

# Spatially and Functionally Distinct Roles of the PI3-K Effector Pathway during NGF Signaling in Sympathetic Neurons

Rejji Kuruvilla, Haihong Ye, and David D. Ginty\*  
Howard Hughes Medical Institute  
Department of Neuroscience  
The Johns Hopkins University School of Medicine  
Baltimore, Maryland 21205

## Summary

NGF is a target-derived growth factor for developing sympathetic neurons. Here, we show that application of NGF exclusively to distal axons of sympathetic neurons leads to an increase in PI3-K signaling in both distal axons and cell bodies. In addition, there is a more critical dependence on PI3-K for survival of neurons supported by NGF acting exclusively on distal axons as compared to neurons supported by NGF acting directly on cell bodies. Interestingly, PI3-K signaling within both cell bodies and distal axons contributes to survival of neurons. The requirement for PI3-K signaling in distal axons for survival may be explained by the finding that inhibition of PI3-K in the distal axons attenuates retrograde signaling. Therefore, a single TrkA effector, PI3-K, has multiple roles within spatially distinct cellular locales during retrograde NGF signaling.

## Introduction

The development and maintenance of the mammalian nervous system is dependent upon the activities of a complex array of extracellular cues. Neurotrophins are a family of target-derived peptide factors that support the growth, differentiation, and survival of developing neurons. Nerve growth factor (NGF) is perhaps the best characterized of all the members of the neurotrophin family, which includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Reichardt and Farinas, 1997). In the peripheral nervous system, sympathetic neurons and cutaneous sensory neurons are dependent upon target-derived NGF for survival during development, while in the central nervous system, NGF is required for growth of cholinergic neurons whose cell bodies are located in the basal forebrain.

The diverse biological responses elicited by neurotrophins are mediated by the action of receptor tyrosine kinases of the Trk family. TrkA is the high-affinity receptor for NGF and is expressed in all NGF-dependent neurons (Reichardt and Farinas, 1997). Ligand-bound TrkA dimerizes and undergoes autophosphorylation on specific tyrosine residues, which serve as docking sites for a variety of effector proteins, including Shc, PLC- $\gamma$ , FRS-2, SH2-B, and rAPS (Kaplan and Miller, 2000). Effector pathways that propagate NGF/TrkA signaling inside the neuron include the Raf-MAPK pathway and the phosphatidylinositol 3-kinase (PI3-K) pathway (Kaplan and Miller, 2000). Recent work has implicated PI3-K as

a key regulator of NGF-dependent survival of PC12 cells (Yao and Cooper, 1995) and sympathetic neurons (Philpott et al., 1997; Crowder and Freeman, 1998). The survival-promoting effects of PI3-K in sympathetic neurons are executed, at least in part, through the actions of the serine/threonine protein kinase Akt (Crowder and Freeman, 1998; Mazzoni et al., 1999).

Since NGF functions as a target-derived survival factor for sympathetic and sensory neurons, it is believed that its chief mode of action for supporting survival of these neurons is retrograde signaling. A role for NGF itself as a carrier of the retrograde signal is supported by the observations that NGF is retrogradely transported in sympathetic neurons *in vivo* and *in vitro* (Hendry et al., 1974; Stockel et al., 1975; Johnson et al., 1978; Korsching and Thoenen, 1983; Palmartier et al., 1984; Nagata et al., 1987). Recent work has indicated that tyrosine-phosphorylated Trk receptors (P-Trk) are also retrogradely transported in neurons (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). In addition, the catalytic activity of TrkA that has been retrogradely transported to the cell bodies and proximal axons is necessary for phosphorylation and activation of the nuclear transcription factor CREB (Riccio et al., 1997). Whether retrograde transport of an NGF/TrkA complex or some other retrograde signal carrier mediates NGF-dependent survival of sympathetic neurons remains unclear.

In addition to supporting survival through retrograde signaling mechanisms, target-derived NGF acts locally to support growth of distal axons. This idea is supported by the observation that both NGF and TrkA are necessary for growth of axons of cutaneous sensory neurons within their target fields *in vivo* (Patel et al., 2000). Likewise, growth of distal axons of sympathetic neurons *in vitro* requires NGF signaling within distal axons; NGF acting exclusively on cell bodies and proximal axons of compartmentalized neurons cannot support growth of distal axons (Campanot, 1977). Further, NGF can act directly on axons of sympathetic neurons to affect changes in synaptic efficacy (Lockhart et al., 1997), and NGF and other neurotrophins can function as potent chemoattractants when applied directly to neuronal growth cones *in vitro* (Ming et al., 1999). Thus, NGF/TrkA signaling within multiple distinct cellular compartments contributes to growth, differentiation, plasticity, and survival of the neurons.

We have begun to characterize the TrkA effector pathways that contribute to both local and retrograde NGF signaling using an *in vitro* model of compartmentalized sympathetic neurons. Here, we provide evidence that PI3-K signaling is essential for survival of sympathetic neurons supported by NGF acting exclusively on distal axons. Compartmentalized inhibition experiments indicate that NGF-dependent survival requires PI3-K within both cell bodies and distal axons. Interestingly, PI3-K signaling in distal axons is critical for initiation, but not propagation, of retrograde transport of NGF and retrograde signaling. These results establish that a single TrkA effector pathway, the PI3-K pathway, has multiple roles within spatially distinct cellular locales during NGF-dependent growth and survival of sympathetic neurons.

\* To whom correspondence should be addressed (dginty@jhmi.edu).

## Results

### NGF Activation of the PI3-K Pathway in Compartmentalized Sympathetic Neurons

We used dissociated sympathetic neurons obtained from newborn rat superior cervical ganglia and grown in compartmentalized cultures (Campenot, 1977; Riccio et al., 1997) to assess the subcellular distribution and state of activation of PI3-K and its downstream effector Akt (protein kinase B). Neurons were maintained under conditions in which cell bodies and proximal axons (hereafter referred to as the cell body compartment) were exposed to medium containing a neutralizing antibody directed against NGF ( $\alpha$ -NGF) while distal axons, which are >1 mm away from cell bodies, were exposed to medium containing NGF. These conditions resemble *in vivo* conditions in which neurons are maintained by NGF acting exclusively on distal axons.

We first asked whether binding of NGF to receptors exclusively on distal axons regulates the activities of PI3-K and Akt in distal axons and/or cell bodies. For these experiments, NGF was removed from medium bathing distal axons for 24 hr. Then, distal axons were exposed to the same medium (control) or medium containing NGF for various times. The activation states of TrkA and Akt were assessed in extracts prepared from cell body and distal axon compartments by immunoblotting using antibodies that recognize the activated, phosphorylated forms of these proteins. P-Trk (Y490) antibodies recognize TrkA when phosphorylated on Tyr-490, which is the Shc recognition site (Kaplan and Miller, 1997). P-Akt antibodies recognize Akt when phosphorylated on Ser-473, which is necessary for its catalytic activity (Datta et al., 1999). Application of NGF to distal axons resulted in increased levels of P-TrkA (Y490) and P-Akt within distal axons, which were maximal after 20 min (Figure 1A and data not shown). Increases in both P-TrkA (Y490) and P-Akt were also detected in cell bodies but with slower kinetics. A small but reproducible increase in both P-TrkA (Y490) and P-Akt was detected in extracts of cell bodies within 20 min, and a more robust increase was seen at 8 hr (Figure 1A). The appearance of P-TrkA (Y490) and P-Akt in both distal axons and cell bodies was coincident with the appearance of PI3-K activity associated with phosphotyrosine immunoprecipitates (Figure 1B). Additionally, withdrawal of NGF from distal axons of neurons, which had been grown with medium containing a high concentration of NGF (100 ng/ml) on distal axons and  $\alpha$ -NGF on cell bodies, led to a decrease in the levels of both P-TrkA (Y490) and P-Akt in distal axons and in cell bodies (Figure 1C). Thus, NGF acting on TrkA receptors on distal axons regulates the phosphorylation/activation of TrkA, PI3-K, and Akt both locally within distal axons and retrogradely to proximal axons and cell bodies of sympathetic neurons.

### TrkA Activity within Both Distal Axons and Cell Bodies Is Necessary for Retrograde Control of PI3-K/Akt Signaling

To determine whether TrkA signaling within distal axons, within cell bodies, or both mediates retrograde activa-

tion of PI3-K/Akt signaling in sympathetic neurons, experiments were done using compartmentalized neurons and the Trk kinase inhibitor K252a (Berg et al., 1992; Ohmichi et al., 1992; Tapley et al., 1992). Neurons were grown in chambers with medium containing  $\alpha$ -NGF bathing cell bodies and medium containing NGF (100 ng/ml) bathing distal axons. Application of K252a to distal axons led to a decrease in levels of both P-Akt (Figure 2) and P-TrkA (Y490) (data not shown) in distal axons and in cell bodies. In contrast, application of K252a exclusively to cell bodies led to a decrease in levels of P-Akt in cell bodies, but not in distal axons. The latter treatment also resulted in a reduction in the amount of P-TrkA (Y490) in cell bodies, but not in distal axons (Riccio et al., 1997). Thus, TrkA activity is required within distal axons for both local and retrograde PI3-K signaling. Moreover, the activity of TrkA within cell bodies, which is derived at least in part from retrogradely transported P-TrkA (Y490) (Tsui-Pierchala and Ginty, 1999), is required for PI3-K/Akt signaling in cell bodies of sympathetic neurons.

### Selective Inhibition of PI3-K Activity within Distinct Cellular Compartments

The availability of wortmannin and LY294002, which are structurally distinct, membrane-permeable inhibitors of PI3-K, allowed us to address the role of the PI3-K effector pathway within cell bodies and distal axons separately during local and retrograde NGF signaling. LY294002 (100  $\mu$ M) and wortmannin (100 nM) inhibit PI3-K, but not PI4-K (Powis et al., 1994; Vlahos et al., 1994). We first asked whether we could apply these pharmacological compounds to individual compartments to selectively block the activity of PI3-K in distal axons, but not cell bodies, and vice versa. When added to the medium bathing either cell bodies or distal axons for 30 min, wortmannin blocked phosphorylation of Akt in cell bodies or distal axons, respectively (Figure 3A). Application of wortmannin to cell bodies did not block phosphorylation of Akt in distal axons, and application of wortmannin to distal axons did not block phosphorylation of Akt in cell bodies. LY294002 was used for experiments requiring long-term inhibition of PI3-K because LY294002, unlike wortmannin, is a reversible inhibitor of PI3-K (Powis et al., 1994; Vlahos et al., 1994), and LY294002 is considerably more stable than wortmannin in aqueous solutions. Remarkably, treatment of cell bodies with LY294002 for 8 hr (Figure 3B) or 2 days (Figure 3C) led to complete inhibition of phosphorylation of Akt within cell bodies, but this treatment did not affect the levels of P-Akt in distal axons. These results indicate that this pharmacological approach is useful for experiments, described below, that address the role of PI3-K signaling in cell bodies versus distal axons during local and retrograde NGF signaling and survival.

### PI3-K Activity Is Necessary for Survival of Sympathetic Neurons Supported by NGF Acting Exclusively on Distal Axons

Recent reports have indicated that activation of PI3-K signaling is sufficient to support survival of sympathetic neurons grown in the absence of NGF (Philpott et al., 1997; Crowder and Freeman, 1998). Yet, whether PI3-K

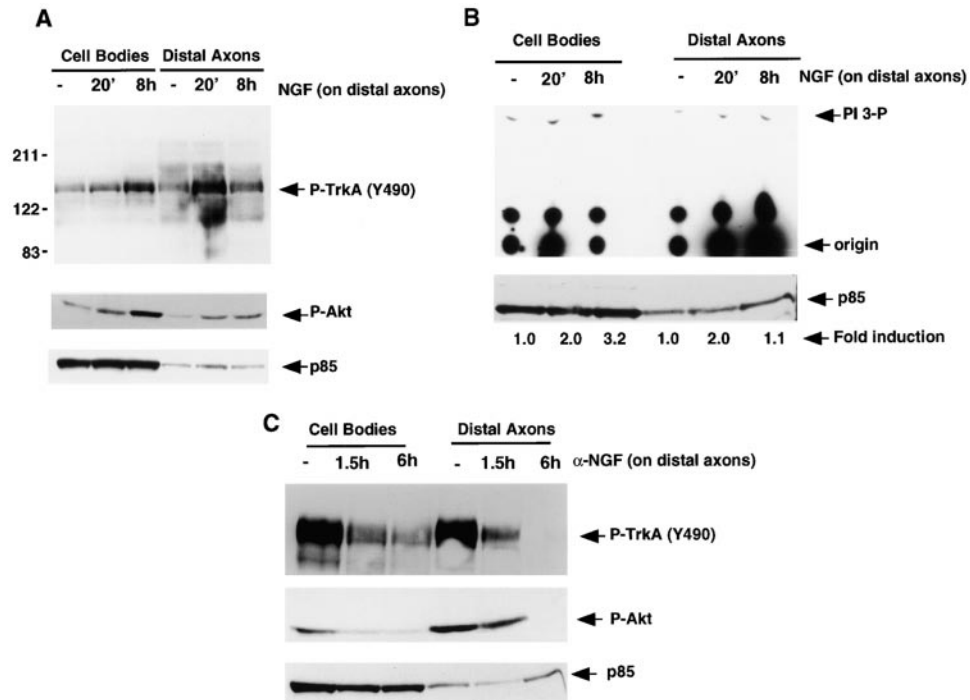


Figure 1. An NGF-Mediated Retrograde Signal Induces TrkA and PI3-K Activity in Cell Bodies of Sympathetic Neurons

(A) Time course of appearance of phosphorylation of TrkA Tyr-490 (P-TrkA [Y490]) and Akt Ser-473 (P-Akt) in cell bodies and distal axons of compartmentalized sympathetic neuronal cultures after application of NGF to the distal axonal processes. The distal axons and terminals of 13 DIV sympathetic neurons grown in biochemistry chambers were maintained in medium lacking NGF for 2 days before treatment with medium alone (minus sign) or medium containing NGF (150 ng/ml) for either 20 min or 8 hr. Cellular lysates were prepared from individual compartments containing cell bodies (CB) and distal axonal processes (DA) by adding boiling laemmli buffer to the compartments. Cell body and distal axonal extracts were subjected to immunoblotting using antibodies specific for P-TrkA (Y490) and P-Akt (Ser-473). The immunoblots were stripped and reprobed with an antibody against p85 to demonstrate equal amounts of total protein in the extracts.

(B) Induction of phosphotyrosine-associated PI3-K activity in cell bodies and distal axons of sympathetic neurons after application of NGF to the distal axons. The distal axons and terminals of 13 DIV sympathetic neurons grown in biochemistry chambers and supported by NGF on distal axons and  $\alpha$ -NGF on cell bodies were placed in medium lacking NGF in distal axonal compartments for 2 days. Then, distal axons were treated with either medium alone (minus sign) or medium containing NGF (150 ng/ml) for 20 min and 8 hr. Lysates were prepared from individual compartments containing either cell bodies or distal axonal processes and subjected to immunoprecipitation with a phosphotyrosine antibody (4G10) for 2 hr at 4°C, and PI3-K assays were carried as described in Experimental Procedures.

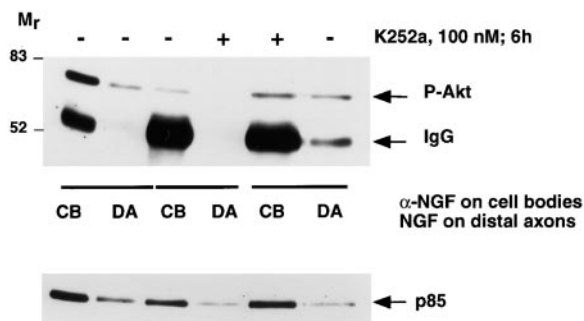
(C) Kinetics of dephosphorylation of P-TrkA (Y490) and P-Akt in the cell bodies and distal axons after withdrawal of NGF from the distal axonal processes. Twelve to thirteen DIV cultures of compartmentalized neurons were maintained in medium containing  $\alpha$ -NGF in the cell body compartment and in medium containing NGF (200 ng/ml) in the distal axon compartment. The NGF-containing medium was removed from the distal axonal compartment, the distal axonal processes were washed once with medium lacking NGF, and then the processes were incubated in medium containing  $\alpha$ -NGF (1:1000) for 1.5 hr and 6 hr, respectively. Lysates were prepared from cell body and distal axonal compartments as described above and subjected to immunoblotting using antibodies against P-TrkA (Y490) and P-Akt. Protein normalization was done by stripping the immunoblots and reprobing with an antibody against p85. All of the above experiments were done at least three times with similar results.

signaling is necessary for NGF-dependent survival of sympathetic neurons is controversial; some groups have reported that PI3-K is required (Crowder and Freeman, 1998; Vaillant et al., 1999) while at least two others have reported that PI3-K is not required (Philpott et al., 1997; Tsui-Pierchala et al., 2000) for NGF-dependent survival of sympathetic neurons. We asked two questions about the role of PI3-K as a mediator of NGF-dependent survival of sympathetic neurons. First, is PI3-K required for survival of sympathetic neurons supported by NGF acting directly on cell bodies and/or exclusively on distal axons? Second, if PI3-K is necessary for survival of neurons supported by NGF acting on distal axons, then in which cellular compartment is PI3-K required?

To address these questions, we compared the effects of LY294002 on survival of sympathetic neurons grown

under three different conditions. The first condition consisted of sympathetic neurons grown in mass culture. Under these conditions, NGF (50 ng/ml) acts on receptors located on both cell bodies and distal axons. The second condition employed compartmentalized cultures of sympathetic neurons in which NGF (50 ng/ml) was present in the medium bathing both cell bodies and distal axons. The third condition consisted of compartmentalized cultures of sympathetic neurons in which NGF (either 50 or 0.5 ng/ml) was present in the medium bathing distal axons, but  $\alpha$ -NGF was present in the medium bathing cell bodies. The latter condition most accurately reflects the natural mode of target-derived NGF signaling in developing sympathetic neurons in vivo.

Interestingly, we found that inhibition of PI3-K had remarkably different effects on NGF-dependent survival



**Figure 2.** TrkA Kinase Activity within Both Distal Axons and Cell Bodies Is Required for the Propagation of a Retrograde Signal to Akt in the Cell Bodies of Sympathetic Neurons

Sympathetic neurons were grown in biochemistry chambers for 13 DIV. Seventy-two hours prior to the experiment, medium containing  $\alpha$ -NGF was added to the cell bodies while the distal axons were maintained in medium containing NGF. Then K252a (100 nM), a specific inhibitor of Trk kinase activity, was added to either cell bodies or distal axons for 6 hr. Cell body and distal axon extracts were prepared as before and subjected to immunoblotting with an antibody against P-Akt. The P-Akt blot was later stripped and reprobed with an antibody against p85 to normalize for protein levels. Representative results from three independent experiments are shown. DA, distal axons; CB, cell bodies.

of neurons depending on their growth conditions. Two assays for assessment of apoptosis were used for these cell survival experiments. One assay employed Hoechst 33258, which is a chromatin stain that can reveal nuclear condensation and fragmentation associated with apoptotic nuclei. The other assay employed immunocytochemistry using an antibody that recognizes the cleaved, catalytically active form of caspase-3 (Srinivasan et al., 1998). Application of LY294002 to mass cultures was much less effective than  $\alpha$ -NGF in promoting apoptosis of sympathetic neurons (Figure 4B). While  $\alpha$ -NGF treatment led to near complete apoptosis of neurons within 48 hr, LY294002 treatment led to  $\sim$ 20% apoptosis in mass cultures at this time point. In addition,  $\sim$ 50% of neurons have normal nuclear morphology and low levels of caspase-3 immunoreactivity after LY294002 treatment for 72 hr (Figure 4B). LY294002 was effective since it completely inhibited phosphorylation of Akt (Figures 3B and 3C), and similar results were seen with multiple lots of the compound (data not shown). Moreover, while neurons exposed to  $\alpha$ -NGF exhibited nuclear condensation and fragmentation as well as strong caspase-3 immunoreactivity, many neurons treated with LY294002 for 72 hr exhibited nuclear condensation and strong caspase-3 immunoreactivity, but relatively few of these neurons exhibited obvious signs of nuclear fragmentation (Figure 4A). Thus, while both LY294002 treatment and  $\alpha$ -NGF treatment led to apoptotic death, the kinetics and magnitude of the apoptotic response were much more dramatic with  $\alpha$ -NGF treatment, and the nuclear response to these treatments was qualitatively different. Very similar results were obtained in experiments using compartmentalized cultures in which NGF was present in media bathing both cell bodies and distal axons (Figure 5A). While  $\alpha$ -NGF treatment was effective at killing most of the neurons grown under these conditions, only 33%–46% of neurons underwent apoptosis after 3 days of exposure of LY294002 to cell

bodies. In contrast, inhibition of PI3-K activity had a more dramatic effect on survival of sympathetic neurons grown under conditions in which they were supported by NGF acting exclusively on distal axons (Figure 5B). Under these growth conditions, application of LY294002 to both cell bodies and distal axons led to rapid apoptotic death that exceeded 70% after 48 hr and was nearly 100% after 72 hr (Figure 5B). The kinetics and magnitude of cell death induced by LY294002 was similar to that seen after NGF withdrawal. Thus, these experiments reveal a more critical role for PI3-K signaling in sympathetic neurons supported by NGF acting exclusively on distal axons than in neurons in which NGF acts directly on cell bodies.

### PI3-K Activity within Both Distal Axons and Cell Bodies Contributes to Retrograde NGF Signaling and Survival

We next asked whether PI3-K signaling in distal axons, cell bodies, or both contributes to survival of neurons supported by NGF acting exclusively on distal axons. Inhibition of PI3-K exclusively in cell bodies for 72 hr led to apoptosis in essentially all neurons (Figure 5B). In addition, LY294002 application to distal axons of neurons supported by high concentration of NGF (50 ng/ml) on distal axons led to significant apoptotic death of neurons (Figure 5B), although the magnitude of the effect was small compared to LY294002 application to cell bodies. The significant amount of cell death observed under the latter conditions prompted us to ask whether inhibition of PI3-K in distal axons has a greater effect on neurons supported by a low concentration of NGF (0.5 ng/ml) in medium bathing distal axons. Indeed, the proapoptotic effect of LY294002 acting on distal axons was much more pronounced when the concentration of NGF in medium bathing distal axons was lowered to 0.5 ng/ml (Figure 5C). It is unlikely that LY294002 acting on distal axons exerts its effects by direct inhibition of PI3-K in cell bodies because application of NGF directly to cell bodies of neurons exposed to PI3-K inhibitors on distal axons effectively activates phosphorylation of Akt in cell bodies (Figure 3A). Moreover, LY294002 applied to distal axons does not kill neurons supported by NGF acting directly on cell bodies (Figure 5A and data not shown). Therefore, PI3-K signaling within both cell bodies and distal axons contributes to survival of sympathetic neurons supported by NGF acting exclusively on distal axons.

### Inhibition of PI3-K Signaling in Distal Axons, but Not Cell Bodies, Attenuates Retrograde Transport of NGF and Retrograde NGF Signaling

Why does inhibition of PI3-K signaling within distal axons lead to apoptosis of sympathetic neurons supported by NGF acting exclusively on distal axons? One possibility is that PI3-K signaling in distal axons is necessary for retrograde transport of an NGF/TrkA signaling complex. In support of this idea, Hendry and colleagues showed that injection of PI3-K inhibitors into the eye attenuates retrograde transport of [ $^{125}$ I]NGF to superior cervical ganglia in vivo (Bartlett et al., 1997). Moreover, PI3-K signaling has been implicated during ligand-dependent trafficking of several receptor tyrosine kinases, including the PDGF receptor (Joly et al., 1994,



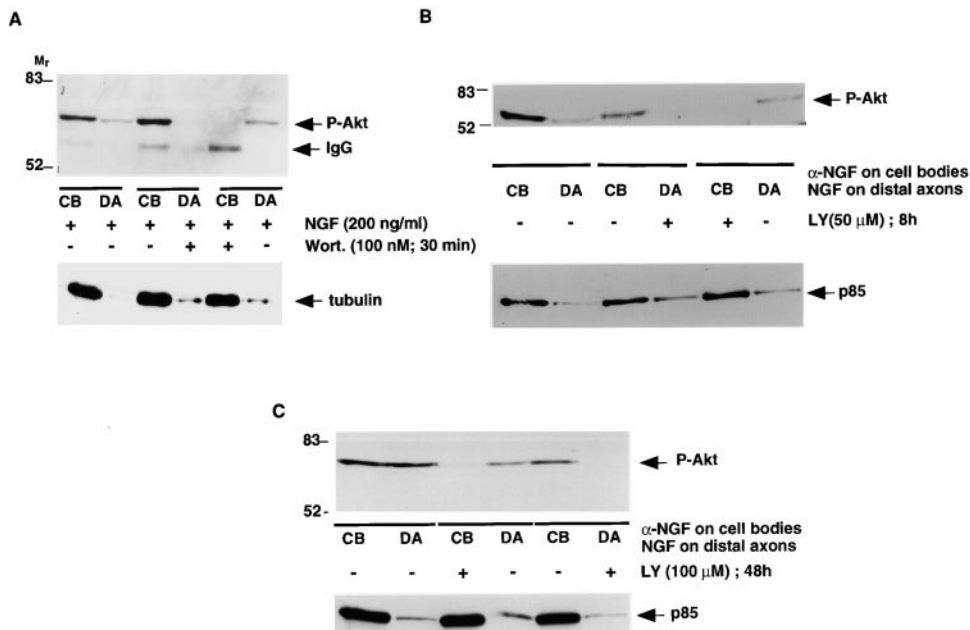


Figure 3. Selective Inhibition of PI3-K within Cell Bodies or Distal Axons of Sympathetic Neurons Grown in Compartmentalized Cultures

(A) Distal axons of 12 DIV sympathetic cultures grown in biochemistry chambers were placed in medium lacking NGF for 2 days. Then, the cell body and distal axonal compartments were treated with either medium alone or medium containing a PI3-K inhibitor, wortmannin (100 nM), for 30 min. Subsequently, both cell bodies and distal axons were treated with NGF (200 ng/ml) for 15 min. Lysates prepared from the cell body and distal axon compartments were resolved by SDS-PAGE and immunoblotted with an antibody against P-Akt. Protein levels were normalized by stripping the P-Akt blot and reprobing with an antibody against  $\alpha$ -tubulin.

(B) Inhibition of PI3-K in the cell bodies has no effect on P-Akt levels in the distal axons of sympathetic neurons. Sympathetic neurons were grown in biochemistry chambers for 13 DIV. Medium containing  $\alpha$ -NGF was added to the cell bodies 72 hr prior to the experiment while the distal axons were maintained in medium containing NGF (200 ng/ml). Then, a specific inhibitor of PI3-K, LY294002 (50  $\mu$ M), was added either to cell bodies or distal axons for 8 hr. Cell body and distal axonal extracts were prepared as before and subjected to immunoblotting with an antibody against P-Akt. The P-Akt immunoblot was then stripped and reprobed with an antibody against p85 to normalize for protein levels.

(C) Twelve DIV sympathetic neurons grown in biochemistry chambers were maintained in medium containing  $\alpha$ -NGF in the cell body compartments and NGF (200 ng/ml) in distal axonal compartments. One set of compartmentalized cultures was then treated with LY294002 (100  $\mu$ M) only in the cell body compartment for 2 days while in another set of biochemistry chambers, LY294002 was added only to the distal axonal processes for 2 days. Boiling laemmli extracts were prepared from the compartments containing cell bodies and distal axons. Western blots were performed using the antibody specific for phosphorylated Akt, followed by stripping and reprobing the blot for p85 to normalize for equal protein amounts. All of the above experiments were performed at least three times with similar results in each case. DA, distal axons; CB, cell bodies.

1995) and the insulin receptor (Sasaoka et al., 1999) and, therefore, may participate in NGF-dependent internalization and/or retrograde movement of P-TrkA. To explore the potential role of PI3-K in retrograde signaling, we performed experiments to address the requirement of PI3-K in cell bodies and/or distal axons for retrograde transport of NGF and retrograde TrkA signaling in compartmentalized sympathetic neurons.

To assess the requirement of PI3-K in cell bodies and/or distal axons for retrograde transport of NGF, we exposed distal axons to [ $^{25}$ I]NGF and then measured the appearance of radiolabeled ligand in extracts prepared from cell bodies. These experiments were done under conditions in which PI3-K was inhibited by LY294002 applied to either cell bodies or distal axons for 8 hr. Similar experiments were done using the Trk inhibitor K252A and the specific MEK inhibitor UO126. Inhibition of PI3-K in distal axons, but not in cell bodies, attenuated retrograde transport of [ $^{25}$ I]NGF by  $\sim$ 80% (Figures 6B and 6C). Likewise, inhibition of TrkA activity in distal axons blocked retrograde transport of [ $^{25}$ I]NGF (Figure 6A). In contrast, complete inhibition of MEK with UO126

(Figure 6E) had no effect on retrograde transport of [ $^{25}$ I]NGF (Figure 6D). Since the above experiments relied upon pharmacological inhibition of PI3-K, we next assessed the necessity for PI3-K activity for retrograde transport of NGF in a complementary set of experiments in which PI3-K was inhibited by a dominant-negative form of p85 ( $\Delta$ p85).  $\Delta$ p85 blocks activation of endogenous PI3-K because it cannot associate with the p110 catalytic subunit. Neurons infected with an adenovirus encoding  $\Delta$ p85 displayed an increase in amounts of p85 and a decrease in amounts of P-Akt compared to neurons infected with a control adenovirus encoding LacZ (Figure 7B). Importantly, neurons expressing  $\Delta$ p85 exhibited a reduction in retrograde transport of [ $^{25}$ I]NGF compared to LacZ-infected neurons (Figure 7A). Taken together with pharmacological experiments described above, we conclude that PI3-K signaling, but not MEK-ERK signaling, is necessary for retrograde transport of NGF.

We next assessed the role of PI3-K signaling in distal axons in retrograde TrkA signaling and retrograde appearance of an NGF/P-TrkA complex in cell bodies.

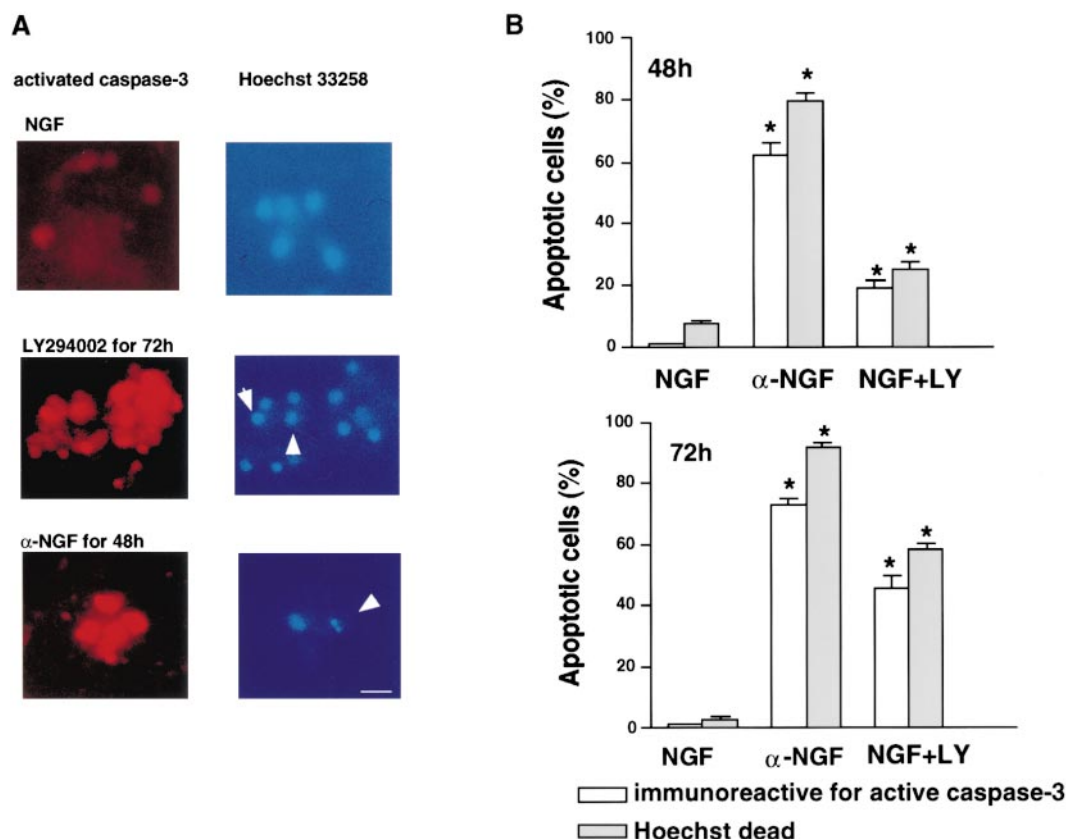


Figure 4. Inhibition of PI3-K Attenuates NGF-Dependent Survival of Sympathetic Neurons Grown in Mass Cultures

(A) Apoptotic cell death of sympathetic neurons treated with  $\alpha$ -NGF and the PI3-K inhibitor LY294002. Sympathetic neurons that were maintained in medium containing NGF (50 ng/ml) (top panel), NGF (50 ng/ml) plus LY294002 (100  $\mu$ M) for 72 hr (middle panel), or  $\alpha$ -NGF for 48 hr (bottom panel) were stained with Hoechst 33258 dye to identify apoptotic neurons. Arrowheads indicate representative apoptotic neurons with shrunken soma and bright, condensed nuclei (middle panel) and fragmented nuclei (bottom panel). The neurons were also stained with an antibody directed against cleaved, activated form of caspase-3. Scale bar = 20  $\mu$ M.

(B) Quantitation of the effects of PI3-K inhibition on NGF-dependent survival of mass sympathetic neuronal cultures. Sympathetic neurons, grown in mass cultures for 10–11 DIV in medium containing NGF (50 ng/ml) were subsequently maintained in NGF-containing medium or treated either with  $\alpha$ -NGF for 48 hr or with NGF (50 ng/ml) and the specific PI3-K inhibitor LY294002 (100  $\mu$ M) for 48 hr or 72 hr, respectively. A neuron was scored as apoptotic if it displayed shrunken soma, fragmented or condensed nuclei, or no nucleus as assessed with Hoechst 33258 dye (Hoechst dead) and was strongly immunoreactive for the cleaved, active form of caspase-3. Results are presented as mean  $\pm$  SEM from two independent experiments done in triplicate for each condition. The data are presented as percent of total number of cells counted for viability. Asterisk,  $p < 0.001$  significantly different from control cultures treated with NGF as assessed by one-way ANOVA followed by a Tukey-Kramer test.

Ligand-dependent retrograde TrkA signaling was assessed by P-Trk immunoblotting using extracts prepared from cell bodies and distal axons of compartmentalized neurons after addition of NGF to distal axons that had been untreated or treated with LY294002. Inhibition of PI3-K in distal axons led to an attenuation of the appearance of P-TrkA (Y490) in cell bodies (Figure 8A). Moreover, inhibition of PI3-K in distal axons led to a decrease in appearance in cell bodies of downstream effectors, including P-MAPK (data not shown) and P-Akt (Figures 3B and 3C). The appearance of an NGF/TrkA complex in cell bodies after treatment of distal axons with NGF was also attenuated after inhibition of PI3-K in distal axons (Figure 8B). For these experiments, distal axons were treated with [ $^{125}$ I]NGF, and TrkA/[ $^{125}$ I]NGF complexes were coprecipitated from extracts prepared from cell bodies using anti-TrkA. We found that the amount of coprecipitation of TrkA and [ $^{125}$ I]NGF from

extracts prepared from cell bodies was less when distal axons were exposed to wortmannin compared to untreated neurons (Figure 8B). Importantly, pharmacological inhibition of PI3-K signaling in distal axons did not affect formation of an [ $^{125}$ I]NGF/TrkA complex (Figure 8B) or the phosphorylation of TrkA (Figure 8A) in distal axons, indicating that TrkA levels on the surface of distal axons are not affected by our treatments. Taken together, these results indicate that PI3-K signaling in distal axons contributes to retrograde transport of NGF, appearance of NGF/TrkA complexes in cell bodies, and retrograde P-TrkA signaling.

#### PI3-K Signaling Is Necessary for Initiation, but Not Propagation, of Retrograde Transport of NGF

The observations that inhibition of PI3-K signaling within distal axons attenuates retrograde transport and retrograde signaling suggest that PI3-K is necessary for ei-

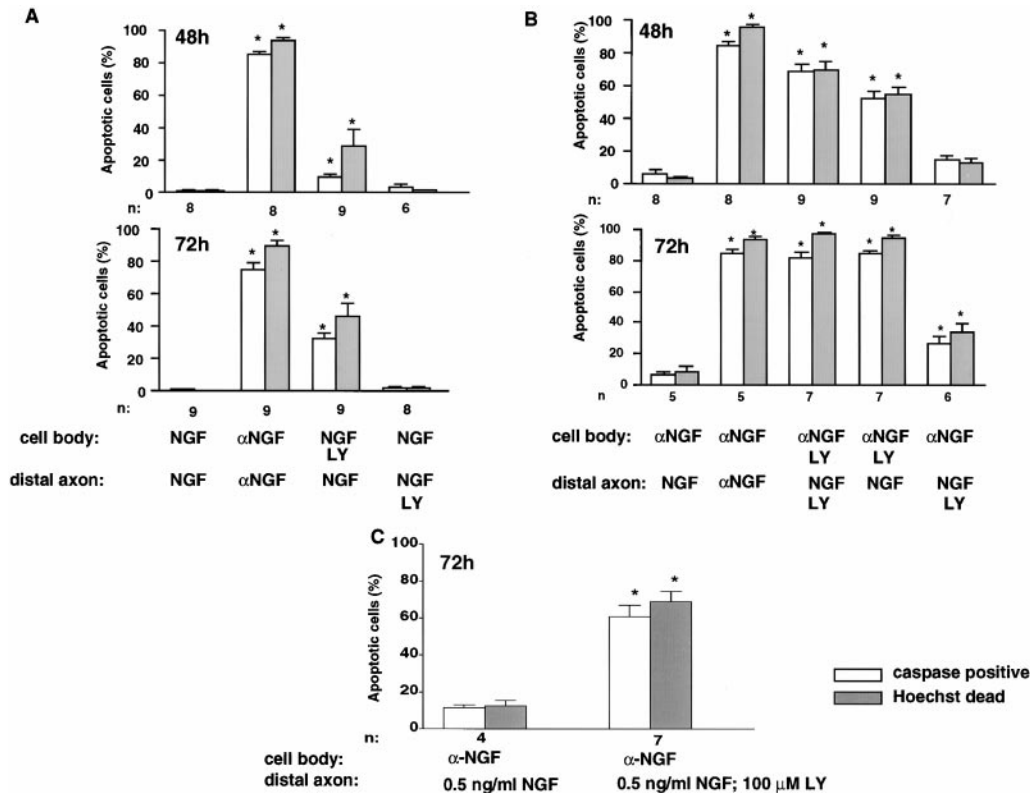


Figure 5. PI3-K Activity in Both Cell Bodies and Distal Axons Contributes to NGF-Dependent Survival of Sympathetic Neurons Supported by NGF Acting Exclusively on Distal Axons

(A) Sympathetic neurons were grown in conventional Campenot chambers for 10–11 DIV and the cell body and distal axon compartments were both incubated with medium containing 50 ng/ml NGF. Under these conditions, cultures were treated with LY294002 (100  $\mu$ M) added exclusively to either the cell body or distal axonal compartments for 48 hr (top panel) or 72 hr (bottom panel), respectively. Some cultures were incubated with medium containing  $\alpha$ -NGF in both cell body and distal axonal compartments for 48 hr. Results are mean  $\pm$  SEM from three independent experiments with a total number of cultures per condition as indicated below each bar. Asterisk,  $p < 0.01$  statistically different from control cultures maintained with NGF in both compartments as determined by one-way ANOVA followed by a Tukey-Kramer test.

(B) In sympathetic neurons maintained with NGF only at the distal axons, PI3-K signaling in cell bodies is crucial for NGF-dependent survival. Sympathetic neurons grown in conventional Campenot chambers were maintained with  $\alpha$ -NGF on cell bodies and NGF (50 ng/ml) on distal axons for 10–11 DIV. Some of the cultures were incubated with medium containing  $\alpha$ -NGF in both cell body and distal axonal compartments. In other conditions, cultures were treated with LY294002 (100  $\mu$ M) applied to either cell body or distal axonal compartments or both for either 48 hr (top panel) or 72 hr (bottom panel). Apoptotic cells were identified as described in Figure 4. Results are mean  $\pm$  SEM from two (bottom panel) or three (top panel) independent experiments using measurements from the number of cultures (n) for each condition as indicated below each bar. Results are presented as percent of total number of cells counted for the viability assays. Asterisk,  $p < 0.001$  statistically different from control cultures maintained with  $\alpha$ -NGF on cell bodies and NGF on distal axons, as determined by one-way ANOVA followed by a Tukey-Kramer test.

(C) Eleven DIV sympathetic neurons were grown in Campenot chambers with medium containing  $\alpha$ -NGF bathing the cell bodies while the distal axons were incubated in medium containing 0.5 ng/ml of NGF. The distal axonal processes were treated with or without LY294002 (100  $\mu$ M) for 72 hr. Asterisk,  $p < 0.001$  significantly different from control cultures maintained with  $\alpha$ -NGF on cell bodies and 0.5 ng/ml NGF on distal axons as determined by a paired Student's  $t$  test.

ther initiation or propagation of retrograde transport within axons, or both. To distinguish between these possibilities, we performed experiments using LY294002 applied to axons of neurons grown in three compartment chambers. In three compartment chambers, axons of sympathetic neurons project underneath two barriers (Figure 9A). Thus, media bathing cell bodies, distal axons (M), and far distal axons (D) can be treated separately. [ $^{125}$ I]NGF added to medium bathing far-distal axons is retrogradely transported to cell bodies of neurons grown in three-compartment chambers (Figures 9B and 9C). LY294002 was added either to medium bathing distal axons or medium bathing far-distal axons. As be-

fore, application of LY294002 to far-distal axons, where NGF binds to its receptors, attenuated retrograde transport of [ $^{125}$ I]NGF to cell bodies. In contrast, application of LY294002 to medium bathing axons within the middle compartment did not affect retrograde transport of [ $^{125}$ I]NGF. These experiments indicate that PI3-K signaling is necessary for initiation, but not propagation, of retrograde transport of NGF in sympathetic neurons.

## Discussion

NGF acting exclusively on distal axons of sympathetic neurons promotes both local axon growth and retro-

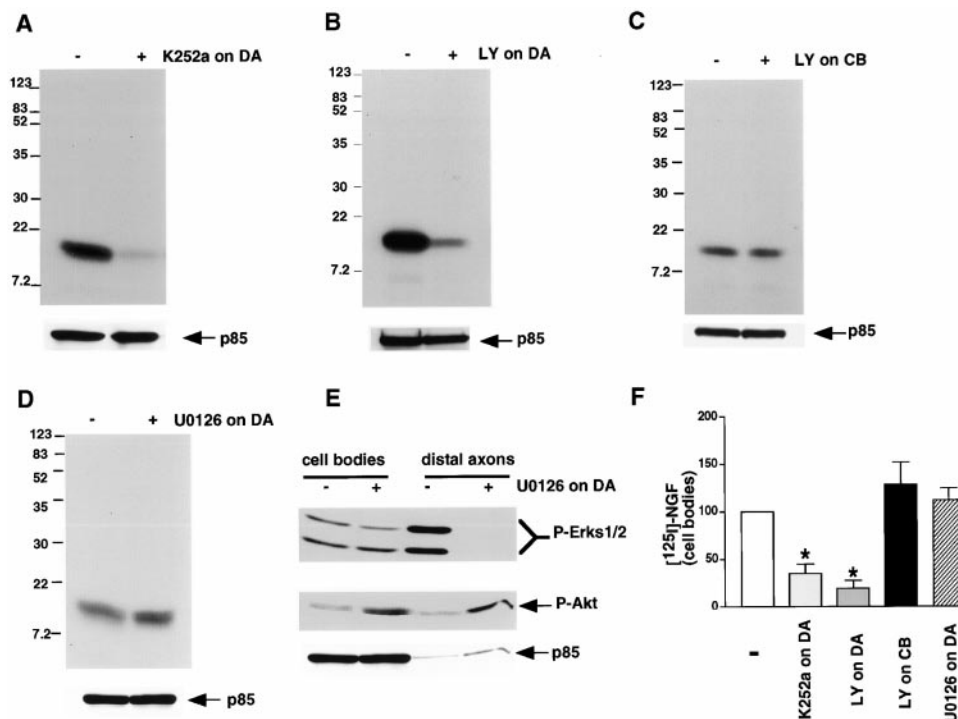


Figure 6. PI3-K Signaling in the Distal Axons Is Necessary for Retrograde Transport of NGF

(A) Inhibition of TrkA kinase activity in the distal axons attenuates the retrograde transport of [ $^{125}$ I]NGF. Distal axons of 12 DIV sympathetic neuronal cultures grown in biochemistry chambers were washed to remove NGF and then either incubated with medium alone (minus sign) or with medium containing K252a (100 nM) for 30 min. Then distal axons were incubated with medium containing [ $^{125}$ I]NGF for 8 hr. Extracts prepared from cell body compartments were resolved by SDS-PAGE gel, dried, and subjected to autoradiography as described in Experimental Procedures.

(B) Inhibition of PI3-K in the distal axons attenuates the retrograde transport of [ $^{125}$ I]NGF. The distal axons of 12 DIV sympathetic neurons grown in biochemistry chambers were washed and then either incubated with medium alone (minus sign) or with medium containing LY294002 (100  $\mu$ M) for 30 min. The distal axons were then incubated with medium containing [ $^{125}$ I]NGF for 8 hr. Shown is [ $^{125}$ I]NGF autoradiography of extracts prepared from cell body compartments. In both (A) and (B), extracts were also subjected to immunoblotting with an antibody against p85 to demonstrate equal protein amounts in the samples.

(C) Inhibition of PI3-K in the cell bodies has no effect on retrograde transport of [ $^{125}$ I]NGF. The cell bodies of 12 DIV sympathetic neurons were incubated with medium containing  $\alpha$ -NGF alone or anti-NGF and LY294002 (100  $\mu$ M) for 8 hr. The distal axonal processes were incubated with medium containing [ $^{125}$ I]NGF. After 8 hr, extracts were prepared from cell body compartments as above and subjected to autoradiography. The remainder of the extracts were subjected to immunoblotting using an antibody directed against p85.

(D) Inhibition of MEK in the distal axons has no effect on retrograde transport of [ $^{125}$ I]NGF. The distal axons of 12 DIV sympathetic neurons grown in biochemistry chambers were washed and then either incubated with medium alone (minus sign) or the MEK inhibitor U0126 (50  $\mu$ M) for 8 hr. Shown is the [ $^{125}$ I]NGF autoradiography using extracts prepared from cell body compartments and normalization for equal protein amounts in the samples by immunoblotting for p85.

(E) Application of the MEK inhibitor U0126 to the distal axons blocks the activation of ERKs but has no effect on P-Akt levels in the distal axons. The distal processes of 12 DIV sympathetic neurons grown in biochemistry chambers were treated as described in (D). Extracts prepared from cell body and distal axonal chambers were subjected to immunoblotting using an antibody specific for the phosphorylated form of ERK1 and ERK2. The immunoblot was later stripped and reprobed for P-Akt and then for p85 to normalize for protein amounts.

(F) Quantitation of the effects of inhibition of TrkA kinase activity, PI3-K and MAPK pathways in distal axons, and PI3-K in cell bodies on the retrograde transport of [ $^{125}$ I]NGF. Results are presented as mean  $\pm$  SEM from three independent experiments. The data are presented as a percent of [ $^{125}$ I]NGF found in cell bodies of chambers that were left untreated. Asterisk,  $p < 0.001$  statistically different from control cultures maintained with  $\alpha$ -NGF on cell bodies and NGF on distal axons, as determined by one-way ANOVA followed by a Tukey-Kramer test. DA, distal axons; CB, cell bodies.

grade signaling, which supports gene expression and survival. We found that NGF acting exclusively on distal axons regulates PI3-K signaling in distal axons and retrogradely in cell bodies. Interestingly, PI3-K signaling within both cell bodies and distal axons contributes to survival of sympathetic neurons. PI3-K signaling within cell bodies is absolutely critical for survival, probably because it promotes activation of Akt and other downstream prosurvival effectors. In contrast, PI3-K signaling in distal axons may indirectly contribute to survival

because it is critical for initiation of retrograde transport in distal axons and retrograde signaling to cell bodies. Thus, a single TrkA effector pathway has multiple roles within spatially distinct cellular locales during NGF-dependent growth and survival of sympathetic neurons.

How does NGF acting on distal axons regulate PI3-K signaling within cell bodies? Our data support the idea that retrograde regulation of PI3-K occurs through a mechanism that is dependent upon retrograde TrkA sig-



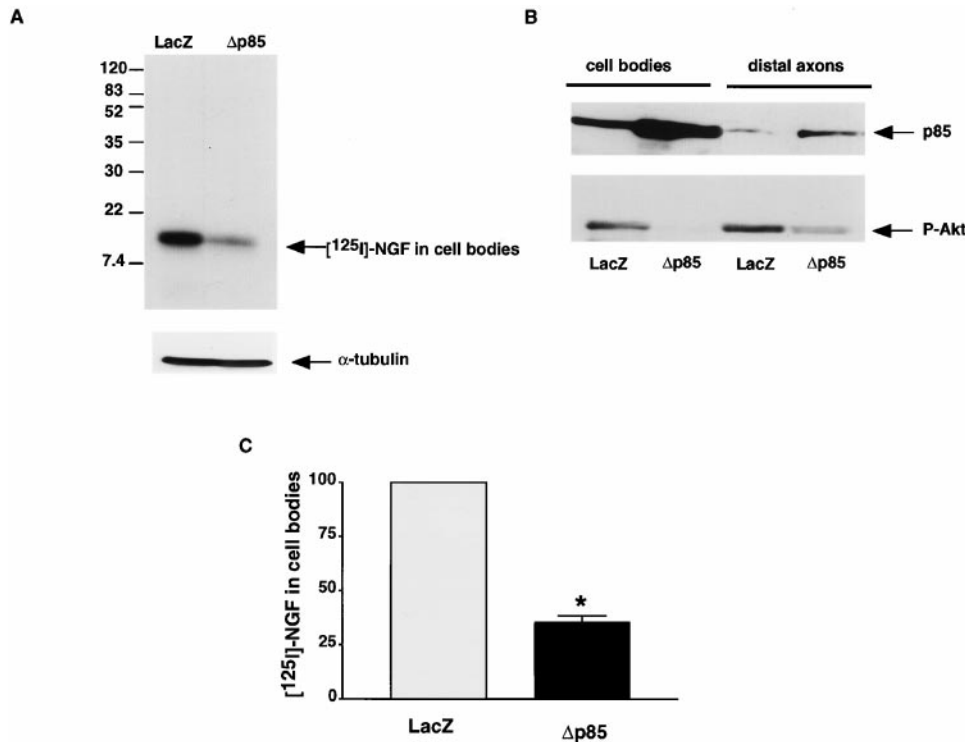


Figure 7. A Dominant-Negative p85 Inhibits Retrograde Transport of [ $^{125}$ I]NGF in Compartmentalized Sympathetic Neurons

(A) Adenovirus-mediated expression of the dominant negative form of the p85 regulatory subunit of PI3-K ( $\Delta$ p85) in sympathetic neurons inhibits the retrograde transport of [ $^{125}$ I]NGF. Eleven to twelve DIV sympathetic neurons grown in biochemistry chambers were infected with adenoviral vectors encoding either  $\beta$ -galactosidase (LacZ) or dominant-negative p85 ( $\Delta$ p85) for 16 hr in DMEM containing 1% FBS and BAF (a nonselective caspase inhibitor, 50  $\mu$ M). The cells were then transferred to regular media supplemented with BAF (50  $\mu$ M), and 36 hr later, the distal axons were washed three times with PBS and incubated with medium containing [ $^{125}$ I]NGF for 8 hr. Extracts prepared from cell body and distal axon compartments were resolved on a 17% SDS-PAGE gel and then subjected to autoradiography or immunoblotting using an antibody against  $\alpha$ -tubulin.

(B) Expression of  $\Delta$ p85 in compartmentalized cultures of sympathetic neurons attenuates the activation of Akt. Extracts prepared from cell body and distal axon compartments were subjected to immunoblotting using antibodies specific for p85 and P-Akt.

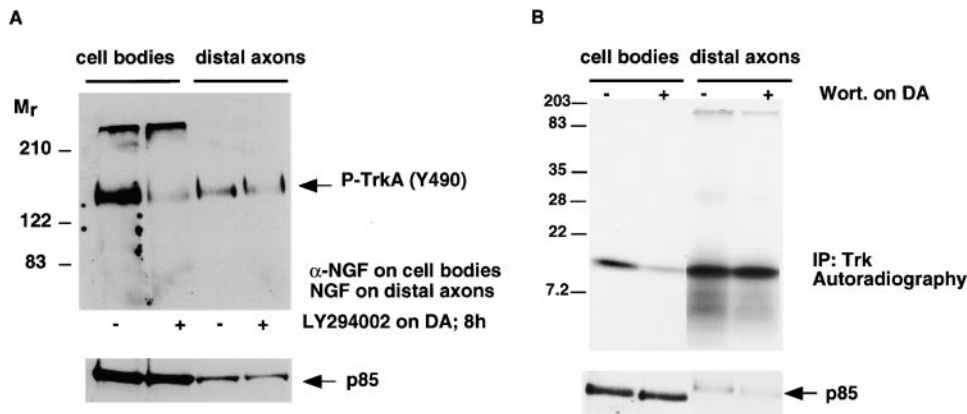
(C) Quantification of the effects of overexpression of dominant-negative p85 on the retrograde transport of [ $^{125}$ I]NGF. Results are mean  $\pm$  SEM from three independent experiments. The data are presented as a percent of the radioactivity found in cell bodies of chambers that were infected with the control LacZ adenovirus. Asterisk,  $p < 0.001$  statistically different from LacZ-infected cultures, as determined by one-way ANOVA followed by a Tukey-Kramer test.

naling. The kinetics of appearance of P-Akt and phosphotyrosine-associated PI3-K activity in cell bodies are coincident with the appearance of P-TrkA in that cellular compartment. Also, inhibition of TrkA activity in cell bodies blocks the appearance of P-Akt in cell bodies. Thus, TrkA signaling exclusively within distal axons is not sufficient to support PI3-K/Akt signaling within cell bodies. Likewise, compartmentalized LY294002 experiments indicate that PI3-K activity within cell bodies is critical for production of P-Akt in cell bodies; P-Akt generated by PI3-K signaling in distal axons does not translocate to cell bodies.

#### PI3-K Signaling Is Required for Survival of Sympathetic Neurons Supported by NGF Acting on Distal Axons

We found that withdrawal of NGF is more effective than inhibition of PI3-K in promoting apoptosis of neurons grown in mass cultures, in which NGF acts directly on cell bodies and distal axons, or of compartmentalized neurons supported by NGF acting directly on cell bod-

ies. Similar results with mass cultures were reported by Crowder and Freeman (1998). These observations indicate that PI3-K-independent mechanisms contribute to survival of sympathetic neurons supported by NGF acting directly on cell bodies. In contrast, inhibition of PI3-K in neurons supported by NGF acting exclusively on distal axons was nearly as effective as  $\alpha$ -NGF in promoting apoptosis. At least two explanations could account for the differential requirement for PI3-K depending on the site of action of NGF. One possibility is that NGF acting directly on cell bodies leads to production of a PI3-K-independent survival signal that is not activated by NGF acting exclusively on distal axons. A second possibility is that signaling pathways activated by NGF acting on cell bodies and distal axons are identical but that the magnitude of activation of a PI3-K-independent survival signal is different. Since the Raf-ERK pathway may contribute to NGF-dependent survival of sympathetic neurons (Mazzoni et al., 1999; Kaplan and Miller, 2000), it is possible that this pathway more effectively supports survival when NGF is acting



**Figure 8.** Inhibition of PI3-K in Distal Axons Attenuates TrkA Signaling and Retrograde Transport of an NGF-TrkA Complex

(A) Inhibition of PI3-K in the distal axons attenuates the appearance of phosphorylated TrkA in cell bodies. Twelve DIV sympathetic neuronal cultures grown in biochemistry chambers were maintained with α-NGF on cell bodies and NGF (100 ng/ml) on distal axons. The distal axons were then either left untreated or treated with LY294002 (50 μM) for 8 hr. Extracts prepared from cell body and distal axonal compartments were subjected to immunoblotting using the P-TrkA (Y490) antibody. The immunoblot was later stripped and reprobed for p85.

(B) Inhibition of PI3-K in the distal axons attenuates the retrograde movement of the NGF/TrkA complex. The distal axons of 12 DIV sympathetic neuronal cultures grown in biochemistry chambers were incubated with medium alone (-) or with wortmannin (100 nM) for 30 min and then treated with [<sup>125</sup>I]NGF for 8 hr as described above. TrkA was then immunoprecipitated from lysates prepared from cell body and distal axonal compartments. The immunoprecipitates were resolved on a 17% gel and subjected to autoradiography to visualize the [<sup>125</sup>I]NGF that coprecipitates with TrkA from the cell bodies and distal axons. Immunoblotting with the p85 antibody was performed on the supernatants to ensure that the samples had equal protein amounts. All experiments were done three times with similar results.

directly on cell bodies. In support of this idea, we have observed that NGF acting directly on cell bodies is more effective than NGF acting exclusively on distal axons for activation of ERK phosphorylation within cell bodies (unpublished data). The identity of PI3-K-independent TrkA survival pathways is of considerable interest because some neurons, such as cortical neurons, may normally respond to neurotrophins acting directly on cell bodies or dendrites. Thus, our observations highlight the importance of the mode of neuronal stimulation for addressing questions regarding the nature of the signaling pathway(s) that support survival. It is clear from the present study that the PI3-K effector pathway is critical for survival of sympathetic neurons supported by NGF acting exclusively on distal axons, its normal site of action *in vivo*.

#### PI3-K in Distal Axons Supports Initiation of Retrograde Transport and Retrograde Signaling

Our results support the idea that PI3-K signaling within both cell bodies and distal axons is necessary for survival of neurons supported by NGF acting on distal axons. Moreover, the requirement of PI3-K signaling in distal axons was more apparent when a submaximal concentration of NGF was used to support survival. How does PI3-K signaling within distal axons contribute to survival? We found that PI3-K activity in distal axons controls retrograde NGF transport and retrograde signaling, which may be critical for survival. Complete inhibition of PI3-K in distal axons, as assessed by levels of P-Akt, attenuated retrograde transport of NGF by ~80% in two compartment chambers and 65% in three compartment chambers. Thus, there is a small but significant amount of retrograde transport that occurs in a PI3-K-independent manner. These observations may account

for the finding that inhibition of PI3-K in distal axons has more dire consequences for neurons supported by 0.5 ng/ml NGF acting on distal axons than for those supported by 50 ng/ml NGF acting on distal axons. Neurons grown in a low, submaximal concentration of NGF are more vulnerable than neurons supported by a high concentration of NGF to a 65%–80% reduction in retrograde signaling.

The precise role of PI3-K signaling in distal axons for ligand-dependent internalization, retrograde transport, and retrograde signaling is not clear. It is possible that products of the PI3-K catalyzed reaction are critical for the ligand-dependent production of clathrin-coated pits, into which NGF and TrkA are initially internalized (Grimes et al., 1996, 1997). In support of this idea, there is an essential role for the pleckstrin homology (PH) domain of the GTPase dynamin for receptor-mediated endocytosis (Achiriloaie et al., 1999). Further, we have recently shown that dynamin is required for retrograde transport of NGF in sympathetic neurons (RK, HY, and DDG; unpublished data). Since the dynamin PH domain binds to phosphoinositide products of the PI3-K-catalyzed reaction (Salim et al., 1996), PI3-K activity associated with TrkA may be critical for recruitment of dynamin to regions of the plasma membrane destined to invaginate to form NGF/TrkA-containing clathrin-coated signaling organelles. Similarly, AP-2, which is involved in clathrin coat formation and vesicle sorting at the plasma membrane, contains an amino-terminal phosphoinositide binding domain that is required for its targeting to the plasma membrane (Gaidarov and Keen, 1999). Thus, it is tempting to speculate that PI3-K signaling in distal axons is needed for survival because this TrkA effector controls membrane recruitment of key regulators of NGF/TrkA endocytosis and retrograde TrkA signaling.

If PI3-K in distal axons is required for retrograde sig-

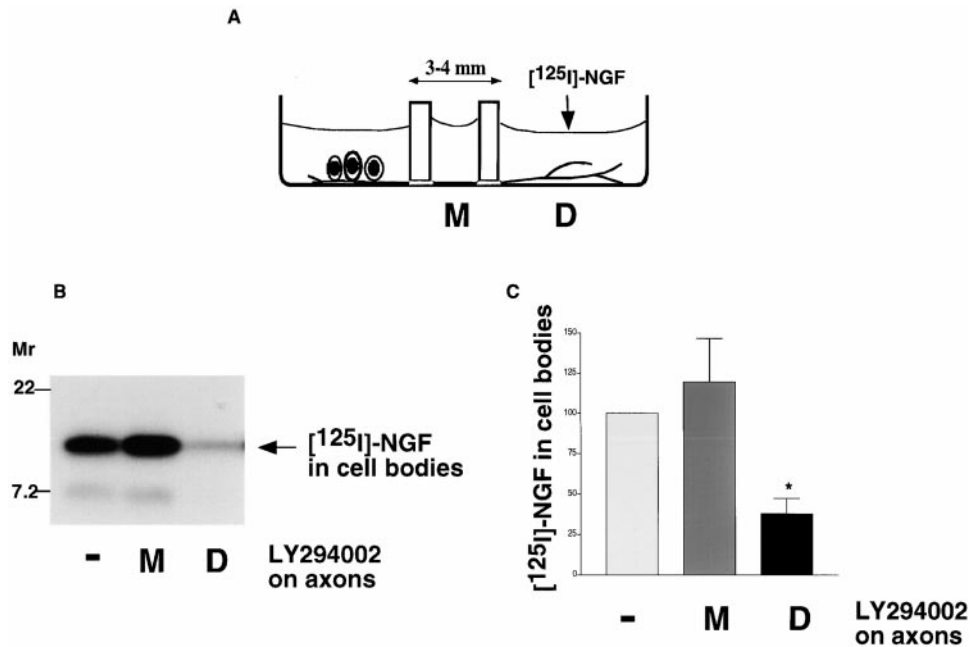


Figure 9. PI3-K Signaling in the Distal Axons Is Required for the Initiation but Not Propagation of Retrograde Transport of [<sup>125</sup>I]NGF

(A) Schematic representation of three-compartmentalized cultures of sympathetic neurons. Dissociated sympathetic neurons were plated in the left-most compartment and the axons projected under two Teflon dividers that are at least 3 mm apart from each other. The left-most compartment contained the cell bodies and proximal axons, the middle compartment contained distal axons (M), while the far-right compartment contained the far-distal axonal processes and axon terminals (D).

(B) The cell body and midaxonal processes of sympathetic neurons plated in three-compartment chambers were bathed in medium containing  $\alpha$ -NGF while the far-distal processes were bathed in medium containing NGF. The cultures were then left untreated or incubated with medium containing LY294002 (100  $\mu$ M) either in the middle compartments (M) or in the far-distal compartments (D) for 30 min. Then far-distal axons and terminals of all cultures were placed in medium containing [<sup>125</sup>I]NGF (10 ng/ml) for 24 hr. Extracts were prepared from the three different compartments and resolved by SDS-PAGE and the gels were subjected to autoradiography to visualize the [<sup>125</sup>I]NGF retrogradely transported to cell bodies.

(C) Quantitation of the effects of PI3-K inhibition in middle and far-distal compartments on retrograde transport of [<sup>125</sup>I]NGF to cell bodies. Quantification of [<sup>125</sup>I]NGF associated with cell bodies was done using Phosphorimager analysis, and the results are mean  $\pm$  SEM from five independent experiments. Results are presented as percent of values obtained from untreated control cultures. Asterisk,  $p < 0.05$  statistically different from both control cultures and cultures treated with LY294002 in the middle compartments as determined by one-way ANOVA followed by a Tukey-Kramer test.

naling, what is the role of PI3-K in cell bodies in neurons supported by NGF acting exclusively on distal axons? Inhibition of PI3-K in cell bodies led to near complete apoptosis of neurons within 48 hr, but inhibition of PI3-K exclusively in cell bodies did not affect retrograde transport of NGF. Under these conditions, P-Akt in cell bodies was completely blocked, but levels of P-Akt in distal axons were unaffected. These observations indicate that PI3-K and Akt signaling in distal axons alone cannot support neuronal survival. Since constitutively active PI3-K and Akt can support survival of sympathetic neurons, we speculate that PI3-K signaling in cell bodies is necessary for survival because it supports Akt signaling and phosphorylation of Akt substrates that mediate the prosurvival effects of PI3-K. Indeed, it seems likely that many of the substrates of Akt function, at least in part, within cell bodies. Substrates of Akt include BAD, caspase-9, IKK, the transcription factor forkhead, and, possibly, CREB (Datta et al., 1999; Kaplan and Miller, 2000). By extension, our data support the idea that phosphorylation of Akt substrates within distal axons cannot support neuronal survival. This may be because critical substrates of Akt are either not present in distal axons or

that they are present in distal axons but cannot move in the phosphorylated forms from distal axons to cell bodies to affect the apoptotic machinery. As mentioned above, P-Akt itself does not move from distal axons to cell bodies to an appreciable extent so the same is likely to be true for products of Akt-catalyzed phosphorylation reactions.

Growth factor signal transduction mechanisms in neurons are arguably more complex than in most other cell types due to the striking morphological specializations of neurons. Most neurons have long axons that can extend centimeters or even one meter from their cell bodies, and target-derived growth factor signals must be propagated over long distances to influence survival and gene expression within cell bodies. These retrograde signals must be integrated with signals coming from dendrites and those emanating from the membrane of the cell body itself. The present study shows that the same NGF effector pathway, the PI3-K pathway, can have different functions in distinct parts of the same neuron during long-range retrograde signaling. Interestingly, the activity of the PI3-K signaling in distal axons indirectly regulates TrkA signaling pathways, including

the PI3-K effector pathway, in cell bodies. Thus, there exists interdependence of TrkA effector pathways in distinct cellular locales whereby ligand-dependent TrkA effector signaling in one compartment, the distal axon, controls effector signaling in another, the cell body.

## Experimental Procedures

### Sympathetic Neuron Cultures

Superior cervical ganglia were dissected from embryonic age 19–20 to postnatal day 1 (P1) rats, enzymatically dissociated, and cells plated in mass cultures or compartmentalized Camp10 or biochemistry chambers as described previously (Tsui-Pierchala and Ginty, 1999). Seventy-two hours prior to experiments, a neutralizing antibody to NGF (1:1000; Sigma, St. Louis, MO) was added to the cell body compartments while the distal axons were maintained in culture medium containing NGF (100–150 ng/ml).

### Immunoprecipitations, Immunoblotting, and Antibodies

For immunoprecipitations, the cell body and distal axonal compartments of compartmentalized cultures were washed three times with ice-cold phosphate-buffered saline (PBS) and then extracts prepared by incubation for 30 min with Tris-buffered saline (TBS) containing Nonidet P-40 (1%), glycerol (10%), leupeptin (10  $\mu$ g/ml), aprotinin (1  $\mu$ g/ml), PMSF (100  $\mu$ M), and sodium orthovanadate (500  $\mu$ M) as described previously (Tsui-Pierchala and Ginty, 1999). Lysates from cell body and distal axon compartments were prepared from 3–4 biochemistry chambers for each condition. The lysates were clarified by centrifugation at 13,000 rpm at 4°C and the supernatants subjected to immunoprecipitation. For PI3-K assays, phosphotyrosine-associated PI3-K was precipitated using a monoclonal phosphotyrosine antibody 4G10 (1:500 dilution; Upstate Biotechnology, Lake Placid, NY) and 60  $\mu$ l of a protein A-agarose slurry (Santa Cruz Biotechnology, Santa Cruz, CA). The suspension was rotated gently at 4°C for 2 hr following which immune complexes were washed 3 times each with ice cold PBS containing Nonidet P-40 (1%), PBS containing 0.5 M LiCl and TBS. The immune complexes were then used for *in vitro* PI3-K assays. Trk immunoprecipitations were done using an affinity-purified pan-Trk polyclonal antibody (Trk C14, 1:100 dilution; Santa Cruz Biotechnology) and protein A-agarose. For immunoblotting of whole-cell extracts, extracts from cell body and distal axonal compartments were prepared by adding boiling laemmli buffer (1 $\times$ ) directly to the compartments. Immunoblots were blocked for 1 hr in TBS containing 5% milk and 0.1% Tween-20, followed by incubation with the primary antibody in the same solution, overnight at 4°C. The immunoblots were washed three times with TBST and then incubated at room temperature for 1 hr with the secondary antibody (1:1000 dilution of  $\alpha$ -rabbit IgG horseradish peroxidase for P490 TrkA, and P-Akt 1:5000 dilution for p85 subunit of PI3-K and 1:5000 dilution of the  $\alpha$ -mouse IgG horseradish peroxidase for P-ERK). The immunoblots were washed three times with TBST after the secondary antibody incubation and detected using ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ). Normalization for equal protein amounts was done by stripping and reprobing immunoblots with a polyclonal antibody directed against p85 (1:1000 dilution, Upstate Biotechnology, Lake Placid, NY) or against  $\alpha$ -tubulin (1:10000, Sigma). The different antibodies used in this study include a P-TrkA (Y490) (Tyr-490) (1:500 dilution), P-Akt (Ser-473) (1:1000 dilution), and a P-p44/42 MAPK (Thr-202/Tyr-204) antibody (1:1000 dilution), all of which were purchased from New England Biolabs (Beverly, MA).

### PI3-K Activity Assay

The PI3-K reaction was initiated by the addition of 10  $\mu$ l (20  $\mu$ g) of a phosphatidylinositol (PI) preparation, 10  $\mu$ l of  $\text{MgCl}_2$  (100 mM), 10  $\mu$ l of solution containing 10  $\mu$ Ci [ $\gamma$ - $^{32}\text{P}$ ]ATP and cold ATP (final concentration 40  $\mu$ M) to phosphotyrosine immunoprecipitates prepared as described above. The reaction was stopped by addition of 6N HCl and chloroform:methanol (1:1). The mixtures were centrifuged and the lower organic phase was applied to a silica gel chromatography plate. The plate was developed in chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2), the chromatograph

dried and subjected to autoradiography to visualize the product, phosphatidylinositol 3-phosphate (PI3-P).

### Cell Survival Assays

Neurons were washed three times with PBS and were then subjected to the various treatments as indicated. Media was changed every 24 hr to maintain efficacy and compartmentalization of treatments. After treatment, neurons were fixed in 4% paraformaldehyde at room temperature for 20 min. Fixed cells were incubated in blocking buffer (PBS containing 2% bovine serum albumin, 0.2% nonfat milk powder, 2% normal goat serum, and 0.4% Triton-100) at room temperature for 1 hr. The cells were then incubated in blocking buffer containing the polyclonal CM1 antibody, which recognizes the cleaved form of caspase-3, (1:2000 dilution; IDUN Pharmaceuticals, La Jolla, CA) after which the cells were washed three times with wash solution (PBS containing 0.2% Tween-20). The cells were then incubated in blocking buffer containing secondary antibody (goat  $\alpha$ -rabbit conjugated to Texas red; 1:500 dilution; Molecular Probes, Eugene, OR) and Hoechst 33258 dye (10  $\mu$ g/ml; Molecular Probes) at room temperature for 1 hr. Then, neurons were washed three times with wash solution, mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and counted for viability as indicated in figure legends.

### Adenoviral Infections

The recombinant adenovirus expressing LacZ was provided by Dr. Jeffrey E. Pessin (University of Iowa, Iowa City, IA) and the recombinant adenovirus encoding dominant-negative p85 (AxCA $\Delta$ p85) was provided by Dr. Wataru Ogawa (Kobe University, Kobe, Japan). The dominant-negative p85 contains a 35 amino acid deletion within the inter-SH2 domain, necessary for binding the catalytic subunit of PI3-K (Kotani et al., 1999). Sympathetic neurons were grown for 11–12 DIV in biochemistry chambers and then infected for 16 hr with the purified recombinant adenoviruses. The nonselective caspase inhibitor BAF (50  $\mu$ M) was included in the medium. Thirty-six hours following infection, the distal axons of the compartmentalized cultures were washed free of NGF and then incubated with medium containing [ $^{25}\text{I}$ ]NGF for 8 hr.

### [ $^{25}\text{I}$ ]NGF Assays

The distal axons and terminals of sympathetic neurons grown in biochemistry chambers were washed three times with PBS to remove NGF. In order to inhibit PI3-K, TrkA, or MEK in the distal axons or cell bodies, medium containing the inhibitors LY294002 (Calbiochem-Novachem, San Diego, CA), wortmannin (Sigma), K252a (Calbiochem), or the specific MEK inhibitor U0126 (Calbiochem) was added to the requisite compartments (see figure legends). Then, the distal axons and terminals were incubated in medium containing [ $^{25}\text{I}$ ]NGF (10 ng/ml; specific activity 2000 Ci/mmol; Amersham) for 8 hr. Extracts from cell body and distal axonal compartments were prepared by adding boiling laemmli buffer directly to the individual compartments and electrophoresed by SDS-PAGE. The dried gels were subjected to autoradiography to visualize [ $^{25}\text{I}$ ]NGF. The amount of [ $^{25}\text{I}$ ]NGF retrogradely transported to cell bodies was quantified using Phosphorimager analysis and a STORM 860 image analyzer. In some experiments, lysates prepared from cell body and distal axonal compartments were subjected to Trk immunoprecipitations as described previously (Tsui-Pierchala and Ginty, 1999). The Trk immunoprecipitates were then washed, resolved by SDS-PAGE, and the amount of [ $^{25}\text{I}$ ]NGF in immune complexes assessed as above.

### Acknowledgments

We thank Bonnie Lonze, Jon Terman, and Catherine Thompson for comments on the manuscript, and members of the Ginty laboratory for helpful discussions. We thank Anu Srinivasan and IDUN Pharmaceuticals for providing the active caspase-3 antibody, Jeffrey E. Pessin and Wataru Ogawa for providing adenoviral constructs, and Brian Tsui-Pierchala and Eugene Johnson for sharing unpublished observations. This work was supported by NIH grant N534814 and



a Pew Scholars Award (DDG). D. G. is an Assistant Investigator of the Howard Hughes Medical Institute.

Received July 25, 2000; revised September 7, 2000.

## References

- Achirioiaie, M., Barylko, B., and Albanesi, J.P. (1999). Essential role of the dynamin pleckstrin homology domain in receptor-mediated endocytosis. *Mol. Cell. Biol.* **19**, 1410–1415.
- Bartlett, S.E., Reynolds, A.J., Weible, M., Heydon, K., and Hendry, I.A. (1997). In sympathetic but not sensory neurones, phosphoinositide-3 kinase is important for NGF-dependent survival and the retrograde transport of 125I-betaNGF. *Brain Res.* **761**, 257–262.
- Berg, M.M., Sternberg, D.W., Parada, L.F., and Chao, M.V. (1992). K-252a inhibits nerve growth factor-induced *trk* proto-oncogene tyrosine phosphorylation and kinase activity. *J. Biol. Chem.* **267**, 13–16.
- Campanot, R.B. (1977). Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **74**, 4516–4519.
- Crowder, R.J., and Freeman, R.S. (1998). Phosphatidylinositol 3-kinase and AKT protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *J. Neurosci.* **18**, 2933–2943.
- Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. *Genes Dev.* **13**, 2905–2927.
- Gaidarov, I., and Keen, J.H. (1999). Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. *J. Cell Biol.* **146**, 755–764.
- Grimes, M.L., Zhou, J., Beattie, E.C., Yuen, E.C., Hall, D.E., Valletta, J.S., Topp, K.S., LaVail, J.H., Bunnnett, N.W., and Mobley, W.C. (1996). Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. *J. Neurosci.* **16**, 7950–7964.
- Grimes, M.L., Beattie, E., and Mobley, W.C. (1997). A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. *Proc. Natl. Acad. Sci. USA* **94**, 9909–9914.
- Hendry, I.A., Stockel, K., Thoenen, H., and Iversen, L.L. (1974). The retrograde axonal transport of nerve growth factor. *Brain Res.* **68**, 103–121.
- Johnson, E.M., Andres, R.Y., and Bradshaw, R.A. (1978). Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [125I]NGF. *Brain Res.* **150**, 319–331.
- Joly, M., Kazlauskas, A., Fay, F.S., and Corvera, S. (1994). Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. *Science* **263**, 684–687.
- Joly, M., Kazlauskas, A., and Corvera, S. (1995). Phosphatidylinositol 3-kinase activity is required at a postendocytic step in platelet-derived growth factor receptor trafficking. *J. Biol. Chem.* **270**, 13225–13230.
- Kaplan, D.R., and Miller, F.D. (1997). Signal transduction by the neurotrophin receptors. *Curr. Opin. Cell Biol.* **9**, 213–221.
- Kaplan, D.R., and Miller, F.D. (2000). Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381–391.
- Korsching, S., and Thoenen, H. (1983). Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. *Neurosci. Lett.* **39**, 1–4.
- Kotani, K., Ogawa, W., Hino, Y., Kitamura, T., Ueno, H., Sano, W., Sutherland, C., Granner, D.K., and Kasuga, M. (1999). Dominant negative forms of Akt (protein kinase B) and atypical protein kinase C do not prevent insulin inhibition of phosphoenolpyruvate carboxykinase gene transcription. *J. Biol. Chem.* **274**, 21305–21312.
- Lockhart, S.T., Turrigiano, G.G., and Birren, S.J. (1997). Nerve growth factor modulates synaptic transmission between sympathetic neurons and cardiac myocytes. *J. Neurosci.* **17**, 9573–9582.
- Mazzoni, I.E., Said, F.A., Aloyz, R., Miller, F.D., and Kaplan, D. (1999). Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. *J. Neurosci.* **19**, 9716–9727.
- Ming, G., Song, H., Berninger, B., Inagaki, N., Tessier-Lavigne, M., and Poo, M. (1999). Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. *Neuron* **23**, 139–148.
- Nagata, Y., Ando, M., Takahama, K., Iwata, M., Hori, S., and Kato, K. (1987). Retrograde transport of endogenous nerve growth factor in superior cervical ganglion of adult rats. *J. Neurochem.* **49**, 296–302.
- Ohmichi, M., Decker, S.J., Pang, L., and Saltiel, A.R. (1992). Inhibition of the cellular actions of nerve growth factor by staurosporine and K252a results from the attenuation of the activity of the *trk* tyrosine kinase. *Biochemistry* **31**, 4034–4039.
- Palmatier, M.A., Hartman, B.K., and Johnson, E.M., Jr. (1984). Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. *J. Neurosci.* **4**, 751–756.
- Patel, T.D., Jackman, A., Rice, F.L., Kucera, J., and Snider, W.D. (2000). Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. *Neuron* **25**, 345–357.
- Philpott, K.L., McCarthy, M.J., Klippel, A., and Rubin, L.L. (1997). Activated phosphatidylinositol 3-kinase promotes survival of superior cervical neurons. *J. Cell Biol.* **139**, 809–815.
- Powis, G., Bonjouklian, R., Berggren, M.M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W.F., Dodge, J., Grindey, G., et al. (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res.* **54**, 2419–2423.
- Reichardt, L.F., and Farinas, I. (1997). Neurotrophic factors and their receptors: roles in neuronal development and function. In *Molecular and Cellular Approaches to Neural Development*, W.M. Cowan, T.M. Jessel, and S.L. Zipursky, eds. (New York: Oxford University Press), pp. 220–263.
- Riccio, A., Pierchala, B., Ciarallo, C., and Ginty, D.D. (1997). An NGF-TrkA-Mediated retrograde signal to transcription factor CREB in sympathetic neurons. *Science* **227**, 1097–1100.
- Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I., Driscoll, P.C., et al. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* **15**, 6241–6250.
- Sasaoka, T., Wada, T., Ishihara, H., Takata, Y., Haruta, T., Usui, I., Ishiki, M., and Kobayashi, M. (1999). Synergistic role of the phosphatidylinositol 3-kinase and mitogen-activated protein kinase cascade in the regulation of insulin receptor trafficking. *Endocrinology* **140**, 3826–3834.
- Srinivasan, A., Roth, K.A., Sayers, R.O., Shindler, K.S., Wong, A.M., Fritz, L.C., and Tomaselli, K.J. (1998). In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ.* **5**, 1004–1016.
- Stockel, K., Schwab, M., and Thoenen, H. (1975). Comparison between the retrograde axonal transport of nerve growth factor and tetanus toxin in motor, sensory and adrenergic neurons. *Brain Res.* **99**, 1–16.
- Tapley, P., Lamballe, F., and Barbacid, M. (1992). K252a is a selective inhibitor of the tyrosine protein kinase activity of the *trk* family of oncogenes and neurotrophin receptor. *Oncogene* **7**, 371–381.
- Tsui-Pierchala, B.A., and Ginty, D.D. (1999). Characterization of an NGF-P-TrkA retrograde signaling complex and age-dependent regulation of TrkA phosphorylation in sympathetic neurons. *J. Neurosci.* **19**, 8207–8218.
- Tsui-Pierchala, B.A., Putcha, G.V., and Johnson, E.M. (2000). Phosphatidylinositol 3-kinase is required for the trophic, but not the survival-promoting, actions of NGF on sympathetic neurons. *J. Neurosci.*, in press.
- Vaillant, A.R., Mazzoni, I., Tudan, C., Boudreau, M., Kaplan, D.R., and Miller, F.D. (1999). Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. *J. Cell Biol.* **146**, 955–966.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269, 5241–5248.

Watson, F.L., Heerssen, H.M., Moheban, D.B., Lin, M.Z., Sauvageot, C.M., Bhattacharyya, A., Pomeroy, S.L., and Segal, R.A. (1999). Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complexes. *J. Neurosci.* 19, 7889–7900.

Yao, R., and Cooper, G.M. (1995). Requirement for phosphatidylinositol-3-kinase in the prevention of apoptosis by nerve growth factor. *Science* 267, 2003–2006.