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Regulation of Neurotrophin-Induced Axonal Responses via Rho GTPases

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Abstract

Nerve growth factor (NGF) and related neurotrophins induce differential axon growth patterns from embryonic sensory neurons. In wholemount explant cultures of embryonic rat trigeminal ganglion and brainstem or in dissociated cell cultures of the trigeminal ganglion, exogenous supply of NGF leads to axonal elongation, whereas neurotrophin-3 (NT-3) treatment leads to short branching and arborization. Axonal responses to neurotrophins might be mediated via the Rho GTPases. To investigate this possibility, we prepared wholemount trigeminal pathway cultures from E15 rats. We infected the ganglia with recombinant vaccinia viruses that express GFP-tagged dominant negative Rac, Rho, or constitutively active Rac or treated the cultures with lysophosphatidic acid (LPA) to activate Rho. We then examined axonal responses to NGF by use of the lipophilic tracer DiI. Rac activity induced longer axonal growth from the central trigeminal tract, whereas the dominant negative construct of Rac eliminated NGF-induced axon outgrowth. Rho activity also significantly reduced, and the Rho dominant negative construct increased, axon growth from the trigeminal tract. Similar alterations in axonal responses to NT-3 and brain-derived neurotrophic factor were also noted. Our results demonstrate that Rho GTPases play a major role in neurotrophin-induced axonal differentiation of embryonic trigeminal axons.

Keywords

Rac; Rho; trigeminal axon growth; NGF; NT-3; BDNF; viral vectors

Primary sensory neurons of the dorsal root and trigeminal (TG) ganglia depend on target-released neurotrophins for survival and express specific Trk receptors (McMahon et al., 1994; Wright and Snider, 1995; Conover and Yancopoulos, 1997; Davies, 1997, Davies, 1998; Enokido et al., 1999; Huang et al., 1999b). Recent studies also suggest that neurotrophins play a major role in axonal differentiation (Hoyle et al., 1993; Lentz et al., 1999; Ulupinar et al., 2000a; see also Gallo and Letourneau, 2000, for a review). During axonal development, elongation and retraction of growth cones are all related to the arrangement of actin cytoskeleton (for reviews, see Lin et al., 1994; Hall, 1998; Gallo and Letourneau, 2000). The Rho family of GTPases is known to play a major role in this process

(for reviews, see Luo et al., 1997; Tapon and Hall, 1997; Aspenstrom, 1999; Mueller, 1999; Song and Poo, 1999; Bishop and Hall, 2000).

Members of the family (Cdc42, Rho, and Rac) are either found in the GTP-bound active state or the GDP-bound inactive state (for reviews, see Van Aelst and D'Souza-Schorey, 1997; Mackay and Hall, 1998; Hall 1999; Kjoller and Hall 1999; Bishop and Hall, 2000). Relative abundance of active Rho and Rac might regulate growth cone behavior during axon navigation (Mueller, 1999). Dominant negative and constitutively active versions of Rho GTPases have been used to study their role in axonal arborization (Threadgill et al., 1997; Nakayama et al., 2000), dendritic growth (Threadgill et al., 1997; Ruchoeft et al., 1999; Nakayama et al., 2000; Li et al., 2000), neuronal remodeling (Luo et al., 1996; Ruchoeft et al., 1999; Li et al., 2000), and growth cone motility (Jin and Strittmatter, 1997; Kozma et al., 1997; Ruckhoeft et al., 1999; Kuhn et al., 2000; Nakayama et al., 2000; Wahl et al., 2000). Dominant negative proteins bind irreversibly to the specific GEF molecules, which mediate the exchange of GDP to GTP and compete for the activation of endogenous small GTPases inside the cell. Thus, expression of dominant negative constructs mimics downregulation of effector molecules for the particular Rho GTPase. Constitutively active constructs are unresponsive to regulatory proteins and are in the activated state at all times.

Although it has also been suggested that neurotrophins regulate the neuronal cytoskeleton (Gallo and Letourneau, 2000), the involvement of Rho GTPases in neurotrophin-mediated axonal changes is largely unknown. To elucidate this issue, we investigated the role of Rac and Rho in axonal responses of central trigeminal axons to NGF, NT-3 and BDNF.

The embryonic rodent trigeminal pathway can be isolated as an intact wholemount preparation. Exogenous application of NGF and NT-3 to these cultures has differential effects on growth parameters of central trigeminal axons in the brainstem. In the presence of NGF, TG axons leave the central trigeminal tract and extend into the surrounding brainstem tissue without branching, whereas NT-3 promotes precocious arborization of tract axons (Ulupinar et al., 2000a). Localized application of neurotrophins also yields similar results (Erzurumlu et al., 2000). In the present study, we prepared TG-brainstem wholemount cultures and infected the ganglion with the recombinant vaccinia viruses that express the dominant negative and constitutively active Rac or Rho GTPases. We then tested the effects of neurotrophins on axon outgrowth from the central trigeminal tract.

MATERIALS AND METHODS

Preparation of trigeminal pathway explant cultures

Embryos from 15-day pregnant Sprague-Dawley rats (day of sperm positivity E0) were removed by cesarean section following euthanasia of the dam. All the experimental procedures were conducted according to NIH and Institutional IACUC guidelines. Trigeminal pathway explants were dissected in ice-cold Gey's balanced salt solution (GIBCO, Gaithersburg, MD) supplemented with D-galactose (6.4 mg/l; Sigma, St. Louis, MO). These ex-plants included the left and right TG and the brainstem from the pontine flexure to the upper cervical levels where the central trigeminal tract lies (Fig. 1A). Wholemount explants were then cultured on microporous Millicell membranes (Millipore,

Bedford, MA) and grown in serum-free culture medium (SFM) at 33°C in a humidified CO₂ incubator (Ulupinar et al., 2000a).

Viral infection and neurotrophin treatment

After 24 hours in culture, TG on one side of each whole-mount explant was infected with recombinant vaccinia viruses (gift of H. Cline), which express GFP-tagged dominant negative (DN) Rho (RhoN19), Rac (RacN17), or constitutively active (AC) Rac (RacV12). The construction and use of these viral vectors have been described in detail by Li et al. (2000). TG on the other side was infected with a GFP-expressing viral construct as a control. TG neurons were infected with high-titer virus (over 10⁶ plaque-forming units [pfu]), and infections were restricted to the ganglion with the help of a Hamilton microliter syringe mounted to a micromanipulator (Fig. 1A). Fast green dye (1%) was used with the viral solution to visualize the injection site. We did not have viral vectors expressing RhoAC. Instead, we added lysophosphatidic acid (LPA; 10μM, Sigma), which is known to activate Rho in neuronal cells (Ridley and Hall, 1992; Jalink et al., 1994). Following viral infections, cultures were transferred to SFM supplemented with NGF 2.5s (50 ng/ml; Collaborative Biomedical Products, Bedford, MA), NT-3, or BDNF (50 ng/ml; Regeneron Pharmaceuticals, Tarrytown, NY) and maintained in vitro for an additional 3 days. GFP expression in the TG was observed after 24 hours under epifluorescence by using a fluorescence isothiocyanate (FITC) filter set. In all cases GFP expression was localized to the ganglion with no or minimal spread to the surrounding brainstem tissue (Fig. 1B). GFP expression within the TG was considered a general indicator of viral infection.

In a separate series of cultures, blue and white sepha-rose beads (200 μm diameter; gift of K. Muneoka) were washed twice with PBS (pH 7.4), air-dried, and soaked in NGF, NT-3, BDNF (100 ng/μl), or bovine serum albumin (BSA; Sigma, 100 ng/μl) overnight at 4°C. A single bead was implanted along the lateral side of the descending trigeminal tract (Fig. 1C). Viral infections were done 24 hours after bead implantation, and wholemounts were kept 3 more days in culture. In each culture, a neurotrophin-loaded bead (blue) was implanted on one side and a BSA-loaded bead (white) on the other side as a control. Bead experiments were carried out to specifically test the localized effects of neurotrophins on trigeminal axon growth patterns, and their regulation by Rho family of GTPases.

Immunohistochemistry and histochemistry

Two of the viral constructs we used (RacV12 and RacN17) were myc-tagged at the amino (N) terminal; the RhoN19 construct expressed β-galactosidase (β-gal) from an independent promoter, and the control viral vector expressed GFP/β-gal fusion protein. Therefore, we performed c-myc immunohistochemistry for RacN17 and RacV12, and β-gal histochemistry for RhoN19 and control GFP viral constructs to further verify viral gene expression in infected TG.

For immunohistochemistry, 20-μm-thick frozen sections taken from the infected explant cultures were first preincubated in a blocking solution containing 0.3% Triton X-100 and 5% horse serum in phosphate buffer (PB, 0.1 M, pH 7.4) for 1 hour followed by overnight incubation in primary antibody solution (monoclonal anti-c-myc, 1:100; Oncogene,

Cambridge, MA) in blocking solution. After several rinses in PB, sections were incubated in Texas Red-conjugated horse anti-mouse antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking solution for 45 minutes. For β -gal staining, frozen sections through the RhoN19-infected TG were rinsed in PB and processed by using the β -gal Staining Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

To further verify that the infected TG explants contained cells that express high-affinity neurotrophin receptors we used TrkA immunohistochemistry. Frozen sections through the virally infected TG explants were preincubated in PB with 10% goat serum and 0.3% Triton X-100 for 45 minutes and then overnight in TrkA antibody (gift of Dr. L. Reichardt) diluted at 1:2500 in blocking solution. After several rinses in PB, the sections were incubated in CY3-goat anti-rabbit secondary antibody (1: 200 dilution in blocking solution; Chemicon, Temecula, CA) for 2 hours. For all immunohistochemical procedures, control sections were treated in the same manner, omitting the primary antibody.

Dil labeling

After 3 days in culture, wholemounts were fixed with 4% paraformaldehyde in PBS) (pH 7.4, 0.1 M), and small crystals of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) were inserted into the ganglion (Fig. 1C) to visualize central trigeminal axons in the brainstem. Cultures were kept at 33°C for 7–10 days for the lipophilic tracer to diffuse along the entire length of the trigeminal tract. DiI-labeled axons in selected cultures were later photoconverted in 0.15% DAB in 0.1 M Tris buffer (pH 8.2; Sandell and Masland, 1988). All the photographic documentation presented in this study was done by the use of a CoolSNAP digital camera attached to a Nikon Microphot-SA or a Nikon Diaphot inverted microscope. Digital images were transferred to a power PC, and the contrast and brightness of the images were adjusted by using the Adobe Photoshop program. In the preparation of the figures the right (control) sides of the explants were inverted to align the right and the left trigeminal tract in comparable positions, and no other alterations were made in the images.

Quantification of axon growth and statistical analysis

The results of each case (NGF [50 ng/ml], RacAC, RacDN, RhoDN, LPA, and NGF [100 ng/ml]) were captured with 10 $\times$ magnification and printed on a full page. A transparent sheet with 50 $\times$ 50- μ m grids was placed on top of the pictures, and the central trigeminal tract was labeled and accepted as zero reference point. For each case a constant length of the central trigeminal tract was used for measurements. Each grid containing an axon was counted as one. Both lateral and medial axon outgrowth was quantified. The number of grids containing axons for each 50- μ m column was counted. The average and standard deviation of each 50- μ m columnar distribution for each case were calculated with the help of the Microsoft Excel program. The result was plotted as the number of grids containing axons versus the length in consecutive 50- μ m increments for both lateral and medial directions. For statistical evaluation, two pairs of metrics were used: mean length of axons and mean axon density in medial and lateral directions. Statistical comparisons were then made by using the one-tailed t-test. Before the application of the proper t-statistic, an F-test was used

to detect any difference in the variance of experimental conditions (Montgomery, 1991). Error bars in the graphs represent one standard deviation.

RESULTS

Effects of NGF on central trigeminal axon growth patterns

The experimental culture model used in the present study is illustrated in Figure 1A. The wholemount trigeminal pathway with both the left and right TG intact allowed us to perform a given experimental procedure on one side and the control procedure on the other side in most experiments. Viral infection within the TG could be detected under epifluorescence due to the presence of GFP (Fig. 1B). In local neurotrophin application experiments, color-coded neurotrophin or carrier solution-loaded control beads were easily identified under microscopic examination (Fig. 1C). The viral constructs we used had a myc tag or β -gal expression in addition to GFP. Immunostaining for c-myc (Fig. 1D) or histochemical staining for β -gal (Fig. 1E) further confirmed the expression of gene products within the TG. In addition, immunostaining for the high-affinity NGF receptor TrkA also indicated that TG cells were not affected adversely by the viral infection, and small-diameter neurons expressed TrkA (Fig. 1F).

During normal development, central trigeminal axons grow along a restricted pathway in the lateral brainstem in an unbranched fashion at E15, begin emitting collaterals into the brainstem trigeminal nuclei by E17, and form terminal arbors that replicate the patterned array of whisker follicles on the snout (Erzurumlu and Jhaveri, 1992). In wholemount cultures derived from E15 rats, most tissue-specific characteristics of the trigeminal pathway are retained. However, central trigeminal axons do not follow the *in vivo* differentiation rate, rather, they remain restricted to the central trigeminal, without any collateral branching or arborization during a 3–5-day culture period (Ulupinar et al., 2000a; see also Fig. 2A). However, when NGF is added to the SFM, many axons grow outside the tract and elongate medially or laterally within the brainstem ($n = 8$; Fig. 2C; see also Ulupinar et al., 2000a). Quantitative and statistical analyses also indicated that there is more growth medially than laterally within the brainstem explants (Fig. 2E). Central trigeminal tract axons also changed their growth patterns when NGF was presented locally ($n = 10$; Fig. 2D). A group of axons, close to the bead, left the tract and extended toward it, forming tangles around the NGF source. In addition, some axons grew away from the bead within the brainstem (Fig. 2D). Control beads loaded with BSA had no effect on central trigeminal axons ($n = 10$; Fig. 2B). To verify that the effect is due to NGF, we added a Trk receptor blocker, K252a (1 μ M; Biomol, Plymouth Meeting, PA), to the culture medium containing NGF. In such cases, the effect of NGF was blocked and the central trigeminal tract axons followed a restricted path as if they were cultured in the presence of SFM (data not shown).

Viral infection and axonal morphology

In wholemount trigeminal pathway explants, infection with the GFP-expressing viral constructs only ($n = 10$; controls) did not affect the morphological features of the central trigeminal axons (data not shown). In such cases, axon growth within the trigeminal tract was indistinguishable from uninfected cases grown in SFM (Fig. 2A). In the presence of

NGF in the medium, and following control viral infection with the GFP expression vector alone, central trigeminal axons left the tract and grew unbranched for long distances, as was seen in uninfected cases maintained in the presence of NGF (data not shown). Similar results were also derived from experiments with neurotrophin-soaked sepharose beads that were placed along the lateral side of the trigeminal tract ($n = 10$). These results indicate that the expression of viral constructs alone does not alter central trigeminal axon growth parameters or their response to neurotrophin treatment. Thus, in the next series of experiments, we used GFP-expressing virus infection on one side of the same wholemount explant culture as a control and GFP-tagged as well as genetically modified Rho GTPase viral constructs on the other side. Central trigeminal axon growth was not altered in cases where TG explants were infected with viral constructs or when LPA was added to the SFM and cultured without any neurotrophins. However, the same constructs or LPA treatment dramatically altered central trigeminal axon growth patterns when neurotrophins were added to the medium or presented locally in sepharose beads.

Rac activity enhances neurotrophin-induced axonal response and is necessary for it

RacAC significantly increased axonal growth outside the trigeminal tract following NGF addition to the culture medium ($n = 5$; Fig. 3A). Similar effects were also seen when a single NGF-loaded bead was placed along the lateral side of the descending trigeminal tract ($n = 12$; Fig. 3B). In these cases, numerous axons left the tract at a 90° angle with respect to the rostrocaudal extent of the descending trigeminal tract. Many axons extended toward the bead and engulfed it. Others grew unbranched in the opposite direction away from the bead (Fig. 3B). Comparison of axon growth outside the boundaries of the tract in NGF-stimulated RacAC cases with NGF stimulation alone cases showed a statistically significant increase ($P < 0.01$, $df = 11$) in axon growth in the former cases where longer distances are covered by axons (Fig. 3C). Axons growing away from the tract were longer ($500\mu\text{m}$) compared with either noninfected or GFP-infected counterparts ($350\mu\text{m}$; Fig. 3C). Conversely, viral infections that led to expression of RacDN completely blocked the axonal responses to NGF ($n = 12$). There were no axons seen leaving the boundaries of the tract medially or laterally following addition of NGF to the medium (Fig. 4A) or placement of NGF beads along the tract ($n = 10$; Fig. 4B). These cases appeared similar to SFM alone, BSA-loaded bead cases, or controls with only GFP-tagged viral construct infections.

Rho activity blocks NGF-induced axon outgrowth from the trigeminal tract

Addition of LPA ($10\mu\text{M}$) to the culture medium containing NGF (50 ng/ml) blocked axonal responses to NGF ($n = 10$; Fig. 5A) in a similar fashion to that seen with RacDN cases. When NGF-loaded beads were placed along the tract in LPA-treated cases ($n = 8$), there was some axonal growth toward the bead (Fig. 5B), but not as much as that seen in cases without LPA treatment. In addition, axonal outgrowth away from the source of NGF was highly reduced or eliminated along the central trigeminal tract (Fig. 5B). In some cultures we added a 100 ng/ml dose of NGF ($n = 5$). Without LPA in the medium, trigeminal tract axons extended beyond the boundaries of the tract and formed a dense, reticular meshwork (Fig. 5C). When LPA was included, this response was largely blocked, and only a few axons were seen to leave the tract in medial or lateral directions ($n = 5$; Fig. 5D). Statistical analysis of axon growth in these cases also confirmed our observations (Fig. 5E): the axon outgrowth in

LPA-treated cases showed a highly significant reduction in axon outgrowth ($P < 0.001$, $df = 8$).

When trigeminal ganglion neurons were infected with the RhoDN viral construct ($n = 10$), we observed axon outgrowth away from the tract following NGF stimulation (Fig. 6A). In these cases, there was dense but short (50–100 μm) axon outgrowth medially. Similarly, with NGF-loaded beads ($n = 11$), there was selective axon growth toward the midline (Fig. 6B). Quantitative comparison of axon outgrowth in NGF (100 ng/ml) only and NGF (100 ng/ml) + RhoDN cases are presented in Figure 6C. F-test analysis verified the equality of variance, and t-test statistics indicated a highly significant decrease in axon outgrowth and an increase in axon number in the first 50 μm columnar distribution ($P < 0.001$, $df = 16$).

Rac and Rho activity and central trigeminal axonal responses to NT-3 and BDNF

Previous studies in explant and dissociated cell cultures documented that, unlike NGF, NT-3 induces short, but highly branched axonal outgrowth (Lentz et al., 1999; Ulupinar et al., 2000a). In the last series of experiments, we implanted NT-3- or BDNF-soaked beads along the lateral trigeminal tract and examined axon growth patterns following the series of Rac and Rho regulation procedures ($n = 10$ for each condition) as described above for NGF cases. NT-3 induced short but highly branched axon growth (Fig. 7A). This effect was more pronounced when TG cells were infected with a RacAC viral construct (Fig. 7B) and was completely blocked upon Rac DN expressing viral vector infection (Fig. 7C). Surprisingly, LPA treatments led to branched axonal growth both toward and away from the NT-3-loaded bead (Fig. 7D). Rho DN, on the other hand, resulted in branched axonal outgrowth outside the tract away from the source of the neurotrophin much like that seen with NGF cases (Fig. 7E). Similar observations were made following NT-3 addition to the medium without bead implants (data not shown).

BDNF-loaded beads induced axonal growth only toward the bead (Fig. 8A), which was not as branched as that seen in NT-3 cases. Some axons extended long distances, as in NGF cases. Upon RacAC viral infection, axonal growth toward the bead was increased (Fig. 8B), whereas RacDN eliminated outgrowth completely (Fig. 8C), as seen in NGF and NT-3 cases. Perturbations in Rho activity did not change the direction of axonal outgrowth in BDNF (Fig. 8D), rather, it seemed to change the level of branching at the site of the bead. Upon RhoDN viral infection (Fig. 8D) or Rho activation via LPA (Fig. 8D), there was more branching in the vicinity of the bead.

DISCUSSION

Wholemount explant cultures of the embryonic rodent trigeminal pathway provide an excellent in vitro model system to study mechanisms underlying axon-target interactions. Previous work from our laboratory showed that NGF and NT-3 induce differential growth responses from central trigeminal tract axons (Ulupinar et al., 2000a). Here, we show that the Rho family of GTPases plays a major role in mediating these responses. Neurotrophin-mediated axonal responses of sensory neurons have been emerging from recent in vitro studies and morphological observations from mice with null mutations for specific

neurotrophins or Trk receptors. However, in such studies, dissociating between the survival-promoting and axonal differentiation effects of neurotrophins has been a challenge.

Mutant mice that lack the BAX protein have provided some clues within this context. Sensory neurons of these mice survive independently of neurotrophins, and naturally occurring cell death is eliminated to a considerable extent (Deckwerth et al., 1996). In dissociated DRG cultures from BAX null mice, NGF promotes neurite elongation, whereas NT-3 induces, short, highly branched neurites (Lentz et al., 1999). Similar results were obtained for the normal rat trigeminal pathway by using both dissociated trigeminal cell cultures and wholemount explant cultures (Ulupinar et al., 2000a). The specific receptor-ligand interactions and associated intracellular signaling pathways underlying such axonal responses to neurotrophins are presently unclear.

It is also important to note that observations from in vitro experiments do not always reflect actual events in vivo. For example, we recently noted that dissociated E15 rat TG cells all express multiple Trk receptors shortly after they settle down in culture regardless of the presence of neurotrophins in the culture medium (Genc and Erzurumlu, 2000). In contrast, in in vivo or explant cultures, different classes of E15 TG cells express only one high-affinity neurotrophin receptor (Genc and Erzurumlu, 2000; see also Huang et al., 1999a). Although explant cultures reflect in vivo conditions more than dissociated cell cultures, they do not perfectly mimic in vivo progression of developmental events. In the rat, central trigeminal axons begin developing collateral branches and terminal arbors within the brainstem by E17, whereas in explant cultures, E15 central trigeminal axons remain in the elongation phase for 3–5 days after the cultures are set up. The most likely explanation for this is that under culture conditions, maturation of the peripheral or central targets of the TG cells and their axonal projections is delayed. However, TG axons readily respond to neurotrophins and show dramatic changes in their growth characteristics.

Developmental regulation of neurotrophin expression in the peripheral targets of TG axons have been documented in a number of previous studies (Ernfors et al., 1992; Arumae et al., 1993; Buchman and Davies, 1993; Davis et al., 1997). Differential expression of neurotrophins has been noted in the developing spinal cord (Maisonpierre et al., 1990; Elkabes et al., 1994) but has not been carefully examined for the developing brainstem trigeminal nuclei. Thus, the question of whether neurotrophin expression in brainstem trigeminal targets plays a role in shifting central trigeminal axons from elongation to arborization still remains open. It is also likely that temporal regulation of neurotrophin expression in peripheral trigeminal targets could switch central trigeminal axons between different growth patterns. In a preliminary study, such effects were observed within the central trigeminal tract following localized applications of neurotrophin-loaded sepharose beads in the whiskerpad in trigeminal pathway wholemount cultures (Ulupinar et al., 2000b). To date, central trigeminal projections in a variety of presently available neurotrophin or Trk receptor knockout mice have not been examined in detail. In BAX and TrkA double knockout mice, peripheral sensory projections are perturbed, but no abnormalities were noted in the central projections of DRG cells at a gross level (Patel et al., 2000). Future detailed examinations of central trigeminal axon morphologies in BAX plus neurotrophin or Trk receptor double knockout mice could shed light on this issue.

Rac and Rho regulate neurotrophin-induced trigeminal axon outgrowth

The signaling cascade mechanisms behind neurotrophin stimulation have been widely studied (for reviews, see Kaplan and Miller, 1997; Kaplan, 1998; Friedman and Green, 1999; Kaplan and Miller, 2000). Neurotrophins initiate two different signaling cascades for survival and axonal differentiation (for reviews, see Klesse and Parada, 1999; Klesse et al., 1999). There is compelling evidence that Rac activity is involved in the dynamics of axonal and dendritic differentiation (Luo et al., 1996; Threadgill et al., 1997; Ruchhoeft et al., 1999; Li et al., 2000; Nakayama et al., 2000; Tashiro et al., 2000). Here we demonstrate that Rac activity is also required for axonal responses of TG cells to neurotrophic factors.

Rho GTPases regulate cytoskeletal dynamics of growth cones and neurite extension. In a previous study, in vivo introduction of the same viral constructs we used led to dramatic effects on dendritic processes of tectal cells in *Xenopus* (Li et al., 2000) without any application of neurotrophins. However, these viral constructs or LPA treatment did not alter the behavior of central trigeminal axons in the absence of neurotrophins. It is most likely that in the in vitro model of the trigeminal pathway employed in our study, TG cells remain in a quiescent state in comparison with their in vivo counterparts. The fact that they do not collateralize into the brainstem trigeminal nuclei and form terminal arbors in a comparable time period to their in vivo counterparts underscores this. However, they readily respond to exogenous or local application of neurotrophins. Our results demonstrate that the Rho family of GTPases regulates this behavior. In the absence of neurotrophic factors, activation or inactivation of one particular Rho GTPase may not be sufficient to change axonal outgrowth properties of the system maintained in vitro.

Rho can bind to the cytoplasmic domain of the p75 receptor in its GTP bound form (Yamashita et al., 1999). Following neurotrophin stimulation, Rho is released to the cytoplasm, where it binds to Rho-GDI and becomes inactive. Inactivation of Rho gives rise to enhanced response to neurotrophic factors (Yamashita et al., 1999). Activation of Rac in the absence of neurotrophic factors did not lead to disruption of the trigeminal tract and axon growth away from it. In addition to what we discussed earlier, this may be due to two other reasons. First, although Rac activity is important for axonal elongation, other cofactors that become available or active upon Trk receptor activation may be required. Second, although Rac is active inside the cell, it may not be able to bind to the downstream signaling proteins due to differences in cellular locations. Docking proteins that become active upon Trk activation might be necessary (Lu et al., 1997).

Rho activity and direction of axonal outgrowth

Activation of Rho induces growth cone retraction (Kozma et al., 1997), alterations in dendritic development (Li et al., 2000; Nakayama et al., 2000), and disruption of axonal path finding (Zipkin et al., 1997). It is suggested that Rho is activated via repulsive axon guidance cues, whereas Rac is activated via attractive cues (Mueller, 1999; Awasaki et al., 2000). Inactivation of Rho initiates neurite outgrowth (Jin and Strittmatter, 1997; Lehmann et al., 1999), and perturbations in Rho activity result in directional changes of growing axons (Awasaki et al., 2000). In our experiments, the RhoDN viral construct induced unidirectional axon growth toward the midline. Despite increased density of axon growth in

this direction, axon length was considerably shorter compared with NGF cases alone. Our findings support the idea that Rho is involved in steering growth cones, because upon RhoDN viral infections we observed unidirectional axonal growth toward the midline. Unilateral axon outgrowth from the tract was pronounced with localized application of neurotrophic factors. When NGF and NT-3 were introduced lateral to the central trigeminal tract, RhoDN viral infection led to unidirectional axonal growth toward the mid-line. This type of outgrowth was not observed in BDNF cases. It is also noteworthy that in several experiments with NGF we observed axon growth toward and away from the source of this neurotrophin. Chemotropic effects of neurotrophins have been recently noted for sensory neurons innervating the limbs (Tucker et al., 2001). A more detailed account of chemotropic and chemorepellent effects of NGF in our system is in preparation as a separate publication.

Balance between Rac and Rho

Recently Trio, a unique GEF that has binding domains for both Rac and Rho, has been studied in detail in *Drosophila* (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). Trio activates Rac selectively by its GEF1 domain, and the GEF2 domain shows specificity for Rho (Debant et al., 1996). Trio is important in mediating axonal development. It has been suggested that Trio is one of the key regulators of Rac and Rho balance inside the cell and that it plays a major role in determining the direction of axonal extension (Lin and Greenberg, 2000). In our culture system, reduction of Rho activity by RhoDN viral infections mimics the situation in which Rho GEFs are reduced in the system. In such cases we observed an abrupt change in direction of axon outgrowth. Activation of Rho, on the other hand, caused decreased axon elongation outside the tract.

Our results underscore the importance of Rac and Rho balance inside the cell for directing axonal outgrowth following neurotrophin stimulation. In *in vivo* and in whole-mount explant cultures of the trigeminal pathway, the descending trigeminal tract follows a highly restricted route along the lateral brainstem. Development of this pathway, and unbranched elongation of axons within it, most likely results from the interplay of numerous molecular signals in the environment. As in other sensory and motor pathways, some of these signals are positive regulators of axon growth, whereas others are negative regulators, which prevent spillover beyond the boundaries of the tract. Neurotrophin stimulation of axon growth and its regulation via Rac and Rho most likely disrupts the balance of other positive and negative axon guidance signals thereby, disrupting the organization of the central trigeminal tract, as documented in the present study. Intracellular changes in RhoGTPase levels could also alter the responsiveness of trigeminal axons to growth-restrictive cues present in the brainstem outside the trigeminal tract boundaries. Thus, whereas the present results demonstrate the involvement of Rho GTPases in neurotrophin responsiveness of TG axons, they do not rule out altered receptiveness of trigeminal axons to many other signals present in the brainstem.

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Abbreviations

β-gal	β-galactosidase
BSA	bovine serum albumin
BDNF	brain-derived neurotrophic factor
DAB	3,39-diaminobenzidine tetrahydrochloride
DiI	1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate
DRG	dorsal root ganglion
Dtr	descending trigeminal tract
E	embryonic day
GEF	guanosine nucleotide exchange factor
GFP	green fluorescent protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
LPA	lysophosphatitic acid
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-3	neurotrophin-3
PB	phosphate buffer
PBS	phosphate-buffered saline
RacAC	constitutively active Rac (RacV12)
RacDN	dominant negative Rac (RacN17)
RhoDN	dominant negative Rho (RhoN19)
SFM	serum-free culture medium
TG	trigeminal ganglion
Trk	receptor tyrosine kinase
WP	whisker pad
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

LITERATURE CITED

- Arumae U, Pirvola U, Palgi J, Kiema TR, Palm K, Moshnyakov M, Ylikoski J, Saarma M. Neurotrophins and their receptors in rat peripheral trigeminal system during maxillary nerve growth. *J Cell Biol.* 1993; 122:1053–1065. [PubMed: 8354693]
- Aspenstrom P. Effectors for the Rho GT Pases. *Curr Opin Cell Biol.* 1999; 11:95–102. [PubMed: 10047515]

- Awasaki T, Saito M, Sone M, Suzuki E, Sakai R, Ito K, Hama C. Drosophila trio plays an essential role in patterning of axons by regulating their directional extension. *Neuron*. 2000; 26:119–131. [PubMed: 10798397]
- Bateman J, Shu H, Van Vactor D. The guanine nucleotide exchange factor trio mediates axonal development in the Drosophila embryo. *Neuron*. 2000; 26:93–106. [PubMed: 10798395]
- Bishop AL, Hall A. Rho GTPases and their effector proteins. *Biochem J*. 2000; 248:241–255. [PubMed: 10816416]
- Buchman VL, Davies AM. Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development*. 1993; 118:989–1001. [PubMed: 8076530]
- Conover JC, Yancopoulos GD. Neurotrophin regulation of the developing nervous system: analyses of knockout mice. *Rev Neurosci*. 1997; 8:13–27. [PubMed: 9402642]
- Davies AM. Studies of neurotrophin biology in the developing trigeminal system. *J Anat*. 1997; 191:483–491. [PubMed: 9449067]
- Davies AM. Developmental changes in the neurotrophin factor survival requirements of peripheral nervous system neurons. *Prog Brain Res*. 1998; 117:47–56. [PubMed: 9932399]
- Davis BM, Fundin BT, Albers KM, Goddness TP, Cronk KM, Rice FL. Overexpression of nerve growth factor in skin causes preferential increases among innervation to specific sensory targets. *J Comp Neurol*. 1997; 387:489–506. [PubMed: 9373009]
- Debant A, Serra-Pages C, Seipel K, O'Brien S, Tang M, Park SH, Streuli M. The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *Proc Natl Acad Sci USA*. 1996; 93:5466–5471. [PubMed: 8643598]
- Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ. BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron*. 1996; 17:401–411. [PubMed: 8816704]
- Elkabes S, Dreyfus C, Schaar DG, Black I. Embryonic sensory development: local expression of neurotrophin-3 and target expression of nerve growth factor. *J Comp Neurol*. 1994; 341:204–213. [PubMed: 8163724]
- Enokido Y, Wyatt S, Davies AM. Developmental changes in the response of trigeminal neurons to neurotrophins: influence of birthdate and the ganglion environment. *Development*. 1999; 126:4365–4373. [PubMed: 10477303]
- Ernfors P, Merlio JP, Persson H. Cells expressing mRNA for neuro trophins and their receptors during embryonic rat development. *Eur J Neurosci*. 1992; 4:1140–1158. [PubMed: 12106420]
- Erzurumlu RS, Jhaveri S. Trigeminal ganglion cell processes are spatially ordered prior to the differentiation of the vibrissa pad. *J Neurosci*. 1992; 12:3946–3955. [PubMed: 1403092]
- Erzurumlu R, Ozdinler H, Jacquin MF. Chemotrophic effects of NGF, NT-3, and BDNF on embryonic central trigeminal tract axons. *Eur J Neurosci*. 2000; 12(suppl 11):124.15.
- Friedman WJ, Greene LA. Neurotrophin signaling via Trks and p75. *Exp Cell Res*. 1999; 253:131–142. [PubMed: 10579918]
- Gallo G, Letourneau PC. Neurotrophins and the dynamic regulation of the neuronal cytoskeleton. *J Neurobiol*. 2000; 44:159–173. [PubMed: 10934319]
- Genç B, Erzurumlu R. TrkA and TrkC co-expression in dissociated trigeminal ganglion cell cultures and axonal responses to NGF and NT-3. *Soc Neurosci Abstr*. 2000; 120:3.
- Hall A. Rho GTPases and the actin cytoskeleton. *Science*. 1998; 279:509–514. [PubMed: 9438836]
- Hall A. Signal transduction pathways regulated by the Rho family of small GTPases. *Br J Cancer*. 1999; 80(suppl 1):25–27. [PubMed: 10466757]
- Hoyle GW, Mercer EH, Palmiter RD, Brinster RL. Expression of NGF in sympathetic neurons leads to excessive axon outgrowth from ganglia but decreased terminal innervation within tissues. *Neuron*. 1993; 10:1019–1034. [PubMed: 8318227]
- Huang EJ, Wilkinson GA, Farinas I, Backus C, Zang K, Wong SL, Reichardt LF. Expression of Trk receptors in the developing mouse trigeminal ganglion: in vivo evidence for NT-3 activation of TrkA and TrkB in addition to TrkC. *Development*. 1999a; 126:2191–2203. [PubMed: 10207144]

- Huang EJ, Zang K, Schmidt A, Saulys A, Xiang M, Reichardt LF. POU domain factor Brn-3a controls the differentiation and survival of trigeminal neurons by regulating Trk receptor expression. *Development*. 1999b; 126:2869–2882. [PubMed: 10357931]
- Jalink K, va Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. Inhibition of lysophosphatidate- and thrombin-induced neu-rite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J Cell Biol*. 1994; 126:801–810. [PubMed: 8045941]
- Jin Z, Strittmatter SM. Rac1 mediates collapsin-1-induced growth cone collapse. *J Neurosci*. 1997; 17:6256–6263. [PubMed: 9236236]
- Kaplan DR. Studying signal transduction in neuronal cells: the Trk/NGF system. *Prog Brain Res*. 1998; 117:35–46. [PubMed: 9932398]
- Kaplan DR, Miller FD. Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol*. 1997; 9:213–221. [PubMed: 9069267]
- Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol*. 2000; 10:381–391. [PubMed: 10851172]
- Kjoller L, Hall A. Signaling to Rho GT Pases. *Exp Cell Res*. 1999; 253:166–179. [PubMed: 10579921]
- Klesse LJ, Parada LF. Trks: signal transduction and intracellular pathways. *Microsc Res Tech*. 1999; 45:210–216. [PubMed: 10383113]
- Klesse LJ, Meyers KA, Marshall CJ, Parada LF. Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. *Oncogene*. 1999; 18:2055–2068. [PubMed: 10321730]
- Kozma R, Sarner S, Ahmed S, Lim L. Rho family GT Pases and neuronal growth cone remodeling: relationship between increased complexity induced by Cdc42Hs, Rac 1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol Cell Biol*. 1997; 17:1201–1211. [PubMed: 9032247]
- Kuhn TB, Meberg PJ, Brown MD, Bernstein BW, Minamide LS, Jensen JR, Okada K, Soda EA, Banburg JR. Regulating actin dynamics in neuronal growth cones by ADF/cofilin and rho family GTPases. *J Neurobiol*. 2000; 44:126–144. [PubMed: 10934317]
- Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L. Inactivation of Rho signaling pathway promotes CNS axon regeneration. *J Neurosci*. 1999; 19:7537–7547. [PubMed: 10460260]
- Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD. Neurotrophins support the development of diverse sensory axon morphologies. *J Neurosci*. 1999; 19:1038–1048. [PubMed: 9920667]
- Li Z, Van Aelst L, Cline HT. Rho GTPases regulate distinct aspects of dendritic arbor growth in *Xenopus* central neurons in vivo. *Nat Neurosci*. 2000; 3:217–225. [PubMed: 10700252]
- Liebl EC, Forsthoefel DJ, Franco LS, Sample SH, Hess JE, Cowger JA, Chandler MP, Shupert AM, Seeger MA. Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. *Neuron*. 2000; 26:107–118. [PubMed: 10798396]
- Lin CH, Thompson CA, Forscher P. Cytoskeletal reorganization underlying growth cone motility. *Curr Opin Neurobiol*. 1994; 4:640–647. [PubMed: 7849519]
- Lin MZ, Greenberg ME. Orchestral maneuvers in the axon: trio and the control of axon guidance. *Cell*. 2000; 101:239–242. [PubMed: 10847678]
- Lu W, Katz S, Gupta R, Mayer BJ. Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr Biol*. 1997; 7:85–94. [PubMed: 9024622]
- Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, Jan YN. Differential effects of the Rac GTPase on Purkinie cell axons and dendritic trunks and spines. *Nature*. 1996; 379:837–840. [PubMed: 8587609]
- Luo L, Jan LY, Jan YN. Rho family GTP-binding proteins in growth cone signalling. *Curr Opin Neurobiol*. 1997; 7:81–86. [PubMed: 9039798]
- Mackay DJ, Hall A. Rho GT Pases. *J Biol Chem*. 1998; 273:20685–20688. [PubMed: 9694808]

- Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM, Yancopoulos GD. NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron*. 1990; 5:501–509. [PubMed: 1688327]
- McMahon SB, Armanini MP, Ling LH, Phillips HS. Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron*. 1994; 12:1161–1171. [PubMed: 7514427]
- Montgomery, DC. Design and analysis of experiments. 3rd ed.. John Wiley & Sons; New York: 1991.
- Mueller BK. Growth cone guidance: first steps toward a deeper understanding. *Annu Rev Neurosci*. 1999; 22:351–388. [PubMed: 10202543]
- Nakayama AY, Harms MB, Luo L. Small GTPases rac and rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J Neurosci*. 2000; 20:5329–5338. [PubMed: 10884317]
- Newsome TP, Schmidt S, Dietzl G, Keleman K, Asling B, Debant A, Dickson BJ. Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila*. *Cell*. 2000; 101:283–294. [PubMed: 10847683]
- Patel TD, Jackman A, Rice FL, Kucera J, Snider WD. Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. *Neuron*. 2000; 25:345–357. [PubMed: 10719890]
- Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 1992; 70:389–399. [PubMed: 1643657]
- Ruchhoeft ML, Ohnuma S, McNeill L, Holt CE, Harris WA. The neuronal architecture of *Xenopus* retinal ganglion cells is sculpted by rho-family GTPases in vivo. *J Neurosci*. 1999; 19:8454–8463. [PubMed: 10493746]
- Sandell JH, Masland RH. Photoconversion of some fluorescent markers to a diaminobenzidine product. *J Histochem Cytochem*. 1988; 36:555–559. [PubMed: 3356898]
- Song HJ, Poo MM. Signal transduction underlying growth cone guidance by diffusible factors. *Curr Opin Neurobiol*. 1999; 9:355–363. [PubMed: 10395576]
- Tapon N, Hall A. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr Opin Cell Biol*. 1997; 9:86–92. [PubMed: 9013670]
- Tashiro A, Minden A, Yuste R. Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of rac and Rho. *Cereb Cortex*. 2000; 10:7–38.
- Threadgill R, Bobb K, Ghosh A. Regulation of dendritic growth and remodeling by Rho, Rac and Cdc42. *Neuron*. 1997; 19:625–634. [PubMed: 9331353]
- Tucker KL, Meyer M, Barde YA. Neurotrophins are required for nerve growth during development. *Nat Neurosci*. 2001; 4:29–37. [PubMed: 11135642]
- Ulupinar E, Jacquin MF, Erzurumlu R. Differential effects of NGF and NT-3 on embryonic trigeminal axon growth parameters. *J Comp Neurol*. 2000a; 425:622–630.
- Ulupinar E, Sendemir E, Ozdinler PH, Jacquin MF, Erzurumlu R. *Soc Neurosci Abstr*. 2000b; 120:1.
- Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. *Genes Dev*. 1997; 11:2295–2322. [PubMed: 9308960]
- Wahl S, Barth H, Ciossek T, Aktories K, Mueller BK. Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J Cell Biol*. 2000; 149:263–270. [PubMed: 10769020]
- Wright DE, Snider WD. Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. *J Comp Neurol*. 1995; 351:329–338. [PubMed: 7706545]
- Yamashita T, Tucker KL, Barde YA. Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron*. 1999; 24:585–593. [PubMed: 10595511]
- Zipkin ID, Kindt RM, Kenyon CJ. Role of a new Rho family member in cell migration and axon guidance in *C. elegans*. *Cell*. 1997; 90:883–894. [PubMed: 9298900]

Fig. 1. Schematic diagram of the wholemount explant cultures and experimental procedures. **A:** The trigeminal pathway between the whiskerpad (WP) and the brainstem is illustrated. In the cultures used, the whiskerpad portion of the pathway was left out (dashed lines). The TG was then infected with GFP-tagged viral vectors. In each culture, one side was infected with control viral construct (GFP expression only) and the other side with a viral construct with GFP plus GTPase expression construct. These cultures were then maintained in either SFM or in medium supplemented with neurotrophins. In some of the cultures a color-coded (blue) bead soaked with a specific neurotrophin was implanted along the lateral side of the descending trigeminal tract (DTr) on one side and a different colored (white), control, BSA-

soaked bead was implanted in a similar position on the other side. Bead implanted cultures were maintained in SFM. **B:** Photomicrograph of a left half of the trigeminal pathway wholemount illustrating GFP expression restricted only to the trigeminal ganglion. **C:** Photomicrograph showing the trigeminal ganglion with a DiI crystal (asterisk) and a bead (arrow) placed along the lateral portion of the trigeminal tract. In B and C, and all the ensuing photomicrographs, rostral is up and medial is to the left. **D:** c-myc immunohistochemistry in RacV12 infected TG sections. Both RacV12 (RacAC) and RacN17 (RacDN) viral constructs show c-myc positive immunohistochemistry. **E:** β -Gal staining in RhoN19-infected TG sections. Both RhoN19 (RhoDN) and GFP (control) viral constructs lead to β -gal expression in TG cells (blue cells). **F:** Photomicrograph showing TrkA-positive cells in a section through a viral infected TG. *, site of DiI labeling; black arrow, site of neurotrophin-loaded bead. Scale bars = 200 μ m for B and C; 30 μ m for D–F.

Fig. 2.

Axon growth in the central trigeminal tract in wholemount cultures and the effects of neurotrophin treatments. **A:** Unbranched, restricted growth of central trigeminal axons along a narrow pathway within the lateral brainstem in wholemount cultures maintained in SFM. **B:** Similar axon growth in the central trigeminal tract on the side of the brainstem with a control, BSA-soaked bead implant (asterisk). **C:** Effects of exogenous application of NGF in the culture medium on central trigeminal axons. Note the profuse outgrowth outside the tract. **D:** Axon outgrowth outside the central trigeminal tract following NGF-soaked bead implantation along the lateral side of the tract. Note axon growth both toward and away from the source of the neurotrophin. Photomicrographs in A and B are from DiI-labeled cases that have been photoconverted and photographed under lightfield illumination; those in C and D were photographed under epifluorescence by using a rhodamine filter set. In this figure and all the ensuing figures, the control side has been flipped over vertically for better comparison. Thus, in all the figures, medial is toward the right and rostral is up. **E:** Quantitative assessment of axon growth away from the trigeminal tract following NGF

treatment. Bar graphs illustrate the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. Asterisks mark the beads. Scale bars = 200 μm .

Fig. 3. Axonal responses to NGF following RacAC (RacV12) viral infections. Infection with RacAC significantly increased axonal responses following NGF addition to the culture medium (**A**) or local application of an NGF-soaked bead (**B**). **C:** Quantitative and statistical comparison of axon growth in NGF-treated cases (in the medium) and RacAC-infected cases in the presence of NGF. Bar graphs illustrate the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. RacAC infection

significantly increases axon outgrowth in both lateral and medial directions ($P < 0.01$). Asterisk marks the bead. Scale bar = 200 μm .

Fig. 4. Axonal responses to NGF following RacDN (RacN17) viral infections. Photoconverted, DiI-labeled central trigeminal axons following RacDN viral infection and NGF stimulation by exogenous (**A**) or local (**B**) supply of the neurotrophin. Note that there are no axons leaving the central trigeminal tract when the ganglion is infected with the RacDN construct. Asterisk marks the bead. Scale bars = 200 μ m.

Fig. 5.

Effects of LPA treatment on NGF-induced axon outgrowth. LPA added to the culture medium, containing 50 ng/ml NGF, completely blocks axon outgrowth from the central trigeminal tract (A). However, relatively low levels of axon outgrowth toward NGF-soaked beads could be seen (B). Increased NGF concentration in the culture medium (100 ng/ml) leads to dense axon outgrowth from the tract and formation of axonal tangles (C). This effect was largely blocked by LPA (D). **E:** Quantitative and statistical comparison of axon growth between cases stimulated with 100 ng/ml NGF and LPA treatment in the presence of NGF (NGF[100 ng/ml] + LPA). Bar graphs illustrating the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial

(positive) directions. Error bars represent one standard deviation. LPA treatment leads to highly significant reduction in axon outgrowth in both directions ($P < 0.001$). All photomicrographs are from DiI-labeled, photoconverted cases. Asterisk marks the bead. Scale bars = 200 μm .

Fig. 6. Reduction of NGF-induced axon outgrowth following RhoDN (RhoN19) viral infections. (A; photoconverted) and (B) illustrate two exemplary cases one with NGF in the medium and the other with an NGF-loaded bead. Note dense but short axon outgrowth medially away from the central trigeminal tract in both cases. C: Quantitative and statistical comparison of axon outgrowth in NGF alone and RhoDN infected cases in the presence of NGF in the medium (NGF + RhoDN). Bar graphs illustrating the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. RhoDN infection leads to highly significant reduction in axon outgrowth and a highly significant increase in the mean axon number ($P < 0.001$). Asterisk marks the bead. Scale bars = 200 μm.

Fig. 7.

Axonal responses to NT-3 following Rac and Rho modulation. **A:** Trigeminal axon growth and branching in response to NT-3 soaked beads. **B:** Enhanced response following RacAC. **C:** Such responses are blocked following RacDN viral infections. **D:** RhoDN viral infection decreases axon growth toward the bead. **E:** Activation of Rho induces trigeminal axons to grow medially away from the source of the NT-3 bead; only a few axons grow toward the bead. Asterisks mark the beads. Scale bars = 100 μm.

Fig. 8. Axonal responses to BDNF following Rac and Rho modulation. **A:** Trigeminal axon growth toward a BDNF-loaded bead. **B:** Increased axon growth toward BDNF following RacAC. **C:** RacDN completely abolishes response to BDNF. **D:** RhoDN or LPA treatments both have similar effects such that trigeminal axon growth is considerable but lesser than that seen with RacAC cases. Asterisks mark the beads. Scale bars = 100 μ m.