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University of Alberta

Retrograde Signaling by Nerve Growth Factor in Cultured Sympathetic Neurons

(Spine Title: Retrograde Signaling by NGF)

by

Donna Lorraine Senger ©

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 and Anatomy

Edmonton, Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, "Retrograde Signaling by Nerve Growth Factor in Cultured Sympathetic Neurons" submitted by Donna Lorraine Senger in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Robert B. Campenot (Supervisor)

Dr. Ellen Shibuya

Dr. Jean Vance

Dr. Manijeh Pasdar

Dr. L. Reichardt (External Examiner)

To Dale

Thank you for giving me the

freedom to grow

ABSTRACT

This thesis examines the roles nerve growth factor (NGF), its tyrosine kinase receptor trkA, and protein tyrosine phosphorylation play in NGF-mediated retrograde signaling in rat sympathetic neurons. According to the current hypothesis of NGF signaling, NGF binds to its receptor(s) at the cell membrane, is internalized, and an endocytic vesicle with NGF in its lumen, bound to an activated receptor in its membrane, moves retrograde to the cell body where it initiates signaling cascades which reach the nucleus. This hypothesis would predict that the activation of signaling proteins at the cell body/nucleus would coincide with the retrograde appearance of NGF-containing vesicles. Data presented in this thesis show that the binding of NGF at the cell surface of distal axons of sympathetic neurons produces rapid tyrosine phosphorylation of several proteins retrograde to the site of NGF application, including proteins apparently localized to the cell bodies. Surprisingly, retrograde phosphorylation of a 140 kDa protein, identified as trkA, occurred within 1 minute of distal application. This rapid appearance of trk phosphorylation remarkably preceded the retrograde transport of ^{125}I-NGF-containing vesicles. Since the retrograde transport of trkA would move at a velocity similar to NGF, these data suggest that the appearance of phosphorylated trkA did not result from the arrival of trkA from the

distal axons. Rather, the presence of phosphorylated trkA in the cell bodies represents the phosphorylation of non-ligand bound trkA already present at the time of distal NGF binding. Therefore, in contrast to the vesicular model for retrograde signaling, these data support the hypothesis that NGF binding to the surface of the distal axons initiates a non-vesicular intracellular mechanism that results in the rapid phosphorylation of trkA and several other proteins proximal to the site of NGF binding.

To identify proteins involved in the propagation of an NGF-induced retrograde signal, experiments investigating the role of the MAPK isoforms, ERK-1 and ERK-2 were performed. Although ERK-1 and ERK-2 were activated locally at the site of NGF binding, no retrograde activation of ERK-1 and ERK-2 was seen. Instead, preliminary data suggest that ERK-1 and ERK-2 are important for local growth mechanisms within the distal axon.

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LIST OF ABBREVIATIONS

Akt	serine/threonine kinase
ApCAM	Aplysia cell adhesion molecule
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CRNF	cysteine-rich neurotrophic factor
CNS	central nervous system
CREB	cyclic adenosine monophosphate response element-binding protein
DRG	dorsal root ganglion
ERK	extracellular signal-regulated kinase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FRS2	fibroblast growth factor receptor substrate 2
$G_{i\alpha}$	α subunit of inhibitory G protein complex
$G_{z\alpha}$	α subunit of G_z protein complex
GRB2	growth factor receptor binding protein 2
IGF-1	insulin like growth factor
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEK	MAPK and ERK kinase
NGF	nerve growth factor
NT-3	neurotrophin 3
NT-4/5	neurotrophin 4
NT-6	neurotrophin 6
N-CAM	neuronal cell adhesion molecule
PBS	phosphate-buffered saline

PC12	pheochromocytoma cell 12
PI3K	phosphatidylinositide 3-kinase
PLC-γ1	phospholipase C-γ1
PNS	peripheral nervous system
PTB	phosphotyrosine-binding domain
Ras	guanine nucleotide-binding protein
SCG	superior cervical ganglion
SH2	Src-homology 2 domain
SHC	Src-homology 2 containing protein
SHP	SH2-containing tyrosine phosphatase
SNT	Suc1-associated neurotrophic factor target
SOS	guanine nucleotide exchange factor
TBS	Tris-buffered saline
TBST	Tris-buffered saline and 0.1% tween 20
trk	tyrosine kinase receptor A
TH	tyrosine hydroxylase

Chapter 1

BACKGROUND

Nerve Growth factor

Neurotrophic factors are important molecules for growth, differentiation and survival of neurons. Nerve growth factor (NGF) was the first discovered, and is the best characterized member of the neurotrophin gene family. This prototypic neurotrophin was discovered close to 50 years ago when embryological experiments based on the grafting of a mouse sarcoma tumor to the body of a chick embryo resulted in a substantial increase in the size of the dorsal root ganglion which innervated it (Bueker, 1948). After extensive and detailed examination of embryos bearing transplants, Rita Levi-Montalcini and Victor Hamburger demonstrated that in addition to the sensory ganglia there was an even larger increase in the size of the sympathetic ganglia (Levi-Montalcini and Hamburger, 1951). Further experimentation showed that implantation of the tumor onto the chorioallantoic membrane produced similar results as direct transplantation into the embryo. Since no direct contact occurred between the embryo and tumor it was hypothesized that a diffusible substance released by the tumor cells was responsible for the effects (Levi-Montalcini, 1952; Levi-Montalcini and Hamburger, 1953). Support for this observation was provided by *in vitro* studies where, in semi-solid medium, sensory and sympathetic ganglion were cultured with either sarcoma tissue (Levi-Montalcini et al., 1954) or tumor extract (Cohen et al., 1954).

Both tissue and extract provoked substantial neurite outgrowth from the ganglionic explants. Subsequent identification and isolation of NGF would have been very difficult due to the low abundance of NGF in target tissues, however purification of NGF was accelerated by the fortuitous discovery of high levels of NGF present in the male mouse submaxillary gland (Cohen, 1960; Levi-Montalcini and Cohen, 1960). Characterization of the salivary NGF revealed that NGF is composed of a 130 kDa multiunit molecule made from three different subunits (α, β and γ) with a molecular stochiometry of $\alpha 2\beta\gamma 2$ and a sedimentation coefficient of 7S. Only one of these subunits (β) contained the specific biological activity with the other subunits acting as regulators (Varon et al., 1967a,b, 1968). Further experimentation unveiled βNGF as a small dimeric molecule consisting of two identical 118 amino acid chains with each chain containing 3 disulfide bonds with a total molecular weight of 26.5 kDa (Angeletti and Bradshaw, 1971).

Tissue culture experiments over several years demonstrated the importance of NGF for the survival and maintenance of a specific population of neurons within the peripheral nervous system (PNS) which included peripheral neural crest-derived sensory and sympathetic neurons. The pattern of NGF specificity in the PNS was confirmed *in vivo* by neutralization of NGF by antibodies which led to the complete loss of sympathetic neurons and loss of a large percentage of the dorsal root ganglion neurons (Levi-Montalcini and Angeletti, 1968). These results were corroborated by the recent development of transgenic knockout mice lacking the gene for NGF. Mice homozygous for the null mutant showed complete loss of sympathetic neurons and a 70% loss of dorsal root ganglion neurons (Crowley et al., 1994). In addition to these

peripheral neurons, NGF is believed to play a similar role for basal forebrain cholinergic neurons of the central nervous system (CNS) (reviewed in Yuen et al., 1996). The strict specificity of NGF for select populations of neurons prompted speculation that there must be other molecules with similar function but distinct specificities. Not surprisingly this led to the discovery of several other proteins homologous to NGF. Along with NGF, these proteins comprise the neurotrophin gene family which includes; 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; Hallböök et al., 1991) and neurotrophin 6 (NT-6) (Götz et al., 1994).

NGF and its receptors

trkA tyrosine kinase receptor

NGF binds to two receptors on the neuronal surface, the trkA receptor tyrosine kinase and the p75 neurotrophin receptor. trkA (also called trk) was the first member of the trk gene family discovered and was initially isolated in its oncogenic form from a solid colon carcinoma (Pulciani et al., 1982). The trk oncogene was produced by somatic rearrangement that spliced a non-muscle tropomyosin to the transmembrane and intracellular domain of a receptor tyrosine kinase, hence the acronym trk meaning tropomyosin receptor kinase (Martín-Zánca et al., 1986). Isolation and cloning of the proto-oncogene revealed a 140 kDa transmembrane receptor tyrosine kinase that was expressed at high levels in NGF-responsive neurons (Martín-Zánca et

al., 1990; Holtzman et al., 1992). Several laboratories identified the 140 kDa membrane-spanning protein tyrosine kinase as a receptor for NGF (Hempstead et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991).

Since the discovery of trk, Barbacid and colleagues demonstrated the existence of two additional gene family members in mammals, trkB (Klein et al., 1989) and trkC (Lambelle et al., 1991). trkA is the only member of the gene family which binds NGF; BDNF and NT-4/5 bind trkB; and NT-3 binds trkC with highest affinity, although NT-3 will also bind trkA and trkB with lower affinity (Berkemeier et al., 1991; Cordon-Cardo et al., 1991; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991).

Activated trk receptors initiate intracellular signaling events that lead to the induction of gene expression and changes in neuronal morphology and function. Several lines of evidence demonstrate that activation of trkA is important for many of the typical neuronal responses to NGF (Loeb and Greene, 1993). Most of our knowledge of NGF signaling mechanisms comes from work with PC12 cells, a clonal cell line derived from adrenal tumors, which grow neurites in response to NGF, but do not require NGF for their survival (Greene and Tischler, 1976). PC12 cells lacking trkA (PC12nnr) showed a lack of response to NGF, while reintroduction of trkA into these cells restored their NGF responsiveness (Loeb et al., 1991). Also, treatment of both PC12 cells and sympathetic neurons *in vitro* with the Fab fragment of anti-RTA (a polyclonal antibody developed against the extracellular domain of trkA which blocks NGF binding to trkA) inhibited NGF-induced biological responses (Clary et al., 1994). In addition, transgenic mice carrying a null

mutation for trkA show marked abnormalities in the development of NGF-responsive neurons both in the PNS and CNS (Smeyne et al., 1994).

trk gene family members have several elements in common: they possess a large extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase catalytic domain (Pérez et al., 1995). Like many other receptor tyrosine kinases, upon binding to its appropriate ligand, receptor aggregation, and tyrosine auto or transphosphorylation occur which result in the activation of the receptor and subsequent phosphorylation of second messenger proteins (Jing et al., 1992; Clary et al., 1994). The trkA receptor is tyrosine phosphorylated on 9 tyrosine residues, 7 of these residues are contained within the kinase domain itself, while two additional residues flank the kinase domain. Important phosphorylation sites in the trk kinase region include: Y674 and Y675, which are required for the activation of the receptor (Segal et al., 1996). Phosphorylation of the receptor on other tyrosine residues provides new binding sites for a distinct set of proteins that initiate signaling cascades which exert the final biological effects. At present, two phosphotyrosine-specific binding domains have been well characterized. The first, SH2 (Src-homology 2) domain, consists of a 100 amino acid domain that has a high affinity for phosphorylated tyrosines present in certain amino acid sequences. Variations in SH2 domains, together with different amino acid sequences surrounding the phosphotyrosine provide specificity of binding to receptor tyrosine kinases (reviewed in Schlessinger, 1994). The second, PTB (phosphotyrosine binding) domain consists of a 100-150 amino acid sequence

which also recognizes phosphorylated tyrosine within specific peptide sequences (Kavanaugh et al., 1995).

The signaling cascade: trkA binding to signaling proteins

To date, NGF-activated trkA has been shown to associate with several cytosolic proteins which include: phospholipase C-γ1 (PLC-γ1) (Obermeier et al., 1994; Stephens et al., 1994), the adaptor proteins SHC and its neuronal specific isoform N-SHC (Nakamura et al., 1996), the phosphatidylinositol-3-kinase (PI3K) (Yao and Cooper, 1995; Nakamura et al., 1996), and the phosphotyrosine phosphatase SHP-1 (Vambutas et al., 1995) (Fig. 2). The best defined trk-induced signal transduction pathway is the Ras/MEK/MAPK pathway that has been shown to be activated via the SHC adaptor protein. Activated trk phosphorylates SHC on Y317 which provides a binding site for the adaptor protein GRB2. In the cytosol, GRB2 constitutively forms a complex with SOS (guanine nucleotide exchange factor for Ras) and recruitment of SOS by GRB2/SHC results in membrane relocation of SOS which is sufficient to induce Ras activation (Obermeier et al., 1994; Stephens et al., 1994). The importance of the Ras pathway in NGF-mediated differentiation in both PC12 and sympathetic neurons has been established by several different types of experiments. First, the introduction of neutralizing anti-Ras antibodies have been shown to block NGF-induced survival of sympathetic neurons (Nobes and Tolkovsky, 1995) and differentiation of PC12 cells (Hagag et al., 1986) by NGF. Second, expression of dominant-inhibitory Ras can inhibit NGF-induced neurite outgrowth (Hagag et al., 1986; Szeberenyi et al., 1990), and third, microinjection or expression of activated

Ras proteins induces neurite formation in PC12 cells (Bar-Sagi and Feramisco, 1985; Noda et al., 1985). Once activated, Ras initiates a signal that passes through a cascade of protein kinases enroute to the activation of the ERK family of serine/threonine kinases (also called MAPK), and subsequent phosphorylation of transcription factors which bring about numerous biological responses (reviewed in Heumann, 1994).

In addition to SHC, PLC-γ1 has also been shown to activate the Ras/MEK/MAPK pathway (Obermeier et al. 1994, Stephens et al., 1994). Since both PLC-γ1 and SHC activation converge on similar signal transduction pathways, it has been hypothesized that there may be redundancy in the signaling mechanisms used by trk. This observation is supported by experiments in which deletion of either the PLC-γ1 or SHC binding site was insufficient to inhibit PC12 cell differentiation, whereas deletion of both PLC-γ1 and SHC binding sites prevented NGF-promoted differentiation (Stephens et al., 1994). Whether these pathways exist as redundant signaling systems in neurons is yet to be determined. One might speculate that in the nervous system these pathways play different roles depending on either the type of neuron or the cellular localization of the receptor itself.

A third signaling pathway believed to activate the Ras pathway has recently been described. PC12nnr cells were transfected with trkA receptors containing a three amino acid deletion (KFG) in the juxtamembrane region. Upon exposure to NGF, these cells were unable to induce neuritogenesis or hypertrophy but were able to promote survival and prolonged activation of ERK-1 and ERK-2 seen in wild-type PC12 cells in response to NGF (Qui and Green, 1992; Peng et al., 1995). Closer examination determined that tyrosine

phosphorylation of SNT (suc-associated neurotrophic factor-induced tyrosine-phosphorylated target) was impaired (Rabin et al., 1993). SNT was identified as a 90 kDa nuclear protein that is rapidly phosphorylated on tyrosine in response to treatment with differentiation factors NGF and FGF but not by mitogens EGF or insulin (Rabin et al., 1993). The fact that the deletion of the KFG sequence could prevent neuritogenesis but not survival or prolonged ERK activation, provides evidence for the discrimination of survival and neurite outgrowth mechanism at the level of the receptor (Peng et al., 1995).

Recent data provides evidence for the existence of a second 90 kDa protein designated FRS2 (fibroblast growth factor receptor substrate 2). FRS2 has also been shown to be tyrosine phosphorylated in response to NGF and FGF but not EGF in PC12 cells (Kouhara et al., 1997). However, in contrast to SNT, activation of FRS2 resulted in the movement of FRS2 to the cell membrane where it was shown to associate with GRB2/SOS (Kouhara et al., 1997; Ong et al., 1996, 1997). In addition, overexpression of FRS2 has been shown to increase tyrosine phosphorylation of MAPK and the phosphorylation of the MAPK substrate MBP (myelin basic protein) (Kouhara et al., 1997). These results suggest that FRS2 may signal via the Ras/MEK/MAPK pathway and that FRS2 may mediate some of the more specific responses of NGF and FGF.

In addition to the activation of kinases, binding of NGF to trkA also activates a tyrosine phosphatase designated SHP-1 (SH2-containing protein tyrosine phosphatase 1). PC12 cells express abundant levels of both protein tyrosine phosphatases 1 and 2 (SHP-1 and SHP-2, respectively) (Vambutas et al., 1995). However, SHP-1, but not SHP-2, was shown to be

phosphorylated following NGF treatment, but not EGF (Vambutas et al., 1995). How SHP-1 becomes activated is not known. Although, NGF has been shown to activate the cytosolic tyrosine kinase Src in PC12 cells (Kremer et al., 1991) and *in vitro*, Src can phosphorylate SHP-1 (Matozaki et al., 1994), suggesting that SHP-1 may be a direct target of Src.

An additional signaling pathway, involving phosphatidylinositol-3-kinase (PI3K) has also been described and is believed to be involved in NGF-induced cell survival (Yao and Cooper, 1995) and neuritogenesis (Kimura et al., 1994). In PC12 cells, the PI3K pathway is activated through the SHC binding site (Baxter et al., 1995), and activation of PI3K initiates a signaling cascade that triggers the activation of the ser/thr kinase, Akt (Dudek et al., 1997; Franke et al., 1997). Activation of Akt has been shown to be important and necessary for IGF-1-induced survival of cerebellar neurons (Dudek et al., 1997). How essential the PI3K/Akt pathway is in neurotrophin-induced survival in neurons is unknown, albeit in PC12 cells, mutation of the SHC site does not abolish NGF-induced survival and neurite outgrowth (Obermeier et al., 1994; Stephens et al., 1994).

The issues regarding which signaling proteins are important for neuronal survival and growth are numerous, and an important first step is to identify the various signaling proteins that are involved. With the discovery of new pathways rapidly emerging, it is evident that the vast complexity underlying NGF-induced biological responses has only begun to be uncovered. A major focus of this thesis is to address the role of NGF-induced retrograde signaling in sympathetic neurons, specifically focusing on signals activated via

trkA, and in part, identify proteins activated either locally or retrograde to the site of NGF binding.

The role of p75

Although many NGF-induced signals occur via the activation of trk, the more abundant p75 neurotrophin receptor was the first NGF receptor isolated. cDNA for p75 was isolated from both PC12 cells (Radeke et al., 1987) and human A875 cells (Johnson et al., 1986). p75 was found to be a 75 kDa transmembrane protein containing a large extracellular domain, a single transmembrane domain and a small cysteine-rich, cytosolic domain. The cDNA encoded a protein with the molecular mass of 45 kDa that was shown to increase to 75 kDa upon glycosylation (Radeke et al., 1987). Originally isolated as an NGF receptor, p75 has subsequently been shown to bind all neurotrophins (Rodríguez-Tébar et al., 1990, 1992; Ernfors et al., 1990; Hallböök et al., 1991). Despite the fact that p75 binds to all neurotrophins, p75 retains the ability to recognize differences in the various neurotrophins (Ibáñez et al., 1990; Rodríguez-Tébar et al., 1990, 1992).

Even though p75 was the first NGF receptor detected, the discovery of the trk family of tyrosine kinases left p75 with a somewhat diminished role in neurotrophin signaling. However, recent studies have revitalized the search for a role for p75. Several lines of evidence provide strong support for p75 maintaining a role in modulating trkA activity. NGF induced trkA tyrosine phosphorylation and c-fos induction were shown to be reduced by both anti-p75 monoclonal antibody, MC192 (Barker and Shooter, 1994) and mutant NGF molecules that retain their ability to interact with trkA but no longer bind

p75 (Ibáñez et al., 1993). Also, competition of NGF/p75 binding with BDNF which only binds to the p75 receptor on PC12 cells, reduced the activation of trkA by NGF (Barker and Shooter, 1994). Furthermore, in sympathoadrenal cells (MAH cells) which only express trkA, NGF induced differentiation and neurite growth. However, if these cells co-expressed both trkA and p75 there was a substantial increase in tyrosine phosphorylation of trkA (Verdi et al., 1994).

In addition to modulating trkA activity, p75 has been implicated in a more direct role in signal transduction. The p75 receptor belongs to a structurally similar family of cytokine receptors that are characterized by a large extracellular domain containing several repeated cysteine clusters (reviewed in Chao, 1994). Despite the structural similarity of the extracellular domain, these receptors have extremely divergent intracellular domains that lack any known catalytic domains. In fact, the only obvious intracellular characteristic is a small domain referred to as the "death domain" (Boldin et al., 1995). Mutations in this domain abolish the activation of apoptosis by these receptors (Brakebusch et al., 1992). In addition to regulation of survival/apoptosis, other cellular responses of this protein family include activation of the transcription factor NFκB (Bothwell, 1996). The structural similarity between p75 and this family of proteins led to the proposal that p75 may also produce similar cellular responses.

The first clear evidence for p75 signaling came from the observation that NGF could activate the sphingomyelin cycle through p75, leading to increases in the lipid messenger ceramide in T9 glioma cells and p75 transfected NIH-3T3 cells (Dobrowsky et al., 1994, 1995). Furthermore, in

Schwann cells, p75 was shown to produce the activation and translocation of NFκB to the nucleus (Carter et al., 1996). In addition, selectivity of p75 signaling between neurotrophins was demonstrated when all neurotrophins activated ceramide turnover in p75 transfected NIH-3T3 cells (Dobrowsky et al., 1995), but only NGF was shown to activate NFκB in Schwann cells (Carter et al., 1996).

While p75 has been linked with apoptosis and cell death in some cellular contexts (Rabizadeh et al., 1993), this would not seem to be a prominent feature. Exogenous NGF has been shown to rescue peripheral NGF-responsive sensory and sympathetic neurons during the period of naturally occurring cell death (Lewin and Barde, 1996). Also, activation of p75 in Schwann cells shows that NGF can activate NFκB without leading to cell death (Carter et al., 1996). Furthermore, Dobrowsky et al., (1995) have shown an inhibitory effect of trkA on p75-mediated ceramide production. On the other hand, both very early retinal cells and mature oligodendrocytes are susceptible to NGF/p75-induced death (Casaccia-Bonnefil et al., 1996; Frade et al., 1996). These data suggest that p75 plays very different roles in response to NGF depending on its molecular environment and that NGF itself mediates both cell survival and cell death during normal neuronal development.

Clues to the role of p75 in cell signaling may best be examined in cells outside the nervous system. *In situ* hybridization shows a widespread expression of p75 in several non-neuronal tissues which do not have full length trk receptors, these include such cells as myoblasts and Schwann cells (Anton et al., 1994; Cartier et al., 1996). At present it is unclear what role p75 plays in the biology of these cells. However the widespread distribution of p75 *in*

vivo has lead to speculation that other naturally-occuring ligands for p75 may yet need to be found. Discovery of a novel non-neurotrophin related ligand, CRNF (cysteine-rich neurotrophic factor), for p75 (Fainzilber et al., 1996) supports the hypothesis that other ligands for p75 unrelated to the neurotrophins exist.

The results in this thesis do not directly address the role of p75 in NGF signaling, but all data must be interpreted with the understanding that p75 does play a role in the development of both sensory and sympathetic neurons. This is best illustrated in p75 knockout mice where both sympathetic and sensory neurons show decreased target innervation in addition to a shift in the dose response curve to lower sensitivities (Lee et al., 1994a,b; Davies et al., 1993). Furthermore, in sympathetic neurons, the ratio of trkA and p75 may be important for NGF signaling since p75 and trkA appear to be independently regulated. mRNA for p75 is dramatically upregulated in sympathetic neurons while trkA mRNA remains unchanged (Ma et al., 1992; Toma et al., 1997). In addition, increases in NGF concentration do not effect trkA protein expression (Belliveau et al., 1997; Toma et al., 1997). Moreover, the upregulation of p75 mRNA is paralleled by a similar increase in p75 protein (Appendix 1). Therefore, data presented in this thesis are interpreted with the awareness that cellular ratio and localization of p75 and trkA in sympathetic neurons may be critical for NGF signaling.

To date, PC12 cells have been by far the most popular tumor cells used to study the mode of action of NGF. Although PC12 cells have proven to be an invaluable tool, the physiological relevance of the observations made in PC12 cells is somewhat obscure and their relationship to *in vivo* or in other

physiological models *in vitro* is not entirely clear. On the other hand, PC12 cells provide sufficient amounts of homogenous material for biochemical analysis. The use of neuronal cultures has been technically and fiscally demanding, however, with the recent development of more sensitive detection methods, utilization of neuronal systems in biochemical analysis has become more approachable. Data presented in this thesis use a compartmented culture system (Campenot, 1992) which permits environmental control and biochemical analysis of the nerve terminals of sympathetic neurons independent of their cell bodies, hence providing a physiologically relevant *in vitro* model that parallels the *in vivo* situation.

Several important features of NGF signaling have been revealed using the compartmented culture system. Previous experiments demonstrated that: first, NGF supplied to the distal axons of sympathetic neurons is sufficient to maintain the viability of the neurons in a retrograde manner (Campenot, 1977, 1982b); second, NGF localized to the distal axons was shown to promote neurite growth and regeneration by mechanisms localized in the distal axons (Campenot 1977, 1982a, 1987; Campenot et al., 1994), with axon growth reaching a maximum of about 1 mm/day at low NGF concentrations and higher concentrations of NGF producing increased axon density (Campenot, 1994); and third, distally applied NGF produces a retrograde signal that regulates the level of mRNA for the p75 neurotrophin receptor and tyrosine hydroxylase (Toma et al., 1997). In the present thesis, compartmented cultures have been used to study NGF-induced signals that occur both locally at the site of NGF application (i.e. distal axons/ axon

terminals) and retrograde towards the cell body (i.e. cell bodies/proximal axons).

Sympathetic Neurons

Neurons contained in the sympathetic chain including the superior cervical ganglia (SCG) are typically dependent on NGF for survival and respond in a similar fashion to NGF (Levi-Montalcini and Cohen, 1960). Sympathetic neurons from postnatal day 1 SCG were shown to provide an easily obtainable and ideal representative model system for studying the mechanisms of NGF (Chun and Patterson, 1977). Sympathetic neurons depend on NGF for growth and survival throughout much of development and postnatal life (Levi-Montalcini and Angeletti, 1968). *In vivo* injections of NGF caused decreased naturally occuring cell death in postnatal superior cervical ganglia (Hendry and Campbell, 1976) and increased size and number of neurons in the sympathetic chain of chick embryos (Oppenheim, 1991). At the same time, anti-NGF injection in late embryogenesis or during neonatal development produced cell death in up to 99% of the neurons in the SCG and sympathetic chain (Cohen, 1960; Levi-Montalcini and Booker, 1960; reviewed in Levi-Montalcini and Angeletti, 1968). While sympathetic neurons are dependent on NGF late in development, survival of sympathetic neurons is initially independent of NGF (Leah and Kidson, 1983; Coughlin and Collins, 1985; Ernsberger et al., 1989). Anti-NGF treatment of sympathetic neurons from mouse embryo SCG (E7-E13) has little effect on sympathetic development (Kessler and Black, 1980). In fact mouse sympathetic neurons do not become dependent on NGF for survival until E16 (Coughlin and Collins, 1985).

Lack of NGF-responsiveness early in sympathetic neuron development implies the possible involvement of other neurotrophins. Recently, evidence supporting a role for NT-3 in sympathetic development was provided from NT-3 knockout mice that were shown to possess 50% fewer sympathetic neurons (Ernfors et al., 1994; Farinas et al., 1994) together with deficits in sympathetic target innervation (El-Shamy et al., 1996). Furthermore, during development, sympathetic neurons express high affinity binding sites for NT-3 (Dechant et al., 1993) and trkC mRNA (Lamballe and Barbacid, 1993). In addition, sympathetic neurons express both BDNF and NT-3 mRNAs (Schecterson and Bothwell, 1992) raising the possibility for autocrine/paracrine interactions.

INTRODUCTION

Retrograde signaling by trophic factors

Since the discovery of nerve growth factor (NGF) in the late 1940s, significant emphasis has centered on understanding the mechanisms used by target-derived growth factors to promote survival of their innervating neurons. Remarkably, after 50 years, the mechanisms underlying trophic-factor-induced retrograde signaling are still not known. A major focus of this thesis was to investigate the mechanisms used by NGF to relay signals from the nerve terminal to the cell body. According to the neurotrophin hypothesis, interactions between a developing PNS neuron and its target play an important role in neuronal competition and cell death. This is best demonstrated in the developing sympathetic nervous system where these neurons are absolutely dependent on NGF once they have innervated their target. In its basic form, the neurotrophin hypothesis predicts that the target tissue produces a trophic factor that can generate a signal in the innervating neuron for the selective control of neuronal cell death during development (Oppenhiem, 1991). Thus, the binding of NGF to its receptor(s) at the nerve terminal is believed to regulate the neuronal input density partly by eliminating neurons that have failed to obtain adequate target territory (Deckwerth and Johnson, 1993a,b). Originally proposed for the PNS, the neurotrophin concept has been extended to neurons of the CNS where it has been shown that target neurons synthesize trophic factors for their afferent neurons (Ernfors et al., 1990).

Because the neurotrophin hypothesis predicts that competition for limiting amounts of NGF during target innervation determines the amount of sympathetic neuronal survival, a signal generated at the nerve terminal must be communicated back to the cell body. Support for this hypothesis came from experiments where radioiodinated NGF injected into the anterior chamber of the eye was found to be taken up by the nerve terminals and retrogradely transported to the cell bodies in the SCG (Hendry et al., 1974; Johnson et al., 1978). Initially it was believed that the transport of NGF itself was responsible for the retrograde signal, but early on it was shown that injection of NGF into the cytosol of a cell was insufficient to elicit a biological response (Heumann et al., 1981; Rohrer et al., 1982). Therefore, if the retrograde transport of NGF were important for retrograde signaling, it must be in complex with other molecules. At present, two prominent hypothesizes have been proposed to explain how signal transduction pathways convey a retrograde signal from the nerve terminal, through the axon to the cell body. The first hypothesis predicts that the transmission of a retrograde signal occurs via the movement of an endocytic vesicle, carrying an activated NGF/receptor(s) complex, which travels to the cell body where it initiates signaling cascades at the nucleus. The second hypothesis theorizes that after a ligand binds to its receptor, the activated receptor initiates a signal that results in the activation of a retrograde messenger, such as a downstream kinase or second messenger molecule, that is transported to the cell body/nucleus where it exerts its biological effect.

Vesicular model for retrograde signaling

Most investigators have favored the view that NGF binds to its receptor(s) at the plasma membrane, is internalized and retrogradely transported to the cell soma where an activated NGF/receptor(s) complex acts on signaling mechanisms in proximity to the nucleus. In addition to signals generated once the vesicle reaches the cell body, catalytically active receptors would be able to activate second messengers as the vesicle moves through the axon. Support for this hypothesis comes from a diverse group of experiments: 1) NGF was shown to be retrogradely transported when ^{125}I-NGF injected into the anterior chamber of the eye was found to be taken up by the axon terminals and transported to the cell somas in the SCG (Hendry et al., 1974; Johnson et al., 1978). In addition, the retrogradely transported ^{125}I-NGF was found to be localized in vesicles, cisternae confined to smooth membranes and lysosomal structures (Thoenen and Barde, 1980). 2) Endogenous NGF produced by target tissues of sympathetic ganglion was retrogradely transported and accumulated in high levels in the SCG (Korsching and Thoenen, 1983a,b; Palmatier et al., 1984). 3) Injection of NGF into the eye of a neonatal rat resulted in massive hypertrophy only in neurons which were shown to transport NGF (Hendry, 1977). 4) *In vivo*, increased activity of the enzyme ornithine decarboxylase (Hendry and Bonhady, 1980) and tyrosine hydroxylase (Hendry 1977; Paravicini et al., 1975) in response to distal NGF coincide with the arrival of NGF at the cell body. 5) Block of retrograde transport of NGF by colchicine (Claude et al., 1982) results in a marked decrease in the level of tyrosine hydroxylase induced by distal NGF (Paravicini et al., 1975). 6) NGF is transported intact to the cell body where it

accumulates prior to degradation (Ure and Campenot, 1997). 7) In addition to NGF, the p75 neurotrophin receptor (Johnson et al., 1987) and trkA (Ehlers et al., 1995) have also been shown to be retrogradely transported. 8) In PC12 cells, binding of NGF to trkA on the cell surface results in the internalization of trkA into endosomes where the trkA has been shown to remain active (Grimes et al., 1996). One prediction of this model for retrograde signaling is that the rate of the retrograde signal will coincide with the arrival of the retrogradely transported NGF.

Evidence that transported signaling molecules produce a retrograde signal

Some neurotrophic factors can exert biological effects on neurons when applied to axon terminals without being retrogradely transported such as acidic and basic fibroblast growth factor (aFGF, bFGF) (Ferguson et al., 1990; Hendry and Belford, 1991). These molecules therefore, require alternative signaling mechanisms that can generate a signal from the nerve terminal to the cell soma in the absence of retrograde transport of the factor itself. It has been hypothesized that binding of neurotrophic factors to their plasma membrane receptor(s) results in the generation of a stable transportable second messenger that moves retrograde to the cell body/nucleus. Initial support for this hypothesis comes from work using 3T3 fibroblasts where stimulation by EGF was shown to induce intracellular translocation of the α subunit of the second messenger protein G_i ($G_{i\alpha}$) from the plasma membrane to the nuclear chromatin (Crouch, 1991). Subsequent experiments using mouse sciatic nerve have shown that $G_{i\alpha}$ (Hendry and Crouch, 1991) and $G_{z\alpha}$ (Hendry, 1992) accumulate within 4 hours on both the

proximal and distal side of a ligated nerve. These data suggest that the transport of $G_{i\alpha}$ and $G_{z\alpha}$ occurs both anterogradely and retrogradely. However, whether or not retrograde transport of $G_{i\alpha}$ and $G_{z\alpha}$ is important for retrograde signaling is not clear.

Similarly, evidence exists that neurotrophins acting through tyrosine kinase receptors may also generate stable second messengers that can be transported back to the cell body. Immunohistochemistry of mouse sciatic nerves, after double nerve crush, showed no phosphotyrosine immunoreactivity 3 hours after nerve crush. However, by 6 hours there was an increase in phosphotyrosine immunoreactivity on the distal side of the nerve crush only (Johanson et al., 1995). In addition, a number of proteins including PI3K, ERK MEK, and MEK kinase, were shown to accumulate on both the proximal and distal sides of the ligations (Johanson et al., 1995). On average, accumulation of these proteins occurred at a later timepoint (18 h), 12 hours after the increase in phosphotyrosine immunoreactivity. The accumulation of phosphotyrosine distal to the ligations suggests that axonal transport of tyrosine kinases and/or tyrosine phosphorylated proteins occurs in a retrograde direction. However, no direct evidence was given to support the identity of the phosphorylated proteins. Taken together, these data suggest that several second messenger molecules have the ability to move within the axon, but whether they are involved in transmission of a signal from the nerve terminal to the ceil body is yet to be determined.

Both models for retrograde signaling predict that the arrival of a transported molecule or complex would coincide with the appearance of the retrograde signaling. Based on this premise, one should be able to test these

hypotheses by comparing the rate of transport with the arrival of a retrograde signal. In the present thesis, experiments were performed to test whether the appearance of tyrosine phosphorylated proteins in the cell bodies of sympathetic neurons is consistent with the appearance of the ^{125}I-NGF that induced them. The compartmented culture system (Campenot, 1992) provided an ideal model system to address this question. The design of the compartmented culture allows manipulation of the microenvironment of the nerve terminal independent of the cell body, hence mimicking the *in vivo* situation. In this approach, sympathetic neurons dissected from the superior cervical ganglia (SCG) of newborn rats are plated in center compartments of a three compartmented chamber (Fig 1) and their neurites extend across the center compartment and enter into the right and left distal compartments. In this way, a stimulus such as NGF, that initiates a retrograde signal, will be supplied to the side compartments containing the distal axons/terminals. The cell bodies/proximal axons are then isolated separately from the distal axons and analyzed. In the experiments reported in this thesis, compartmented cultures were used to apply NGF locally to the distal axons of sympathetic neurons and to observe the appearance of tyrosine phosphorylated proteins in the cell bodies/proximal axons. In addition, these cultures provided a system in which the movement of ^{125}I-NGF-containing vesicles could be study in detail.

Since one model for retrograde signaling proposes that NGF signals from the plasma membrane to the nucleus following internalization and retrograde transport of an NGF/receptor(s) complex to the cell soma, the rate of internalization, loading on to the transport system and transport of

NGF are all important components of any signal. In sympathetic neurons, NGF is retrogradely transported at a rate of 10-20 mm/hr (Ure and Campenot, 1997). At this rate, NGF can move across a 1 mm barrier (Fig 1) in 3 to 6 minutes. However, this rate does not include the time that is required for internalization and loading of NGF on to the transport system. Previous experiments in sympathetic neurons have shown that following binding of ^{125}I-NGF to its receptors on the plasma membrane there is a substantial lag (of approximately 1 hr) prior to the movement of NGF molecules retrogradely toward the cell body (Ure and Campenot, 1997). Therefore, according to the vesicular hypothesis, the appearance of an NGF-induced retrograde signal in the cell bodies/proximal axons of sympathetic neurons in compartmented cultures should not occur within the first hour after NGF binding to the distal axons.

Surprisingly, data presented in this thesis show that several phosphorylated proteins are seen in the cell bodies/proximal axons of sympathetic neurons within 15 minutes of distal NGF application. In addition, retrograde phosphorylation of trkA in the cell bodies/proximal axons occurred within 1 minute. This data is not consistent with the vesicular hypothesis. Moreover, the appearance of phosphorylated trkA is too rapid to be accounted for by the movement of any molecule. Therefore, this data supports a new model for NGF-induced retrograde signaling. In contrast to the present retrograde signaling models, data presented here suggest that the transport of molecules within the neuron is not required to generate a signal from the nerve terminal to the cell body. Instead, this data supports the hypothesis that binding of NGF to the distal axons of sympathetic neurons

initiates an intracellular signal that propagates along the axon to the cell body at a rate that precludes the mass transport of any molecules. Moreover, additional experiments suggest that the propagation of this signal does not appear to involve the activation of ERK-1 and ERK-2 within the cell bodies and proximal axons. Therefore, the binding of NGF to the distal axons of sympathetic neurons initiates the propagation of a signal that travels via a non-vesicular intracellular mechanism that appears to activate a distinct set of second messenger proteins. Whether these proteins are specific for the propagation of a retrograde signals or are a subset of proteins activated at the site of NGF binding is yet to be determined. Furthermore, these data in no way rule out the possibility that NGF-induced signals are carried by the retrograde transport of NGF/receptor(s) or other signaling molecules. However, they do suggest that some retrograde signals may travel via an alternative mechanism to retrograde transport. Knowledge of the underlying mechanisms mediated by NGF will provide critical information for the development of therapies to promote neuronal survival and regeneration after injury or disease. In addition, information obtained for NGF-induced signaling in sympathetic neurons can serve as a model for many other types of neurons and trophic factors.

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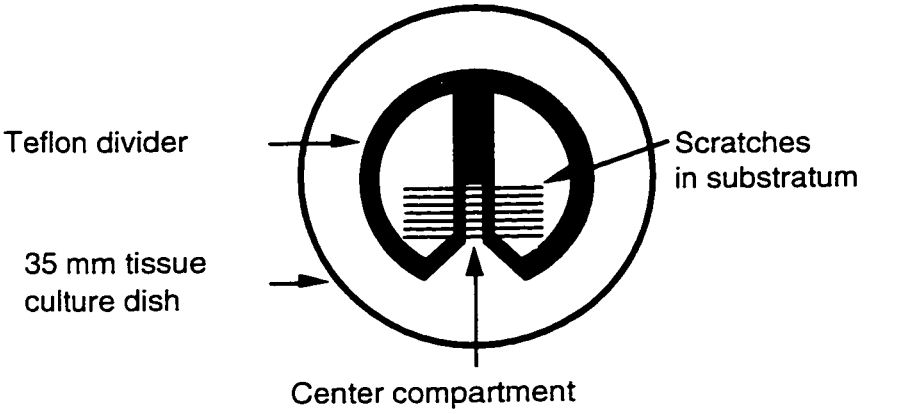
Figure 1. Compartmented culture system.

a is a schematic of an entire compartmented culture showing a 35 mm plastic tissue culture dish which has a series of parallel scratches made in the collagen coating the floor of the dish extending under a Teflon divider that has been applied to the dish with silicone.

b is an enlargement of a single track between two scratches. Each track is about 200 μM wide, the center compartment is 1.5 mm wide and the barriers between the center compartment and distal compartments are about 1.0 mm wide. One culture can contain up to 20 tracks occupied by neurons.

a. Compartmented culture



b. Enlargement of a single track

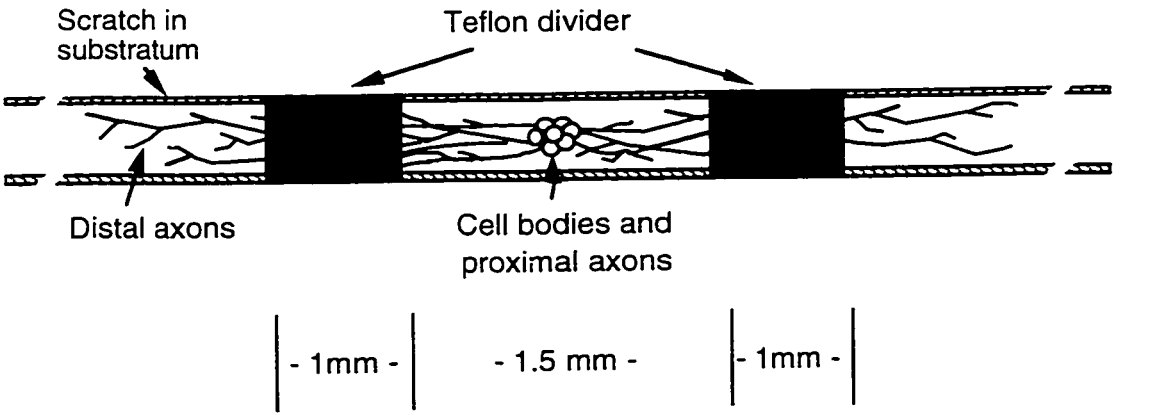
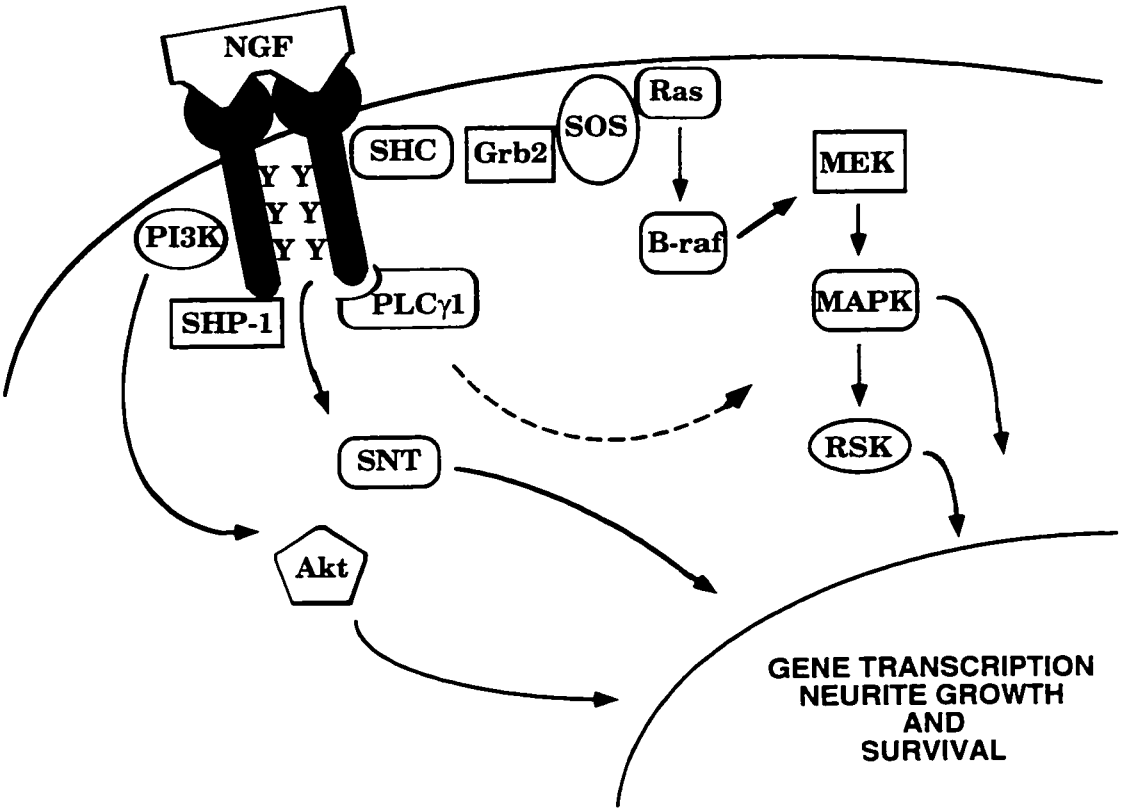


Figure 2. Summary of NGF induced intracellular signaling via the trk tyrosine kinase receptor.

In response to NGF binding, activated trkA associates with several cytosolic proteins which include: phospholipase C-γ1 (PLC-γ1), the adaptor protein SHC, phosphatidylinositol-3-kinase (PI3K), and the phosphotyrosine phosphatase SHP-1. SHC has been shown to bind to the adaptor protein GRB2. In the cytosol, GRB2 constitutively forms a complex with the Ras GTP exchange factor, SOS. Recruitment of SOS to the plasma membrane is sufficient to induce Ras activation. Activation of Ras leads to the phosphorylation and activation of the dual specific kinase MEK via an intermediate MEK kinase designated B-raf. Activated MEK then phosphorylates and activates MAPK (also known as extracellular regulated kinase or ERK). The MAPKs are believed to be central regulators of signal transduction that activate and phosphorylate a number of signaling enzymes, which include ribosomal S6 kinase (RSK), as well as several transcription factors. A third transducer of the Ras signal leads from the activation of the phospholipase PLC-γ1.

Two trkA-dependent, Ras-independent pathways have also been described. The first pathway involves the activation of PI3K, a lipid protein kinase which is believed to be involved in NGF-induced cell survival. Activation of PI3K initiates a signaling cascade that triggers the activation of the ser/thr kinase Akt. The second pathway initiates the activation of SNT, a nuclear protein believed to regulate genes that control withdrawl from the cell cycle.


NGF
Y Y
Y Y
Y Y
SHC
Grb2
SOS
Ras
MEK
B-raf
PI3K
SHP-1
PLCγ1
MAPK
SNT
RSK
Akt
GENE TRANSCRIPTION
NEURITE GROWTH
AND
SURVIVAL

Chapter 2

Rapid retrograde tyrosine phosphorylation of trkA and other proteins in rat sympathetic neurons in compartmented cultures

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INTRODUCTION

Nerve growth factor (NGF), the best characterized neurotrophin, elicits differentiation, survival, and neurite growth in sympathetic neurons. Many NGF effects are mediated by binding of NGF to the receptor tyrosine kinase, trkA (trk) (Loeb et al., 1991; Loeb and Greene, 1993; Ibáñez et al., 1992), which induces rapid tyrosine autophosphorylation of trk and subsequent tyrosine phosphorylations of several second messenger proteins (Kaplan et al., 1991a,b; Klein et al., 1991; Jing et al., 1992). Activation of these proteins by tyrosine phosphorylation is believed to play an important role in mediating biological responses of neurons to NGF.

Evidence indicates that the immediate neurite-growth-promoting action of NGF involves mechanisms at or near the site of NGF binding to the nerve terminals (Campenot 1977; 1982; 1987; and Campenot et al., 1994). This suggests that trk phosphorylation leads to the activation of second messenger systems in the growth cones that directly couple to local growth mechanisms. In contrast, biological effects of NGF such as promotion of cell survival (Levi-Montalcini, 1976; Levi-Montalcini, 1987) and changes in gene expression (Mathew and Miller, 1990; Miller et al., 1991; Ma et al., 1992; Wyatt and Davies, 1995; Toma et al., 1997) involve retrograde signals that travel from the nerve terminals to the cell body and nucleus.

NGF is retrogradely transported along axons of sympathetic neurons and neural-crest-derived sensory neurons (Hendry et al., 1974a; Stöckel et al., 1975; Claude et al., 1982; Korsching and Thoenen, 1983; Palmatier et al., 1984). These observations support a favorite model for retrograde signaling:

NGF binds to and activates trk receptors on the nerve terminals and is internalized by receptor-mediated endocytosis. Then, the endocytotic vesicles carrying trk in their membranes, activated by NGF in their lumens, are retrogradely transported to the cell body. Once in the cell body, activated trk phosphorylates second messenger proteins which transmit signals to the nucleus resulting in altered gene expression (see reviews by Korshing, 1993 and Campenot, 1994). Recent evidence that phosphorylated trk is transported in the axonal retrograde transport system supports this theory of retrograde signaling by NGF (Ehlers et al., 1995; Grimes et al., 1996).

Discovering the mechanisms of retrograde signaling in NGF-responsive neurons is of vital importance, serving as a model for many other types of neurons and trophic factors. This information is indispensable for understanding neural development and will help in efforts to devise ways to promote neuronal survival and repair after disease or injury. The compartmented culture model is an ideal means to investigate the mechanisms of retrograde signaling. In compartmented cultures the cell bodies and proximal neurites reside in center compartments while distal neurites extend into left and right distal compartments. We used these cultures to apply NGF locally to distal neurites and observe the appearance of NGF-induced tyrosine phosphorylations and the arrival of ^{125}I-NGF in the cell bodies/proximal neurites. Our results indicate that NGF binding to distal neurites induces the tyrosine phosphorylation of trk and other proteins in the cell bodies/proximal neurites long before the NGF is internalized and delivered by retrograde transport. While our results do not rule out the possibility that some NGF-induced retrograde signals could be carried by retrograde NGF

transport, retrograde transport cannot be the only mechanism. Rather, our results suggest that at least some retrograde NGF signals are carried by a propagation mechanism.

MATERIALS AND METHODS

Culture Procedures

Superior cervical ganglia were dissected from newborn rats (Sprague-Dawleys supplied by the University of Alberta Farm) as previously described (Campenot et al., 1991), and subjected to trypsin and mechanical dissociation and plated into collagen-coated culture dishes. For mass cultures neurons were plated into 24-well Linbro tissue culture dishes at a density of 1 ganglion/well. Neurons were plated into compartmented cultures as previously described (Campenot et al., 1994). For most experiments compartmented cultures were maintained for 2 weeks after plating with NGF supplied in all compartments at 10 ng/ml. Figure 1 shows a single track from a culture raised under these conditions and retrogradely labeled overnight with the lipophyllic, fluorescent dye, FM-143 (Molecular Probes, Eugene OR).

Culture Media

L15 medium without antibiotics (Gibco Laboratories, Grand Island, NY) was supplemented with 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) was supplied in medium given to mass neuronal cultures and medium given to the center compartments of the compartmented cultures containing the cell bodies. Culture medium was routinely changed every 3-7 days. Non-neuronal cells were virtually eliminated by supplying 10 mM cytosine arabinoside in the mass neuronal cultures and center compartments of compartmented cultures during the first 6 days. Mass cultures were grown in

medium containing 200 ng/ml NGF for 10 - 14 days prior to experimental treatment. Compartmented cultures were grown in 10 ng/ml NGF in all three compartments for 10 - 14 days.

Experimental Treatments

The experimental variable in these experiments consisted of various concentrations of NGF, K-252a and anti-NGF. NGF (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) stock was 20 mg/ml in phosphate buffered saline. The standard NGF concentration used in cultures ranged from 10 ng/ml to 200 ng/ml. K-252a (Kamiya Biomedical Co., Thousand Oaks, CA) was prepared as a 2 mM stock in DMSO (dimethyl sulfoxide) and stored at 4°C. The 2 mM stock was diluted to 500 nM in culture medium. The DMSO concentration with 500 nM K-252a was 0.025%. Anti-NGF affinity purified sheep IgG (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) was used at a final concentration of 24 nM.

Protein Tyrosine Phosphorylation

Following experimental treatment cultures were washed twice with ice-cold Tris-buffered saline (TBS), and cell extracts from both the cell body/proximal axon compartments and the distal axon compartments were collected separately into sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, 0.001% bromophenol blue). Extracts were run on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose (hyper-bond, Amersham, Oakville, ON, Canada) using a Hoeffer semi-dry transfer unit and immunoblotted using anti-phosphotyrosine

4G10 (UBI, Lake Placid, NY). Immunoreactivity was determined using enhanced chemiluminescence (ECL; Amersham). Data were quantified using a LKB Ultroscan XL laser densitometer.

Immunoprecipitation

Following treatment cultures were washed with ice-cold TBS and solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet p-40, 10% glycerol, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 5 ug/ml leupeptin, 5 ug/ml aprotinin). Extracts from mass neuronal cultures were pooled in microcentrifuge tubes, homogenized using a micro-homogenizer pestle (Mandel Scientific company Ltd.) and centrifuged in an Eppendorf microcentrifuge for 30 seconds to remove cell debris. Extracts were then normalized for total protein by Bicinchoninic acid protein determination kit (Sigma, St. Louis, MO) and immunoprecipitated with a polyclonal antibody against trk (anti-trk 203B provided by David Kaplan, Montreal Neurological Institute, Montreal, Canada). Immunoprecipitates were then run on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, immunoblotted using anti-phosphotyrosine 4G10, and detected using ECL as described above. Immunoprecipitations for trk in compartmented cultures were performed on extracts from the cell body/proximal axon compartment of 14-18 cultures. Data was quantified using a LKB Ultroscan XL laser densitometer.

Equalization of Sample Loading

Attempts to detect trk by Western blotting with anti-trk 203B were unsuccessful, presumably because compartmented cultures, which contain about 1,500 neurons per dish, do not provide sufficient trk protein for detection. Therefore, it was not feasible for us to reprobe our anti-phosphotyrosine blots to verify equal amounts of trk between control and experimental groups. Therefore, to equalize sample loading we always used an equal number of sister cultures for control and experimental groups which had been treated identically from the initial day of plating. We have evidence that using equal culture numbers is effective since our previous observations showed that activation of trk by global application of 200 ng/ml NGF followed by immunoprecipitation with anti-trk and immunoblot with anti-phosphctyrosine reproducibly detected equal amounts of trk protein under a variety of experimental conditions (Toma et al., 1997). In the present study, this was confirmed in 4 replicate experiments in which equal aliquots of extracts from control and NGF-treatment groups taken prior to trk immunoprecipitation were immunoblotted with anti-α-tubulin and anti-ERK. Immunoblots showed that control and experimental groups had equal amounts of tubulin and ERK, while retrograde tyrosine phosphorylation of trk was increased after 1 min of NGF exposure at the distal axons. Moreover, the NGF-induced retrograde tyrosine phosphorylation was highly repeatable, replicated 7 times for the retrograde increase in trk phosphorylation at 1 minute and 12 times for the retrograde increase in p140 phosphorylation at 10 minutes after distal NGF application.

Immunoblotting of tubulin and ERK

In some experiments following treatment aliquots of cell extracts were collected and analyzed on immunoblots using either 1 ug/ml monoclonal α-tubulin (Clone DM 1A; Sigma) or 0.1 ug/ml polyclonal ERK antibody (691; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The ERK antibody detects both p44 and p42 ERKs. Except for the use of these antibodies the procedure was the same as previously described above for protein tyrosine phosphorylation.

Radioiodination of NGF and Retrograde Transport Assay

Radioiodination of NGF and retrograde transport assays were performed as previously described by Ure and Campenot (1997). All transport assays were performed on compartmented cultures of sympathetic neurons grown for 10 - 14 days in 10 ng/ml NGF in all compartments. ^{125}I-NGF was applied at a concentration of 40 x 10^6 cpm/ml (200 ng/ml) to distal axon compartments for times ranging from 1 minute to 24 hours. Radioactivity present in both the center compartment medium and the cell bodies/proximal axon extracts was quantified using a Wallac 1470 gamma counter.

RESULTS

Concentration Dependence and Timecourse of trk Activation by NGF in Mass Cultures

Before attempting to investigate retrograde tyrosine phosphorylations in compartmented cultures, the NGF-induced trk phosphorylation was characterized in mass cultures of rat sympathetic neurons. Cultures initially grown for two weeks in medium supplied with 200 ng/ml NGF were given NGF-free media for 2-4 hours and then given medium containing NGF at concentrations ranging from 10 - 200 ng/ml. Cell extracts were collected, samples containing equal amounts of protein were immunoprecipitated with anti-trk and analyzed for tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine (see Materials and Methods). Tyrosine phosphorylation of trk increased substantially with concentrations of NGF ranging from 10 - 100 ng/ml (Fig. 2a). This contrasts with PC12 cells which show maximal trk autophosphorylation at 10 ng/ml NGF (Kaplan et al., 1991b). We used 200 ng/ml NGF in all experiments to ensure maximal activation of trk.

To assess the time course of NGF-induced trk phosphorylation, cultures grown in 200 ng/ml NGF were given NGF-free medium for two hours and then given 200 ng/ml NGF for various times. trk tyrosine phosphorylation was detected within 5 minutes and persisted for at least 24 hours (Fig. 2b) consistent with a role for trk autophosphorylation in mediating long-term, not just transient, signals.

Local and Retrograde NGF-induced Protein Tyrosine Phosphorylations

In order to analyze the tyrosine phosphorylations that occur both locally and retrograde to the binding of NGF, we utilized three-compartmented cultures. Neurons were plated in center compartments, their axons extended under silicone grease barriers, and entered into separate distal axon compartments (Campenot 1992). Using this system, distal axons could be locally exposed to increased NGF, and protein tyrosine phosphorylation could be measured in cell extracts separately obtained from distal axons and from cell bodies/proximal axons. Initially, neurons were grown in 10 ng/ml NGF in all compartments for two weeks (Fig. 1). Then 200 ng/ml NGF was given either only in the distal compartments or in all compartments for 10 minutes. Control cultures received the same changes of medium, but with NGF maintained at 10 ng/ml in all compartments. Each group consisted of 3 cultures, extracts of which were analyzed by anti-phosphotyrosine immunoblot. Ten minute exposure to distal NGF produced tyrosine phosphorylations locally in the distal neurites as well as retrograde phosphorylations of proteins in the cell bodies/proximal neurites not directly exposed to increased NGF (Fig. 3b). Proteins displaying a retrograde tyrosine phosphorylation included a band at 140 kD, the apparent molecular weight of trk, and several other proteins with apparent molecular weights ranging from 30-190 kD (indicated by arrows). The pattern of tyrosine-phosphorylated proteins in the cell bodies/proximal axons was similar whether NGF was given only to the distal axons or globally to the entire surface of the neurons (Fig. 3c). The cell bodies/proximal axons displayed 5 tyrosine-phosphorylated bands that were not present in the distal axons (apparent molecular weights

30, 36, 38, 50 and 55 kD, indicated by asterisks). Their relative absence from the distal compartments containing axons alone suggests that these phosphorylated proteins are localized to the neuronal cell bodies.

This experiment was repeated several times and the retrograde tyrosine phosphorylation of p140 and of the cell-body-localized bands, p38, p36, and p30, was quantified by densitometry (Fig. 6). Application of 200 ng/ml NGF to distal axons for 10 minutes produced a 3-4.5-fold increase in the tyrosine phosphorylation density of these proteins.

To determine to what extent the phosphorylation and activation of trk was involved in the retrograde tyrosine phosphorylation of these proteins, trk autophosphorylation was blocked by application of K-252a (Berg et al., 1992; Ohmichi et al., 1992; Tapley et al., 1992). Two groups of cultures given 200 ng/ml NGF in distal compartments were also treated with 500 nM K-252a either only in the center compartments containing cell bodies and proximal axons (Fig. 3d) or in all compartments (Fig. 3e). K-252a was given 30 minutes prior to distal application of NGF. When present in all compartments, K-252a blocked the NGF-induced tyrosine phosphorylations of all proteins. K-252a given only to the cell bodies/proximal axons selectively blocked retrograde phosphorylations without any apparent effect on the NGF-induced tyrosine phosphorylations in the distal axons.

Since we observed retrograde tyrosine phosphorylations 10 minutes after exposure of distal axons to NGF, we conducted an experiment to define the timecourse of tyrosine phosphorylations from 1 minute (the shortest time practical in our system) to 30 minutes after NGF was given to distal axons. Neurons grown for two weeks with 10 ng/ml NGF in all compartments were

supplied with either 10 or 200 ng/ml NGF on their distal axons. Local tyrosine phosphorylation of the 140 kD protein in distal axons occurred within 1 minute of distal NGF treatment, reaching a maximum by 5 minutes and was maintained for 30 minutes (Fig. 4a). Phosphorylations of proteins at 65, 70, 85 and 180 kD in distal axons also occurred within one minute of distal NGF application. Tyrosine phosphorylation of two additional proteins at 42 and 44 kD occurred within 5 minutes. Tyrosine phosphorylation of all proteins observed in the distal axons was maintained for 30 minutes.

Retrograde tyrosine phosphorylation of the 140 kD protein appeared within 1 minute of application of NGF to the distal axons followed by several other proteins within 10 (Fig. 4b, filled arrows) and 15 minutes (open arrows) with increasing phosphorylation throughout the 30 minutes of observation. In addition to phosphorylation, dephosphorylation of some proteins occurred within 5 to 10 minutes (asterisks). Similar results were obtained in experiments in which 24 nM of anti-NGF was present in the center compartments to ensure that the retrograde phosphorylation did not result from direct exposure to extracellular NGF (Fig. 4c).

Retrograde transport of ^{125}I-NGF

To determine the relationship between the appearance of retrograde tyrosine phosphorylations and the appearance of retrogradely transported NGF, the retrograde transport of ^{125}I-NGF was measured in experiments similar to the retrograde tyrosine phosphorylation experiments. Compartmented cultures were grown with 10 ng/ml NGF in all compartments for 10 -14 days, then given 200 ng/ml ^{125}I-NGF in distal compartments for

times ranging from 1 minute to 24 hours. Extracts of cell bodies/proximal axons from the center compartments and the medium bathing them were collected and assayed separately. Previous results have shown that the ^{125}I accumulated in the cell bodies/proximal axons represents intact NGF, and the ^{125}I in the medium represents low-molecular weight breakdown products released into the medium after breakdown of transported NGF (Ure and Campenot, 1994, 1997). We observed no retrogradely transported ^{125}I-NGF within 10 minutes, and little, if any, during the first hour (Fig. 5). After 1 hour the retrograde transport of ^{125}I-NGF greatly increased. The transport was specific since a 100-fold excess of unlabeled NGF reduced the accumulation of ^{125}I-NGF in cell bodies and proximal axons at 24 hours by 95%.

Rapid Retrograde Tyrosine Phosphorylation of trk

We performed experiments to confirm that the 140 kD protein that is phosphorylated retrograde within 1 minute of distal application of NGF is trk. Cultures grown under two different NGF regimes were used. In some experiments the cultures were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments, and after 1 week NGF was withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments. In other experiments cultures were grown with 10 ng/ml NGF in all compartments for the two weeks. This variation in initial conditions had no effect on subsequent results.

In all experiments cultures were given NGF-free medium for two hours prior to experimental treatment. Following the incubation with NGF-free medium, distal axons of control cultures were treated for 1 minute with

medium containing 0 ng/ml NGF, and distal axons of experimental cultures were treated for 1 minute with medium containing 200 ng/ml NGF. At the end of the 1 minute incubation, the cell bodies/proximal axons were immediately lysed in immunoprecipitation buffer. In each experiment lysates were harvested from equal numbers of control cultures and NGF-treated cultures. The number of cultures per treatment group ranged from 14-18 among experiments. Immunoprecipitates were prepared from the cell bodies/proximal axons lysates, and immunoblotted with anti-phosphotyrosine.

Fig. 7 shows representative results for the 7 experiments that were performed. In every case, increased trk phosphorylation was observed in cell bodies/proximal axons within one minute of application of NGF to distal axons. Figure 7C is representative of 4 experiments in which equal aliquots of extracts from control and NGF-treatment groups were removed prior to trk immunoprecipitation and immunoblotted with anti-α-tubulin (7C(b)) and anti-ERK (7C(c)). These blots confirmed that equal amounts of cellular material were harvested from control and NGF-treated groups. Tyrosine phosphorylation density scans were obtained for 6 of the experiments and revealed that 1 minute application of NGF to distal axons resulted in a 2.4-fold increase in trk phosphorylation density in the cell bodies/proximal axons which was highly significant ($P< 0.004$) (Fig. 6). These results clearly indicate that tyrosine phosphorylated trk appeared in the cell bodies/proximal axons within one minute of distal application of NGF.

DISCUSSION

We utilized compartmented cultures of sympathetic neurons to investigate the appearance of retrograde tyrosine phosphorylations in cell bodies/proximal axons in response to an increase in NGF supplied to distal axons. In this way we began to address the mechanisms of retrograde signaling by neurotrophic factors. Since the discovery that NGF is taken up by nerve terminals and retrogradely transported to cell bodies (Hendry et al., 1974a; Stöckel et al., 1975; Claude et al., 1982; Korsching and Thoenen, 1983; Palmatier et al., 1984) it has been theorized that NGF is, itself, involved in carrying retrograde signals. The current version of the NGF transport hypothesis is that NGF binds to trk receptors on the nerve terminals and is internalized by receptor-mediated endocytosis. Then, vesicles with NGF in their lumens, activating trk in their membranes, travel retrograde along the microtubule-based transport system to the cell body where activated trk initiates signaling cascades which carry the signals into the nucleus. The NGF transport hypothesis is supported by recent evidence: In PC12 cells NGF stimulates the internalization of trk into endosomes in which trk remains activated (Grimes et al., 1996), and phosphorylated trk is retrogradely transported in sciatic nerve (Ehlers et al., 1995).

According to the above hypothesis, in the present experiments in which NGF is applied only to distal axons, the phosphorylated trk appearing in the cell bodies/proximal axons should represent the retrograde transport of activated trk bound to NGF from the distal axons. We observed that 200 ng/ml NGF applied to distal axons induced the retrograde appearance of

phosphorylated trk within 1 minute (Fig. 7). The retrograde phosphorylations of several other proteins were detected within 5-15 minutes after distal NGF administration (Fig. 4). These included several bands not observed in the distal axons (e.g. 30, 36, 38, 50 and 55 kD). Since distal compartments contain axons and the center compartments contain axons, cell bodies, and dendrites, we conclude that these bands likely represent cell body-associated proteins localized to the cell bodies and/or dendrites but absent or in low abundance in axons. This suggests that these retrograde phosphorylations reach the cell bodies. Moreover, since the proximal axons appear to be a very small fraction of material relative to the cell bodies (Fig. 1), it is unlikely that phosphorylations in the proximal axons alone would be detectable. This suggests that all of the retrograde phosphorylations that we observed are occurring in the cell bodies.

In contrast to retrograde tyrosine phosphorylation, retrogradely transported ^{125}I-NGF was not detected for at least 30-60 minutes after application of 200 ng/ml ^{125}I-NGF to distal axons (Fig. 5). These data are consistent with previous evidence in compartmented cultures of sympathetic neurons indicating a 1 hour lag between binding of ^{125}I-NGF to distal axons and internalization and loading of NGF onto the retrograde transport system (Ure and Campenot, 1997). These data suggest that the retrograde tyrosine phosphorylations that we observed at 1-15 minutes preceded the retrograde transport of the NGF that induced them.

A recent study has shown that unprimed PC12 cells treated with NGF at 4°C internalized about 37% of their surface trk receptors within 10 minutes and about 66% within 20 minutes of rewarming to 37°C (Grimes et

al., 1996). This is faster internalization than observed in distal axons of sympathetic neurons (Ure and Campenot, 1997). However, many differences in NGF responses exist between sympathetic neurons and PC12 cells. Therefore, it is quite possible that PC12 cells, especially cells which have not grown neurites or differentiated other neuronal properties, may internalize NGF into their cell bodies with different kinetics than distal sympathetic axons.

Our conclusion that NGF-induced retrograde tyrosine phosphorylations precede the arrival of retrogradely transported NGF rests upon the assumption that retrograde tyrosine phosphorylations of trk and other proteins could not have arisen by NGF diffusion across the barrier from the distal compartments and activating surface trk receptors on the cell bodies/proximal axons. Two observations rule out this possibility: Analysis of the retrograde transport of ^{125}I-NGF shown in Fig. 5 indicates that if we assume that all NGF appearing in the center compartment medium and cell extracts had resulted from diffusion, it would have produced NGF concentrations of only 2.4 pg/ml at 1 minute and 28 pg/ml at 60 minutes. These would represent minuscule increases, especially considering that in most experiments the cell bodies and proximal axons were exposed to 10 ng/ml NGF, and these increases would amount to 0.024% and 0.28% respectively. Also, retrograde tyrosine phosphorylations were observed in experiments in which 24 nM anti-NGF was present in the center compartments to block any direct action of NGF (Fig. 4).

Fast retrograde tyrosine phosphorylations could not have arisen from contamination with distal axon lysates because: Several of the retrograde phosphorylations were cell-body-localized proteins not observed in distal

axons. When the tyrosine kinase inhibitor, K-252a, was applied to cell bodies/proximal axons, it blocked the retrograde phosphorylations without affecting tyrosine phosphorylations in the distal compartments (Fig. 3). The effectiveness of our harvesting procedures has been verified by experiments in which the neurons were completely labeled with the fluorescent dye, FM143. The axons under the barrier remained after the axons in the distal compartments and the cell bodies and proximal axons in the center compartments had been harvested with either immunoprecipitation (IP) buffer or SDS sample buffer. Thus, the center compartment cell extracts are not contaminated with distal axon material.

The fact that the axons under the barrier remain after harvesting indicates that the barrier is sealed its entire length of approximately 1 mm. Our previous observations estimate the velocity of retrograde transport in compartmented cultures at 10-20 mm/h (Ure and Campenot, 1997). This is significantly higher than the 2-3 mm/h velocity of NGF retrograde transport reported for adult rat sympathetic axons *in vivo* (Hendry et al., 1974a,b; Johnson et al., 1978), but is within the reasonable biological range since sensory neurons *in vivo* have been reported to transport NGF at 7-13 mm/h (Stöckel et al., 1975; Yip and Johnson, 1986), and sympathetic axons of the sciatic nerve have been reported to transport dopamine β-hydroxylase at 12 mm/h (Brimijoin and Helland, 1976). Using our figures, it would require 3-6 minutes for NGF that binds to receptors on distal axons just outside the barrier (see Fig. 1) to cross the barrier between compartments and reach the proximal axons just inside the barrier. The distal axonal material extends many mm from the barrier (Fig. 1), therefore, most NGF-trk receptor

complexes that give rise to the trk phosphorylations in distal axons would have to travel much farther than 1 mm to reach the center compartment and be detected as retrograde phosphorylations on our Western blots. Thus, it is extremely doubtful that the tiny fraction of trk receptors on distal axons immediately adjacent to the barrier could alone produce a measurable retrograde trk phosphorylation within 1 minute even if they were internalized and transported to the center compartment within this time. These considerations rule out the possibility that a fast wave of NGF transport undetected in our ^{125}I-NGF studies could have produced the retrograde trk phosphorylation observed at 1 minute.

We conclude that the appearance of tyrosine phosphorylated trk receptors in proximal compartments within 1 minute of NGF application occurred before the arrival of activated trk receptors from the distal axons. Instead we propose that this represents the phosphorylation of trk already present in the cell bodies/proximal axons at the time of distal NGF application. Since other retrogradely-transported molecules are likely to travel at a similar velocity as NGF, we further believe that the extreme speed of this response precludes the mass transport of any molecular species. Rather, our results suggest that NGF binding to receptors on the surfaces of distal axons initiates a propagated signal resulting in the rapid tyrosine phosphorylation of trk proximal to the site of NGF binding to receptors on the axon surface.

Our results imply that the trk molecules phosphorylated retrograde of NGF application were not bound to NGF, but were phosphorylated by an intracellular mechanism which, in effect, bypassed the ligand binding step. Cell bodies and proximal axons have trk receptors on their surfaces which

respond with tyrosine autophosphorylation when increased NGF is applied directly to them (Toma et al., 1997). Since the cell bodies/proximal axons in the present experiments were not exposed to increased extracellular NGF, most of the surface trk would not be bound to NGF. Presumably there are also intracellular organelles containing trk not bound to NGF. Any or all could be substrates for tyrosine phosphorylation by a propagated signal.

Activation of receptor tyrosine kinases without ligand binding has precedents: Increased activation of trk in PC12 cells can be produced by overexpression of trk (Hempstead et al., 1992) or treatment with the ganglioside, GM1 (Ferrari et al., 1995; Mutoh et al., 1995). Rosen and Greenberg (1996) showed that Ca^{+2} influx can produce tyrosine phosphorylation of the EGF receptor in the absence of EGF. Although, Ca^{+2} influx through voltage-gated Ca^{+2} channels in PC12 cells did not result in tyrosine phosphorylation of trk, the PC12 cells had no prior exposure to NGF and had not developed neuron-like properties. Therefore, when considering possible mechanisms, it would be premature to rule out Ca^{+2} as playing a role in propagating the NGF-induced retrograde phosphorylation of trk. Other speculative possibilities could include phosphorylations that are self propagated and may travel through the axon toward the cell body, or self-propagated inhibition of phosphotyrosine phosphatases.

The proteins displaying retrograde NGF-induced tyrosine phosphorylations included several low molecular weight bands not observed in distal axons, i.e. 30, 36, 38, 50 and 55 kD. Interestingly, Cabrera et al., (1996) have shown that PC12 cells given NGF and other treatments generate a 41 kD fragment of trk from which the extracellular domain has been

cleaved. The truncated trk displays increased kinase activity and autophosphorylation compared to intact trk, and they suggest that generation of phosphorylated, truncated trk may be part of the NGF signal transduction mechanism in PC12 cells. Also, Zhou et al., (1995) have found a 38 kD tyrosine-phosphorylated protein in PC12 cells, that appears on anti-phosphotyrosine blots in response to NGF treatment and may represent a fragment of the intracellular domain of trk.

These previous observations raise the possibility that the tyrosine-phosphorylated proteins that we observed in the cell bodies in response to distal NGF may include truncated forms of trk containing the cytosolic, but not the extracellular, domain. Since we did not observe these proteins in the distal axons, it is unlikely that they are directly involved in propagating the retrograde signal. Rather, it seems that truncated forms of the cytosolic domain of NGF, if present, would be more likely part of the transduction mechanism that receives the retrograde signal after it reaches the cell body and carries it to the nucleus.

Aithough the appearance of the tyrosine phosphorylation of trk in Fig. 4 suggests a biphasic response of trk phosphorylation, this was not supported by densitometric analysis of all results which indicated that retrograde trk tyrosine phosphorylation nearly doubled between 1 minute and 10 minutes after application of NGF to distal axons (Fig. 6). However, it would be premature to rule out a biphasic response, especially since observations in PC12 cells overexpressing trk indicate that individual tyrosines are differentially phosphorylated by NGF with maximal phosphorylation of Y674

and Y675 preceding phosphorylation of the Y490 SHC binding site (Segal et al., 1996).

A question also arises as to why the propagated signal that we observed initially involved the tyrosine phosphorylation of trk at 1 minute and only later involved the other proteins. This may not be the case. Since trk has 5 tyrosine phosphorylation sites (Kaplan and Stephens, 1994) it is possible that retrograde tyrosine phosphorylations of other proteins with fewer sites were present but below detection at 1 minute after NGF administration. In fact, in a few experiments we did observe increased tyrosine phosphorylation at 1 minute of several other proteins (Fig. 4c).

It has been hypothesized that prolonged activation of trk and downstream second messengers by NGF may be one of the deciding factors between induction of a proliferative pathway by growth factors such as EGF and initiation of a differentiation pathway by NGF (reviewed in Chao, 1992). A prolonged activation of trk would also be needed to mediate the long-term promotion of neuronal survival and other trophic effects of NGF. Our mass culture experiments showed that an increase in NGF produced a rapid and prolonged increase in tyrosine phosphorylation of trk that lasted at least 24 hours. In addition, we have presented data showing that trk receptors respond to increases in NGF over a broad concentration range (10 - 100 ng/ml; 0.4 nM - 3.9 nM) (also see Belliveau et al., 1997).

These results contrast results with wild-type PC12 cells where trk tyrosine phosphorylation was maximal at 10 ng/ml NGF (Kaplan et al., 1991b) and returned to basal levels after only 2 hours of NGF exposure (Hempstead et al., 1992; Kaplan et al., 1991b). However, overexpression of

trk in PC12 cells produced a sustained activation of trk (Hempstead et al., 1992), similar to our observations in sympathetic neurons. Thus, the machinery for a sustained trk phosphorylation response to NGF is present in PC12 cells and likely reflects the mechanisms operative in sympathetic neurons.

The fact that sympathetic neurons respond to a broad range of NGF concentrations would enhance their ability to sense changes in the availability of NGF in the *in vivo* environment. While NGF has been measured in target tissues, the results have been, and remain, controversial (Zettler et al., 1996). Thus, the levels of NGF in the target tissues of sympathetic neurons are not established. Even if they were established, it is likely that extracellular NGF is not uniformly distributed in the target cell environment, e.g. it is possible that NGF release sites could be localized near nerve terminals and expose them to a much higher NGF concentration than the target tissue average. In this regard, it is relevant that glutamate released by hippocampal neurons reaches concentrations as high as 1.1 mM in the synaptic cleft (Clements et al., 1992). Thus, although we used a high NGF concentration of 200 ng/ml NGF in our experiments to saturate the NGF receptors on the distal axons, it cannot be concluded a priori that this concentration is beyond the biological range. In fact, previous experiments investigating the induction of mRNAs for Tα1 α-tubulin, tyrosine hydroxylase, and p75 neurotrophin receptor in sympathetic neurons in mass culture show that gene expression increases over the range of 10-200 ng/ml NGF (Ma et al., 1992). Moreover, experiments also showed that the retrograde induction of gene expression when 200 ng/ml NGF was only applied locally to distal axons was not a

maximal response since substantial additional increases were observed when 200 ng/ml NGF was also applied to the cell bodies and proximal axons (Toma et al., 1997). Thus, the concentrations used in the present experiments were in the biologically effective range, and application of 200 ng/ml NGF to distal axons did not saturate the ability of the neurons to respond to NGF.

While our results indicate that retrograde signaling by NGF must include other mechanisms besides the retrograde transport of NGF-containing vesicles, they by no means rule out that signals are also carried by NGF-containing vesicles. In fact, previous results indicate that NGF is not degraded during retrograde transport and accumulates in the neuronal cell bodies where it resides with a half-life of about 3 hours which is consistent with a retrograde signaling role (Ure and Campenot, 1997). On the other hand, under steady state transport conditions, only a small fraction of the axon-bound NGF was delivered to the cell bodies each hour, and a far greater fraction of the neuron-associated NGF was bound to distal axons than was present in the cell bodies (Ure and Campenot, 1997). This is consistent with recent *in vivo* observations suggesting that a large fraction of NGF in target tissues may be associated with sympathetic axons (Zettler et al., 1996). NGF bound to axonal receptors undoubtedly has other functions besides retrograde signaling, e.g. the activation of local signaling pathways that regulate neurite growth (reviewed in Campenot, 1994) and presumably regulate other local functions of the axon. However, the present results raise the possibility that a major function of NGF bound to axonal trk receptors is to give rise to intracellular signals that reach the cell body by mechanisms not involving NGF transport.

In conclusion, we have presented evidence that application of NGF to distal axons of rat sympathetic neurons in compartmented cultures results in the appearance of tyrosine phosphorylated trk and other proteins in the cell bodies before the arrival of the NGF that induced them. These data do not support the concept of retrograde transport of NGF and associated signaling molecules as the only mechanism of retrograde signaling along axons. Our data suggest, rather, that binding of NGF to trk receptors on nerve terminals generates intracellular tyrosine phosphorylations of trk and other proteins by a rapid propagation mechanism. Our results have broad implications for the mechanisms of retrograde signaling by all neurotrophic factors, raising the possibility that many kinds of retrograde signals may reach the neuronal cell bodies without the retrograde transport of signaling molecules.

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FIGURES

Figure 1. Sympathetic neurons in compartmented cultures

Photomicrographs show a single track in a compartmented culture of sympathetic neurons raised for 14 days with 10 ng/ml NGF in all compartments. The culture was labeled overnight with the lipophyllic fluorescent dye, FM-143 (4 μM), added to the left and right compartments. Labeling of cell bodies and proximal axons occurred by retrograde transport from labeled distal axons. The letters on photomicrographs are keyed to the schematic diagram. Panel b shows a cluster of cell bodies in the center compartment. Fine bundles of proximal axons connect the cell bodies to the neurites under the barrier which tend to hug the scratches. Upon emerging into the distal compartments the neurites spread out to cover the collagen track and extend many mm into the left (panel a) and right (panel c) compartments.

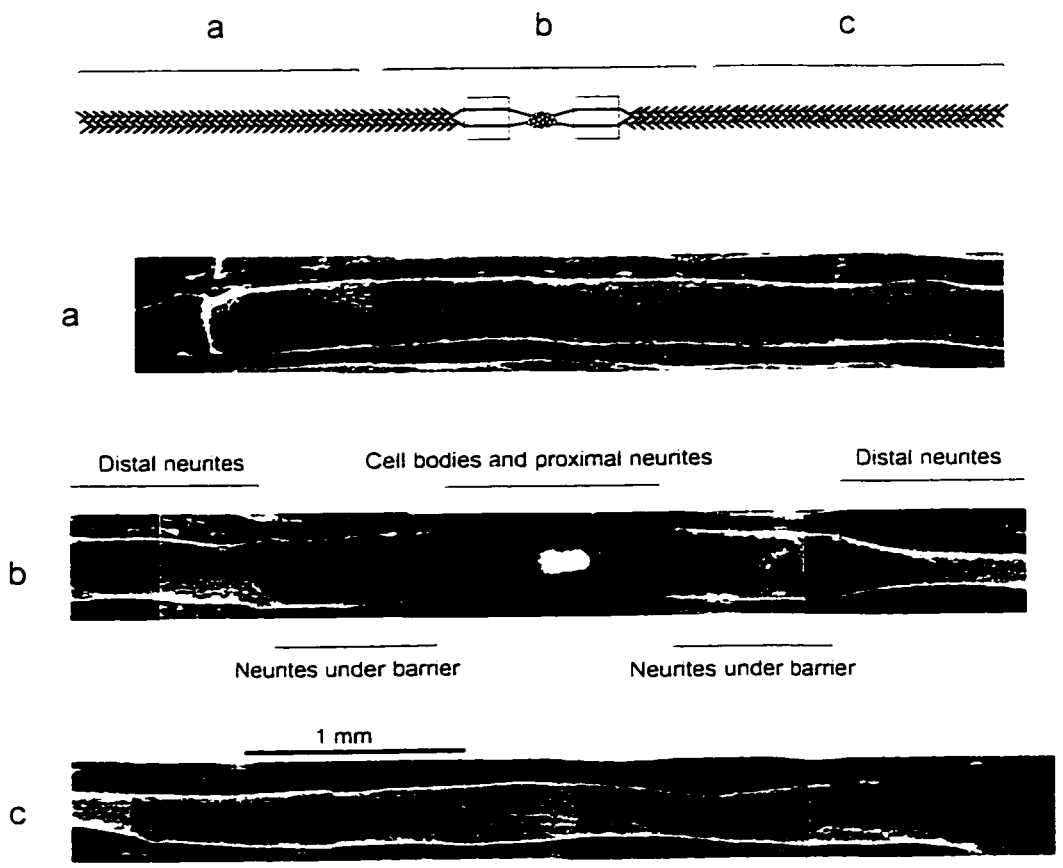

a
b
c
a
Distal neurites
Cell bodies and proximal neurites
Distal neurites
b
Neurites under barrier
Neurites under barrier
1 mm
c

Figure 2. NGF-dependent tyrosine phosphorylation of trk in mass cultures

Cultures of rat sympathetic neurons grown in 200 ng/ml NGF were given NGF-free media for 2-4 hours prior to experimental treatment. In each experiment samples containing equal amounts of protein were immunoprecipitated using anti-trk and immunoblotted using anti-phosphotyrosine. Molecular weight markers in kilodaltons are indicated on the left of each blot. a) Dose-response of trk tyrosine phosphorylation. Cultures were given varying concentrations of NGF (0-200 ng/ml) for 10 minutes. b) Timecourse of trk tyrosine phosphorylation. Cultures were given 200 ng/ml NGF for times ranging from 0 to 24 hours.

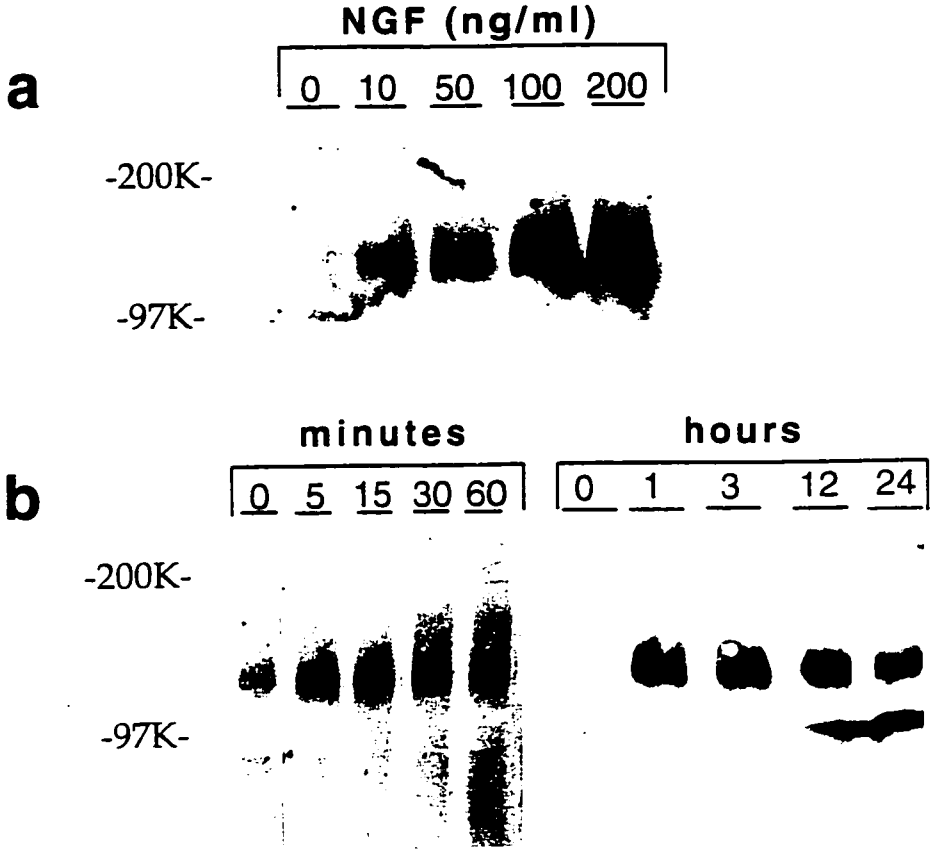
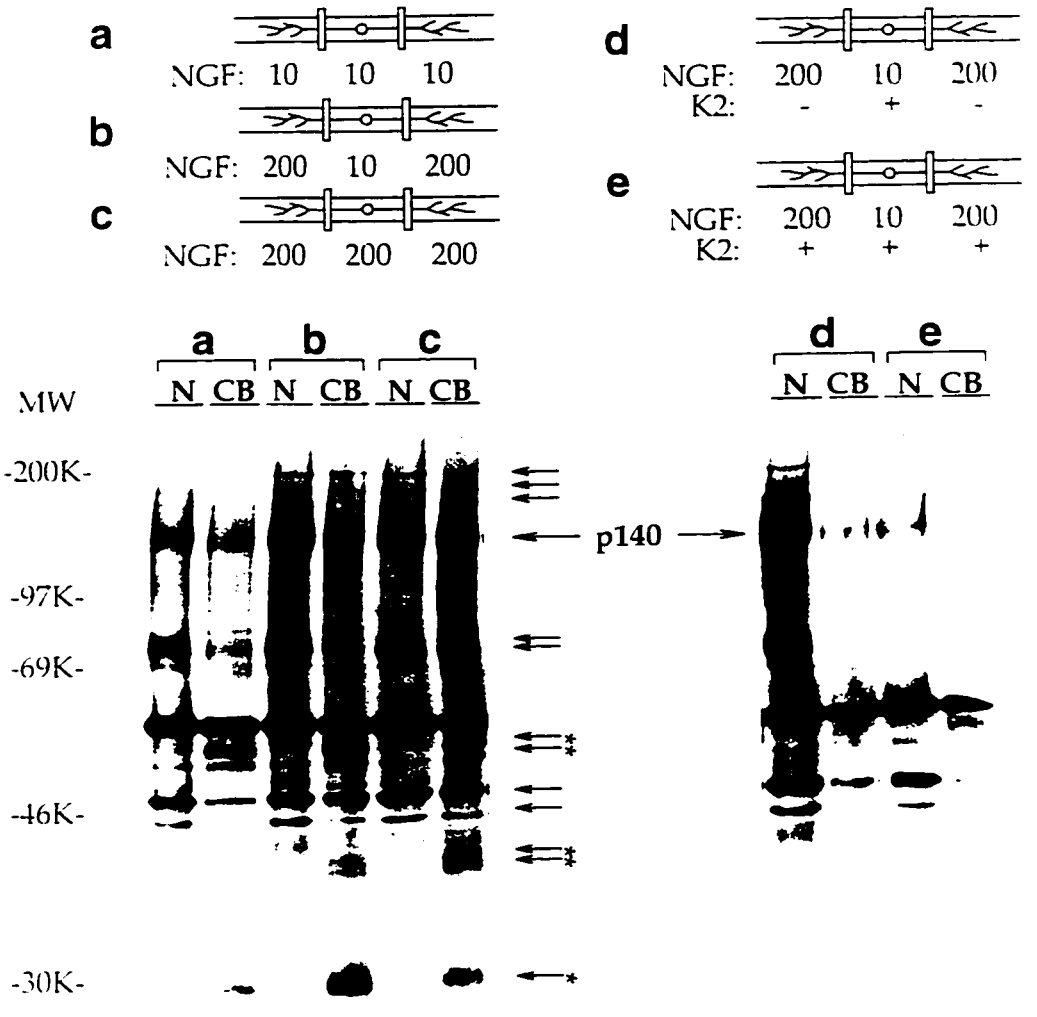

a
NGF (ng/ml)
0 10 50 100 200
-200K-
-97K-
b
minutes
hours
0 5 15 30 60
0 1 3 12 24
-200K-
-97K-

Figure 3. Protein tyrosine phosphorylation in response to different distributions of NGF

Compartmented cultures of rat sympathetic neurons were grown for two weeks in 10 ng/ml NGF in all compartments. Cultures were treated for 10 minutes with either; (a) 10 ng/ml NGF applied to all compartments; (b, d, e) 10 ng/ml NGF applied to cell bodies/proximal axons and 200 ng/ml NGF applied to distal neurites; or (c) 200 ng/ml NGF applied to all compartments. In (d) center compartments and in (e) all compartments were supplied with 500 nM K-252a (K2) starting 30 minutes prior to the NGF treatments. Cell extracts were collected from the cell body/proximal neurite compartments (CB) and the distal neurite compartments (N). To insure comparability between treatments, all cultures used were sister cultures, and each group contained the extracts pooled from 3 cultures. The extracts were analyzed by immunobloting with anti-phosphotyrosine (4G10). Tyrosine phosphorylation of proteins produced by distally applied NGF are indicated by arrows. Asterisks (*) indicate tyrosine phosphorylated proteins found only in cell bodies/proximal axons. The position of trk migration is indicated. Molecular weight markers are indicated on the left.



ANTI-PHOSPHOTYROSINE

Figure 4. Time course of local and retrograde NGF-induced tyrosine phosphorylations

Compartmented cultures of rat sympathetic neurons grown for two weeks in 10 ng/ml NGF in all compartments were supplied with 200 ng/ml NGF in distal compartments for the times indicated. All cultures were sister cultures. Cell extracts were collected directly into sample buffer and analyzed by immunoblotting with anti-phosphotyrosine. Molecular weight markers in kilodaltons for all gels are indicated on the left. Increasing tyrosine phosphorylation of proteins is indicated by arrows (filled arrows are proteins appearing within 10 minutes and open arrows are proteins appearing by 15 minutes). Asterisks (*) indicate dephosphorylation of proteins. The position of trk is indicated. (a) shows results from extracts of distal neurites of 3 cultures for each timepoint. (b) shows results from cell bodies and proximal neurites (CB) of 5 cultures for each timepoint. The neurites in (a) were from a subgroup of the cultures used in (b). (c) shows results from cell bodies and proximal neurites of 5 cultures for each timepoint which were given 24 nM anti-NGF to the center compartment 10 min prior to distal application of 200 ng/ml NGF.

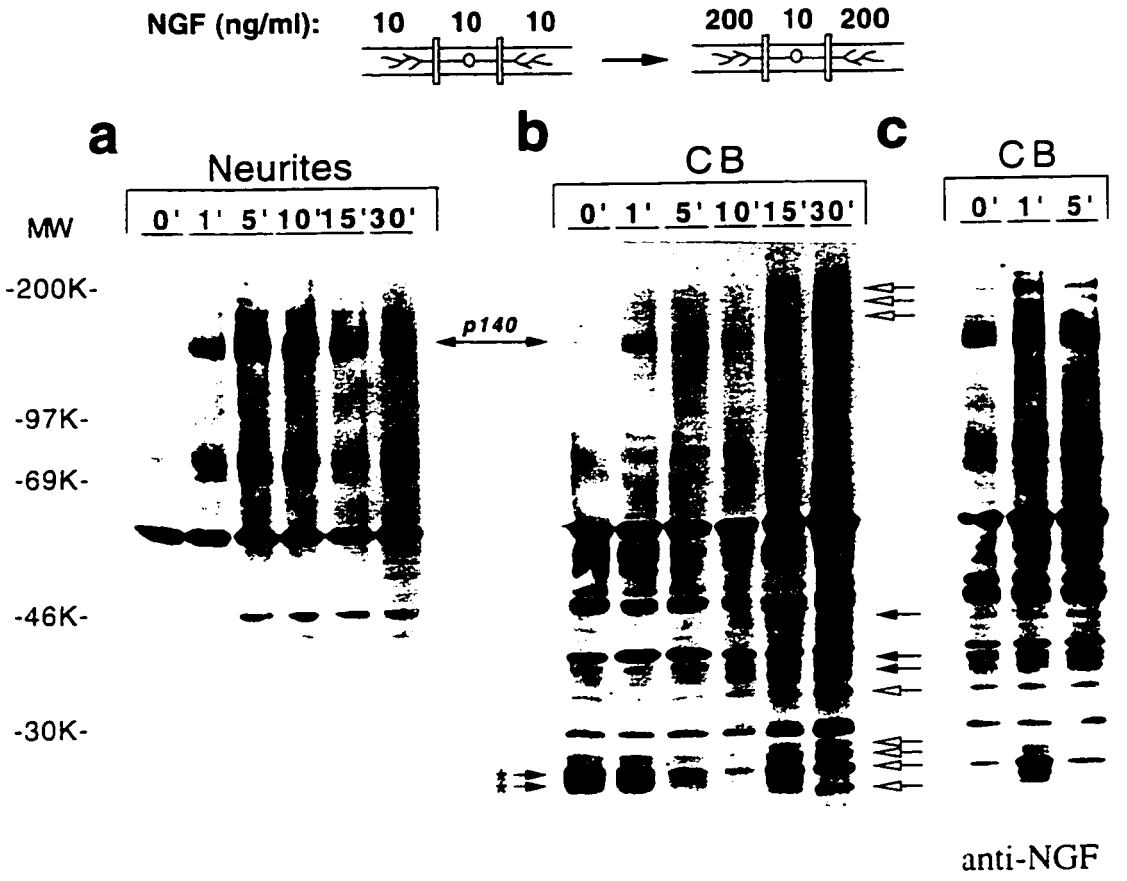

NGF (ng/ml): 10 10 10 200 10 200
a
Neurites
0' 1' 5' 10' 15' 30'
b
CB
0' 1' 5' 10' 15' 30'
c
CB
0' 1' 5'
MW
-200K-
-97K-
-69K-
-46K-
-30K-
p140
anti-NGF
ANTI-PHOSPHOTYROSINE

Figure 5. Timecourse of radiolabel accumulation in center media and cell extracts following addition of ^{125}I-NGF to distal compartments

Compartmented cultures of rat sympathetic neurons grown for 10 -14 days in the presence of 10 ng/ml NGF where given 200 ng/ml ^{125}I-NGF to distal compartments for times ranging from 1 minute to 24 hours. All cultures were sister cultures. After the addition of ^{125}I-NGF to distal compartments, medium (stippled bars) and cell extracts (filled bars) from the center compartment were collected and assayed separately for radiolabel content for each individual culture (cpm; primary Y axis). Equivalent ^{125}I-NGF concentration (pg) was calculated and indicated on the secondary Y axis. Bars represent means (± SEM). The number of cultures (N) for each timepoint was 7-10 except 18 h which was 3 cultures.

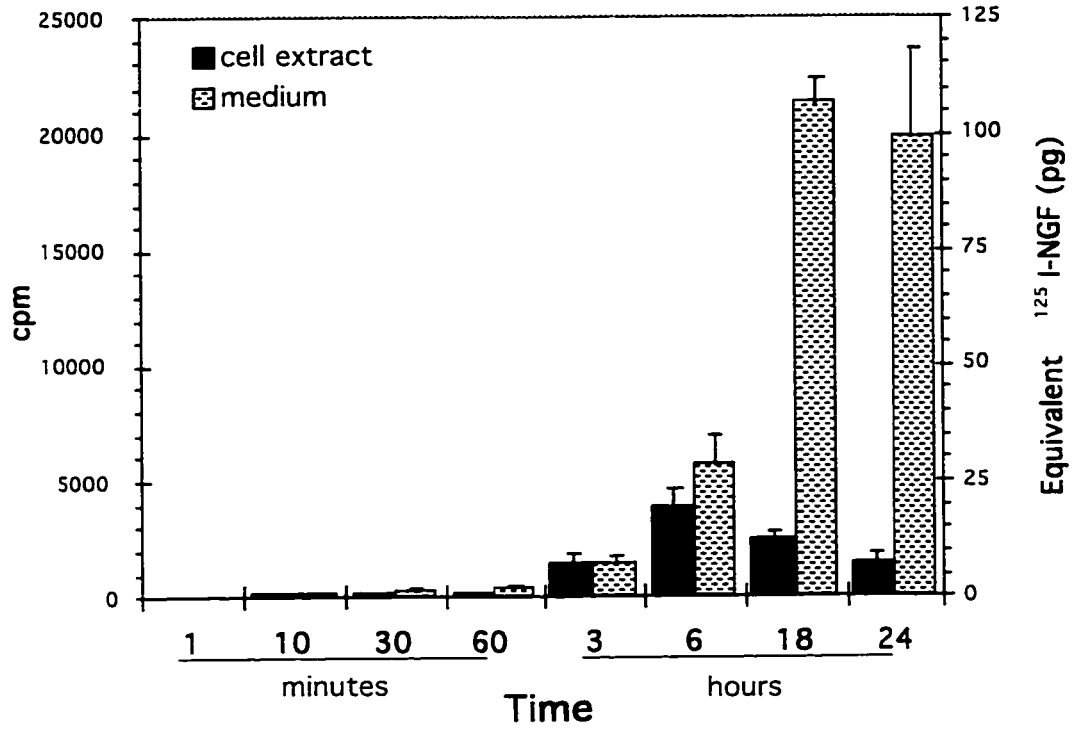

25000
20000
15000
10000
5000
0
cpm
cell extract
medium
125
100
75
50
25
0
Equivalent ^{125}I-NGF (pg)
1
10
30
60
3
6
18
24
minutes
hours
Time

Figure 6. Quantitative analysis of retrograde phosphorylated proteins

Retrograde tyrosine phosphorylation occuring in the cell bodies/proximal axons of control neurons and neurons with 200 ng/ml NGF supplied to their distal axons were quantified by densitometry. The results are expressed relative to the values of the control response which was set as 1.0. The stippled bars represent the means of the NGF-treated groups and solid bars represent the control levels. The proteins and time of NGF exposure are indicated on the x axis. The sample sizes were 6, 6, 3, 3, and 4 respectively and error bars are ±SEM. The results of one experiment were not included in the analysis of p30 because of a low control density which gave an outlying NGF-induced increase of 62-fold. The statistical significance between NGF-treated and control neurons for each protein was tested by the paired sample t-test and is indicated for each protein as the probability value (P). ** $P<0.01$ and * $P<0.05$ relative to control neurons.

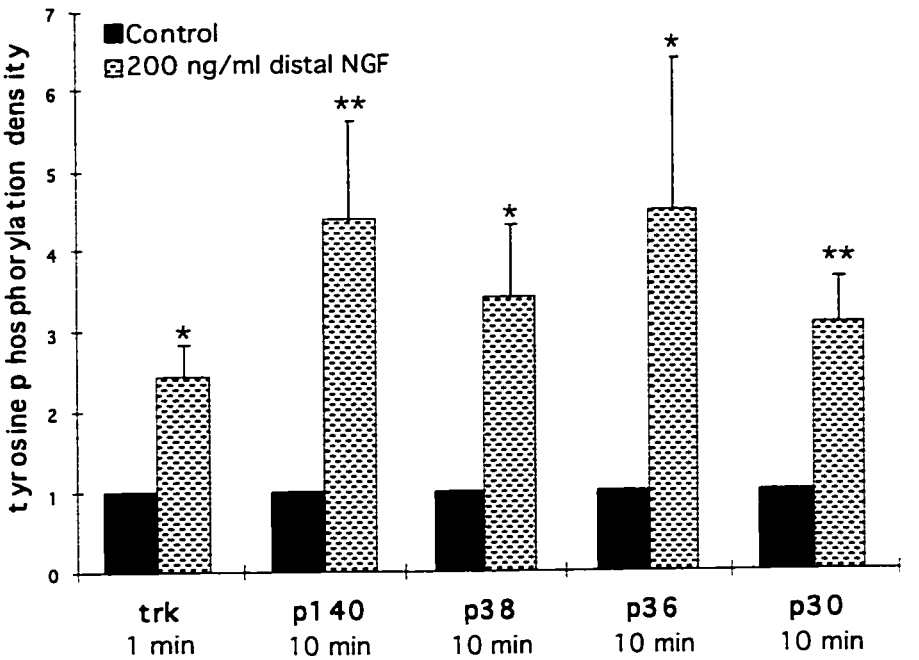
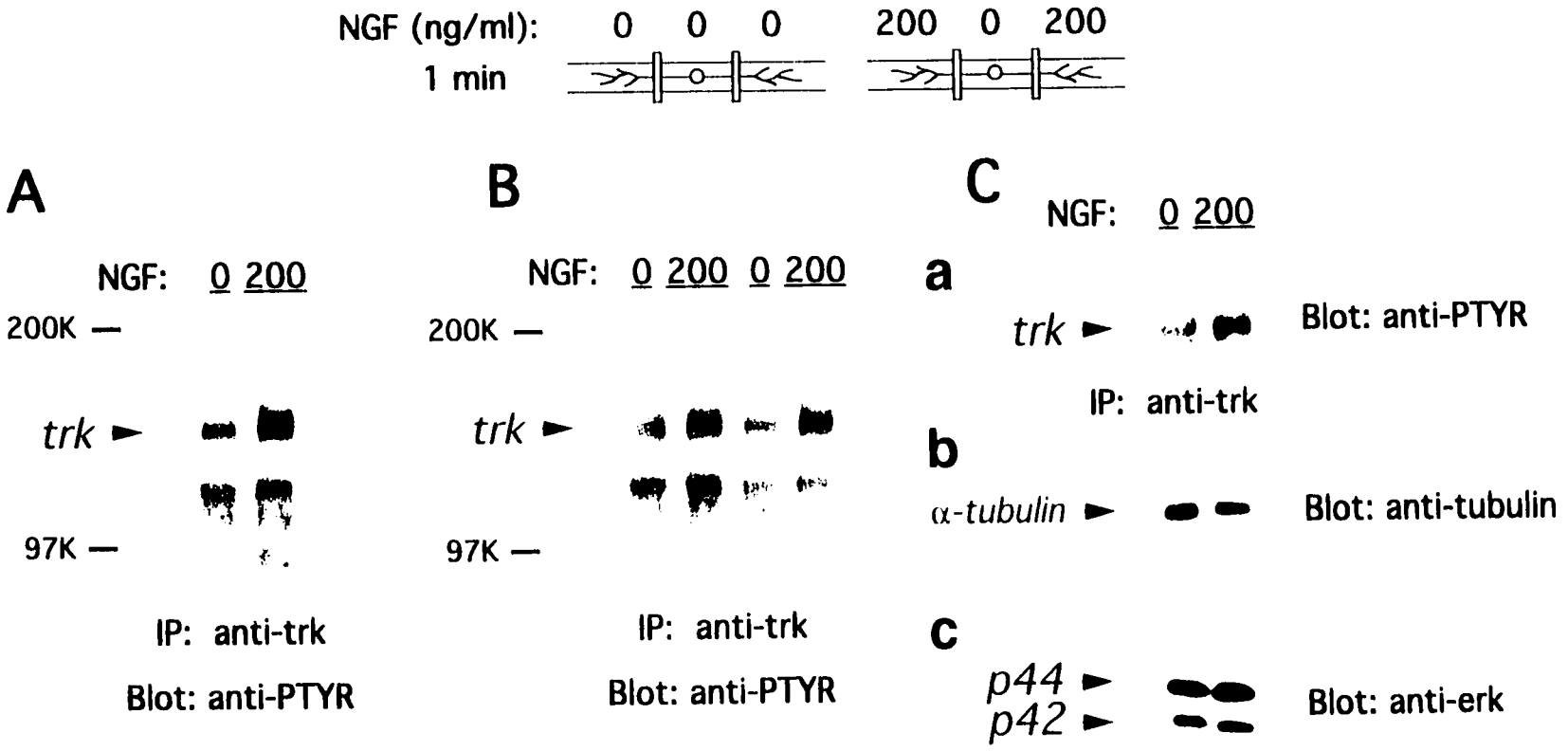

Control
200 ng/ml distal NGF
tyrosine phosphorylation density
0
1
2
3
4
5
6
7
*
**
*
*
**
trk
1 min
p140
10 min
p38
10 min
p36
10 min
p30
10 min

Figure 7. Retrograde phosphorylation of trk in response to distally applied NGF

(A) Cultures in this experiment were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments, and after 1 week, NGF was withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments. (B and C) Cultures in these experiments were supplied with 10 ng/ml NGF in all compartments for two weeks. Cultures in all experiments were given NGF-free medium for two hours followed by treatment for 1 minute with either 0 ng/ml NGF to distal neurites (0), or 200 ng/ml NGF to distal neurites (200). In each experiment extracts of cell body/proximal axon compartments were collected from equal numbers of control and NGF-treated cultures. The number of cultures per treatment group ranged from 14-18 between experiments. In experiments (A), (B) and (C(a)) extracts were immunoprecipitated using anti-trk and analyzed by immunoblotting with anti-phosphotyrosine (anti-PTYR). To verify that the observed NGF-induced trk phosphorylation could not have arisen from unequal loading of extracts, in experiment (C) equal aliquots of extracts from control and NGF-treatment groups removed prior to trk immunoprecipitation were immunoblotted with anti-α-tubulin (C(b)) and anti-ERK (C(c)). Neither showed an increase in the NGF treatment group. Molecular weight markers are indicated on the left of (A) and (B).


NGF (ng/ml): 0 0 0 200 0 200
1 min
A
NGF: 0 200
200K —
trk ►
97K —
IP: anti-trk
Blot: anti-PTYR
B
NGF: 0 200 0 200
200K —
trk ►
97K —
IP: anti-trk
Blot: anti-PTYR
C
NGF: 0 200
a
trk ►
Blot: anti-PTYR
IP: anti-trk
b
α-tubulin ►
Blot: anti-tubulin
c
p44 ►
p42 ►
Blot: anti-erk

Chapter 3

ERK-1 and ERK-2 activity involved in regulating axon growth is localized to distal axons of cultured rat sympathetic neurons

[Results presented in this paper have not yet been submitted for publication]

INTRODUCTION

Nerve growth factor (NGF) is the best characterized member of the neurotrophin family and is important for development (Crowley et al., 1994), survival (Levi-Montalcini, 1976, 1987), and growth (Campenot, 1977, 1982a,b) of sympathetic neurons. NGF exerts its biological effects by binding to two cell surface receptors, the trkA tyrosine kinase receptor, and the p75 neurotrophin receptor. Many of the biological effects of NGF are believed to occur via the trkA receptor (also called trk; Loeb et al., 1991; Ibanez et al., 1992; Loeb and Greene, 1993). Binding of NGF to trkA induces receptor dimerization, activation, and autophosphorylation on tyrosine residues (Jing et al., 1992, Kaplan et al., 1991a,b; Klein et al., 1991). Phosphorylation of trkA, couples the receptor to intracellular signal transduction pathways by providing binding sites for several adaptor proteins and enzymes which subsequently initiate signaling cascades that produce many prominent biological events (Ohmichi et al., 1992; Obermeier 1993a,b; Stephens et al., 1994; Vetter et al., 1991).

In sympathetic neurons, binding of NGF to its receptor(s) on the cell surface of distal axons, produces rapid tyrosine phosphorylation of a large number of proteins retrograde from the site of NGF binding (Senger and Campenot, 1997). In addition, application of NGF to distal axons produces increases in mRNAs for tyrosine hydroxylase (TH), and the p75 neurotrophin receptor (Toma et al., 1997). In order to understand the mechanisms underlying NGF-induced retrograde signals that produce these changes in

protein phosphorylation and gene expression, it is important to link extracellular NGF signals produced at the nerve terminal with downstream effectors. Like other growth factors, NGF binds to its receptor(s) on the cell surface and activates an array of downstream second messengers, which subsequently activate transcription factors that control changes in gene expression at the nucleus. However, unlike other growth factors, binding of NGF to its receptor(s) *in vivo* may occur at substantial distances from the cell soma, as nerve terminals can reside anywhere from a few millimeters to over a meter from the cell body. A key question in NGF-mediated signaling is how do signals travel from the nerve terminal to the cell body/nucleus?

To address this question, it is important to identify and characterize the proteins activated in response to NGF, and to distinguish their biological functions. Most of our knowledge of NGF signaling mechanisms comes from work with PC12 cells, a pheochromocytoma cell line which is used extensively to investigate ligand-receptor interactions, intracellular signaling, and cell differentiation in response to NGF (Greene and Tischler, 1976). Several experiments using PC12nnr cells (trk deficient PC12 cells) expressing various mutant trk receptors, have identified a number of cytosolic signaling proteins that associate with trkA, including: phospholipase C-γ1 (PLC-γ1) (Obermeier et al., 1993a; Stephens et al., 1994; Vetter et al., 1991), phosphatidylinositol-3-kinase (PI3K) (Ohmichi et al., 1992; Obermeier et al., 1993a; Yao and Cooper, 1995), the adaptor protein SHC (Nakamura et al., 1996; Obermeier et al., 1994; Stephens et al., 1994), and the phosphotyrosine phosphatase SHP-1 (Vambutas et al., 1995). Binding and activation of these proteins in PC12 cells, initiates signaling via Ras-dependent and -independent second

messenger cascades (reviewed in Heumann, 1994). One distinct trkA-dependent, Ras-independent pathway occurs via the activation of PI3K which has been implicated in neurotrophin-induced survival via the activation of a ser/thr kinase designated Akt (Dudek et al., 1997; Yao and Cooper, 1995). In addition, a second Ras-independent pathway leads to the activation of the SNT (Rabin et al., 1993; Peng et al., 1995). At present, little is known about the second messenger proteins associated with the PI3K and SNT pathways. On the other hand, the role of the Ras pathway in PC12 cell differentiation has been well studied. Phosphorylation of trkA on Y490 provides a binding site for the adaptor protein SHC (Stephens et al., 1994). SHC then recruits a second adaptor protein GRB2 which constitutively forms a complex with the Ras GTP exchange factor, SOS. Movement of SOS to the plasma membrane is sufficient to induce Ras activation (Obermeier et al., 1994; Stephen et al., 1994). Ras promptly initiates the activation of a series of protein kinases which include: B-Raf and MAPK kinase kinase (MEKK) (Cowley et al., 1994; Jaiswall et al., 1994; Lange-Carter and Johnson, 1994; Oshima et al., 1993; Traverse and Cohen, 1994). One or more of these kinases then activate MAPK kinase (MEK) (Jaiswal et al., 1994; Lange-Carter and Johnson, 1994) which then activate the MAPK isoforms, ERK-1 and ERK-2 (Boulton et al., 1991; Lange-Carter and Johnson, 1994; Robbins et al., 1992). The MAPKs are believed to be central regulators of signal transduction that activate and phosphorylate a number of signaling enzymes (reviewed in Blenis, 1993) and nuclear transcription factors (reviewed in Davis, 1993). Two additional transducers of the Ras signal lead from the activation of PLC-γ1 (Obermeier

et al., 1994; Stephens et al., 1994) and the adaptor protein FRS2 (Kouhara et al., 1997; Ong et al., 1996, 1997).

Although PC12 cells have provided a useful tool to study the effects of NGF, the physiological relevance of these observations to NGF-mediated signaling in neurons is not entirely clear. In fact, several studies have demonstrated that it is possible for a single receptor to activate different signal transduction pathways depending on its cellular environment. For example, the fibroblast growth factor receptor can signal proliferation in fibroblasts and differentiation in PC12 cells (reviewed in Marshall, 1995), thus demonstrating the importance of cellular context when attempting to understand cell signaling. In addition, the *in vivo* environment of neurons is unique in the sense that neurons send axons toward their target tissues that may be substantial distances from the cell body. Therefore, a signal that is transmitted along the length of the axon may require distinct mechanisms or signaling proteins to reach its target.

Our results indicate that in sympathetic neurons, NGF induces the sustained tyrosine phosphorylation of 10 major proteins in response to NGF. Here we have identified four of these proteins as: ERK-1 (44 kDa), ERK-2 (42 kDa), SHC (66 kDa) and PLC-γ1 (170 kDa). In addition, we have demonstrated that these proteins are present in both cell bodies/proximal axons and distal axons. In order to understand the role of the Ras/MEK/MAPK pathways in sympathetic neurons, we examined the role of MAPK proteins ERK-1 and ERK-2 using the compartmented culture system (Campenot, 1992). In PC12 cells, activation of the Ras/MEK/MAPK pathway concludes in the activation of transcription factors at the nucleus (reviewed in

Davis, 1993), we therefore hypothesize that NGF-induced activation of MAPK may provide a mechanism for retrograde signaling in sympathetic neurons. Surprisingly, binding of NGF to the distal axons of sympathetic neurons did not produce activation of ERK-1 and ERK-2 in the cell bodies/proximal axons. Furthermore, inhibition of ERK-1 and ERK-2 by the MEK inhibitor PD98059 in the cell bodies/proximal axons had no effect on distal axon growth. In contrast, preliminary data show that inhibition of ERK-1 and ERK-2 by PD98059 locally within the distal axons severally disrupted NGF-induced axon growth. While our results do not rule out the possibility that retrograde activation of ERK-1 and ERK-2 occurs at later times, they suggest that ERK is not involved in the propagation of a rapid retrograde signal in response to NGF. Moreover, these data support an alternate role for ERK-1 and ERK-2 in sympathetic neurons and suggest that growth mechanisms at or near the site of NGF interaction with the cell surface may require the activation of ERK-1 and/or ERK-2.

MATERIALS AND METHODS

Culture Procedures

Superior cervical ganglia were dissected from new born rats (Sprague-Dawleys supplied by the University of Alberta Farm) as previously described (Campenot et al., 1991), and subjected to a trypsin and mechanical dissociation procedure. Cells were plated into collagen-coated 24 well Linbro tissue culture dishes (ICN Biomedical Inc., Aurora, OH) (Campenot, 1977) at a density of 2 ganglia /well. Neurons were plated into compartmented cultures (Campenot, 1992) at a density of 1 ganglion/dish. For most experiments neurons grown in compartmented cultures were plated into center compartments containing 10 ng/ml NGF and were allowed to establish axons in right and left compartments containing 200 ng/ml NGF. After 1 week NGF was withdrawn from the center compartments and cultures were maintained for an additional week. Fig. 6 shows a single track from a culture raised under these conditions and stained with a monoclonal α-tubulin antibody (Sigma) and visualized by Rhodamine. Alternatively, in some experimental conditions, compartmented cultures of sympathetic neurons were treated on day four (Fig. 11), or were maintained for 2 wk after plating with 10 ng/ml NGF in all compartments (Fig. 10).

Culture Media

L15 medium without antibiotics (Gibco Laboratories, Grand Island, NY) was supplemented with additives described 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) was supplied in medium given to mass neuronal cultures, and only to the medium given to center compartments containing cell bodies of compartmented cultures. Culture medium was routinely changed every 3 to 6 days. Non-neuronal cells were eliminated by supplying 10 mM cytosine arabinoside in the neuronal cultures for 5 to 7 days.

Experimental Treatments

The experimental variables in these experiments consisted of various concentrations of the MEK inhibitor PD98059 and NGF. PD98059 (Calbiochem, San Diego, CA) was prepared as a 10 mM stock in DMSO (dimethyl sulfoxide) and stored at minus 20°C. The 10 mM stock was then diluted in L15 medium to produce 25 to 100 μM working solutions. Control culture medium contained 0.75% DMSO. NGF (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) stock was 20 mg/ml in phosphate buffered saline. NGF concentrations ranged from 10 ng/ml to 200 ng/ml, with the standard NGF concentration at 200 ng/ml.

Protein tyrosine phosphorylation

Following experimental treatment, cultures were washed twice with ice-cold Tris-buffered saline (TBS) and cell extracts were lysed directly into sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, 0.001% bromophenol blue). Extracts from equal numbers of cultures were run on SDS-polyacrylamide gels (10%). To equalize for sample loading, all experiments were performed using an equal number of sister cultures for control and experimental groups that had been treated the

same from the initial day of plating. The proteins were transferred to nitrocellulose (hyper-bond, Amersham, Oakville, Ontario, Canada) using a Hoefer Semi-dry transfer unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA) and incubated for 2 hrs at 37°C in TBS, pH 7.6, containing 2% bovine serum albumin (BSA) and 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO). Immunoblots were probed with 1 µg/ml monoclonal phosphotyrosine antibody, 4G10 (Upstate Biotechnology Inc. (UBI), Lake Placid, NY) in TBS, pH 7.6, containing 0.1% Tween 20 overnight at 4°C. Nitrocellulose was washed once for 15 minutes and twice for 5 minutes in TBS containing 0.1% Tween 20 prior to incubation for 1 hour with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). Immunoreactivity was determined using enhanced chemiluminescence (ECL, Amersham Corp., Arlington Heights, IL).

Tyrosine phosphorylation of trk

Following experimental treatment cultures were washed twice with ice-cold Tris-buffered saline (TBS) and solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate, 5 mg/ml leupeptin, 5 mg/ml aprotinin) and left on ice for 10 minutes. Extracts were pooled in microcentrifuge tubes, homogenized using a micro-homogenizer pestle (Mandel Scientific Co. Ltd., Guelph, Ontario, Canada) and centrifuged in an Eppendorf microcentrifuge (Eppendorf North American Inc., Madison, WI) for 30 seconds to remove cell debris. Extracts were then normalized for total protein by Bicinchoninic acid protein determination kit (Sigma) and immunoprecipitated with a polyclonal antibody against trk (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or anti-

trk polyclonal antibody 203B (provided by David Kaplan, Montreal Neurological Institute, Montreal, Canada). Immunoprecipitates were then run on SDS-polyacrylamide gels (8%), transferred to nitrocellulose, immunoblots were probed with 1 μg/ml phosphotyrosine 4G10 monoclonal antibody (UBI) and detected by ECL (Amersham) as described above.

Phosphotyrosine immunoprecipitations and immunoblotting

Following experimental treatment equal numbers of cultures were washed twice with ice-cold TBS and solubilized in lysis buffer as described above. Cell extracts were immunoprecipitated with agarose conjugated phosphotyrosine 4G10 monoclonal antibody (UBI) overnight at 4°C. Immunoprecipitates were washed twice with lysis buffer, boiled for 7 minutes in sample buffer and separated by SDS polyacrylamide gel. Proteins were then transferred to nitrocellulose and incubated with one of the following antibodies: 1 μg/ml phosphotyrosine 4G10 monoclonal antibody (UBI); 0.1 μg/ml phospholipase C-γ1 monoclonal antibody (Transduction Laboratories, Lexington, KY (Trans. Lab.)); or 1 μg/ml SHC polyclonal antibody (Trans. Lab.) overnight at 4°C and detected by ECL as described above.

Immunoblotting: PLC-γ1, SHC, ERK, MEK, GRB2, active ERK

Following experimental treatment, cell extracts were collected as previously described for protein tyrosine phosphorylation and processed for immunoblotting with one of the following antibodies: 0.1 μg/ml phospholipase C γ1 monoclonal antibody (Trans. Lab.); 1 μg/ml SHC polyclonal antibody (Trans. Lab.); 1 μg/ml ERK polyclonal antibody (Santa Cruz Biotechnology

Inc., Santa Cruz, CA) which detects both 44 and 42 kDa ERK proteins; 1:20,000 dilution of active ERK polyclonal antibody (Promega Corporation, Madison WI) which detects only the activated forms of the 44 and 42 kDa ERK by binding specifically to the 44 and 42 Kda proteins that have been phosphorylated on both tyrosine and threonine; 1 μg/ml ERK-1 (MK12) monoclonal antibody (Trans. Lab.); 1 μg/ml ERK-2 polyclonal antibody (Trans. Lab.) which detects both ERK-1 and ERK-2; 1 μg/ml ERK-3 polyclonal antibody (Trans. Lab); 0.25 μg/ml MEK1 monoclonal antibody (Trans. Lab.); 0.25 μg/ml MEK2 monoclonal antibody (Trans. Lab.); 1 μg/ml MEK 1 and 2 polyclonal antibody (Trans. Lab.); and 1 μg/ml GRB2 monoclonal antibody (UBI).

Tubulin immunocytochemistry

Cultures of rat sympathetic neurons were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments. After 1 week, NGF was withdrawn from the center compartments. After two weeks the axons from the right and left compartments were mechanically removed by a jet of sterile distilled water. Distal axons were allowed to regenerate for 3 days. The cultures were then labeled with a monoclonal antibody to α-tubulin (Sigma) and visualized by Rhodamine (Jackson ImmunoResearch Lab. Inc. Mississauga, Ont.).

Results

NGF induces the sustained tyrosine phosphorylation of 10 major proteins

Since NGF produces sustained biological effects on sympathetic neurons, we wanted to determine if other signaling proteins, in addition to trkA, remained phosphorylated in response to NGF. Mass cultures of rat sympathetic neurons initially grown in 200 ng/ml NGF were given NGF-free medium for two hours followed by medium containing 200 ng/ml NGF for various times ranging from 0 to 72 hours. Cell extracts were collected as described in experimental procedures and equal aliquots of cell extract were separated on SDS-polyacrylamide gels (10%), and immunoblotted using phosphotyrosine 4G10 monoclonal antibody (UBI). As shown in Fig. 1a, NGF induced sustained phosphorylation of 10 major proteins with apparent molecular weights 38, 42, 44, 65, 70, 85, 110, 140, 170 and 190 (indicated by arrows). Tyrosine phosphorylation occurred within 15 minutes and was maintained up to 24 hours with most phosphorylations returning to control levels by 72 hours. It was also observed that the 38 kDa protein was dephosphorylated by 3 hours and was rephosphorylated by 6 hours. It is possible that this protein is in some way cycling between a phosphorylated and unphosphorylated state. Immunoblots for PLC-γ1 (Fig. 1b), and ERK (691) (Fig. 1c) were also performed on equal aliquots of cell extract, confirming that equal amounts of protein were loaded on each lane. These results show that sympathetic neurons not only maintain a sustained phosphorylation of trkA, but also prolonged phosphorylation of several other proteins.

NGF induces protein tyrosine phosphorylation of PLC-γ1 and SHC.

As a first step toward characterizing the proteins which are tyrosine phosphorylated in response to NGF in sympathetic neurons, mass cultures of sympathetic neurons were given NGF-free medium for 2 to 4 hours. Cultures were then incubated in the absence or presence of 200 ng/ml NGF for 10 minutes. Cell extracts from an equal number of cultures were immunoprecipitated (IP) using anti-phosphotyrosine 4G10 (PTYR) conjugated to agarose beads as described in Material and Methods. Immunoprecipitates were separated on SDS-polyacrylamide gels (8%) and immunoblotted (IB) with PTYR (Fig 2a), PLC-γ1 (Fig. 2b) or SHC (Fig. 2c) antibodies. Anti-SHC recognizes the 66, 52 and 46 kDa SHC proteins. PLC-γ1 and the three SHC proteins were all shown to be phosphorylated on tyrosine residues in response to NGF. Note that PLC-γ1 and the 66 kDa SHC correspond to the 170 and 65 kDa proteins shown to be phosphorylated in Fig. 1.

NGF induces the activation of ERK-1 (p44) and ERK-2 (p42).

One of the major pathways by which NGF is known to exert its biological effects is the Ras/MEK/MAPK pathway which leads to the activation of the MAPK isoforms ERK-1 and ERK-2 (Gomez and Cohen, 1991; Thomas et al., 1992; Wood et al., 1992). However, the role ERK activation plays in postmitotic, differentiated neurons is not clear. To examine the role of MAPK proteins ERK-1 and ERK-2 in sympathetic neurons, a timecourse of ERK activation was established using a polyclonal antibody (anti-active ERK) that has been affinity purified using a dually phosphorylated peptide that corresponds to the active form of the MAP kinase enzymes. Thereby

recognizing only the activated form of ERK-1 and ERK-2. Mass cultures of sympathetic neurons were given NGF-free medium for two hours prior to treatment with NGF-containing medium (200 ng/ml) for various times ranging from 0 to 24 hours. Cultures were isolated directly into sample buffer and equal aliquots were separated on SDS polyacrylamide gels and immunoblotted using anti-active ERK (Fig. 3a) and anti-ERK (691) which recognizes ERK 1 and 2 protein (Fig. 3b). Both ERK 1 and 2 were shown to be phosphorylated rapidly (within 1 minute) and remained phosphorylated up to 24 hours. This time course is consistent with the 44 and 42 kDa tyrosine phosphorylated proteins in Fig. 1.

To establish that the 44 and 42 kDa proteins which are tyrosine phosphorylated in response to NGF in Fig. 1, are ERK-1 and ERK-2, cultures of sympathetic neurons were incubated with the MAPK kinase (MEK) inhibitor PD98059. The protein kinase inhibitor PD98059 has been shown to inhibit MEK activation and hence the phosphorylation of MAPK (Alessi et al., 1995). First, the optimum concentration for PD98059 inhibition of MAPK in sympathetic neurons was determined (Fig. 4). Two-week old mass cultures of sympathetic neurons were given NGF-free medium for two hours followed by a 30 minute incubation with increasing concentrations of PD98059 ranging from (0 to 100 μM). Cultures were then induced with 200 ng/ml NGF for 10 minutes. Cells were lysed and equal aliquots of cell extracts were immunoblotted using anti-active ERK. As shown in Figure 4, a PD98059 concentration of 75 μM was effective in blocking the rapid activation of ERK-1 and ERK-2 by NGF. Thus, PD98059 provides us with a tool to block the activation of MAPK in response to NGF.

Mass cultures of sympathetic neurons were then treated with NGF in the absence or presence of 75 μM PD98059 (Fig. 5). Cultures were incubated in NGF-free medium for two hours followed by 30 minute treatment with 75 μM PD98059 or DMSO (as control). Control or inhibitor treated cultures were then given medium with or without 200 ng/ml NGF for 10 minutes. Total cell extracts were prepared as previously described, and immunoblotted using anti-active ERK (Fig. 5a), anti-ERK-1 (Fig. 5b) and anti-PTYR (Fig. 5c). In the presence of PD98059, tyrosine phosphorylation of the 44 and 42 kDa proteins were inhibited by the MEK inhibitor PD98059. In addition, tyrosine phosphorylation of some proteins in cultures treated with PD98059 followed by NGF treatment showed substantially higher phosphorylation. Since the PD98059 drug by itself does not produce increased tyrosine phosphorylation of these particular proteins, we can assume that the increase in tyrosine phosphorylation resulted from inhibition of ERK-1 and ERK-2. A simple explanation for this result may be that activation of ERK-1 or ERK-2 activates a downstream phosphatase. When activation of either ERK-1 and/or ERK-2 is blocked, the phosphatase remains inactive resulting in increased phosphorylation. Together with previous results, our data strongly suggest that the 44 and 42 kDa tyrosine phosphorylated proteins are indeed the MAPK isoforms ERK-1 and ERK-2. Although there are Ras and Raf independent pathways leading to ERK activation (Lange-Carter and Johnson, 1994), the data presented here show that in the presence of 75 μM of the MEK inhibitor PD98059, NGF-induced activation of ERK-1 and ERK-2 in sympathetic neurons is almost completely blocked.

Localization of NGF-induced signaling proteins.

NGF-induces retrograde tyrosine phosphorylation of several proteins including some proteins localized to the cell body (Senger and Campenot, 1997). In an attempt to identify proteins involved in the propagation of NGF-induced retrograde phosphorylations, localization of several known NGF-induced second messenger proteins was investigated. Using sympathetic neurons grown in three-compartmented cultures (Campenot, 1992), extracts from cell bodies and proximal axons can be collected separately from distal axons, and the presence of NGF-induced signaling proteins can be detected using Western blot. Cultures used for these experiments were initially plated in 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments for one week which allows establishment of axons in the side compartments. NGF was then discontinued in the center. Fig. 6 shows a photomicrograph of a culture grown under similar conditions which has been labeled with a monoclonal antibody to α-tubulin and visualized by Rhodamine. Panel b displays a large cluster of cell bodies connected by a fine network of proximal axons that extend under the barrier and into the left and right compartments. Once the axons have passed into the side compartments, panels a and c, they produce a highly branched network of fibers that extends several mm. Cell extracts collected from the center compartments of these cultures are highly enriched in cell body proteins while extracts from the distal compartments contain axons only. Western blot analysis of these extracts would identify any proteins that are present exclusively at the cell body.

Cultures, grown under similar conditions as used for Fig. 6, were given NGF-free medium for two hours followed by 200 ng/ml NGF for 10 minutes in all compartments. Extracts were collected from both cell body/proximal axon compartments (CB) and distal axon compartments (N) either directly into sample buffer and analyzed by immunoblotting with anti-PTYR (Fig. 7a), or into lysis buffer, immunoprecipitated using anti-trk, and immunoblotted for anti-PTYR (Fig. 7b). Extracts equivalent to two compartmented cultures were loaded per lane for Western blots (Fig. 7a), and extracts from twelve compartmented cultures were used for each trk immunoprecipitation (Fig. 7b). Even though trkA activation was detected in both compartments, the protein tyrosine phosphorylation profile for the cell bodies/proximal axons was different from that in the distal axons. This difference in protein phosphorylation may reflect variations of spatial localization of cytoplasmic signaling proteins along the length of the neuron, or variations in regional protein or receptor concentration. Tyrosine phosphorylation of two proteins with apparent molecular masses of 36 and 38 kDa (indicated by arrows) in the cell body/proximal axon compartments only, may represent proteins that are present exclusively at the cell body.

In an effort to identify proteins present exclusively at the cell bodies, paired extracts of cell bodies/proximal axons (CB) and the distal axons (N) were processed for immunoblotting with PLC-γ1, SHC, GRB2, ERK (1,2 and 3) and MEK (1 and 2) antibodies (Fig. 8). Extracts equivalent to two compartmented cultures were loaded per lane. Even though NGF was shown to induce tyrosine phosphorylation of 36 and 38 kDa proteins apparently localized to the cell body (Fig. 7), PLC-γ1, SHC, GRB2, ERK (1,2 and 3) and

MEK (1 and 2) were found to be present in both the cell bodies/proximal axons and the distal axons.

Activation of ERK-1 and ERK-2 in compartmented cultures of sympathetic neurons.

Considering that many of the proteins associated with the Ras pathway appear to have a broad distribution in growing sympathetic neurons (Fig. 8), we wanted to assess the level of MAPK activity in response to NGF either in the cell bodies/proximal axons or the distal axons. Compartmented cultures grown as described for Fig. 6, were given NGF-free medium for 2 to 4 hours in all compartments followed by a 30 minute incubation in the absence or presence of 75 μM PD98059 in all compartments. Control and PD98059 treated cultures were then incubated for 10 minutes in the absence or presence of 200 ng/ml NGF in all compartments. Extracts were collected from both the cell bodies/proximal axon compartments and the distal axon compartments and equal aliquots were immunoblotted using anti-active ERK (Fig. 9a) or anti-ERK-1 (Fig. 9b). NGF induced local activation of ERK-1 and ERK-2 in both the cell bodies/proximal axons, and in the distal axons, and this activation was inhibited by 75 μM PD98059.

NGF does not induce retrograde activation of ERK-1 and ERK-2.

To test whether the MAPK proteins ERK-1 and ERK-2 are involved in the propagation of a rapid retrograde signal in response to NGF, cultures were grown under two different NGF regimes. In some experiments the cultures were grown similar to the cultures used for Fig. 6. In other experiments the

cultures were grown in 10 ng/ml NGF in all compartments, similar to previous retrograde phosphorylation experiments in sympathetic neurons (Senger and Campenot, 1997). This variation in initial conditions had no effect on the results. All cultures were incubated in NGF-free medium for 2 to 4 hours prior to treatment with 200 ng/ml NGF in the distal compartments only for times ranging from 0 to 3 hours. An additional group received NGF in all compartments for 30 minutes (G_{30m}). Extracts were collected from both the cell bodies/proximal axon compartments and the distal axon compartments. Fig. 10 shows the results of an experiment using cultures grown for 2 weeks in 10 ng/ml NGF in all compartments. Equal aliquots of cell extracts from either distal axons (Fig. 10 a and b) or cell bodies/proximal axons (Fig. 10 c and d) were processed for immunoblotting with anti-active ERK (Fig. 10 a and c), and anti-ERK-1 (Fig. 10 b and d). Substantial activation of ERK-1 and ERK-2 proximal to the site of NGF application was not seen within the first three hours. Therefore, rapid activation of tyrosine phosphorylated proteins retrograde to the site of NGF binding do not appear to include ERK-1 and ERK-2. However, these data do not rule out the possibility that retrograde activation of ERK proteins occurs at a later time.

Inhibition of ERK activity by PD98059 severely impairs local axon growth in sympathetic neurons

Since ERK-1 and ERK-2 did not appear to play a role in generating the rapid retrograde signal, experiments were performed to investigate a possible role for NGF-induced ERK proteins locally within the growing axon terminal. Compartmented cultures of rat sympathetic neurons used for these

experiments were initially plated in 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments for four days at which time the axons from the center compartments had grown 1 to 2 mm into the side compartments. Cultures were divided into 3 treatment groups (Fig. 11): group 1 received media containing 0.075% DMSO in all compartments (control, Fig. 11a), group 2 received 75 μM PD98059 in center compartment (Fig. 11b) and group 3 received 75 μM PD98059 in the distal compartments (Fig. 11c). All compartments that did not receive 75 μM PD98059 were given matched DMSO (as control). Within 18 hours, local exposure of distal axons to PD98059 (Fig. 11c) resulted in severe disruption of axon growth as compared to control DMSO-treated cultures, and by 48 hours the distal axons had completely degenerated. In contrast, distal axons of neurons given 75 μM PD98059 only to the cell bodies/proximal axons were normal in appearance (Fig 11b). Thus, preliminary data suggests that the inhibition of MEK and hence ERK-1 and ERK-2 activation, by 75 μM PD98059 in the distal axons of sympathetic neurons, severely disrupted local axon integrity. However, when the same concentration of PD98059 was applied to the cell bodies and proximal axons, there was no effect on distal axon growth or axon appearance (Fig. 11b). These data suggest that activation of ERK-1 and ERK-2 in the distal axons may play a role locally within the growing axon terminal, since local inhibition of ERK activity (within 18-48 hours) produced complete degeneration of distal axons.

DISCUSSION

NGF induces the sustained phosphorylation of PLC-γ1, SHC, ERK-1 and ERK-1 in sympathetic neurons

In spite of the fact that several second messenger proteins involved in NGF-induced signals in PC12 cells have been identified, a remarkably small number of proteins have been identified in primary neurons. Therefore, initial experiments focused on identifying proteins that are phosphorylated in response to NGF. Using mass cultures of sympathetic neurons we were able to identify several major proteins tyrosine phosphorylated in response to NGF including: PLC-γ1, SHC (46, 52, 66 kDa), ERK-1 and ERK-2. Identification of these proteins in sympathetic neurons was not surprising in view of the fact that NGF-induced Ras activation via PLC-γ1 and SHC is important for differentiation of PC12 cells (Obermeier et al., 1994; Stephens et al., 1994). However, considering that neurons are highly differentiated cells, it is probable that this pathway evokes different biological functions in sympathetic neurons than in PC12 cells. In addition, the duration of tyrosine phosphorylation of these proteins was substantially longer (up to 24 hours). Although, the exact nature of the sustained signal is not known, one might speculate that it serves to maintain catalytically active forms of proteins that can enhance signaling in cells that are chronically exposed to NGF.

PLC-γ1, SHC, MEK, ERK and GRB2 are present in both cell bodies/proximal axons and distal axons of sympathetic neurons.

Previous results have shown that NGF supplied to the distal axons of sympathetic neurons produced tyrosine phosphorylation of several proteins within the distal axons. In addition, NGF produced retrograde tyrosine phosphorylation of proteins in the cell bodies and proximal axons, including several proteins not seen in the distal axons (Senger and Campenot, 1997). Since we were interested in investigating the mechanisms involved in NGF-induced signaling, it seemed potentially informative to establish the localization of signaling proteins that may be involved in either local or retrograde signals. Using compartmented cultures, where the cell bodies/proximal axons can be collected separately from the distal axons, we were able to establish the localization of several signal transduction proteins. Compartmented cultures used for these experiments were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments, and after 1 week NGF was withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments. Figure 6 shows a photomicrograph of a compartmented culture which has been stained for the microtubule protein, α-tubulin. The distal compartment contains axons and the center compartment contains axons, cell bodies, and dendrites. Therefore, any proteins present exclusively at the cell bodies/dendrites would be detected only in the center compartment extracts. Local activation of proteins in either the cell bodies/proximal axons or the distal axons, produced regional differences in the proteins tyrosine phosphorylated in response to NGF (Fig. 7). At least two proteins, with apparent molecular weights of 36 and 38 (Fig.

7; indicated by arrows) are localized to the cell bodies and/or dendrites. This difference in tyrosine phosphorylation may result from variations of spatial localization of signaling proteins along the length of the neuron or possibly a regional variation in protein or receptor concentrations. Despite the regional difference in proteins tyrosine phosphorylated in response to NGF, PLC-γ1, SHC (46, 52, 66 kDa), GRB2, ERK (1,2 and 3), and MEK (1 and 2) were all shown to be present in both the cell bodies/proximal axons and the distal axons. While our results indicate the presence of these proteins along the entire neuron, two differences are worth noting. First, in addition to SHC proteins 46, 52 and 66, a protein of 64 kDa present only in the cell bodies/proximal axons and a protein of apparent molecular mass of 48 kDa in the distal axons were detected. Further investigation is required to determine if these proteins are other SHC-like proteins or the result of differential activation of SHC proteins in response to NGF. In addition, the ERK-3 antibody detected an additional band with the apparent molecular mass of 66 kDa present exclusively in the cell bodies/proximal axons. It is possible that this is an ERK-like protein present exclusively in the cell bodies/proximal axons. Although, at present, no evidence exists to support a role for ERK-3 in NGF-induced signaling maybe other ERK-like proteins present in the cell bodies are important.

Localization of signaling proteins along the neuron is consistent with the presence of NGF receptors on the nerve terminals, cell bodies, and along the axons of sympathetic neurons (Kim et al., 1979; Toma et al., 1997; see also Fig. 7b). Thus, sympathetic neurons should be capable of responding to NGF presented anywhere on the surface of the neuron, not just at the

terminal. Bearing in mind that neurotrophins are not only made by target cells but are also supplied by glial cells and other neurons (Acheson et al., 1991; Heumann et al., 1987a,b; Schecterson and Bothwell, 1992; Yoshida and Gage, 1992), localization of signaling proteins along the neuron may be important for normal maintenance and/or repair of the neuron.

ERK-1 and ERK-2 are not involved in NGF-induced retrograde signaling

In view of the fact that the activation and location of signaling proteins investigated to this point provided no substantial clues to their biological function in sympathetic neurons, we elected to study in more detail the function of the MAPK isoforms, ERK-1 and ERK-2. Our decision to investigate the role(s) of ERK-1 and ERK-2 was based on the following reasons: First, compartmented cultures of sympathetic neurons only contain about 1500 neurons per dish, limiting the amount of available material thereby making immunoprecipitation for signaling proteins difficult. The availability of an antibody (anti-active ERK) that only recognizes ERK-1 and ERK-2 once they have been activated by phosphorylation on both tyrosine and threonine provided a convenient tool to investigate their role(s) in NGF-induced signaling. Second, MAPK is believed to be a critical transducer of growth factor signaling to the nucleus in mammalian cells (Segal and Greenberg, 1996). Phosphorylation and activation of MAPK at the cell surface results in the translocation of MAPK to the nucleus where it has been shown to phosphorylate several transcription factors that control immediate early gene expression (reviewed in Johnson and Vaillancourt, 1994). In addition, in PC12 cells, translocation of MAPK to the nucleus is associated with sustained

activation of MAPK (Traverse et al., 1992, 1994; Nguyen et al., 1993). Since we have shown that binding of NGF to the cell surface of sympathetic neurons results in sustained activation of ERK-1 and ERK-2 (Fig 3), we hypothesized that ERK-1 and ERK-2 may be involved in the transmission of a NGF-induced signal from the nerve terminal retrograde to the cell body/nucleus.

According to the above hypothesis, application of NGF to distal axons of sympathetic neurons should result in the activation of ERK-1 and ERK-2 not only locally within the distal axon but also in the cell bodies/proximal axons retrograde to the site of NGF binding. Moreover, previous experiments indicate that NGF supplied to the distal axons of sympathetic neurons results in tyrosine phosphorylation of trkA in the cell bodies/proximal axons within one minute (Senger and Campenot, 1997). Tyrosine phosphorylation of proximal proteins was not limited to trkA, but included several other proteins within 5-15 minutes. These observations were confirmed by an independent study that showed, in addition to trkA, application of NGF to the distal axons of sympathetic neurons in compartmented cultures produced retrograde phosphorylation of the transcription factor CREB (cyclic AMP regulatory element-binding protein) in the nucleus within 10 minutes (Riccio et al., 1997). However, when NGF was given locally to the distal axons for times ranging from 0 to 3 hours, no activation of ERK-1 and/or ERK-2 was seen within the cell bodies/proximal axons (Fig. 10). This was extremely surprising, especially since application of NGF to either the distal axons or the cell bodies/proximal axons did result in activation of ERK-1 and ERK-2 locally at the site of NGF binding. These data suggest that the retrograde phosphorylation of proteins observed at 1-15 minutes does not include ERK-1 and ERK-2. Furthermore,

these observations support the hypothesis that the rapid retrograde phosphorylation of proteins in the cell bodies/proximal axons is different from those activated locally. However, if only a small fraction of ERK is required to initiate a signal, we cannot rule out the possibility that ERK-1 and ERK-2 were retrogradely activated but were below detectable levels. Nonetheless, the fact that NGF applied directly to the cell bodies/proximal axons produces a large increase in ERK-1 and ERK-2 activities suggests that even if a small, undetectable fraction of ERK is activated, the bulk of the activatable ERK in the cell bodies/proximal axons is not involved in the propagation of a retrograde signal.

Since previous studies have shown that trkA appears to be involved in the generation of a retrograde signal (Riccio et al., 1997; Senger and Campenot, 1997), how can we explain the difference in ability of local and retrogradely-activated trkA to activate ERK-1 and ERK-2? One explanation maybe that NGF-induced retrograde signaling pathway(s) result in the inhibition of ERK activation by activating a phosphatase that either directly dephosphorylate ERK-1 and ERK-2, or dephosphorylate one of the upstream kinases leading to the activation of ERK-1 and ERK-2. Or the possibility exists that the activation of some substrates such as ERK-1 and ERK-2 require a signal to reach a certain threshold before they can be activated. If the retrograde signal is attenuated as a function of distance, it is possible that this threshold is not reached. A third possibility could be that not all sites on the trkA molecule are phosphorylated thereby resulting in the activation of only a subset of signaling pathways. Assuming that the propagation of a rapid retrograde signal does not require the activation of ERK-1 and ERK-2,

then NGF has the ability to differentially activate local and retrograde signals. Separate retrograde signaling pathways could provide a neuron with the ability to respond to target-derived NGF in one way and NGF supplied at the cell body by glial cells or presynaptic neurons in another. If this interpretation is extended to include other neurotrophins, then any given neuron could interpret differences in spatial localization of different neurotrophins. By way of illustration, a recent study has shown that postnatal sympathetic neurons respond to both NGF and NT-3 predominantly through the trkA receptor (Belliveau et al., 1997). Therefore, a sympathetic neuron could differentiate between these two neurotrophins by regulation of signaling pathways activated in response to different spatial localization of the neurotrophins. i.e. NGF from the target tissue could signal to the cell body using one mechanism, whereas binding of NT-3 to trkA present at the cell body could activate a second mechanism in proximity to the nucleus. As intriguing as these results are, it is obvious that substantially more information is required before we can fully understand the differences between these NGF-induced signaling mechanisms.

Role of ERK-1 and ERK-2 in sympathetic axon growth

By using the MEK inhibitor PD98059 (Dudley et al., 1995) we were able to investigate the role of ERK-1 and ERK-2 further by selectively blocking upstream activation of MEK (Moriguchi et al., 1995). PD98059 was shown to inhibit activation of ERK-1 and ERK-2 in a concentration dependent manner (0 to 100 μM) (Fig. 4). At 75 μM concentration PD98059 inhibited both the tyrosine phosphorylation (Fig. 5c) and activation (Fig. 5a) of ERK-1 and ERK-

2. In contrast, PD98059 did not inhibit tyrosine phosphorylation of several other proteins including trkA, PLC-γ1 and SHC (66 kDa) (Fig. 5c). In fact, the level of tyrosine phosphorylation of these proteins by NGF was higher in the presence of PD98059. Since PD98059 in the absence of NGF did not induce increases in tyrosine phosphorylation, we can assume that this increase in phosphorylation is the direct result of MEK inhibition. Although the mechanism underlying this increase in phosphorylation is not known, one might speculate that activation of ERK-1 and/or ERK-2 results in the activation of a phosphatase(s) which in the absence of ERK-1 and/or ERK-2 activity remains inactive and thus results in an increased level of phosphorylation. Application of PD98059 to the center compartment, which locally inhibited ERK-1 and ERK-2 in the cell bodies/proximal axons had no effect on distal axon growth (Fig. 11b). Conversely, when PD98059 was applied to the distal compartment only, it blocked activation of ERK-1 and ERK-2 in the growing axon terminal and severely disrupted NGF-induced axon growth (Fig. 11c). Absence of any inhibitory effect of PD98059 on axon growth when applied to the cell body/proximal axon compartment suggests that the disruption of axon growth when PD98059 was applied locally to the distal axons does not involve mechanisms at the cell body, but instead is affecting mechanisms which are localized to the distal axons. Moreover, since both compartments contained segments of axon, it is probable that the disruption of axon growth occurred by interruption of signal transduction mechanisms within the growth cone. Therefore, it is possible that the inhibitory effect of PD98059 directly results in interference with the intracellular signaling pathways linking the NGF receptor(s) to mechanisms essential for growth cone motility.

Furthermore, we know that the degeneration of distal axons in response to PD98059 did not result from blocking NGF-survival-promoting mechanisms, since PD98059 present at the cell body did not affect distal axon elongation. These results agree with a recent study that showed inhibition of ERK-1 and ERK-2 by PD98059 was insufficient to inhibit neuronal survival of sympathetic neurons in the presence of NGF (Creedon et al., 1996). Because PD98059 had no effect on axon elongation when present on the cell bodies/proximal axons, we can also assume that mechanisms localized to the cell body such as gene expression or those present in axons such as axoplasmic transport, are not involved in the local growth inhibitory response. Therefore, these data are consistent with the observation that ERK-1 and ERK-2 are involved with local mechanisms present within the distal axons, possibly at the growth cone, and that ERK-1 and ERK-2 present at the cell bodies/proximal axons does not appear to play a role in the immediate local growth response elicited by NGF.

Conversely, neurite outgrowth in chick sympathetic neurons (E12) was not effected by the MEK inhibitor PD98059 (Klinz et al., 1996). Although this result is in direct contrast to data presented here, we believe that there are significant differences between the two systems that can account for this discrepancy. During sympathetic neuron development, neurotrophin responsiveness changes from neurotrophin independent at early stages in development to neurotrophin dependent late in development (Coughlin and Collins, 1985; Ernsberger et al., 1989; Fagan et al., 1996; Wyatt and Davies, 1995). Rat neonatal sympathetic neurons do not survive in culture without NGF (Chun and Patterson, 1977). On the other hand, survival of early chick sympathetic neurons *in vitro* has been shown to be NGF independent

(Ernsberger et al., 1989). In addition, NGF-induced signaling in chick sympathetic neurons (E12) results in the transient tyrosine phosphorylation of two major proteins: ERK-2 and a 105 kDa protein (Klinz and Heumann, 1995). This is in striking contrast to rat sympathetic neurons, where NGF-induced the sustained tyrosine phosphorylation of 10 major proteins (Fig. 1), and at least two MAPK proteins, ERK-1 and ERK-2 (Fig. 3). Our data imply that mechanisms activated at these two stages in development are extremely different and therefore cannot be directly compared.

On the other hand, two recent studies support a role for MAPK in the regulation of processes localized to the growth cone. First, MAPK within the axons of Aplysia sensory neurons were shown to be involved in the regulation of synaptic remodeling by affecting the cell adhesion molecule apCAM (N-CAM-like protein in Aplysia) (Bailey et al., 1997). Therefore, one possible mechanism by which ERK-1 and ERK-2 could control neuronal growth is by the regulation of adhesion molecules present at the growth cone. In a second study, PC12 cells overexpressing the adaptor protein c-Crk spontaneously sprouted neurites in the absence of NGF (Tanaka et al., 1993; Matsuda et al., 1994). In addition, PC12 cells expressing v-Crk have been shown to have enhanced responses in the presence of NGF that correlate with prolonged MAPK activation (Hempstead et al., 1994; Teng et al., 1995). Crk is believed to target ligand bound receptor complexes such as NGF/receptor to neuronal cytoskeleton (Teng et al., 1996). In this way, the enhanced response to NGF in PC12 cells is thought to occur via increased internalization and association of Crk with paxillin at the growth cone (Teng et al., 1996). Although no direct evidence for an association of MAPK and Crk exists, one might speculate that

in a growing nerve terminal MAPK may phosphorylate a downstream kinase or phosphatase that directly regulate the formation of the Crk/paxillin complex.

Finally, how do we incorporate the data presented in this paper into our present knowledge of NGF-induced signaling in primary neurons. One might predict that retrograde phosphorylation of proteins is important in relaying signals to the cell body that are important for the survival of the neurons, while the activation of proteins such as ERK-1 and ERK-2 in the axon terminal are essential for local mechanisms important for growth or synaptic efficacy. Moreover, the molecular context of a given signaling protein may influence its biological role. For example, MAPK present within the nerve terminal may be critical for the regulation of local growth mechanisms. On the other hand, activation of MAPK at the cell body, where it is in close proximity to transcription factors, may be necessary for the induction of gene expression in response to neurotrophins from presynaptic neurons or glia in the vicinity of the cell body. Clearly these results carry important implications for neuronal signaling and support the idea that differential activation of signaling pathways can occur by a given neurotrophin depending on cellular localization.

With the discovery of new pathways still emerging, it is evident that we are just beginning to dissect the mechanisms underlying NGF-induced biological responses. Moreover, it is likely that many proteins involved in these processes have not yet been discovered. However, regardless of the mechanism, the findings presented here have two significant biological implications: First, rapid retrograde phosphorylation can activate trkA but not ERK-1 or ERK-2 providing support for differential activation of local and

retrograde signaling pathways. Second, activation of ERK-1 and ERK-2 appears to play an important role locally within the growing nerve terminal, possibly at the growth cone. Understanding the molecular basis of NGF-induced local and retrograde mechanisms in neurons is of vital importance for the understanding of neuronal survival and repair.

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FIGURES

Figure 1. Time course of protein tyrosine phosphorylation in response to NGF.

Cultures of rat sympathetic neurons grown in 200 ng/ml NGF were incubated in NGF-free medium for two hours. Cultures were then incubated in medium containing 200 ng/ml NGF for times ranging from 0 to 72 hours. An equal number of cultures were collected directly into SDS sample buffer and equal aliquots of total cell extracts were separated on SDS-polyacrylamide gels (10%) and immunoblotted with anti-phosphotyrosine (4G10: UBI) (a), anti-phospholipase C gamma 1 (PLC-γ1) (b) and anti-ERK (691) which recognizes both ERK-1 (44 kDa) and ERK-2 (42 kDa) (c). Immunoblots were detected by enhanced chemiluminescence (Amersham). The position and approximate molecular weight of the tyrosine phosphorylated proteins are indicated on the right. Molecular weight markers in kilodaltons are indicated on the left.

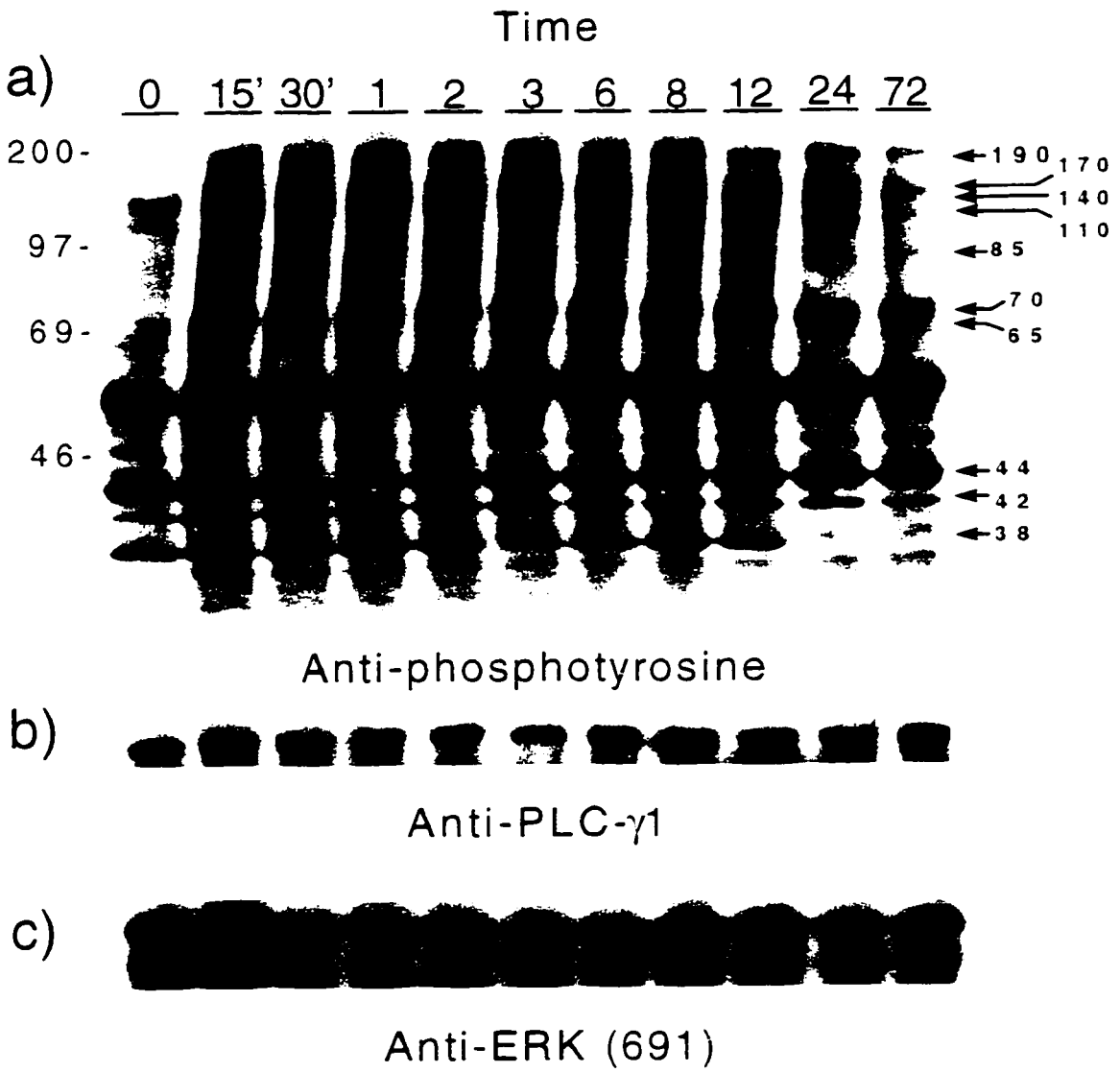

Time
a)
0
15'
30'
1
2
3
6
8
12
24
72
200-
97-
69-
46-
190
170
140
110
85
70
65
44
42
38
Anti-phosphotyrosine
b)
Anti-PLC-γ1
c)
Anti-ERK (691)

Figure 2. NGF-induced protein tyrosine phosphorylation of PLC-γ1 and SHC (66, 52 and 46) in sympathetic neurons.

Cultures of sympathetic neurons were given NGF-free medium for 2-4 hours prior to incubation with media in the absence (-) or presence (+) of 200 ng/ml NGF for 10 minutes. Equal numbers of cultures were immunoprecipitated (IP) using anti-phosphotyrosine (PTYR) and immunoblotted (IB) with anti-PTYR (a), anti-phospholipase C γ1 (PLC-γ1) (b) and anti-SHC (c). The SHC antibody recognizes the 66 kDa, 52 kDa and 46 kDa SHC proteins. Molecular weight markers in kilodaltons are indicated on the left.

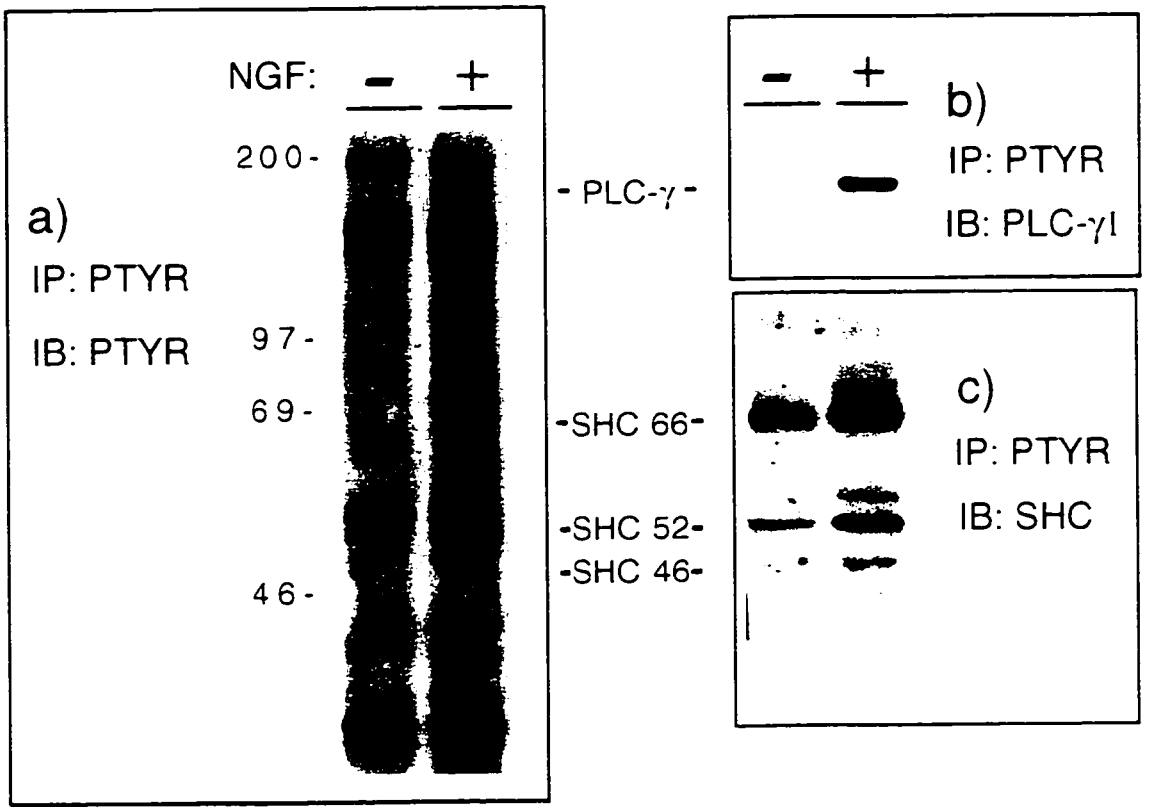

NGF: - +
200-
97-
69-
46-
a)
IP: PTYR
IB: PTYR
- PLC-γ -
-SHC 66-
-SHC 52-
-SHC 46-
- +
b)
IP: PTYR
IB: PLC-γ1
c)
IP: PTYR
IB: SHC

Figure 3. NGF-induced activation of ERK-1 and ERK-2 in sympathetic neurons.

Cultures of sympathetic neurons were given NGF-free medium for 2-4 hours prior to induction with NGF (200 ng/ml) for times ranging from 0-24 hours. Equal numbers of cultures were isolated directly into SDS sample buffer. Equal aliquots were separated on SDS-polyacrylamide gels (10%) and immunoblotted using anti-active ERK which recognizes only the activated form of ERK-1 and ERK-2 (a) and anti-ERK (691) which recognizes both ERK-1 and ERK-2 (b).

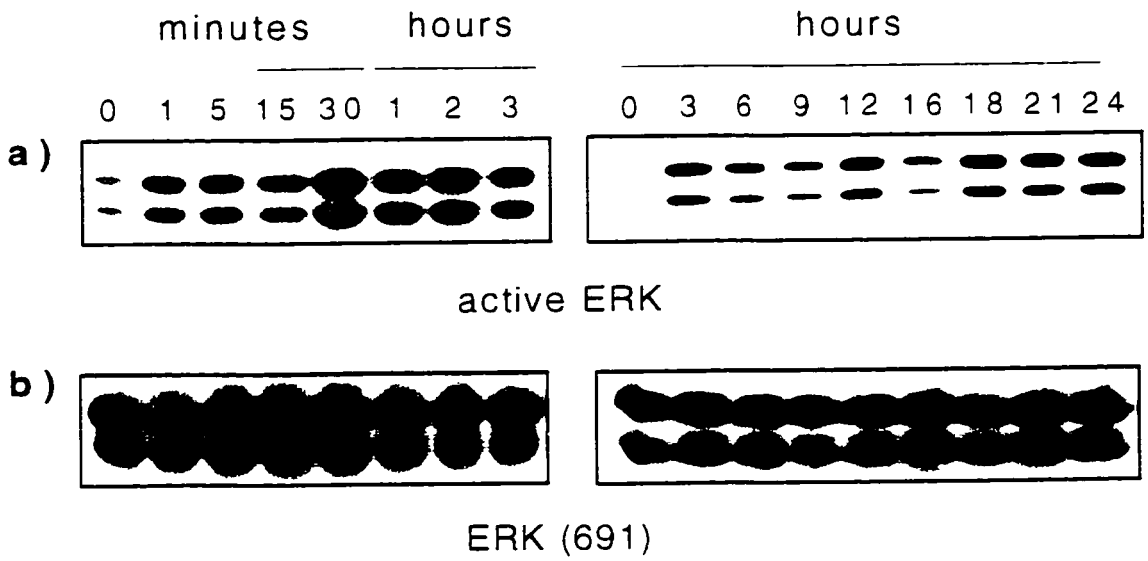
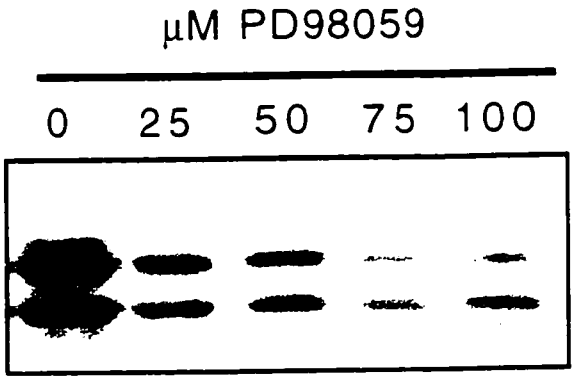

minutes
hours
hours
0 1 5 15 30 1 2 3
0 3 6 9 12 16 18 21 24
a)
active ERK
b)
ERK (691)

Figure 4. Concentration-dependent inhibition of ERK-1 and ERK-2 activation by MEK inhibitor PD98059.

Mass cultures of sympathetic neurons were grown in 200 ng/ml NGF for two weeks. Cultures were incubated in NGF-free medium for 2-4 hours followed by incubation in the same medium containing various concentrations of the MEK inhibitor PD98059 (0 to 100 μM) for 30 minutes. Cultures were then induced with 200 ng/ml NGF for 10 minutes. Cell extracts were collected into SDS sample buffer and equal aliquots were run on SDS-polyacrylamide gels (10%) and immunoblotted using anti-active ERK. NGF-induced activation of ERK-1 and ERK-2 was inhibited by 75 μM PD98059.



active ERK

Figure 5. Inhibition of NGF-induced ERK activation by MEK inhibitor PD98059 in sympathetic neurons.

Mass cultures of rat sympathetic neurons were grown for two weeks in 200 ng/ml NGF. Cultures were given NGF-free medium for two hours. Cultures were incubated in the presence (+) or absence (-) of 75 μM PD98059 or DMSO for 30 minutes. Cultures were then incubated in the absence (-) or presence (+) of 200 ng/ml NGF for 10 minutes. Cell extracts were collected directly into SDS sample buffer and equal aliquots of total cell extract were run on SDS polyacrylamide gels (10%) and immunoblotted using anti-active ERK (a), anti-ERK-1 (b) and anti-phosphotyrosine (4G10; UBI) (c). Immunoblots were developed using enhanced chemiluminescence (Amersham). (c) Tyrosine phosphorylation of ERK-1 and ERK-2 is indicated by arrows.

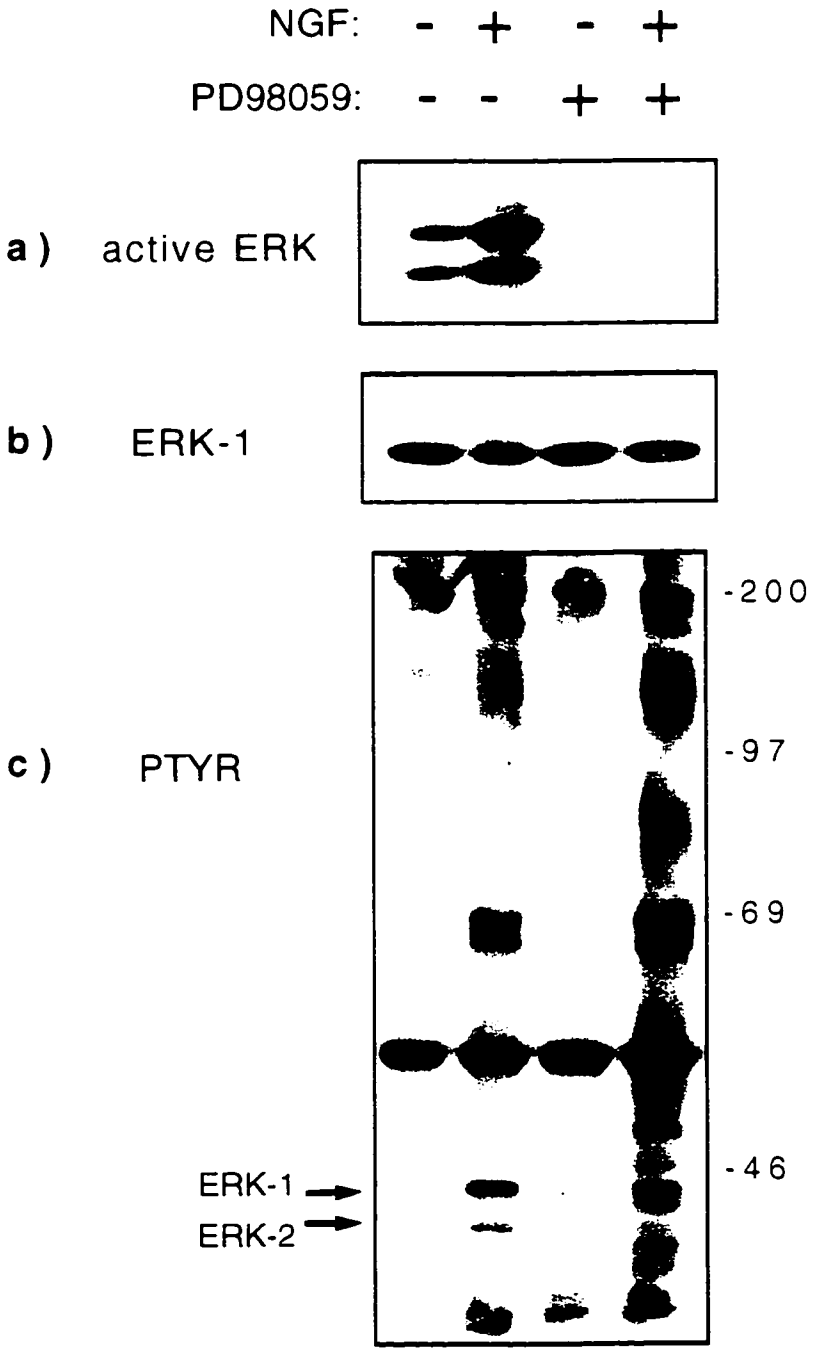

NGF: - + - +
PD98059: - - + +
a) active ERK
b) ERK-1
c) PTYR
-200
-97
-69
-46
ERK-1
ERK-2

Figure 6. Sympathetic neurons in compartmented cultures visualized by α-tubulin immunoreactivity.

Photomicrograph of a single track in a compartmented culture of sympathetic neurons. Cultures of rat sympathetic neurons were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments. One week later, NGF was withdrawn from the center compartments. After two weeks the axons from the right and left compartments (a and b) were mechanically removed by a jet of sterile distilled water. Distal axons were allowed to regenerate for 3 days. The culture was labeled with a monoclonal antibody to α-tubulin and visualized by Rhodamine. Panel b displays a large cluster of cell bodies connected by a fine net work of proximal axons that extend under the barrier and into the left and right compartments. Once the axons have passed into the side compartments, panels a and c, they produce a highly branched network of fibers that extends several mm. Cell extracts collected from center compartments are highly enriched in cell body proteins while extracts from the left and right compartments contain protein present only in the distal axons and growth cones.

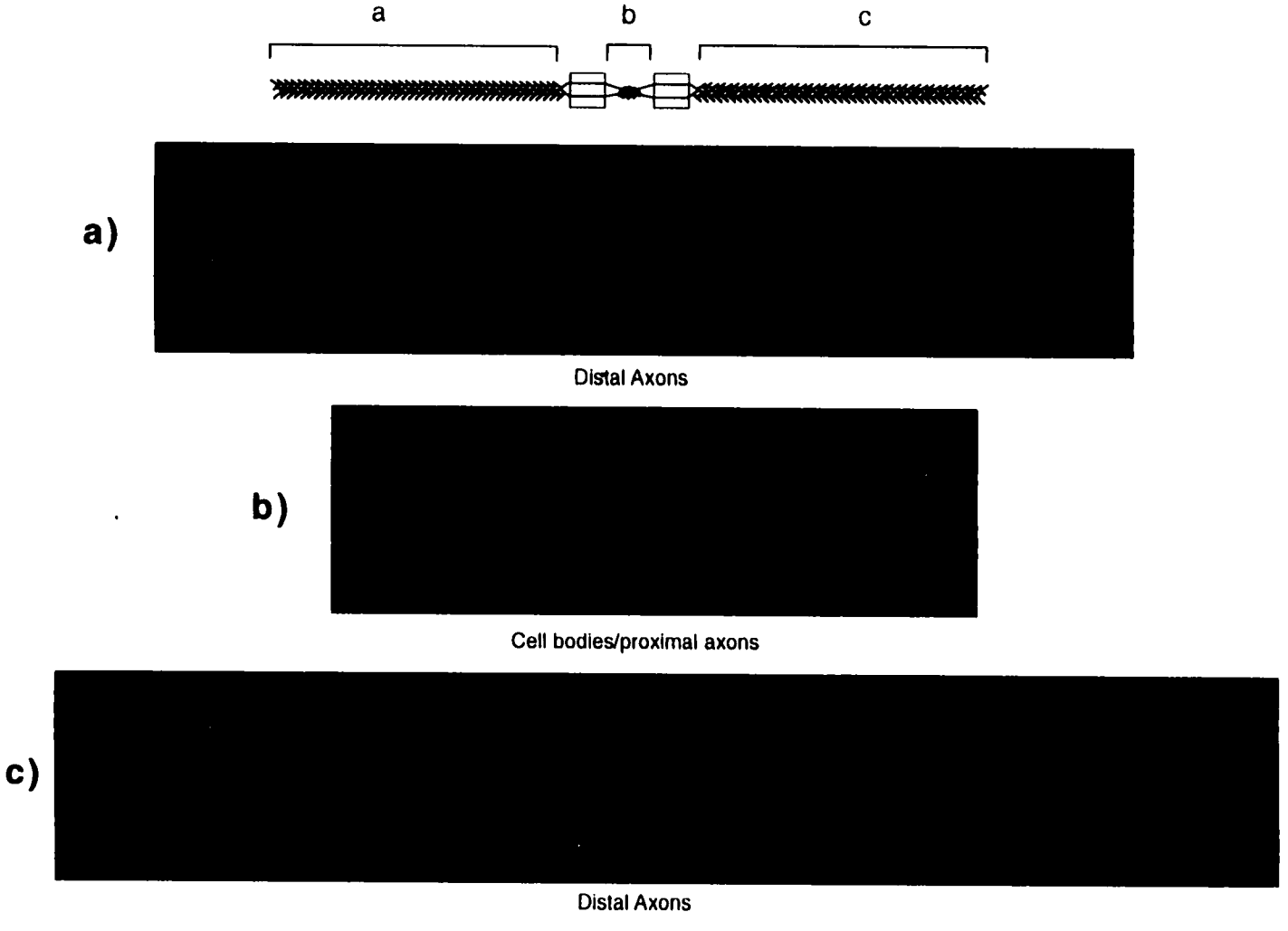

a
b
c
a)
Distal Axons
b)
Cell bodies/proximal axons
c)
Distal Axons

Figure 7. Localization of NGF-induced tyrosine phosphorylated proteins in cell bodies/proximal axons and distal axons of sympathetic neurons grown in compartmented cultures.

Cultures were initially plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments for 1 week. NGF was then withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments for an additional week. Prior to isolation, cultures were given 200 ng/ml NGF to all compartments for 10 minutes. Extracts obtained from both cell body/proximal axon compartments (CB) and distal axon compartments (N) were collected either directly into SDS sample buffer and analyzed by immunoblotting (IB) with anti-PTYR (a) or collected into lysis buffer and immunoprecipitated (IP) using anti-trk and immunoblotted with anti-PTYR (b). Two compartmented cultures were loaded per lane in (a) and extracts from twelve compartmented cultures were used for the trk immunoprecipitation (b). The position of two tyrosine phosphorylated proteins of apparent molecular weights 38 and 36 are indicated by arrows. Molecular weight markers are indicated in kilodaltons on the left.

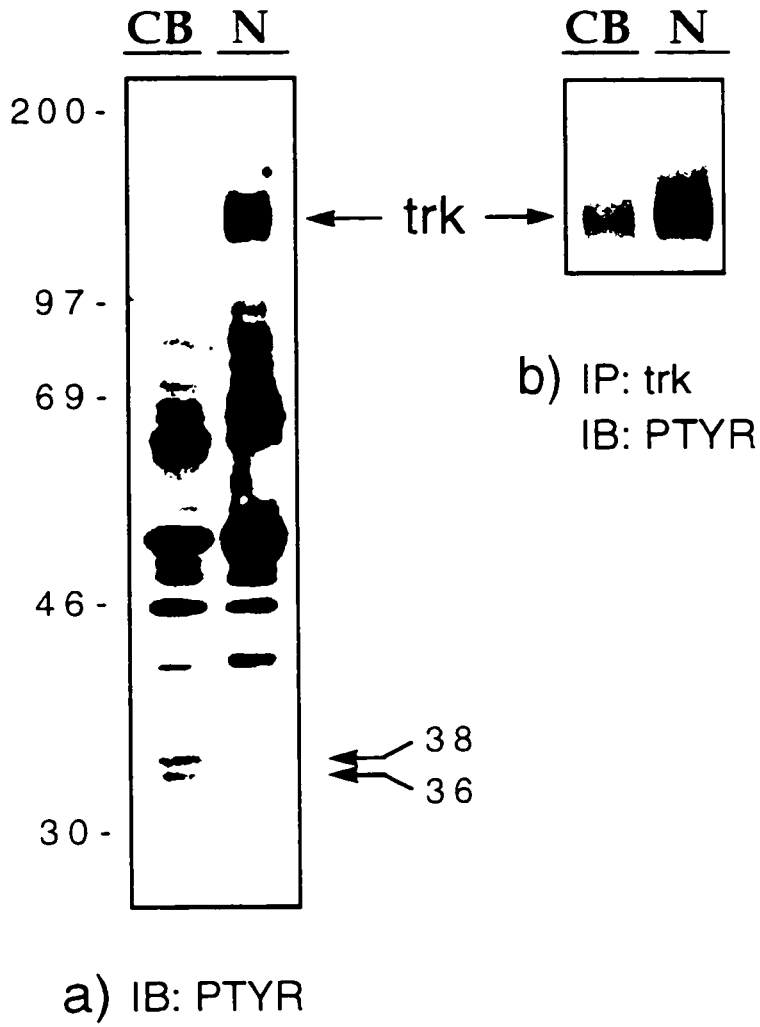

CB N
CB N
200-
97-
69-
46-
30-
trk
38
36
b) IP: trk
IB: PTYR
a) IB: PTYR

Figure 8. Spatial localizationof PLC-γ1, SHC, GRB2, ERK, and MEK proteins in sympathetic neurons.

Compartmented cultures of sympathetic neurons were grown for two weeks as described in the legend of Figure 7. Cell extracts were obtained from both the cell body/proximal axon compartments (CB) and the distal axon compartments (N). Cultures were collected directly into SDS sample buffer and paired extracts equivalent to two compartmented cultures were loaded per lane and separated on SDS polyacrylamide gels (10%) and immunoblotted with anti-PLCγ1, anti-SHC and anti-GRB2 (a), anti-ERK (691) and anti-ERK 3 (b) and anti-MEK1, anti-MEK2 and anti-MEK 1 and 2 (c). Molecular weight markers in kilodaltons are indicated on the left.

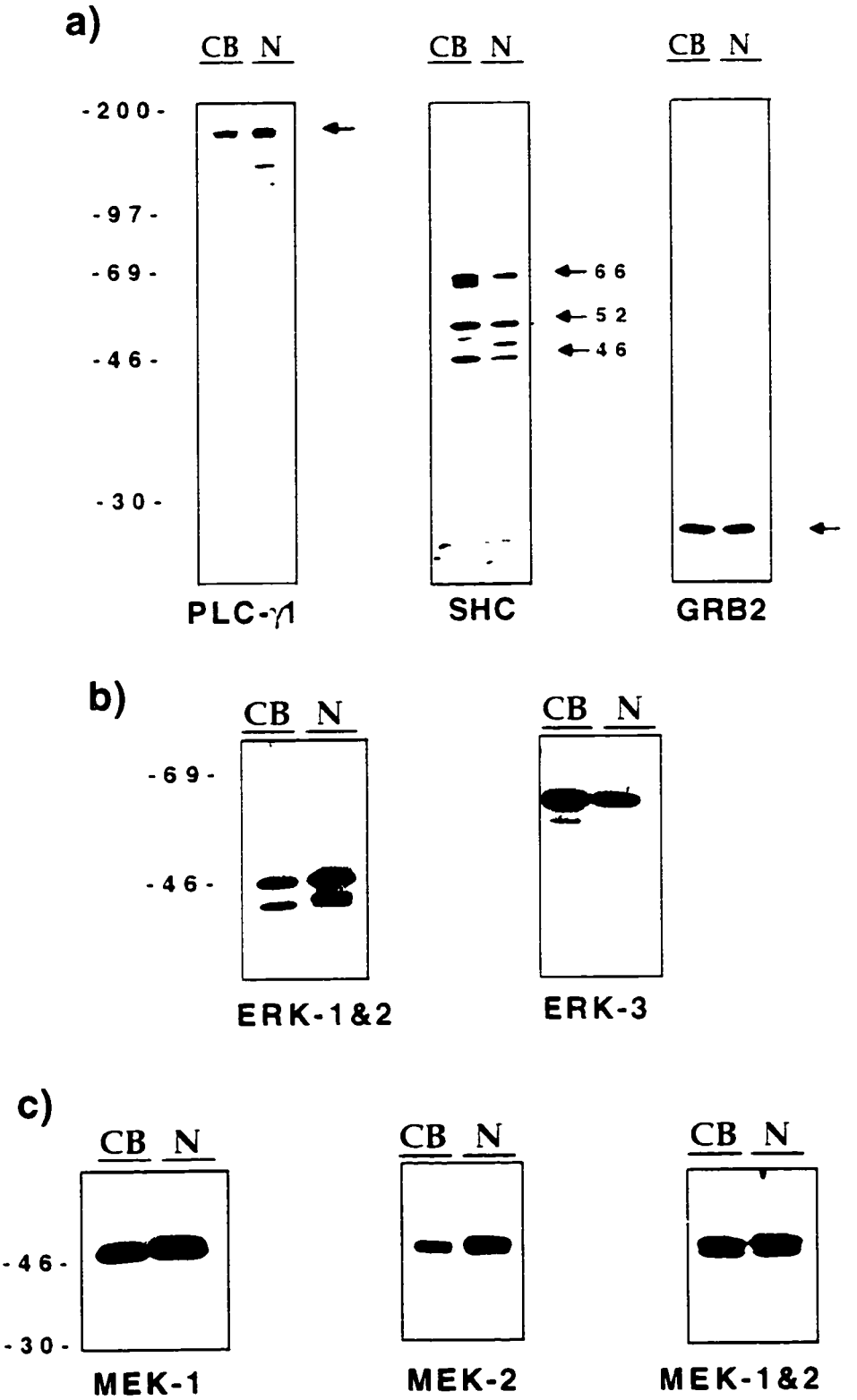

a)
CB N
CB N
CB N
-200-
-97-
-69-
-46-
-30-
66
52
46
PLC-γ1
SHC
GRB2
b)
CB N
CB N
-69-
-46-
ERK-1&2
ERK-3
c)
CB N
CB N
CB N
-46-
-30-
MEK-1
MEK-2
MEK-1&2

Figure 9. Local inhibition of NGF-induced ERK activation by MEK inhibitor PD98059 in compartmented cultures of rat sympathetic neurons.

Compartmented cultures of rat sympathetic neurons were given NGF-free medium for two hours prior to incubation with either 75 μM PD98059 or equivalent DMSO (control) for 30 minutes. Cultures were then given 100 ng/ml NGF for 10 minutes. Cell extracts were collected directly into SDS sample buffer, separated on SDS polyacrylamide gels (10%) and immunoblotted with anti-active ERK (a) and anti-ERK-1 (b). To insure comparability between treatments, all cultures used were sister cultures and each group contained extracts pooled from 2 cultures. Cell extracts equivalent to one culture was loaded on each lane.

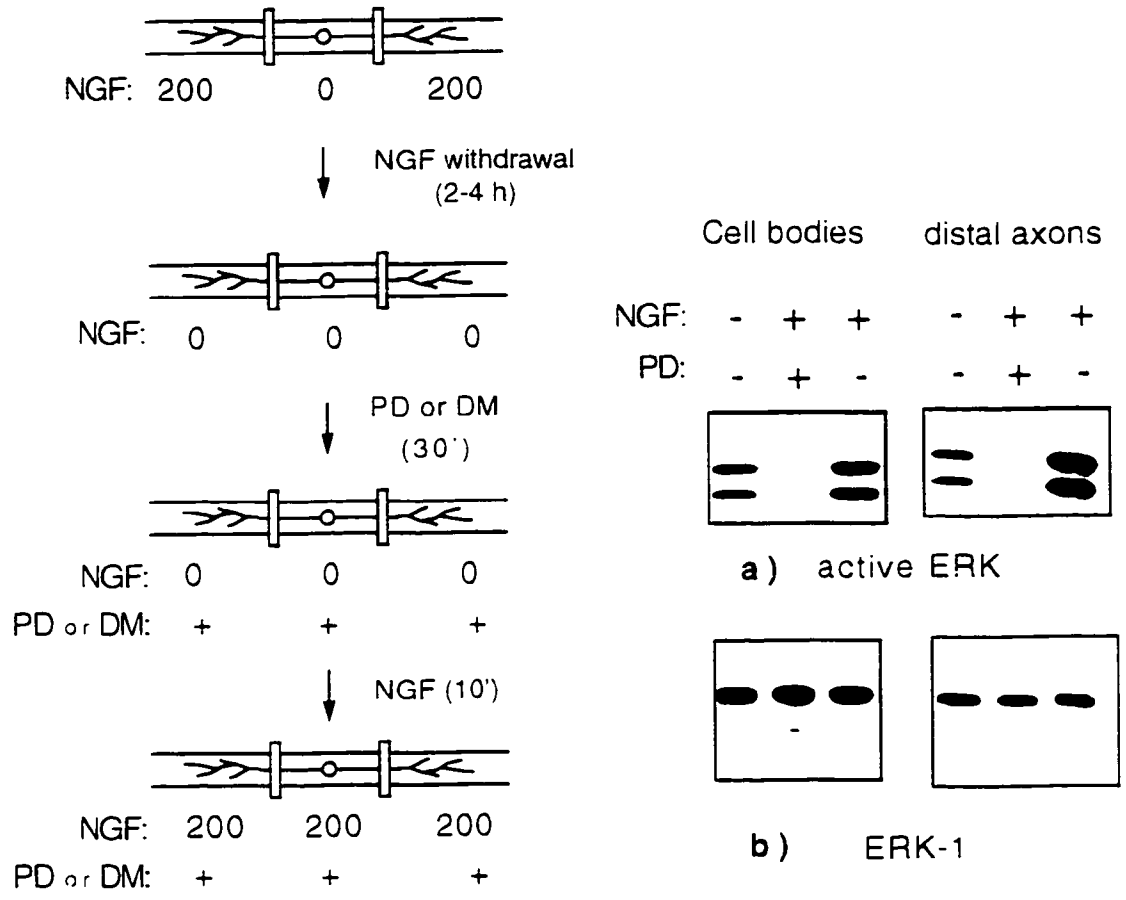

NGF: 200 0 200
NGF withdrawal
(2-4 h)
NGF: 0 0 0
PD or DM
(30')
NGF: 0 0 0
PD or DM: + + +
NGF (10')
NGF: 200 200 200
PD or DM: + + +
Cell bodies
distal axons
NGF: - + + - + +
PD: - + - - + -
a) active ERK
b) ERK-1

Figure 10. Time course of ERK-1 and ERK-2 activation in cell bodies/proximal axons and distal axons of sympathetic neurons in response to distal application of NGF.

Cultures grown for two weeks in 10 ng/ml NGF in all compartments were given NGF-free medium for 2-4 hours followed by either 0 NGF to distal axons, 200 ng/ml NGF to distal axons for times indicated or 200 ng/ml NGF for 30 minutes in all compartments (G_{30m}). Extracts from cell bodies/proximal axon (PAx) compartments and distal axon compartments were collected directly into sample buffer, separated on SDS polyacrylamide gels (10%) and immunoblotted with anti-active ERK (a and b) and anti-ERK-1 (c and d).

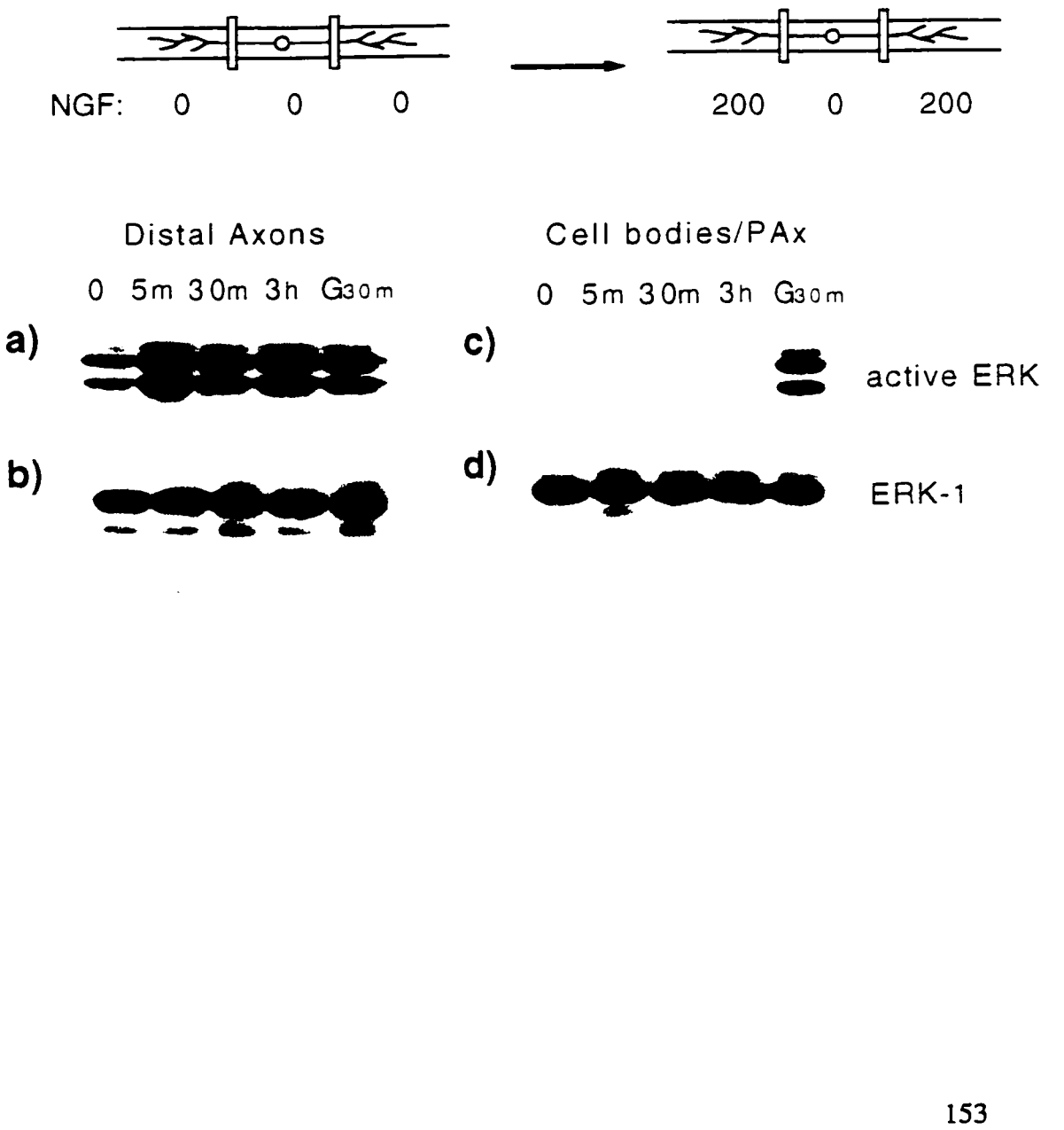
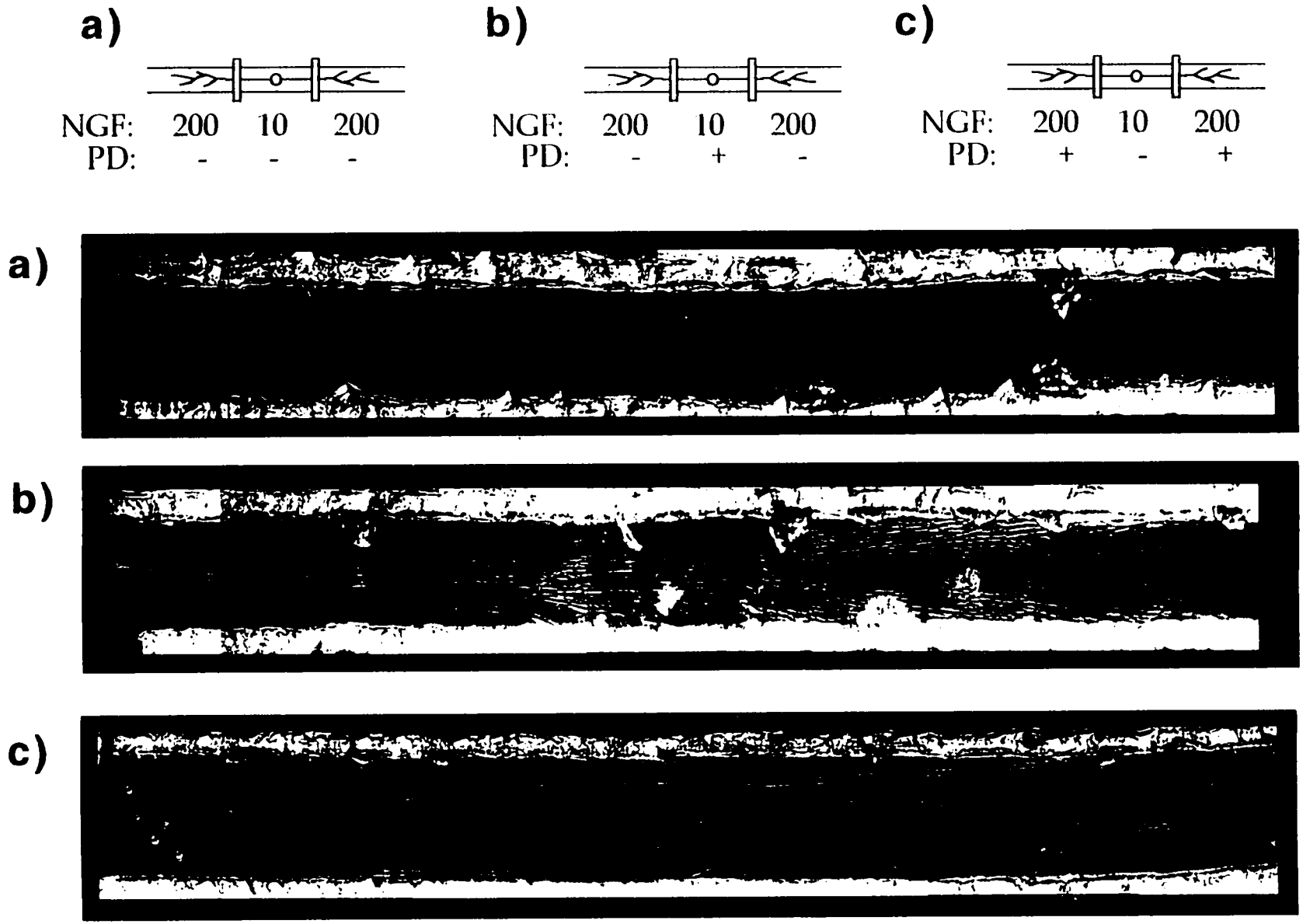

NGF: 0 0 0
200 0 200
Distal Axons
0 5m 30m 3h G30m
Cell bodies/PAx
0 5m 30m 3h G30m
a)
b)
c)
d)
active ERK
ERK-1

Figure 11. Local inhibition of ERK-1 and ERK-2 activation by PD98059 severely disrupts sympathetic axon growth.

Photomicrograph of a single track in compartmented cultures treated with: 0.75% DMSO in all compartments (control) (a), 75 μM PD98059 in the center compartments (b), or 75 μM PD98059 in the distal compartments (c), for 18 hours. Cultures of rat sympathetic neurons were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the left and right compartment. On day 4 when axons from the center plated neurons had grown 1 to 2 mm into the distal compartments, cultures were given medium containing 0.75% DMSO (as a control) or 75 μM PD98059. To ensure the stability of the PD98059 inhibitor, media were replaced every 12 hours.


a)
NGF: 200 10 200
PD: - - -
b)
NGF: 200 10 200
PD: - + -
c)
NGF: 200 10 200
PD: + - +
a)
b)
c)

Chapter 4

Conclusions and Summary

Retrograde Signaling

Neurotrophin hypothesis revisited

In view of the vast number of publications in the neurotrophin field over the past 10 years, the simplistic model of NGF produced by a target tissue is sequestered by innervating neurons and transported to the cell body where it exerts its biological effect, appears to be a somewhat limited assessment. In fact, the number of cellular processes that are regulated by neurotrophins are much greater than previously believed. Peripheral neurons can derive trophic support from several sources including neuronal-target tissue (retrograde mechanisms), afferent neurons (anterograde mechanisms), glial cells or even themselves (autocrine or paracrine) (reviewed in Korsching, 1993). Furthermore, data presented in this thesis raise questions as to the necessity of retrograde transport for neurotrophin signaling. Therefore, it is important to re-examine the neurotrophin hypothesis and incorporate new evidence and ideas into our working model.

Retrograde mechanisms: vesicular, transported or propagated

At present, two prominent models for the transmission of a retrograde signal have been proposed. First, NGF binds to its receptor on the axon terminal, is internalized, and an endocytotic vesicle carrying an activated NGF/receptor(s) complex, is transported via a microtubule-based mechanism to the cell body where the NGF/receptor complex can activate signaling

proteins within proximity to the nucleus (vesicular hypothesis). This hypothesis predicts that the arrival of a retrograde signal would coincide with the retrograde transport of NGF. Previous experiments using sympathetic neurons grown in 5-compartmented cultures established that the rate of retrograde transport of ^{125}I-NGF calculated over a 5 mm segment of axon, was 10-20 mm/hr (Ure and Campenot, 1997). Therefore, it would require 3 to 6 minutes for NGF to cross a 1 mm barrier. This rate however, does not include the time necessary for internalization and loading of NGF onto the retrograde transport mechanism. Data presented here, showed that no retrogradely transported ^{125}I-NGF was detected until 30 to 60 minutes after NGF binding to the distal axon of sympathetic neurons. This is consistent with previous evidence in sympathetic neurons showing a 1-h lag between the time of ^{125}I-NGF binding to the distal axons and internalization and loading of ^{125}I-NGF on to the retrograde transport system (Ure and Campenot, 1997). Therefore, according to the vesicular hypothesis, the appearance of tyrosine phosphorylated trkA in the cell bodies/proximal axons should not be detected within the first hour following NGF binding to the distal axons. Remarkably, the retrograde appearance of trkA was seen within 1 minute of distal NGF application. In addition, several other tyrosine phosphorylated proteins were seen within 5-15 minutes including several proteins apparently localized to the cell bodies. Furthermore, since the proximal axons make up only a very small fraction of the total cell body extract, it is likely that all retrograde phosphorylations observed occur in the cell bodies. Therefore, these data suggest that the appearance of tyrosine phosphorylated proteins in the cell bodies of sympathetic neurons preceded the retrograde movement of the NGF

that induced them. Moreover, even if the time necessary for internalization and loading onto the transport mechanism is excluded, the transport of activated trkA from just outside the compartment barrier to just inside the barrier would take 3 to 6 minutes. Thus it is extremely unlikely that the small amount of trkA just outside the barrier alone could produce a measurable amount of trkA phosphorylation within 1 minute. If the appearance of phosphorylated trkA does not occur via the delivery of trkA from the distal axons, then the phosphorylated trkA in the cell bodies/proximal axons must have occurred via the phosphorylation of trkA already present. Moreover, these results imply that the phosphorylated trkA molecules in the cell bodies/proximal axons were not bound to NGF. Rather, it would appear that the phosphorylation of trkA in the cell bodies occurred via an intracellular mechanism that did not require ligand binding.

The second hypothesis states that the binding of NGF to its receptor(s) on the cell surface results in the generation of an active second messenger molecule that is transported to the cell body where it exerts its biological effect (transport hypothesis). According to this hypothesis, the rate by which the retrograde signal would move along the axon does not include the time required for internalization and loading onto the transport system. Therefore, transmission of a retrograde signal should correspond with the time required to transport an activated molecule. Assuming that all retrogradely transported molecules move at a similar velocity i.e. 10-20 mm/hr, the transport of any molecule across the 1 mm compartment barrier would require 3 to 6 minutes. Therefore, these results suggest that the appearance of tyrosine phosphorylated trkA in the cell bodies/proximal axons, within one

minute, precedes the arrival of any molecules. These data provide compelling evidence that the rapid phosphorylation of proteins retrograde to the site of NGF binding occurs via a mechanism that can not be accounted for by conventional retrograde transport. Instead, I propose that the phosphorylation of trkA in the cell bodies/proximal axons occurred via a propagated signal.

The data presented in this thesis provides the first evidence for a rapid NGF-induced phosphorylation signal that precedes the arrival of endocytotic vesicles containing the NGF that induced them. Consistent with these results, a recent *in vivo* study showed that BDNF injected into the gastrocnemius muscle produced a rapid increase in both the catalytic activity and the phosphorylation state of trkB in rat sciatic nerve (Bhattacharyya et al., 1997). Following injection of BDNF into the gastrocnemius muscle, the sciatic nerve innervating the muscle was collected at 10, 30 and 60 minutes after injection. Protein extracts from sciatic nerve segments were immunoprecipitated with anti-pY490 (antibody developed against a peptide corresponding to the phosphorylated SHC binding site of the trk receptor) and immunoblotted with anti-phosphotyrosine and anti-trkB. Phosphorylated trkB receptors were found in sciatic nerve segments 1 to 3 cm away from the injection site within 10 minutes. The appearance of phosphorylated trkB receptors occurred at a rate of 8 to 16 μm/sec. This rate is consistent with the appearance of retrogradely phosphorylated trkA in sympathetic neurons where the phosphorylation of trkA, 1 mm away from the site of NGF binding, occurred within 1 minute. In addition, this rate is substantially faster then the 0.7-2 μm/sec velocity of retrograde transport reported in sciatic nerve *in vivo*

(Richardson and Riopelle, 1984). Moreover, the phosphorylated trkB was shown to bind the signal-generating molecule SHC suggesting that these receptors are catalytically active. These data therefore suggest that the activation of trkB occurred too rapidly to be accounted for by conventional retrograde transport. Instead, these results support the hypothesis that retrogradely phosphorylated trk occurs via a mechanism that has a velocity substantially faster then vesicular transport. Bhattacharyya et al., (1997) have also shown that 24 hours after ligation of the sciatic nerve, activated trk receptors are present distal to the ligation. The appearance of the phosphorylated trk colocalized with clathrin, a protein component of coated vesicles. Therefore they conclude that the activated trk receptors are moving by retrograde vesicular transport. This result is similar to previous observations showing that activated trkA receptor accumulates proximal to a sciatic nerve ligation after 18 hours (Ehlers et al., 1995). However, the fact that activated trk is vesicle associated after 24 hours has no bearing on the appearance of retrogradely phosphorylated trkB seen within 10 minutes. In fact, it is possible that trk is involved in the transmission of two different retrograde signals, one that rapidly propagates along the axon and a second slower signal that is carried via an endocytotic vesicle.

In an independent study using the compartmented culture system, Riccio et al., provided further support for a rapid retrograde signal. They demonstrated that in addition to the activation of trkA phosphorylation at the cell bodies of sympathetic neurons, distal NGF also produced phosphorylation of the nuclear transcription factor CREB (cyclic adenosine monophosphate response element-binding protein) (Ricco et al., 1997). Previous evidence has

shown that CREB is a key target of NGF-induced signaling in PC12 cells (Bonni et al., 1995; Ginty et al., 1994). Immunohistochemistry using an antibody that recognizes the phosphorylation of CREB on ser^{133} (pCREB), showed that direct exposure of cell bodies to NGF resulted in 30% of the neurons pCREB positive within 5 minutes and 75% positive by 10 minutes. When NGF was supplied to the distal axons (1-2 mm away from the cell bodies), 20% of the neurons showed pCREB nuclear staining within 10 minutes of distal NGF and 75% by 20 minutes. Even though the activation of CREB occurred at a slower rate than trkA phosphorylation, the initial phosphorylation of CREB nevertheless precedes the arrival of the NGF-containing vesicles that induced them (Senger and Campenot, 1997; Ure and Campenot, 1997).

The rapidity of the pCREB signal disputes the argument that the phosphorylation of CREB occurred via the vesicular transport of an activated NGF/trkA complex. However, Ricco et al., present additional data suggesting that the activation of pCREB does occur via a vesicular mechanism. For these experiments NGF was covalently coupled to 1 μm-diameter microspheres to prevent both internalization and transport of NGF. NGF-coupled beads applied to the distal axons of sympathetic neurons in compartmented cultures failed to induce the phosphorylation of CREB (Ricco et al., 1997). However, several issues arise with respect to the bead experiment. The most significant was that when a direct comparison of native NGF and NGF-coupled beads was performed, the NGF-coupled beads were shown to activate trkA to a substantially lower level then the native NGF (Ricco et al., 1997). Therefore, it is possible that the retrograde activation of pCREB was not seen because a fewer number of receptors were activated and in this way, a threshold for

retrograde signaling was not reached. Furthermore, substantial evidence supports the hypothesis that the ratio between p75 and trkA plays a significant role in NGF-induced signaling (reviewed in Kaplan and Miller, 1997). If the binding of NGF to the microsphere in any way alters the relative affinity of NGF for p75 or trkA, the final outcome could have significant impact on the NGF-induced signaling pathways initiated. Thus, it is hard to judge the validity of this experiment. Even though, the interpretation of these studies vary, two important points are clear: First, this evidence indicates that trk acts as a retrograde signal carrier (Bhattacharyya et al., 1997; Riccio et al., 1997; Senger and Campenot, 1997) and second, trk is involved in the propagation of a signal to the celi body (Riccio et al., 1997; Senger and Campenot, 1997).

While the extreme speed of these retrograde phosphorylations can not be accounted for by the present NGF hypothesis, these data do not rule out the possibility that some retrograde signals are carried via molecular or vesicular transport. In fact, two recent studies investigating the role of trkA in NGF-induced signaling provide data that support the NGF vesicular hypothesis. Using rat sciatic nerve as a model, Ehlers et al. demonstrated that following nerve ligation, injection of NGF into the footpad of a rat enhances accumulation of trkA distal to a ligation (Ehlers et al., 1995). This accumulation of trkA is thought to result from the build up of retrogradely transported vesicles carrying activated NGF/trkA receptors. To ensure that the accumulation of tyrosine phosphorylated trkA at the site of the ligation was due to retrograde transport of activated trkA, anti-NGF injected into the footpad was shown to reduce the accumulation of tyrosine phosphorylated trkA, providing evidence that trkA is retrogradely transported.

In addition, in PC12 cells, evidence was provided for the continuation of trkA signaling following internalization and endocytosis of the trkA receptor. Grimes et al. (1996) have shown that unprimed PC12 cells treated with NGF, internalized their surface trkA receptors upon warming from 4°C to 37°C. Using fractionation techniques they were able to show that the cellular fraction enriched in intracellular endocytotic organelles was also enriched in tyrosine phosphorylated trkA. In addition, the tyrosine phosphorylated trkA was bound to PLC-γ1 suggesting that the receptors were competent to initiate signaling.

Taken together, these data provide added support for the vesicular hypothesis and suggest that vesicle associated trkA retains the ability to initiate signals as it moves retrograde through the axon toward the neuronal cell body. However, it is important to point out that trkA activation in the adult sciatic nerve and trkA activation in neonatal DRG, SCG or PC12 cells, have been shown to express different forms of trkA distinguished by their mobility on polyacrylamide gels (Ehlers et al., 1995; Grimes et al., 1995; Senger and Campenot, 1997). Immunoprecipitation with trkA specific antibodies demonstrated that the majority of trkA in adult DRG and sciatic nerve occurs as a 180 kDa molecule while neonatal DRG and sympathetic neurons express the 110 and 140 kDa forms of trkA (Ehlers et al., 1995; Senger and Campenot, 1997). The 110 kDa trkA species is presumed to be an intermediate between the 80 kDa polypeptide chain and the 140 kDa glycosylated protein (Martín-Zánca et al., 1989). The basis of the 180 kDa protein seen in adult DRG and sciatic nerve is thought to occur by differential post translational modification of the 140 kDa protein (Ehlers et al., 1995). It

is possible that the 180 kDa form of trkA and the 140 kDa form have different roles in NGF-induced signaling.

The mechanisms of rapid signal propagation

Although it has previously been proposed that phosphorylation reactions can provide a mechanism for the interaction of growth factors at the nerve terminal to transmit a signal to the cell body via the specific alterations of second messenger proteins along the axons (Greengard, 1987), very little evidence has been provided to support this concept. Rather, the fact that a phosphorylation signal in neurons may have to travel distances in excess of a meter, has lead to the hypothesis that these signals require a more stable mechanism of transmission. Therefore, for many years the idea that NGF complexed with its receptor(s) must be transported to the cell body to exert its biological effects has maintained substantial support as the mechanism by which neurotrophins elicit cell body responses. Data presented in this thesis, provide the first evidence that some trophic factors may have the ability to activate phosphorylated signals that can rapidly propagate along the axon toward the cell body. In addition, these data suggest that this rapid propagation involves the activation of proximal trk receptors via a mechanism that does not require ligand binding. The idea of a cell surface receptor being activated in the absence of ligand binding has some precedence. In PC12 cells, Ca^{++} influx through voltage-gated calcium channels results in the phosphorylation of the EGF receptor (EGFR) to a level that can induce the activation of MAPK in the absence of EGF (Rosen and Greenberg, 1996). Calcium influx was shown to lead to tyrosine phosphorylation of the EGFR

within 20 seconds. Although the underlying mechanism is not known, activation of a cytoplasmic tyrosine kinase such as Src may lead to the phosphorylation of the intracellular domain of the EGFR. Therefore, a possible mechanism by which the rapid retrograde phosphorylation of proteins in sympathetic neurons occurs may be via the propagation of a calcium wave that results in the activation of an intracellular tyrosine kinase that in turn activates trk present at the cell body via a ligand-independent mechanism. Differential expression of calcium-responsive signaling intermediates or different modes of calcium entry into the cell i.e. extracellular or intracellular, could provide differential regulation of signaling responses (Ghosh and Greenberg, 1995). The entry of calcium into the cytosol can be regulated by multiple mechanisms, thus allowing for the spatial and temporal control of Ca^{++} by growth factors that act at the cell surface.

trk receptors themselves can also be activated in the absence of ligand. Overexpression of trk in PC12 cells results in the increased phosphorylation of trk in the absence of NGF (Hempstead et al., 1992). This increase in trkA phosphorylation is thought to occur due to an increased probability of trk dimerization in the plasma membrane. Also PC12 cells exposed to the ganglioside, GM1 in the absence of NGF were shown to have increased activation of trk (Ferrari et al., 1995; Mutoh et al., 1995). Gangliosides are a class of glycosphingolipids mainly associated with the plasma membrane and particularly abundant in the nervous system (Skaper et al., 1989). GM1 has been shown to maintain the long-term survival of NGF-deprived sympathetic neurons (Ferrari et al., 1993) due to the stimulation of trk receptor dimerization and autophosphorylation (Ferrari et al., 1995; Mutoh et al.,

1995). Since trk can be activated in the absence of ligand, other speculative scenarios for the rapid retrograde phosphorylation of trk may include self-propagated phosphorylation of trk molecules along the length of the axon, or the self-propagated inhibition of a tyrosine phosphatase such as SHP-1.

Evidence that more than one retrograde signal exists

One of the intriguing aspects of neurotrophic proteins is that a single trophic factor such as NGF can elicit such a large variety of responses in a single neuron (Levi-Montalcini, 1987). Therefore, it is important to realize that several different retrograde mechanisms likely exist simultaneously to control the numerous biological effects initiated by neurotrophic factors. Abundant evidence already exists to support the hypothesis that there is more than one retrograde signal. For example, target-derived NGF regulates the amount of target innervation which determines the amount of sympathetic neuron survival, with excess NGF leading to developmental rescue of sympathetic neurons (target-derived neurotrophin) (Hendry and Campbell, 1976). In contrast, sympathetic-neuron-derived BDNF regulates neuronal hypertrophy and preganglionic innervation without effecting the number of neurons that survive (neuron-derived neurotrophin) (Causing et al., 1997). Although both neurotrophins are producing retrograde signals, the neuronal response to these neurotrophins is significantly different. Therefore, neuron-derived neurotrophin may use an alternative mechanism to produce dynamic remodeling of preganglionic innervation. Moreover, neuron-derived BDNF can act directly on the sympathetic neuron itself. Exposure of sympathetic neurons to BDNF produced c-Jun phosphorylation at the nucleus via the p75

neurotrophin receptor (Kaplan and Miller, 1997). These data suggest that the sympathetic neuronal response to BDNF occurred via a non-vesicular mechanism since BDNF is not retrogradely transported by sympathetic neurons (Ure, 1997). In addition, it is becoming more apparent that the regulation of survival by neurotrophins can be separated from the regulation of other functions such as synaptic efficacy. Differential control of survival and synaptic input is evident in basal forebrain cholinergic neurons, the prototypical NGF-responsive neuron in the CNS. In NGF and trkA knockouts the basal forebrain cholinergic neurons survive for at least the first month of life (Crowley et al., 1994; Smeyne et al., 1994). However, these neurons are atrophied and synthesis of the neurotransmitter enzyme choline acetyltransferase is decreased (Crowley et al., 1994). One suggestion for this difference may be that the retrograde transport of a neurotrophin itself is important for survival promoting effects, while the rapid propagation of a non-vesicular non-transported retrograde signaling via the same neurotrophin or even a different neurotrophin may be more appropriate for the activation of signals important for the regulation of dynamic remodeling. Existence of a rapid mechanism for synaptic rearrangement would be appropriate in the CNS where synaptic connections between a neuron and its target are constantly changing (reviewed in Lo et al., 1995; Thoenen et al., 1995).

Although the data presented in this thesis support the hypothesis that the generation of a retrograde signal occurs via a propagated mechanism, it is abundantly apparent that this will not be the only mechanism by which NGF evokes retrograde effects. Rather, it seems that multiple retrograde mechanisms may be used to initiate any number of NGF-induced retrograde

signals. In this way, the neuron would have several different mechanisms that can be used to relay a signal from the neuron terminal to the cell body. Moreover, these signals may even act synergistically to elicit the full biological response.

Activation of local mechanisms by NGF

Traditionally NGF is considered a target-derived growth factor that binds to the distal terminals of neurons and initiates signaling pathways that are important for survival. However, many of NGF's biological effects may be mediated at the level of the plasma membrane via the generation of one or more second messengers. Experiments examining the fate of NGF following its binding to cell surface receptor(s) show that a very large percentage of NGF bound to the plasma membrane of sympathetic neurons is not transported back to the cell bodies, but instead, remains bound to the distal axons (Liu et al., 1996; Ure and Campenot, 1997). NGF bound to distal axons may therefore have other functions in addition to retrograde signaling. Previous experiments using sympathetic neurons grown in compartmented cultures have shown that NGF-induced axon growth occurs via mechanisms localized within the distal axons (Campenot 1977, 1982, 1987, Campenot et al., 1994). These observations support the hypothesis that some biological effects of NGF are mediated at the level of the plasma membrane via the production of one or more second messenger(s) important for the regulation of NGF-mediated growth mechanisms. Data presented in this thesis provides further support for the idea that some signals important for axon growth are present locally within the growing axon terminal.

Role of ERK-1 and ERK-2 in sympathetic neurons

The impetus for investigating the role of ERK-1 and ERK-2 in sympathetic neurons began with the observation that in non-neuronal cells, activation of MAPK at the plasma membrane results in its translocation to the nucleus (Chen et al., 1992) where nuclear accumulation of MAPK results in phosphorylation of transcription factors (Blenis et al., 1993). In this way, MAPK activation translates into changes in gene expression at the nucleus. Therefore, it was quite surprising when activation of ERK-1 and ERK-2 in distal axons of sympathetic neurons did not result in the movement or activation of ERK-1 or ERK-2 in the cell bodies/proximal axons. Instead, evidence provided here suggests that NGF-induced activation of ERK-1 and/or ERK-2 is important locally within the growing nerve terminal. Substantial evidence from several different neuronal cell systems suggests that the pathways leading to axon growth and survival diverge. For example, the inhibitor 6-thioguanine which blocks the activation of some NGF-induced pathways in PC12 cells (Volonté et al., 1989) was shown to inhibit growth but not survival in rat sympathetic neurons (Greene et al., 1990). Conversely, in E8 chick nodose ganglia, a chimeric pan-neurotrophin was shown to induce axon growth but not survival (Ibáñez et al., 1993). Consistent with this evidence, inhibition of ERK-1 and ERK-2 activation via the MEK inhibitor PD98059 locally within the distal axons of sympathetic neurons, severely disrupted distal axon growth. However, PD98059 applied to the cell bodies/proximal axons had no apparent inhibitory effects on axon growth. Furthermore, a recent study showed that blocking the activation of ERK-1 and ERK-2 via PD98059 had no effect on sympathetic

neuron survival (Creedon et al., 1996). Therefore, it would appear that ERK-1 and/or ERK-2 may be downstream messengers that are involved in the regulation of growth mechanisms localized within the nerve terminal, possibly at the growth cone. These data in no way rule out the possibility that ERK-1 and ERK-2 have alternative roles in sympathetic neurons, but they do suggest that at least one role for ERK-1 and ERK-2 could conceivably involve the control of growth mechanisms locally within the nerve terminal.

Differential regulation of trkA and p75

Mass culture experiments showed that in cultured sympathetic neurons NGF induced a long-lasting trkA autophosphorylation that in addition to sustained activation, increased over a broader range of NGF concentrations than observed in PC12 cells. trkA responded to increasing concentration of NGF ranging from 10 to 100 ng/ml and once activated remained phosphorylated upwards of 24 hours. These results contrast those obtained in wild-type PC12 cells, where trkA tyrosine phosphorylation was maximal at 10 ng/ml NGF (Kaplan et al., 1991), highest at 5 minutes following NGF treatment, and declined to basal level in untreated cells after exposure to NGF for 2 hours (Hempstead et al., 1992; Qui and Green, 1992; Kaplan et al., 1991). A more recent study suggests that this decrease in trkA phosphorylation is due to increased degradation of trkA in PC12 cells rather than dephosphorylation (Zhou et al., 1995). Despite the large response to increasing NGF concentrations and the sustained tyrosine phosphorylation of trkA in sympathetic neurons, levels of trkA protein do not change (Belliveau et al., 1997; Toma et al., 1997). Also, mRNA for trkA remains unaffected (Toma et

al., 1997). A graded response of sympathetic neurons to NGF would provide the neuron with the ability to modulate the level of trk activation in response to differences in the amount of available NGF supplied either by a target tissue or by surrounding Schwann cells during regeneration. Moreover, sustained activation of trkA is consistent with mediating a long-lasting trophic signal.

Conversely, mRNA for p75 increases substantially with NGF concentrations ranging from 10 to 200 ng/ml (Ma et al., 1992, Toma et al., 1997), and data presented here show that the level of p75 protein also increased with increasing concentrations of NGF, paralleling the mRNA results. The ratio of trkA to p75 may effect the response of the neuron to NGF either by enhancing the activation of trkA (Barker and Shooter, 1994; Verdi et al., 1994), or by cross talk between the two pathways initiated by the individual receptors (Kaplan and Miller, 1997). Therefore, the cellular ratio and localization of the receptors may be critical for NGF signaling, especially when the two receptors can be independently regulated as shown in sympathetic neurons. In the present thesis, compartmented cultures were grown under two different NGF regimes: 1) cultures were grown in 10 ng/ml NGF in all compartments for two weeks or 2) cultures were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments, and after 1 week NGF was withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments. Therefore the ratio between trkA and p75 in cultures grown in low NGF (10 ng/ml) will be substantially different than in the cultures grown in high NGF (200 ng/ml), as the cultures grown in high NGF will have a substantially higher concentration of p75. Despite this variation, no obvious difference was seen in tyrosine phosphorylated proteins

in response to NGF. Since many proteins are believed to be phosphorylated in response to trkA activation this was not surprising. However, it is important to be aware that differences may exist but were undetected by the assays used here.

The basis for the specificity of neurotrophin action

Most studies of neuronal proliferation and differentiation pathways in PC12 cells, have found very few differences in second messengers that are activated even though the final outcome is substantially different, the production of mitogenesis rather than differentiation. A key question in developmental neurobiology is what can account for these differences? Two hypothesis have been proposed: 1) the duration of response to the growth factor, i.e. sustained vs transient signals; 2) the existence of unique targets.

First hypothesis: duration of signal activation: sustained vs transient signal

It has been hypothesized that prolonged activation of second messengers by NGF may be one of the deciding factors between a mitogenic and differentiation pathway (reviewed in Chao, 1992; Marshall, 1995). In support of this hypothesis, studies using PC12 cells transfected with trk (trk-PC12; overexpressing trk by 20-fold) (Hempstead et al., 1992) or oncogenic N-Ras (UR61J cells) (Qui and Green, 1992) showed increased neuronal differentiation along with sustained tyrosine phosphorylation of a number of trk kinase targets including PLC-γ1, ERK-1 and ERK-2. Consistent with this idea is the observation that sympathetic neurons induced by NGF not only give rise to sustained phosphorylation of trkA (upwards of 24 hours), but also the

sustained phosphorylation of several other proteins including: PLC-γ1, SHC, ERK-1 and ERK-2. The fact that trkA and several additional proteins remain phosphorylated may be pertinent to the initiation of NGF-mediated biological responses.

Although sustained activation of signaling proteins by trkA has been hypothesized to be an important component of differentiation, not all evidence agrees with this. For example, sustained activation of the Ras signaling pathway in embryonic chick sympathetic neurons is insufficient for neurotrophin-mediated neuronal differentiation events (Borasio et al., 1993). Previous evidence has shown that differences in cell type and developmental stage can influence the outcome of neurotrophin signaling (discussed in chapter 3). Therefore, it is highly probable that sustained phosphorylation alone may be insufficient for making the decision between proliferation and differentiation. Moreover, once the neuron is fully differentiated, it is likely that sustained phosphorylation has a different biological function. For example, prolonged activation of signaling pathways in neuroblasts may be important for the induction of differentiation pathways. Whereas, prolonged activation of proteins in fully differentiated sympathetic neurons may serve to maintain catalytically active forms of proteins that can enhance signaling in cells chronically exposed to neurotrophin. Many questions surrounding the actual underlying mechanisms for sustained phosphorylation, still need to be answered.

Second hypothesis: unique targets of trk

In addition to sustained activation of signaling pathways, it has been hypothesized that NGF-induced differentiation may occur via the activation of differentiation specific pathways. Two examples of such pathways have been discovered. The first came with the discovery of a 90 kDa protein, designated SNT that was shown to be tyrosine phosphorylated in response to NGF in PC12 cells and cortical neurons, but not by EGF (Rabin et al., 1993). The second, and more recent discovery, is a protein tyrosine phosphatase (SHP-1) which in PC12 cells is also phosphorylated on tyrosine in the presence of NGF, but not EGF (Vambutus et al., 1996).

In sympathetic neurons, several proteins were shown to be tyrosine phosphorylated in response to NGF. The identification of some of these proteins have been determined, including trkA, PLC-γ1, SHC, ERK-1, and ERK-2. However, several proteins are yet to be identified. The possibility exists that some of these proteins are unique targets of NGF-induced signaling. Identification of these proteins will be an important focus for future research and may provide substantial clues to the understanding of neuronal cell fate. Moreover, the issues regarding which of these hypotheses are correct remain to be resolved. Factors leading to the decision between mitogenesis and differentiation will most likely be decided by a composite of both hypotheses.

Localization of signaling proteins

Using compartmented cultures, the localization of several second messenger signaling proteins including: trkA, PLC-γ1, SHC (46, 54 and 66 kDa), MEK (1 and 2), and ERK (1, 2 and 3) was determined. All of these proteins

were found to be present in both the cell bodies/proximal axons and the distal axons/terminals. This is consistent with the localization of NGF receptors on the nerve terminals, along the axon and at the cell body in cultured sympathetic neurons (Kim et al., 1979; Toma et al., 1997). Thus the neuron should be capable of responding to neurotrophin present anywhere along the neuron. For example, after nerve injury, Schwann cells surrounding the injured neuron quickly upregulate their expression of NGF (Bandtlow et al., 1987) providing a source of trophic factor to the injured neuron. One would predict, that the regenerating nerve could sense increases of NGF at the site of injury, and instantly activate local signaling proteins required for biological responses important for growth and survival of the injured neuron. On the other hand, distribution of signaling proteins along the length of the neuron, may be important for the transmission of a retrograde signal from the nerve terminal to the cell body. Either way, the relative uniform distribution of signaling proteins within the neuron suggests that these proteins are potential candidates for the generation of both local and retrograde signals.

Conclusion

The experiments presented in this thesis have brought into question the role of retrograde transport of NGF/receptor complexes as a mechanism for carrying a retrograde signal. Here I have provided evidence to support the hypothesis that at least some signals generated by the binding of NGF at the nerve terminal precede the retrograde movement of NGF. Although evidence to support the physiological importance of a rapid retrograde phosphorylation signal is yet to be determined, these data provide evidence that there may be

mechanisms in place that can deliver many kinds of retrograde signals to the cell bodies/nucleus of neurons without the retrograde transport of signaling molecules. In addition, these data provide additional support for the local nature of NGF-induced growth mechanisms. It is important to think of NGF signaling as a multifaceted process where a variety of mechanisms may be involved in producing any number of biological effects. In any given neuron, NGF may participate in local signals either at or near the site of NGF interaction with the cell surface or retrograde signals elicited directly by NGF/receptor(s) complexes or via a non-vesicular propagation mechanism back to the cell body.

The fact that NGF belongs to a family of structurally similar growth factors, and that all of these factors bind to receptors that are either structurally similar or share a receptor, suggests that the generality of the mechanisms used for NGF retrograde signaling may be broadened to incorporate signaling by other neurotrophins as well. Discovery the mechanisms underlying NGF-induced retrograde signals is of vital importance for the enhancement of neuronal repair following spinal cord injury or the clinical treatment of degenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS). Ultimately this knowledge will provide insight for the development of new strategies that have critical and effective therapeutic value.

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APPENDIX 1

Figure 1. NGF induced a concentration-dependent increase of the p75 neurotrophin receptor in sympathetic neurons.

Two week old cultures grown from first day of plating in varying concentrations of NGF ranging from 10 to 200 ng/ml were isolated into RIPA buffer (1% triton X-100, 1% deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM tris (pH 7.2), 1mM sodium orthovanadate, 1 mM PMSF), and normalized for total protein using Bicinchoninic acid protein determination kit (Sigma). Equal amounts of protein were run on 10% SDS-polyacrylamide gels, and immunoblotted using p75 polyclonal antibody supplied by Phil Barker (Montreal Neurological Institute, Montreal, Quebec). Antibody was raised against a peptide with a sequence of amino acids in the rat p75 intracellular domain (SATLDALLAALRRIQR) located near the C-terminus. L cells (LC) were run as a negative control. Schwann cells (SC) and pB124 (pB) construct were run as positive controls. pB124 is an E. coli-derived fusion protein consisting of the intracellular domain of p75 fused to maltose binding protein. Molecular weight markers in kilodaltons are indicated on the right.

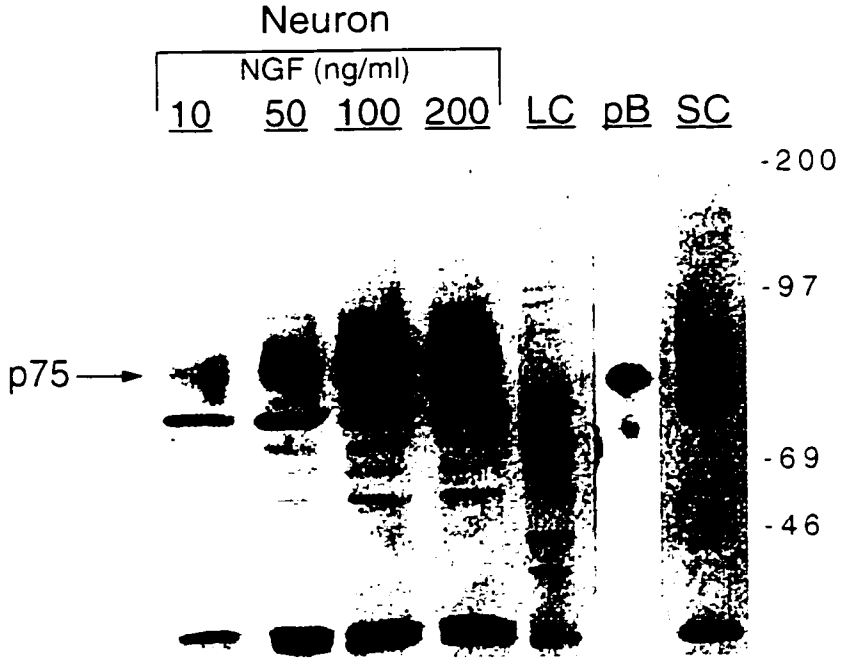
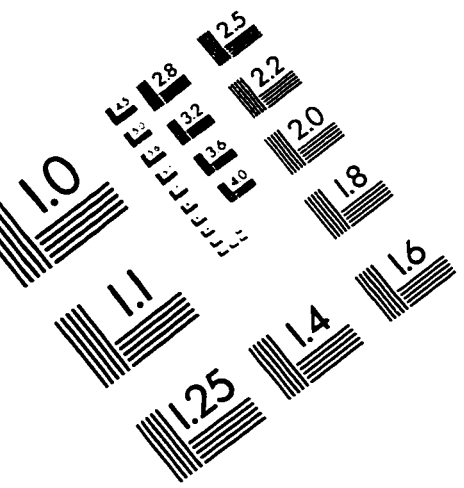
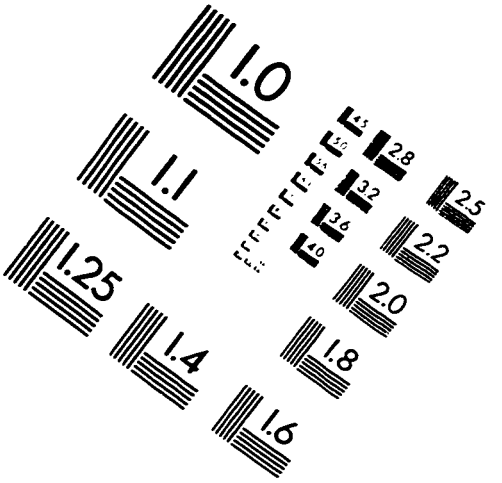
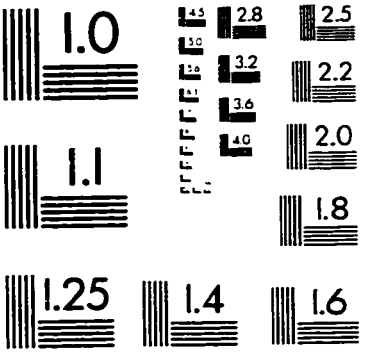
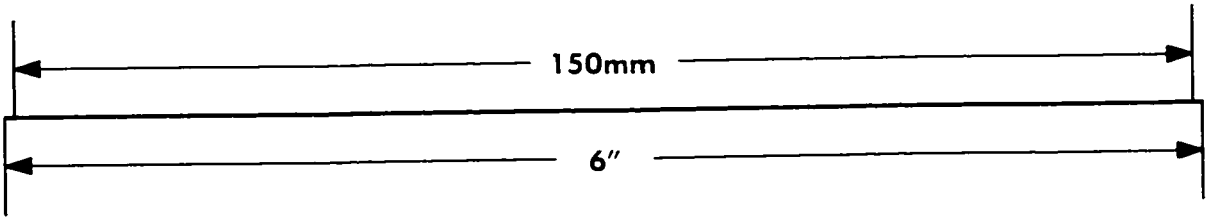

Neuron
NGF (ng/ml)
10
50
100
200
LC
pB
SC
-200
-97
-69
-46
p75

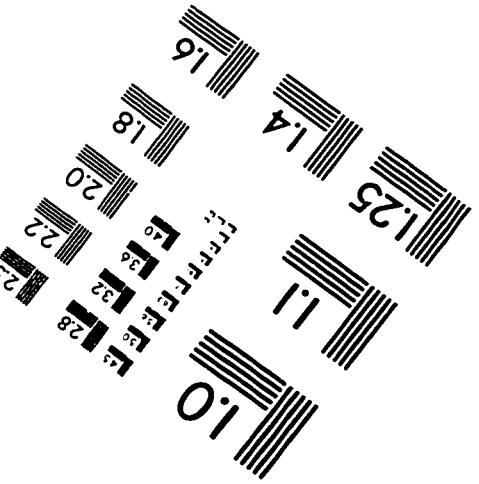
IMAGE EVALUATION
TEST TARGET (QA-3)

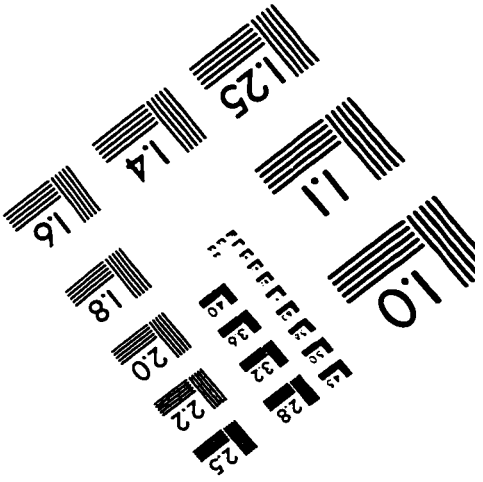












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