A Neurotrophin Signaling Cascade Coordinates Sympathetic Neuron Development through Differential Control of TrkA Trafficking and Retrograde Signaling

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Summary
A fundamental question in developmental biology is how a limited number of growth factors and their cognate receptors coordinate the formation of tissues and organs endowed with enormous morphological complexity. We report that the related neurotrophins NGF and NT-3, acting through a common receptor, TrkA, are required for sequential stages of sympathetic axon growth and, thus, innervation of target fields. Yet, while NGF supports TrkA internalization and retrograde signaling from distal axons to cell bodies to promote neuronal survival, NT-3 cannot. Interestingly, final target-derived NGF promotes expression of the p75 neurotrophin receptor, in turn causing a reduction in the sensitivity of axons to intermediate target-derived NT-3. We propose that a hierarchical neurotrophin signaling cascade coordinates sequential stages of sympathetic axon growth, innervation of targets, and survival in a manner dependent on the differential control of TrkA internalization, trafficking, and retrograde axonal signaling.

Introduction
Postganglionic sympathetic neurons have long served as an amenable model to study the molecular events underlying neuronal survival, axon growth, and the elaboration of dendrites. While the requirement of the neurotrophin Nerve Growth Factor (NGF) as a final target-derived survival factor for sympathetic neurons is well established (Levi-Montalcini, 1987; Crowley et al., 1994), the relative contribution of factor(s) released by intermediate targets, such as the vasculature, to growth and survival of these neurons is less clear. Intriguingly, the related neurotrophin NT-3 is crucial for sympathetic neuron survival in vivo (Ernfors et al., 1994; Farinas et al., 1994; Francis et al., 1999), which suggests that NT-3 may act as an intermediate and/or final target-derived survival factor for these neurons. Consistent with this idea is the finding that NT-3 is expressed at high levels in intermediate targets, particularly in the vasculature, as well as in various end organs receiving sympathetic innervation (Francis et al., 1999). Interestingly, both genetic and biochemical analyses have shown that NT-3, like NGF, promotes survival of sympathetic neurons via the receptor tyrosine kinase TrkA rather than its high-affinity receptor TrkC. Indeed, no deficits in sympathetic neuron survival have been observed in TrkC null mice (Fagan et al., 1996; Tessarollo et al., 1997), sympathetic neurons derived from TrkC null mice can survive in vitro in the presence of NT-3 (Davies et al., 1995), and NT-3 activates TrkA in sympathetic neurons (Belliveau et al., 1997). In contrast, an NT-3 variant that activates TrkC but not TrkA is incapable of supporting survival of sympathetic neurons (Belliveau et al., 1997). Furthermore, sympathetic neurons die in both NT-3 null and NGF null mice at a time when these neurons express high levels of TrkA and low or negligible levels of TrkC (Fagan et al., 1996; Francis et al., 1999). These observations support a model in which both NGF and NT-3 signal via TrkA at the same developmental stage to support survival of sympathetic neurons.

Along with TrkA, the p75 neurotrophin receptor, which binds with comparable affinity to each of the neurotrophins (Bibel and Barde, 2000), also plays an essential role in the development of the sympathetic nervous system. Genetic evidence has revealed that p75 modulates neurotrophin-dependent survival in vivo (Brennan et al., 1999), and unliganded or BDNF bound p75 may directly promote apoptosis of sympathetic neurons (Bamji et al., 1998). Interestingly, in vitro experiments indicate that p75 modulates NT-3’s ability to activate TrkA. In cell lines, NT-3 activates TrkA efficiently when TrkA is overexpressed (Hempstead et al., 1992) or when p75 levels are reduced (Benedetti et al., 1993; Clary and Reichardt, 1994). In addition, NT-3 can more effectively support survival of sympathetic neurons established from embryonic mice at times when p75 expression is low (Davies et al., 1995) or from p75-deficient mice (Lee et al., 1994b). These findings, taken together, have led to the idea that both NT-3 and NGF are required for survival of sympathetic neurons when their axons are extending toward and innervating target fields. Since NT-3 is highly expressed by blood vessels along which sympathetic axons extend, intermediate target-derived NT-3 may provide retrograde trophic support until sympathetic axons innervate final targets and acquire target-derived NGF. Yet, such a model is inconsistent with the finding that the periods of NT-3 and NGF dependence for survival are essentially identical (Francis et al., 1999). It is also unclear how these neurotrophins and other growth factors coordinate the extension of axons within intermediate and, ultimately, in final target fields. Nor do current models fully define a role for p75, which, in addition to its effects on survival, is crucial for innervation of at least some sympathetic targets (Lee et al., 1994a). Thus, a unifying model for how NGF, NT-3, TrkA, and p75 coordinate axon growth along intermediate targets, final target innervation, and retrograde survival of sympathetic neurons remains to be established.

At the molecular level, NGF acting through TrkA receptors initiates both growth- and survival-promoting signal...
transduction pathways, which include the Ras/Rap1-Erk and PI3-kinase effector pathways (Huang and Reichardt, 2003). Yet, NGF, which acts on TrkA receptors on distal axons, must support survival of morphologically complex neurons whose cell bodies are located millimeters, centimeters, or more from distal axons. Thus, the mechanisms by which NGF and other growth factors support long-range retrograde survival signaling, from distal axons to cell bodies, have been the topic of much interest, and several models have been proposed to explain the basis of retrograde signaling (Ginty and Segal, 2002). Of these, the “Signaling Endosome” model, in which retrograde signals are carried by endosomes containing NGF and activated TrkA, has gained considerable support. Neurotrophins promote internalization of Trks, some of which are then retrogradely transported in their activated, autophosphorylated state to cell bodies (Delcroix et al., 2003; Tsui-Pierchala and Ginty, 1999; Watson et al., 2001). Importantly, Trk catalytic activity and autophosphorylation within cell bodies is crucial for several tyrosine phosphorylation events (Senger and Campenot, 1997), CREB Ser133 phosphorylation and gene expression (Riccio et al., 1997; Watson et al., 1999), and survival (Ye et al., 2003). The evidence that NT-3 is critical for sympathetic neuron survival in vivo raises the possibility that this intermediate target-derived neurotrophin employs signaling endosomes to support survival and gene expression in developing sympathetic neurons in a manner similar to target-derived NGF. Here, we ask how the related neurotrophins NGF and NT-3 and their receptors TrkA and p75 orchestrate the establishment of the sympathetic nervous system.

Results

Both NGF and NT-3 Contribute to Growth of Sympathetic Neuron Axons In Vivo

We sought to understand how the related neuronal growth factors NGF and NT-3 coordinate the establishment of the sympathetic nervous system, since both of these neurotrophins are crucial for survival of sympathetic neurons in vivo. Recent findings indicate that NGF is critical for sympathetic innervation of target organs by neurons whose cell bodies reside within both paravertebral and prevertebral sympathetic ganglia but not for proximal projections of sympathetic axons along the vasculature (Glebova and Ginty, 2004). To determine whether NT-3 is crucial for sympathetic axon outgrowth in vivo, a whole-mount tyrosine hydroxylase immunohistochemistry assay was employed. This assay allows us to visualize the extent of axonal growth along intermediate targets at E15.5 and innervation of several peripheral target tissues at E16.5 in both NT-3 null mice and their control littersmates. These early stages of sympathetic neuron development are ideal for our measurements, because intermediate target innervation can be assessed prior to the period of excess sympathetic neuronal death in NT-3 null mice, which is first evident at E17.5 (Francis et al., 1999; Wyatt et al., 1997).

We find that growth of proximal sympathetic axons along the vasculature around the superior cervical ganglia at E15.5 is impaired in NT-3 null mice (Figures 1A and 1B). Fewer axons from the SCG are found associated with the carotid artery in NT-3null mice, and those present are shorter than axons of littermate controls. Moreover, projections from another paravertebral sympathetic ganglion, the stellate ganglion, which extend toward the heart and other thoracic targets, are shorter in NT-3null mice as compared to their littersmates. While sympathetic axons course along the ventricles of E15.5 wild-type mice, few if any fibers are observed approaching the hearts of NT-3 mutants (Figures 1C and 1D). The NT-3 dependence of axon growth is also observed for neurons whose cell bodies are located in prevertebral sympathetic ganglia, including mesenteric ganglia, since sympathetic axons extending along the mesenteric arteries are shorter and less developed in E15.0 NT-3null embryos (Figure 1F) as compared to control littersmates (Figure 1E). This failure of neurons to properly extend along intermediate targets is not a reflection of excess neuronal loss at these early time points, since nearly identical numbers of sympathetic neurons are found in E15.5 NT-3 null and wild-type controls (Figure 1G), as previously described (Francis et al., 1999). These axon growth deficiencies stand in contrast to those observed in NGF null mice, which exhibit target organ innervation defects but relatively normal axonal growth along the vasculature around the SCG, stellate, and mesenteric ganglia (Glebova and Ginty, 2004). Taken together with the finding that the vasculature expresses high levels of NT-3 (Francis et al., 1999; Scarisbrick et al., 1993), these observations indicate that NT-3 supports growth of proximal sympathetic axons along the vasculature, an intermediate target.

As expected because of the proximal projection defects, sympathetic innervation of many end organs, examined at later gestational ages, is substantially attenuated in the absence of NT-3. Reduced levels of sympathetic innervation were observed in E16.5 NT-3null mice in the heart, duodenum, brown fat (Figures 2A–2F), thymus, stomach, pancreas, kidney, and bladder (data not shown). Moreover, at postnatal day 0.5, the hearts of NT-3null; Baxnull mice, and, as recently reported, NGFnull; Baxnull mice (Glebova and Ginty, 2004) in which sympathetic neuron apoptotic cell death is eliminated because of deletion of the gene encoding proapoptotic factor Bax showed markedly reduced levels of sympathetic innervation as compared to Baxnull controls (Figures 2G–2J). Together with our recent findings (Glebova and Ginty, 2004), these results indicate that both NT-3 and NGF are critical for sympathetic axon growth in vivo. NT-3 supports proximal axon extension along the vasculature and subsequent innervation of several effector organs, whereas NGF is crucial for final target innervation.

NT-3, like NGF, Can Activate TrkA Locally in Distal Axons of Sympathetic Neurons to Mediate Neurite Outgrowth

The findings that NT-3 is expressed along intermediate and within final targets and that axon growth is attenuated in mice lacking NT-3 suggest that its in vivo function in the developing sympathetic nervous system is to provide direct support of axon growth. We therefore used
Figure 1. NT-3 Is Required for Outgrowth of Proximal Projections from Sympathetic Ganglia

(A–F) NT-3 is required for the growth of proximal projections from paravertebral and prevertebral sympathetic ganglia. Whole-mount tyrosine hydroxylase (TH) immunohistochemistry reveals a deficit in sympathetic axonal growth from the superior cervical ganglia (B) and stellate ganglia (D) at E15.5 and along the mesenteric arteries at E15.0 (F) of NT-3/−/− mice, as compared to littermate controls (A, C, and E). Dashed lines outline the ganglia, while arrows/arrowheads indicate the proximal projections. (G) Cell counts reveal no deficits in cell number in the superior cervical ganglia at E15.5 in NT-3/−/− mice as compared to littermate wild-type controls. Results are presented as mean ± SEM of neuron number from three wild-type and three NT-3/−/− mice. Scale bars, 100 μm (A–B, C–D, E–F). n = 2 (NT-3+/+; E15.0) and n = 4 (NT-3+/−; E15.0); n = 3 (NT-3−/−; E15.0) and n = 3 (NT-3−/−; E15.5).
compartmentalized cultures of sympathetic neurons to test whether NT-3 added directly and exclusively to distal axons supports axonal extension. This model system allows for the separation of distal axons from cell bodies and proximal axons and the application of growth factors exclusively to distal axons (see Figure 4A). The broad-spectrum caspase inhibitor BAF (50 μM) was added to cell bodies in these experiments so that axon growth responses could be assessed in the absence of complications of apoptosis. We found that NT-3, like NGF, added exclusively to distal axons of compartmentalized sympathetic neurons, promotes robust growth of axons (Figure 3A). The axon growth-promoting effect of NT-3 is evident at relatively low concentrations (10 ng/ml; Figure 3A). TrkA, not TrkC, mediates this effect of NT-3, because immunoprecipitation and immunoblot experiments indicate that, while TrkC levels in sympathetic neurons are undetectable, these neurons express high levels of TrkA, which can be activated effectively by NT-3 (Figures 3B and 3C). In addition, an NT-3 variant (NT-3mut; Ryden and Ibanez, 1996) that binds TrkC but not TrkA is incapable of promoting axon outgrowth when applied to distal axons (Figure 3D), although NT-3mut does support growth and survival of TrkC-expressing sensory neurons (data not shown). Thus, both NGF and NT-3 signal via TrkA locally in distal axons to promote axonal growth.

p75 Modulates NT-3/TrkA Signaling in Distal Axons and Is Expressed in a Manner Dependent upon Target-Derived NGF

The observation that NT-3 is less potent at promoting axonal outgrowth than NGF (Figure 3A) led us to question whether axonal sensitivity to NT-3 is modulated by p75. It is interesting to note, in this regard, that levels of p75 are very low in developing sympathetic neurons as they project along NT-3-expressing vasculature and other intermediate targets and rise sharply upon innervation of final target fields (Verdi and Anderson, 1994; Wyatt and Davies, 1995). Our finding that NT-3 is required for growth of sympathetic axons in vivo at times when expression of p75 is very low, i.e., E15.0 and the previous findings that p75 may decrease responsiveness to NT-3 (Davies et al., 1995; Lee et al., 1994b; Wyatt et al., 1997), prompted us to test whether p75 modulates NT-3’s ability to promote axon growth. Indeed, we found that NT-3 (10–100 ng/ml) elicits a 2- to 3-fold increase in axonal extension rates when added to distal axons of compartmentalized sympathetic neurons obtained from p75 mutant mice as compared to wild-type controls (Figure 3E). The ability of NT-3 to evoke an augmented axonal growth response in the absence of p75 is specific to NT-3, since axon extension rates in response to NGF were similar in the presence or absence of p75 (Figure 3E). Thus, p75 inhibits NT-3-mediated axonal extension in sympathetic neurons.

The findings that p75 expression peaks as axons innervate NGF-expressing target fields and that sympathetic axons become less sensitive to NT-3 upon expression of p75 (Figure 3E) implicate p75 as a candidate for switching sympathetic neuron responsiveness from NT-3 to NGF. We therefore considered the possibility that one of the functions of target-derived NGF and retrograde NGF/TrkA signaling is to control expression...
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Figure 3. Both NGF and NT-3 Signal Through TrkA in Distal Axons to Promote Axon Extension

(A) NT-3 and NGF promote axon outgrowth when added exclusively to distal axons of compartmentalized rat sympathetic neurons. Distal axon length was measured using OpenLab software and expressed as average axon growth per day (μm/day). (B) Both NGF and NT-3 can induce TrkA phosphorylation in sympathetic neurons. Neurons were treated with media alone, NGF (10 ng/ml), or NT-3 (500 ng/ml) for 20 min. The amount of phosphorylation of TrkA, Akt, and Erk1/2 in response to NGF and NT-3 at multiple time points including 5 min, 1 hr, 3 hr, and 8 hr was similar (data not shown). Neuronal extracts were subjected to immunoprecipitation with an antibody directed against phosphotyrosine followed by SDS-PAGE and immunoblotting with a TrkA-specific antibody. (C) Postnatal rat sympathetic neurons express high levels of TrkA but little or no TrkC as compared to rat cortical cultures (compare second and third panels from top). Cortical cultures were established from embryonic day 18 rat embryos and cultured for 3 days in vitro (DIV). Neuronal extracts were subjected to immunoblotting using antibodies against P-Trk (Y490), TrkA, TrkC, and tubulin. (D) A mutant form of NT-3 (NT-3mut R31A/H33A; Ryden and Ibanez, 1996) that abolishes NT-3 binding to p75, TrkA, and TrkB but maintains its ability to bind TrkC demonstrates a lack of TrkC involvement in NT-3-mediated axon growth. Experiments were done as described above with NT-3mut (100 ng/mL), NT-3 (100 ng/mL), or NGF (10 ng/mL) added exclusively to distal axons. (E) NT-3-mediated axonal growth is enhanced in the absence of p75. Sympathetic neuronal cultures were established from postnatal day 0.5 BalbC and p75−/−/− mice and grown in compartmentalized cultures in NGF-containing media for 7–10 DIV. Axonal growth assays were performed as described above (A). For (A), (D), and (E), results are presented as mean ± SEM from three independent experiments. Asterisk, p < 0.01; one-way ANOVA (for [A] and [D]) and two-way ANOVA (for [E]) followed by Tukey’s Multiple Comparisons Test. (F) NGF is necessary for expression of p75 in vivo. RT-PCR results showing reduced levels of p75 mRNA in SCGs from NGF−/−; Bax−/− mice as compared to NGF+/+; Bax−/− littermates, while, in contrast, levels of TrkA mRNA are similar in these mice. Thus, target-derived NGF but not NT-3 supports p75 expression in developing sympathetic neurons in vivo which, in turn, triggers a reduction in NT-3-mediated axon growth.

NGF but Not NT-3 Promotes Retrograde Survival and Hypertrophy of Sympathetic Neurons

It is striking that NT-3 null mice, like NGF null mice, exhibit excess sympathetic neuronal cell death, and this dramatically reduced in the absence of NGF is p75 (average 6-fold reduction, n = 2). This result was obtained in two separate microarray experiments and was confirmed using a sensitive RT-PCR assay (Figure 3F). SCGs from P0.5 NGF−/−; Bax−/− mice have markedly reduced levels of p75 mRNA as compared to their control NGF+/+; Bax−/− littermates, while, in contrast, levels of TrkA mRNA are similar in these mice. Thus, target-derived NGF but not NT-3 supports p75 expression in developing sympathetic neurons in vivo which, in turn, triggers a reduction in NT-3-mediated axon growth.
Figure 4. NGF but Not NT-3 Supports Retrograde Survival and Soma Hypertrophy of Compartmentalized Sympathetic Neurons

(A) Compartmentalized sympathetic neuronal culture. Top panel, schematic view of compartmentalized culture chamber. Bottom panel, neurofilament immunofluorescence of 7 DIV compartmentalized culture showing cell bodies (CB) and distal axons (DA). Scale bar, 1 mm.

(B and C) NT-3 cannot support survival of sympathetic neurons when applied exclusively to distal axons (B) but can when applied directly to cell bodies (C). NT-3mut added directly to cell bodies does not promote survival (C, last bar). Sympathetic neurons grown in compartmentalized cultures for 7–10 DIV in media containing NGF (100 ng/mL) were subjected to NGF withdrawal and treated with either NGF or NT-3 added exclusively to distal axons (B) or cell bodies (C) at indicated concentrations (ng/mL) for 48 hr. Results are presented as mean ± SEM from three independent experiments. Asterisk, p < 0.01; one-way ANOVA followed by Tukey’s Multiple Comparisons Test.

(D and E) NT-3 applied directly to cell bodies (E) but not distal axons (D) of compartmentalized sympathetic neurons promotes soma hypertrophy. The broad spectrum caspase inhibitor BAF (50 μM) was added to cell bodies to prevent cell death, and media alone or containing NGF or NT-3 was added to distal axons (D) or cell bodies (E) at indicated concentrations (ng/ml) for 72 hr. Cell volume was determined by measuring soma diameter using OpenLab software. Asterisk, p < 0.01; one-way ANOVA followed by Tukey’s Multiple Comparisons Test.

(F) p75 does not modulate the ability of NT-3 to support retrograde survival. Sympathetic neuron cultures were established from embryonic day 18.0 BalbC and p75−/− mice and grown in compartmentalized cultures in NGF-containing media for 7–10 DIV. Neuronal survival was assessed as described (B), and results are presented as mean ± SEM from three independent experiments. Asterisk, p < 0.05 significantly different from untreated controls; two-way ANOVA followed by Tukey’s Multiple Comparisons Test.

may reflect survival-promoting roles of intermediate- and/or target-derived NT-3. Our finding that NT-3 is a critical intermediate target-derived axon growth factor raises the alternate possibility, however, that neurons may die in NT-3 null mice because of deficiencies in target innervation and acquisition of target-derived NGF. We therefore used compartmentalized cultures of sympathetic neurons to determine the extent to which these neurotrophins can directly support retrograde survival. Compartmentalized cultures were established using NGF-containing media for 7 days in vitro (DIV) prior to washing to remove NGF and then switching to media containing either fresh NGF or NT-3. As reported previously (Ye et al., 2003), 10 ng/ml NGF applied exclusively to distal axons supports maximal retrograde survival (Figure 4B). In sharp contrast, NT-3 is unable to support survival when added exclusively to distal axons, even at an extremely high concentration (5000 ng/ml) (Figure 4B). This is remarkable in light of the findings that NT-3 added either directly to mass cultures of sympathetic neurons (Belliveau et al., 1997; Davies et al., 1995) or to cell bodies of compartmentalized sympathetic neurons (Figure 4C) can support survival, and NT-3 added to distal axons can support axonal outgrowth (Figure 3A). Similarly, NT-3 added directly to cell bodies supports soma hypertrophy (Figure 4D), while, in marked contrast, NT-3 added to distal axons is incapable of promoting soma hypertrophy (Figure 4D). Hence, while NGF promotes robust survival and soma hypertrophy through retrograde signaling, NT-3 cannot.

We next considered the possibility that NT-3’s ability to support retrograde survival is modulated or perhaps inhibited by p75. This appears not to be the case, however, since a comparable amount of cell survival is ob-
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Figure 5. NGF but Not NT-3 Signals Retrogradely via TrkA to Cell Bodies of Sympathetic Neurons

(A) Compartmentalized sympathetic neuronal culture. Top panel, schematic view of compartmentalized biochemistry chamber. Bottom panel, neurofilament immunofluorescence of 7 DIV compartmentalized culture showing cell bodies (CB) and distal axons (DA). Scale bar, 1 mm.

(B) NGF and NT-3 promote phosphorylation of TrkA, Akt, and Erk1/2 in distal axons, but only NGF promotes retrograde activation of these molecules in cell bodies of compartmentalized sympathetic neurons. Distal axons were treated with media alone or media containing NGF (100 ng/ml) or NT-3 (500 ng/ml) for 8 hr. Cell body/proximal axon and distal axon lysates were prepared and subjected to immunoblotting using antibodies against P-TrkA (Y490), P-Akt (Ser473), P-Erk 1/2, and p85.

(C) NGF but not NT-3 supports retrograde accumulation of P-TrkA in vivo. SCGs were dissected from NGF+/−; Bax+/− and NGF−/−; Bax−/− mice at postnatal day 0.5, homogenized in boiling Laemmli buffer, and proteins were resolved by SDS-PAGE and subjected to immunoblotting using anti-P-Trk. The immunoblot was stripped and reprobed with anti-tubulin to normalize for protein amounts.

NGF but Not NT-3 Supports Retrograde TrkA Signaling to Cell Bodies of Sympathetic Neurons

The striking differences in the abilities of NGF and NT-3 to support retrograde survival and hypertrophy, despite both being potent axon outgrowth factors, prompted us to ask about potential differences in their capacities to control TrkA signaling and trafficking. Employing compartmentalized cultures (Figure 5A), we asked whether differences in NGF and NT-3’s capacity to support retrograde survival and hypertrophy are due to differences in their abilities to activate retrograde TrkA signaling. Addition of either NGF or NT-3 exclusively to distal axons of compartmentalized cultures induced robust phosphorylation of TrkA and its effectors Erk1/2 and Akt within distal axons (Figure 5B). However, in stark contrast to NGF, NT-3 stimulation of distal axons does not promote retrograde accumulation of P-TrkA, P-Erk-1/2, and P-Akt within cell bodies (Figure 5B). Thus, while NT-3 is a potent activator of TrkA in distal axons and can support TrkA-mediated axon outgrowth, it cannot promote retrograde TrkA signaling. These in vitro findings are corroborated and extended by results of in vivo experiments. In mice lacking both NGF and Bax (NGF−/−; Bax−/− mice), neurons are found to project axons along intermediate targets (Glebova and Ginty, 2004), which express high levels of NT-3 (Francis et al., 1999). Yet, the amount of P-TrkA in cell bodies of neurons in the SCG of NGF−/−; Bax−/− mice is essentially undetectable while, in contrast, robust P-TrkA is observed in neuronal cell bodies of Bax−/− littermate controls (Figure 5C). Taken together, these results indicate that the inability of NT-3 to promote retrograde TrkA signaling is likely to explain its inability to support retrograde survival and hypertrophy.

NT-3 and NGF Differ in Their Ability to Support TrkA Internalization and Signaling from TrkA-Containing Endosomes

How can two neurotrophins, each of which promotes TrkA autophosphorylation and signaling in axons and
axonal growth, differ in their capacities to support retrograde TrkA signaling? It is possible that NGF and NT-3 differ in their abilities to promote TrkA internalization, sorting of TrkA into transport or signaling vesicles, and/or retrograde transport of TrkA-containing signaling endosomes. It is also possible that NGF and NT-3 activate distinct sets of TrkA effectors, the NT-3-activated set being incapable of supporting retrograde signaling. Other possibilities exist, and they are not mutually exclusive.

To begin to distinguish among these possibilities, we next asked whether NGF and NT-3 might signal from distinct subcellular platforms, i.e., endosomes versus the plasma membrane. To address this question, an immunofluorescence assay was employed to detect both TrkA and phosphorylated TrkA in cultured sympathetic neurons. Sympathetic neurons transfected with a TrkA expression vector elevate the levels of TrkA were used for immunolocalization of the activated receptor using P-Trk antibodies and confocal microscopy. Consistent with our biochemical data (Figure 5B), both NGF and NT-3 stimulation result in activation of TrkA along axons and at growth cones (Figure 6A). This analysis of axons, however, does not allow us to assess the subcellular localization and trafficking of P-TrkA. We therefore examined the effects of ligand stimulation on cell bodies, a method previously used to study TrkA receptor internalization and trafficking in PC12 cells (Shao et al., 2002). Consistent with previous PC12 cell experiments, NGF application to sympathetic neurons results in a rapid and sustained intracellular accumulation of phosphorylated TrkA (Figure 6B). After 60 min of NGF treatment, little or no P-TrkA remains on the plasma membrane, while robust punctae of P-TrkA immunoreactivity are found associated with intracellular organelles. Similar to NGF treatments at short time points, exposure of sympathetic neurons to NT-3 results in rapid phosphorylation of TrkA localized to the cell surface (Figure 6B). Yet, in dramatic contrast to NGF, NT-3 treatment results in sustained localization of activated TrkA at the cell surface (Figure 6B). Equally dramatic differences in TrkA trafficking were observed in experiments in which PC12 cells and 293T cells overexpressing TrkA were exposed to either NGF or NT-3 (Supplemental Figures S3A and S3B). Interestingly, NT-3 is highly effective at activating ectopically expressed TrkC at the surface of sympathetic neurons within 5 min, while, at 60 min, phosphorylated TrkC accumulates within intracellular organelles with a concomitant loss of activated receptor from the cell surface (Figure 6C). Thus, while both NGF and NT-3 activate TrkA on the plasma membrane, these neurotrophins have markedly different effects on trafficking of the activated receptor.

To further test the possibility that NGF and NT-3 differ in their ability to promote internalization of P-TrkA and the formation of TrkA-containing signaling endosomes, we employed a cell surface biotinylation assay, which provides a measure of ligand-dependent internalization of endogenous TrkA in cultured sympathetic neurons. We found that NGF induces robust internalization of biotinylated TrkA receptors in sympathetic neurons (Figure 7A), while, in sharp contrast, NT-3 cannot. Both NGF and NT-3 activated TrkA signaling under the conditions of this assay as indicated by comparable amounts of TrkA phosphorylation (Figure 7B). Thus, although NT-3 can bind and activate cell surface TrkA, it clearly differs from NGF in its inability to induce TrkA internalization and, therefore, the formation of TrkA-containing signaling endosomes. The lack of NT-3-mediated internalization of TrkA can explain why this neurotrophin is incapable of supporting retrograde TrkA signaling, survival, hypertrophy, and gene expression in vitro and in vivo.

Since p75 has been shown to modulate the ability of TrkA to be internalized in response to NT-3. Using the confocal immunofluorescence assay to visualize trafficking of activated TrkA in 293T cells which do not express p75, we found that NGF but not NT-3 induced internalization of phosphorylated TrkA (Supplemental Figures S3B and S3C), suggesting that the presence of p75 alone does not regulate the differential internalization of TrkA. However, it is possible that 293T cells may lack other cellular components that facilitate the ability of p75 to mediate signaling. Thus, in order to directly ask whether differential regulation of TrkA internalization by NGF and NT-3 is altered in neurons lacking p75, the cell surface biotinylation assay using mass cultures of sympathetic neurons established from p75 KO mice was employed. Analogous to the results in rat sympathetic neurons (Figure 7A), which express high levels of p75, we found that, while NGF promotes internalization of TrkA in p75 KO mice sympathetic neurons, NT-3 cannot (Figure 7C), despite the fact that both ligands are equally capable of inducing TrkA signaling, as seen by the equivalent levels of Akt activation (Figure 7C). These results suggest that the mechanisms underlying the differential trafficking of TrkA by NT-3 and NGF appear to be either intrinsically related to TrkA or to be influenced by cellular components other than the p75 neurotrophin receptor.

Discussion

A fundamental question in developmental biology is how a limited repertoire of growth factors and their receptors act in concert to coordinate the construction of morphologically and functionally complex organs and tissues. In this study, we show that the related neurotrophins NGF and NT-3 signal through the same receptor, TrkA, to coordinate distinct stages of sympathetic neuron development. Remarkably, NGF and NT-3 control unique aspects of sympathetic development through differential control of TrkA internalization and retrograde signaling. Thus, intermediate target-derived NT-3 signals via cell surface TrkA to support axon growth but not retrograde survival, whereas final target-derived NGF supports not only local axon growth but also survival, anabolic responses, and gene expression through retrograde signaling. Interestingly, retrograde NGF/TrkA signaling leads to upregulation of p75, which in turn diminishes NT-3/TrkA signaling in axons. We propose that this hierarchical neurotrophin signaling cascade facilitates the extension of axons from intermediate targets into nearby target fields. Furthermore, the differential control of TrkA trafficking ensures that target-derived NGF and not intermediate target-derived NT-3 is solely responsible for retrograde survival signaling, thereby enabling proper systems matching.
Figure 6. NGF and NT-3 Differentially Regulate Trafficking of Phosphorylated TrkA

(A and B) Both NGF and NT-3 promote phosphorylation of TrkA at sympathetic neuron growth cones (A) but induce differential P-TrkA localization at cell bodies following ligand treatment (B). Sympathetic neurons grown in mass culture were transfected with TrkA to enhance detection of its subcellular distribution in neurons treated with either NGF or NT-3. Following NGF withdrawal, neurons were treated with NGF (50 ng/mL), NT-3 (500 ng/mL), or media alone for 60 min (A and B) or 5 min (B) and processed for TrkA and P-Trk immunofluorescence. (C) NT-3 induces trafficking of activated TrkC from the cell surface to intracellular organelles in sympathetic neurons transfected with GFP and TrkC. Neurons were treated with NT-3 (50 ng/mL) for either 5 or 60 min and then immunostained for P-Trk and GFP. All images were obtained using a laser scanning confocal microscope (Zeiss LSM510) with a 100× Apochromat oil immersion objective lens at 3× zoom.

Both NGF and NT-3 Activate TrkA in Distal Axons to Support Axon Growth

Both NT-3 and NGF activate TrkA and its downstream effector pathways locally in axons, and both ligands promote robust axon outgrowth when added exclusively to distal axons of sympathetic neurons. Moreover, NT-3 is required in vivo for sympathetic axon extension along intermediate targets, whereas both NT-3 and NGF are critical for end-organ innervation. While NT-3 is a key regulator of sympathetic axon growth, this neurotrophin...
is unlikely to be the sole axonal growth factor for sympathetic neurons extending along intermediate targets, since a substantial amount of axonal growth remains in NT-3 null animals, and approximately 50% of neurons from NT-3 null mice do ultimately acquire target-derived NGF and survive (Ernfors et al., 1994; Farinas et al., 1994; Francis et al., 1999). Thus, NT-3 likely acts in concert with other growth-promoting factors to support axonal extension along the vasculature. One excellent candidate is artemin, a member of the GDNF family, because, like NT-3, artemin is expressed along the vasculature, and exogenous artemin promotes sympathetic axonal growth in vitro and in vivo (Honma et al., 2002). It will be important in future studies to assess the relative in vivo contributions of artemin and other factors, such as VEGFs, to growth of axons as they extend along the vasculature and into final target fields.
that NGF but not NT-3 promotes the retrograde accumulation of phosphorylated TrkA and at least some of its effectors within cell bodies of sympathetic neurons. Surprisingly, the inability of NT-3 to support retrograde biochemical signaling, survival, and p75 expression can be explained by the finding that NT-3 cannot support internalization of TrkA. This was unexpected, because other findings have indicated that TrkA catalytic activity is required for its internalization (data not shown), and, as shown here, both NGF and NT-3 can promote TrkA autophosphorylation in distal axons. Thus, autophosphorylation is necessary but not sufficient for TrkA internalization in sympathetic neurons. It must be noted, however, that we assessed tyrosine phosphorylation of TrkA using a Y490-specific antibody, and it remains possible that phosphorylation of other crucial tyrosine residues on TrkA are differentially regulated by NGF and NT-3. There is precedence for multiple EGF-like ligands inducing differential patterns of tyrosine phosphorylation of a common receptor kinase, ErbB4, leading to the recruitment of unique sets of cytoplasmic effectors and activation of different intracellular signaling cascades (Sweeney et al., 2000). A similar mechanism leading to recruitment of distinct signaling effectors or endocytic adaptors to trafficking motifs within TrkA may underlie the differential effects of NGF and NT-3 on TrkA internalization. In any case, the inability of NT-3 to support TrkA internalization is likely to account for its inability to promote retrograde TrkA signaling, expression of p75, and retrograde control of survival and hypertrophy.

Our finding that NT-3 supports local axonal signaling but not retrograde TrkA signaling or survival is consistent with the previous findings that massive neuronal apoptosis within the SCG of NGF null mice is observed at E17.5, a time at which NT-3 is both expressed in the vasculature and found in the present study to support local axonal growth in vivo. Moreover, loss of one or both alleles of NT-3 does not exacerbate cell death caused by the absence of NGF (Francis et al., 1999). These findings, taken together, indicate that NT-3 cannot substitute for NGF to support sympathetic neuron survival, and, therefore, the physiological function of NT-3 is to support axonal growth but not survival of developing sympathetic neurons. This stands in marked contrast to NGF, which serves as both an essential target-derived axonal growth factor (Glebova and Ginty, 2004) and a retrograde survival factor. Our findings, therefore, support the idea that excess sympathetic neuron cell death observed in NT-3-/- mice (Emfors et al., 1994; Farinas et al., 1994; Francis et al., 1999) occurs not because NT-3 is a survival factor but rather because it is required for axonal growth and target innervation and, hence, the acquisition of target-derived NGF. These findings also raise an important question regarding the extent to which intermediate targets, such as the vasculature, are needed for neuronal survival. Indeed, it seems to us advantageous that intermediate target-derived growth factors such as NT-3 not serve as retrograde survival factors in order to ensure robust competition for final target-derived survival factors such as NGF. In this way, the size of the end-organ target field dictates the appropriate size of the neuronal population, whereas the size of intermediate targets, which for the vasculature can vary dramatically in both mass and length depending on the distance between the soma and target organ, does not. Thus, a scenario whereby retrograde survival of sympathetic neurons is supported solely by end organ-derived NGF and not intermediate target-derived NT-3 enables efficient and accurate systems matching.

### p75 Expression Enables Sympathetic Neurons to Switch Sensitivity from NT-3 to NGF

A central principle of axon guidance is that axons of long projection neurons navigate in a stepwise fashion from a series of intermediate targets to reach final targets (Tessier-Lavigne and Goodman, 1996). A growing body of evidence indicates that, as axons extend toward final targets, they employ mechanisms that dynamically modulate their responses to extracellular cues en route. For example, as vertebrate commissural axons cross the midline, they switch responsiveness from the attractant netrin to the repellent slit, an effect that is mediated at least in part by the silencing effect of slit on netrin/DCC signaling (Stein and Tessier-Lavigne, 2001). In *Drosophila*, an intracellular protein trafficking-based mechanism delivers the slit receptor Robo to the cell surface after commissural axons cross the midline, so that these axons respond to slit only after midline crossing (Dickson, 2002). Commissural axons crossing the midline may also use local protein synthesis to gain responsiveness to a new set of guidance cues after they have crossed (Brittis et al., 2002). A central theme in all of these examples is that axons must make complex decisions at intermediate targets, and growth cones must employ resourceful mechanisms to advance along their trajectories toward final target fields.

Many sympathetic axons grow in close proximity to blood vessels, an intermediate target that expresses abundant levels of NT-3, en route to their final targets. What makes sympathetic axon growth cones leave intermediate target regions to invade final peripheral targets? We propose a model in which a hierarchical neurotrophin signaling cascade coordinates the projection of sympathetic neurons. In this model, axons exiting developing sympathetic ganglia closely follow blood vessels and other intermediate targets toward the periphery. NT-3 derived from vascular smooth muscle cells promotes activation of TrkA on sympathetic axons, allowing for rapid and robust axonal extension along the vasculature at times when p75 levels are low. As axons approach end organs and begin to acquire target-derived NGF, the ensuing retrograde NGF/TrkA signaling promotes survival, anabolic responses, and expression of p75. The increase in p75, in turn, diminishes axonal responsiveness of TrkA to NT-3, enabling target-derived NGF to become the dominant axonal growth factor. One key prediction of this model is that sympathetic innervation of end organs would be diminished in the absence of p75. Indeed, decreased sympathetic innervation of several targets, including pineal gland, sweat glands, and brown fat, have been reported in postnatal and adult p75-/- mice (Brennan et al., 1999; Lee et al., 1994a). Moreover, we have found major deficits in innervation of the heart and small intestines in E16.5 p75 mutant mice (Supplemental Figure S4). Thus, through retrograde activation of expression of p75, NGF/
TrkA signaling dominantly represses axonal NT-3/TrkA signaling, thereby facilitating the extension of axons into final target fields.

Taken together, our findings indicate that NT-3 and NGF coordinate sympathetic axon growth, innervation of targets, and survival in a manner dependent on the differential control of TrkA internalization, trafficking, and retrograde signaling. In a broader sense, it is remarkable that emerging evidence indicates that many or perhaps most growth factor receptors appear to have evolved to mediate responses of more than one ligand. We propose that distinct growth factors acting upon a receptor evolved to mediate responses of more than one ligand.

Experimental Procedures

Animals

p75 mutant mice on a mixed background of 129 and Balb/C strains (targeted deletion of exon III; Lee et al., 1992) and control Balb/C mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Embryos homozygous for the p75 mutation were obtained by crosses between p75 mutant animals, while control embryos were obtained by crosses from inbred Balb/C mice. NT-3/− mice were obtained from Dr. Luis Parada (Liebl et al., 1997), and NT-3/−embryos and wild-type littermates were obtained from crosses between heterozygous mutant mice, while NT-3/−; Bax−/− null mice and Bax−/− controls were obtained by crossing NT-3/−; Bax−/− mice.

RT-PCR

mRNA was isolated from SCG from NGF+/−; Bax−/− and NGF−/−; Bax−/− mice at postnatal day 0.5 using RNAeasy (Qiagen, Inc.) and treated with RNaseaase-free DNase I (Roche) for 30 min at 37°C, and mRNA was then subjected to RT-PCR using the SuperScript first-strand synthesis system (Invitrogen) with specific primers for p75 and TrkA.

Sympathetic Neuron Survival, Cell Body Diameter Measurements, and Axon Outgrowth Assays

Sympathetic neurons were obtained by enzymatic dissociation of superior cervical ganglia from postnatal day 1 (P1) rats or P1 p75−/− and Balb/C mice and grown in compartmentalized cultures as described previously (Kuruvilla et al., 2000). Cell survival, cell body measurements, and axonal growth were assessed as described previously (Ye et al., 2003).

Neurotrophins and Antibodies

NGF was purified from mouse salivary glands, while recombinant NT-3 was a kind gift from Amgen. Western blot analysis revealed that these preparations contained little or no proneurotrophins (Supplemental Figure S2). Cell lysates from cultured neurons or SCG explants were prepared either by using NP-40 lysis buffer for immunoprecipitations or boiling Laemmli buffer, resolved by SDS-PAGE, and immunoblotted with antibodies against P-TrkA (Y490; Cell Signaling), TrkA and TrkC (Chemicon), P-Akt (Ser473; Cell Signaling), and P-Erk1/2 (Cell Signaling), the p85 subunit of PI3-kinase (UBI) or tubulin (Sigma).

Transfection and Immunofluorescence

Plasmids encoding either TrkA or TrkC were transfected into sympathetic neurons, PC12 cells, or 293T cells as previously described (Lein et al., 2002). Cells were incubated in NGF-free media for 6–8 hr prior to addition of NT-3 (500 ng/mL), NGF (50 ng/mL), or media alone. Antibodies to P-Trk (Cell Signaling), TrkA (Santa Cruz Biotechnology), and GFP (Sigma) as well as the secondary antibodies anti-mouse AlexaFluor 488 (Molecular Probes) and anti-rabbit AlexaFluor 594 (Molecular Probes) were utilized for immunofluorescence detection as previously described (Shao et al., 2002). All images were processed with Adobe PhotoShop.

Internalization Assay

Internalization of TrkA was analyzed using a cell-surface biotinylation assay. Briefly, sympathetic neurons were grown in mass cultures in NGF-containing media for 7 DIV and then starved of neurotrophins in media containing BAF (50 μM) for 2 days. Neurons were subjected to biotinylation on ice with the reversible membrane-impermeable derivative of biotin (sulfo-NHS-S-S-biotin from Pierce; 1.5 mg/mL in PBS). Internalization was allowed to occur by incubation at 37°C with media containing no added neurotrophins, NGF (50 ng/mL), or NT-3 (1000 ng/mL). The remaining cell-surface biotin was cleaved by reducing its disulfide linkage with glutathione cleavage buffer and neurons lysed with RIPA buffer. Biotylated proteins were precipitated using UltraLink immobilized Neutravidin beads (Pierce, Rockford, IL), eluted from the beads with boiling Laemmli buffer, resolved by SDS-PAGE, and immunoblotted with an antibody directed against TrkA (Chemicon).

Whole-Mount Tyrosine Hydroxylase Immunohistochemistry

Whole-mount tyrosine hydroxylase (TH) immunohistochemistry was performed as previously described (Glebova and Ginty, 2004). We did not detect any differences in TH levels in SCGs isolated from NGF+/−; Bax−/− and NGF−/−; Bax−/− mice (Supplemental Figure S1).

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