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Raf and Akt Mediate Distinct Aspects of Sensory Axon Growth

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Summary

Nerve growth factor (NGF) induces dramatic axon growth from responsive embryonic peripheral neurons. However, the roles of the various NGF-triggered signaling cascades in determining specific axon morphological features remain unknown. Here, we transfected activated and inhibitory mutants of Trk effectors into sensory neurons lacking the proapoptotic protein Bax. This allowed axon growth to be studied in the absence of NGF, enabling us to observe the contributions of individual signaling mediators. While Ras was both necessary and sufficient for NGF-stimulated axon growth, the Ras effectors Raf and Akt induced distinct morphologies. Activated Raf-1 caused axon lengthening comparable to NGF, while active Akt increased axon caliber and branching. Our results suggest that the different Trk effector pathways mediate distinct morphological aspects of developing neurons.

Introduction

Members of the neurotrophin family of neuronal growth factors play crucial roles in peripheral neuron development. The complex set of signal transduction pathways activated by neurotrophins in responsive cells has been intensely studied, and substantial progress has been made, particularly in elucidating the pathways contributing to neurotrophin-dependent cell survival (for a review see Kaplan and Miller, 2000). Because of this neurotrophin survival dependence, however, it has been difficult to define the roles of the neurotrophins and their signaling effectors in axon growth and morphological differentiation (for a review, see Bibel and Barde, 2000).

The neurotrophins influence multiple aspects of axon morphological development, including axon extension (Patel et al., 2000; Tucker et al., 2001; Goldberg et al., 2002), axon branching (Wright et al., 1997; Lentz et al., 1999), and axon caliber (Munson et al., 1997). It is as yet unknown whether these morphological effects require activation of the entire signaling network stimulated by neurotrophins or whether individual aspects of morphology can be assigned to delineable signal transduction pathways.

Axon growth signaling pathways in primary neurons, as well as growth factor signaling in general, might well be heavily redundant. The many downstream pathways being activated by neurotrophins could contribute incrementally to the overall morphology such that interference with just one of these pathways might not lead to clear-cut effects. For example, a recent study of PDGF-β receptor signaling by Fambrough et al. (1999) found that suppression of single pathways emanating from this receptor affected the induction of immediate early genes by PDGF stimulation quantitatively to some extent, but not qualitatively.

On the other hand, different neurotrophin signaling pathways might each regulate a specific feature of axon morphology. Importantly, the signaling mediators involved are likely to be activated by growth factors other than neurotrophins present in the trajectories and targets of peripheral neurons, such as members of the glial cell-line-derived neurotrophic factor (GDNF) family, hepatocyte growth factor (HGF), and insulin-like growth factors (IGFs). Therefore, defining morphological regulation by the major Trk signal transduction pathways may provide novel insights into how growth factors cooperate in the regulation of axon morphology.

Most of the positive neurotrophin effects are attributed to signaling via the Trk family of receptor tyrosine kinases. Upon stimulation, Trk kinases activate several intracellular pathways that have been studied in considerable detail in the NGF-responsive PC12 cell line and recently in sympathetic neurons (for reviews see Segal and Greenberg, 1996; Kaplan and Miller, 2000). One major pathway involving the small GTPase Ras, phosphatidylinositol-3′-kinase (PI3K), and the protein kinase Akt has been shown to be essential in the NGF-dependent survival of sympathetic (Nobes and Tolkovsky, 1996; Markus et al., 1997; Datta et al., 1997) and sensory neurons (Borasio et al., 1993; Klesse and Parada, 1998), as well as in the IGF-1-dependent survival of cerebellar granule cells (Dudek et al., 1997). Recent studies have also shown a requirement for B-Raf in NGF-mediated sensory neuron survival (Wiese et al., 2001).

Specific pathways related to morphological responses have been more difficult to define. Activation of Ras, the serine/threonine kinase Raf, and the subsequent cascade of Mek and Erk kinases are required for neurite outgrowth of PC12 cells treated with NGF (Cowley et al., 1994). Furthermore, overexpression of active forms of Ras, Raf, and Mek induces neurite extension in these cells (Wood et al., 1993; Klesse et al., 1999). In neurons, the role of this pathway remains unclear. More than a decade ago, Borasio et al. (1989) showed that activated Ras could induce neurite growth in BDNF-responsive nodose ganglion neurons in the absence of neurotrophins. However, a study using Mek inhibitors in dense cultures reported that the extracellular signal-regulated kinases (Erks) are not required for either axon outgrowth or survival of embryonic sympathetic neurons (Klinz et al., 1996). Furthermore, Klesse and Parada (1998), using adenoviral transfection, reported that dominant-negative Ras, Raf, or Erk-2 did not noticeably affect axon morphology. On the other hand, recent work by Atwal et al. (2000), using transfection of TrkB mutants, demonstrated that Mek-Erk signaling is, in fact, required at least for BDNF-TrkB-mediated elongation of sympathetic axons. Thus, the published data concerning Raf-

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Erk function in embryonic axon growth remain contradictory.

There is evidence that the PI3K-Akt pathway, in addition to its importance in survival signaling, may also contribute to morphological responses induced by neurotrophins. PI3K activity is required for NGF-induced differentiation of PC12 cells (Jackson et al., 1996). Studies using compartmentalized cultures of sympathetic neurons have shown that the PI3K pathway is required to enable NGF-induced distal axon growth (Kuruvilla et al., 2000; Atwal et al., 2000). PI3K is also necessary for growth cone responses to neurotrophins and other chemotropic regulators (Ming et al., 1999). Finally, activated Akt has recently been shown to differentiate PC12 cells and accelerate motor axon regeneration in vivo (Namikawa et al., 2000). However, it has also been reported that selective activation of PI3K in PC12 cells does not result in neurite growth (Ashcroft et al., 1999) and that overexpression of Akt may even inhibit NGF-induced differentiation of PC12 cells (Bang et al., 2001). Thus, the precise roles played by PI3K and Akt signaling in the development of axonal morphology remain undefined.

It has been difficult to study the morphological effects of the neurotrophins separately from their survival effects in embryonic neurons because they are absolutely required for neuronal survival. However, sensory neurons taken from mice lacking the apoptotic protein Bax (Deckwerth et al., 1996) survive in culture in the absence of neurotrophins (Lentz et al., 1999; Liu and Snider, 2001). Transfection of these "apoptosis resistant" cultures with dominant-negative signal transduction intermediates allows us to interfere with pathways essential for survival and assess their specific contributions to axon growth and morphology. Additionally, we can selectively activate and study morphological effects of pathways that do not by themselves support survival.

With this approach, we show that while all signaling intermediates investigated—Ras, Raf, PI3K, and Akt—appear necessary for axon growth, they each lead to unique morphologies in gain-of-function experiments. Our results suggest that morphological responses to neurotrophins are not achieved by accumulation of incremental effects of the major Trk effector pathways but, rather, that each pathway regulates specific morphological features.

Results

Ras, Raf, MEK, PI3K, and Akt Are Required for NGF-Induced Axon Elongation

We overexpressed Myc- or HA-tagged constructs of dominant-inhibitory Ha-Ras (S17N mutant; Feig and Cooper, 1988), Raf-1 (Stokoe et al., 1994, Leevers et al., 1994), the p85 regulatory subunit of PI3K (Kotani et al., 1994), and Akt (the PH domain only, Crowder and Freeman, 1998) in embryonic day (E)13 dorsal root ganglion (DRG) neurons and assessed the effects on NGF-induced axon elongation. Examples of neurons transfected with Myc-tagged constructs are shown in Figure 1B.

In the absence of neurotrophic factors, cultured E13 $Bax^{-/-}$ DRG neurons grew rudimentary axons of 566 $\pm$ 42 μm length over a period of 3 days. In the presence of NGF, the cells grew axons of 2179 $\pm$ 187 μm total length (Figure 1A). Inhibition of Ras signaling completely abrogated NGF-stimulated axon growth without killing the cells, resulting in 536 $\pm$ 54 μm axon length. Inhibition of Raf in sensory neurons by overexpression of a dominant-inhibitory mutant also resulted in nearly complete suppression of axon growth (to 543 $\pm$ 157 μm), as did inhibition of the Raf effector Mek with the pharmacological inhibitor U0126 (448 $\pm$ 70 μm, data not shown).

The $Bax^{-/-}$ cultures also allowed us to inhibit the PI3K-Akt pathway and assess its contribution to axon growth. We found that both inhibition of PI3K or Akt by overexpression of dominant-negative constructs completely inhibited NGF-induced axon growth (Figure 1). Pharmacological inhibition of PI3K with LY294002 had a similar inhibitory effect (351 $\pm$ 29 μm, data not shown).

These results clearly indicate that Ras activity and activation of both the Raf-MEK and the PI3K-Akt pathways are required for axon growth in cultures of embryonic sensory neurons.

Activation of the Ras-Raf-ERK Cascade Results in Axon Elongation

To elucidate the differential contributions of different NGF signaling effectors to axon growth, we performed a series of experiments overexpressing activated signaling molecules in embryonic DRG cultures.

Activated Ras, which has been shown to enable the survival and axon growth of wild-type sensory neurons in the absence of NGF (Borasio et al., 1989), was transfected into $Bax^{-/-}$ cultures under conditions that allow evaluation of the axonal arbors of single cells. We found that cells expressing Ras(G12V) in the absence of neurotrophins were indistinguishable from control cells maintained with NGF. Axons grew to the same length (2389 $\pm$ 130 μm for Ras[G12V] versus 2185 $\pm$ 219 μm for NGF) and in a similar arborization pattern (Figure 2). In our cultures, about 20%–30% of EYFP-transfected control neurons did not respond to NGF with significant elongation, which is to be expected since the DRG at E13 contains about 20% neurons that do not express the NGF receptor TrkA (Fariñas et al., 1998; Molliver et al. 1997). Interestingly, at least 90% of the neurons expressing Ras(G12V) grew long axons resembling NGF-treated cells, and none developed the highly branched morphology typical of NT3-responsive E13 sensory neurons described by Lentz et al. (1999).

Raf-1 can be activated by localization to cell membranes via fusion with the CAAX motif derived from K-Ras (Stokoe et al., 1994). Overexpression of this construct in our cultures led to strong axon elongation, resulting in an average total length of 2180 $\pm$ 250 μm (Figure 2). These axons exhibited a different morphology from NGF- or Ras-induced axons. Raf-induced axons are less branched (9.7 branches per cell versus 12.6 for NGF, Figure 5B). Additionally, Raf overexpression led to larger cell bodies than either NGF or Ras (see Figure 4).

The Erk kinases are the best understood downstream effectors of Raf. Recently, activated Erk constructs have been generated by Robinson et al. (1998) through fusion of Erk-2 with its activating kinase Mek-1. The Erk-Mek fusion results in a purely cytoplasmic protein, while mutation of the nuclear export signal on Mek allows the fusion protein to enter the nucleus (Robinson et al., 1998). Overexpression of the nuclear Erk-Mek fusion

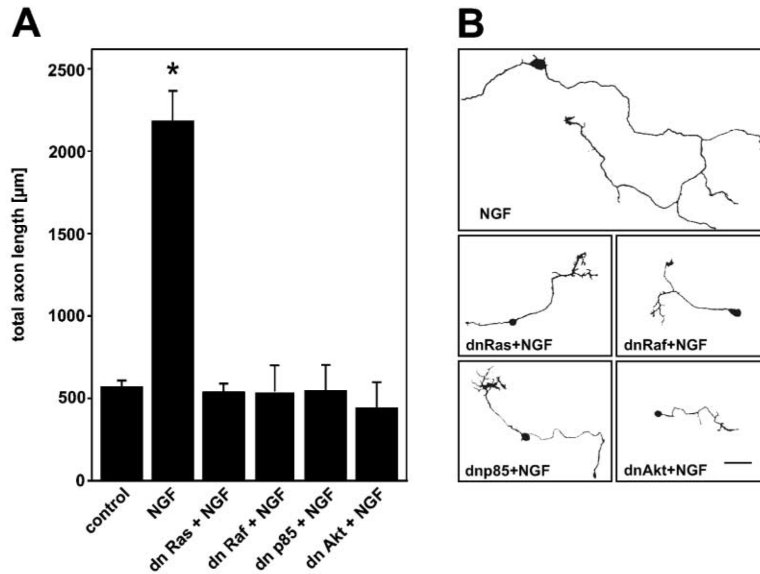


Figure 1. Both the Ras-PI3K-Akt and the Ras-Raf Pathway Are Required for Axon Elongation

(A) Axons of transfected cells were traced in camera lucida and measured using NIH Image. All inhibitory constructs completely suppressed NGF-induced axon growth without killing the apoptosis-deficient $Bax^{-/-}$ neurons. Bars represent mean ± SEM from four independent experiments for control, dnRas, and dnRaf and from three independent experiments for dn p85 and dnAkt. The asterisk designates a significant difference relative to unstimulated, control-transfected cells ($p < 0.001$, Student's t test).

(B) Cells were transfected with Myc-tagged constructs, cultured for 3 days, and stained for the Myc antigen. Myc-tagged EYFP was used as a control plasmid.

Scale = 50 μm.

protein resulted in axon growth to 1536 ± 225 μm, corresponding to 70% of the axon elongation achieved by NGF. Overexpression of the cytoplasmic construct did not stimulate axon outgrowth at all (454 ± 132 μm), suggesting that Erk regulation of gene transcription plays a major role in developmental axon growth.

PI3K Overexpression Increases Cell Body Size and Axon Caliber

PI3K is an important effector of Ras and activator of Akt in neurotrophic survival signaling. We have overexpressed a membrane-targeted form of its p110 catalytic domain to assess its specific contribution to sensory axon morphology ($PI3K^{CAAX}$, Klippel et al., 1996). The effects of PI3K on axon morphology were very different from those of Raf. In particular, axon caliber was doubled compared to NGF treatment or Raf^{CAAX} expression (Figure 3), while there was no effect of $PI3K^{CAAX}$ on axon length (see below). The axon caliber was measured at 20 μm (shown) and 50 μm from the cell soma. At 20 μm from the cell body, $PI3K^{CAAX}$ expression resulted in a 2-fold increase over controls in axon diameter (Figure

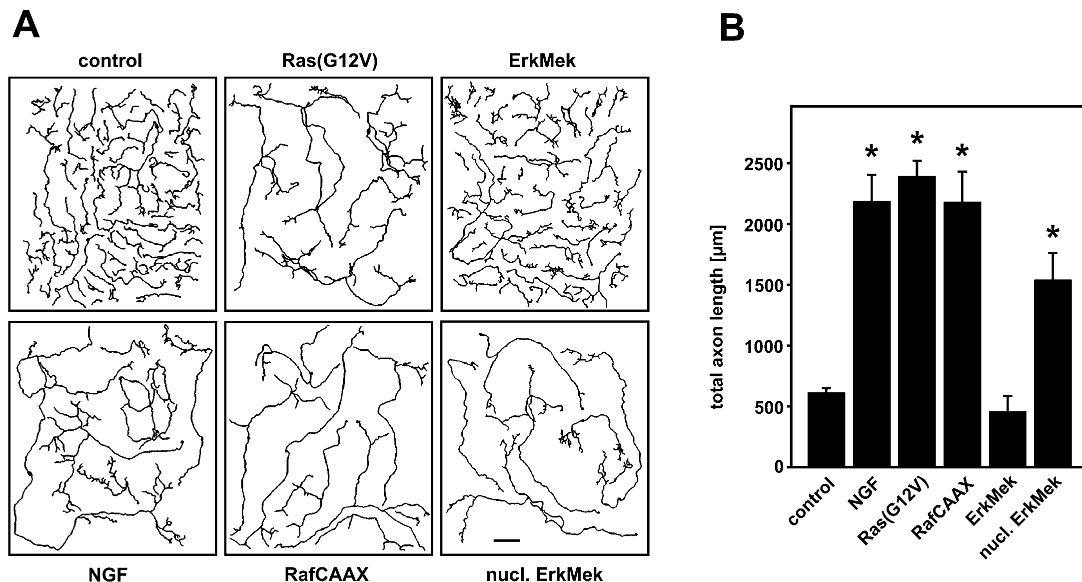


Figure 2. Activation of Ras, Raf, or Erk Leads to Axon Growth

(A) Axons of transfected cells were traced in camera lucida as outlined in Experimental Procedures. Representative pages of tracings are shown. Cells were transfected with Myc-tagged constructs (Myc-tagged EYFP in the controls), cultured for 3 days, and stained for the Myc antigen with DAB.

(B) Axons of transfected cells were traced in camera lucida and measured using NIH Image. Activated Ras and Raf could, by themselves, completely replicate NGF-induced axon elongation. Erk had a less robust effect and had to be able to translocate into the nucleus. Bars represent the average (± SEM) from four independent experiments. Asterisks denote a significant difference relative to unstimulated, control-transfected cells ($p < 0.001$, Student's t test).

Scale = 200 μm.

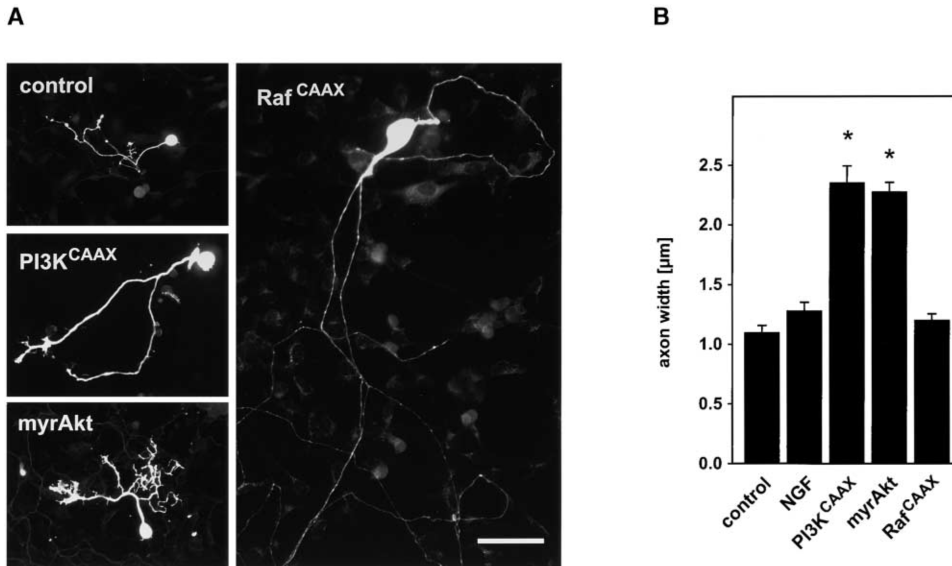


Figure 3. Transfection with Different Activated Signaling Intermediates Causes a Variety of Axon Morphologies

(A) Cells were transfected with the tagged constructs indicated, cultured without NGF for 3 days, and immunostained with anti-Myc or anti-HA antibody. PI3K led to enlarged axon calibers and cell bodies but had little effect on axon length. Note the extensive growth of relatively thin axonal processes after transfection of activated Raf-1, as well as the enlarged cell body. In contrast, activated Akt causes axon thickening and branching, but no significant elongation.

(B) Axon calibers were measured from high-magnification photomicrographs of DAB-stained neurons at a distance of approximately 20 μm from the cell soma. Asterisks denote a significant difference relative to unstimulated, control-transfected cells ($p < 0.002$, Student's t test). Scale = 100 μm.

3B). At 50 μm from the soma, there was still a significant increase by 1.5-fold (control, 1.16 ± 0.05 μm; NGF, 1.12 ± 0.04 μm; and $PI3K^{CAAX}$, 1.76 ± 0.08 μm; $p < 0.005$). It was our strong impression that the axon caliber in distal portions of the arbor (>50 μm) was reduced with Raf^{CAAX} expression compared to NGF treatment, but we found this difficult to quantify since NGF calibers are already quite small.

NGF treatment increased the axon calibers of EYFP-transfected neurons only slightly to 1.29 ± 0.07 μm, as compared to 1.19 ± 0.06 μm in control cultures at a distance of 20 μm from the cell soma. Transfection with any of the inhibitory constructs in the presence of NGF resulted in values for axon caliber not significantly different than controls (dnRas-expressing axons, 1.15 ± 0.05 μm; dnRaf axons, 1.14 ± 0.02 μm; dn p85 axons, 1.12 ± 0.04 μm; and dnAkt axons, 1.08 ± 0.02 μm; all values are derived from three independent experiments, means ± SEM).

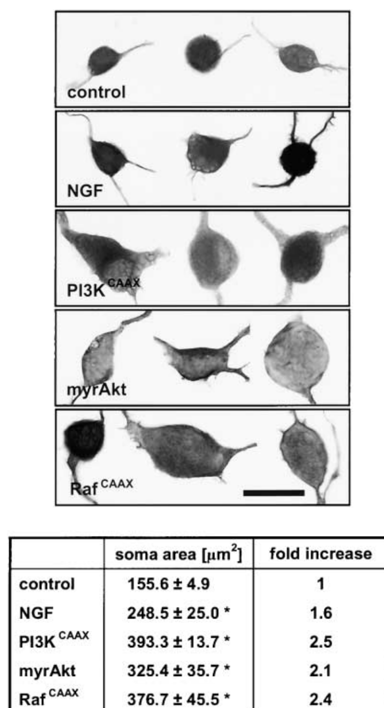
Cell body size of $PI3K^{CAAX}$-overexpressing neurons, gauged by soma footprint area, was increased 2.5-fold over controls (Figure 4). PI3K-transfected cell bodies were on average 393.3 ± 13.7 μm^2 in size. Control cells were 155.6 ± 4.9 μm^2, and NGF treatment increased this to only 248.5 ± 25.0 μm^2. These results suggest that NGF does not activate PI3K signaling to its maximum extent (see below).

The cell body sizes of neurons cultured with NGF were reduced by all inhibitory constructs except the dominant-negative Raf. NGF-treated neurons transfected with inhibitory constructs attained cell soma footprints of 194.1 ± 11.1 μm^2 (dnRas), 144.6 ± 6.8 μm^2 (dn p85), and 181.4 ± 9.8 μm^2 (dnAkt), but transfection with dnRaf allowed the somata to grow to 267.1 ± 11.7 μm^2, which is statistically no different from NGF-treated control cells (all values are derived from three independent experiments, means ± SEM). This result suggests that NGF increases soma size exclusively through the PI3K pathway. However, Raf may play a role in other soma growth paradigms since we observed a significant increase in soma size with overexpression of the activated Raf construct (Figure 4).

We found that $PI3K^{CAAX}$ expressed at low levels after transfection, making accurate measurements of total axon length difficult. To visualize the full length of the axonal arbor, we used a double promotor vector to express $PI3K^{CAAX}$ from a CMV promotor and, simultaneously, EGFP from an SV40 promotor. Neurons transfected with this construct also exhibited enlarged cell somata and axon width, but no axon elongation was observed (control, 827 ± 209 μm; NGF, 2517 ± 435 μm; and $PI3K^{CAAX}$, 783 ± 38 μm; three independent experiments).

Overexpression of Akt Increases Axon Caliber and Distal Branching

The serine/threonine kinase Akt is essential for the growth factor-dependent survival of several classes of embryonic neurons (Dudek et al., 1997; Datta et al., 1997; Crowder and Freeman, 1998). Here, we examined the morphological effects of selective Akt activation. Overexpression of myristylated Akt (Kohn et al., 1996) again led to a morphology very different from activation of Ras or Raf. As with PI3K, there was very little effect on axon length. Instead, myr-Akt overexpression dramatically enlarged axon caliber by 1.9-fold to 2.27 ± 0.08



	soma area [μm^2]	fold increase
control	155.6 ± 4.9	1
NGF	248.5 ± 25.0 *	1.6
PI3K ^{CAAX}	393.3 ± 13.7 *	2.5
myrAkt	325.4 ± 35.7 *	2.1
Raf ^{CAAX}	376.7 ± 45.5 *	2.4

Figure 4. Soma Size Is Increased through Both the PI3K/Akt and the Raf Pathways

Cells were transfected with the constructs as indicated and cultured for 3 days. NGF was added to half of the Myc-EYFP-transfected control cultures 8 hr after seeding. The cells were fixed and stained for the Myc-tag or, in case of myr-Akt, the HA tag. The cell body circumference was traced from photomicrographs and cell body footprint area was measured in NIH Image.
The table lists data collected from three independent experiments, as means ± SEM. The asterisk designates a significant difference relative to unstimulated, control-transfected cells ($p < 0.02$, Student's t test).
Scale = 25 μm.

μm compared to 1.18 ± 0.05 μm in the EYFP-transfected controls.

Furthermore, myr-Akt increased the number of axon branches by 2.6-fold. Although there was some increase in total axon length as compared to control transfected cells (1057 ± 119 μm versus 566 ± 42 μm), this was not due to growth of the main axon shaft but, instead resulted from the extensive elaboration of short branches (Figures 5 and 3A). Myr-Akt-expressing neurons had, on average, 20.3 branch points per cell while control cells had 7.7. The branch-inducing activity was equally pronounced in NGF-treated cultures, with an average of 32.5 branch points in myr-Akt-expressing cells and 12.6 in control transfected cells (Figure 5B). Overexpression of the dominant-inhibitory Akt PH domain construct interfered with branching in the presence of NGF, resulting in only 5.6 ± 0.7 branch points per cell.

Coexpression of Raf^{CAAX} and Myr-Akt

Crosstalk between the Raf and Akt pathways has recently been demonstrated (Zimmermann and Moelling, 1999; Rommel et al., 1999; Reusch et al., 2001) with Akt phosphorylating and inhibiting Raf in myotubes in vitro, but not in their myoblast precursors. To see whether there might be similar crosstalk in our cultured neurons, we cotransfected myr-Akt and Raf^{CAAX} into embryonic sensory neurons in the absence of NGF. Cotransfection resulted in long, thick, and moderately branched axons (Figure 6). The average axon length of the doubly transfected neurons was equal to that of NGF-treated cells (Figure 6, table). The axon caliber was increased 2.5-fold compared to control-transfected, unstimulated neurons, and 2-fold larger than in NGF-treated control-transfected cells (Figure 6, table). Cotransfection of myr-Akt and Raf^{CAAX} enhanced branching 1.7-fold, as compared to neurons transfected with Raf^{CAAX} alone. Thus, for the most part, the effects of Raf and Akt appeared independent, and no inhibition of Raf by Akt was apparent. The relatively small caliber of axons in response to NGF, compared to transfection with PI3K^{CAAX}, myr-Akt, or Raf^{CAAX} plus myr-Akt again suggests that NGF may not activate the PI3K-Akt pathway to its fullest extent at this stage of sensory neuron development.

TrkC Signaling Activates Akt More Strongly Than TrkA Signaling

We and others have previously shown that NT3 causes responsive sensory neurons in vitro to elaborate thicker, more highly branched axons than does NGF (Lentz et al., 1999; Ulupinar et al., 2000). Here, we report that overactivation of Akt leads to thickening and branching of axons of transfected neurons. To test the hypothesis that NT3 might activate Akt more strongly than NGF and thereby lead to increased caliber and branching, we looked at NGF- and NT3-induced Akt phosphorylation in transiently transfected PC12nnr5 cells, as well as in primary sensory neurons.

PC12nnr5 cells are derived from the NGF-responsive neuroendocrine cell line PC12, but lack Trk expression (Green et al., 1986). Expression levels of transfected TrkA and TrkC were equivalent and levels of Akt protein were the same in TrkA versus TrkC-expressing cells (Figure 7A). A potential complication is that Akt might be activated by p75^{NTR} (Roux et al., 2001). To guard against the possibility that NGF and NT3 might differentially affect the low-affinity neurotrophin receptor p75^{NTR}, we used a mixture of 100 ng/ml NGF and 100 ng/ml NT3 to stimulate the transfected cultures after 4 hr of serum deprivation. In three independent experiments, we found that Akt was phosphorylated more strongly in TrkC-expressing cells than in TrkA-expressing cells (Figure 7A). Similar results of stronger activation of Akt by TrkC than TrkA in stably transfected SH-SY5Y neuroblastoma cells were recently reported (Edsjö et al., 2001).

To determine whether Akt is more strongly activated by NT3 than NGF in DRG neurons, we looked at Akt phosphorylation in explant cultures of E13 mouse DRG maintained in either NGF or NT3 for 4 days to select for a relatively pure population of either TrkA or TrkC-expressing neurons. Again, we used a mixture of 100 ng/ml NGF and 100 ng/ml NT3 to stimulate the cultures after a 4 hr period of neurotrophin and serum deprivation. Because there are different ratios of neurons to nonneuronal cells under these conditions, we adjusted

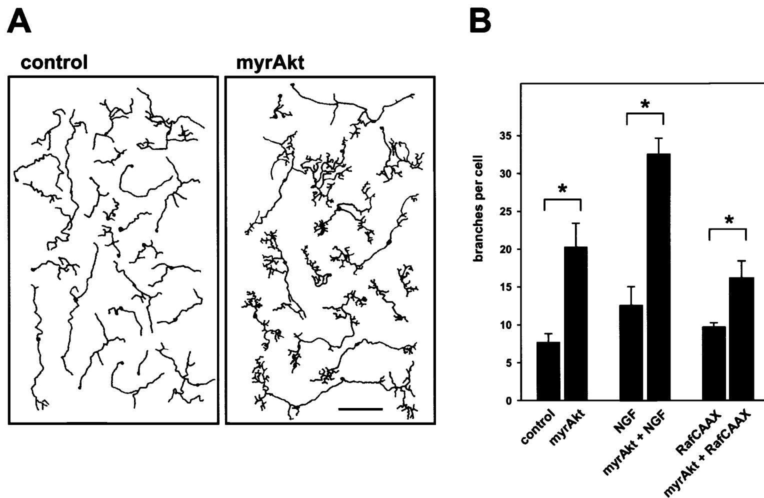


Figure 5. Activation of Akt Enhances Axon Branching

(A) Axons of transfected cells were traced in camera lucida, as outlined in Experimental Procedures; representative tracings are shown. Control cells were transfected with Myc-tagged EYFP, and myr-Akt cells were transfected with HA-tagged myristylated Akt. They were cultured for 3 days without NGF, then immunostained for the Myc or HA antigen.

(B) The number of branches per cell was counted from camera lucida axon traces. Myristylated Akt increased the number of branches per cell 2.6-fold in the absence or presence of NGF. In cells overexpressing membrane-tagged Raf-1, cotransfection with myristylated Akt increased branching 1.7-fold. Bars represent average ± SEM from five independent experiments for the first five bars and three independent experiments for the Raf^{CAAX}-myr-Akt cotransfection. Asterisks indicate statistical significance (Student's t test, $p < 0.02$).

Scale = 200 μm.

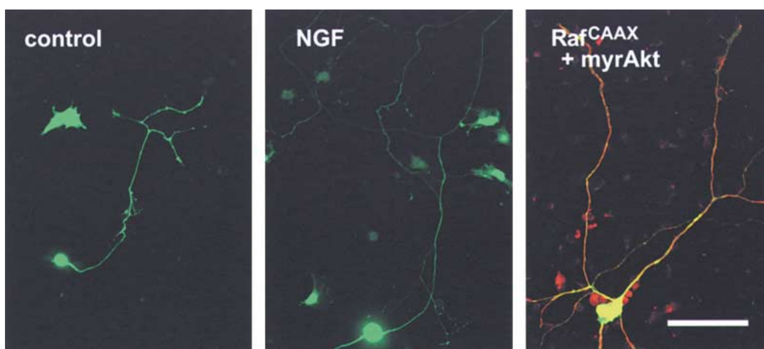
the amounts of total protein electrophoresed to arrive at comparable Trk levels for NGF/TrkA and NT3/TrkC samples.

Figure 7B (top) shows that NT3/TrkC signaling caused much stronger Akt activation than NGF/TrkA signaling in embryonic DRG neurons. For quantitation, phospho-Akt levels were normalized to the amount of TrkA or TrkC in the same lanes. The histograms (Figure 7B, bottom) show mean normalized values of three independent experiments. After subtraction of baseline phospho-Akt levels from the respective stimulated levels, we conclude that NT3 leads to a 2.69 ± 0.13-fold stronger activation of Akt than NGF.

Interestingly, baseline levels of phospho-Akt were significantly higher in NT3-maintained cultures than in NGF-maintained cultures (Figure 7B, bar graph). Although some of this effect could be due to the presence of greater amounts of nonneuronal Akt in the NT3 lysates, preliminary data (not shown) suggest that NT3-induced Akt phosphorylation may be more stable than that induced by NGF, thus not declining to the same extent after the 4 hr of neurotrophin and serum starvation preceding stimulation.

Discussion

In this study, we analyzed the contributions of single downstream effectors of the NGF signaling pathway to axon growth of embryonic sensory neurons in vitro. We found that all effectors investigated (Ras, Raf, PI3K, and Akt) are required for NGF-induced axon growth. But, selective activation of these signaling molecules by overexpression of active mutants in the absence of NGF revealed specific functions associated with each pathway. Overexpression of Ras mimicked the effect of NGF, while selective activation of Raf or Akt led to strikingly different morphologies with Raf producing lengthening and Akt producing increased caliber and branching.



	axon length [μm]	axon caliber [μm]	no. of branches per cell
control	610 ± 39	1.09 ± 0.06	7.7 ± 1.2
NGF	2185 ± 219 *	1.28 ± 0.07	12.6 ± 2.5 *
Raf^{CAAX} + myrAkt	2062 ± 100 *	2.53 ± 0.09 *	16.2 ± 2.3 *

Figure 6. Cotransfection of Active Raf and Akt Results in Additive Effects on Axon Length, Caliber, and Branching

Cells were transfected with Myc-tagged EYFP for the controls or simultaneously with Myc-tagged Raf^{CAAX} and HA-tagged myristylated Akt. After 3 days in culture, they were stained for the Myc- (green) and HA- (red) tags. Scale = 100 μm. The table lists data collected from three independent experiments as means ± SEM. Asterisks designate a significant difference relative to unstimulated, control-transfected cells ($p < 0.02$, Student's t test).

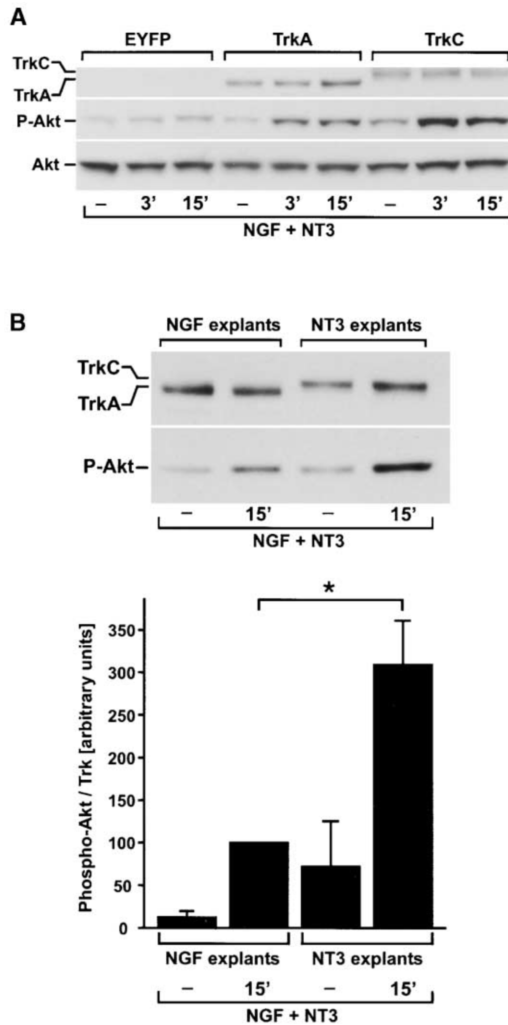


Figure 7. TrkC Signaling Activates Akt More Strongly Than TrkA Signaling

(A) PC12nnr5 cells were transfected with EYFP, TrkA, or TrkC and stimulated with a mixture of 100 ng/ml NGF and 100 ng/ml NT3 for the times indicated. Lysates were separated by SDS-PAGE, blotted, and detected using anitbodies to Akt, Phospho-Ser437-Akt, and pan-Trk, as described in Experimental Procedures. Akt phosphorylation after 3 or 15 min was stronger in TrkC-expressing cells than in those expressing TrkA. Levels of Trk expression and total Akt were comparable in TrkA versus TrkC transfected cells. One of three independent experiments is shown.

(B) DRG explant cultures were maintained in the presence of either NGF (left lanes) or NT-3 (right lanes) for four days. Explants were starved of serum and neurotrophins for 4 hr, stimulated with a mixture of 100 ng/ml NGF and 100 ng/ml NT3 for 15 min, and then lysed. Western blots were probed with anti-phospho-Ser473-Akt and anti-pan-Trk. The bar graph represents three independent experiments (means $\pm$ SD), and the asterisk denotes $p < 0.005$ (Student's t test). The lysate amounts electrophoresed were adjusted to contain approximately equal amounts of the respective Trks, as detected by a pan-Trk antibody. For quantification, amounts of phosho-Akt were divided by the amounts of Trk in the same lanes.

Thus, Akt and Raf stimulate two independent sets of morphology generating mechanisms.

A key feature of our study is the use of $Bax^{-/-}$ neurons. Conclusions about the morphological role of specific Trk effector pathways have been hampered by the fact that NGF or another neurotrophin is always required for neuronal survival. Thus, in all previous experiments in neurons, Trk effector pathways have been strongly stimulated at baseline conditions. Although the use of Campenot chambers has recently allowed the dependence of axon growth on particular pathways to be addressed (Atwal et al., 2000; Kuruvilla et al., 2000), these chambers still require NGF in distal compartments to attract axons. Thus, whether specific morphological features could be attributed to specific pathways was not addressed in these studies.

The use of $Bax^{-/-}$ neurons invites concerns about whether Bax may itself play a role in axon elaboration and, therefore, whether our own results might be compromised by its absence. In fact, the Bax-related Bcl-2 gene has been implicated in axon growth (Hilton et al., 1997; Chen et al., 1997; although see Goldberg et al., 2002). However, previous studies have shown that sensory neuron cultures derived from $Bax^{-/-}$ mice grow axons normally in response to NGF (Lentz et al., 1999) and that $Bax^{-/-}$ mice do not display axonal defects (White et al., 1998).

Since all of the Trk signaling mediators we investigated are required for axon growth, gain-of-function studies represent the most reasonable initial approach to assessing their effects on specific aspects of morphology. However, the results of such studies must be interpreted carefully. For example, activation of the signaling molecules may be far greater in our experiments than those observed in vivo. Also, the CAAX- and myr-tagged constructs may not accurately model the localization of signals in physiological situations. Recently, the importance of subcellular localization in neurotrophin signaling has been highlighted by studies demonstrating a requirement for endocytosis of the NGF-TrkA receptor complex for NGF-induced neurite extension from PC12 cells (Zhang et al., 2000), as well as for NGF-dependent Erk1/2 activation in DRG neurons (Howe et al., 2001).

Although we have not determined localization of the constitutively active constructs used here, those that carry a Ras-derived CAAX signal (i.e., Raf^{CAAX} and $PI3K^{CAAX}$) should be found in clathrin-coated vesicles, as is Ras itself (Howe et al., 2001). Indeed, K-ras and H-ras CAAX boxes have been shown to localize fusion proteins to endomembrane compartments such as Golgi and ER, in addition to the plasma membrane (Choy et al., 1999; Michaelson et al., 2001). The N-myristylation sequence is derived from Src, which itself is not a strictly membrane-bound protein (see Resh, 1996, for a review). N-myristylated Akt has recently been shown to differentiate PC12 cells (Namikawa et al., 2000) and should therefore be present in appropriate locations in neurons.

Axon Elongation Is Mediated through the Ras-Raf-Erk Pathway

The importance of Ras, Raf, and Mek in differentiation and neurite extension of PC12 cells has been well established (Bar-Sagi and Feramisco, 1985; Cowley et al., 1994; Korhonen et al., 1999; Klesse et al., 1999). We therefore might expect the Ras-Raf-Erk pathway to be involved in axon growth of primary neurons. Surprisingly

though, earlier studies have reported no effect of Mek inhibitors (Klinz et al., 1996) or dominant-negative Ras, Raf, Mek, or Erk adenoviruses (Klesse and Parada, 1998) on axon growth of embryonic peripheral neurons.

While the role of the Ras-Raf-Erk pathway in axon growth remains controversial, Ras (Borasio et al., 1993) and B-Raf (Wiese et al., 2001) are clearly required in the neurotrophin-dependent survival of embryonic sensory neurons, making interference with this pathway problematic. Our system of transfection of apoptosis-impaired Bax null neurons allows us to circumvent the survival problem and to evaluate the complete axonal arbors of single cells in medium-density cultures. We find now that inhibition of Ras or Raf activity by overexpression of dominant-inhibitory constructs or pharmacological inhibition of Mek completely inhibit NGF-induced axon growth of embryonic sensory neurons.

We also report that activation of either Ras or Raf is sufficient for sensory axon elongation. Both constructs lead to as much axon elongation as NGF treatment. Furthermore, Raf was capable of inducing this axon extension in the absence of additional PI3K stimulation. Our results thus establish a role for Raf as a morphological regulator in peripheral neurons. Neurons prominently express two isoforms of Raf, Raf-1, and B-Raf, with B-Raf being the most important activator of the Mek-Erk cassette, at least in PC12 cells (Jaiswal et al., 1994, 1996). Though we used dominant-negative and constitutively active constructs derived from Raf-1 in our studies, these mutants might inhibit or mimic both isoforms to some extent. The issue of whether specific morphological effects can be attributed to Raf-1 or B-Raf remains to be investigated.

The kinases Erk-1 and Erk-2 are generally considered to be the most important downstream effectors of Raf activation. Indeed, in our experiments, an activated Erk-2 fusion protein able to enter the nucleus resulted in substantial axon elongation. A purely cytoplasmic form of activated Erk-2 was unable to trigger any axon growth. These results agree with previous findings in PC12 cells, where it has been shown that only the nuclear form of activated Erk-2 could induce neurite outgrowth in the absence of NGF (Robinson et al., 1998).

However, in our study, activated Erk-2 resulted in only 70% of the axon growth achieved by NGF, Ras, or Raf, suggesting that other effectors besides Erk-2 are likely to contribute to Raf-triggered axon growth. In fact, there are numerous clues in recent literature suggesting that Raf works through other effectors besides the Erks. Hüser et al. (2001) report that a kinase-defective Raf-1, which is unable to activate Erks, can completely rescue the embryonic lethal phenotype of Raf-1 knockout mice. Pearson et al. (2000) found that an activated Raf-1 mutant defective in Mek binding (and therefore unable to activate Erks) could nevertheless induce neurite outgrowth in PC12 cells, possibly through activation of NF-κB and Rsk. A role of these or other Raf effectors besides Erk in neuronal morphology has not been reported, but could readily be investigated in our system.

Activation of the PI3K-Akt Pathway Increases Axon Caliber

While activated Ras and Raf strongly promote axon elongation, we did not find a significant effect on axon diameter or branching. This is surprising because evidence implicates growth factors released from targets and possibly from Schwann cells in the regulation of these parameters (Gold et al., 1991). In adult animals, the reduction in axon caliber that results from separation from targets by axotomy can be reversed by administration of neurotrophin family and GDNF family growth factors (Munson and McMahon, 1997; Munson et al., 1997).

Investigating the second major signaling pathway downstream of Ras, PI3K, and its effector Akt, we found morphological effects completely different from those induced by Raf. Atwal et al. (2000) and Kuruvilla et al. (2000) have shown that PI3K is required for axon elongation in sympathetic neurons, and our experiments confirm this for sensory cultures. We also find that Akt is required. But overexpression of active, membrane-tagged forms of either PI3K or Akt do not cause significant axon elongation. Instead, we find that selective activation of PI3K or Akt doubles the diameter of E13 axons. Interestingly, this effect of PI3K or Akt far surpasses axon caliber gain by NGF treatment, suggesting that NGF does not fully activate this pathway at embryonic day 13.

The increase in axon caliber mediated by the PI3K-Akt pathway may be analogous to the recently established role of PI3K, Akt, and other components of the insulin signaling pathway in the regulation of cell size in invertebrate development (see Stocker and Hafen, 2000, for a review) as well as in a myotube cell line (Rommel et al., 1999) and mouse cardiac myocytes (Shioi et al., 2000). Indeed, Tsui-Pierchala et al. (2000) have recently suggested that regulation of cell size and metabolism, rather than promotion of survival, may be the most prominent function of PI3K signaling in developing peripheral neurons.

We suspect, however, that the roles of Akt and PI3K are more specific than just regulating metabolism. For example, we find that axon caliber and cell body size are not regulated concomitantly. Both PI3K and Raf, when activated, strongly increase the soma size of cultured sensory neurons, with a more modest effect by Akt or NGF. But Raf and NGF, in contrast to Akt and PI3K, do not significantly affect axon calibers.

The differential regulation of axon length and caliber is not altogether surprising because axon lengthening and caliber growth involve different subsets of cytoskeletal elements. Axon elongation depends on microtubule polymerization (reviewed in Cleveland and Hoffman, 1991), while axon caliber is controlled through the expression of neurofilaments and regulation of neurofilament architecture (reviewed by Lee and Cleveland, 1996). We would predict that transcription of these cytoskeletal proteins during axonal development would be regulated by different signal transduction mediators.

Akt Enhances Branching

Another important feature of axon morphology is the massive branching that occurs once axons have arrived in target fields. It has long been suspected that this branching is regulated by target-derived supplies of growth factor (Korsching and Thoenen, 1983).

Perhaps surprisingly, morphological changes induced by Ras, Raf, and Erk were associated with very little

branching. However, NGF-responsive cells at these early stages also do not exhibit extensive branching. In contrast, embryonic TrkC-expressing DRG and trigeminal neurons respond to NT3 with thick and highly branched axons (Lentz et al. 1999; Ulupinar et al., 2000). Furthermore, administration of GDNF in vivo leads to excessive branching of motor axons (Keller-Peck et al., 2001). Since GDNF and NT3, as NGF, engage tyrosine kinase receptors (which are assumed to activate the Ras-Raf pathway), it is surprising that we do not observe even a subset of DRG neurons responding to Ras or Raf overexpression with increased branching.

Among the constructs we tested, Akt alone increased terminal branching of sensory axons, and it did so by 2.6-fold in naïve, as well as NGF-treated, cultures. Though Akt has been implicated in neurite extension of PC12 cells, as well as axon growth of regenerating motor neurons in vivo (Namikawa et al., 2000), an effect on branching has not been described. One plausible hypothesis to explain our current results, as well as the previous observations of branch induction by NT3, is that NT3 may activate Akt more strongly than NGF. We have tested this hypothesis in transiently transfected PC12nnr5 cells, as well as in primary DRG explants, and we found 2- to 3-fold increased Akt phosphorylation by NT3-TrkC signaling, as compared to NGF-TrkA signaling in both systems. Though we cannot be sure of the exact mechanisms leading to stronger activation of Akt in NT3- versus NGF-responsive sensory neurons, our results are consistent with the idea that many details of axon morphology may be determined by how strongly different growth factors activate the PI3K/Akt versus the Raf/Erk pathways.

The mechanism of Akt-induced axon branching remains unclear, but Akt phosphorylates a number of known partners that may interact with the actin cytoskeleton. For example, regulation of the small G protein Rac1 by Akt has been reported in vitro (Kwon et al., 2000). The kinase Pak is another Akt substrate (Tang et al., 2000) that may be involved in the regulation of axon morphology. It has been implicated in both neurite extension of PC12 cells (Daniels et al., 1998) and axon guidance in *Drosophila* (Hing et al., 1999). Additionally Akt can phosphorylate GSK-3β during Wnt signaling (Fukumoto et al., 2001), which may be another possible pathway for it to influence axon branching. Inhibition of GSK-3β through phosphorylation is a major mechanism in Wnt signaling, and Wnt-7a reportedly causes unbundling of axonal microtubules and is essential in the remodeling of mossy fiber axons during cerebellar development (Hall et al., 2000).

Terminal branching, as well as cell soma and axon caliber growth, are relatively late steps in the differentiation of peripheral neurons. Our results demonstrate a capability of the PI3K-Akt pathway to regulate these characteristics of embryonic neurons in culture. We therefore hypothesize that this pathway, which is essential in the early phases of sensory axon growth for neuronal cell survival as well as receptor internalization (York et al., 2000; Kuruvilla et al., 2000), also plays an important part in the morphological differentiation of axons later in development. In these stages the PI3K-Akt pathway is probably activated by additional growth factors and signals besides neurotrophins emanating from glial cells as well as the axon's target tissue.

Experimental Procedures

Plasmid Constructs

Signal tranduction constructs were kind gifts of C.J. Der (Raf^{CAAX}, dnRaf, $PI3K^{CAAX}$), P.F. Maness (Δp85), A. Wittinghofer (Ha-Ras[G12V], Ha-Ras[S17N]), R.S. Freeman (AktPH), A.S. Baldwin (myr-Akt), M.H. Cobb (Erk-Mek fusion constructs), and P.A. Barker (CMX-TrkA and CMX-TrkC).

We subcloned all constructs to be transfected into primary neurons into the pCMV-Myc vector (Clontech, Palo Alto, CA) using standard techniques. For myristylated Akt, which was HA-tagged on the C terminus, the Myc-tag of pCMV-Myc was removed by primer-directed mutagenesis. $PI3K^{CAAX}$ was additionally subcloned into pCMS-EGFP (Clontech). The CMX-TrkA and CMX-TrkC clones were used as received for transfection of PC12nnr5 cells.

Transfection and Culture of E13 $Bax^{-/-}$ Sensory Neurons

Male mice lacking Bax are infertile (Knudson et al. 1995). Therefore, $Bax^{-/-}$ females were bred with $Bax^{+/-}$ males to obtain E13 litters consisting of $Bax^{-/-}$ and $Bax^{+/-}$ embryos. Dorsal root ganglia were dissected and kept at 4°C until genotyping was completed. Genotyping was essentially done as described in White et al. (1998).

$Bax^{-/-}$ ganglia were treated with collagenase and trypsin (see Lentz et al. 1999), then washed in complete medium (MEM with 5% FBS and antibiotics) containing 0.1 mg/ml DNAase (Roche) and incubated at 37°C for 5 min. They were washed twice with cold, calcium-free ACSF (157.7 mM NaCl, 3.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES [pH 7.4]) and kept on ice. For transfection (adapted from Teruel et al., 1999), the ACSF was removed and 40 μl of plasmid DNA solution (0.5–1 μg/μl in ACSF) were added. The ganglia, suspended in the DNA solution, were pipetted onto a dry 35 mm petri dish.

The electrode consisted of two parallel 6 mm long platinum wires 6 mm apart, fastened to a disk of about 8 mm diameter, and connected to a Grass S-44 stimulator. This was lowered into the drop containing ganglia and DNA, and four square pulses of 140 V and 60 ms were applied with 20 s intervals, with switching of polarity between pulses. The ganglia were washed off the dish with complete medium, returned to ice, and dissociated by gentle trituration.

This method reliably achieved transfection rates of 0.2%–1%, with some variability between constructs. Expression levels (as estimated from immunostaining, data not shown) varied between constructs, but not from experiment to experiment. We also found that in contrast to the results reported by Teruel et al. (1999), electroporation with equimolar amounts of two constructs simultaneously led to coexpression of the two proteins in nearly all (>95%) transfected cells.

7500 neurons per well were seeded into 24 well plates containing 12 mm glass coverslips coated with 100 μg/ml poly-D-lysine (Sigma, St. Louis, MO) and 4 μg/ml laminin (BD Biosciences, Bedford, MA). 25 ng/ml NGF was added to cultures where appropriate 8–12 hr after seeding to allow for transgene expression before stimulation. The cells were cultured for a total of 72 hr.

Immunocytochemistry

The cultures on coverslips were fixed with 4% paraformaldehyde in PBS and stained according to standard protocols. Primary antibodies were used at the following dilutions: 1:250 for mouse-anti-Myc 9E10 (Oncogene, Boston, MA), 1:400 for mouse-anti-HA (Roche, Indianapolis, IN), and 1:200 for rabbit anti-HA (Clontech, Palo Alto, CA). Secondary antibodies employed were goat anti-mouse IgG-HRP (1:500), goat anti-mouse IgG-Cy2 (1:500), and goat anti-rabbit Fab_2'-RedX (1:100), all from Jackson Immuno (West Grove, PA). HRP was detected by incubation for 2–10 min in 0.5% DAB (3,3′-diaminobenzidine tetrahydrochloride, Polysciences, Inc., Warrington, PA) in 100 mM acetate buffer (pH 5.5).

Image Acquisition and Morphometry

To measure axon length, full axon arbors of DAB-stained neurons were traced onto plain paper using a camera lucida setup (Nikon).

The tracings were then scanned for measurement in NIH Image version 1.62. To trace the axonal arbors of fluorescently labeled neurons (those transfected with pEGFP-CMS-PI3K^{CAAX} and those coexpressing Myc-tagged Raf^{CAAX} and HA-tagged myristylated Akt), photomicrographs were captured at 50× magnification with a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Where necessary, several photomicrographs were joined in Adobe Photoshop to reproduce the complete axonal arbor. The axons were then traced in Adobe Photoshop and again measured in NIH Image.

Starting with the left-most of four coverslips per microscope slide, all the stained neurons on one or several coverslips were traced until at least 20, or at most, 50 neurons per condition were documented. The tracings were measured in NIH Image using the perimeter/length function. Axon branching was quantified from the tracings using a custom-written branchpoint counting program (P. Pomorski, UNC).

To determine axon caliber and soma size, pictures of 20 stained neurons per condition were taken at 150× magnification. To achieve a random sample, we again documented the first 20 transfected cells on the slides. The width of axons was measured in Adobe Photoshop at 20 μm and 50 μm from the cell body. In case of cells bearing more than one axon, the thickest axon was measured. For soma size, the circumference of the cell bodies was traced and the footprint area measured in NIH Image.

Western Blot Analyses

E13 DRG explants were cultured on 6 cm dishes coated as described above, in complete medium supplemented with antimitotics (20 μM 5-fluoro-2′-deoxyuridine, 20 μM uridine) and either 50 ng/ml NGF or 50 ng/ml NT3 for 4 days to enrich for TrkA- or TrkC-positive neurons. PC12nnr5 cells (a kind gift of P.A. Barker, Montreal) were cultured in 6 cm dishes in RPMI medium supplemented with 10% horse serum, 5% FBS, and antibiotics. They were grown to approximately 30% confluency and then transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Preceding neurotrophin stimulation, the complete medium was removed from DRG explants, as well as transfected PC12nnr5 cells, and replaced with basal medium (MEM or RPMI) supplemented with only 50 μg/ml BSA for 4 hr. The cultures were then stimulated with a mixture of 100 ng/ml each of NGF and NT3 for the times indicated. Cells were lysed in a standard lysis buffer supplemented with protease and phosphotase inhibitors, as described (Markus et al., 1997). Lysates were electrophoresed on 4%–12% gradient minigels (Invitrogen, Carlsbad, CA) and blotted onto PVDF membranes (Amersham, Piscataway, NJ). The membranes were blocked with 5% ECL-Blocking Agent (Amersham) in TBST, then incubated at 4°C overnight with anti-Phospho-Akt (Ser473) or anti-Akt (both from Cell Signaling Technology, Beverly, MA) or anti-pan-Trk (C-14, Santa Cruz Biotechnology, CA; Edsjö et al., 2001). All first antibodies were diluted 1:1000 in TBST with 5% BSA. Detection was performed using peroxidase-labeled anti-rabbit antibody diluted 1:20000 (Amersham) and a chemiluminescent substrate (ECLplus, Amersham). For quantification, films (BioMax MR, Kodak) were scanned and densitometry was performed using NIH Image.

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