of TrkA phosphorylation, could also transmit survival signals or other retrograde signals under other cellular conditions is not ruled out by the present results.

Our results contrast with the conclusions by Riccio *et al.* (1997) that NGF retrograde signaling requires the retrograde transport of NGF. They observed that application of NGF beads for 20 min to distal axons of rat sympathetic neurons in compartmented cultures was ineffective in inducing the nuclear accumulation of phosphorylated CREB that was observed when free NGF was applied to distal axons. They further reported that retrograde activation of CREB was inhibited when retrograde TrkA kinase activity was inhibited by K252a. These observations are not necessarily in conflict with the present results because the failure of distal axon-applied NGF beads to produce nuclear accumulation of pCREB within 20 min does not rule out the possibility that it could produce a retrograde survival signal by another mechanism. Additionally, the level of TrkA tyrosine phosphorylation induced by their preparation of NGF beads, tested in PC12 cells, was significantly less than that induced by the concentration of free NGF that they used. Thus, the magnitude of TrkA activation by NGF beads applied to distal axons could have been too small to produce a detectable retrograde phosphorylation of CREB. Although they did observe nuclear accumulation of pCREB in response to NGF beads applied to cell bodies/proximal axons, cell body responses to NGF applied to cell bodies/proximal axons are generally larger than produced by the same concentration of NGF applied to distal axons (Toma et al., 1997). Finally, longer timepoints of retrograde CREB phosphorylation in the presence of K252a applied to the cell bodies/proximal axons, as detected here, were not presented by Riccio *et al.*

Although it is clear that NGF that has dissociated from NGF beads is not transported to the cell bodies in biologically significant or detectable quantity, we cannot rule out the possibility that internalization and retrograde transport of activated TrkA occurs in the absence of NGF internalization. This seems unlikely in light of the general theory that dissociation from ligand, which typically occurs in the acidic environment of the late endosome, results in down-regulation of receptor tyrosine kinase activity. However, Verveer *et al.* also showed that after focal stimulation with EGF beads,

phosphorylated but non-ligand-bound ErbB1 receptors were endocytosed (Verveer et al., 2000). Thus, NGF may be required simply to bring the TrkA molecules into close enough proximity of one another to initiate receptor autophosphorylation and kinase activity, which may be sufficient to induce endocytosis and transport of pTrkA to the cell body. We attempted to address this issue by biotinylating proteins at the axonal plasma membrane (including TrkA) prior to addition of free NGF or NGF beads, and following a period of incubation, recovering retrogradely transported, biotinylated proteins from the cell bodies/proximal axons by precipitation with streptavidin-conjugated sepharose beads. In these experiments, retrograde transport of biotinylated TrkA was not induced by distal application of NGF beads, relative to cultures given no NGF, while free NGF induced a 2.5-fold increase in biotinylated TrkA in the cell bodies/proximal axons. These results are consistent with the hypothesis that NGF beads do not induce retrograde transport of TrkA, however, the difference in transported TrkA between neurons treated with or without free NGF was relatively small, and we can not exclude the possibility that the relatively weak sensitivity of this assay precludes the detection of a low but biologically significant level of transported TrkA in the NGF bead-treated group. Nonetheless, it also seems unlikely that the intracellular trafficking of TrkA is normal under conditions in which internalization of NGF is blocked, as the phosphorylation of Erk1/2 is largely inhibited in response to NGF beads. Furthermore, similar results were found neurons were stimulated with NGF in the presence of inhibitors of clathrin-mediated endocytosis, which would presumably prevent the internalization of pTrkA in addition to NGF.

If activated TrkA at the cell body is not required to communicate retrograde signals promoting survival and growth triggered by distal NGF, it seems likely that the signals that are required can be activated within the axon prior to internalization of TrkA. Interestingly, most signaling molecules known to be activated by TrkA are present in axons, including PI3-kinase, Erk1/2, PLC-γ (Tsui-Pierchala and Ginty, 1999), Akt (Atwal et al., 2000, Kuruvilla, 2000), and Erk5 (Watson et al., 2001). The PI3-kinase/Akt pathway is particularly significant because it is implicated in supporting neuronal survival (Brunet et al., 2001; Miller and Kaplan, 2001) and in receptor-mediated endocytosis and endosomal trafficking (Takenawa and Itoh, 2001). Kuruvilla *et al.* (2000) reported that pharmacological inhibition of PI3-kinase activity in either the distal axons or in the cell

bodies/proximal axons of neurons supported by distal NGF induced significant apoptosis. They also reported that inhibition of PI3-kinase activity in axons prevented the retrograde transport of NGF/pTrkA complexes. Based on these findings the authors concluded that neuronal survival requires PI3-kinase activity in axons to permit receptor-mediated endocytosis and thus subsequent retrograde transport of NGF/pTrkA complexes, and requires PI3-kinase activity in cell bodies to activate Akt-mediated survival signaling in proximity of the nucleus.

In contrast to the findings of Kuruvilla *et al.*, our results imply that the arrival of pTrkA in cell bodies is not required to induce retrograde phosphorylation of Akt or to mediate neuronal survival. Futhermore, we have found that inhibition of PI3-kinase activity in distal axons given free NGF had little effect on neuronal survival. Taken with the finding that PI3-kinase activity is required to maintain neuronal survival when NGF is restricted to the axonal plasma membrane, but not when internalization is permitted, these data raise the possibility that PI3-kinase-mediated activation of Akt in the axon is required to communicate the retrograde survival signal in the absence of retrograde transport of NGF. However, inhibition of PI3-kinase activity in the cell bodies/proximal axons also had a partial inhibitory effect on NGF bead-mediated survival, suggesting that retrograde activation of PI3-kinase occurs in response to stimulation of distal axons with NGF beads and that this activity contributes to NGF bead-mediated survival. Our results suggest that under conditions were retrograde TrkA kinase activity is inhibited, distal TrkA kinase activity is sufficient to maintain retrograde Akt signaling and thus neuronal survival. Whether a component of retrograde activation of Akt reflects transported or cell body-localized Akt is unknown. Our results do suggest that a significant component of free NGF-mediated retrograde survival is induced by transported or cell body-localized retrograde PI3-kinase activity, implicating retrograde PI3-kinase activity in the retrograde activation of Akt. Whether activated PI3-kinase and/or Akt are transported from distal axons to cell bodies in response to NGF bead stimulation of distal axons may reveal important clues regarding the nature of the mechanism by which NGF beads mediate survival.

We have found that inhibiting endocytosis of NGF (by NGF beads) or NGF/pTrkA complexes (by MDC and CPZ) internalization does not prevent NGF-

induced phosphorylation of Akt, indicating that this pro-survival signaling pathway can be activated from the plasma membrane. This result is consistent with findings of Zhang *et al* (2000), who showed that phosphorylation of Akt was increased both in magnitude and duration in NGF-treated PC12 cells when TrkA signaling was concentrated to the plasma membrane by inhibiting endocytosis of TrkA with dominant-negative dynamin (Zhang et al., 2000). Significantly, the authors further reported that cell survival is enhanced under conditions where NGF/pTrkA signaling is confined to the plasma membrane. Since constitutive activation of Akt has been shown to promote survival of NGF-deprived PC12 cells (Dudek *et al.*, 1997) and sympathetic neurons (Crowder and Freeman, 1998), the authors suggest that the persistent activation of Akt, generated by NGF-induced activation of TrkA at the plasma membrane, may account for the enhanced survival response. We have found that the survival of NGF-dependent sympathetic neurons is maintained under conditions where internalization of NGF is blocked and that NGF-induced phosphorylation of Akt is not impeded under these conditions. These results suggest that NGF-induced activation of Akt occurs primarily by TrkA at the plasma membrane prior to internalization. However, the present data are also consistent with the possibility that PI3-kinase is retrogradely activated in a manner that is not inhibited by K252a-induced loss of TrkA kinase activity in the cell body. It is important to consider that none of these possibilities are mutually exclusive and that multiple mechanisms may be important at different times during development and/or may act in parallel thereby safe-guarding the viability of the neuron.

K252a is a well-known inhibitor of Trk kinase activity and has been shown to block many biological functions of NGF, including NGF-induced neurite outgrowth in PC12 cells and NGF-induced survival and neurite outgrowth in dorsal root ganglion neurons (Doherty and Walsh, 1989; Koizumi et al., 1988). Paradoxically, however, K252a has also been shown to have neurotrophic properties. K252a promoted the survival of several populations of CNS neurons in various experimental paradigms *in vitro* (Borasio, 1990; Cheng et al., 1994; Glicksman et al., 1995) and protected against ischemic brain damage *in vivo* (Hada and Miyamoto, 1990; Ohno et al., 1991). The mechanisms by which these neuroprotective effects are mediated have not been elucidated. Interestingly, it has recently been reported that K252a treatment induced

activation of the PI3-kinase/Akt pathway and of the MEK/Erk pathway in primary cortical neurons and PC12nnr cells, and that pharmacological inhibitors of these pathways reduced the survival promoting effects of K252a (Roux et al., 2002). In light of our findings that K252a application to cell bodies of sympathetic neurons supported by distal NGF does not cause apoptosis, we have considered the possibility that K252a may be supporting survival independently of NGF in our experiments. Under various experimental conditions in both mass and compartmented cultures, we found that treatment of sympathetic neurons with K252a at concentrations of 100 and 200 nM in the absence of NGF exhibited no survival- or growth-promoting effects above background, while at 500 nM and 1000 nM K252a there was a small but consistent increase in neuronal survival and axon growth in the absence of NGF, although the effects were marginal compared to those induced by NGF. Furthermore, at concentrations ranging from 100 nM to 1000 nM, and over treatment times of 0.5 to 15 h, K252a did not induce phosphorylation of Akt or Erk1/2 in mass cultures, nor did 24 h treatment with 200 nM K252a induce phosphorylation of Akt or CREB in the cell bodies/proximal axons of NGF-deprived neurons in compartmented cultures. Thus, in contrast to results obtained with other neuronal types (Borasio, 1990; Cheng et al., 1994; Glicksman et al., 1995; Hada and Miyamoto, 1990; Maroney et al., 1995; Ohno et al., 1991; Roux et al., 2002), the neurotrophic effect of K252a on sympathetic neurons *in vitro* is relatively weak and does not appear to support robust survival or survival-associated signaling in these cells in the absence of NGF.

The specificity of action of K252a is not a major concern, since the main conclusions of the present experiments are drawn from the absence of an effect of K252a applied to cell bodies/proximal axons. Since K252a: produces rapid dephosphorylation of TrkA, prevents the accumulation of distal NGF-induced pTrkA in cell bodies/proximal axons, inhibits NGF-induced axon growth when directly applied to distal axons, and does not itself promote significant neuronal survival, the survival of neurons treated with K252a at the cell bodies/proximal axons cannot have arisen from a lack of effect of K252a or a side effect. In our system, K252a treatment did not reduce neuronal survival as extensively as was achieved in the absence of NGF. The weaker effect of K252a may reflect incomplete inhibition of TrkA kinase activity at the lower concentrations used,

and a slight survival-promoting effect at higher concentrations. However, it is difficult to reconcile this explanation with our observation that the phosphorylation state of TrkA in neurons treated with 200 nM K252a in the presence of NGF was consistently at or below the level detected in neurons maintained in the absence of NGF (Fig. 1D and 5A,B). Several previous reports indicate that K252a-mediated inhibition of Trk kinase activity and neurotrophin withdrawl produce similar levels of neuronal death in other systems (Lee and Chao, 2001; Lee et al., 2002; Orike et al., 2001). It is difficult to speculate about the cause of this discrepancy with our results, or the explanation for the reduced efficacy of K252a-meditated death, as it does not appear to be attributable to incomplete inhibition of TrkA kinase activity or to a neurotrophic property of the compound at the concentrations used.

In addition to initiation of retrograde signaling, local NGF signaling in distal axons is necessary for axonal growth (Campenot, 1977; Campenot, 1982a; Campenot, 1987; Campenot, 1994). However, continued axonal extension requires an ongoing production and delivery of cytoskeletal proteins (such as Tα1 α-tubulin (Toma et al., 1997)) and cholesterol (Posse de Chaves et al., 1997; Vance et al., 1994). The stimulation of synthesis of such materials likely involves retrograde NGF signaling to the cell body (Toma et al., 1997). We have found that inhibition of TrkA kinase activity in cell bodies had minimal effects on the rate and magnitude of axonal extension. This suggests that direct TrkA signaling in the cell bodies is not required to mediate the growth-promoting effects of NGF, and lends further support to the conclusion that the viability of these cells is not compromised under conditions of prolonged inhibition of TrkA kinase activity at the cell bodies, as complex biological functions such as axonal growth are sustained. Paradoxically, however, our results demonstrate a clear role for internalization of NGF/pTrkA complexes in promoting local axon growth. Pharmacological inhibition of clathrin-mediated endocytosis at the site of NGF application at distal axons significantly reduced the rate and the magnitude of local axon growth induced by NGF. In addition to attenuating the growth-promoting effect of NGF in axons, inhibition of endocytosis also prevented the NGF-induced phosphorylation of Erk1/2. Similar effects have been reported in studies in which clathrin mediated endocytosis was prevented by pharmacological inhibitors (including MDC and CPZ) or by expression of function-

blocking dynamin mutants in PC12 cells and dorsal root ganglion neurons (Howe et al., 2001; Rakhit et al., 2001; York et al., 2000). These findings are consistent with the emerging role for endocytosis in the regulation of activation of molecules in the MEK/Erk pathway by several receptor tyrosine kinases and G protein-coupled receptors (reviewed in: (Ceresa and Schmid, 2000; (Grimes and Miettinen, 2003)). However, contradictory results were obtained by Zhang *et al.* (2000), who found that NGF-induced signals derived only from the plasma membrane in PC12 cells produced prolonged activation of Erk1/2, although the initial magnitude of Erk1/2 activation was significantly less than when endocytosis was not blocked. Reasons for this discrepancy in the results are not immediately clear.

An important biological function of signaling by the MEK/Erk pathway in neurons is in the regulation of axon growth. Activation of this pathway is required for neurite outgrowth in NGF-treated PC12 cells (Cowley et al., 1994), and over-expression of active forms of Ras, Raf, and MEK induces neurite extension in these cells (Klesse et al., 1999; Wood et al., 1993). Over-expression of constitutively-active Ras induced axon growth in BDNF-responsive nodose ganglion neurons in the absence of neurotrophins (Borasio et al., 1989), while similar results were found when Raf-1 was expressed in sensory neurons in the absence of neurotrophin (Markus et al., 2002b). Thus, it is reasonable to hypothesize that the disruption of axon growth caused by inhibition of clathrin-mediated endocytosis was a result of attenuation of NGF-induced Erk1/2-mediated signaling. Consistent with this hypothesis, we found that inhibition of MEK kinase activity (and thus inhibition of Erk1/2 phosphorylation) at the site of NGF uptake in the distal axons disrupted axon growth, decreasing the rate of axon elongation and the magnitude of growth to a similar extent as observed when endocytosis was inhibited in distal axons. No change in the nature of axon growth was observed when MEK kinase activity was inhibited in cell bodies/proximal, suggesting that internalization of NGF/pTrkA complexes is required to activate signaling via the MEK/Erk pathway locally within the axon. These results are consistent with another study in which pharmacological inhibition of MEK were used to show MEK/Erk signaling locally in distal axons, but not retrogradely in cell bodies/proximal axons, is required for BDNF/TrkB-mediated elongation of axons of sympathetic neurons in compartmented cultures (Atwal et al.,

2000) (described in more detail in Chapter 5: General Discussion). Notably, Atwal *et al.* also reported that inhibition of MEK kinase activity in cell bodies/proximal axons of neurons supported by distal NGF did not cause apoptosis, which was also suggested in the present study since axon growth, and thus likely neuronal survival, was not affected by the application of U0126 to cell bodies/proximal axons.

The results of this study provide strong evidence for a mechanism of NGF-mediated retrograde survival of sympathetic neurons that does not require retrograde transport of NGF. Furthermore, we present evidence that inhibition of TrkA kinase activity in the cell bodies of sympathetic neurons supported by NGF applied only to distal axons has no significant effect on two of the fundamental biological functions of NGF in these neurons: survival and axon growth. The findings suggest that NGF-induced phosphorylation of TrkA at the plasma membrane of distal axons is sufficient to support survival of sympathetic neurons, which contradicts the long-standing belief that internalization and retrograde transport of NGF/pTrkA complexes are required to transmit NGF-mediated retrograde signals. This effect may be mediated in part by intact activity of the pro-survival factor Akt maintained by PI3-kinase activity at the plasma membrane, and does not require MEK kinase activity or phosphorylation of Erk1/2. The existence of a mechanism for NGF-mediated survival that is independent of NGF transport has broad implications in the approaches taken to understand how target derived neurotrophins maintain neuronal survival. There are many speculative scenarios as to how such a mechanism may operate, but clearly the assumption of over 30 years that retrograde transport of NGF (and all other neurotrophic factors) is the only way that retrograde signals can reach the cell bodies needs continued re-examination.

FIGURES

Figure 2.1 NGF beads stimulate phosphorylation of TrkA and Akt, but not Erk1/2

Neurons in 24-well plate mass cultures were deprived of NGF for 6 h by exposure to medium containing no NGF and 24 nM anti-NGF antibodies. The cultures were then treated for 1 h with medium lacking NGF (lane 1), or medium containing free NGF (lanes 2-4), or NGF beads (lanes 5-7) at the concentrations indicated. Cell lysates were then prepared for immunoblotting. Lanes in A and B shown are from the same immunoblot, each consisting of pooled extracts from 2 culture wells. *A)* The blot was first probed with anti-phosphotyrosine (4G10) (upper panel) and then stripped and reprobed with anti-phosphoTrkA (Y490) (middle panel). The blot was stripped again and reprobed with antibodies total TrkA protein (anti-Trk (C-14)) to confirm equalization of protein loading (lower panel). *B)* The portion of the blotted membrane containing proteins less than 100 kDa was probed with anti-phosphoAkt antibodies (upper panel), and then probed with anti-phosphoErk1/2 antibodies (lower panel). Finally, the blot was stripped and re-probed with anti-Erk1 antibodies to confirm equal protein loading. These results are representative of three experiments with similar results.

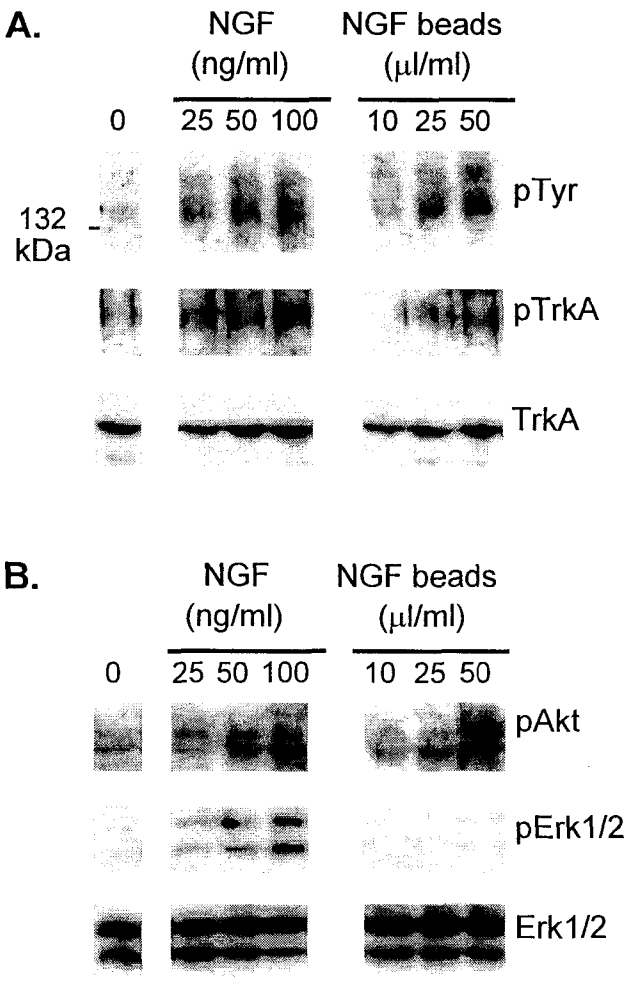


Figure 2.2 NGF beads support retrograde survival of sympathetic neurons in compartmented cultures

Neurons in compartmented cultures were depleted of the free NGF (50 ng/ml) in which they were raised by incubating all compartments with 24 nM anti-NGF for 3 h. Cell bodies/proximal axons (CB/PAx) were given anti-NGF for the remainder of the experiment. Distal axons (DAx) were rinsed twice with unsupplemented culture medium to remove the antibodies, and cultures were divided into treatment groups of 3 cultures/group and distal axons were given medium with or without 50 ng/ml free NGF, 50 µl/ml NGF beads, or 50 µl/ml control beads which were prepared in the absence of NGF or in the presence of NGF but without the EDAC cross-linking agent. After 30 h of treatment the neurons were fixed with 4 % paraformaldehyde and stained with Hoechst DNA stain. *A)* Representative fluorescence confocal micrographs of neurons from each treatment group after Hoechst DNA staining. *B)* Quantification of Hoechst DNA staining from neurons in each treatment group. For quantification, nuclei were visualized by ultraviolet florescence using a Nikon inverted epi-florescence microscope. The nuclei of at least 1000 neurons were counted per treatment group. Data are presented as the mean of the percentages of live neurons in each treatment group (+/- SEM, n=3). Results are representative of three experiments performed under similar conditions. *C)* Stage 1: Survival of neurons in compartment cultures exposed to no NGF, NGF or NGF beads at the distal axons was assessed as described in Fig. 2.2A and B. Stage2: Following the stage 1 incubation, medium bathing the distal axons in each group was collected, subjected to centrifugation at 100,000 x g for 1 h to remove beads, and supernatants were exposed to the distal axons of a second set of cultures (*vertical lines*). Survival was assessed as outlined in Fig. 2.2A and B. Stage 2 also shows the survival of neurons exposed to medium that was incubated in compartmented cultures lacking neurons ("mock" cultures) during the stage 1 incubation (*horizontal lines*).

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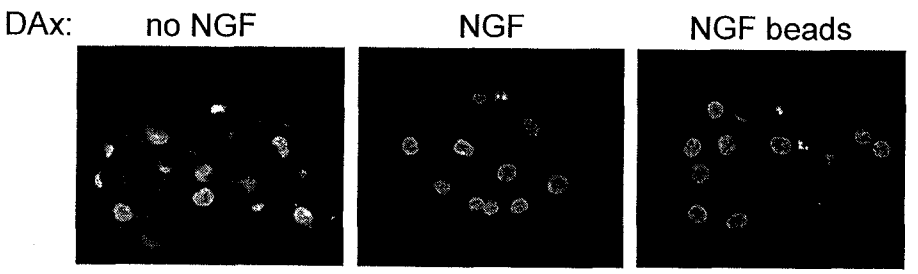

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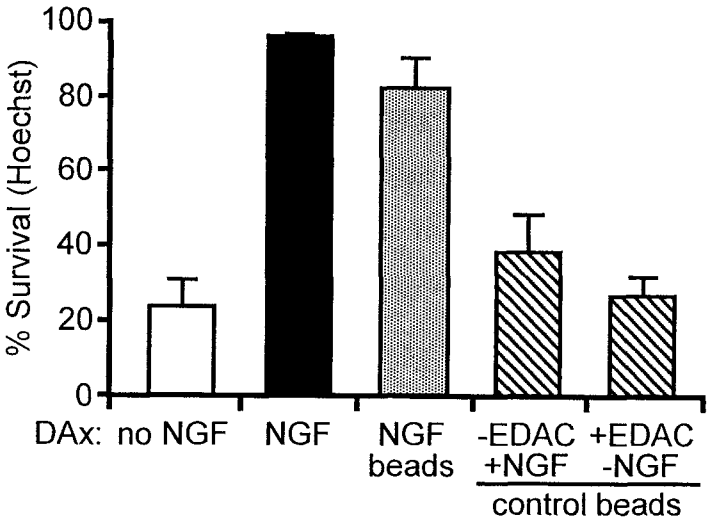

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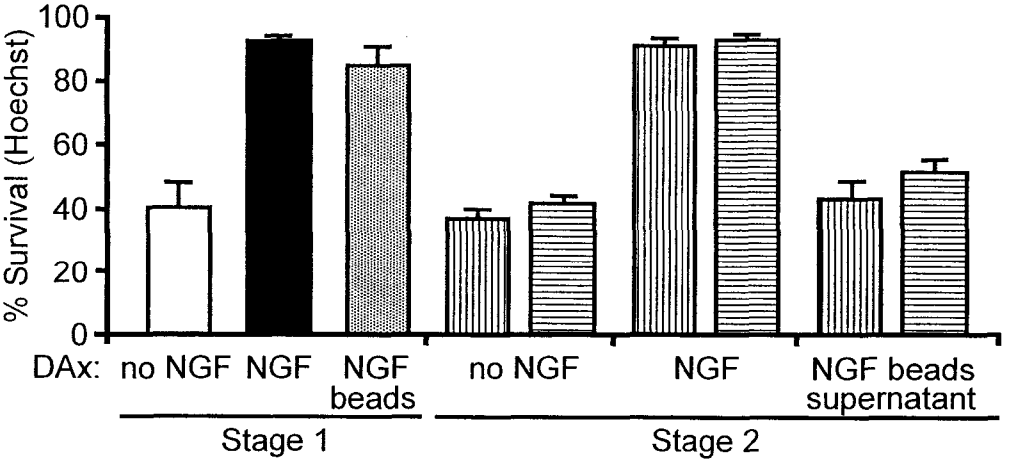

Figure 2.3 [^{125}I] NGF from is not detectably transported from [^{125}I] NGF beads applied to distal axons

A) Neurons in 24-well plate mass cultures were deprived of NGF for 6 h with 24 nM anti-NGF. Cultures were then divided into 2 wells per treatment group and treated for 1 h with medium containing no NGF, 50 µl/ml [^{125}I] NGF beads, 50 ng/ml free [^{125}I] NGF (an aliquot from the same [^{125}I] NGF preparation used to make [^{125}I] NGF beads), or 50 ng/ml unlabeled free NGF, as shown in the figure. Cell extracts were prepared for immunoblotting and probed with anti-phosphoTrkA (Y490) antibodies (top panel). The blot was then stripped and reprobed with antibodies to total TrkA protein to confirm equal protein loading (bottom panel). *B and C)* Neurons in compartmented cultures were deprived of NGF in all compartments as described in Figure 2.2A. Cultures were then divided into treatment groups of 3 cultures/group, and distal axons were given medium from the same preparations as in 2.3A: no NGF, 50 ng/ml soluble [^{125}I] NGF, or 50 µl/ml [^{125}I] NGF beads, for 30 h. Control cultures for non-specific [^{125}I] transport received the same concentration of soluble or bead-bound [^{125}I] NGF plus a 100-fold excess unlabeled NGF in the distal axon compartments for the same period. After this incubation medium bathing the cell bodies/proximal axons was collected and gamma-counted to determine transported [^{125}I] NGF. *B)* Average transported NGF (equivalent pg) minus average non-specific transported NGF for soluble [^{125}I] NGF and [^{125}I] NGF beads-treated cultures (+/- SEM, n=3). *C)* The neurons were then prepared for Hoechst staining as described in Figure 2.2A. Nuclei were visualized by ultraviolet florescence using a Nikon inverted epi-florescence microscope. At least 500 nuclei were counted per treatment group. The average percentage of live cells in each group (+/- SEM, n=3) is presented in Figure 2.3C. The data shown in Figure 2.3 is representative of 2 experiments performed under similar conditions.

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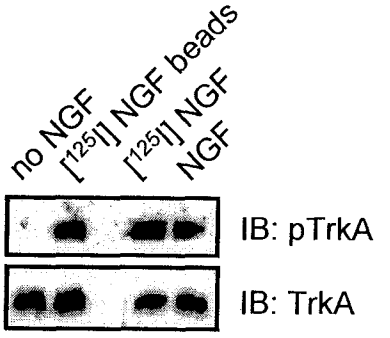

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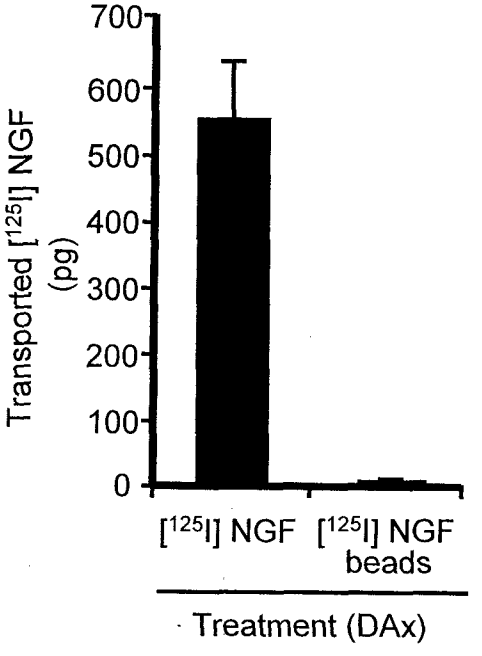

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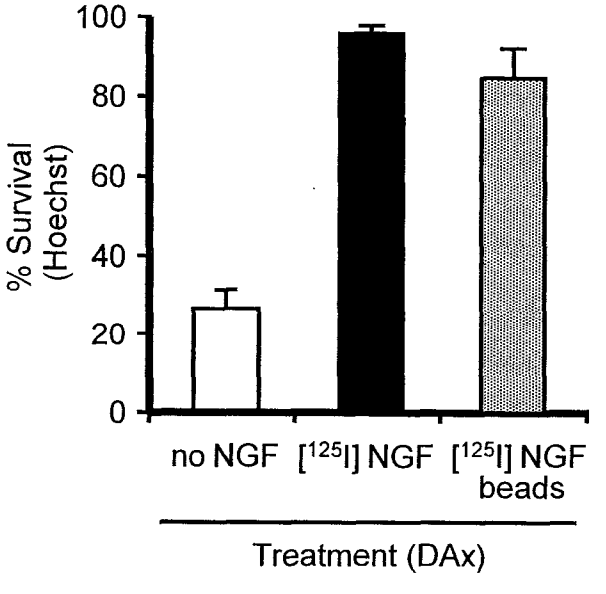

Figure 2.4 Relationship of NGF retrograde transport and neuronal survival for free NGF and NGF beads

Neurons in compartmented cultures were deprived of NGF in all compartments as described in Figure 2.2A. Cultures were then divided into treatment groups of 3 cultures per group, and distal axons were given medium containing no NGF, 50 µl/ml [^{125}I] NGF beads, or soluble [^{125}I] NGF in concentrations ranging from 0.0005 ng/ml to 50 ng/ml. Soluble [^{125}I] NGF was from the same [^{125}I] NGF preparation used to produce [^{125}I] NGF beads. For each group, control cultures for non-specific [^{125}I] transport received the same concentration of soluble or bead-bound [^{125}I] NGF plus 100-fold excess unlabeled NGF in the distal axon compartments. Cultures were incubated for 30 h, at which time medium bathing the cell bodies/proximal axons was collected and transported radioactivity was determined by gamma-counting. Average non-specific transport was subtracted from average transported counts for each concentration of free NGF and for NGF beads. The neurons were then prepared for Hoechst staining as described in Figure 2.2A. Nuclei of at least 250 neurons were counted per culture, and the percentage of live neurons was plotted against the specific [^{125}I] NGF transport for each culture. Figure 2.4 shows the combined data from 2 experiments performed under similar conditions using [^{125}I] NGF from the same preparation. Radioactive decay of the [^{125}I] during the time between experiments, based on its half-life of 59.7 d, accounts for a difference of less than 4 % between experiments and was considered insignificant. Each data point shown represents the [^{125}I] NGF transport and survival data for one culture. The average survival of neurons receiving no NGF was 21 %.

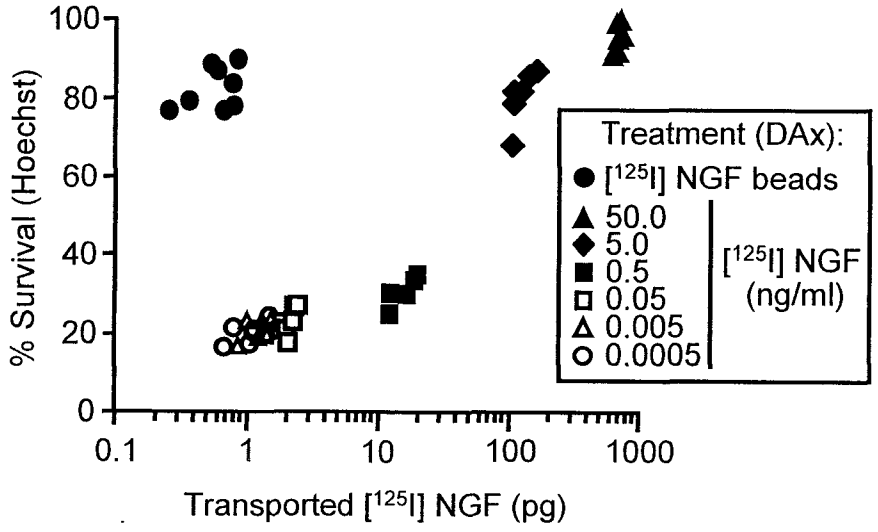

Figure 2.5 Retrograde transport of TrkA from the axonal plasma membrane is not detectably induced by NGF beads applied to distal axons.

Distal axons of neurons in compartmented cultures were rinsed with ice-cold PBS and incubated with 2 mM sulfo-NLS-LC-biotin for 30 min at 4 °C to biotinylate proteins exposed at the surface of the axonal plasma membrane. Distal axons were then rinsed and cultures were divided into treatment groups of 10 cultures/group, given medium with or without 50 ng/ml NGF or 50 μl/ml NGF beads at the distal axons, and retrurned to 37 °C for 8 h. Transported, biotinylated proteins in the cell bodies/proximal axons were then recovered by precipitation with streptavidin beads and resolved by SDS PAGE. Transported, biotinylated TrkA was identified by immunoblotting with anti-Trk antibodies. Equal aliquots of the cell bodies/proximal axons lysates collected after the streptavidin precipitation were also resolved by SDS PAGE and immunoblotted with anti-Trk antibodies to confirm the presence of equal protein levels in the lysates.

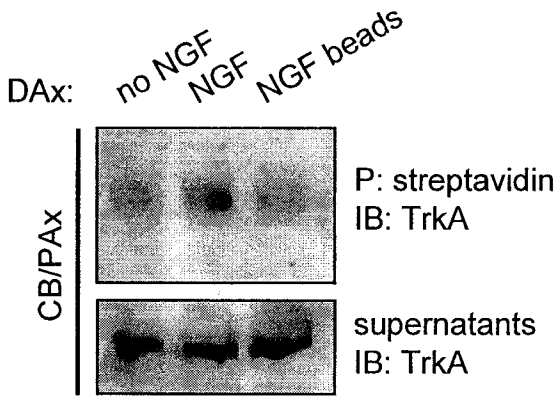

Figure 2.6 Characterization of the inhibitory effect of K252a on TrkA kinase activity in mass and compartmented cultures of sympathetic neurons

A) Concentration-dependent inhibition of TrkA kinase activity by K252a in mass cultures. Mass cultures were given medium containing 24 nM anti-NGF for 3 h, then given medium lacking NGF or anti-NGF but containing 0, 100, 200, or 500 nM K252a for 1 h, and finally exposed to 0 or 100 ng/ml NGF for 16 h, while the same concentration of K252a was maintained. The phosphorylation state of TrkA, Akt, and Erk1/2 was assessed by immunoblotting with anti-pTrkA (Tyr674/675), anti-pAkt (Ser473) and anti-pErk1/2 (Thr202/Tyr204) antibodies, respectively. The blot was then reprobed with anti-β-tubulin antibodies, which indicated that the lack of pTrkA, pAkt, or pErk1/2 observed in the presence of K252a could not be attributed to reduced protein levels loaded on the gel. *B)* The effect of K252a remains localized to the compartment to which it is applied. Neurons in compartmented cultures were deprived of NGF in all compartments for 12 h with anti-NGF, while 200 nM K252a was applied to the cell bodies/proximal axons, distal axons, or neither. All compartments were then stimulated with 200 ng/ml NGF for 15 min, while maintaining the same K252a distribution. TrkA phosphorylation was assessed by immunoblotting with anti-pTrkA (Tyr674/675) antibodies. Results are representative of 2 similar experiments. *C)* K252a treatment results in loss of TrkA kinase activity after NGF stimulation. Neurons in mass cultures in the presence of 50 ng/ml NGF were treated with 200 nM K252a for the indicated times. TrkA phosphorylation was assessed by immunoblotting with anti-pTrkA (Tyr674/675) antibodies. The upper band is a protein that cross-reacts with the antibody and demonstrates that the decrease in pTrkA immunoreactivity in lanes 3-5 is not attributable to reduced protein loading. Results shown are representative of 4 similar experiments. *D)* K252a prevents accumulation of pTrkA in the cell bodies induced by distal NGF. Neurons grown in compartment cultures were deprived of NGF in all compartments for 12 h using anti-NGF. While maintaining anti-NGF at the cell bodies/proximal axons (CB/PAx), cultures were then treated with or without K252a (200 nM) at the cell bodies/proximal axons and with or without NGF (200 ng/ml) at the distal axons (DAx), as indicated, for 12 h. TrkA phosphorylation in cell bodies/proximal axons was assessed by immunoprecipitation with antibodies to TrkA followed by immunoblotting with antibodies to phosphotyrosine (upper panel). The blot was reprobed with antibodies to total Trk protein to ensure similar protein levels in each lane (lower panel). Results shown are representative of 2 similar experiments.

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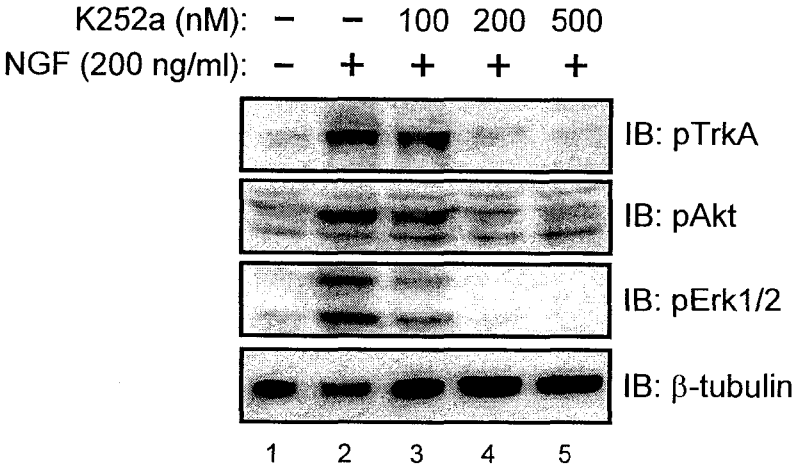

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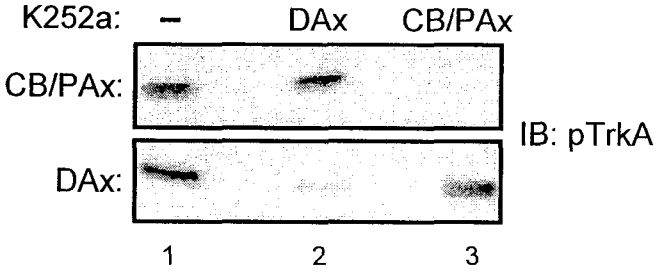

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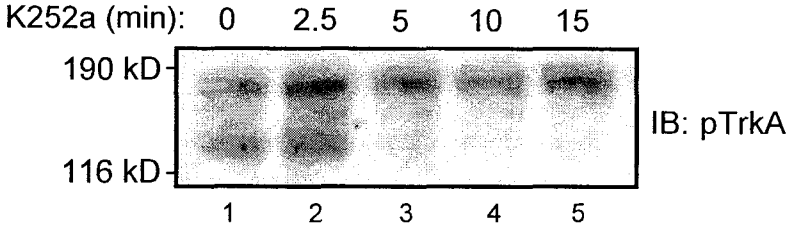

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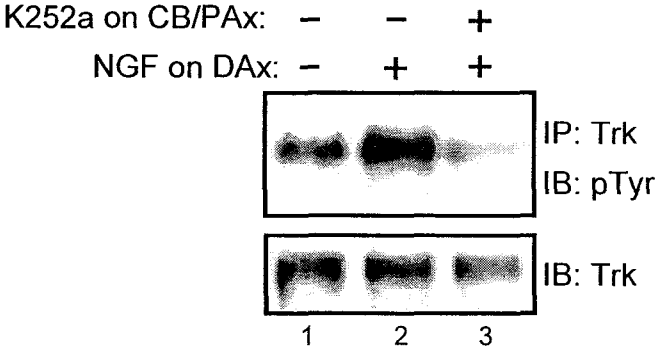

Figure 2.7 Effect of K252a-mediated inhibition of TrkA kinase activity on neuronal survival in mass cultures

A and B) Mass cultures were treated with the indicated concentrations of K252a in the presence of 10 ng/ml NGF. Negative control cultures received anti-NGF and no K252a. After 48 h of treatment, neuronal viability was determined by MTT assay (A) followed by Hoechst staining (B) in which nuclei of at least 400 neurons were counted per treatment group. Results are presented as the mean +/- SEM combined from 4 similar experiments, each comprised of 4-6 culture wells per treatment group. Statistically significant differences between each K252a-treated group and the NGF-treated control group were detected by the paired sample *t*-test and are indicated for each group by one ($p<0.05$) or two ($p<0.01$) astericks. *C)* Mass cultures were treated with anti-NGF and the indicated concentrations of K252a. Positive control cultures were given 10 ng/ml NGF and no K252a. After 48 h neuronal viability was assessed by MTT assay. Results are presented as mean +/- SEM (n=4 wells/group) and are representative of 3 similar experiments. Statistically significant differences between each K252a-treated group and the NGF-deprived (anti-NGF treated) control group as assessed by the paired sample *t*-test are indicated by an asterick ($p<0.05$).

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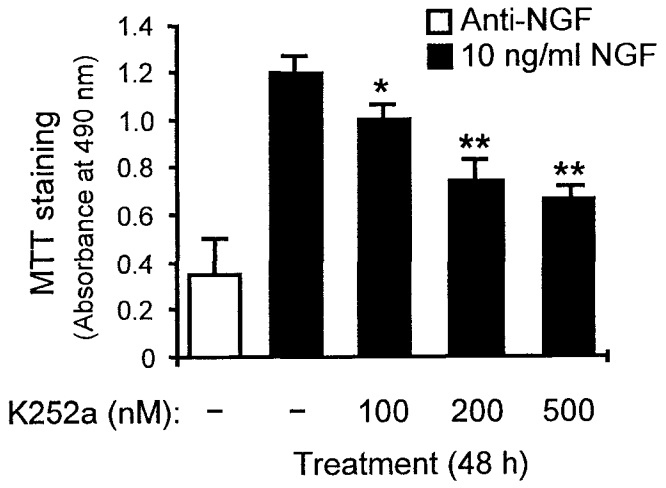

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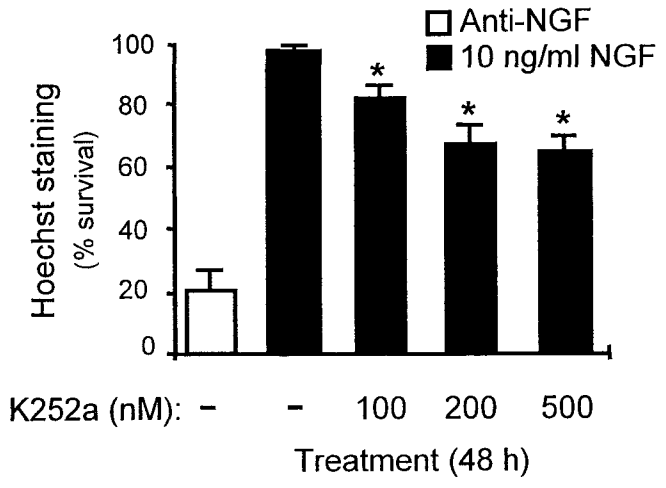

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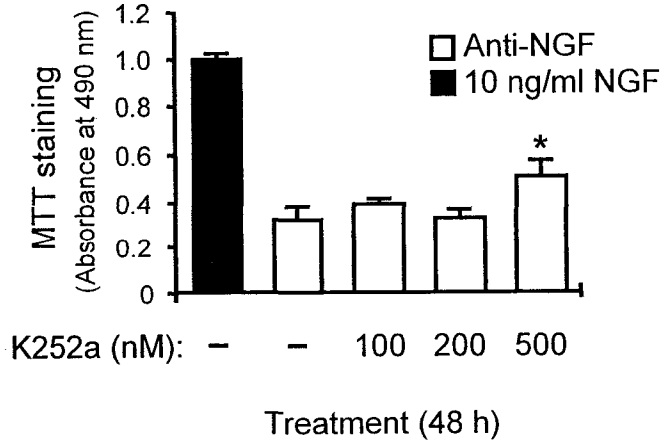

Figure 2.8 Effect of local K252a-mediated inhibition of TrkA kinase activity on neuronal survival mediated by free NGF or NGF beads

A and B) Compartmented cultures were provided with anti-NGF at their cell bodies/proximal axons (CB/PAx) and 10 ng/ml NGF at their distal axons (DAx). Simultaneously, either the distal axons or the cell bodies/proximal axons were given K252a. Control cultures were given anti-NGF at the cell bodies/proximal axons and either 10 ng/ml NGF, or anti-NGF at the distal axons. Neuronal survival was assessed after 48 h of treatment. *A)* Representative images of MTT and Hoechst staining (visualized by phase contrast microscopy and UV fluorescence, respectively) of neurons from control groups and 200 nM K252a treatment groups. *B)* Quantification of neuronal survival assessed by Hoechst staining. The combined data of 7-9 cultures per group from 3 similar experiments are presented as the mean percentage of live neurons out of at least 300 neurons counted per culture in each treatment group (+/- SEM). Statistically significant differences between each K252a-treated group and the NGF-treated control group were detected by the paired sample *t*-test and are indicated for each group by an asterick ($p<0.05$). *C)* Neurons in compartmented cultures were deprived of NGF in all compartments as described in Figure 2.2A. Cell bodies/proximal axons were then given anti-NGF for and distal axons were given no NGF, NGF (50 ng/ml), or NGF beads (50 µl/ml) for 30 hours. Concurrently, cell bodies/proximal axons or distal axons were treated with or without 500 nM K252a as indicated. Then the neuronal survival of at least 250 neurons per culture was determined by quantification of Hoechst stained nuclei. The mean percentage of live neurons in three replicate experiments is shown (+/-SEM, $n = 9$ cultures). *D)* Neurons in compartmented cultures were given anti-NGF in all compartments and K252a at the cell bodies/proximal axons. Positive control cultures were given anti-NGF at the cell bodies/proximal axons and 10 ng/ml NGF at the distal axons. Neuronal survival was assayed after 48 h by Hoechst staining. Results are shown as the mean from 6 cultures per group from 3 similar experiments are presented as the mean percentage of live neurons out of at least 300 neurons counted per culture in each treatment group (+/- SEM). A statistically significant difference was detected between the NGF-deprived control group and 500 nM K252a-treated group by the paired sample *t*-test and is indicated by an asterick ($p<0.05$).

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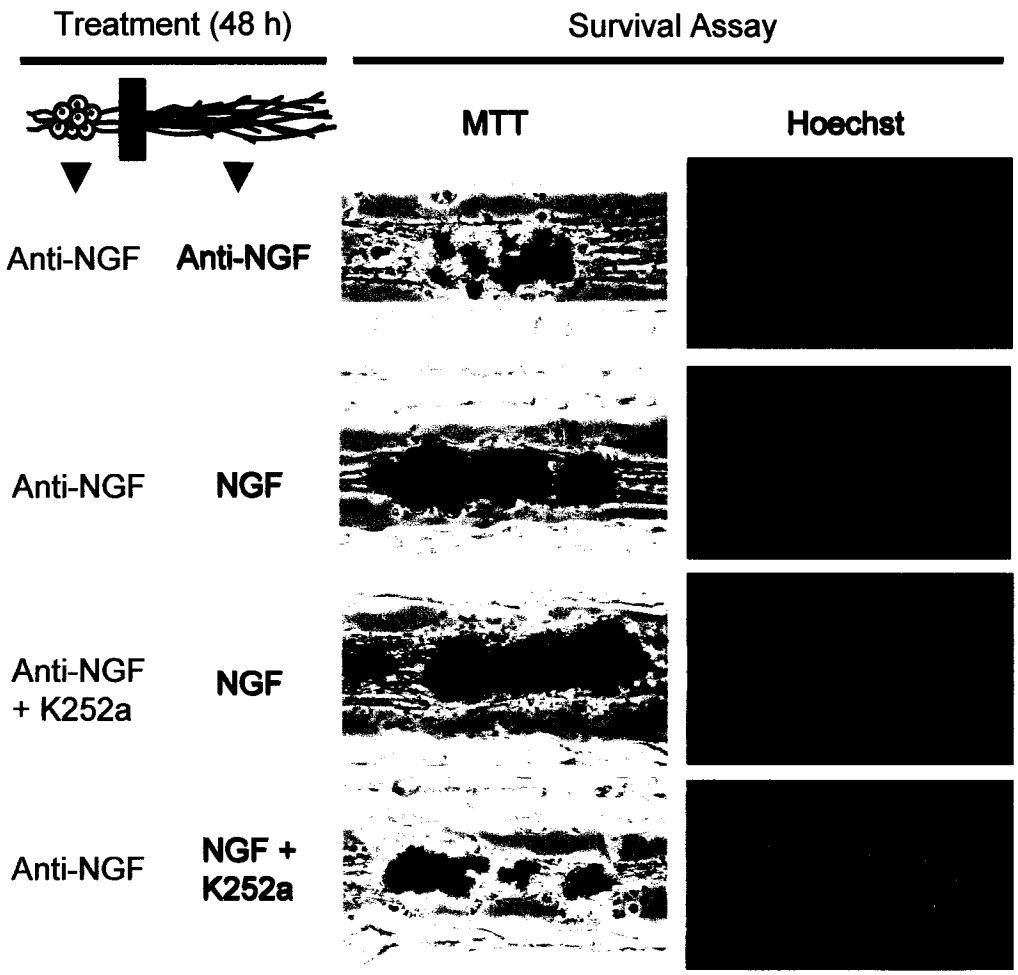

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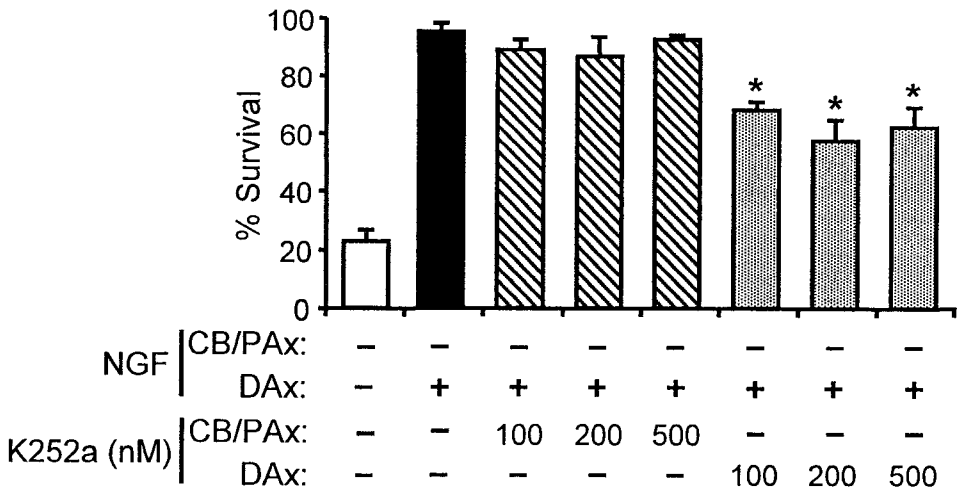

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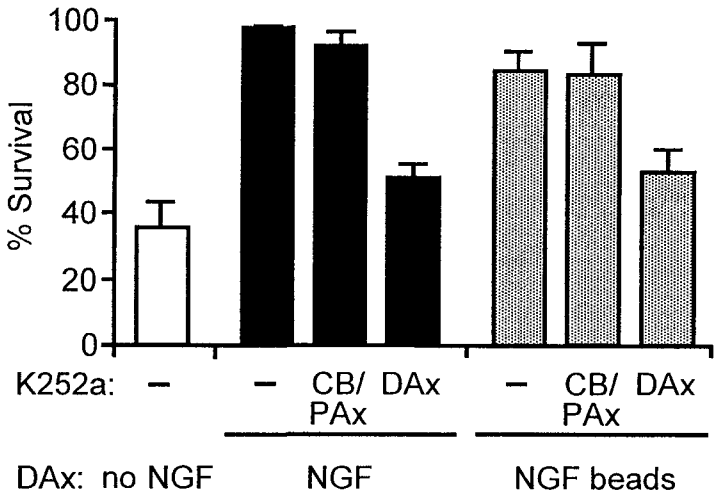

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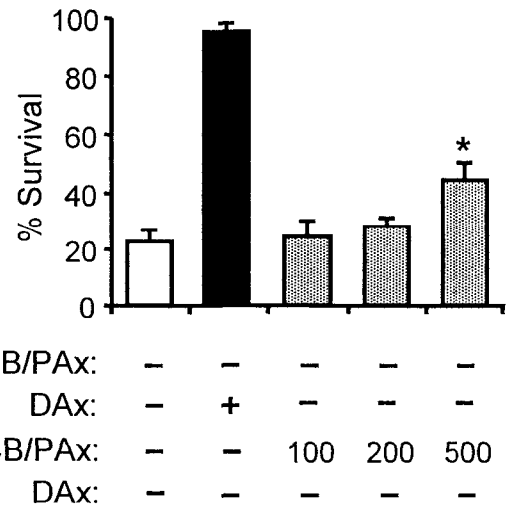

Figure 2.9 Retrograde phosphorylation of Akt and CREB is maintained in the absence of TrkA kinase activity

A and B) Cultures were given anti-NGF with or without 200 nM K252a at the cell bodies/proximal axons (CB/PAx). Simultaneously, distal axons (DAx) were given anti-NGF or 10 ng/ml NGF. After 24 h of incubation, the phosphorylation state of TrkA, Akt and CREB in the cell bodies/proximal axons was analyzed by immunoblotting with anti-pTrkA (Tyr674/675), anti-pAkt (Ser473) and anti-pCREB (Ser133) antibodies, respectively (5 cultures per lane). Equivalent protein loading was confirmed by immunoblotting with antibodies to total Erk1. *A)* Immunoblot of a representative experiment. *B)* Quantitative densitometric analysis of the effect of K252a applied to the cell bodies/proximal axons on the levels of pTrkA, pAkt, and pCREB in the cell bodies/proximal axons (combined results of 3-4 experiments, +/-SEM).

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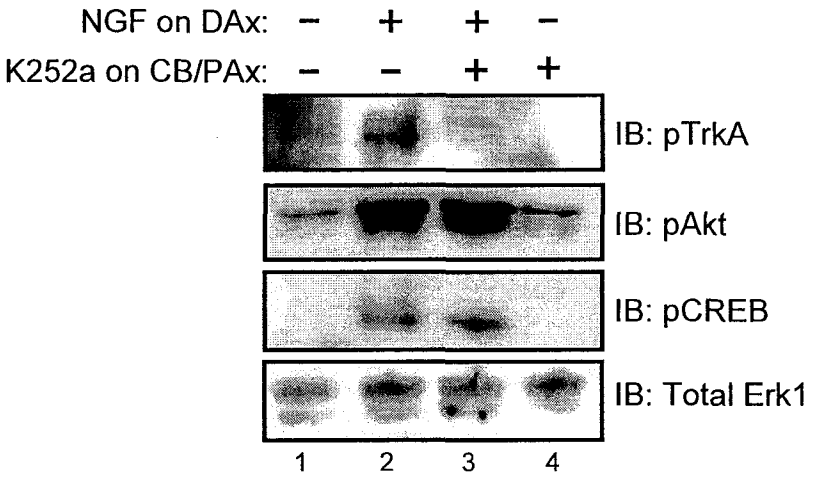

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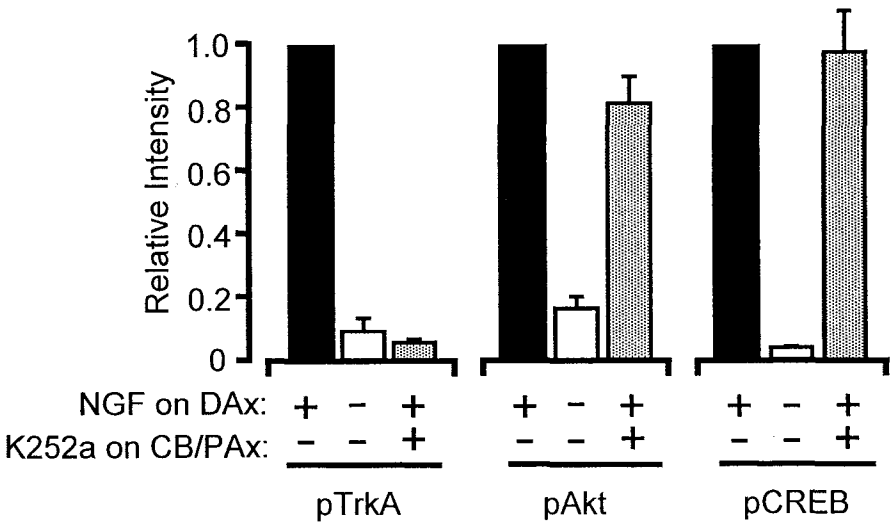
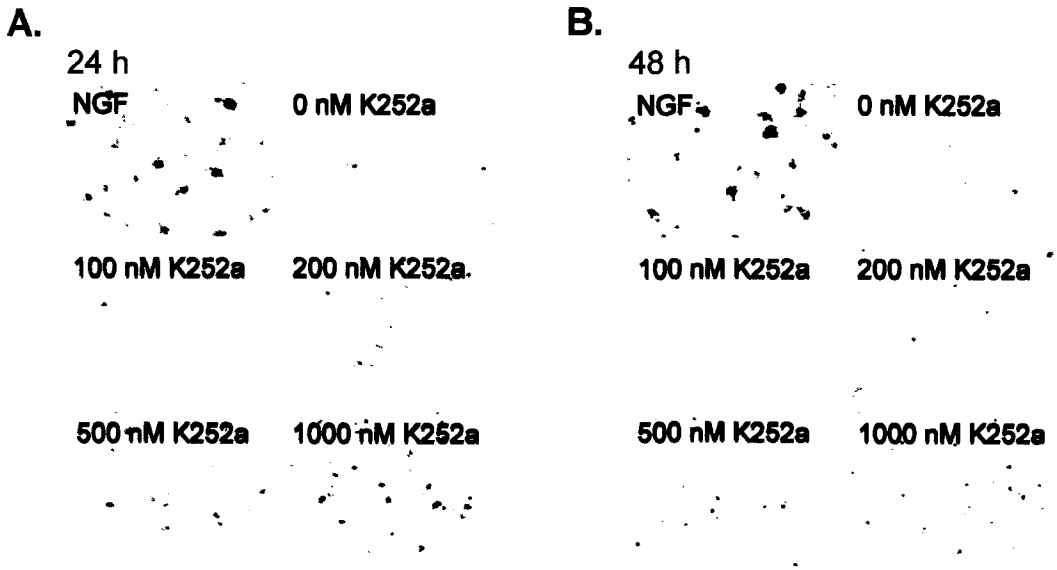
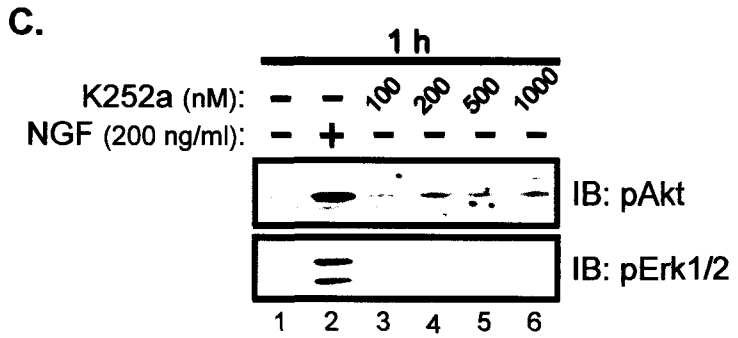


Figure 2.10 K252a does not promote significant survival, or induce phosphorylation of Akt or Erk1/2 in sympathetic neurons

A and B) Dissociated neurons from newborn rats were plated in mass cultures with 0 or 10 ng/ml NGF and containing 0 – 1000 nM K252a, as indicated. After 24 h (*A*) and 48 h (*B*) of treatment, neuronal survival was assayed by MTT staining. Results are representative of 2 – 4 similar experiments. *C*) Mass cultures were given medium lacking NGF for 6 h and then exposed to medium containing 0 or 200 ng/ml NGF and 0 – 1000 nM K252a, for 1h. Cell lysates of two culture wells per group were harvested, pooled, and the phosphorylation of Akt and Erk1/2 was determined by immunoblotting with the respective phospho-specific antibodies. Results are representative of 3 similar experiments. *D*) Mass cultures were given 0 NGF for 6 h and then 0 or 200 ng/ml NGF, along with 0, 200, or 1000 nM K252a, for 0.5 h, 5 h, or 15 h, as indicated. Cell lysates of two culture wells per group were harvested, pooled, and the phosphorylation of Akt and Erk1/2 was determined by immunoblotting with the respective phospho-specific antibodies. Results are representative of 2 similar experiments.





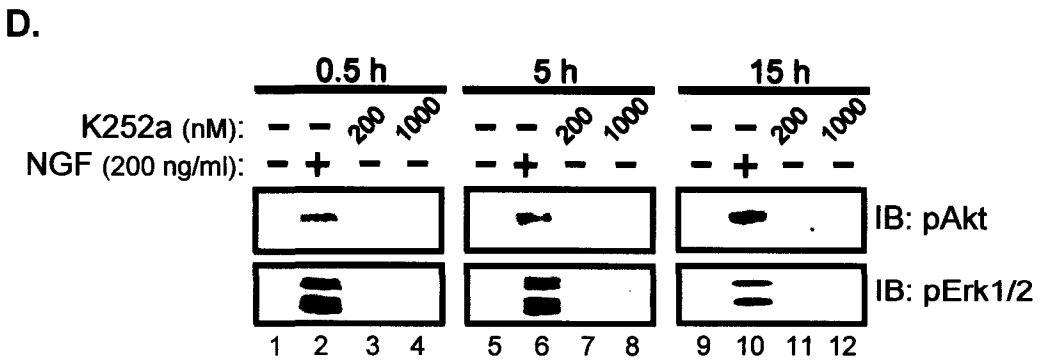
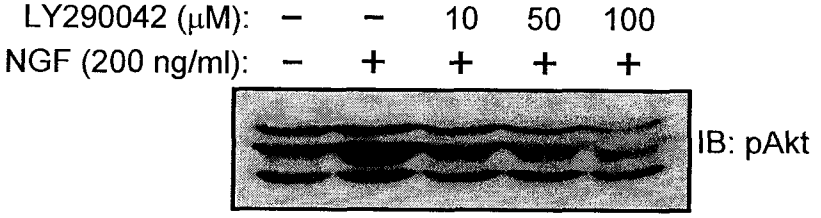


Figure 2.11 Effect of LY294002 on retrograde survival supported by NGF and NGF beads

A) LY290042 prevents NGF-induced phosphorylation of Akt. Neurons in mass cultures were deprived of NGF using anti-NGF antibodies for 6 h. During this period neurons were exposed to LY290042 (0 – 100 μM, as shown in the figure). Neurons were then given medium with or without 200 ng/ml NGF for 15 min, while maintaining the same concentration of LY290042. The phosphorylation state of Akt was assessed by immunoblotting with anti-phosphoAkt antibodies. *B)* Neurons in compartmented cultures were deprived of NGF in all compartments as described in Figure 2.2A. Cell bodies/proximal axons (CB/PAx) were then given anti-NGF for and distal axons (DAx) were given no NGF, NGF (50 ng/ml), or NGF beads (50 μl/ml) for 30 hours. Concurrently, cell bodies/proximal axons or distal axons were treated with or without 50 μM LY290042 (LY) as indicated. Then the neuronal survival of at least 250 neurons per culture was determined by quantification of Hoechst stained nuclei. The mean percentage of live neurons (+/- SEM, $n = 3$ cultures). Similar results were obtained in two replicate experiments.

A.



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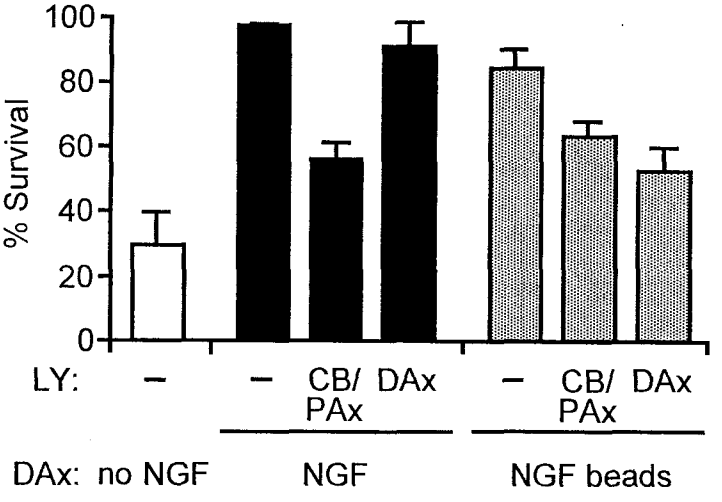
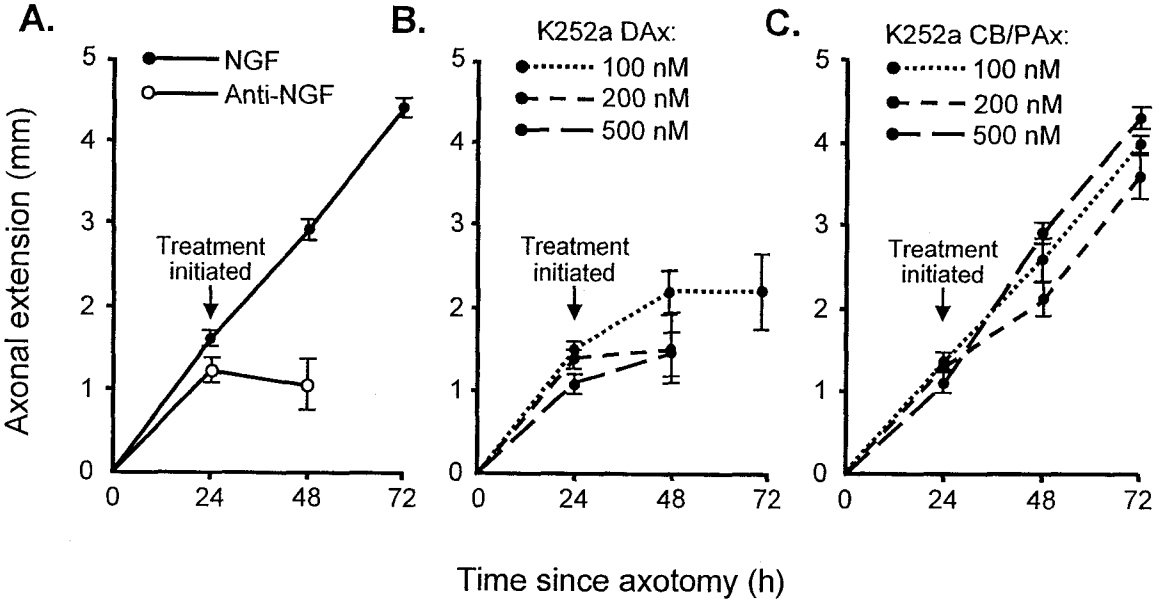
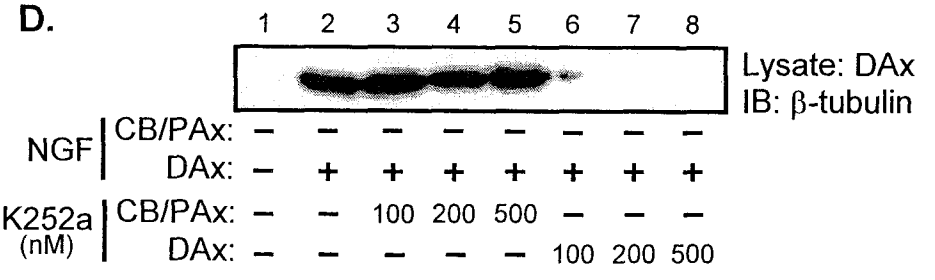
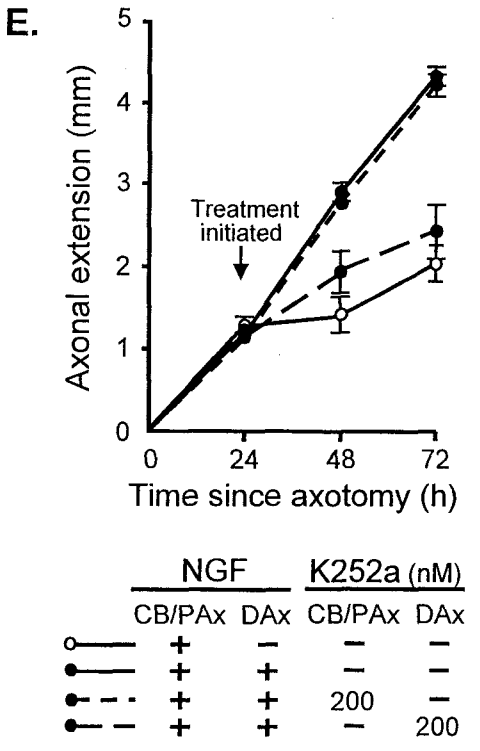


Figure 2.12 Effect of local inhibition of TrkA kinase activity on axonal growth

A, B, C) Neurons in compartmented cultures were axotomized and regenerated for 24 h with 10 ng/ml NGF in all compartments at which time experimental treatments were established. Cell bodies/proximal axons (CB/PAx) of all groups were given anti-NGF during the treatments. *A)* Axonal extension in control cultures in which distal axons (DAx) were given 10 ng/ml NGF or anti-NGF. *B)* Axonal extension in cultures in which distal axons were given K252a at the indicated concentrations with 10 ng/ml NGF. *C)* Axonal extension in cultures in which distal axons were given 10 ng/ml NGF while cell bodies/proximal axonswere exposed to K252a at the indicated concentrations. The mean axonal extension from a total of at least 60 tracks from 3 cultures per group at each time point are shown (+/- SD, track n= 60-80). Results are representative of 3 similar experiments. *D)* Immunoblots of β-tubulin from distal axons of all treatment groups harvested 72 h after axotomy. Results are representative of similar 3 experiments. *E and F)* Results of a similar experiment in which the cell bodies/proximal axons were given 10 ng/ml NGF during the experimental treatments. *E)* Mean axonal extension from a total of at least 60 tracks from 3 cultures per treatment group at each time point are shown (+/- SD, track n= 60-80). *F)* Immunoblots of β-tubulin from distal axons and cell bodies/proximal axons from all treatment groups harvested 72 h after axotomy. Results are representative of 2 similar experiments.







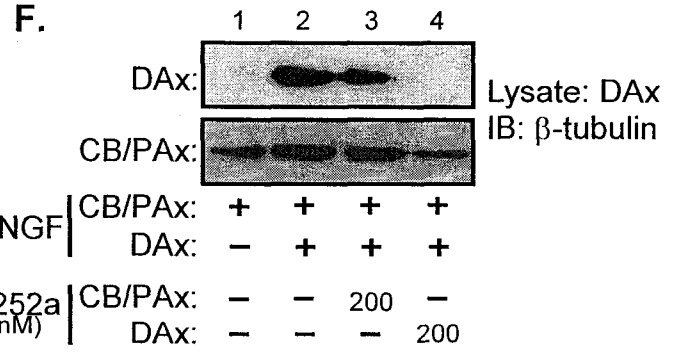
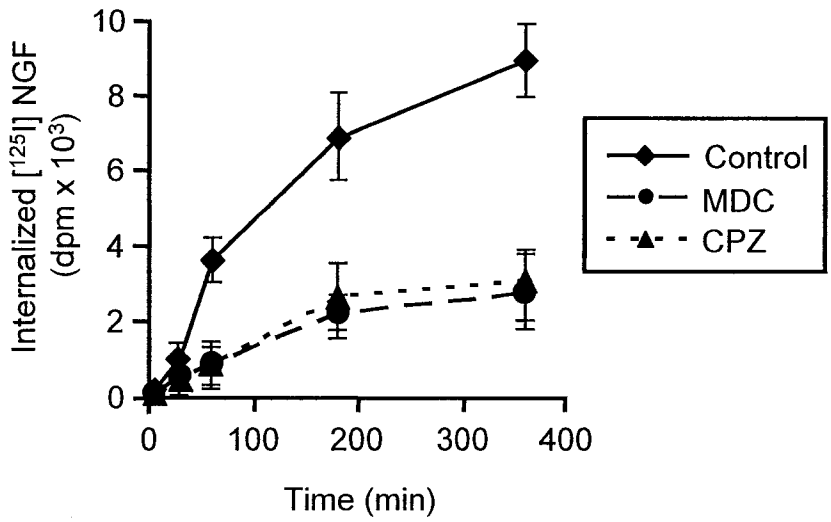


Figure 2.13 Inhibition of clathrin-mediated endocytosis inhibits internalization and inhibits retrograde transport of [^{125}I] NGF.

A) Neurons in 96-well plate mass cultures were pre-treated for 30 min with or without 50 μM MDC or 50 μM CPZ and then, while maintaining the same drug treatments, were given medium containing 5 ng/ml [^{125}I] NGF for 5 min, 30 min, 1 h, 3 h, or 6 h. At each time point neurons were rinsed 3 times with ice-cold Krebs-HEPES buffer and then incubated in Krebs-HEPES buffer containing trypsin (1 μg/ml) for 30 min at 4 °C. The trypsin-containing buffer (pooled with buffers from 2 subsequent rinses) and cellular material were collected separately and gamma-counted. The specificity of [^{125}I] NGF binding/internalization was assessed by subtracting from each group the trypsin-sensitive and -insensitive counts obtained in a control group given 5 ng/ml [^{125}I] NGF plus 100-fold excess unlabeled NGF. Data are presented as the mean +/- SEM of 5 wells per group and are representative of 3 experiments performed under similar conditions. *C)* Neurons in compartmented cultures were pre-treated with or without 50 μM MDC or 50 μM CPZ at the distal axons for 30 min and then, while maintaining the same drug treatments, were given medium containing 50 ng/ml [^{125}I] NGF or 50 ng/ml [^{125}I] NGF plus 100-fold excess unlabeled NGF (for determination of non-specific binding) for 15 h. Transported [^{125}I] NGF was determined by collecting the culture medium and the cellular material from the cell bodies/proximal axons and gamma-counting. Data are presented as the mean +/- SEM of the specific transported radioactivity of 6 cultures/group and are representative of 3 experiments performed under similar conditions.

A.



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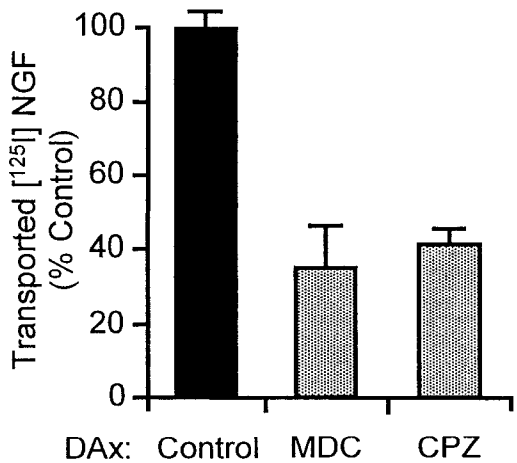


Figure 2.14 Inhibition of clathrin-mediated endocytosis does not affect NGF-induced phosphorylation of TrkA or Akt, but attenuates phosphorylation of Erk1/2

Neurons in 24-well plate mass cultures were given medium containing 24 nM anti-NGF for 6 h, then given medium lacking NGF or anti-NGF with or without 50 μM MDC or 50 μM CPZ for 30 min, and then exposed to 0 or 50 ng/ml NGF for 1 h, while the same concentration of MDC and CPZ was maintained. The phosphorylation state of TrkA, Akt, and Erk1/2 was assessed by immunoblotting with anti-pTrkA (Tyr674/675), anti-pAkt (Ser473) and anti-pErk1/2 (Thr202/Tyr204) antibodies, respectively. The blot was then reprobed with anti-β-tubulin antibodies, which indicated that the lack of pErk1/2 observed in the presence of MDC or CPZ could not be attributed to reduced protein levels loaded on the gel.

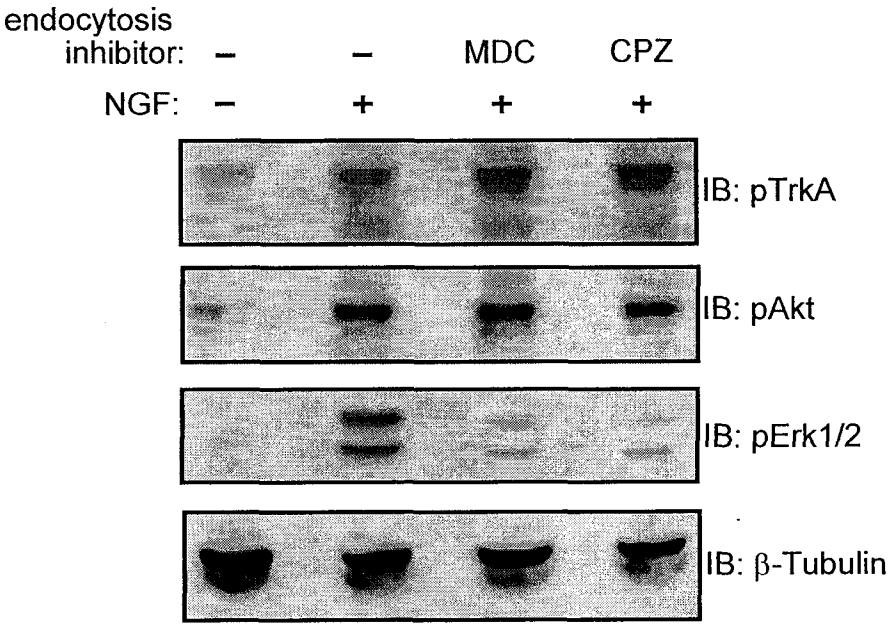


Figure 2.15 Inhibition of clathrin-mediated endocytosis by MDC or CPZ at the site of NGF application at distal axons attenuates axon growth

Neurons in compartmented cultures were axotomized and allowed to regenerate for 24 h with 10 ng/ml NGF in the cell bodies/proximal axons (CB/PAx) and 50 ng/ml in the distal axons (DAx), at which time experimental treatments were established. Cell bodies/proximal axons of all groups were given anti-NGF and distal axons were given medium lacking NGF, or containing 50 ng/ml NGF with or without 50 μM MDC or 50 μM CPZ for 48 h. *A)* Axonal extension was measured at 24 h intervals throughout the experiment. Data are shown as the mean axonal extension from a total of at least 80 tracks from 3 cultures per group at each time point (+/- SEM, track n= 95-110). Results are representative of 3 experiments performed under similar conditions. *B)* Immunoblots of β-tubulin from distal axons of all treatment groups harvested 72 h after axotomy. Results are representative of 2 experiments performed under conditions.

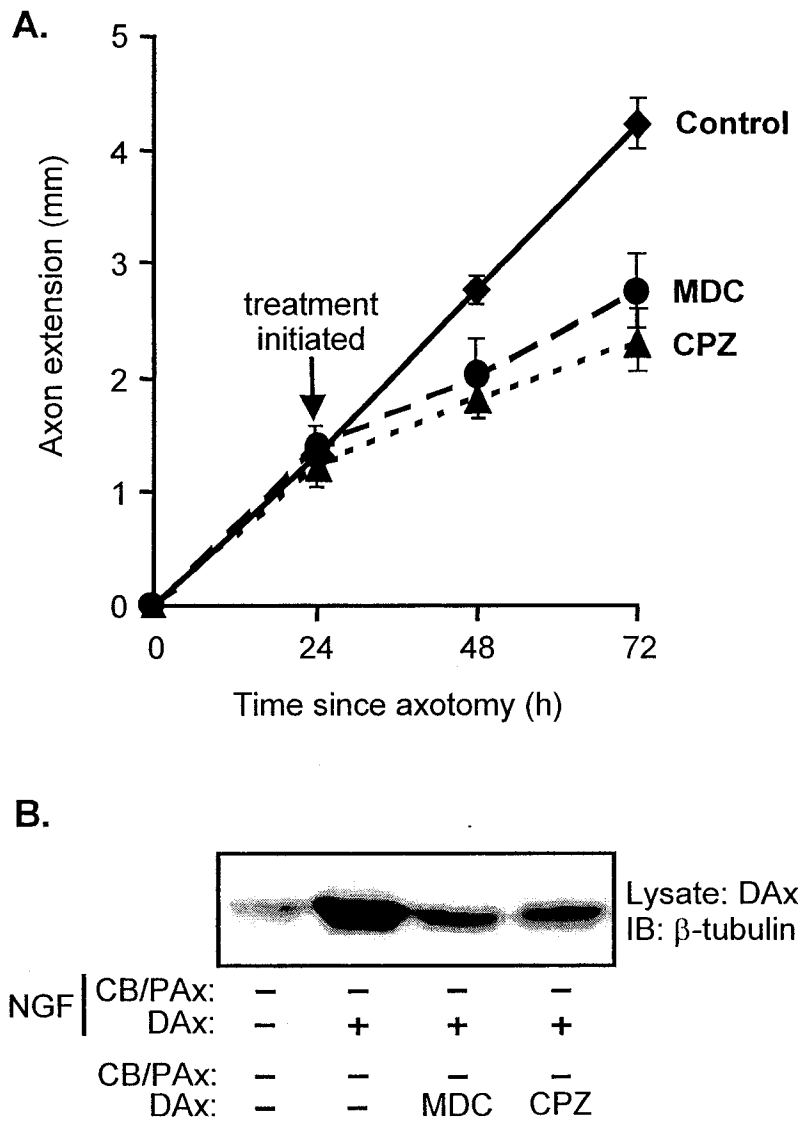
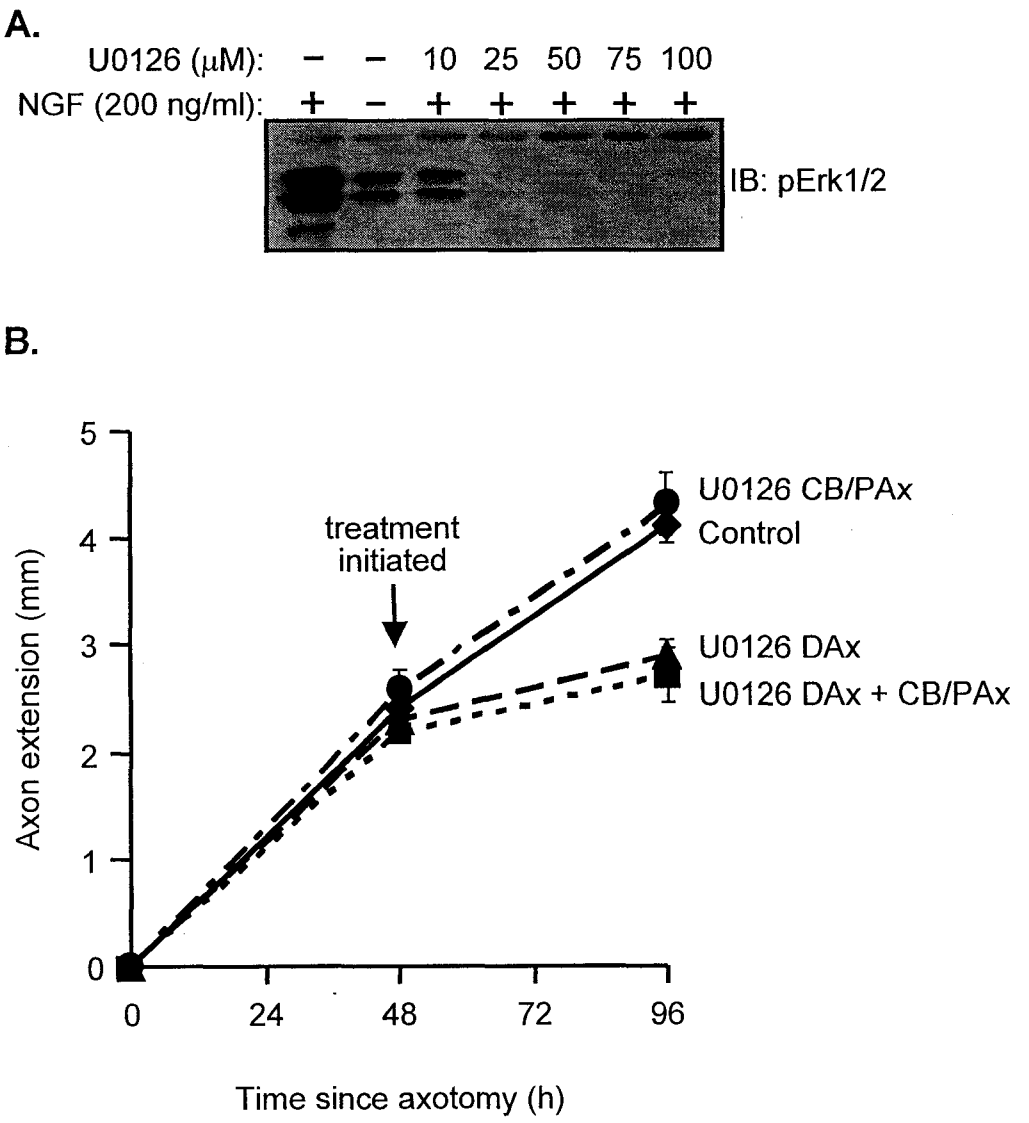


Figure 2.16 Local inhibition of NGF-induced Erk1/2 activation by U0126 in distal axons, but not cell bodies/proximal axons, inhibits axon growth

A) U0126 prevents NGF-induced phosphorylation of Erk1/2. Neurons in mass cultures were deprived of NGF using anti-NGF antibodies for 6 h. During this period neurons were exposed to U0126 (0 – 100 μM, as shown in the figure). Neurons were then given medium with or without 100 ng/ml NGF for 15 min, while maintaining the same concentration of U0126. The phosphorylation state of Erk1/2 was assessed by immunoblotting with anti-phosphoErk1/2 antibodies. *B and C)* Neurons in compartmented cultures were axotomized and allowed to regenerate for 24 h with 10 ng/ml NGF in the cell bodies/proximal axons (CB/PAx) and 50 ng/ml in the distal axons (DAx), at which time experimental treatments were established. Cell bodies/proximal axons of all groups were given anti-NGF and distal axons were given medium lacking NGF, or containing 50 ng/ml NGF with or without 50 μM U0126 for 48 h. *B)* Axonal extension was measured again 48 h after experimental treatments were established. Data are shown as the mean axonal extension from a total of at least 80 tracks from 3 cultures per group at each time point (+/- SEM, track n= 80-110). Results are representative of 3 experiments performed under similar conditions. *C)* Immunoblots of β-tubulin from distal axons of all treatment groups harvested 72 h after axotomy. Results are representative of 2 experiments performed under conditions.



CHAPTER 3

Inhibition of Proteasome-Mediated but not Caspase-Mediated Proteolysis Protects Axons from Wallerian Degeneration and Degeneration Caused by Local and Global NGF Withdrawal

[Data in this chapter are being prepared for submission for publication in Journal of Neuroscience; authors: BL MacInnis and RB Campenot]

INTRODUCTION

The previous chapter investigated the spatial organization of mechansisms by which NGF/pTrkA signaling promote the survival of sympathetic neurons. These experiments show that, in the absence of NGF, sympathetic neurons undergo apoptotic cell death. The apoptotic program has been shown to require caspase-mediated proteolytic activity, such that inhibition of caspases delays or inhibits apoptotic neuronal death (reviewed in: (Deshmukh and Johnson, 1997)). Apoptotic neuronal death is also a feature of several neurodegenerative diseases and neuropathological disorders (Raff et al., 2002). In addition to apoptotic death of the entire neuron, the axons of neurons have the ability to degenerate or "die" in a manner that is independent of the death of the cell body. Little is known about how axon degeneration is controlled, however, evidence suggests that the process is mechanistically distinct from neuronal apoptosis (Finn et al., 2000) (discussed in detail below). In this chapter we examine the relationship between apoptotic neuronal death and axon degeneration by investigating the role of caspase activity in the latter process, and identify a critical role for proteasome-mediated protein degradation in mediating axon degeneration.

The process of axonal degeneration is critical to nervous system development and maintenance, and is a potentially causative element of many neurodegenerative disorders (Raff et al., 2002). During development, many neurons initially overproduce axons or send axons to inappropriate targets. These superfluous axon branches are then eliminated by a process known as "branch elimination" or "pruning"(Nakamura and O'Leary, 1989; O'Leary and Koester, 1993; Truman, 1990). Intriguingly, this degeneration of specific axons is accomplished without spreading to kill the entire neuron or causing degeneration of the parent axon. Thus, axons possess the intrinsic ability to self-destruct independently, by mechanisms that appear to be distinct from the apoptotic program that mediates death of the entire neuron. These mechanisms are virtually undefined.

The extensive neuronal death associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease has traditionally been regarded as a primary cause of their pathology. Correspondingly, efforts have focused on developing therapies aimed at protecting neurons from apoptosis. However, it is unknown whether

pathological death of the neurons causes, or is a consequence of, axonal degeneration. Accumulating evidence suggests that in many neurodegenerative diseases degeneration of the axon precedes death of the cell body, and therefore therapies that prevent neuronal apoptosis may be too late to be of functional significance to the neuron (Coleman and Perry, 2002; Raff et al., 2002). Furthermore, pathological axon degeneration is the hallmark feature of "dying back"-type neuropathies, such as diabetic neuropathy, in which the axons of affected neurons degenerate from the distal end toward the cell body over the course of several weeks or months (Cavanagh, 1964; Schaumburg et al., 1974). Thus, understanding of the mechanisms by which axonal degeneration occurs, and how it can be prevented, is critical to the development of effective clinical therapies for several neurodegenerative and neuropathological diseases.

Until recently, axonal degeneration was viewed as a passive wasting process, resulting primarily from the loss of supply of axonal components from the dying or disconnected cell body. The discovery and characterization of the naturally occurring *Wld*^{S} mutant strain of mice, in which Wallerian degeneration of transected axons is slowed dramatically, has triggered a new outlook of axonal degeneration as an active process (Lunn et al., 1989; Perry et al., 1991). Whereas the distal portion of axons in wild-type mice degenerate rapidly after transection, in *Wld*^{S} mice severed axons remain viable and able to conduct action potentials for up to 2 weeks (Lunn et al., 1989). This protection is conferred by an intrinsic property of the axon and is not dependent on macrophages or glial cells (Glass et al., 1993; Perry et al., 1991). In addition to Wallerian degeneration, the axons of *Wld*^{S} neurons are also protected from other degenerative insults, including NGF withdrawal and vincristine toxicity (Deckwerth and Johnson, 1994; Wang et al., 2001), indicating that a common underlying mechanism in axons promotes their degeneration in response to a variety of noxious stimuli. The *Wld*^{S} mutant suggests that selective axonal degeneration is an actively regulated, auto-destructive process, analogous to apoptosis of the cell body.

Although the mechanism by which *Wld*^{S} axons are protected from degeneration remains unknown, the identification of the genetic mutation resulting in this phenotype may reveal some clues. The novel chimeric gene encodes an in-frame fusion protein of the N-terminus of the ubiquitination factor E4B (Ube4B), an E4 ubiquitin ligase, with

the complete coding region of the nicotinamide mononucleotide adenylyltransferase (Nmnat), an adenine nicotinamide dinucleotide (NAD) synthesizing enzyme (Conforti et al., 2000; Fernando et al., 2002). As the product of the *Wld*^{S} gene is a fusion protein, either component of the chimera, or elements of both, may be responsible for the phenotype. However, evidence suggests that the protective mechanism may involve an alteration in the ubiquitin-proteasome protein degradation pathway, as transgenic expression of a truncated version of the *Wld*^{S} Ube4b sequence as a fusion with full length Nmnat does not confer delay in Wallerian degeneration (Coleman and Perry, 2002).

The highly conserved ubiquitin-proteasome pathway is the predominant extralysosomal system by which the intracellular proteolysis is mediated. Proteins to be destroyed by this pathway are targeted for recognition and subsequent degradation by the 26S proteasome, a multi-catalytic protease complex, by the covalent attachment of multiple monomers of ubiquitin, a 76 amino acid polypeptide (Naujokat and Hoffmann, 2002). Proteasomal protein degradation is known to be involved in diverse cellular activities including signal transduction, cell cycle progression, differentiation, and apoptosis. Furthermore, in addition to its potential role in mediating axonal protection in *Wld*^{S} mice, dysregulation of ubiquitin-proteasome function is a hallmark of neurodegenerative disorders including gracile axonal dystrophy, recessive juvenile parkinsonism, and others (Coleman and Perry, 2002; Raff et al., 2002). Here we add to the growing list of cellular functions mediated by this pathway by demonstrating a role for the proteasome in regulating axonal degeneration, using *in vitro* models of Wallerian degeneration, of local NGF withdrawal from axons, and of global NGF withdrawal from the entire neuron.

We also use these models to investigate whether axon degeneration is influenced by some of the same machinery that mediates neuronal apoptosis. Caspases are a family of cysteine proteases that activate and effect intracellular proteolysis during apoptotic cell death by cleavage of multiple target proteins at specific aspartate residues (Stennicke and Salvesen, 2000). Inhibition of caspase activity delays or prevents apoptosis in response to various lethal stimuli in various cell types, including NGF withdrawal from sympathetic neurons (McCarthy et al., 1997). The role of local caspase activity in axonal degeneration has been examined in several systems, however, under which conditions, if any,

caspases are activated and/or required to mediate axonal degeneration is unclear (Cowan et al., 2001; Finn et al., 2000; Ivins et al., 1998; Mattson et al., 1998a; Mattson et al., 1998b). In the present study we asked whether inhibition of broad-spectrum caspase activity in sympathetic axons protects them from degeneration caused by transection or NGF withdrawal.

MATERIALS AND METHODS

Culture Methods

Superior cervical ganglion neurons were obtained from newborn Sprague-Dawley rats supplied by the University of Alberta Health Sciences Lab Animal Services, and plated into the center compartments of three-compartmented dishes supplied with L15 medium (Gibco Laboratories, Grand Island, NY) as previously described (Campenot, 1992; Campenot and Martin, 2001). Non-neuronal cells were eliminated by addition of 10 μM cytosine arabinoside in the center compartments and in mass cultures during the first 7 days. The 2.5S form of NGF (Alamone Laboratories, Jerusalem, Israel) was initially supplied in center compartments at 10 ng/ml. Throughout the culture period the medium in distal compartments was supplied with 50 ng/ml NGF. Treatment groups within all experiments consisted of cultures from the same plating of neurons maintained under identical conditions until the time of the experiment. Neurons ranged from 7 – 11 days *in vitro* at the time they were used in experiments which made no apparent difference in the results.

Reagents and Inhibitor Treatments

All reagents were obtained from Sigma (St. Louis, MO) unless indicated otherwise. Stock solutions of inhibitors were prepared as follows: MG132 (2.5 mM in DMSO; Biomol, Plymouth Meeting, PA), lactacystin (10 mM in DMSO; Biomol), BocD-fmk (50 mM in DMSO; Kamiya Biomedical, Seattle, WA), U0126 (50 mM in DMSO; Promega Corporation, Madison, WI), AEBSF (50 mg/ml in TBS), apoprotinin (10 mg/ml in TBS), chymostatin (10 mM in DMSO), and pepstatinA (2 mg/ml in ethanol), and cycloheximide (350 mM in TBS). Dilutions were made directly into working culture medium. Control groups were given equivalent concentration of vehicle which had no apparent detrimental effects on the neurons. Function-blocking anti-NGF antibodies were from Cedarlane Laboratories (Hornby, ON).

Axonal transection

Cell bodies/proximal axons were detached by a jet of sterile distilled water delivered via syringe fitted to a 22 gauge needle to the center compartment of compartmented cultures. All cultures were visually inspected to ensure that all cell bodies/proximal axons were removed.

MTT and Mitotracker Mitochondrial Assays

CellTiter 96 Cell Proliferation Assay (a derivative of the MTT assay (Mosmann, 1983); Promega Corporation, Madison, WI), and Mitotracker-Green FM and Mitotracker-Orange-CM-H_2TmRos (Molecular Probes, Eugene, OR) were resuspended in DMSO, added directly to the culture medium bathing the axons/neurons at the concentrations suggested by the manufacturer, and incubated for 1 h at 37 ^{o}C. Images were obtained using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with Nikon digital camera DXM-1200 (Nikon Canada, Toronto ON). Images were analyzed using Northern Elite V6.0 image capture and analysis software (Empix Imaging, Missisauga, ON).

Measurement of Axon Extension

Distal axons of neurons in compartmented were axotomized with a jet of sterile water delivered via syringe fitted with a 22 gauge needle. Culture medium was then restored to allow axonal regeneration. Axon extension was measured on individual tracks using an inverted microscope fitted with a stage digitizer (AccuStage, Shoreview, MN) as previously described (Campenot, 1992).

Immunoblotting

Following experimental treatment, cultures to be analyzed by immunoblotting were rinsed with ice-cold TBS containing 1 mM sodium orthovanadate and 10 mM sodium fluoride. Axonal material was then harvested directly into SDS sample buffer, boiled for at least 5 min and run on 10 % SDS PAGE gels. Each group contained pooled material from an equal number of distal axon compartments (3-5 per experiment). Proteins were transferred to Immobilon-P PVDF membrane (Millipore Corporation,

Bedford, MA) and immunoblotted using the following antibodies at the indicated dilutions: monoclonal anti-phospho-Erk1/2 (Thr202/Tyr204) (1:2000, New England Biolabs, Beverly, MA); monoclonal anti-Erk1 (detects both Erk1 and Erk2) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was determined was determined by enhanced chemiluminescence (SuperSignal West Dura Substrate, Pierce, Rockford, IL).

RESULTS

Inhibition of proteasome-mediated protein degradation protects transected axons from Wallerian degeneration

Following removal of the cell bodies/proximal axons of neurons in compartmented cultures, the transected axons remaining in the distal axon compartments rapidly die by a process analogous to the Wallerian degeneration of severed axons *in vivo*. Morphological indication of this degeneration is first detectable by visual inspection at the light microscopy level by approximately 10 h after transection by collapsing of the axon terminals and loss of adhesion to the substratum. By 15-18 h after transection, most of the axons have completely disintegrated. This process occurs at approximately the same rate independently of the presence of NGF in the culture medium.

Evidence of dysregulation of the ubiquitin-proteasome pathway in conditions of axonal degeneration prompted us to investigate the role of proteasome-mediated protein degradation in the Wallerian degeneration of transected axons. Using MG132 and lactacystin, mechanistically distinct inhibitors of the 26S proteasome, we tested whether transected axons were protected from Wallerian degeneration in the absence of proteasome activity. Cell bodies/proximal axons were removed (Fig. 4.1A) and transected axons were cultured for 24 h in the presence or absence of 5 μM MG132 or 10 μM lactacystin. Intriguingly, while untreated control axons had disintigrated extensively by this time, axons in which proteasomal activity was inhibited showed virtually no morphological sign of degeneration (Fig 4.1B). The protective effect of MG132 was somewhat more robust than that of lactacystin, likely due to the relatively poorer stability in solution and reduced cell permeability of the latter compound (Manenti et al., 2002). To determine if the axonal protection confered by proteasome inhibition was due to a non-specific effect of blocking protease activity, we tested whether inhibition of non-proteasomal proteases had a similar effect. Using the same experimental paradigm described above it was found that a cocktail of cell-permeant non-proteasomal protease inhibitors (AEBSF, apoprotinin, chymostatin, and pepstatin A) did not delay Wallerian degeneration (Fig 4.1B). Similarly, inhibition of caspase activity with a broad-spectrum caspase inhibitor, BocD-fmk (100 μM), conferred no protective effect against

degeneration of transected axons (Fig 4.1B), although it did prevent DNA condensation and fragmentation due to NGF withdrawal assessed by Hoechst-staining in intact sister-plated neurons. Treatment of transected axons with 1 mg/ml cycloheximide in addition to MG132 did not disrupt the protective effect of the proteasome inhibitor, suggesting that local protein synthesis in axons was not required (Fig 4.1B).

To determine if proteasome inhibitor-treated axons may have been retracting and dying in a manner than was morphologically distinct from the characteristic degeneration pattern of transected axons *in vitro*, the length of the longest axon in each track was measured before and again 24 h after transection. There was no significant change in axon length in MG132 or lactacystin-treated axons over the 24 period, indicating that the axons had not retracted, which is typically a hallmark feature of early stages of axon degeneration (Fig 4.1C). In contrast, untreated axons could not be measured in this manner, as 24 h after transection there were virtually no intact axon fibers.

Transected axons protected by proteasome inhibition are metabolically active

Morphologically, inhibition of proteasome-mediated protein degradation appears to significantly delay Wallerian degeneration of transected axons. To determine if the protected axons were viable, the metabolic activity of proteasome inhibitor-treated axons was assessed. Two assays of axonal mitochondrial function were used: the MTT assay, a measure of mitochondrial activity and cell viability (Mosmann, 1983), and Mitotracker-Orange staining, each of which label metabolically active mitochondria *in situ*. Mitotracker-Orange selectively labels metabolically-active mitochondria in which the membrane potential is maintained, whereas Mitotracker-Green labels all mitochondria regardless of their viability.

Consistent with the lack of morphological deterioration of proteasome-inhibited axons, both assays of mitochondrial function indicated that these axons were metabolically active for at least 24 h after transection. In contrast, as early as 12 h after transection untreated axons showed a dramatic decrease in reactivity by either MTT or Mitotracker-Orange staining, and no indication of mitochondrial function by either assay was detected by 24 h (Fig. 4.2A and B, respectively). As suggested by the degeneration of transected axons in the presence of caspase inhibitors, treatment of axons with

BocD-fmk also did not significantly delay the loss of mitochondrial activity, and by 24 h after transection there was virtually no indication of metabolic function in either assay (Fig. 4.2A and B, respectively).

Proteasome inhibition-mediated protection of transected axons requires MEK kinase activity

The MEK/Erk1/2 signal transduction pathway is well-known to be regulated by protein phosphorylation, mediated by the activity of the kinases and phosphatases in the cascade. However, recent evidence has highlighted a role for the ubiquitin-proteasome system as an additional level of control of Erk1/2 activity, likely by regulating the abundance of the molecules in the pathway. For example, activation of MEK1/2, the upstream kinase of Erk1/2, was found to be required for proteasome inhibition-mediated induction of interleukin-6 and interleukin-8 gene expression in human endothelial cell lines (Shibata, 2002; Wu, HM 2002), and proteasome inhibition decreased the loss of Erk1/2 phosphorylation induced by disrupting cell adhesion in NIH3T3 cells (Manenti, 2002). Proteasome-mediated downregulation of the Erk1/2 pathway has also been described. MEKK1, an upstream activator of MEK, has been shown to act as a E3 ubiquitin ligase that induces ubiquitination and degradation of Erk1/2 (Lu et al., 2002), and proteasome inhibition resulted in decreased phosphorylation of Erk1/2 associated with increased activity of Erk1/2 phosphatases-1 and -2 in breast epithelial and carcinoma cell lines (Orlowski, 2002).

We thus asked if the protective effect of proteasome inhibition in axonal degeneration was sensitive to loss of activity of the Erk1/2 pathway, using U0126 to inhibit MEK kinase activity. Cell bodies/proximal axons of neurons in compartmented cultures were removed, and the transected axons were treated for 24 h with MG132 (5 μM), MG132 and U0126 (50 μM), U0126 alone, or not treated. Independent experiments confirmed that 50 μM U0126 effectively inhibits NGF-induced phosphorylation of Erk1/2 (see Fig. 2.10 A). Axonal integrity was assessed using the Mitotracker-Orange mitochondrial activity assay and MTT assay described in the previous section. It was found that inhibition of MEK kinase activity by U0126 blocked the protective effect of down-regulating proteasomal protein degradation in transected axons (Fig. 4.3A and B,

respectively). The effect of MEK inhibition was robust, as there was no discernible difference between axons treated with both MG132 and U0126, and untreated control transected axons. There was no apparent difference between the Wallerian degeneration of untreated axons and those treated with U0126 alone (not shown). Inhibition of MEK activity for 24 h in axons of intact neurons did not cause axonal degeneration (Fig. 4.3C), indicating that loss of MEK function does not promote axonal death in healthy neurons and that the degeneration observed in response to U0126 in transected axons was not due to a toxic, non-specific effect of the compound. Thus, protection from axonal degeneration by proteasome inhibition was dependent upon MEK kinase activity, suggesting that a sustained activation of MEK is induced by inhibition of proteasomal protein degradation, and that this MEK activity is required for axonal protection.

Inhibition of proteasomal protein degradation preserves phosphorylation of Erk1/2 in transected axons

Inhibition of MEK kinase activity completely blocked the protective effect of proteasome inhibition-mediated axonal protection, suggesting that a sustained activation of MEK is induced by inhibition of proteasomal protein degradation, and that this activity is required for axonal protection. To verify that MEK kinase activity could play a role in supporting axonal survival, we asked whether MEK kinase activity was maintained in transected axons in which Wallerian degeneration was delayed by proteasomal inhibition. The phosphorylation state of Erk1/2 (the downstream targets of MEK1/2) was determined by immunoblot analysis of lysates from intact, untreated distal axons and axons treated with or without MG132 for 4, 8, or 18 h after transection. At 4 h post-transection there was no significant change in the levels of phosphorylated Erk1/2 in axons in the presence or absence of MG132 (Fig. 4.4 *upper panel*). However, by 8 h post-transection, phosphorylated Erk1/2 was maintained at the same level in MG132-treated axons but was greatly reduced in untreated axons, and by 18 h persistent Erk1/2 phosphorylation was still detectable in MG132-treated axons but was undetected in untreated axons, which had severely degenerated. Treatment with MG132 did not appear to induce phosphorylation of Erk1/2, rather it prevented the loss of activated Erk1/2 that was associated with the transection of untreated axons. Furthermore, the loss of phosphorylated Erk1/2 in

untreated axons at 8 h was not attibutable to a decrease in total Erk1/2 protein level due to general protein degradation of the degenerating axon (Fig. 4.4 *lower panel*) indicating that the reduction in pErk1/2 is a specific, and suggesting that it may be a causative element of Wallerian degeneration.

Proteasome inhibition, but not caspase inhibition, protects axons from local and global NGF deprivation

Selective axon degeneration also occurs during development of the nervous system, when neurons initially overproduce axons or send axons to inappropriate targets, and are later eliminated. Local axon degeneration of this nature may occur due to failure of specific axons or axon branches to obtain sufficient neurotrophic support. Axon degeneration due to lack of neurotrophic support may also occur after injury. We thus asked whether axon degeneration due to local NGF withdrawal from distal axons of neurons in compartmented cultures was delayed by inhibition of proteasome or caspase-mediated proteolytic activity. Neurons in compartmented cultures were given 10 ng/ml NGF in the center compartment to maintain their survival, while distal axons were given medium lacking NGF and containing function-blocking anti-NGF antibodies (anti-NGF), or medium containing 50 ng/ml NGF as a control, for 30 h. During this period, NGF-deprived axons were also treated with or without 5 μM MG132 or 100 μM BocD-fmk. Following this treatment, axonal viability was assessed morphologically and by MTT staining. Consistent with the protective effect of MG132 in transected axons, NGF deprived, MG132-treated axons showed little evidence of morphological deterioration and were darkly stained by MTT (Fig. 4.5A). In contrast, BocD-fmk treatment provided little protection to NGF-deprived axons, which were severely retracted and degenerated and displayed no MTT reactivity, similar to untreated NGF-deprived axons (Fig. 4.5A).

It has been hypothesized that the mechanism of axon degeneration under conditions in which survival of the cell body is maintained is distinct from the apoptotic mechanism that executes death of the entire neuron, such that the cell body is spatially and/or functionally protected from the local program of axon degeneration. Consistent with this hypothesis, Finn *et al.* (2000) found that NGF withdrawal from the entire neuron induced caspase-3 activation in both cell bodies and axons, whereas no axonal

caspase-3 activity was detected when axons were locally deprived of NGF suggesting that the two processes are mechanistically distinct. Based on this hypothesis it would be expected that, under conditions of global NGF withdrawal from the entire neuron, inhibition of caspase activity would protect axons from degeneration, whereas inhibition of proteasome activity would not, as the latter clearly prevents local axon degeneration. Thus we tested whether inhibition of proteasome or caspase activity in axons prevented their degeneration when neurons were deprived of NGF at both the cell bodies/proximal axons and the distal axons.

Neurons in compartmented cultures were given medium lacking NGF and containing anti-NGF in all compartments for 30 h. During this time the neurons were also treated with or without 5 μM MG132 or 100 μM BocD-fmk in all compartments. Following this treatment, axonal viability was assessed morphologically and by MTT staining. Again in this model of axon degeneration, NGF deprived, MG132-treated axons were morphologically intact and and were darkly stained by MTT, whereas BocD-fmk treatment exerted no protective effect on NGF-deprived axons and appear similar to NGF deprived, untreated axons (Fig. 4.5B). It has previously been shown that inhibition of either proteasome-mediated or caspase-mediated proteolysis protects sympathetic neurons from NGF-withdrawal induced apoptosis, as evidenced by Hoechst-staining, for 48 h (Sadoul et al., 1996).

DISCUSSION

Here we report evidence that the proteasomal protein degradation pathway is a key mechanism by which axonal degeneration is locally regulated. Local inhibition of proteasome function in axons of sympathetic neurons in compartmented cultures protects these axons from three degenerative insults: Wallerian degeneration, degeneration due to local NGF withdrawal from axons, and degeneration due to global NGF withdrawal from the entire neuron. In contrast, inhibition of caspase-mediated proteolytic activity does not confer axon protection in any of the paradigms tested. The protective effect of proteasome inhibition is reversed by co-incident inhibition of MEK kinase activity, and correspondingly, persistent phosphorylation of Erk1/2 was observed in proteasome inhibitor-treated transected axons at time points that preceded loss of total Erk1/2 protein in untreated control axons. Together these results suggest that proteasome activity in transected axons mediates down-regulation of the MEK/Erk pathway leading to rapid axonal degeneration.

The ubiquitin-proteasome pathway was implicated in axonal degeneration by the identification and characterization of the genetic mutation that confers the slow Wallerian degeneration phenotype of the *Wld*^{S} mouse. Although the *Wld*^{S} mutation encodes a fusion protein, it appears that the component of the chimera responsible for the *Wld*^{S} phenotype is the N-terminal region of Ube4b, an enzyme in the multi-ubiquitination process that targets proteins to be degraded by the proteasome (Coleman and Perry, 2002). However, the mechanism by which this protein confers axonal protection is unknown.

Paradoxically, in immunolocalization studies the *Wld*^{S} protein was found primarily in neuronal nuclei, and could not be detected in motor neuron or sciatic nerve axons, even though Wallerian degeneration was delayed in these axons (Mack et al., 2001). This suggests that the protective effect of the *Wld*^{S} protein is indirect, potentially functioning in the regulation of expression levels of an unidentified protective protein that is targeted to axons. Thus, a mechanistic relationship between protection from Wallerian degeneration by the *Wld*^{S} mutation, and the protection derived from inhibition of proteasome activity in the axons themselves described here, is not immediately clear. We performed the obvious test of whether pretreatment of cell bodies/proximal axons

with MG132 or lactacystin for 24 h prior to transection conferred protection to the severed axons, but no protection was observed (not shown). Furthermore, a direct mechanistic relationship between the current data and the mechanism of the *Wld*^{S} protein is unlikely in light of the finding that, although the axons of *Wld*^{S} mouse sympathetic neurons are protected from degeneration due to NGF withdrawal, the *Wld*^{S} mutation does not delay apoptosis of their cell body (Deckwerth and Johnson, 1994). In contrast, however, proteasome inhibition by the inhibitors used here was found to protect the cell bodies of sympathetic neurons from NGF withdrawal (Sadoul et al., 1996). It is nonetheless possible that: the *Wld*^{S} protein, though present, is not functional in cell bodies; that it is in fact present in axons at low levels and may itself be the protective factor in these mice; and that the two observations are related mechanistically, suggesting that the Ube4b sequence may act in a dominant-negative manner to inhibit protein multi-ubiquitylation, and subsequently proteasomal degradation, in *Wld*^{S} axons.

The findings presented here suggest that the protective effect of proteasome inhibition in transected axons is mediated by preventing loss of activity of the MEK/Erk pathway, potentially highlighting another role for this pathway in axonal physiology. Although the targets of MEK/Erk pathway that maintain axonal integrity in the absence of connection to the cell body are unknown, possibilities include Erk1/2-mediated regulation of cytoskeletal structure thus preserving axonal morphology, and/or Erk1/2-mediated protection of mitochondrial function thus maintaining axonal metabolic function. It will be interesting to determine if the protective effect of the *Wld*^{S} protein also acts by preserving MEK/Erk signaling in axons.

Selective axonal degeneration also occurs under physiological circumstances. During development of the nervous system axonal branches are overproduced and inappropriate or excess fibers are eventually eliminated. For example, axons may be lost due to failure to obtain sufficient neurotrophic support, as neurotrophins are believed to be released by target tissues of innervation in limiting quantities. Intriguingly, axonal degeneration of this nature occurs in a very specific manner, without spreading to cause death of the cell body, or even of the parent axon. In addition to conferring protection from Wallerian degeneration, inhibition of proteasome function also protects axons from degeneration due to local NGF withdrawal. This finding provides direct support to the

hypothesis that the process of Wallerian degeneration and of axonal degeneration due to NGF withdrawal are mediated by a common mechanism.

Our results, consistent with those of Finn *et al.* (2000), indicate that Wallerian degeneration of transected axons and axonal degeneration due to local neurotrophin withdrawal are not delayed in the presence of broad-spectrum caspase inhibitors. We further report that inhibition of caspase activity in NGF-deprived neurons protects cell bodies from apoptosis but does not significantly impede axonal degeneration. These findings suggest that the caspase-mediated apoptotic mechanism of the cell body is universally distinct from mechanisms of axonal degeneration. Several studies have assessed the role of caspase proteolytic activity in axonal degeneration in response to a variety of degenerative insults. However, the results have been seemingly inconsistent, and a general contribution of caspases to mechanisms of axonal degeneration is not established. Consistent with our observation that caspase inhibition does not protect axons from NGF withdrawal-induced degeneration, over-expression of Bcl-2, an inhibitor of caspase-mediated apoptosis, was found to protect cell bodies but not axons of sympathetic neurons from death due to NGF withdrawal, nor did it protect axons from Wallerian degeneration. In contrast, Finn *et al.* demonstrated that global NGF withdrawal activates axonal caspase-3, a key effector caspase in NGF-withdrawal induced apoptosis, while no active caspase 3 was detected in axons locally deprived of NGF. They suggest that under conditions in which the entire neuron is deprived of NGF, caspase activity is induced globally, and inhibition of global caspase activity could thus protect the entire neuron. They did not, however, test this hypothesis explicitly by examining the viability of caspase inhibitor-treated axons in response to global NGF withdrawal. Activated caspase-3 has also been detected in axons of neurons undergoing developmental apoptosis. Furthermore, caspase activity has been shown to be required for the exposure of phosphatidylserine at axonal membranes in response to local exposure of axons to toxic β-amyloid peptide (Ivins et al., 1998). Caspase activity was also found to be responsible for apoptotic-like features, including phosphatidylserine exposure and mitochondrial membrane permeabilization, elicited by treatment of axon terminal-derived synaptosomes with β-amyloid peptide, staurosporine, and Fe^{2+} (Mattson et al., 1998a; Mattson et al., 1998b).

It has been hypothesized that axons possess a unique self-destruction program that is mechanistically distinct from the programs that mediate apoptosis of the cell body, and that operates under multiple circumstances of local axon degeneration, including local NGF deprivation, local exposure to neurotoxic agents, axonal injury, or Wallerian degeneration (Finn et al., 2000; Raff et al., 2002). Such a mechanism could thus provide spatial control of degeneration within the neuron, being activated in situations when death of the axon, without death of the cell body, is warranted. It has been speculated that the axonal degeneration program is independent of caspases, whereas the cell body program, as well as global degeneration of the entire neuron, is caspase-dependent (Finn et al., 2000; Raff et al., 2002). In contrast, the data presented here are consistent with the notion that caspase-mediated proteolysis is not an effector of axon degeneration under any circumstance, including death of the entire neuron. Instead, these data highlight an important role for proteasome-mediated proteolysis in axonal degeneration in response to both local and global degenerative insults. Taken with the previous report that inhibition of proteasome function protects the cell bodies of sympathetic neurons from NGF withdrawal (Sadoul et al., 1996), it appears that this program does not represent a mechanism of axonal death that is inoperative in the cell body. Thus, under what physiological circumstances proteasome-mediated degeneration functions, and whether or not its effects can spatially segregated in circumstances of selective axon death, are important questions for future research.

Understanding of the mechanisms that regulate axonal degeneration will likely be of significant clinical importance, as axon degeneration, rather than death of the entire neuron, may be the causative element of many neurodegenerative disorders. Of particular relevance are "dying back"-type neuropathies, in which axons degenerate progressively, starting from the most distal end and spreading toward the cell body, a pathology that is manifested in disorders including diabetes- and AIDS-associated peripheral neuropathies, as well as centrally in Parkinson's, Alzheimer's, and motor neuron diseases (Azzouz et al., 1997; de la Monte et al., 1988; Iseki et al., 2001; Sima et al., 1983). Dysregulation of ubiquitin-proteasome function has been implicated in the pathology of several neurodegenerative disorders (Coleman and Perry, 2002; Keller et al., 2002;

Klimaschewski, 2003; Raff et al., 2002), suggesting that axon protection by proteasomal inhibition may represent a valuable therapeutic target.

FIGURES

Figure 4.1 Inhibition of proteasome-mediated degradation protects transected axons from Wallerian degeneration

A) Cell bodies/proximal axons of neurons in compartmented cultures before (intact) and after (transected) mechanical removal to induce Wallerian degeneration of transected axons in the adjacent distal axon compartments. B) Representative phase contrast images of axons from intact or transected neurons in which all axons were given NGF (50 ng/ml) and otherwise left untreated, or treated with MG132 (5 μM), lactacystin (10 μM), BocD-fmk (100 μM), a mixture of non-proteasomal protease inhibitors (AEBSF (100 μM), apoprotinin (1 μg/ml), chymostatin (10 μM) , and pepstatin A (1 μM)), or MG132 plus cycloheximide (350 μM), for 24 h. Axons of intact neurons from sister-plated cultures are shown as a control (intact). The experiment shown is represented of results that were replicated at least 3 times. C) Axons of intact neurons were axotomized and allowed to regenerate for 3 d, at which time axon extension was measured. Neurons were then left intact (not transected; *filled squares*), or cell bodies/ proximal axons were removed and transected axons were left untreated (*circle*) or treated with MG132 (5 μM) (*diamonds*) or lactacystin (10 μM) (*triangles*) for 24 h, at which time axon length was measured again. The mean axonal extension of at least 60 tracks from 3 cultures per group at each time point are shown. SD of $n > 60$ falls within data symbols. Results shown are from a representative experiment that was repeated with similar results.

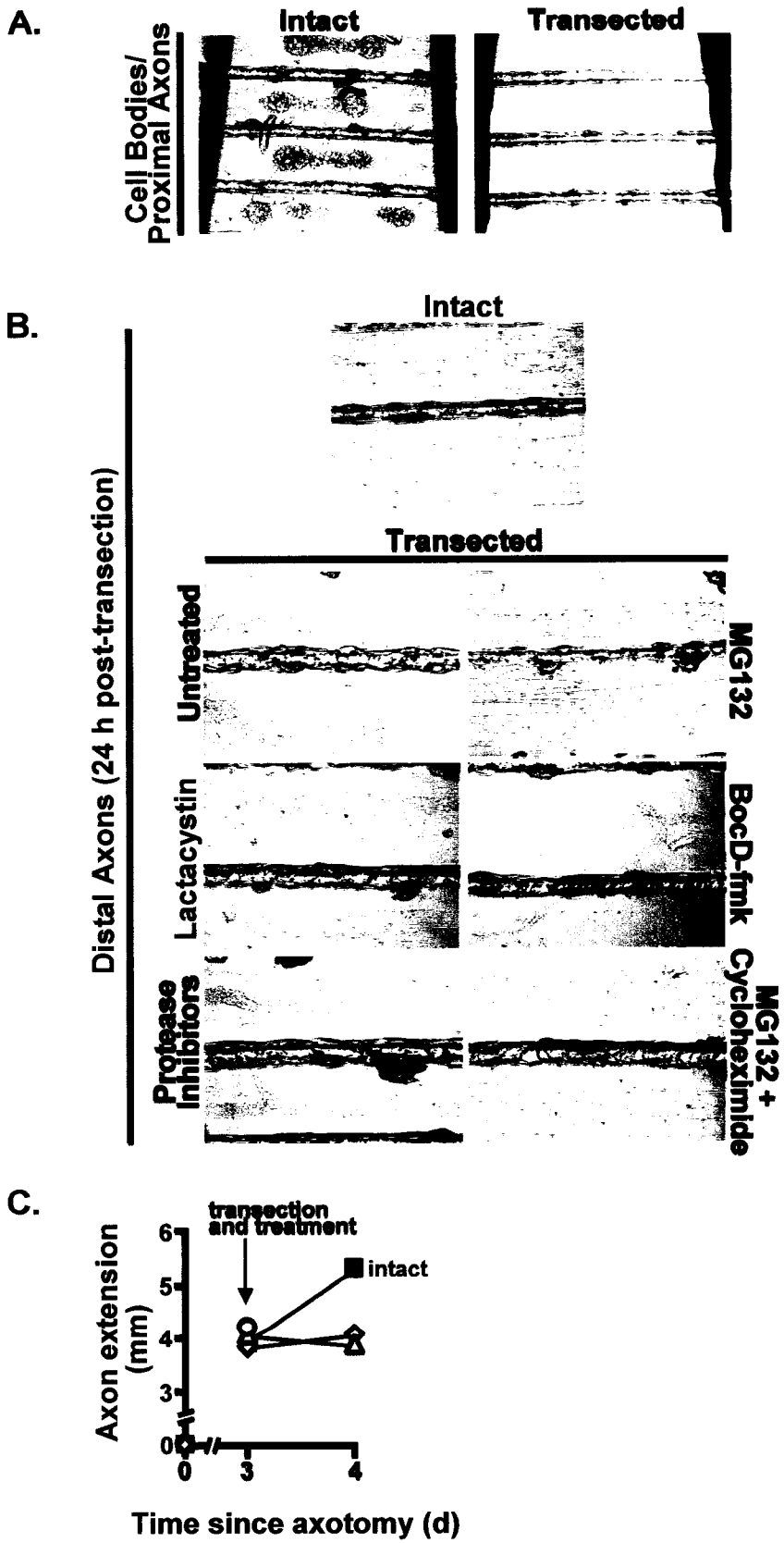


Figure 4.2 Transected axons protected by proteasome inhibition are metabolically active

A and B) Cell bodies/proximal axons were removed and transected axons were left untreated, or treated with MG132 (5 μM) or BocD-fmk (100 μM), for 24 h. Axons of intact neurons from sister-plated cultures are shown as a control. *A)* Fluorescence images of axons stained with Mitotracker-Orange (labels only mitochondria with active membrane potential, top panels), Mitotracker-Green (labels all mitochondria irrespective of membrane potential; middle panels) and merged images (axons with active mitochondria appear orange, those in which mitochondrial membrane potential is lost appear green; bottom panels). *B)* Bright field images of axons stained with MTT (labels active mitochondria). Images are representative of three experiments with similar results.

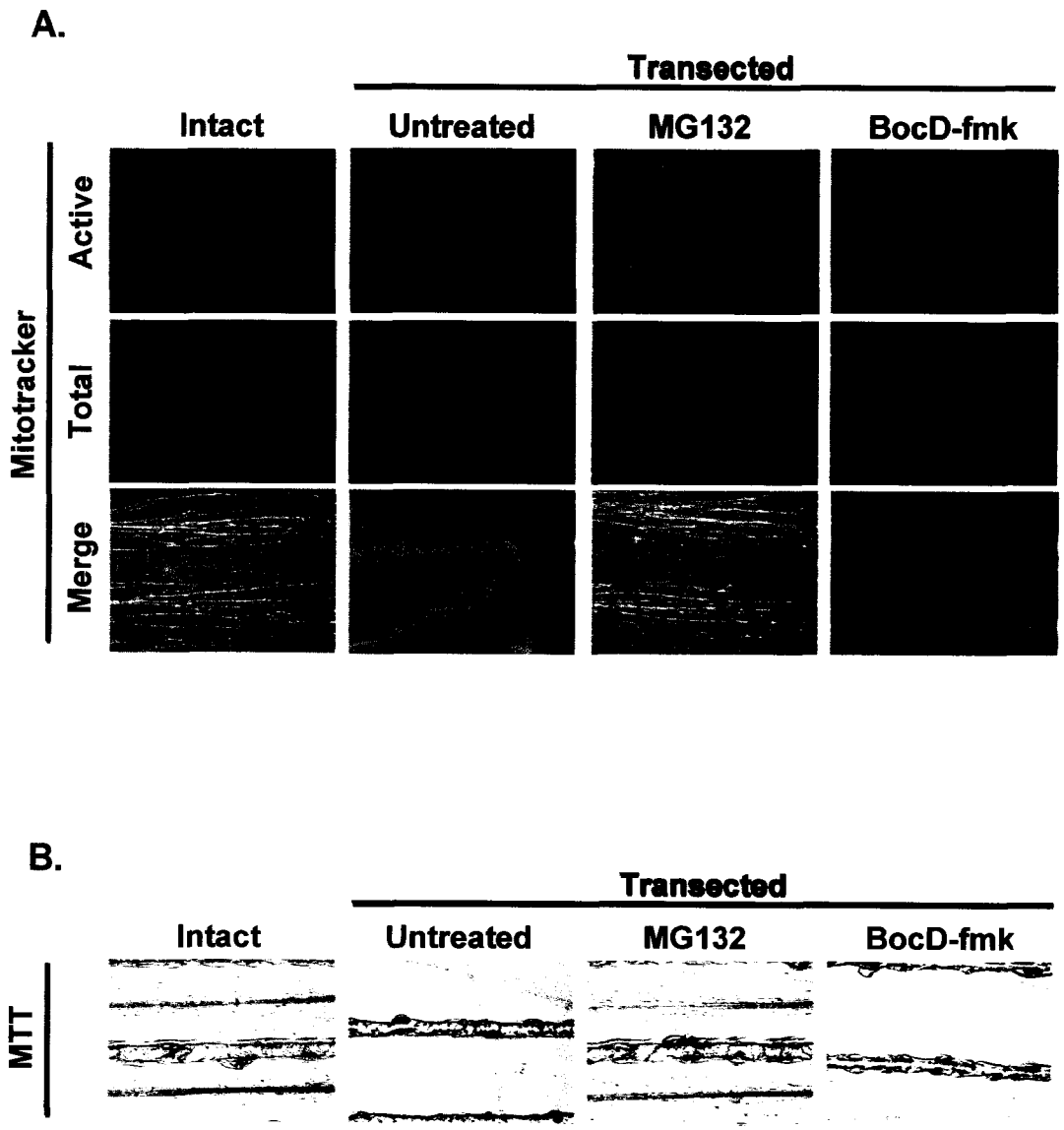


Figure 4.3 Proteasome inhibition-mediated protection of transected axons requires MEK kinase activity

A and B) Cell bodies/proximal axons were removed and transected axons were left untreated, or treated with MG132 (5 μM), MG132 and U0126 (50 μM), or U0126 alone which had no effect (not shown), for 24 h. Axons of intact neurons from sister-plated cultures are shown as a control. *A)* Fluorescence images of axons stained with Mitotracker-Orange (top panels), Mitotracker-Green (middle panels) and merged images (bottom panels). *B)* Bright field images of axons stained with MTT. Images are representative of three experiments with similar results. *C)* Axons of intact neurons were given NGF (50 ng/ml) or neutralizing anti-NGF antibodies (24 nM) with or without U0126 (50 μM), for 24 h. Shown are fluorescence images of axons stained with Mitotracker-Orange (top panels), Mitotracker-Green (middle panels) and merged images (bottom panels).

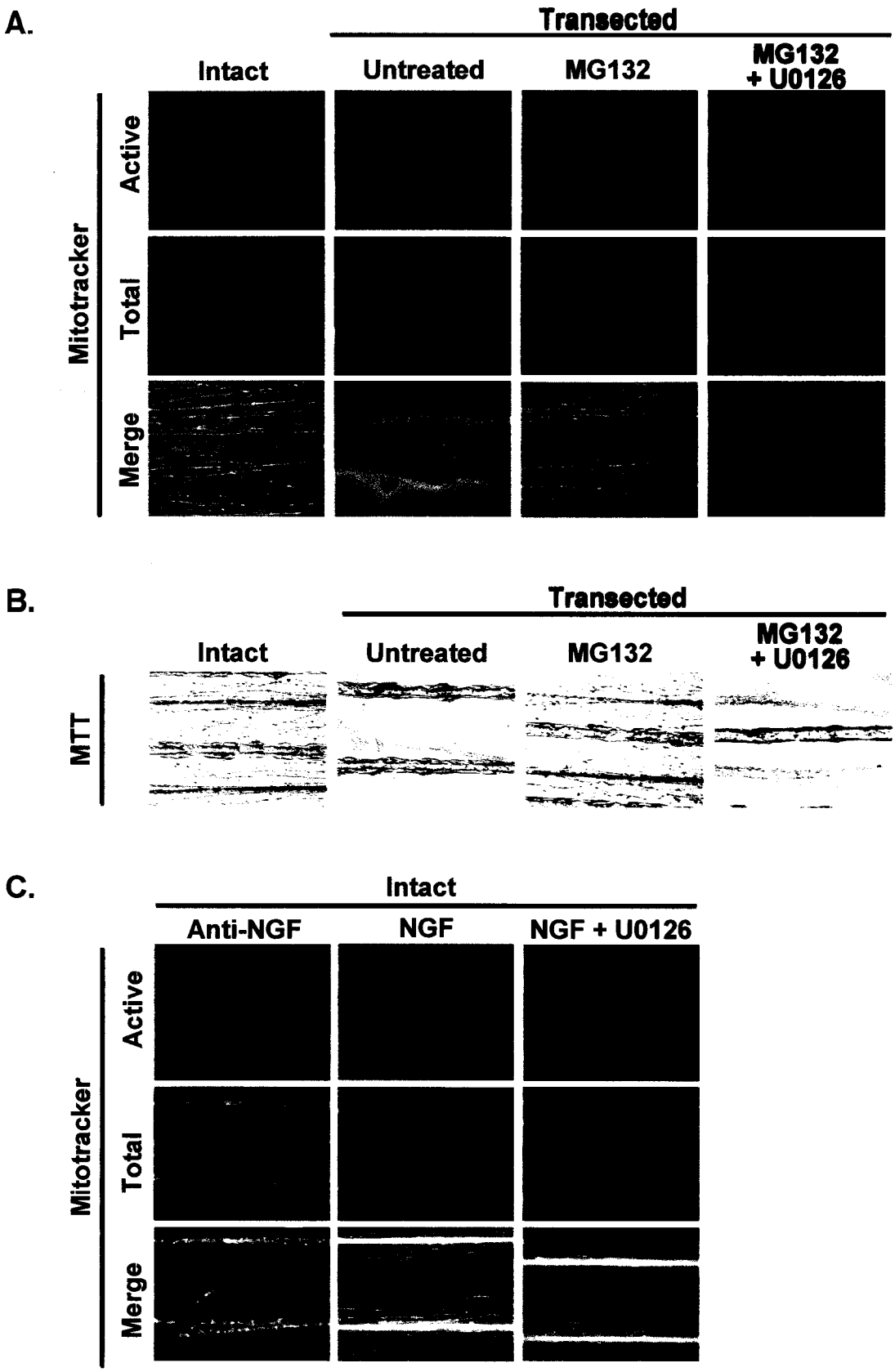


Figure 4.4 Inhibition of proteasome-mediated degradation induces persistent phosphorylation of Erk1/2 in transected axons

Lysates were prepared from axons of intact neurons (0 h), or from transected axons treated with or without MG132 (5 μM) for up to 18 h, as indicated in the figure. The level of phosphorylated Erk1/2 *(upper panel)* and total Erk 1 (to confirm equivalent protein loading) *(lower panel)* at each time point was determined by sequentially immunoblotting the same membrane with anti-phosphoErk1/2 (Thr202/Tyr204) and anti-Erk1 antibodies, respectively. Results are representative of four experiments with similar results.

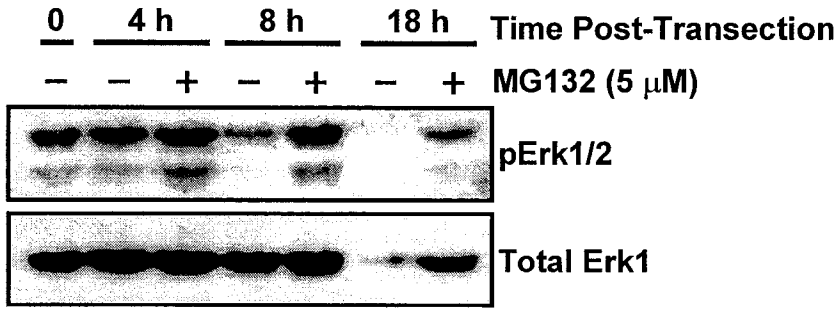
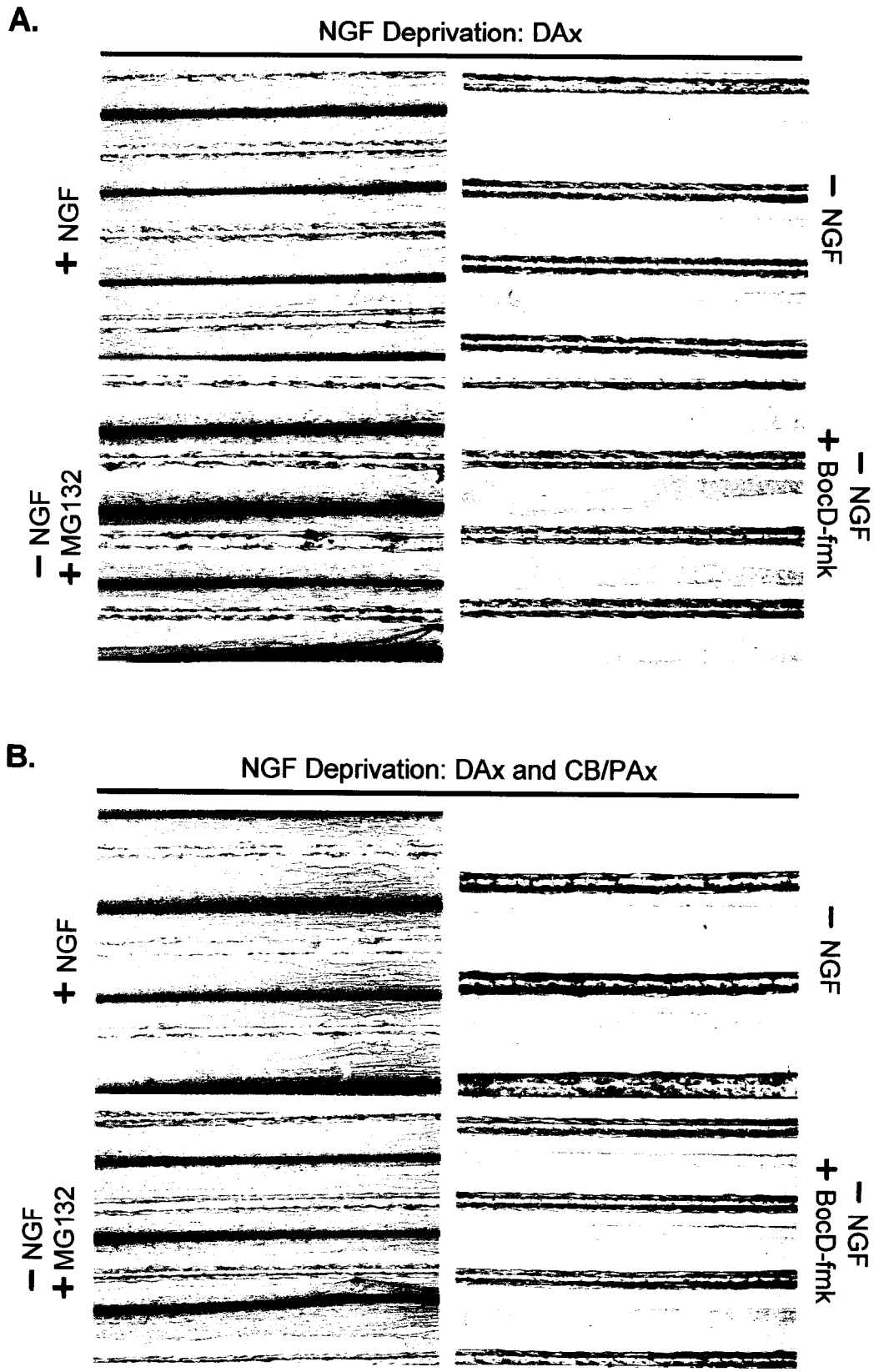


Figure 4.5 Proteasome inhibition, but not caspase inhibition, protects axons from local and global NGF deprivation

A) Neurons in compartmented cultures were incubated for 30 h with 10 ng/ml NGF at the cell bodies/proximal axons and 24 nM anti-NGF antibodies at the distal axons, to induce local axon degeneration. Simultaneously axons were also treated with 5 μM MG132 (-NGF +MG132), 100 μM BocD-fmk (-NGF +BocD-fmk), or given no other treatment (-NGF). Control cultures were given -50 ng/ml at the distal axons (+ NGF). Following the incubation axon viability was assessed by MTT staining. *B)* Neurons in compartmented cultures were incubated for 30 h with 24 nM anti-NGF antibodies in all compartments to induce apoptosis of the neurons. Simultaneously, cell bodies/proximal axons and distal axons were also treated with 5 μM MG132 (-NGF +MG132), 100 μM BocD-fmk (-NGF +BocD-fmk), or given no other treatment (-NGF). Control cultures were given 50 ng/ml at the distal axons (+ NGF). Following the incubation axon viability was assessed by MTT staining.



CHAPTER 4

GENERAL DISCUSSION

The prevalent hypothesis of neurotrophin-mediated retrograde signaling, known as the signaling endosome hypothesis, asserts that retrograde signaling is achieved by the retrograde transport of endosomes that carry NGF/pTrkA complexes to the cell body, where they are in close enough proximity to signal to the nucleus and thus regulate the activity of gene expression that controls neuronal survival (reviewed in: (Ginty and Segal, 2002)). The results presented in this thesis discredit this theory as being the exclusive mechanism by which retrograde signals are communicated. Specifically, this thesis establishes that retrograde transport of NGF, and retrograde arrival of pTrkA in the cell bodies, are not required to maintain the survival of neurons dependent on a distal source of NGF *in vitro*. This conclusion is supported principally by two observations: NGF presented to distal axons in a non-internalizable, bead-bound form maintained survival of the neurons without detectable retrograde transport of NGF; and inhibition of TrkA kinase activity locally in the cell bodies/proximal axons of neurons given NGF only at their distal axons did not induce significant death of the neurons, or inhibit pro-survival signaling. It is thus appropriate to re-examine the signaling endosome hypothesis of retrograde signaling in light of the data presented in this thesis, incorporating it into a revised working model of NGF-mediated retrograde signaling.

Possible Mechanisms for Retrograde Signaling

Currently several mechanisms for retrograde signaling by NGF have been proposed (reviewed in: (Miller and Kaplan, 2002)). These include: 1) the canonical signaling endosome hypothesis; 2) serial phosphorylation of TrkA at the plasma membrane; 3) retrograde transport of downstream second messenger signaling molecule(s). It is important to acknowledge that these mechanisms are not mutually exclusive, and there may be other mechanisms involved. The data presented in this thesis address predictions made by each of these hypotheses, and although it does not rule out the existence of any, it does eliminate the requirement for retrograde transport of NGF and retrograde TrkA kinase activity to promote neuronal survival, elements that are generally believed to be critical for retrograde signaling.

It is commonly believed that neurotrophin/pTrk complexes are themselves the basic units of retrograde signaling, and that ligand-bound, catalytically active Trks carried in signaling endosomes are required to reach the cell body to tell neuron that a distal source of neurotrophin is present (reviewed in: (Barker et al., 2002; Ginty and Segal, 2002; Heerssen and Segal, 2002)). This hypothesis is supported by several experimental observations, namely: neurotrophins have been shown to be retrogradely transported in multiple experimental paradigms (Hendry et al., 1974; Stoeckel et al., 1976; Claude et al., 1982; Palmatier et al., 1984; Ure and Campenot, 1997), and a fraction of transported neurotrophin has been found to co-precipitate with pTrk (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999); local inhibition of Trk kinase activity in the cell bodies/proximal axons has been shown to inhibit retrograde signaling responses to distal neurotrophin stimulation (Riccio et al., 1997; Senger and Campenot, 1997; Watson et al., 1999; Kuruvilla et al., 2000; Watson et al., 2001); and NGF treatment of PC12 cells has been shown to induce the formation of putative signaling endosomes containing NGF/pTrkA complexes and associated with PLC-γ, PI3-kinase, and several components of the MEK/Erk signaling pathway (Grimes et al., 1996; Grimes et al., 1997; Howe et al., 2001; Wu et al., 2001).

The data presented in this thesis contradict several predictions of the signaling endosome model. Foremost, it refutes the hypothesis that retrograde transport of NGF is a requisite component of the mechanism by which NGF-induced survival signals are communicated. The predicted role of the retrograde transport of NGF in signaling endosomes is to maintain TrkA in its ligand-bound, activated form, such that it is still catalytically active when it reaches the cell body. However, exposure of distal axons to NGF beads, preventing retrograde transport of NGF, was sufficient to maintain survival of approximately 80 % of the neurons for 30 h. Several control experiments confirmed that NGF was not released from the beads in concentrations and/or species that could account for the observed survival-promoting effect. In the most decisive of these experiments, [^{125}I] NGF of at least 97 % purity was used to prepare NGF beads, and the survival and retrograde transport of NGF induced by [^{125}I] NGF beads was compared to a range of free [^{125}I] NGF concentrations. In these experiments, retrograde transport of NGF from NGF beads was virtually undetectable, falling in the range of the lower limit

of detectability of the gamma-counter, while the survival of approximately 80 % of these neurons was maintained. In contrast, application of 0.5 ng/ml [^{125}I] NGF to distal axons resulted in approximately 20-fold more retrograde transport than achieved with [^{125}I] NGF beads, while survival was reduced to 29 %. Thus, the possibility that retrograde transport of a smaller amount of [^{125}I] NGF released from NGF beads than from 0.5 ng/ml free [^{125}I] NGF accounts for the survival-promoting effect they induce is virtually eliminated. Since the discovery that NGF is retrogradely transported from the site of uptake at pre-synaptic nerve terminals to the cell body almost 35 years ago, it has been believed that retrograde transport of NGF is critical to the mechanism by which retrograde signals are communicated. The finding that retrograde transport of NGF is not critical to NGF-mediated survival dispels not only a prediction of the signaling endosome hypothesis, but a fundamental theory that has been entrenched in the field for more than three decades.

These experiments established conclusively that application of NGF beads to distal axons did not permit retrograde transport of biologically significant levels of NGF. It remained possible, however, that NGF stimulation of TrkA was sufficient to induce internalization and retrograde transport of signaling endosomes containing pTrkA in the absence of the NGF that initiated the signal. There is experimental precedence for such a mechanism, as application of EGF beads to MCF7 cells induced internalization of phosphorylated ErbB1 receptors in the absence of EGF (Verveer et al., 2000). However, the results of two experiments suggest that TrkA was not internalized in response to NGF bead stimulation. In mass culture experiments, signaling downstream of TrkA was altered when NGF was restricted to the plasma membrane: NGF beads did not induce phosphorylation of Erk1/2, although robust phosphorylation of these proteins was observed in response to concentrations of free NGF that induced a similar level of TrkA activation as was achieved with NGF beads. This suggests that primarily endosomal TrkA signals to Erk1/2, and that prevention of internalization of NGF also prevents the internalization of TrkA. To directly assess whether NGF beads induced retrograde transport of TrkA, surface proteins at the axonal plasma membrane were biotinylated and, after incubation of axons with or without NGF or NGF beads, retrogradely transported proteins were recovered from cell bodies/proximal axons by streptavidin precipitation.

In these experiments, free NGF induced a 2.5-fold increase in the appearance of biotinylated TrkA in the cell bodies/proximal axons over the level detected in cultures given no NGF. In contrast, there was no increase in biotinylated TrkA in the cell bodies/proximal axons of neurons given NGF beads, again suggesting that NGF beads did not permit internalization of TrkA, and furthermore, that survival induced by NGF beads was generated by NGF/pTrkA signals derived from the axonal plasma membrane.

A propagative mechanism has also been proposed for retrograde signaling by TrkA in response to NGF stimulation. This mechanism also implicates Trks as the principal signal carriers from the axons to the cell body. However, rather than vesicular transport of neurotrophin/pTrk complexes, it predicts that retrograde propagation of Trk phosphorylation at the plasma membrane passes the signal in a serial manner (from receptor to receptor) along the axon to the cell body. In this way, neurotrophin is required distally to initiate the signal, whereas proximal receptors become activated in the absence of ligand binding. This mechanism was first suggested by Senger and Campenot (1997), who found that retrograde activation of TrkA in compartmented cultures of sympathetic neurons was induced within 1 min of distal NGF application. The rapid retrograde phosphorylation of TrkA was too fast to be attributed to internalization and retrograde transport of NGF/pTrkA complexes, as retrograde transport of [^{125}I] NGF to the cell bodies/proximal axons was not detected for at least 30 – 60 min after its addition to the distal compartments. Evidence of the existence of such a cellular mechanism was later presented by Verveer *et al.* (2000), who demonstrated that focal application of EGF beads to MCF7 cells induced rapid and extensive lateral propagation of ErbB1 phosphorylation to unoccupied receptors at the plasma membrane.

Both the signaling endosome model and the propagated phosphorylation of TrkA model implicate retrograde activation of TrkA as the principal conveyer of the distal NGF signal. The prediction follows that receptor kinase activity is required in the cell body to access local signaling pathways that mediate transcriptionally-controlled aspects of NGF signaling. Lateral propagation of TrkA phosphorylation at the plasma membrane could conceivably provide a mechanism by which survival signals induced by NGF beads at distal axons could be communicated to the cell body. However, several findings presented here argue that local signaling by TrkA in the cell body is neither required to

mediate retrograde signaling, nor to produce biological outcomes that are regulated by gene expression, induced by free or bead-bound NGF at distal axons. K252a-mediated inhibition of TrkA kinase activity in the cell bodies/proximal axons of neurons given NGF or NGF beads at the distal axons did not induce apoptosis of the neurons, as would be predicted if TrkA kinase activity is required locally in the cell bodies to mediate survival. Nor did inhibition of TrkA kinase activity in the cell bodies/proximal axons significantly affect axon growth induced by distal NGF, further supporting the conclusion that the viability of these cells was not compromised, and suggesting that TrkA kinase activity is not required in the cell bodies to mediate these effects mediated by distally-applied NGF. Furthermore, inhibition of accumulation of pTrkA in the cell bodies/proximal axons of neurons supported by distal NGF did not result in a corresponding inhibition of the activity of Akt or CREB, two key pro-survival signaling molecules that are known to be activated retrogradely by distal NGF stimulation (Riccio et al., 1997; Riccio et al., 1999; Kuruvilla et al., 2000).

The validity of the interpretations of the K252a experiments presented here relied on the ability to establish that K252a effectively inhibits TrkA kinase activity in the cell bodies/proximal axons of neurons given NGF at the distal axons. This was confirmed by two methods: Trk immunoprecipitation followed by phospho-tyrosine immunoblot of lysates from K252a-treated cell bodies/proximal axons, and direct analysis of the phosphorylation state of key tyrosine residues on TrkA by immunoblot analysis using phospho-specific Trk antibodies. Both methods demonstrated that in the presence of 200 nM K252a, TrkA kinase activity in the cell bodies/proximal axons induced by distal NGF was effectively blocked. In fact, TrkA kinase activity in the cell bodies/proximal axons of K252a-treated cultures was consistently at or below levels detected in cultures deprived of NGF for the experimental period, although neurons exposed to the former treatment survived, while those exposed to the latter treatment did not. In other control experiments it was confirmed that K252a effectively blocked the NGF-induced phosphorylation of Akt, eliminating the possibility that pro-survival signaling may occur by a TrkA-independent mechanism, and that K252a itself did not exert significant survival- or growth-promoting effects, or induce activation of Akt or CREB, in the absence of NGF.

Although it is unlikely to account for the survival-promoting effects, serial propagation of TrkA phosphorylation at the plasma membrane may account for the robust phosphorylation of TrkA induced by NGF beads. The 1 μm-diameter beads would be expected to access fewer receptors at the plasma membrane than would unbound NGF, however, the magnitude of TrkA phosphorylation induced by NGF beads was not significantly lower than that induced by near-saturating concentrations of free NGF after 1 hour of treatment. NGF beads could induce activation of TrkA directly at the sites of membrane contact, and subsequently induce extensive TrkA phosphorylation indirectly by serial propagation of TrkA kinase activity to unoccupied receptors, in a manner analogous to lateral propagation of ErbB1 phosphorylation induced by focal stimulation with EGF beads. NGF bead-induced phosphorylation of TrkA may also induce increased lateral diffusion of TrkA molecules within the plasma membrane, thus enhancing the formation of TrkA homodimers. It is known that TrkA dimerization is sufficient to induce phosphorylation of TrkA, as over-expression of the receptor in PC12 cells was found to induce TrkA kinase activity in the absence of NGF, which was suggested to be due to induced proximity of the TrkA at the plasma membrane, resulting in an increased probability of receptor dimerization (Hempstead et al., 1992). TrkA has been shown to be capable of autophosphorylation in the absence of NGF in sympathetic neurons by treatment with the ganglioside GM1. Furthermore, GM1-mediated activation of TrkA was sufficient to maintain neuronal survival in the absence of NGF (Ferrari et al., 1995; Ferrari and Greene, 1996).

Other Evidence for Alternatives to the Signaling Endosome Model of Retrograde Signaling

The data presented in this thesis suggest that sympathetic neurons are capable of transmitting retrograde signals without the necessity for retrograde transport of NGF, or for catalytically active TrkA in the cell body, to communicate the signal. Independent investigations have also produced evidence supporting the existence of mechanisms for retrograde signaling that do not involve retrograde transport of neurotrophin/neurotrophin receptor complexes. Perhaps most relevant to the current results, it has demonstrated that the survival-promoting effect of ceramide on NGF-deprived sympathetic neurons can

be mediated by increasing ceramide content exclusively in distal axons of sympathetic neurons in compartmented cultures, either by local addition of exogenous short chain (C_6) ceramide, or by induction of endogenous long chain ceramide production locally in axons (Song and Posse De Chaves, *Submitted*) (personal communication). These findings suggest that elevation of ceramide levels in axons initiates a local pro-survival signal that is then transmitted to the cell body. It is possible that this survival signal may be mediated by TrkA kinase activity, as long-term exposure of C_2-ceramide to sympathetic neurons induces phosphorylation of TrkA, possibly by inducing receptor homodimerization (MacPhee and Barker, 1999). Interestingly, however, it is unlikely that this signal would require retrograde transport of pTrkA to the cell body to convey the ceramide-induced survival signal, as addition of C_6-ceramide to distal axons prevents the internalization and retrograde transport of [^{125}I] NGF (Posse De Chaves et al., 2001). In basal forebrain cholinergic neurons, distal NGF released by target hippocampal neurons is essential to maintain cell body morphology and choline acetyltransferase production and activity. These neurons normally transport NGF, however, in p75NTR knockout mice retrograde transport of NGF was undetectable, yet the NGF-regulated characteristics were not compromised (Kramer et al., 1999; Yeo et al., 1997). These results suggest that NGF-mediated retrograde signals that control both structural and functional aspects of basal forebrain cholinergic neurons do not require retrograde transport of NGF. The findings described above are consistent with the possibility that retrograde signals can be carried by the retrograde transport of activated downstream second messenger signaling molecules, although the identity of such molecules is unknown.

Further evidence supporting the hypothesis that the signaling endosome model is not the sole mechanism for retrograde signaling comes from detailed analysis of the characteristics and kinetics of [^{125}I] NGF trafficking in sympathetic neurons in compartmented cultures (Ure and Campenot, 1997). The rate of [^{125}I] NGF retrograde transport was determined to be 10 – 20 mm/hr, derived from the difference in the time of first detection of [^{125}I] NGF degradation products released into the medium in the center compartment of neurons in 3-compartmented cultures versus 5-compartmented cultures, in which the difference in the distance of the source of NGF to the center compartment is 3 mm. Based on this transport rate, 3 – 6 min are required for NGF to cross the 1 mm

partition between the distal and center compartments in the 3-compartment culture, the shortest possible distance between the two compartments. Interestingly, however, almost no retrograde transport of [^{125}I] NGF was observed in the first hour after its addition to the distal compartments, suggesting a significant lag period exists between binding of NGF and its internalization and/or loading onto the retrograde transport system. Furthermore, when [^{125}I] NGF reached steady state distribution in the neurons after its addition to distal axons, approximately 80 % of the total neuron-associated NGF was associated with the distal axons, and approximately 85 % of that was at the axonal plasma membrane rather than in internalized pools. A similar phenomenon was observed in a study in which binding and internalization of NGF in cultured sympathetic neurons was directly visualized using rhodamine-labeled NGF (Weible et al., 2001). It was found that vesicle-like structures containing NGF in the axon terminals were not detectable until 1 h after addition of NGF. Furthermore, in the same study it was reported that the retrograde transport of [^{125}I] NGF *in vivo* could be prevented by addition of excess unlabeled NGF for up to 4 hours after addition of [^{125}I] NGF to the anterior chamber of the rat eye, indicating that an extended period of time exists prior to the initiation of retrograde transport, during which [^{125}I] NGF remains at or near the nerve terminal where it can be competed with unlabeled NGF. It is known that axonal NGF regulates processes other than retrograde signaling, such as the local control of axon growth. However, in conjunction with the present results, it is reasonable to speculate that the vast association of NGF at the axonal membrane may also function to generate intra-axonal signals that are communicated to the cell body without the retrograde transport of NGF or pTrkA.

Several studies in which neurotrophin-mediated retrograde signaling were investigated using the compartmented culture model system have shown that distal neurotrophin stimulation induces the appearance of phosphorylated proteins, including Trk receptors, in the cell bodies/proximal axons faster than could be attributed to the retrograde transport of the neurotrophin that initiated the signal, based on the lag period and velocity of [^{125}I] NGF transport established in this system. As previously described, Senger and Campenot (1997) observed retrograde tyrosine phosphorylation of TrkA and several other proteins as early as 1 min after application of NGF to distal axons of sympathetic neurons in compartment cultures. The authors interpreted these signals as

being too fast to be attributed to retrograde transport of any molecule, and instead suggested that the signal was carried by retrograde propagation of TrkA phosphorylation at the plasma membrane.

Riccio *et al.* (1997) found that addition of NGF to distal axons of sympathetic neurons in compartmented cultures induced rapid activation of the nucleur transcription factor CREB. By immunocytochemical analysis using antibodies specific to phosphorylated CREB, the authors reported that distal NGF stimulation induced CREB phosphorylation in 20 % of neurons within 10 min, which increased to 80 % within 20 min. These time points significantly precede the earliest detection of [^{125}I] NGF transported from distal axons of sympathetic neurons in compartment cultures described by Ure and Campenot (1997). However, the authors attributed retrograde phosphorylation of CREB to retrograde transport of NGF/pTrkA complexes, as the effect was blocked by inhibition of NGF internalization at distal axons using NGF beads, or by inhibition of TrkA kinase activity at cell bodies/proximal axons using K252a. The relative effectiveness in inducing axonal TrkA phosphorylation of NGF beads and free NGF was not tested, but NGF beads were considerably less effective in inducing phosphorylation of TrkA in PC12 cells than free NGF at the concentration that was used (Riccio et al., 1997). It has been established that cell-body responses are more sensitive to NGF applied directly to the cell bodies than to NGF applied to distal axons (Toma et al., 1997). Therefore, these data are also consistent with the hypothesis that NGF beads were not an adequate stimulus to axons to induce retrograde signaling to the cell bodies regardless of its mechanism.

In similar experiments using compartmented cultures of dorsal root ganglion neurons, Watson *et al.* (Watson et al., 1999) reported that phosphorylation of Trk (these neurons express TrkA and TrkB) and CREB in cell bodies/proximal axons reached greater than 90 % of the maximum level achieved within 5 min of distal neurotrophin (NGF and BDNF) stimulation and that maximal levels of pTrk and pCREB were achieved within 10 – 20 min. The authors interpret these rapid retrograde signals as being carried by the retrograde transport of neurotrophin/pTrk complexes because: pTrk could be co-immunoprecipitated with BDNF from cell bodies/proximal axons; green fluorescent protein-labeled TrkB (TrkB-GFP) was found to accumulate in the cell body

after addition of BDNF to distal axons; disruption of microtubule-dependent retrograde transport by application of colchicine (a microtubule depolymerizing agent) inhibited the retrograde accumulation of TrkB-GFP, pTrk, and pCREB; and, treatment of cell bodies/proximal axons with K252a inhibited retrograde accumulation of pCREB. However, the validity of this interpretation is questionable given the data provided. The level of pTrk that co-precipitated with BDNF appears to be very low, and no indication of the fraction of total pTrk or BDNF in the cell bodies/proximal axons this represents is given. The increase in TrkB-GFP fluorescence induced by 10 min of distal neurotrophin stimulation was only 12 – 16 % above untreated controls. Furthermore, removal of 90 % of background fluorescence by pre-bleaching of the cell bodies prior to the distal addition BDNF caused a decrease the BDNF-induced recovery of TrkB-GFP fluorescence, to the extent that there was no significant increase in the level of TrkB-GFP accumulation between between experiments in which pre-bleaching was or was not used. As well, essential control experiments demonstrating that neurotrophin stimulation induces Trk phosphorylation locally in colchicine-treated axons are not presented, thus raising the possibility that inhibition of pTrk accumulation in the cell bodies of these neurons is due to compromised Trk phosphorylation in the distal axons rather than specific inhibition of retrograde transport. Moreover, generalized depolymerization of the microtubule network by colchicine likely would cause widespread non-specific disruption of anterograde and retrograde transport in the neurons. Furthermore, the present results suggest that the inhibitory effect of K252a on retrograde activation of CREB observed in this and the Riccio *et al.* study may have been overcome if longer time points of neurotrophin stimulation had been examined.

Spatial Organization of TrkA Signaling

Results presented in this thesis also address several aspects of the spatial segregation of NGF-induced signaling pathways in sympathetic neurons. Research in the broader field of signal transduction by transmembrane growth factor and G-protein coupled receptors has recently focused on how the sub-cellular location and compartmentalization of signaling molecules, including the receptors and downstream effector proteins, affect the nature of the signals they generate (reviewed in: (Grimes

and Miettinen, 2003)). The spatial organization of signal transduction is especially important in neurons, in which target-derived neurotrophins mediate local activation of signaling pathways at the nerve terminal, and retrograde activation of molecules in the cell bodies. The spatial organization of signal transduction induced by distal NGF stimulation can be sub-divided into two major categories of spatial compartmentalization: 1) signals activated by pTrkA at plasma membrane and versus signals activated by internalized TrkA in signaling endosomes, and 2) signals activated locally at the site of NGF stimulation in the axons versus signals activated retrogradely in the cell bodies.

Spatial Organization of TrkA Signaling: *NGF/pTrkA-mediated signaling from the plasma membrane versus from signaling endosomes*

In this thesis, pharmacological inhibition of clathrin-mediated endocytosis was used to assess the ability of TrkA signaling predominantly from the plasma membrane to activate downstream pathways. It was found that inhibition of clathrin-mediated endocytosis did not affect the phosphorylation levels of TrkA at tyrosines 674/675, in the catalytic kinase domain, or at tyrosine 490, the Shc binding site. Likewise, there was no effect on the ability of NGF to induce activation of Akt when internalization of TrkA was inhibited. In contrast, inhibition of clathrin-mediated endocytosis significantly impaired the NGF-induced phosphorylation of Erk1/2. Similarly, phosphorylation of TrkA and Akt were induced, while phosphorylation of Erk1/2 was virtually undetected, when NGF was provided to the neurons in bead-bound form to inhibit endocytosis. Thus it appears that NGF can induce full activation of TrkA and Akt prior to internalization, whereas endosomal pTrkA couples NGF to the activation of MAPK.

The results of several studies investigating the role of internalization in neurotrophin-mediated signaling have produced data that are consistent with this model. In primary cultures of dorsal root ganglion neurons and PC12 cells, inhibition of clathrin-mediated endocytosis by treatment with the pharmacological inhibitors used in the present experiments, or by expression of dominant-negative dynamin, has been shown to block neurotrophin-induced activation of Erk1/2 (York et al., 2000; Howe et al., 2001; Rakhit et al., 2001). Another study in which clathrin-mediated endocytosis was inhibited in PC12 cells by expression of a temperature-sensitive dynamin mutant in PC12 cells

reported that phosphorylation of MEK1/2 and Erk1/2 was inhibited at 5 min, but was increased at 60 min after NGF stimulation at the non-permissive temperature (Zhang et al., 2000). These results suggest that internalized, NGF/pTrkA-containing signaling endosomes are the predominant platform for NGF-mediated signaling to Erk1/2. Further supporting this hypothesis, signaling endosomes isolated from PC12 cells were shown contain several members of the MEK/Erk pathway, including Shc, Ras, Rap-1, b- and c-Raf, pMEK, and pErk1/2 (Howe et al., 2001; Wu et al., 2001). Furthermore, it was shown that this pathway is capable of signaling from endosomes *in vitro*, as signaling endosomes induced by NGF treatment of PC12 cells could be used to phosphorylate Elk, a direct target of Erk1/2 kinase activity (Howe et al., 2001).

In contrast, evidence to date indicates that PI3-kinase-mediated signaling is derived primarily from the plasma membrane and does not require endocytosis of NGF/pTrkA complexes (York et al., 2000; Zhang et al., 2000). In PC12 cells expressing a temperature-sensitive dynamin mutant, NGF stimulation at the temperature that is non-permissive for internalization caused an increase in both the magnitude and the duration of Akt phosphorylation. Based on these findings the authors suggest that internalization terminates PI3-kinase/Akt signaling (Zhang et al., 2000). Furthermore, although PI3-kinase was enriched in NGF/pTrkA-containing signaling endosomes purified from PC12 cells, the presence Akt or pAkt could not be detected (Howe et al., 2001). As well, evidence from other growth factor/receptor systems suggests that activation of Akt occurs at the plasma membrane and is not affected by inhibition of clathrin-mediated endocytosis (Ceresa et al., 1998; Kao et al., 1998; Di Fiore and De Camilli, 2001).

Spatial Organization of TrkA Signaling: *Local signaling in distal axons versus retrograde signaling in cell bodies/proximal axons*

Binding of NGF to TrkA at distal axons activates local signaling mechanisms in the axons and retrograde signaling mechanisms in the cell bodies. Activation of local signaling mechanisms in axons has been shown to control processes that do not require changes in gene expression, such axon growth and motility. However, signals activated in axons are believed to be out of range of the nucleus in the cell body, thus necessitating the retrograde transport of NGF/pTrkA-containing signaling endosomes to convey

signals that influence transcriptionally-regulated processes. Notably, to date no signaling proteins have yet been identified as being retrogradely co-transported with NGF/pTrkA-containing endosomes *in vivo*, or neurons in compartmented cultures *in vitro*.

The local control of axon growth by NGF was demonstrated using the compartmented culture system, in which it was found that NGF promoted growth of axons only in the compartment(s) to which it was supplied (Campenot, 1977; Campenot, 1982a). However, the molecular nature of the signaling mechanisms in axons that mediate this process are just beginning to be delineated. Evidence presented here indicates that local activation of Erk1/2 signaling in axons is required to promote normal axon growth. In compartmented cultures of sympathetic neurons it was found that pharmacological inhibition MEK, the upstream kinase of Erk1/2, in distal axons caused a reduction in the rate of axonal extension, and a reduction in the total accumulation of axonal tubulin. In contrast, no significant effect on axon growth was detected when MEK signaling was inhibited locally in the cell bodies/proximal axons. Furthermore, it was found that inhibition of clathrin-mediated endocytosis in distal axons also inhibited axon growth, suggesting that local internalization of NGF/pTrkA in axons is required for normal axon growth, likely to induce local activation of Erk1/2 signaling. Consistent with these findings, inhibition of clathrin-mediated endocytosis by expression of dominant-negative dynamin inhibited NGF-induced neurite outgrowth in PC12 cells (Zhang et al., 2000). The specific targets of local Erk1/2 signaling in axons that promotes axon growth are unknown. However, likely candidates include neurofilament proteins and microtubule-associated proteins (such as Tau) which are phosphorylated by Erk1/2, promoting microtubule stability and axonal elongation.

The requirement for local activation of MEK/Erk signaling in axon growth was also identified in a study in which contributions of the MEK/Erk, PI3-kinase/Akt and PLC-γ1 signaling pathways to axon growth were investigated in detail (Atwal et al., 2000). Adenovirus-mediated gene transfer was used to express constructs encoding wild-type TrkB, kinase-dead TrkB, or TrkB that had been specifically mutated at the Shc or PLC-γ1 binding site, in sympathetic neurons which have no endogenous TrkB. The effects of the mutations on survival were distinguished from effects on axon growth using the compartmented culture system, such that neuronal survival was maintained by

addition of NGF to the cell bodies/proximal axons, while axon growth was stimulated by addition of BDNF to the distal axon compartments. It was found that disruption of the Shc binding site inhibited BDNF-dependent axon growth, as fewer axons that lacked Shc-mediated signaling grew into the distal compartments, and the average length of the axons that did grow was one third of that observed in axons expressing wild-type TrkB. In contrast, disruption of the PLC-γ1 binding site had no effect on axon growth. As the Shc is the principal effector site leading to activation of both the MEK/Erk and PI3-kinase/Akt pathways, the authors then assessed the contribution of each of these pathways to axon growth. Pharmacological inhibitors of these pathways were applied to distal axons of sympathetic neurons expressing wild-type TrkB, while NGF was provided to the cell bodies/proximal axons to maintain survival, and BDNF was provided to distal axons to stimulate axon growth. Interestingly, inhibition of either of these pathways severely disrupted axon growth (shown as frequency distribution plots of the number of tracks containing axons of a given length), although inhibition of MEK kinase activity using U0126 had a more profound effect than did inhibition of PI3-kinase activity using LY290042. A more recent study in which the contribution of these pathways to axon growth was investigated in sensory neurons suggests that activation of the MEK/Erk pathway promotes axon lengthening *per se*, whereas PI3-kinase/Akt activity controls axon caliber and branching (Markus et al., 2002b).

As previously discussed, local application of neurotrophins to distal axons also induces retrograde activation of several signaling proteins in the cell body, highlighting the unique relevance of the spatial organization of signal transduction in neurons. Data presented here confirm previously published observations that distal application of NGF induces retrograde accumulation of phosphorylated TrkA, Akt and CREB (Riccio et al., 1997; Senger and Campenot, 1997; Riccio et al., 1999; Atwal et al., 2000; Kuruvilla et al., 2000). Notably, this is the first evidence that these molecules are chronically activated under basal culture conditions. Previous studies of neurotrophin-mediated retrograde signaling have used acute stimulation paradigms, in which the neurons are temporarily deprived of, or maintained in a low concentration of neurotrophin, to attenuate the basal signal levels, and then stimulated for relatively short time periods (minutes to a few hours) to induce robust signal activation. Interestingly, no investigation of the effect of

the deprivation conditions on expression levels of the receptor or its downstream targets has accompanied these studies.

Inhibition of TrkA kinase activity in the cell bodies/proximal axons had little effect on the retrograde phosphorylation of Akt and CREB. Interestingly, however, K252a-mediated inhibition of TrkA kinase activity in cell bodies/proximal axons of sympathetic neurons in compartment cultures has also been reported to prevent the retrograde phosphorylation of Akt and CREB induced by distal NGF (Riccio et al., 1997; Kuruvilla et al., 2000). Although these findings are in apparent contradiction, the latter studies were performed by the same laboratory, and several technical differences in culturing conditions may account for these discrepancies. Protein loading controls in immunoblot experiments indicate that the vast majority of cellular material is in the center compartment and less protein is contained in the distal axon compartments. This is likely because both the center and distal compartments are given relatively high concentrations of NGF, and thus profuse axon growth in the distal compartments is not promoted. Furthermore, not only is the absolute level of axonal protein decreased, but the axons that are present have been reported to grow at a rate that is approximately 10 % of that typically observed under our culture conditions (Ginty, 2002). These findings suggest that the neurons used in these experiments are not healthy under basal culture conditions, and thus may be more adversely affected when experiments involving significant periods of NGF deprivation are preformed.

It is noteworthy that there are no published reports that distal neurotrophin stimulation induces retrograde activation of Erk1/2 in any neuronal population to date, consistent with the hypothesis that the primary role of Erk1/2 signaling induced by distal NGF exposure is in mechanisms related to local axon growth. This hypothesis is also supported by the observation that inhibition of MEK kinase activity in the cell bodies/proximal axons of neurons supported by distal NGF did not cause significant apoptosis. Interestingly, Watson *et al.* (1999) reported that addition of neurotrophin to distal axons of dorsal root ganglion neurons in compartmented cultures induced local but not retrograde activation of Erk1/2, but that retrograde activation of another MAPK family member, Erk5, was induced. The authors went on to show that retrograde activation of Erk5 is required for the survival of these neurons, and that retrograde

activation of Erk5 was inhibited by disrupting clathrin-mediated endocytosis, suggesting retrograde transport of neurotrophin/pTrk complexes is required to activate this pro-survival signaling pathway. This study illustrates that neurotrophin/pTrk signaling can access distinct pathways, generating distinct biological outcomes, depending on whether the signal is locally or retrogradely derived. However, no role for Erk5 in NGF-mediated signaling in sympathetic neurons has been identified. Furthermore, it is unlikely that Erk5 is required for retrograde survival signaling in these cells, as inhibition of MEK kinase activity in cell bodies/proximal axons, which prevents Erk5 activation and induces apoptosis in dorsal root ganglion neurons, is shown here and elsewhere (Creedon et al., 1996; Klesse and Parada, 1998; Mazzoni et al., 1999; Virdee et al., 1999; Atwal et al., 2000; Xue et al., 2000) to not cause significant death in sympathetic neurons.

Possible Mechanisms for Retrograde Signaling Suggested by the Present Findings

The results presented in this thesis indicate that the signaling endosome model, as defined by signaling achieved by the retrograde transport of NGF/pTrkA-containing vesicles, is not the exclusive mechanism by which NGF-mediated retrograde signals are communicated in sympathetic neurons. Although it is not clear that the same mechanism accounts for all retrograde signals observed here, there are several features that are consistent between the experimental paradigms used. Taken together with the findings of other investigations presented above, it is possible to speculate on possible mechanisms of retrograde signaling that are independent of retrograde transport of signaling endosomes. Perhaps paradoxically, the data presented here are consistent with a model in which clathrin-mediated endocytosis of NGF/pTrkA complexes is required for the local growth-promoting effects of NGF in distal axons, whereas retrograde survival signaling to the cell body is mediated without the need for internalization and retrograde transport of NGF/pTrkA-containing signaling endosomes. This interpretation is derived primarily from the observations that neither inhibition of internalization of NGF, nor inhibition of retrograde TrkA kinase activity, results in significant apoptosis of neurons supported by NGF supplied only to distal axons, whereas clathrin-mediated endocytosis at distal axons is required for the local control of axon growth.

Several characteristics of NGF/pTrkA-mediated signal transduction, identified here and elsewhere, are consistent with this model. It is well established that Erk1/2 activity is required for axonal growth in sympathetic neurons, dorsal root ganglion neurons, and PC12 cells (reviewed in: (Markus et al., 2002a)), and in each of these cell types it has been shown that inhibition of clathrin-mediated endocytosis attenuates NGF-induced activation of Erk1/2 (York et al., 2000; Rakhit et al., 2001). Furthermore, NGF/pTrkA-containing signaling endosomes purified from PC12 cells are preferentially enriched in components of the MEK/Erk pathway, including phosphorylated and activated Erk1/2 (Howe et al., 2001; Wu et al., 2001). Together these data suggest that internalization of NGF/pTrkA complexes in axons is required to induce local activation of Erk1/2, which in turn regulates mechanisms that control axon growth and morphology. As there is no evidence of retrograde accumulation of activated Erk1/2 induced by distal NGF stimulation, and since Erk1/2 signaling is not required for NGF-mediated survival of sympathetic neurons (Creedon et al., 1996; Klesse and Parada, 1998; Mazzoni et al., 1999; Virdee et al., 1999; Atwal et al., 2000; Xue et al., 2000), it is not clear whether signaling endosomes of this nature have any direct role in retrograde survival signaling by NGF in these neurons.

In contrast, several lines of evidence suggest that NGF/pTrkA signaling at the axonal plasma membrane is sufficient to generate retrograde survival signals. Activation of Akt is believed to be necessary and sufficient for NGF-mediated survival of sympathetic neurons (Crowder and Freeman, 1998; Vaillant et al., 1999; Virdee et al., 1999). It has been shown here and elsewhere that NGF-induced activation of Akt is not reduced when clathrin-mediated endocytosis is inhibited. In PC12 cells, inhibition of clathrin-mediated endocytosis actually increased the magnitude and duration of NGF-induced Akt phosphorylation, suggesting that endocytosis attenuates signaling to Akt (Zhang et al., 2000). Furthermore, Akt and pAkt were reported to be undetectable in NGF/pTrkA-containing signaling endosomes isolated from PC12 cells (Howe et al., 2001). In the present experiments, it was found that inhibition of PI3-kinase activity locally in distal axons reduced the survival-promoting effects of NGF beads but had little effect on survival mediated by free NGF, suggesting that survival signaling restricted to the axonal plasma membrane is more dependent on PI3-kinase-mediated signals. Also,

inhibition of TrkA phosphorylation in the cell bodies/proximal axons of neurons supported by distal NGF did not result in a significant reduction in the phosphorylation levels of Akt or CREB in this compartment, suggesting that the arrival of catalytically active TrkA in the cell bodies is not required to mediate the retrograde activation of these pro-survival signaling proteins.

These data are consistent with the hypothesis that Akt can be activated by PI3-kinase at the axonal plasma membrane, and then transported to the cell bodies independently of the retrograde transport of NGF/pTrkA-containing signaling endosomes. It is noteworthy that remarkably little evidence exists for Akt signaling from endosomes in any ligand/receptor system including NGF-stimulated PC12 cells (Ceresa et al., 1998; Di Fiore and De Camilli, 2001; Howe et al., 2001; Kao et al., 1998), and in contrast, Akt has been shown to disassociate from the plasma membrane and to translocate to the nucleus, after its activation (Ferrigno and Silver, 1999; Neri et al., 2002). Providing support that a mechanism for directed retrograde transport and nuclear-translocation of a signaling protein such as Akt may exist, retrograde transport from axons to cell bodies and subsequent nuclear translocation of activating transcription factor (ATF)-2 has been shown to occur in an NGF-dependent manner in nociceptive neurons (Delcroix et al., 1999). Similarly, the transcription facor NF-kappaB is redistributed from axons and dendrites to the nucleus in response to glutamatergic stimuli in hippocampal neurons (Wellmann et al., 2001). NF-kappaB has recently been shown to promote NGF-dependent survival of sympathetic neurons (Kramer et al., 1999; Maggirwar et al., 1998; Sarmiere and Freeman, 2001), raising the possibility that a similar retrograde transport process could occur in response to NGF. It is possible that pAkt and pCREB act in parallel, independent pathways of NGF-induced survival. However, evidence from other systems indicates that CREB is a direct target of Akt kinase activity in the nucleus (Du and Montminy, 1998; Pugazhenthi et al., 2000; Brami-Cherrier et al., 2002; Hayakawa et al., 2002; Perkinton et al., 2002), and therefore it is possible that the Akt- and CREB-dependent survival of sympathetic neurons reflects a single survival pathway.

However, the present data are not consistent with the hypothesis that retrograde transport of pAkt is the exclusive mechanism by which retrograde survival signals are

carried. A component of the survival mediated by NGF beads, and a somewhat greater proportion of the survival mediated by free NGF, was found to require the activity of PI3-kinase in the cell bodies/proximal axons. These results suggest that PI3-kinase activity in the cell bodies/proximal axons contributes to the transmission of retrograde signals from the axon, and/or reception of signals in the cell body. It is possible that active PI3-kinase and pAkt, are retrogradely transported as a component of a yet-unidentified signaling endosome that contains NGF/pTrkA complexes, but that the K252a-induced loss of TrkA phosphorylation at the endosome does not result in a significant loss PI3-kinase activity or associated Akt phosphorylation. Distal NGF stimulation may also induce activation of PI3-kinase that is resident in the cell body, which could in turn promote survival by activation of cell body-localized Akt. Although a mechanism by which such retrograde activation of PI3-kinase activity could occur is not known, it has been reported that insulin stimulation can induce rapid activation of endoplasmic reticulum-associated PI3-kinase in rat liver hepatocytes (Phung et al., 1997; Daniele et al., 1999), suggesting that PI3-kinase can be targeted to internal membranes in response to external ligand stimulation. It is important to consider that each of these mechanisms is conceivable, multiple mechanisms for retrograde signaling clearly exist, and it is reasonable to expect that they are not mutually exclusive.

Potential Roles for Multiple Mechanisms of Retrograde Signaling

Neurotrophins elicit a wide range of biological functions depending on cell type, cellular context, and stage of development. In peripheral neurons, NGF regulates multiple biological effects including survival, axon growth, target innervation and nociception (Farinas, 1999). As a single growth factor controls so many aspects of the survival and function of these neuronal populations, redundant mechanisms may be advantageous. Similarly, this principle could be extended to any neuronal population responding to a distal neurotrophin stimulus. There is clearly substantial evidence, including the findings presented in this thesis, that neurons posses the ability to transmit retrograde signals by means other than the canonical signaling endosome mechanism. Nonetheless, the existence of alternative mechanisms for retrograde signaling does not preclude the presence or potential importance of signaling endosomes: there is considerable

evidence that retrograde transport of signaling endosomes carrying NGF/pTrkA complexes is a bona fide mechanism by which retrograde signals are communicated. Under what circumstances distinct retrograde signaling mechanisms are operative is unknown. The maintenance of survival in the absence of retrograde transport of NGF or of retrograde TrkA kinase activity observed here was achieved in neurons with relatively short axons (approximately 7-10 mm), and such that the minimum distance a retrograde signal would have to travel was across the 1 mm barrier between the distal axon and cell bodies/proximal axon compartments. Thus, a plausible hypothesis is that retrograde signals operating under these conditions are effective over relatively shorter distances, whereas communication of longer range signals may require retrograde transport of NGF/pTrkA complexes. The distance that retrograde signals need to travel to elicit cell body responses varies extensively between neuronal populations and throughout development: from several millimeters in the developing embryo to one meter or more in adults of larger vertebrate species. Retrograde signaling mechanisms that are unique to relatively short axons may also function after axonal injury.

In addition to the possibility that distinct retrograde signaling mechanisms are operative over short distances, unique signaling mechanisms in relatively short axons may function to generate a signal proportional to the NGF available to the low axonal surface area. The extent of NGF uptake in any one axon branch is likely to be independent of the surface area of the entire axonal tree, however, the cell body receives transported NGF and associated signals accumulated from all branches, which likely depends on the total axonal surface area. Thus, when the axonal surface area is relatively small, such as during development or after injury, the decreased level of transported NGF and associated signals that accumulate in the cell body may be too low and thus it may be necessary for the axon to send an amplified signal to the cell body via distal activation of downstream second messengers. On the other hand, a signal mediated by the retrograde transport of NGF/pTrkA that is proportional to the NGF available to the axon may be more appropriate when axons have produced a highly branched array of terminals at the target cells which are competing with the terminals of other neurons for limited supplies of NGF (Campenot, 1994).

Axonal Degeneration is Mediated by Proteasome Activity

The identification of the naturally occurring *Wld*^{S} mutant mouse, in which Wallerian degeneration of the distal stump of transected axons is remarkably delayed, suggests that axonal degeneration is an active and regulated process (Lunn et al., 1989; Perry et al., 1991). However, the mechanism(s) by which localized axonal degradation is effected has received relatively little experimental attention. Experiments presented here suggest that the proteasomal protein degradation pathway is a critical effector of two mechanisms of selective axon degeneration: Wallerian degeneration of transected axons and degeneration due to local NGF withdrawal. Under these conditions, inhibition of the proteolytic activity of the 26S proteasome protected axons for at least 24 h *in vitro*, preserving fine axon morphology and maintaining axonal mitochondrial function. In contrast, untreated severed or NGF deprived axons were severely retracted and degenerated, displaying little sign of mitochondrial activity by this time. The presence of a local proteasome-mediated mechanism of axon degeneration in axons is interesting in view of the recent cloning and characterization of the *Wld*^{S} gene, which encodes a chimeric protein comprised of the N-terminal 70 amino acids of UbE4b, an enzyme involved in the polyubiquitinylation of proteins destined for proteasomal degradation, and the complete sequence of Nmnat, an enzyme involved in NAD^+ synthesis (Conforti et al., 2000; Fernando et al., 2002). It appears that the UbE4b component of the chimera is primarily responsible for the *Wld*^{S} phenotype, as transgenic expression of Nmnat fused to a truncated UbE4B sequence does not delay Wallerian degeneration (Coleman and Perry, 2002).

However, a connection between the proteasomal inhibition-mediated axonal protection observed here, and the molecular function of the *Wld*^{S} protein, is not immediately clear. Intriguingly, immunolocalization studies revealed that the *Wld*^{S} protein is predominantly nuclear (Mack et al., 2001). While *Wld*^{S} protein may be present in axons below the level of detection by this assay, its nuclear localization suggests that the mechanism by which it acts is indirect, possibly by regulating the abundance of a transcription factor that controls the expression of an unidentified protein that directly mediates axon protection. In contrast, inhibition of proteasomal protein degradation in axons directly protected them from degeneration, and 24 h treatment of cell

bodies/proximal axons of intact neurons with proteasome inhibitors prior to transection conferred no protective effect to the axons.

The protective effect of proteasomal inhibition on transected axons was almost completely prevented by coincident inhibition of MEK kinase activity. Correspondingly, phosphorylation of Erk1/2 was significantly decreased in untreated axons within 8 h of transection prior to detectable degradation of total Erk protein or morphological evidence of axonal degeneration. In contrast, Erk1/2 phosphorylation in proteasome inhibitor-treated transected axons persisted at similar levels to intact axons at 8 h after transection, and was still readily detectable 20 h after separation from the cell bodies. Importantly, axon degeneration was not due to a general degenerative effect of MEK inhibition, or a toxic effect of the compound itself, as inhibition of MEK in axons of intact neurons had no significant effect on their viability.

Taken together, these results suggest that Wallerian degeneration is induced by proteasome-mediated loss of axonal MEK/Erk pathway activity, highlighting again the critical role of this pathway in axon physiology. The level at which the MEK/Erk pathway is regulated by proteasomal degradation during Wallerian degeneration is unknown. However, because the protective effect of proteasome inhibition is reversed by MEK inhibition, the target of the proteasome is presumably upstream of MEK. The mechanism by which activation of the MEK/Erk pathway confers axonal protection is unknown. Persistent Erk1/2 activation could maintain morphological integrity by phosphorylation and stabilization of cytoskeletal proteins including Tau and neurofilament proteins (Roder et al., 1993; Garcia Rocha and Avila, 1995; Veeranna et al., 1998). Preservation of mitochondrial activity could also be directly coupled to Erk1/2 activity, as activated pp90Rsk, a direct target of Erk1/2 kinase activity, sequesters Bad in its inactive state in the cytoplasm, preventing its translocation to the mitochondria which induces loss of mitochondrial cytochrome c and leads to cell death (Brunet et al., 2001; Masters et al., 2001; Datta et al., 2002; Germain and Shore, 2003). The evidence presented here indicates that both of these mechanisms may be operative, as proteasome inhibition protected both the physical morphology and the mitochondrial metabolic function of transected axons.

Axon degeneration is emerging as a key target in the treatment of various neurodegenerative diseases for two important reasons: First, treatments that protect the neurons from apoptosis, such as caspase inhibition and overexpression of Bcl-2 (a well known inhibitor of caspase-dependent apoptosis), generally do not protect axons from degeneration (Dubois-Dauphin et al., 1994; Sagot et al., 1995; Burne et al., 1996), and will thus likely be of little clinical value in preserving neuronal function. In the present experiments, inhibition of axonal caspases had exerted no protective effect on transected or NGF-deprived axons. Second, in several neuropathological disorders including multiple sclerosis, AIDS, and diabetic neuropathy, axonal degeneration precedes apoptosis of the neuron, suggesting that axonal degeneration *per se* is the cause of neuronal death (Raff et al., 2002). Axons of *Wld*^{S} mice are protected from degeneration caused by various insults, including transection (Perry et al., 1991), NGF withdrawal (Deckwerth and Johnson, 1994) and vincristine toxicity (Wang et al., 2001), suggesting a common underlying mechanism executes axonal degeneration. Thus, understanding of the molecular regulation of Wallerian degeneration and degeneration due to NGF withdrawal is likely to be broadly applicable to the development of effective of clinical therapies for the treatment of axonal degeneration and thus neurodegenerative disease.

Closing Comments

Broadly, this thesis highlights the unique nature and added complexity of cell biological processes bestowed upon the neuron as a result of its morphological polarity. Although clearly they are intimately co-dependent, the cell body and its axons also function in many ways as distinct cellular entities. One of the key questions raised by the polarity of the neuron is how survival signals generated by the binding of neurotrophin at axon terminals are communicated to the nucleus in the cell body. The data presented in this thesis indicate that at least one mechanism for retrograde signaling by NGF in sympathetic neurons exists that is independent of the canonical signaling endosome mechanism. It is clear from the present data that retrograde signals that promote survival can be mediated without the retrograde transport of NGF. Furthermore, as retrograde inhibition of TrkA kinase activity by K252a did not inhibit retrograde pro-survival signaling or survival itself, the evidence here is consistent with the hypothesis that pro-

survival signaling can be mediated by pTrkA signals derived from the axonal plasma membrane, without the need for retrograde TrkA kinase activity in the cell body. Verification of the hypothesis that pTrkA can signal from the axonal plasma membrane to promote survival be facilitated by the development of techniques that specifically and definitively block the internalization of pTrkA at the axonal plasma membrane, without inhibiting downstream signaling that normally occurs from this location. Alternatively, methods that specifically block pTrkA-mediated signal transduction from signaling endosomes may prove to be equally valuable. Furthermore, development of the signaling endosome hypothesis will be dependent on the ability to isolate and characterize neurotrophin/pTrk-containing signaling endosomes from primary neuronal cultures, and preferably retrogradely transported endosomes from compartmented neuronal cultures, to overcome the inherent limitations of extrapolating data from the PC12 cell system. In addition, further characterization of the nature of pro-survival signaling by the PI3-kinase/Akt pathway will aid in clarifying the current understanding of retrograde signaling. Important issues in this regard include whether retrograde transport of activated PI3-kinase and/or Akt acts as a carrier of retrograde signals, whether retrograde transport of Akt is dependent on retrograde transport of signaling endosomes, and whether CREB is a target of Akt signaling.

The discovery that axonal degeneration is an intrinsically regulated process further illustrates the semi-autonomous nature of this region of the neuron, and opens an exciting new direction in the study of neuronal cell biology. The finding that Wallerian degeneration of transected axons is actively controlled by proteasome-mediated inhibition of the MEK/Erk pathway is among the first mechanistic characterizations of this process. It will now be important to determine whether this mechanism functions in other, more physiologically relevant forms of local axon degeneration, such as proteasome-mediated degeneration of NGF-deprived axons. As well, elucidation of the targets of Erk1/2 kinase activity that promote axonal protection will provide important mechanistic information.

The results presented here have broad implications in many aspects of neurobiology. Understanding the nature of the signaling pathways that promote neuronal survival and axon growth is fundamental to improving our knowledge of the basic mechanisms that control neuronal development. The findings presented here will

certainly generate new directions in the study of neurotrophic factor-mediated retrograde signaling, that until now may have been precluded by the overwhelming bias towards mechanisms that involve retrograde transport of neurotrophic factors. This knowledge may also lead to clinical advances in the treatment of neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis, and neurotraumatic injury, in which neuronal degeneration may occur in part because the affected neurons fail to obtain sufficient neurotrophic factor support to maintain survival. Furthermore, the recognition that axon degeneration is a regulated process, and the elucidation of mechanisms that control it, will allow for the new targets in the development therapies to treat neuropatholological disorders in which axonal degeneration is a causative elements of disease progression.

CHAPTER 5

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