

Evidence in Support of Signaling Endosome-Based Retrograde Survival of Sympathetic Neurons

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Summary

The mechanism by which target-derived Nerve Growth Factor (NGF) signaling is propagated retrogradely, over extremely long distances, to cell bodies to support survival of neurons is unclear. Here we show that survival of sympathetic neurons supported by NGF on distal axons requires the kinase activity of the NGF receptor, TrkA, in both distal axons and cell bodies. In contrast, disruption of TrkA activity exclusively in proximal axonal segments affects neither retrograde NGF-TrkA signaling in cell bodies nor neuronal survival. Ligand-receptor internalization is necessary for survival of neurons supported by NGF on distal axons. Furthermore, antibody neutralization experiments indicate that retrogradely transported NGF, within cell bodies, is critical for neuronal survival but not for growth of distal axons. Taken together, our results indicate that retrogradely transported NGF-TrkA complexes promote sympathetic neuron survival.

Introduction

It is well established that limiting amounts of target-derived growth factors are crucial mediators of survival of neurons within the peripheral nervous system. NGF, the prototypic target-derived growth factor, is a member of the neurotrophin family, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5) (Huang and Reichardt, 2001; Sofroniew et al., 2001). Each neurotrophin binds with high affinity to a specific Trk receptor tyrosine kinase: NGF to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC. In addition, each of the neurotrophins binds to the p75 neurotrophin receptor. Upon ligand binding, Trk receptors undergo dimerization and autophosphorylation on several key tyrosine residues, which then serve as docking sites for a variety of effector proteins including Shc, PLC- γ , FRS-2, SH2-B, and rAPS. Major signaling pathways initiated by activated Trks in neurons include the Raf-MAPK pathway and the PI3-K/Akt pathway (Miller and Kaplan, 2001).

Antibody neutralization and genetic ablation studies have firmly established that NGF and its receptor, TrkA, are necessary for the development and survival of postganglionic sympathetic neurons and cutaneous sensory neurons (Huang and Reichardt, 2001; Sofroniew et al., 2001). NGF, which is produced and released by target

cells innervated by peripheral neurons, acts on TrkA receptors located on axon terminals of innervating neurons, initiates biochemical signals locally within distal axons, and sends signals retrogradely to neuronal cell bodies and ultimately to their nuclei. The nature of the retrograde signal and how it is transduced over long distances in neurons, from distal axons to cell bodies, has emerged as a significant and intriguing neurobiological question that remains unresolved.

Early work to define the nature of the retrograde signal showed that NGF itself is retrogradely transported in sympathetic and sensory neurons *in vivo* and *in vitro* (Claude et al., 1982; Hendry et al., 1974; Johnson et al., 1978; Schwab, 1977; Stöckel et al., 1975a, 1975b). More recent studies have demonstrated that phosphorylated TrkA (P-TrkA) is also retrogradely transported in neurons *in vivo* in a manner dependent on endogenous target-derived NGF (Bhattacharyya et al., 1997; Ehlers et al., 1995). In addition, application of neurotrophins exclusively to distal axons of compartmentalized neuronal cultures results in the accumulation of P-Trks in cell bodies (Riccio et al., 1997; Senger and Campenot, 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999), and coprecipitation studies performed by two independent laboratories showed that Trk receptors are retrogradely cotransported with neurotrophins (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). Moreover, pharmacological inhibition experiments demonstrated that the catalytic activity of TrkA in cell bodies is required for retrograde phosphorylation and activation of TrkA and several signaling effectors (Kuruvilla et al., 2000; Riccio et al., 1997; Senger and Campenot, 1997; Watson et al., 1999, 2001). Finally, retrograde signaling experiments employing compartmentalized neurons and either NGF crosslinked to beads that are unable to internalize or a dominant-negative form of dynamin that inhibits ligand-receptor internalization suggested that internalization is required for at least some aspects of retrograde NGF signaling including the appearance of phosphorylated CREB (P-CREB) and phosphorylated Erk5 (P-Erk5) in cell bodies (Riccio et al., 1997; Watson et al., 2001). These findings, taken together, have provided support for a "Signaling Endosome Model" in which NGF-TrkA complexes undergo internalization in distal axons and are then physically translocated along the axonal microtubule network to cell bodies where they can initiate or propagate pro-survival signals (Ginty and Segal, 2002; Miller and Kaplan, 2001; Reynolds et al., 2000; Sofroniew et al., 2001). More recently, however, considerable attention has been focused on alternative modes of retrograde signaling that mediate retrograde survival. MacInnis and Campenot (2002) reported that NGF immobilized on beads is capable of supporting survival when applied to distal axons of compartmentalized sympathetic neuronal cultures. The authors argued that retrograde neuronal survival is mediated by mechanisms that do not require internalization and retrograde transport of NGF.

What, then, are the other possible modes of retrograde signaling generated in response to target-derived

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neurotrophins? At least three additional models have been proposed to explain retrograde signaling. In a second model, which is a subtle variation of the Signaling Endosome Model, a TrkA-containing signaling endosome without neurotrophin ligand is the retrograde signal carrier. This seems plausible since a high concentration of Trk receptors, as may be found in Trk receptor-containing vesicles, can support receptor autophosphorylation and signaling independent of ligand, at least in PC12 cells (Hempstead et al., 1992). In a third model, the “Domino Model” or “Wave Propagation Model” (Ginty and Segal, 2002; Miller and Kaplan, 2001; Senger and Campenot, 1997), NGF binding to TrkA on distal axons initiates a wave of TrkA phosphorylation that is propagated along the axonal plasma membrane independent of ligand. Support for this model has come from the reported rapid kinetics of retrograde TrkA phosphorylation (Senger and Campenot, 1997) as well as from experiments showing that focal application of EGF can elicit a ligand-independent wave of EGF receptor tyrosine kinase activation for several microns along the plasma membrane of transformed A431 cells (Verveer et al., 2000). Whether lateral propagation of TrkA phosphorylation along the length of the axonal plasma membrane accounts for long-range retrograde signaling in neurons (a distance of millimeters, centimeters, or longer) is not known. A fourth “Retrograde Effector Model” (Ginty and Segal, 2002) proposes that TrkA effectors activated in distal axons are themselves retrogradely propagated independent of retrograde transport of both ligand and receptor.

In the present study, we employed compartmentalized cultures of sympathetic neurons to distinguish between the various models of retrograde NGF signaling. We provide evidence that the catalytic activity of TrkA is essential in both cell bodies and distal axons to mediate retrograde neuronal survival. Furthermore, inhibition of TrkA activity exclusively in proximal axonal shafts of neurons supported by NGF in distal axons neither disrupted NGF-TrkA signaling in cell bodies nor compromised survival. Moreover, blocking ligand-receptor internalization and antibody-mediated neutralization of retrogradely transported NGF resulted in significant cell death when NGF was added exclusively to distal axons. Taken together, these results provide direct evidence in support of the Signaling Endosome Model of retrograde neuronal survival.

Results

Compartmentalized cultures of sympathetic neurons were used to address the roles of retrogradely activated TrkA and retrograde NGF transport for survival signaling. We first assessed the viability and axonal growth properties of compartmentalized sympathetic neurons supported by NGF applied exclusively to distal axons. While NGF withdrawal led to soma atrophy and apoptosis, NGF concentrations of 10 ng/ml and 100 ng/ml, applied exclusively to distal axons for 3 days, supported soma hypertrophy and survival to the same extent as NGF (100 ng/ml) applied directly to cell bodies (Figures 1B

and 1C). Moreover, 10 ng/ml and 100 ng/ml NGF applied to distal axons promoted similar axon extension rates of 250–450 $\mu\text{m}/\text{day}$ (Figure 1D), a rate slightly less than that reported by others (Atwal et al., 2000), probably due to the presence of serum in the medium in the distal compartments in our cultures. Nevertheless, since 10 ng/ml NGF in distal axon compartments supports robust axon growth, maximum survival, and soma hypertrophy, while NGF withdrawal led to marked apoptosis, these conditions were ideal for subsequent retrograde survival experiments.

NGF applied to distal axons of sympathetic neurons results in activation of TrkA locally in distal axons and retrogradely in cell bodies and axons in the same compartment (hereafter referred to as “cell bodies”) (Kuruville et al., 2000; Riccio et al., 1997; Senger and Campenot, 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). Therefore, we next performed experiments to determine whether retrogradely activated TrkA, within cell bodies, is necessary for survival. In initial experiments, we confirmed the efficacy and specificity of the Trk kinase inhibitor K252a (100 nM) (Ohmichi et al., 1992; Tapley et al., 1992) for inhibiting NGF-induced TrkA activation and signaling in cultured sympathetic neurons. NGF-induced phosphorylation of TrkA, Akt, Erk1/2, and survival of sympathetic neurons grown in mass cultures were partially blocked by K252a (Figures 2A–2C). This drug did not block phosphorylation of Akt and Erk1/2 or survival of sympathetic neurons treated with the GDNF family member Neurturin (NTN, 10 ng/ml), which signals through the receptor tyrosine kinase c-Ret (Figures 2B and 2C). Since TrkA and c-Ret use similar if not identical downstream signaling pathways to promote survival of sympathetic neurons (Atwal et al., 2000; Crowder and Freeman, 1998; Encinas et al., 2001), these results confirm that K252a specifically inhibits TrkA but not other signaling intermediates necessary for survival of these neurons. Therefore, K252a (100 nM) was used to determine whether TrkA activity in cell bodies, distal axons, or both is required for survival when neurons are supported by NGF added exclusively on distal axons. As shown in Figure 2E, K252a added either to cell bodies or distal axons led to significant cell death over the 72 hr treatment period. The kinetics of cell death are comparable to those observed upon addition of K252a to both cell body and distal axon compartments and are somewhat slower than those observed upon complete NGF withdrawal, probably due to the incomplete inhibition of TrkA signaling during the long treatment period (Figures 2B, 2D, and 2E). Importantly, cell death was not observed when NGF was applied directly to cell bodies and K252a to distal axons (Figure 2E), and K252a applied exclusively to the cell body compartment inhibits phosphorylation of TrkA in that compartment but not the distal axon compartment (Figure 2D), indicating that pharmacological inhibition of TrkA was effectively compartmentalized. Together, these data indicate that TrkA kinase activity in both cell bodies and distal axons is necessary for retrograde signaling-mediated survival of sympathetic neurons. The requirement of activated TrkA in cell bodies suggests that downstream effectors of TrkA, activated in distal axons, are by themselves not sufficient to support neuronal survival.

We next investigated the mechanism by which acti-

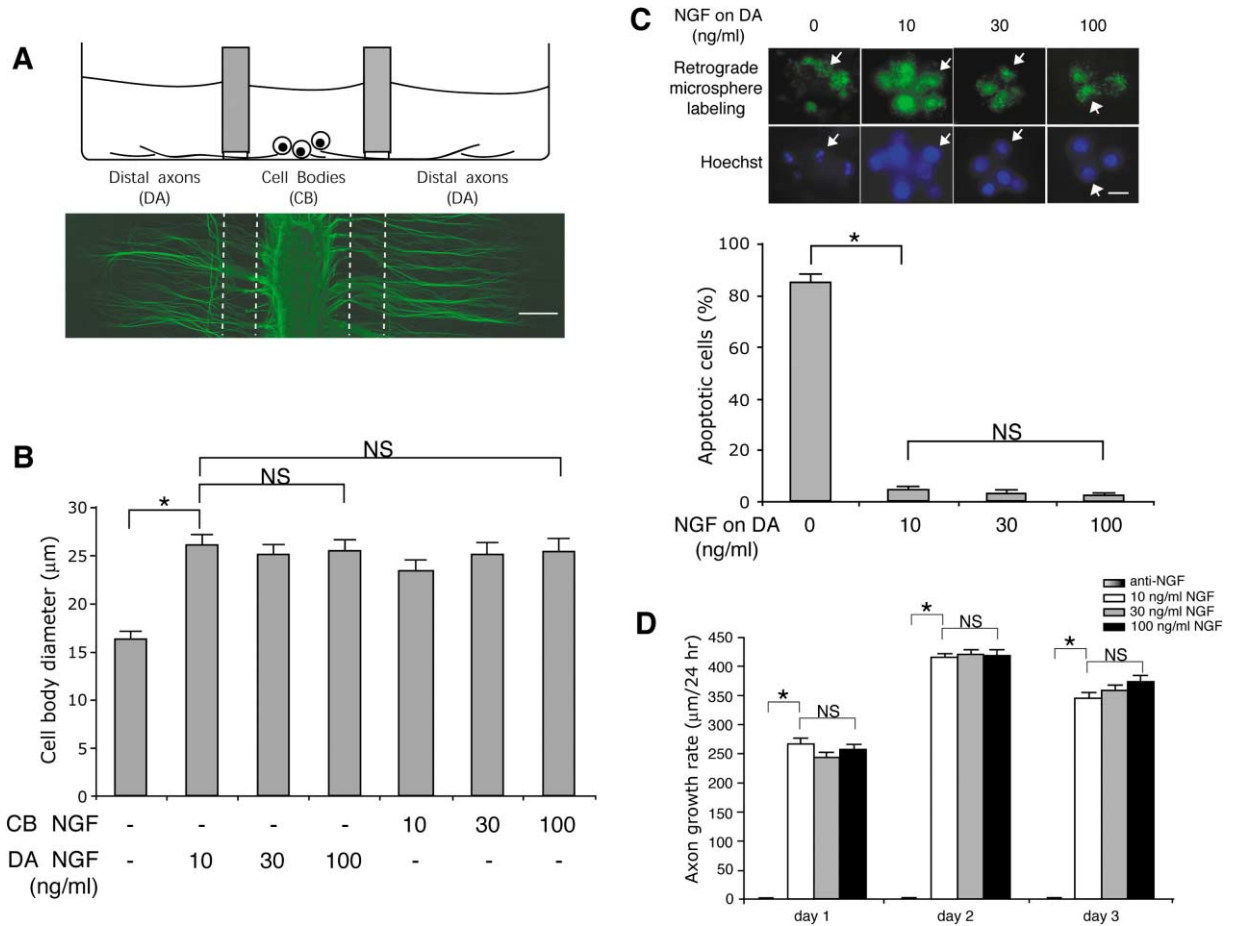


Figure 1. Soma Hypertrophy, Axon Extension Rates, and Survival of Compartmentalized Sympathetic Neurons

(A) Compartmentalized sympathetic neuronal cultures. Top panel, schematic view of the culture. Bottom panel, neurofilament staining of sympathetic neurons grown in compartmentalized culture for 12 days. Scale bar equals 1 mm.

(B) Sympathetic neurons grown in medium containing NGF (10, 30, or 100 ng/ml) exclusively in the distal axon compartments exhibit soma hypertrophy to the same extent as those grown with medium containing NGF directly added to cell bodies. Sympathetic neurons grown in compartmentalized culture for 10–12 days were treated for 72 hr. BAF (50 μM) was added to cell body compartments to prevent cell death. Medium was changed every 24 hr. After treatment, cells were fixed with 4% paraformaldehyde and cell body diameter was measured. Note that sympathetic neurons deprived of NGF show significantly smaller cell body diameter than those treated with NGF. The cell body diameter is presented as mean ± SEM from three independent experiments. Asterisk, $p < 0.001$; NS, not significantly different; one-way ANOVA followed by a Tukey's Multiple Comparison Test.

(C) NGF (10 ng/ml) on distal axons supports survival of sympathetic neurons grown in compartmentalized culture. Sympathetic neurons were grown in compartmentalized culture for 10–12 days and then subjected to treatment for 72 hr. Medium was changed every 24 hr. Fluorescent microspheres (40 nm) were added to the distal axon compartment to retrogradely label neurons projecting axons into the distal axon compartment. After treatment, cells were fixed with 4% paraformaldehyde and stained with Hoechst 33258 dye. Microsphere-labeled neurons were scored based on nuclear morphology. The amount of neuronal cell death is presented as mean ± SEM from three independent experiments. Asterisk, $p < 0.001$; NS, not significantly different; one-way ANOVA followed by a Tukey's Multiple Comparison Test. Scale bar equals 20 μm.

(D) Sympathetic neurons exhibit robust axon extension upon exposure of distal axons to medium containing either 10, 30, or 100 ng/ml NGF. Sympathetic neurons were grown in compartmentalized culture for 10–12 days. Distal axons were washed away with distilled water, and medium containing either anti-NGF or NGF was added to distal axon compartments. Anti-NGF and BAF (50 μM)-containing medium was added to all cell body compartments. The length of the longest axons in each track in the distal axon compartments was measured every 24 hr, and the axon growth rate was calculated. Note that there was no axon growth in the distal compartment of anti-NGF-treated cultures. The rate of axon growth is presented as mean ± SEM from three independent experiments. Asterisk, $p < 0.001$; NS, not significantly different; two-way ANOVA followed by Duncan multiple range test.

vated TrkA propagates along axons of sympathetic neurons over a long distance. While NGF present on distal axons resulted in retrograde appearance of phosphorylated TrkA, Akt, and Erk1/2 in cell bodies, the presence of NGF on cell bodies failed to promote anterograde appearance of phosphorylated TrkA and downstream effectors in distal axons (Figure 3). Similar results were

reported previously for the unidirectional propagation of P-Trk receptors in sensory neurons (Watson et al., 1999). Thus, like the unidirectional nature of NGF transport (Claude et al., 1982), TrkA phosphorylation and TrkA signaling propagates retrogradely, but not anterogradely. This observation is not consistent with the ligand-independent Domino Model of TrkA autophosphorylation

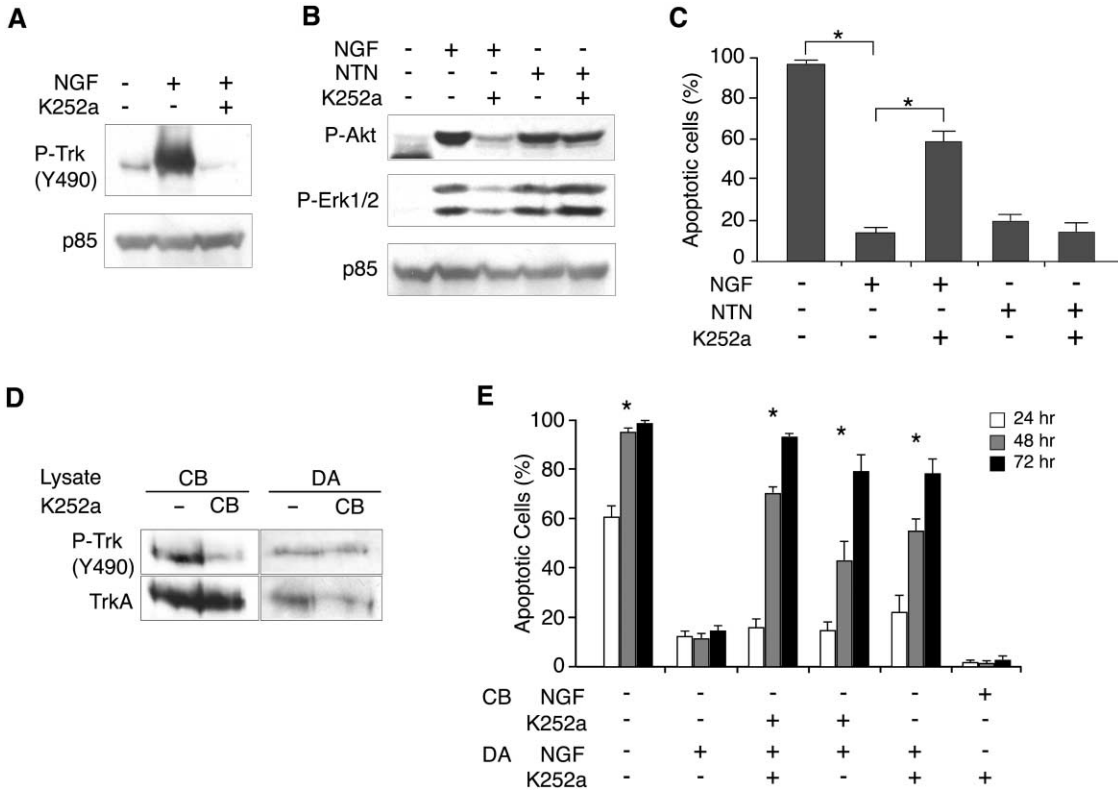


Figure 2. TrkA Kinase Activity in Both Cell Bodies and Distal Axons Is Necessary for Survival of Sympathetic Neurons Supported by NGF Added to Distal Axons

(A–C) K252a (100 nM) is a specific inhibitor of TrkA in sympathetic neurons. For sympathetic neurons grown in mass cultures (7 DIV), 24 hr treatment of K252a (100 nM) decreased the levels of NGF (10 ng/ml)-induced P-Trk(Y490) (A), P-Akt, and P-Erk1/2 (B), but had no effect on Neurturin (NTN, 10 ng/ml)-induced P-Akt and P-Erk1/2 (B), as shown by immunoblot. (C) K252a (100 nM) kills sympathetic neurons supported by NGF (10 ng/ml) but has no effects on survival of neurons supported by NTN (10 ng/ml). Sympathetic neurons grown in mass cultures (7–10 DIV) were treated for 72 hr as indicated. Media were changed every 24 hr, and neuronal death was assessed based on morphology of nuclei stained with Hoechst 33258. The amount of cell death is presented as mean \pm SEM from three independent experiments. Asterisk, $p < 0.001$; one-way ANOVA followed by a Tukey's Multiple Comparison Test.

(D) Application of K252a (100 nM) to the cell body compartment of sympathetic neurons grown in compartmentalized cultures inhibits P-Trk(Y490) within that compartment without affecting P-Trk(Y490) levels in distal axons. Compartmentalized sympathetic neurons (14 DIV) grown with NGF (100 ng/ml) on distal axons were treated with medium containing anti-NGF with or without K252a (100 nM) added to cell bodies for 8 hr. Cell body/proximal axons and distal axonal extracts were prepared as before and subjected to immunoblotting with an antibody against P-Trk(Y490) followed by reprobing the blot with an antibody against TrkA (Chemicon) to normalize for protein amounts.

(E) Inhibition of TrkA kinase activity by K252a (100 nM) either in cell bodies, in distal axons, or in both leads to apoptotic cell death of sympathetic neurons supported by NGF (10 ng/ml) applied on distal axons. Compartmentalized sympathetic neuronal cultures (10–12 DIV) were treated as indicated for 72 hr. Media were changed every 24 hr, and neuronal death was assessed. Results are presented as mean \pm SEM from four independent experiments. Asterisk, $p < 0.001$; two-way ANOVA followed by the Duncan multiple range test. DIV, days in vitro.

along the membrane, which would predict bidirectional propagation.

To more directly test the validity of the Wave Propagation or Domino Model for retrograde propagation of TrkA activity, we cultured neurons in three-compartment chambers and inhibited TrkA kinase activity exclusively in proximal axons in the middle compartment (Figure 4A, inset), which should disrupt this mode of TrkA signal propagation. As observed previously, TrkA kinase activity in distal axons is required for initiation of retrograde transport of [¹²⁵I]NGF (Figure 4A; Kuruville et al., 2000; Reynolds et al., 1998). However, inhibition of TrkA activity solely in proximal axons in the middle compartment had no effect on the retrograde transport of [¹²⁵I]NGF (Figure 4A). Thus, TrkA kinase activity is crucial for the initiation but not propagation of retrograde transport

of [¹²⁵I]NGF, suggesting that P-TrkA signaling does not serve to maintain unidirectional transport of NGF-containing vesicles in axons. Interestingly, application of K252a exclusively to the middle compartment blocked the retrograde appearance of phosphorylated TrkA, Akt, and Erk1/2 in proximal axons within that compartment, but had no effect on the levels of these activated molecules and the phosphorylated form of the transcription factor CREB detected in cell body compartments (Figures 4B and 4C). Importantly, while inhibition of TrkA catalytic activity either in the distal axon compartment or in the cell body compartment resulted in significant cell death after 3 days of K252a application, K252a applied to proximal axons in the middle compartment did not compromise neuronal survival (Figure 4D). Together, these data indicate that TrkA kinase activity and signal-

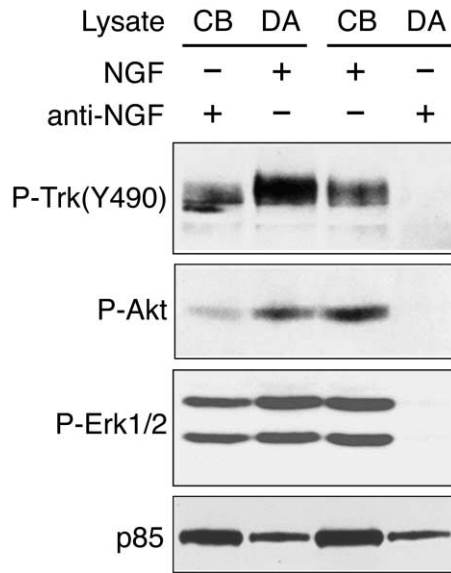


Figure 3. TrkA Signaling Can Propagate Retrogradely, but not Anterogradely, in Sympathetic Neurons

Sympathetic neurons were grown in medium containing NGF in both CB and DA compartments for 10–12 days. Then NGF was withdrawn by adding a neutralizing NGF antibody to either the cell body compartments (lanes 1 and 2) or distal axon compartments (lanes 3 and 4), while NGF (100 ng/ml) was maintained in the other compartments as indicated. Twenty-four hours later, protein extracts were collected from cell body and distal axon compartments for immunoblot analysis using antibodies against P-Trk(Y490), P-Akt, P-Erk1/2, and p85 (for normalization).

ing in proximal axons is dispensable for NGF retrograde transport and signaling to cell bodies and neuronal survival. Moreover, retrogradely propagating TrkA and its downstream effectors can be newly phosphorylated and activated within the cell bodies. Thus, a ligand-independent Domino mode of propagation of TrkA phosphorylation along the axonal membrane cannot account for retrograde signaling and neuronal survival.

Another possible mode of retrograde propagation of activated TrkA, described by the Signaling Endosome Model, requires internalization and retrograde transport of ligand-receptor complexes in signaling endosomes. To assess the necessity of NGF-TrkA internalization for neuronal survival, we used sympathetic neurons infected with an adenovirus encoding a dominant-negative form of dynamin, K44A dynamin. The GTPase activity of dynamin is required for membrane scission in vesicle formation during clathrin-mediated endocytosis of receptors (McPherson et al., 2001), including Trk receptors (Zhang et al., 2000). As expected, K44A dynamin effectively blocked the appearance in cell bodies of [¹²⁵I]NGF added to distal axons (Figure 5A). Expression of K44A dynamin resulted in apoptosis of approximately 70% of sympathetic neurons supported by NGF on distal axons (Figure 5B). Apoptotic cell death caused by inhibition of internalization could be prevented by including a broad-spectrum caspase inhibitor (BOC-Asp(Ome)-Fluoromethyl Ketone, BAF) in the medium bathing cell bodies. NGF added directly to cell bodies of neurons expressing K44A dynamin also effectively supported

survival. These data indicate that internalization is required for survival of neurons supported by NGF on distal axons, but not neurons supported by NGF acting directly on cell bodies, consistent with the idea that formation of NGF/TrkA-containing endosomes is critical for retrograde NGF-TrkA signaling.

The above observations, together with previous findings that neurotrophins and Trk receptors can be internalized into signaling endosomes in PC12 cells (Grimes et al., 1996, 1997; Howe et al., 2001; Shao et al., 2002) and retrogradely cotransported in neurons (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999), support the possibility that retrogradely transported NGF sustains both TrkA phosphorylation and downstream survival signaling in cell bodies. To test this idea, we sought to neutralize NGF activity in the cell body compartment and to assess the consequences on neuronal survival. We used a protein delivery system employing a peptide (Pep-1) that can form noncovalent complexes with proteins and facilitate their delivery across membranes of cultured cells (Morris et al., 2001). Pep-1 could effectively deliver both β -gal protein and rabbit IgG into sympathetic neurons (Figure 6A). Pep-1 is also capable of delivering an affinity-purified neutralizing antibody against NGF into NGF-containing transport vesicles, since in Pep-1/anti-NGF-treated cell bodies, the antibody was found complexed with [¹²⁵I]NGF that had been retrogradely transported from distal axons (Figure 6C). Therefore, Pep-1 was used to deliver anti-NGF into NGF-containing endosomes within cell bodies of sympathetic neurons supported by NGF (10 ng/ml) on distal axons to assess the requirement of retrogradely transported NGF for neuronal survival. Twenty-four hours after adding the Pep-1/anti-NGF complex to cell bodies, we detected significantly more apoptotic cells compared to neurons receiving the same amount of anti-NGF, but without Pep-1 (Figure 6D). The difference in cell death between Pep-1/anti-NGF delivery and complete NGF withdrawal may be explained by incomplete delivery of anti-NGF (delivery efficiency ranged from 29% to 70%, mean = 53% \pm 3% as assessed by Pep-1-mediated delivery of the β -gal protein in parallel experiments).

Several experiments indicate that apoptosis observed in neurons exposed to the Pep-1/anti-NGF complex was the result of NGF neutralization and not a nonspecific toxic effect of the manipulation. Pep-1-mediated delivery of preimmune rabbit IgG into cell bodies did not cause apoptosis (Figure 6D). Also, Pep-1/anti-NGF delivered to distal axons did not affect the survival of sympathetic neurons supported by NGF applied directly to cell bodies (data not shown). Additional control experiments were done using a TrkA antibody, generated against an extracellular TrkA epitope, to substitute for NGF as a ligand for TrkA. This antibody can effectively activate TrkA (Clary et al., 1994) and can support survival of sympathetic neurons when applied to mass cultures (unpublished observations) or distal axons of compartmentalized neurons (Figure 6D). Importantly, survival of sympathetic neurons supported by anti-TrkA (20 μ g/ml) applied exclusively to distal axons was not affected by the Pep-1/anti-NGF complex delivered to cell bodies (Figure 6D), indicating that Pep-1/anti-NGF specifically blocks NGF-dependent signaling and not some other component of TrkA signaling or neuronal survival.

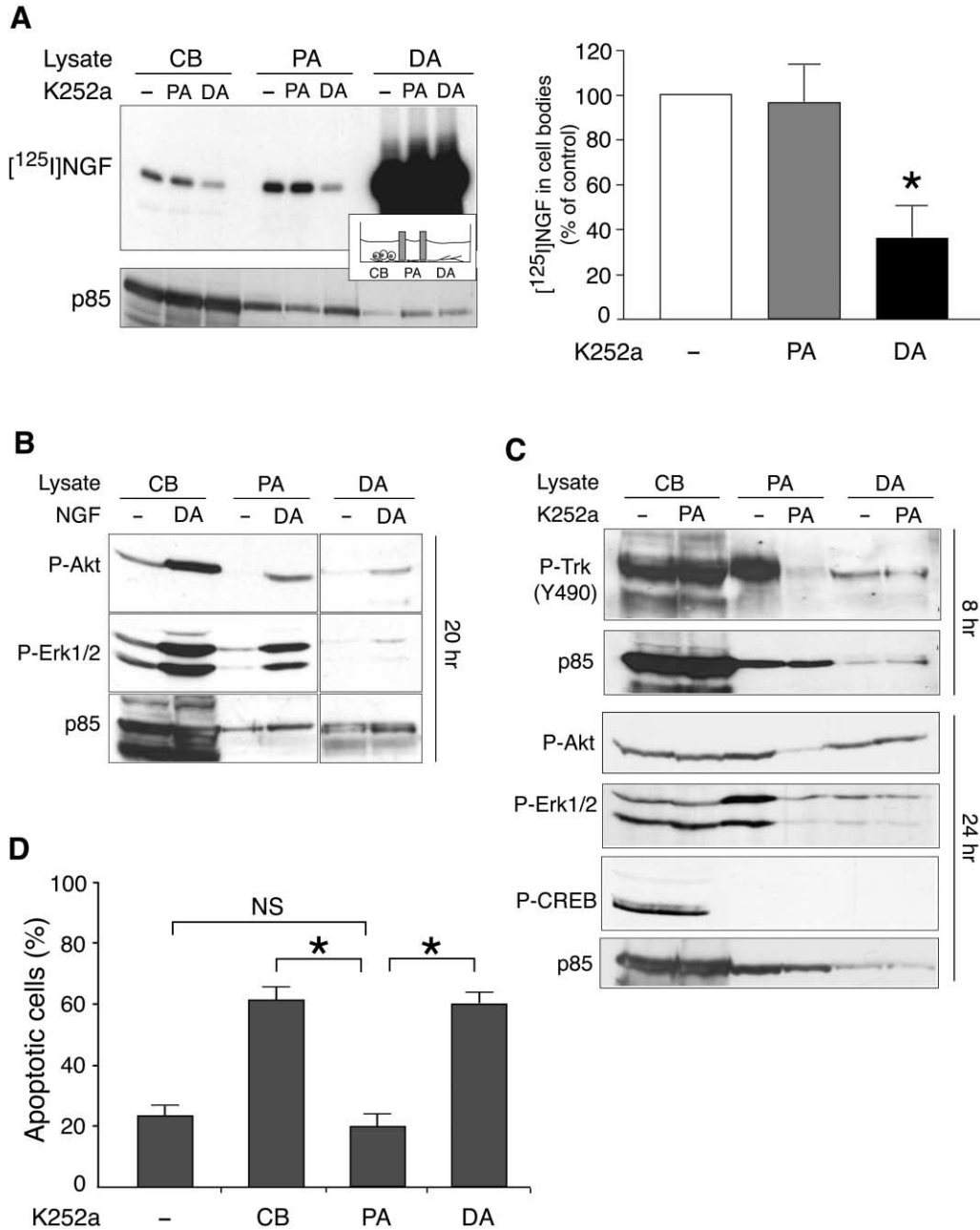


Figure 4. TrkA Activity in Proximal Axons within Middle Compartments Is Not Required for Retrograde Accumulation of [¹²⁵I]NGF, TrkA Signaling in Cell Bodies, or Survival of Sympathetic Neurons Grown in Three-Compartment Chambers

(A) K252a (100 nM) in distal axons (DA), but not proximal axons (PA), attenuates retrograde transport of [¹²⁵I]NGF (0.5 μCi/ml) from distal axons to cell bodies (CB). Extracts were prepared from CB, PA, and DA compartments, resolved by SDS-PAGE, and subjected to autoradiography or immunoblotting for p85. Inset in left panel: schematic diagram of sympathetic neurons grown in three-compartment chambers. Right panel: quantitation of [¹²⁵I]NGF in extracts prepared from cell body compartments is presented as mean ± SEM from three independent experiments. Asterisk, *p* < 0.05; one-way ANOVA followed by a Tukey's Multiple Comparison Test.

(B) NGF (100 ng/ml) applied to far-distal axons of sympathetic neurons grown in three-compartment chambers for 20 hr induces the retrograde appearance of P-Akt and P-Erk1/2 in proximal axons and cell bodies. 15 DIV sympathetic neurons in three compartment chambers were grown in medium containing anti-NGF applied only to cell bodies and proximal axons, and medium lacking NGF to far-distal axons for 3 days. BAF (50 μM) was added exclusively to the cell body compartment to prevent cell death during this period. Three days later, axons in the far-distal axonal compartment was treated with NGF (100 ng/ml) for 20 hr, and lysates were prepared from cell body, proximal axonal, and far-distal axonal compartments and subjected to immunoblotting using antibodies against P-Akt and P-Erk1/2 and p85.

(C) Inhibition of TrkA activity in proximal axons does not affect TrkA signaling in cell bodies. Immunoblots for P-TrkA, P-Akt, P-Erk1/2, P-CREB, and p85 from sympathetic neurons (11–15 DIV) grown in three-compartment chambers and treated with or without K252a (100 nM) added only to proximal axons, and stimulated with NGF (100 ng/ml) added only to distal axons for 8 hr (top two panels) or 24 hr (bottom four panels).

(D) 72 hr inhibition of TrkA activity with K252a (100 nM) in distal axons and cell bodies, but not in proximal axons, kills sympathetic neurons

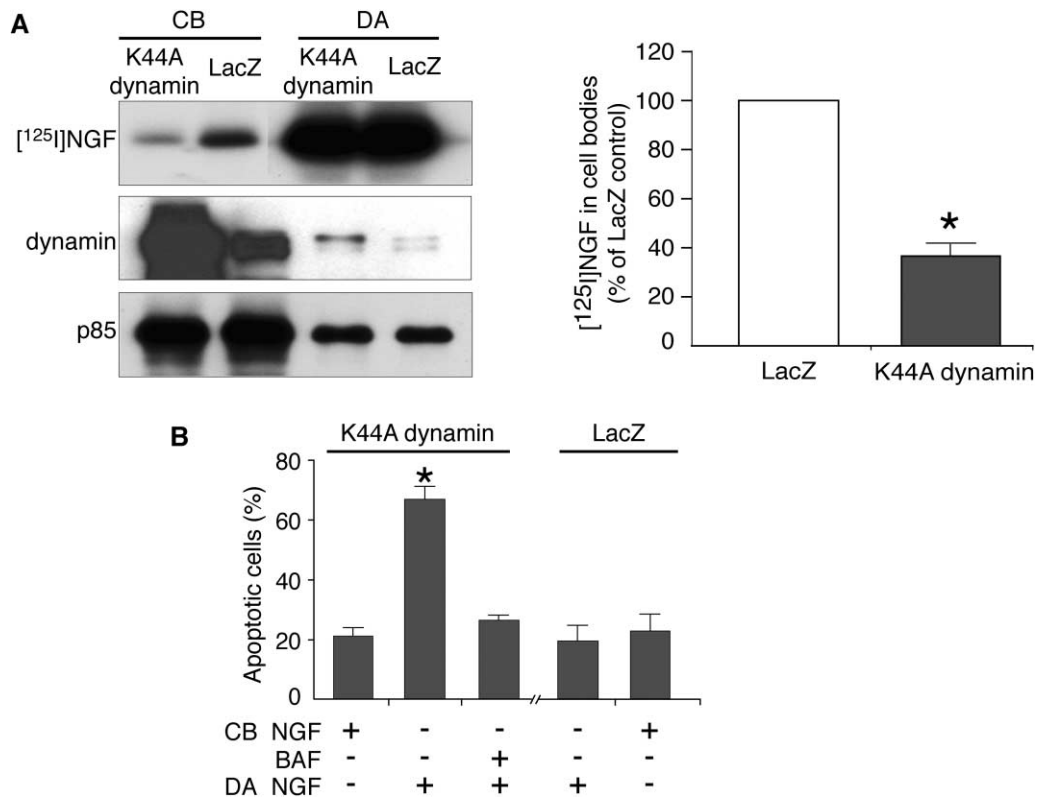


Figure 5. Blocking Internalization and Retrograde Transport of NGF by K44A Dynamin Results in Increased Apoptotic Cell Death in Sympathetic Neurons Supported by NGF Added only to Distal Axons

(A) K44A dynamin blocks retrograde accumulation of [¹²⁵I]NGF in sympathetic neurons grown in compartmentalized cultures. The [¹²⁵I]NGF retrograde transport assays were performed on sympathetic neurons infected with adenoviruses encoding either K44A dynamin or LacZ (as control). Right panel: quantitation of the relative amount of accumulation of retrogradely transported [¹²⁵I]NGF in cell body compartments, from four independent experiments expressed as mean ± SEM. Asterisk, $p < 0.001$; paired t test.

(B) K44A dynamin expressing neurons exhibit increased cell death compared to LacZ-expressing neurons when supported by NGF (10 ng/ml) acting on distal axons. This cell death could be prevented either by adding NGF (10 ng/ml) directly to the cell body compartment or by inhibiting caspase activity in cell bodies with BAF (50 μM). Sympathetic neurons were infected with adenoviruses encoding either K44A dynamin or LacZ and were subjected to treatment as indicated for 72 hr. Apoptosis of infected neurons was assessed based on Hoechst 33258 staining of nuclei. Results are presented as mean ± SEM from four independent experiments. Asterisk, $p < 0.001$; one-way ANOVA followed by a Tukey's Multiple Comparison Test. K44A, K44A dynamin encoding adenovirus; LacZ, LacZ encoding adenovirus.

We also performed two complementary sets of experiments to confirm that the Pep-1/anti-NGF complex applied to cell bodies did not diffuse the length of the axons to inhibit NGF signaling in distal axons. In one set of experiments, delivery of β-gal protein into cell body compartments of sympathetic neurons resulted in robust β-galactosidase activity within the cell bodies and most proximal axons within that compartment after 24 hr of delivery. In contrast, we were unable to detect any β-galactosidase activity in axons underneath the barriers or in the distal axon compartments (Figure 6B). Similar results were observed when Pep-1/rabbit IgG was applied to cell body compartments (data not shown). Since axon growth is critically dependent upon local NGF signaling within distal axons (Campenot, 1977), a second set of experiments was performed to

assess the rates of extension of individual axons in distal axon compartments during the 24 hr period following Pep-1/anti-NGF delivery to cell bodies. For these axon outgrowth experiments, the caspase inhibitor BAF was included in the medium to prevent cell death associated with neutralization of NGF in cell bodies. The average values and distribution profiles of axon extension rates were comparable for neurons treated on cell bodies with either anti-NGF alone or the Pep-1/anti-NGF complex (Figure 6E). These control experiments indicate that Pep-1/anti-NGF delivery to cell bodies neutralizes NGF signaling within cell bodies but not NGF signaling in distal axons. Together, these experiments indicate that retrogradely transported NGF, within cell bodies, is required for neuronal survival but not growth of distal axons.

(14 DIV) grown in three-compartment chambers and maintained with NGF (10 ng/ml) added only to distal axons. Neuronal death was assessed based on Hoechst 33258 staining of nuclei. Results are mean ± SEM from three independent experiments. Asterisk, $p < 0.001$; NS, not significantly different; one-way ANOVA followed by a Tukey's Multiple Comparison Test.

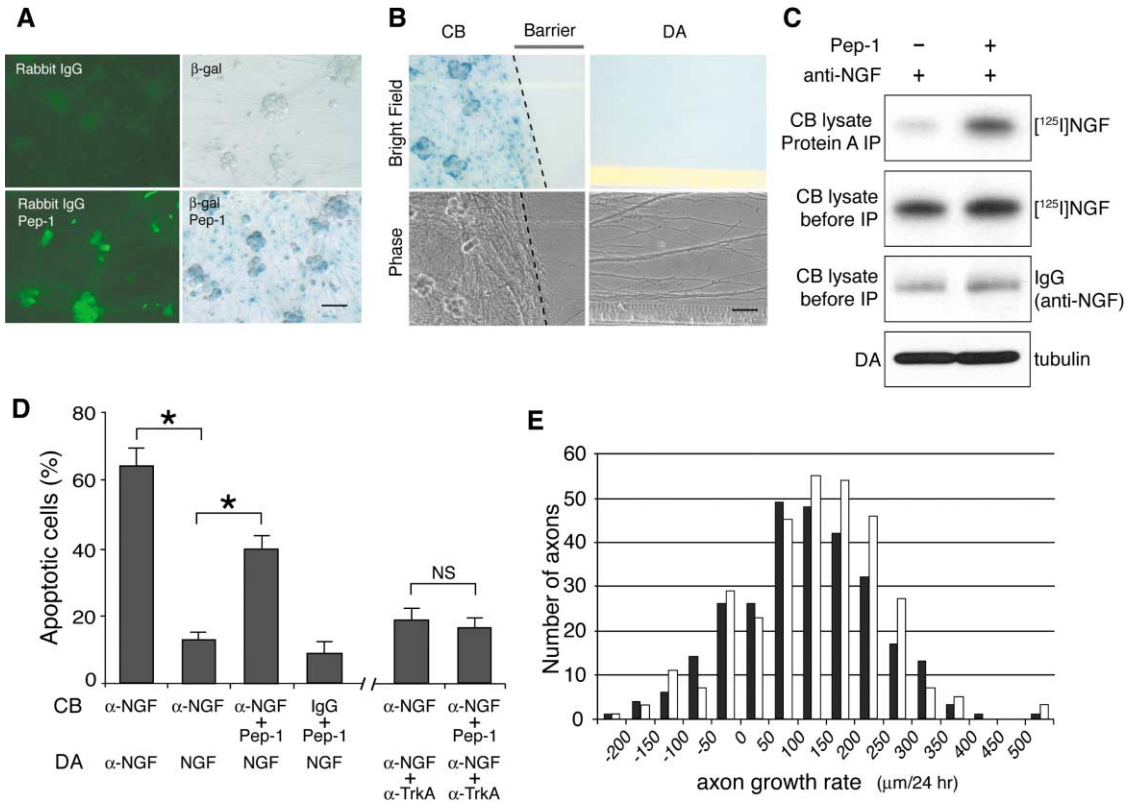


Figure 6. Retrograde Transport of NGF Is Necessary for Survival of Sympathetic Neurons

(A) Left panels: anti-rabbit IgG staining of sympathetic neurons exposed to rabbit IgG in the presence or absence of Pep-1 for 4 hr. Right panels: X-gal staining of sympathetic neurons exposed to β-galactosidase in the presence or absence of Pep-1 for 24 hr. Scale bar equals 100 μm.

(B) X-gal staining of sympathetic neurons grown in the compartmentalized culture exposed to β-galactosidase in the presence or absence of Pep-1 in the cell body compartment for 24 hr. Note the X-gal staining is restricted to the cell body compartment. Scale bar equals 100 μm.

(C) Pep-1 can deliver affinity-purified anti-NGF into NGF-containing vesicles. [¹²⁵I]NGF was added to the distal axon compartment for 12 hr to allow [¹²⁵I]NGF-containing vesicles to accumulate in cell bodies. Three hours after addition of either anti-NGF alone or the Pep-1/anti-NGF complex to cell bodies, protein-A immunoprecipitation (IP) was performed using 83% of cell body lysates, and precipitated complexes were resolved by SDS-PAGE. For comparison, 17% of cell body lysates was directly resolved by SDS-PAGE (CB, before IP). As collagen was used as the substrate for these cultures, some antibody remained in the collagen, resulting in similar amounts of antibody in IP lysates. This serves as an internal control, providing assurance that the association of [¹²⁵I]NGF and antibody did not occur during the preparation of the lysates and IP process.

(D) Neutralization of retrogradely transported NGF in cell bodies promotes neuronal death. Compartmentalized sympathetic neuronal cultures (10–12 DIV) were treated with conditions as indicated in the panel. Neuronal death was assessed 24 hr following Pep-1 delivery based on Hoechst 33258 staining of nuclei. Results are presented as mean ± SEM from seven independent experiments. Asterisk, *p* < 0.001; NS, not significantly different; one-way ANOVA followed by a Tukey's Multiple Comparison Test. NGF, 10 ng/ml; α-NGF, affinity-purified rabbit anti-NGF; IgG, purified preimmune rabbit IgG; α-TrkA, rabbit anti-TrkA.

(E) Local NGF signaling in distal axons is not affected by Pep-1-mediated delivery of anti-NGF into cell bodies. Histogram of extension rates of individual axons in the distal axon compartments for the 24 hr period following addition of either anti-NGF alone (filled bar) or the Pep-1/anti-NGF complex (open bar) to cell body compartments and NGF (10 ng/ml) to distal axon compartments. Results are from three independent experiments. (Not significantly different, Student's *t* test).

Discussion

An unresolved issue in retrograde neurotrophin survival signaling has been the precise mechanism by which signals initiated at distal axons are propagated over long distances back to neuronal cell bodies. Although several models have been proposed to explain long-range retrograde survival signaling, the available evidence has neither been conclusive nor has it discounted the existence of any of the models. Recent evidence suggesting the existence of nonvesicular modes of retrograde signaling has prompted us to rigorously examine each of the current models to explain retrograde neurotrophin survival

signaling in sympathetic neurons. We found that survival of neurons supported by NGF on distal axons requires the activity of TrkA in both distal axons and cell bodies. In contrast, disruption of TrkA activity exclusively in proximal axons of neurons grown in three-compartment cultures affected neither retrograde NGF-TrkA signaling in cell bodies nor neuronal survival. Also, ligand-receptor internalization is necessary for survival of neurons supported by NGF on distal axons. Furthermore, Pep-1-mediated delivery of a neutralizing NGF antibody into cell bodies, which was shown to bind to retrogradely transported NGF, resulted in apoptosis. Interestingly, antibody-mediated neutralization of NGF in cell bodies

did not affect growth of distal axons. Taken together, these results provide strong support for a Signaling Endosome Model in which retrogradely transported NGF-TrkA complexes support neuronal survival.

TrkA as a Retrograde Signal Carrier

It is now well established that, upon binding their ligands, receptor tyrosine kinases are rapidly internalized through clathrin-mediated endocytosis (McPherson et al., 2001). There are several intriguing possibilities as to why an activated receptor tyrosine kinase is transported to the interior of the cell: attenuation of plasma-membrane receptor signaling; delivery of activated receptors directly to the nucleus; and continued signaling from intracellular receptors, which may control the specificity of biological responses to growth factors. Recent exciting evidence indicates that internalized receptor tyrosine kinases continue to signal and that endosomal-based signaling is sufficient to support the survival of MDCK and BT20 cells (Wang et al., 2002). Moreover, internalization appears to be necessary for NGF-induction of neurite outgrowth in PC12 cells (Zhang et al., 2000). In neurons, neurotrophin bound Trk receptors are internalized and at least some of these internalized receptors are retrogradely transported to cell bodies (Bhattacharyya et al., 1997; Ehlers et al., 1995; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). Indeed, P-Trk receptors are associated with vesicles bound to dyneins in sciatic nerve (Bhattacharyya et al., 2002), and Trk-dynein complexes accumulate distal to a sciatic nerve ligation (Yano et al., 2001). Do retrogradely transported Trk containing vesicles serve as platforms for signaling? The answer to this question is likely to be yes, since TrkA kinase activity in cell bodies is necessary for the activities of PI3-K and Akt, Erk5, and CREB, for multiple tyrosine phosphorylation events, and for IEG induction (Kuruvilla et al., 2000; Riccio et al., 1997; Senger and Campenot, 1997; Watson et al., 1999, 2001). In addition, downstream TrkA effectors are clearly activated locally, within distal axons, and so it is possible that one or more of these active effectors functions as a retrograde survival signal. However, we have found that TrkA activity is required in both distal axons and cell bodies for survival of sympathetic neurons. Thus, TrkA effectors that support survival, including Akt and Erks, are unlikely to propagate long distances retrogradely independent of activated TrkA, as suggested by the earlier studies (Kuruvilla et al., 2000; Maclinnis and Campenot, 2002; Watson et al., 2001). This idea is further supported by two additional observations of the present study. First, inhibition of TrkA kinase activity exclusively in the proximal axons of three-compartment cultures leads to decreased phosphorylation of Akt and Erk1/2 in axons within that compartment, but not in either the cell body or distal axon compartments. Second, like NGF transport, TrkA phosphorylation and activation of its effectors are only propagated unidirectionally—retrogradely—in axons. Activated PI3-K and Erk1/2 cannot propagate anterogradely, from cell bodies to distal axons. These observations support the idea that PI3-K, Akt, and Erks cannot propagate in their active states far (i.e., millimeters or more) from their initiating source, the activated receptor tyrosine kinase. It is cer-

tainly possible that other types of intracellular signals (for example, transcription factors that dissociate from complexes and translocate to the nucleus) could travel much further distances from active receptors. Indeed, NF- κ B, a transcription factor complex which, in its latent form, is cytoplasmic and also found in neuronal processes, can dissociate upon glutamate receptor activation. The free transcription factor subunit is then able to translocate to the cell body and nucleus (Wellmann et al., 2001). Whether dissociation/activation of NF- κ B or other transcription factors occurs in distal axons far from cell bodies is not yet known but if so, then they could, in principle, be long-range retrograde signal carriers. However, our results suggest that this type of signal is, by itself, not able to support survival. Finally, the finding that inhibition of TrkA kinase activity in proximal axons of three-compartment chambers affects neither the survival of neurons nor downstream signaling in cell bodies suggests that retrogradely transported TrkA activates downstream effectors in a dynamic manner and may serve as a platform for the initiation of signal transduction events once it reaches the cell body. Thus, our results support the idea that activated TrkA itself is a retrograde carrier of prosurvival signals in neurons.

TrkA Is Retrogradely Transported in a Vesicle-Dependent Manner

We found that survival of neurons supported by NGF acting on distal axons is unique in its requirement for dynamin-mediated internalization, as inhibition of internalization does not result in death of neurons supported by NGF acting directly on cell bodies. These results are most consistent with an internalization- and vesicle transport-dependent mechanism of retrograde propagation of phosphorylated TrkA and retrograde signaling. An alternate model, the Domino or Wave Propagation Model, of Trk propagation was proposed to explain the findings that the initial appearance of phosphorylated TrkA can be detected in cell bodies of sympathetic neurons prior to the detection of retrogradely transported [¹²⁵I]NGF (Senger and Campenot, 1997). It was speculated that fast propagation of phosphorylated receptor tyrosine kinase activity could arise through a wave of ligand-independent spontaneous receptor dimerization (Ginty and Segal, 2002; Miller and Kaplan, 2002; Senger and Campenot, 1997). However, this form of RTK activation may only happen in transformed cells that express unusually high levels of receptors (Sawano et al., 2002; Schlessinger, 2002; Verveer et al., 2000). Thus, spontaneous dimerization of TrkA receptors is unlikely to propagate Trk signaling along the axonal membrane of sympathetic neurons. Consistent with this view are the findings of previous cell surface biotinylation experiments that showed that NGF acting on distal axons cannot promote phosphorylation of TrkA associated with the plasma membrane of the cell body (Tsui-Pierchala and Ginty, 1999). The present study also suggests that a Domino mode of propagation of TrkA phosphorylation is untenable, because (1) inhibition of TrkA kinase activity in the proximal axons of three-compartment chambers, which should disrupt any lateral, membrane propagation of TrkA activity, has no effect on either the level of phosphorylated TrkA in cell body compartments

or the survival of these neurons; and (2) ligand-independent spontaneous dimerization of TrkA receptors should show no directional preference, while we and others (Watson et al., 1999) observed unidirectional propagation of phosphorylated TrkA—only from distal axons to cell bodies. Taken together, a Domino or Wave Propagation mode of retrograde TrkA signaling, if it does exist, is unlikely to account for retrograde survival signaling.

Retrogradely Transported NGF Is Necessary for Survival

In the Signaling Endosome Model, P-TrkA associated with endosomes must travel a very long distance, millimeters or more, to reach the cell body and, therefore, a key question is how TrkA activity is maintained in endosomes. One possibility is that NGF, within the endosome, binds to and maintains Trk receptor dimerization and activity. A second possibility is that a high concentration of receptors within endosomes supports ligand-independent signaling. Our results from NGF neutralization experiments support the former possibility and indicate that retrogradely transported NGF within the endosomes is necessary for maintenance of TrkA signaling during the retrograde transport and survival of sympathetic neurons. This finding, together with the observation that K252a applied to proximal axons in the middle compartment of three-compartment cultures has no effect on retrograde NGF-TrkA signaling to the cell body compartment, suggests that as long as the NGF-TrkA interaction in endosomes is maintained, TrkA can autophosphorylate and recruit local adaptor proteins that activate downstream signaling pathways. Such a dynamic interaction between TrkA and its effectors during retrograde transport may allow for propagation of distinct signaling pathways in different cellular compartments which may, in turn, mediate distinct functions, such as axonal extension in distal axons and gene expression and survival in cell bodies.

In contrast to our findings, MacInnis and Campenot (2002) found that NGF covalently coupled to large microspheres, preventing internalization, is capable of supporting survival of sympathetic neurons when added to distal axons, raising the possibility of NGF-independent retrograde signaling for survival. However, in those experiments, a small amount of soluble NGF (0.2–1 pg) released from the beads was retrogradely transported. To address whether this small amount of leakage could account for retrograde survival, the authors bathed distal axons with a medium containing a low concentration of soluble NGF (0.5 ng/ml or less) to achieve an amount of retrograde NGF transport comparable to or more than that which had resulted from leakage from the beads. Soluble NGF (0.5 ng/ml) applied to distal axons could not support retrograde survival, leading the authors to conclude that the small amount of leakage of NGF from the beads could not contribute to the retrograde survival signal. This control experiment, however, may not adequately assess the effects of “leaked” NGF in the bead experiments since in the control experiments, the low concentration of soluble NGF is very unlikely to effectively activate TrkA receptors, while NGF bound microspheres do effectively activate TrkA receptors (MacInnis and Campenot, 2002; Riccio et al., 1997). Therefore, a

small amount of soluble NGF leaked from the beads together with robust NGF-bead-mediated TrkA activation could lead to formation of competent retrograde signaling endosomes containing ligand, while in contrast, addition of small amounts of soluble NGF alone may not. Thus, the nature and functional capacity of retrogradely transported NGF may be very different under the experimental and control conditions used in MacInnis and Campenot’s study, rendering the interpretation of those results potentially problematic. Is there any other evidence to support transport-independent survival signaling? We note that in each of the experiments reported in the present study, perturbations that affected retrograde signaling did not completely prevent survival. Thus, the K252a, K44A dynamin, and Pep-1/Anti-NGF treatments resulted in cell death that was less than that observed following NGF withdrawal. While we favor the idea that incomplete cell death in these experiments is due to either incomplete infection efficiency (K44A experiments) or delivery (Pep-1 experiments) or poor long-term efficacy (K252a experiments), we cannot rule out the possibility that other modes of signaling contribute a minor component of retrograde survival signaling under our conditions. If, however, other modes of retrograde survival signaling do exist, the potential mechanism to account for a ligand-independent, non-vesicular mode of signal propagation remains unknown. Further studies would be required to establish the mechanism of any such transport-independent mode of retrograde survival.

The results of the present study provide support for the idea that ligand-receptor complexes are transported retrogradely, over long distances, to support neuronal survival. Since Trk receptors and their ligands are retrogradely transported *in vivo*, the mechanism of survival signaling defined here is likely to account for some or all of retrograde NGF survival signaling *in vivo*. Thus, major future challenges will be to define the molecular composition of NGF/TrkA transport vesicles, to establish their mechanisms of formation, sorting within axons, and the basis of unidirectional trafficking along axons, and to understand the ways in which Trk-containing endosomes serve as signaling platforms. Vesicle purification strategies and proteomic studies are likely to take center stage in the attempts to provide answers to these questions.

In summary, we have addressed the mechanism of retrograde neurotrophin signaling for the survival of sympathetic neurons. Our results provide evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. In this model, retrograde transport of NGF is required for survival of sympathetic neurons, most probably by maintaining TrkA activity in signaling endosomes during the long-distance retrograde transport from distal axons to cell bodies. Thus, NGF-TrkA-containing signaling endosomes, upon arrival in cell bodies, serve as platforms for TrkA signaling and the generation of nascent prosurvival signals.

Experimental Procedures

Sympathetic Neuron Survival Assays

Sympathetic neurons were obtained by enzymatic dissociation of superior cervical ganglia from postnatal day 1 (P1) rats. Cells were

grown in mass cultures or compartmentalized cultures in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with fetal bovine serum (10%), penicillin-streptomycin (1 U/ml), and NGF as described (Kuruville et al., 2000). For compartmentalized cultures, fluorescent microspheres (40 nm; Molecular Probes) were added to distal axon compartments to retrogradely label neurons that projected axons into side chambers, and only the labeled neurons were scored in cell survival assays. Cells were washed three times with DMEM and then subjected to the various treatments as indicated. Medium was changed every 24 hr during the treatment period to maintain efficacy and compartmentalization. After treatment, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, then stained with Hoechst 33258 (1 µg/ml; Molecular Probes) at room temperature for 5 min. A neuron was scored as apoptotic if it displayed fragmented or condensed nuclei, or no nucleus as described (Kuruville et al., 2000).

Cell Body Diameter Measurement

Sympathetic neurons grown in compartmentalized cultures for 10–12 days were treated for 3 days as indicated. BAF (50 µM) was added to cell body compartments to prevent cell death. Medium was changed every 24 hr to maintain the compartmentalization. After treatment, cells were fixed with 4% paraformaldehyde and photographs of cell body compartments were taken using a Hamamatsu Orca-ER digital camera. Cell body diameter was measured using Openlab software.

Axonal Growth Assays

For axon growth measurements shown in Figure 1, distal axons of sympathetic neurons were washed away using distilled water as described (Atwal et al., 2000), and neurons were treated immediately as described in the figure legend. The length of the longest axons in each track in the distal axon compartment was measured every 24 hr, and axon growth rate was calculated. Since the efficiency of Pep-1 delivery of protein is below 100%, we used a different axon growth assay for experiments reported in Figure 6 to better reflect the distribution of axon growth rates of all axons in the distal compartments. Photographs of axon terminals in distal axon compartments were taken using a Hamamatsu Orca-ER digital camera immediately before adding either anti-NGF alone or the Pep-1/anti-NGF complex to the cell body compartment and NGF to the distal axon compartments. Twenty-four hours later, a second set of pictures was taken for the same fields, and the length of individual axons was measured using the Openlab software.

Immunoblotting and Antibodies

After treatment, cells were lysed using boiling Laemmli buffer. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against P-TrkA(Y490), P-Akt (Ser473), P-Erk1/2, or P-CREB (Ser133). Normalization for protein amounts was done by reprobing the immunoblots with an antibody against the p85 subunit of PI3-kinase or tubulin. The anti-TrkA polyclonal antibody (Chemicon) used in some experiments to support survival of sympathetic neurons was diluted in complete growth medium to achieve a final working concentration of 20 µg/ml.

Sympathetic Neuron Cultures in Three-Compartment Chambers

When cultured in three-compartment chambers, cells were plated in the left-most compartment and axons projected under two teflon dividers that are at least 3 mm apart. For survival assays, cells were grown for 11 DIV and then treated with K252a (Calbiochem) added either exclusively to cell bodies, proximal axons, or distal axons for another 3 days, with NGF present only on distal axons. For biochemical experiments, neurons were cultured for 11–15 DIV to allow sufficient axonal growth into far-distal axonal compartments. The cultures were then maintained in medium lacking NGF for 3 days (BAF, 50 µM was added to cell bodies to prevent cell death), and proximal axons were subsequently treated with or without K252a (100 nM) for 2 hr, followed by addition of NGF to distal axons for either 8 hr or 24 hr. Extracts were prepared from all three compartments, resolved by SDS-PAGE, and subjected to immunoblotting. For retrograde transport of [¹²⁵I]NGF in three-compartment chambers, sympathetic neurons were cultured for 21 DIV, [¹²⁵I]NGF

(0.5 µCi/ml) was added exclusively to far-distal axons, and retrograde appearance of [¹²⁵I]NGF in cell bodies was assessed as described (Kuruville et al., 2000).

Adenoviral Infections

The recombinant adenoviruses encoding K44A dynamin and LacZ were provided by Dr. Jeffrey E. Pessin (University of Iowa). Adenoviral infection of sympathetic neurons was performed as described (Kuruville et al., 2000). For survival assays, cells were subjected to treatments immediately after infection. Infected neurons were identified by either dynamin immunostaining (1:500 dilution of monoclonal anti-dynamin antibody; Transduction Laboratories) for K44A dynamin virus-infected neurons or X-gal staining for lacZ virus-infected neurons. [¹²⁵I]NGF retrograde transport assays were performed 48 hr after infection as described.

Pep-1 Delivery of Affinity-Purified Anti-NGF

Affinity-purified anti-NGF was purified from polyclonal anti-NGF serum (Sigma) by affinity chromatography using NGF-coupled Affi-Gel 10 (Bio-Rad). Pep-1 was obtained from Active Motif (product name Chariot™). Transfections were performed according to the manufacturer's instructions. Pep-1 (2 µl) and affinity-purified anti-NGF (16 µg/ml) in a final volume of 200 µl was used for each transfection. Pep-1-mediated transfection is independent of the endosomal pathway (Morris et al., 2001), although the involvement of endocytosis in the cellular internalization of cell-penetrating peptides has been implicated (Richard et al., 2003).

[¹²⁵I]NGF Immunoprecipitation Assays

Cells in biochemistry chambers (Kuruville et al., 2000) were washed three times with DMEM, and complete growth medium containing [¹²⁵I]NGF (0.5 µCi/ml) was added to the distal axon compartment. Twelve hours later, medium containing either anti-NGF alone or the Pep-1/anti-NGF complex was added to the cell body compartment. Three hours later, plasma membrane bound anti-NGF was removed by two acid washes and three DMEM washes at 4°C. For each condition, extracts from cell body compartments were prepared by incubating for 15 min with ice-cold NP-40 lysis buffer. Lysates were clarified by centrifugation at 12,000 × g at 4°C and either directly resolved by SDS-PAGE or subjected to immunoprecipitation using Protein A-agarose at 4°C for 1 h and then resolved by SDS-PAGE.

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