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Activity Regulates Positive and Negative Neurotrophin-Derived Signals to Determine Axon Competition

Report

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Summary

Developmental axon competition plays a key role in sculpting neural circuitry. Here, we have asked how activity and neurotrophins could interact to select one axon over another. Using compartmented cultures of sympathetic neurons, we show that, in the presence of NGF, local depolarization confers a competitive growth advantage on the depolarized axon collaterals and at the same time disadvantages the growth of unstimulated axons from the same and competing neurons. Depolarization mediates the competitive advantage by activating a CaMKII-MEK pathway, which converges to enhance local NGF-mediated downstream growth signals. Patterned electrical stimulation also acts via this pathway to enhance NGF-promoted axonal growth. In contrast, the competitive disadvantage is due to BDNF secreted from and acting on the unstimulated, competing axons through p75NTR. Thus, activity regulates both positive and negative neurotrophin-derived signaling cascades to confer a competitive growth advantage on one axon versus another, thereby providing a cellular mechanism for developmental axon selection.

Introduction

One strategy used by the developing mammalian nervous system to establish neural circuitry is the overproduction of both neurons and axons, and the subsequent selection of only those neurons and axons that are appropriately connected. This selection occurs in many, if not all, populations of CNS and PNS neurons and is thought to be a key mechanism for environmental/extrinsic input in the development of neural connectivity (Kantor and Kolodkin, 2003). The mechanisms underlying neuronal selection during the developmental cell death period are becoming increasingly well-understood (Kaplan and Miller, 2000), but much less is known about the process of axonal competition.

Axon competition has been extensively studied in the visual system (Sengpiel and Kind, 2002), at the neuromuscular junction (Sanes and Lichtman, 1999) and in the peripheral nervous system (Purves, 1988). For peripheral neurons, it is clear that target-derived neurotrophins such as NGF play a key role in regulating competition between axons of both the same and different populations of neurons (reviewed in Purves, 1988). For developing sympathetic neurons, activity is also essential both for appropriate target innervation (Black and Mytilineou, 1976; Lawrence et al., 1979) and for developmental axon competition. For example, developing sympathetic eye-projecting neurons initially extend axon collaterals to two different eye compartments; these are largely maintained throughout the sympathetic neuron death period, but then axon elimination occurs so that any individual sympathetic neuron only ultimately projects to one eye compartment. If either circuit activity or target-derived NGF are perturbed during this time period, then this axon selection does not occur (Vidovic et al., 1987; Vidovic and Hill, 1988; Hill and Vidovic, 1989).

How could activity and neurotrophins select one axon collateral over another? Two mechanisms are commonly thought to underly axon competition. In the first, activity enhances trophic factor-mediated axonal growth, thereby providing active axons with a competitive advantage (Goldberg et al., 2002). In the second, inactive axons are actively pruned or retracted in response to negative signals such as, for example, the semaphorins (Bagri et al., 2003). Here, we have modeled axon competition using compartmented cultures of sympathetic neurons and have defined a third potential mechanism. Specifically, we show that local activity enhances local axonal growth, but that at the same time it actively disadvantages the growth of inactive axon collaterals deriving from the same and neighboring neurons. This distal growth inhibition is at least partially mediated via secretion of the neurotrophin BDNF acting through the growth-inhibitory p75 neurotrophin receptor. These findings therefore provide a mechanism for the selection of one axon collateral over another and raise the possibility that activity-dependent neurotrophin secretion may allow active axons to inhibit incoming, competing axons from growing into the same target territory.

Results

Local Depolarization Confers a Competitive Growth Advantage on Axon Collaterals and Disadvantages Unstimulated Axons

To model activity and neurotrophin-dependent axon selection, we first asked whether depolarization regulated local sympathetic axon growth in compartmented cultures (Figure 1A), a system where neurons are plated in the center of a divider and axons grow into sealed side compartments, thereby allowing independent manipulation of distal axons. Neonatal sympathetic neurons were established in these cultures in 10 ng/ml NGF for 6–7 days, axons in side compartments were removed and regenerated back, and a 4 day growth measurement was obtained by measuring axons on days 1 and 5. Double retrograde labeling with DiI and Fluorogold demonstrated that 40%–50% of neurons extended

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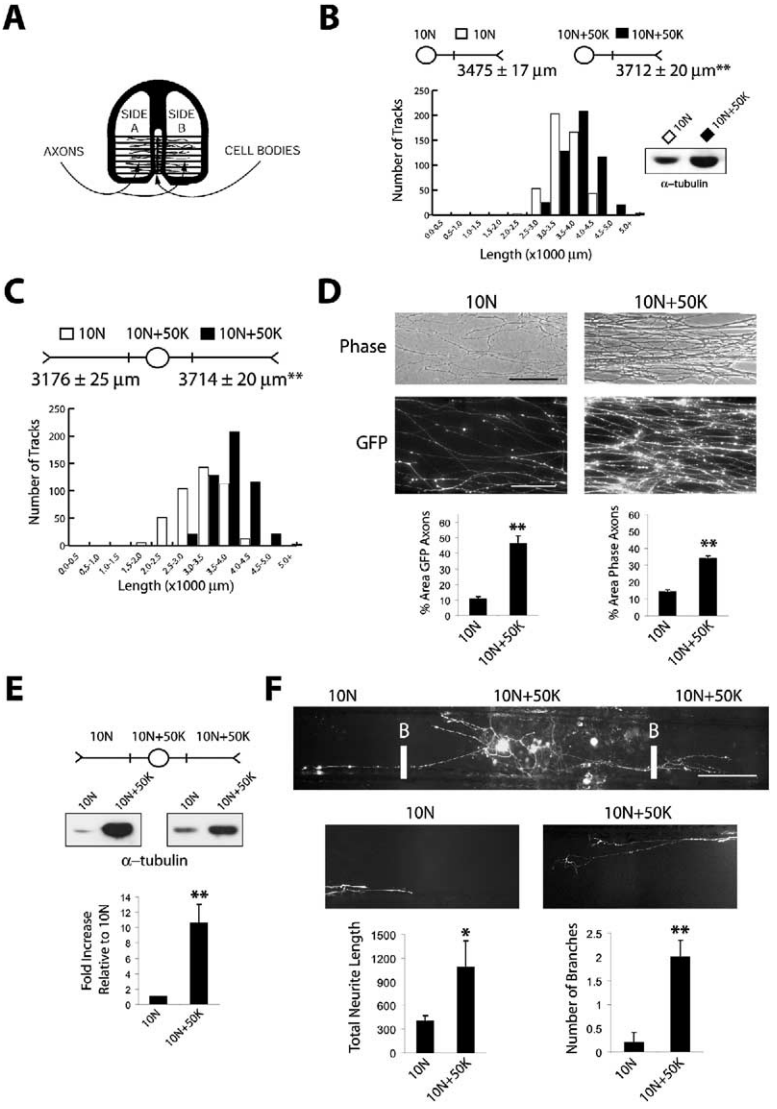

Figure 1. Local Depolarization Confers a Competitive Growth Advantage to Stimulated versus Unstimulated Axons

(A) Schematic diagram of compartmented Campenot chambers. Neurons are plated in the center compartment, and the axons grow into sealed side compartments. (B) Four day axon growth measurements for compartments containing either 10 ng/ml NGF or 10 ng/ml NGF plus 50 mM KCl in all compartments. Results are pooled from five experiments and are shown as a distribution histogram of the number of tracks containing axons of a given length. Numbers under the schematic diagrams are mean axonal length ± standard error of the mean (SEM). **p < 0.005. The right panel shows a Western blot for α-tubulin in side compartments grown as in the schematics. n = 2. (C) Four day axon growth measurements in competition cultures (schematic on top). Results from five experiments were pooled and expressed as in (B). **p < 0.005. (D) The top two panels are phase micrographs of uninfected axons, and the middle two panels are fluorescence micrographs of GFP-expressing axons. The graphs represent quantitation of three similar experiments and show the percentage of area covered by axons. **p < 0.005. Scale bar, 125 μm (upper panels), 50 μm (middle panels). (E) α-tubulin immunoblots of total axonal material from side compartments of competition cultures. The graph represents scanning densitometry of five experiments. **p < 0.005. (F) The top panel shows a single GFP-labeled neuron and its axonal arbor in a competition culture with "B" indicating the boundary between center and side compartments. The middle panel shows competing, unstimulated (10N) and stimulated axons (10N+50K) of a second neuron. The graphs show total axonal length and number of branches added in side compartments over two days. n = 3 experiments. *p < 0.05, **p < 0.005. Scale bar, 250 μm. In (D)–(F), error bars = SEM.

axon collaterals into both sides (data not shown). To depolarize axons locally, we utilized 50 mM KCl, which causes sustained membrane depolarization and increased intracellular calcium in sympathetic neurons (Franklin et al., 1995).

As previously reported (Campenot, 1984), when 10 ng/ml NGF was present in the center, axons grew into side compartments containing NGF, but not into those containing 0 NGF, 50 mM KCl, or 10 ng/ml NGF plus 50 mM KCl (data not shown). In contrast, when all compartments contained 10 ng/ml NGF plus 50 mM KCl, the 4 day axonal growth was increased relative to NGF alone (Figure 1B). This increase was confirmed by Western blots for total α-tubulin (microtubules are the major protein component of axons), levels of which were higher in side compartments of cultures containing NGF plus KCl versus NGF alone (Figure 1B). Thus, depolarization enhances NGF-mediated axonal growth, results similar to those previously obtained in retinal ganglion cells (Goldberg et al., 2002).

To ask whether depolarization provided an advantage for one axon versus another, we developed a competition paradigm. Cultures were established in NGF, axons were removed, and then we put NGF in one side and NGF plus KCl into the center and other side (Figure 1C). Three different measurements demonstrated enhanced growth of depolarized, competing axons growing into NGF plus KCl versus unstimulated, competing axons growing only into NGF. First, the mean 4 day axon growth length was significantly increased (Figure 1C). Second, image analysis of neurons infected with a recombinant adenovirus expressing GFP (Figure 1D) (Atwal et al., 2003) revealed that axonal density was increased 3- to 5-fold for depolarized versus unstimulated axons (Figure 1D). Finally, Western blots revealed that α-tubulin levels were 10-fold higher in depolarized versus unstimulated side compartments (Figure 1E).

Since these are population measurements, we confirmed our results with single identified neurons that

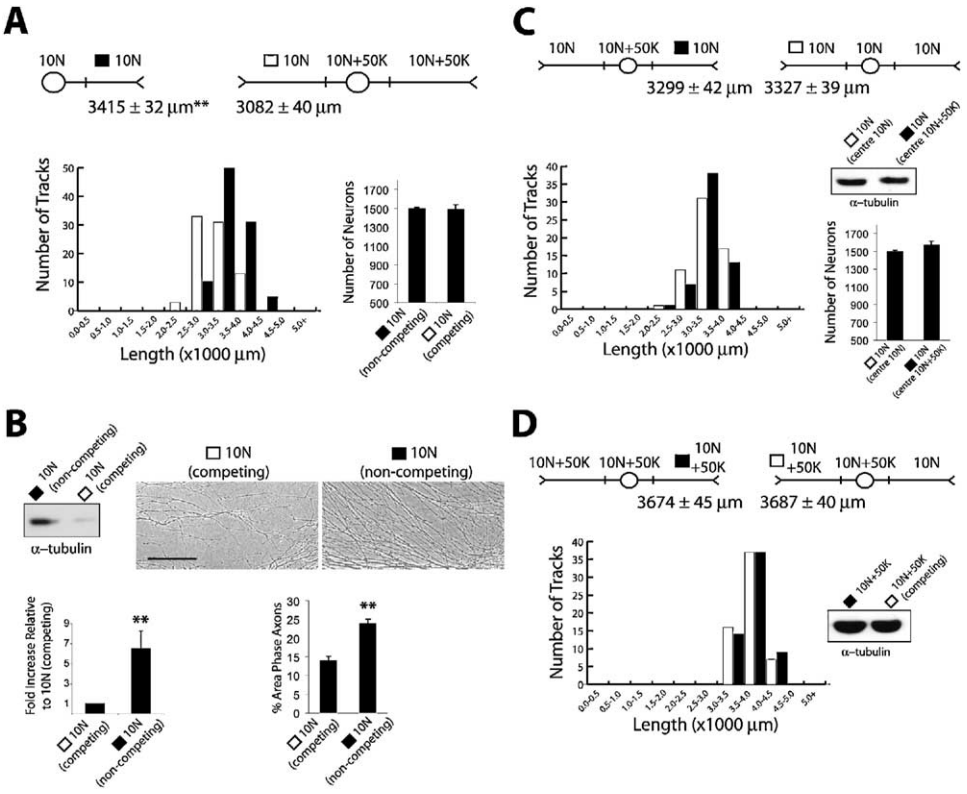

Figure 2. Local Depolarization of Axon Collaterals Disadvantages Unstimulated Axons

(A) Four day growth measurements for axons growing into 10 ng/ml NGF in competition versus control cultures (schematic at the top). Results are expressed as in Figure 1B. The bottom right panel shows mean number of neurons ± SEM in these same cultures. **p < 0.005. n = 3 experiments. (B) The left top panel is a Western blot for α-tubulin in 10 ng/ml NGF-containing side compartments from competition versus control cultures. The graph represents quantification of three experiments. *p < 0.05. The right top panels are micrographs of axons in NGF-containing side compartments of competition versus control cultures, with quantification of three similar experiments shown in the graph below. **p < 0.005. Scale bar, 125 μm. (C) Measurement of axonal growth in compartments where only the center compartment was depolarized. Results are expressed as in Figure 2A. p = 0.621. n = 3. The right inset shows an α-tubulin Western blot of total axonal material in side compartments of similar cultures. (D) Measurement of axonal growth in competition cultures versus cultures with 10 ng/ml NGF plus 50 mM KCl everywhere, with results expressed as in Figure 2A. p = 0.827. The bottom right panel shows an α-tubulin Western blot of total axonal material in the side compartments containing 10 ng/ml NGF plus 50 mM KCl from both culture configurations. In (A)–(C), error bars = SEM.

were infected with GFP adenovirus for 1 day and plated in low numbers with unlabeled neurons. One day after plating, we identified cultures with single GFP-expressing neurons that extended axon collaterals into both sides (Figure 1F), switched them into competition conditions, and quantitated growth of GFP-positive axons after 2 days (Figure 1F). This analysis confirmed that the total length of depolarized versus unstimulated axon collaterals was increased 3- to 4-fold and that depolarized collaterals added on average two new branches, while most unstimulated collaterals added none. Thus, depolarization locally enhances growth of one axon collateral over another.

We also compared growth of unstimulated axons in competition cultures versus control cultures containing NGF alone, ensuring that cultures contained similar neuron numbers (Figure 2A). Three measurements demonstrated that unstimulated, competing axons grew less than did unstimulated, control axons, even though both populations were growing into 10 ng/ml NGF (Figures 2A and 2B). First, the 4 day mean axon growth was decreased (Figure 2A). Second, axonal density, as assessed by phase microscopy, was decreased at least 2-fold (Figure 2B). Third, total α-tubulin levels, assessed by immunoblots, were markedly diminished (Figure 2B).

One explanation for this finding is that axons may not grow as well into side compartments containing NGF when the center contains NGF plus KCl. To test this, we generated cultures with NGF plus KCl in the center, and NGF in both sides. For comparison, we used cultures with NGF everywhere. Measurement of the 4 day axon growth and total α-tubulin levels (Figure 2C) showed that axons grew equally well into NGF whether or not the center contained KCl. A second possible explanation is that neurons have a finite capacity for growth, and maximal growth into the side containing NGF plus KCl limits growth into the other side. To test this, we compared competition cultures with cultures that had NGF plus KCl everywhere (Figure 2D). Measurement of the 4 day axonal growth and total α-tubulin levels revealed that axons grew equally well into NGF plus KCl in either culture configuration. Thus, the de-

creased growth of competing, unstimulated axons was not due to a limitation in the amount of growth these neurons could sustain.

Local CaMKII-MEK Pathway Activation Is Necessary for the Depolarization-Induced Competitive Growth Advantage

Since NGF promotes local axonal growth via a MEK-ERK pathway (Atwal et al., 2000) and since depolarization can enhance this activation via CaMKII (Rosen et al., 1994; Vaillant et al., 1999; Vaillant et al., 2002), we asked whether MEK was involved in the competitive local growth advantage. We first determined whether depolarization enhanced NGF-mediated ERK activation locally in axons as it does in mass neuronal cultures (Vaillant et al., 1999; Vaillant et al., 2002; data not shown). Compartments were established in NGF, and the center and one side were acutely stimulated with KCl for 30 min. Alternatively, axons were regenerated for 3 days into NGF plus KCl on one side and NGF only on the other side. Western blots with an antibody specific to activated, phosphorylated ERKs revealed that depolarization locally increased ERK activation ~2-fold (Figure 3A) and that this enhancement was not transduced distally to unstimulated axons.

To determine whether the local depolarization-induced ERK activation required calcium influx through L-type calcium channels and subsequent CaMKII activation (Vaillant et al., 1999), axons were exposed for 30 min to NGF plus KCl with or without a pharmacological inhibitor on one side (Figure 3A). Alternatively, axons were removed and then regenerated for 3 days in the constant presence of a pharmacological inhibitor (Figure 3A). Western blots revealed that the long- and short-term depolarization-induced increases in ERK activation required L-type calcium channels and CaMKII, since they were blocked by nifedipine and the CaMKII inhibitors KN62 and mAIP (Figure 3A). Quantitation demonstrated that these inhibitors all reduced ERK phosphorylation ~2-fold to levels seen in 10 ng/ml NGF.

To ask whether the depolarization-induced CaMKII-ERK activation was necessary for the growth advantage, we performed similar experiments and measured 4 day growth and α-tubulin levels. These experiments showed that inhibition of L-type calcium channels or CaMKII completely abolished the competitive advantage provided by local depolarization (Figure 3B). We then asked whether downstream MEK activation was involved by exposing depolarized axons to PD98059, a MEK inhibitor that effectively inhibited local axonal ERK activation (Atwal et al., 2000; data not shown). The 4 day growth measurements and α-tubulin immunoblots (Figure 3C) demonstrated that MEK inhibition actually reduced growth of the depolarized axons relative to their unstimulated counterparts. Since NGF promotes axonal growth via MEK, this result would be explained if the growth advantage was due to convergence of NGF and depolarization on MEK. We tested this by putting PD98059 in both depolarized and unstimulated sides and showed that axon growth was now reduced to the same low level in both sides (Figure 3C).

These experiments indicate that the competitive axon growth advantage was conferred by local depolarization- and neurotrophin-dependent MEK signaling and that the depolarization-induced growth advantage did not involve MEK-independent signals. To solidify these conclusions, we performed a few additional control experiments. First, to ask whether KCl in the center compartments activated MEK anterogradely, cultures were washed free of NGF, and NGF plus KCl was added for 30 min to the center compartments. Immunoblotting showed that phosphorylated ERK was only detected in the center compartment, arguing that MEK was only activated locally (see Figure S1A in the Supplemental Data available with this article online). This conclusion was confirmed in growth experiments where PD98059 in the center compartment of competition cultures had no effect on axonal growth in the sides (Figure S1B). Second, to confirm the necessity for local CaMKII-MEK signaling, cultures were established with NGF plus KCl everywhere, and CaMKII or MEK were inhibited in one side. In these experiments, the drugs only inhibited local axon growth, with no effect on depolarization-induced growth in the distal compartment (Figure S1C).

Patterned Electrical Stimulation Enhances NGF-Promoted Axon Growth through CaMKII-MEK Activation

To confirm the conclusions from the depolarization studies, we performed experiments using patterned electrical stimulation at 5 Hz, a more physiologically relevant paradigm that causes pulses of increased intracellular calcium (Brosenitsch and Katz, 2001). Neurons were established in 10 ng/ml NGF in mass cultures for 4 days, were stimulated for 3 additional days, as previously described (Vaillant et al., 2002; Brosenitsch and Katz, 2001), and neuronal growth was measured by assessing α-tubulin levels (Figure 4A). In five independent experiments, stimulation increased α-tubulin levels ~2-fold. To visualize this effect, newly isolated neurons were infected with a GFP-expressing adenovirus, a small number were plated for 24 hr on previously-plated uninfected neurons, and cultures were stimulated for 3 days. Image analysis revealed that individual stimulated neurons grew much more extensive neuritic arbors (under these conditions, the vast majority of neurites are axons and not dendrites [Vaillant et al., 2002]) (Figure 4B); both total neurite length and number of branches per neuron were increased ~2.5-fold.

To ask whether this growth enhancement involved the CaMKII-MEK pathway, cultures were stimulated in the presence or absence of CaMKII or MEK inhibitors, which do not affect sympathetic neuron survival in the presence of NGF (Vaillant et al., 1999; Vaillant et al., 2002). Three days later, analysis of either α-tubulin levels (Figure 4C) or of the morphology of individual, GFP-labeled neurons (Figure 4D) revealed that blocking CaMKII completely inhibited the stimulation-dependent increase in neuritic growth. With regard to MEK inhibition, neuronal growth was even less in stimulated cultures treated with PD98059 than in unstimulated cultures (Figure 4C). However, as in the competition experiments, α-tubulin levels were similar in stimulated and unstimulated cultures when MEK was inhibited in both, confirming that neuronal activity and NGF converge on MEK to enhance axonal growth.

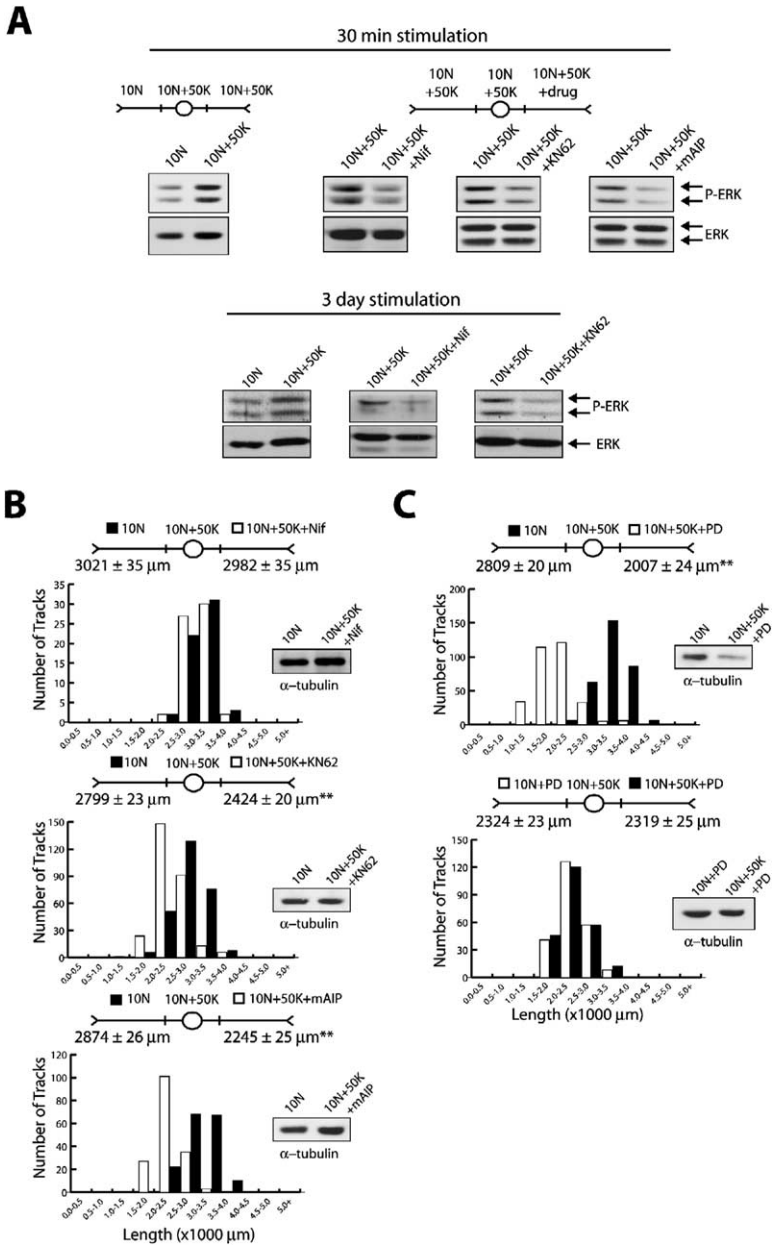

Figure 3. Local CaMKII-MEK Activation Mediates the Depolarization-Induced Competitive Growth Advantage

(A) Western blots for phosphorylated ERKs (P-ERK) in the side compartments of cultures stimulated as in the schematic for 30 min (top panels) or 3 days (bottom panels). The same blots were reprobed for total ERK to demonstrate equal protein loading. Inhibitors were used at concentrations of 1 μM nifedipine (Nif), 10 μM KN62, or 100 μM mAIP. (B) Four day axon growth measurements in competition cultures where nifedipine, KN62, or mAIP were present in the depolarized side compartment for the entire time. Results from three experiments were pooled and expressed as in Figure 1B. **$p < 0.005$. The insets show α-tubulin Western blots of total axonal material from side compartments of similar cultures. (C) Four day growth measurements in compartmented cultures grown as in (B) except that 50 μM PD98059 was present in one or both sides. Results from three experiments were pooled and expressed as in Figure 1B. **$p < 0.005$. The insets are α-tubulin Western blots of total axonal material from side compartments of similar cultures. n = 2.

We next asked whether MEK activation was sufficient to promote axonal growth. Neurons were infected with an adenovirus expressing constitutively activated HA-tagged MEK, which increases downstream neuronal ERK phosphorylation (Subramaniam et al., 2004). Transduced neurons were plated at low numbers with uninfected neurons and were grown for 3 days in 10 ng/ml NGF alone. Immunocytochemical analysis for the HA tag revealed that constitutively activated MEK was transported into axons (Figure 4E) and that it significantly enhanced NGF-mediated axon growth and branching relative to GFP-expressing neurons (Figure 4E). Thus, increased MEK activation is both necessary and sufficient for the activity-dependent axon growth enhancement.

The Growth of Unstimulated, Competing Axons Is Inhibited by a BDNF-p75NTR-Dependent Mechanism

Our data indicate that depolarization of one set of axon collaterals actively inhibits the growth of competing, unstimulated axons, many of them deriving from the same neurons. One candidate for such a distal effect is the neurotrophin BDNF, since (1) neuronal BDNF synthesis and secretion are activity dependent (Tao et al., 1998; Shieh et al., 1998; Fawcett et al., 1997; Aloyz et al., 1999; Balkowiec and Katz, 2002), (2) BDNF is synthesized by sympathetic neurons (Causing et al., 1997; Kohn et al., 1999), and (3) BDNF, acting through p75NTR, is a growth inhibitory cue for sympathetic axons (Kohn et al., 1999). We therefore hypothesized that neuronal depolarization would globally enhance BDNF

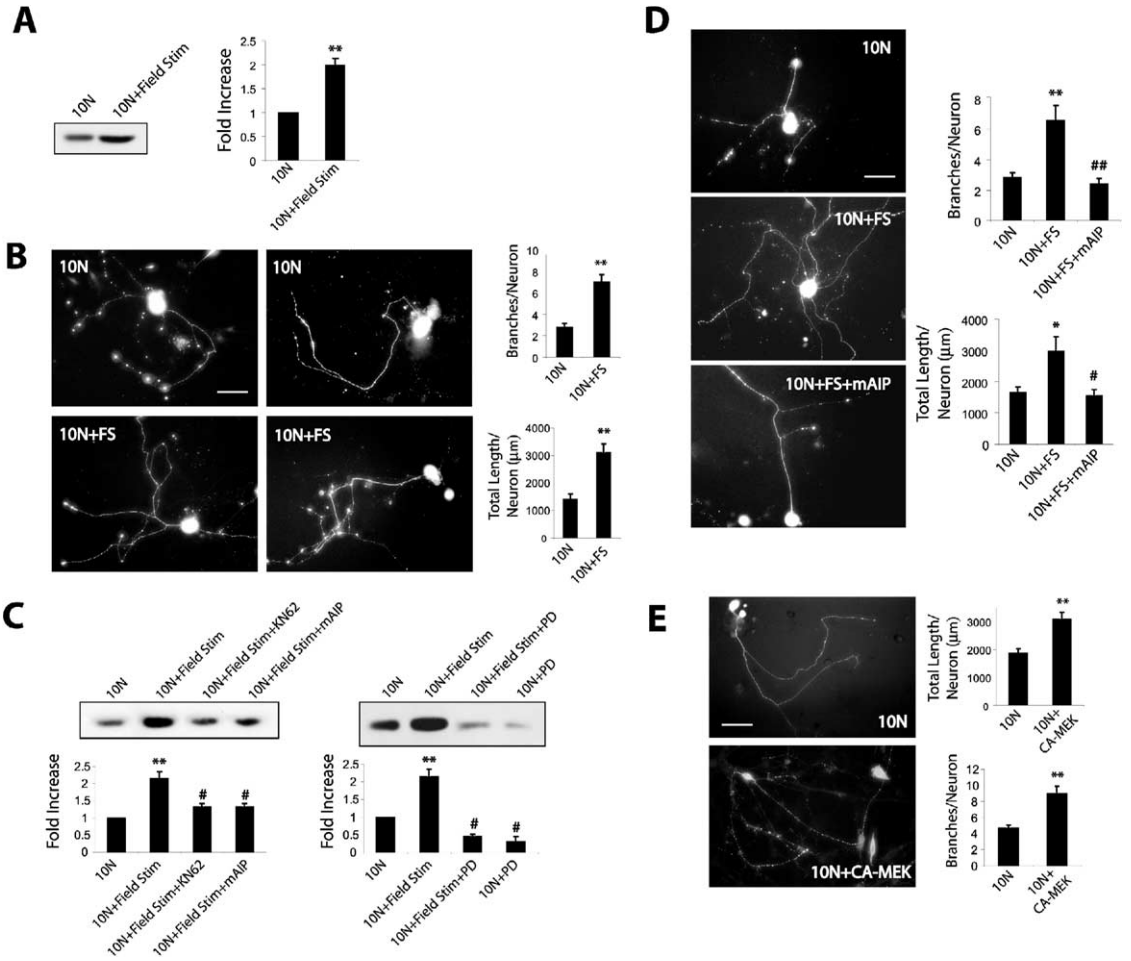

Figure 4. Patterned Electrical Stimulation Enhances NGF-Promoted Axon Growth and Branching via a CaMKII-MEK Pathway

(A) Western blot analysis for α-tubulin in mass neuronal cultures stimulated at 5 Hz for 3 days. Quantitation of five experiments is shown in the graph. **p < 0.005. (B) Photomicrographs of individual, isolated GFP-expressing sympathetic neurons that were grown as in (A) for 3 days either in 10 ng/ml NGF (10N) or in 10 ng/ml NGF with patterned field stimulation (10N+FS). The graphs show quantitation of branch number and total neuritic length per neuron. **p < 0.005, n = 4. Scale bar, 50 μm. (C) Western blots for α-tubulin in neurons grown as in (A), with or without 10 μM KN62, 100 μM mAIP CaMKII, or 50 μM PD98059. Graphs represent quantitation of three experiments. **p < 0.005 relative to 10 ng/ml NGF, #p < 0.05 relative to 10 ng/ml NGF plus field stimulation. (D) Photomicrographs of individual, isolated GFP-expressing neurons grown for 3 days in either 10 ng/ml NGF (10N) or 10 ng/ml NGF plus field stimulation (10N+FS) with or without 100 μM mAIP. The graphs are as in (B). *p < 0.05, **p < 0.005 relative to NGF alone, #p < 0.5, ##p < 0.005 relative to NGF plus field stimulation, n = 3 experiments. Scale bar, 50 μm. (E) Photomicrographs of neurons in 10 ng/ml NGF that were infected with adenoviruses expressing either GFP (top) or HA-tagged, constitutively activated MEK (bottom) and then analyzed for GFP or immunocytochemically for the HA tag, respectively. The right panels show quantitation of similar micrographs. **p < 0.005. Scale bar, 50 μm. In all panels, error bars = SEM.

levels throughout the entire axonal arbor, thereby explaining the growth disadvantage we observed for competing, unstimulated axons. To test this hypothesis, we first localized BDNF in sympathetic neurons. Immunocytochemistry revealed specific, punctuate BDNF immunoreactivity in axons, and bright, more diffuse immunostaining in cell bodies (Figure 5A). We then asked whether inhibition of BDNF or of its receptor, p75NTR, could rescue the decreased growth of unstimulated, competing axons by adding function-blocking antibodies for either BDNF (Kohn et al., 1999) or p75NTR (REX; Weskamp and Reichardt, 1991) to the unstimulated side of competition cultures. Measurement of the 4 day growth and of total α-tubulin revealed that anti-BDNF and anti-p75NTR significantly rescued the growth of competing, unstimulated axons relative to sister cultures treated with a control antibody (Figures 5B and 5C). Thus, BDNF secretion from sympathetic axons provides one mechanism whereby active axons disadvantage inactive axons from the same and neighboring neurons.

Discussion

In this study, we have investigated the cellular mechanisms that regulate developmental axon competition. Our findings support five major conclusions. First, using compartmented cultures, we show that, in the presence of NGF, local axonal depolarization and the consequent increased intracellular calcium confers a major growth advantage to the stimulated axon collaterals, findings similar to those previously reported for

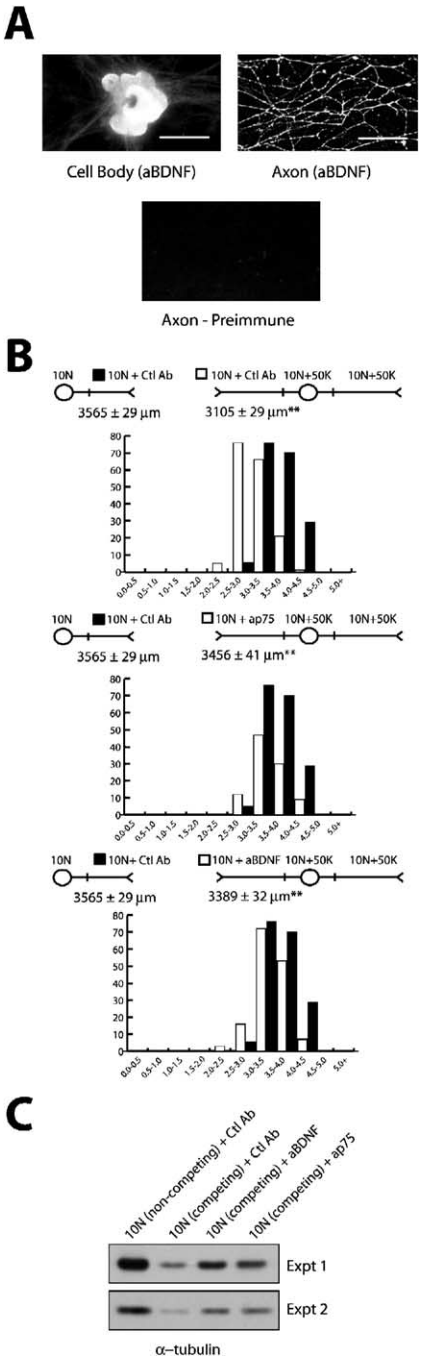

Figure 5. The Growth of Unstimulated, Competing Axons Is Inhibited by a BDNF-p75NTR Autocrine/Paracrine Mechanism

(A) Photomicrographs of neurons grown in compartments and immunostained with anti-BDNF (aBDNF) or with control preimmune serum (bottom). The photomicrographs of axons were obtained using a confocal microscope. Scale bar (cell body picture), 50 μm. Scale bar (axon pictures), 25 μm. (B) Four day growth measurements of competition cultures with function-blocking antibodies specific for BDNF (aBDNF), the p75 neurotrophin receptor (ap75), or a control, nonspecific antibody (Ctl Ab) present in the unstimulated side compartment. For comparison, control cultures contained 10 ng/ml NGF everywhere and the control antibody in the quantitated side compartment. Results from three experiments were pooled and analyzed as in Figure 1B. **$p < 0.005$. (C) Western blot analysis for total α-tubulin in side compartments of cultures similar to those shown in (B). The two blots derive from independent experiments.

retinal ganglion cells (Goldberg et al., 2002). Second, and somewhat surprisingly, our data indicate that depolarization of one set of axon collaterals actively inhibits the growth of unstimulated, competing axons. Third, we show that the competitive growth advantage is conferred by depolarization-induced CaMKII-MEK activation in the axons themselves, which converges intracellularly with local NGF-mediated MEK activation to directly promote local growth. Fourth, we show that patterned electrical stimulation can enhance NGF-mediated axonal growth and branching and that this too is dependent upon a CaMKII-MEK pathway. Finally, we provide evidence that the decreased growth of unstimulated, competing axon collaterals is at least partially mediated by a BDNF-p75NTR autocrine/paracrine loop. Thus, neuronal activity regulates both positive and negative neurotrophin-derived signals to promote the growth of one axon over another, providing a potential cellular mechanism for developmental axon selection.

One of the assumptions of our model is that different collaterals from the same developing neuron would have different levels of activity. Is there precedent for this? In mature neurons, conduction failure at axonal branches is well-documented. For example, collaterals emanating from the same muscle spindle afferent are differentially depolarized in vivo (Lomeli et al., 1998). Importantly, a similar phenomenon has been documented during development; in developing insect giant interneurons, morphological changes caused the selective failure of impulse propagation in only some afferents and thereby led to functional afferent elimination (Spira and Yarom, 1983; Yarom and Spira, 1983). Since sympathetic axon growth is locally regulated by NGF (Campenot, 1982), two different collaterals that were exposed to different amounts of target-derived NGF might differ in terms of their size and safety factor for impulse propagation, and thus in their relative level of activity.

Our studies clearly demonstrate that the competitive growth advantage is conferred by intracellular convergence on MEK, a key determinant of local sympathetic axon growth (Atwal et al., 2000; Atwal et al., 2003). Since the MEK-ERK pathway regulates proteins associated with both microtubules and actin filaments (Sanchez et al., 2000; Houle et al., 2003; Nebl et al., 2004), the growth advantage is likely mediated at the level of the local cytoskeleton. In contrast, our findings indicate that unstimulated axons are disadvantaged as a consequence of an active mechanism requiring stimulation of other axon collaterals from the same or neighboring neurons. We propose that BDNF-mediated p75NTR activation provides one such active mechanism for the following reasons. First, sympathetic neurons synthesize and secrete BDNF (Causing et al., 1997; Kohn et al., 1999), and we show here that BDNF is present in sympathetic axons. Second, we previously showed that BDNF, either exogenous or made by sympathetic neurons, binds to p75NTR and inhibits NGF-promoted axon growth (Kohn et al., 1999), and here we show that this autocrine/paracrine BDNF-p75NTR loop is at least partially responsible for the decreased growth of unstimulated, competing axons. Third, both BDNF and p75NTR are essential for development of the

appropriate density and pattern of sympathetic innervation in vivo (Lee et al., 1994; Kohn et al., 1999).

Thus, we propose that, in our competition experiments, increased calcium influx in cell bodies and depolarized axons causes enhanced transcription of the activity-dependent BDNF gene (Shieh et al., 1998; Tao et al., 1998) and increased BDNF secretion from all parts of the axonal network, including the unstimulated axons. This increased BDNF then signals growth inhibition via p75NTR. For the stimulated axons, this negative growth signal would be more than compensated by the depolarization-mediated positive growth signals. In contrast, the unstimulated axons would be disadvantaged in terms of their growth in response to a given amount of NGF. This mechanism explains our culture findings, and, as described below, provides the basis for a model to explain selection of one collateral over another. Interestingly, this mechanism also predicts that active axons could secrete BDNF to repel incoming, competing axons from growing into the same target territory. In this regard, work by Gan and Lichtman (1998) demonstrated that, at the neuromuscular junction, axon branches close to successful competing axons were removed first, suggesting the existence of such short-distance negative signals in vivo.

In summary, we propose a feedforward model of sympathetic axon competition. In this model, the first axons to reach an appropriate target would not only sequester limiting amounts of target-derived NGF, but because these neurons have already received synaptic input from their preganglionic afferents (Rubin, 1985), they would also become the first axons to participate in an active circuit. The increased calcium influx in these active, firing axons would converge with the local NGF-mediated activation of TrkA to enhance their sprouting into the target, leading to sequestration of additional synaptic territory and additional NGF, and thereby reinforce their position as the "winning" axons in the competition. In contrast, a collateral that was unsuccessful in competing for target-derived NGF would also be actively inhibited from growing into the target by the local secretion of BDNF from active successful axons. Such an axon would likely be smaller and would therefore display a higher impulse conduction failure, as discussed above. Moreover, since sympathetic axons degenerate in the absence of NGF, then this inability to compete for target territory would over time lead to axonal degeneration, thereby effectively pruning that collateral. Thus, the biasing seen here to make the strong stronger and the weak weaker would ultimately ensure the selection of only those axon collaterals that were appropriately connected. From a broader perspective, since interactions between growth factors and neural activity are important for the selection and reinforcement of many active neural circuits, we propose that the cellular mechanisms we have defined here for sympathetic neurons may well be relevant for other developing neural systems.

Experimental Procedures

Neuronal Cultures and Axon Growth Measurements

Sympathetic neurons were dissected from the superior cervical ganglion (SCG) of P1 Sprague-Dawley rats. Mass (Vaillant et al., 2002) and compartmented cultures (Atwal et al., 2003; Campenot, 1992) were prepared as described. In all cases, NGF refers to 10 ng/ml NGF and KCl to 50 mM KCl. For axon growth rates, neurites in side compartments were axotomized (Campenot, 1992), and de novo growth was assessed by measuring the longest axon in each track at days 1 and 5 (20 tracks per side) using the Neurotrack program (Northern Eclipse) on a Zeiss inverted microscope. Inhibitors were nifedipine (Biomol), PD98059 (Biomol), KN62 (Calbiochem) and myristolated AIP (mAIP; Calbiochem). Antibodies were replaced every 24 hr and included function-blocking anti-BDNF (Kohn et al., 1999) (1:500, Promega), function-blocking anti-p75NTR (REX; Weskamp and Reichardt, 1991) (1:200, the kind gift of L. Reichardt), and control, nonspecific IgG (1:250, Promega). In some experiments, neurons were infected overnight with adenovirus expressing either GFP or constitutively activated MEK, as previously described (Vaillant et al., 1999; Subramaniam et al., 2004; Atwal et al., 2003). Field stimulation experiments were performed as described with 1.5 ganglia/well (Vaillant et al., 2002; Brosenitsch and Katz, 2001); neurons were stimulated with 0.2 ms pulses of alternating polarity delivered at 5 Hz.

Immunocytochemistry and Image Analysis

Immunocytochemistry was performed as previously described (Atwal et al., 2003) using anti-HA (1:400, Covance), anti-BDNF (the kind gift of David Kaplan) (Friedman et al., 1998), or preimmune serum. Fluorescent images were captured using Northern Eclipse software on an inverted Zeiss microscrope or with a Zeiss Pascal confocal microscope. Quantification of branch number and total axon length was performed using Northern Eclipse software. Quantification of phase and GFP axon densities were performed by chosing random fields, thresholding the images using Northern Eclipse software, and then determining the percentage of total area covered by thresholded axons. Statistics were performed using Student's t test.

Western Blot Analysis

Neurons were lysed and Western blots performed and quantitated by scanning densitometry as previously described (Vaillant et al., 1999; Vaillant et al., 2002). For all α-tubulin Westerns, the total axonal material from two side compartments of equivalent cultures were analyzed. In long-term experiments, inhibitors were changed every 24 hr. Antibodies included anti-phospho-ERK (1:5000 mass culture, 1:2000 compartment culture, Promega), anti-ERK (1:5000 mass culture, 1:2500 compartment culture, Santa Cruz), and anti-α-tubulin (1:100000 mass culture, 1:5000 compartment culture, Sigma).

Supplemental Data

The Supplemental Data that accompanies this article can be found at http://www.neuron.org/cgi/content/full/45/6/837/DC1/.

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