

Phospholipase C- γ and Phosphoinositide 3-Kinase Mediate Cytoplasmic Signaling in Nerve Growth Cone Guidance

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Summary

Expression of rat TrkA in *Xenopus* spinal neurons confers responsiveness of these neurons to nerve growth factor (NGF) in assays of neuronal survival and growth cone chemotropism. Mutational analysis indicates that coactivation of phospholipase C- γ (PLC- γ) and phosphoinositide 3-kinase (PI3-kinase) by specific cytoplasmic domains of TrkA is essential for triggering chemoattraction of the growth cone in an NGF gradient. Uniform exposure of TrkA-expressing neurons to NGF resulted in a cross-desensitization of turning responses induced by a gradient of netrin-1, brain-derived neurotrophic factor (BDNF), or myelin-associated glycoprotein (MAG) but not by a gradient of collapsin-1/semaphorin III/D or neurotrophin-3 (NT-3). These results, together with the effects of pharmacological inhibitors, support the notion that there are common cytosolic signaling pathways for two separate groups of guidance cues, one of which requires coactivation of PLC- γ and PI3-kinase pathways.

Introduction

Growing axons in developing nervous systems are guided to their targets by diffusible or substrate-bound guidance molecules (Tessier-Lavigne and Goodman, 1996; Mueller, 1999). The neuronal surface receptors for many guidance molecules have recently been identified, but the cytoplasmic events triggered upon activation of these receptors are largely unknown. In cell cultures, growth cones of *Xenopus* spinal neurons exhibit attractive and repulsive turning responses in a gradient of netrin-1, collapsin-1/semaphorin III (Sema III), brain-derived neurotrophic factor (BDNF), or neurotrophin-3 (NT-3) but not in a gradient of nerve growth factor (NGF) (Ming et al., 1997a, 1997b; Song et al., 1997b, 1998). In the present study, we found that expression of exogenous rat TrkA in these *Xenopus* spinal neurons promotes

their survival in medium supplemented with NGF and confers a chemoattractive response of their growth cones in a gradient of NGF. The apparent lack of endogenous TrkA in these neurons offered an opportunity for a systematic mutational analysis of the cytoplasmic domain of TrkA responsible for downstream signaling that leads to the turning response of the growth cone. Since many other factors have been shown to induce turning of growth cones of these neurons, such studies of TrkA signaling may reveal cellular transduction mechanisms triggered by a variety of guidance cues.

Based mainly on the studies of NGF-dependent neuronal differentiation of PC12 cells, previous studies have delineated the sequence of events associated with TrkA receptor signaling (Barbacid, 1995; Segal and Greenberg, 1996; Kaplan and Miller, 1997). Binding of NGF to TrkA on the cell surface causes receptor dimerization and leads to activation of its intrinsic tyrosine kinase activity and autophosphorylation of TrkA. Tyrosine-phosphorylated TrkA serves as a scaffold for the recruitment of a variety of adaptor proteins and enzymes that further transduce the NGF signal. By expressing mutant or chimeric Trk receptors lacking specific tyrosine residues that are potential interaction sites for intracellular substrates in PC12 cells, previous studies have identified tyrosine Y794 in rat TrkA as a site for PLC- γ interactions and Y499 as a site for association with Shc (Obermeier et al., 1993, 1995; Loeb et al., 1994; Stephens et al., 1994). In vitro binding studies have also identified tyrosines Y499 and Y760 as the association site for Shc and the p85 subunit of PI3-kinase (Obermeier et al., 1993, 1995), respectively, although there is evidence that PI3-kinase activity might be controlled through the Shc-binding site Y499 in PC12 cells (Baxter et al., 1995). Each of the binding molecules activates distinct signaling pathways that are responsible for different cellular functions. Recent evidence indicates that the Ras-MAP kinase pathways initiated by Shc or PLC- γ may be involved in neuronal differentiation, while the PI3-kinase pathway may be important for survival (Segal and Greenberg, 1996). Activation of all three pathways may contribute to neurite outgrowth (Inagaki et al., 1995). TrkA-mediated increase in neurite branch initiation by NGF-coated beads also requires PI3-kinase activation (Gallo and Letourneau, 1998). Turning of growth cones induced by NGF-coated beads was blocked by k252a, suggesting involvement of TrkA tyrosine kinase activity (Gallo et al., 1997). In the present study, by using mutated forms of TrkA in which specific tyrosine residues have been substituted singly or in combination, we found that coactivation of PLC- γ and PI3-kinase pathways, but not the Shc pathway, are essential for triggering the turning response of the growth cones toward NGF.

Recent studies on the role of Ca²⁺ and cyclic nucleotides in regulating growth cone turning behaviors of *Xenopus* spinal neurons have shown that all guidance cues examined for these *Xenopus* neurons can be classified into two groups (Ming et al., 1997b; Song et al., 1997b, 1998). In group I, which includes netrin-1, BDNF, acetylcholine (ACh), and myelin-associated glycoprotein (MAG),

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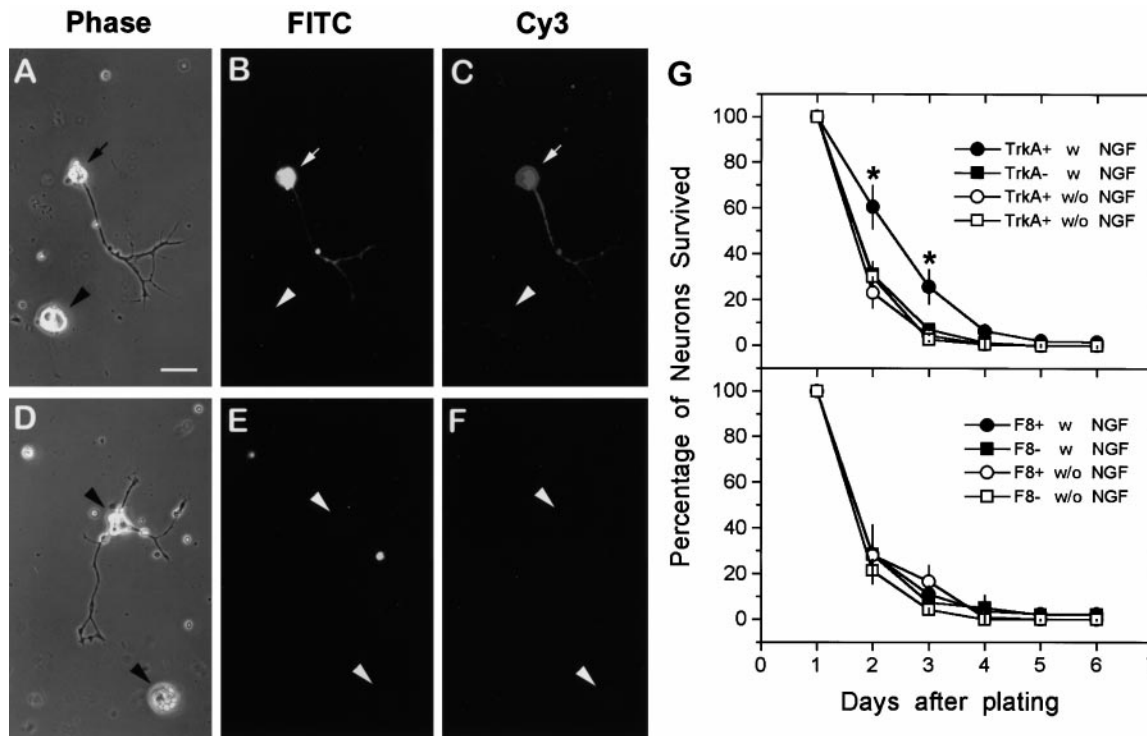


Figure 1. Expression of Wild-Type and Mutant Forms of Rat TrkA in *Xenopus* Spinal Neurons and Their Effects on Neuronal Survival (A–F) Sample images of *Xenopus* spinal neurons cultured in the same dish shown in phase contrast (A and D) and in fluorescence for FITC-dextran (B and E) and for immunofluorescence staining of TrkA (C and F). Arrow marks a TrkA-expressing neuron and arrowheads mark cells not expressing TrkA. Scale bar, 50 μ m. (G) Effects of NGF on survival of *Xenopus* spinal neurons. (Top) Survival of control and TrkA⁺ neurons after various days in culture. The number of neurite-bearing cells at different days after cell plating was normalized to that observed in 1-day-old culture (100%). The data represent mean \pm SEM (n = 6–11 cultures). Closed and open symbols represent cultures with and without NGF (100 ng/ml) in the medium, respectively. An asterisk indicates significant difference from all other three groups (p < 0.05, Kruskal-Wallis test). (Bottom) Survival of neurons expressing TrkA-F8.

the turning responses are abolished by depleting extracellular Ca²⁺, and the level of cytosolic cAMP or the activity of protein kinase A (PKA) is critical in determining whether the turning response is attractive or repulsive. Inhibition of PKA converts attraction induced by a gradient of netrin-1, BDNF, or ACh into repulsion, while activation of PKA converts repulsion induced by MAG into attraction. Similarly, elevation of endogenous cAMP levels can overcome BDNF-induced growth cone collapse of young *Xenopus* spinal neurons in culture (Wang and Zheng, 1998) and block the inhibition of axon regeneration of cerebellar and dorsal root ganglion neurons by MAG and myelin (Cai et al., 1999). In group II, which includes Sema III and NT-3, the turning responses are independent of extracellular Ca²⁺ and are regulated by cGMP or protein kinase G (PKG) (Song et al., 1998). Activation of PKG converts repulsive turning induced by Sema III into attraction, while inhibition of PKG converts NT-3-induced attraction into repulsion. In the present study, we found that NGF-induced attractive responses required extracellular Ca²⁺ and could be converted into repulsion by inhibition of PKA, indicating NGF as a group I guidance cue. This prompted us to study the involvement of PI3-kinase and PLC- γ in mediating the actions of other guidance cues. We found that

uniform exposure of TrkA-expressing neurons to extracellular NGF resulted in a cross-desensitization of the turning responses to other group I, but not group II, guidance cues. Conversely, uniform presence of other group I cues resulted in the loss of turning responses in an NGF gradient. In addition, pharmacological inhibition of PI3-kinase activity abolished the turning responses induced by all group I cues but not group II cues. Taken together, these results support the notion of two separate groups of guidance cues, each sharing different signal transduction pathways, with the action of group I cues transduced by PLC- γ and PI3-kinase pathways.

Results

Neuronal Survival and Chemotropic Responses Induced by NGF

The neurotrophins BDNF and NT-3 promote survival and induce chemotropic responses of *Xenopus* spinal neurons in culture, but NGF is ineffective (Ming et al., 1997a), suggesting that these neurons lack functional TrkA, a specific high-affinity receptor for NGF (Segal and Greenberg, 1996). In the present study, we first examined whether introduction of exogenous TrkA into these

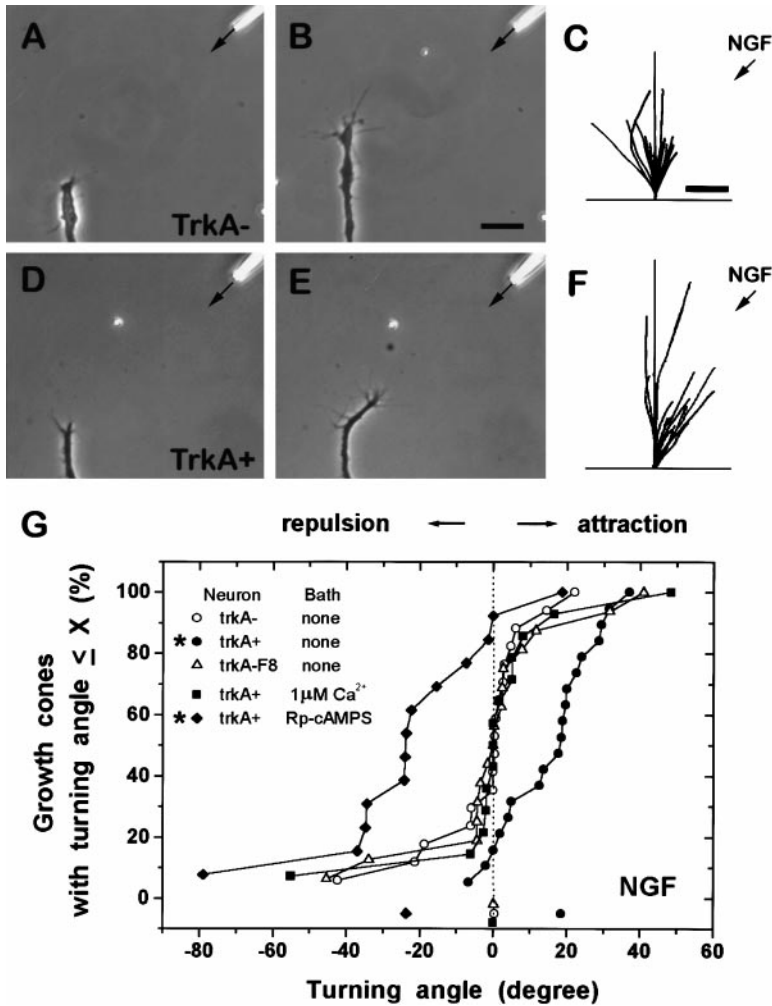


Figure 2. Turning of Growth Cones of *Xenopus* Spinal Neurons Induced by NGF

(A–C) A gradient of NGF was applied to TrkA⁻ control neurons by pulsatile application of picoliters of NGF solution (50 μg/ml) from a micropipette (shown at the right upper corner). Microscope images were recorded at the onset (A) and the end (B) of the 1 hr exposure to the NGF gradient. In (C), superimposed traces depict the trajectory of neurite extension during a 1 hr period for a random sample of 15 neurons. The origin is the center of the growth cone at the onset of the experiment and the original direction of growth was vertical. The arrow indicates the direction of the gradient. Scale bars, 20 μm (A and B) and 10 μm (C). (D–F) Experiments were similar to those in (A) through (C), except that TrkA⁺ neurons were studied.

(G) Distribution of turning angles. For each experimental condition, angular positions of all growth cones at the end of a 1 hr exposure to an NGF gradient are shown in a cumulative frequency plot. The percent value refers to the percentage of growth cones with angular position less than or equal to a given angular value. Data shown are the turning observed for TrkA⁻ neurons in normal culture medium (1 mM [Ca²⁺]_o) as the control group and that observed for TrkA⁺ neurons in normal medium, medium containing 1 μM [Ca²⁺]_o, or 10 μM Rp-cAMPS. Also shown are data for TrkA-F8⁺ neurons in normal culture medium. Isolated data points along the abscissa are median values for corresponding data shown above. An asterisk indicates significant difference from the control group ($p < 0.01$, Kolmogorov-Smirnov test).

neurons confers sensitivity of these neurons to NGF. In vitro synthesized mRNA encoding rat TrkA was injected into one of the two blastomeres of *Xenopus* embryos at the two-cell stage, with FITC-dextran coinjected as a marker for progeny cells derived from the injected blastomere (Alder et al., 1995; Song et al., 1997a). Cultures of dissociated spinal neurons were made from 1-day-old injected embryos. The expression of TrkA in *Xenopus* cells was confirmed by immunostaining (Figures 1A–1F). We found that application of NGF (100 ng/ml) increased the survival of cultured neurons expressing exogenous TrkA (TrkA⁺) but not that of the control neurons (TrkA⁻) derived from noninjected blastomeres in the same culture (Figure 1G). No effect of NGF was found for neurons expressing a mutated form of TrkA (TrkA-F8), in which all conserved tyrosine residues (except Y679, Y683, and Y684 located within the catalytic loop of the tyrosine kinase domain) in the cytoplasmic domain of rat TrkA (Y499, Y594, Y643, Y704, Y726, Y732, Y760, and Y794) had been changed to phenylalanine (Figure 1G). In addition to the effect on neuronal survival, we found that NGF induced a significant increase in the rate of neurite growth of TrkA⁺ neurons but not TrkA-F8⁺ neurons (data not shown), suggesting that downstream activation of cytosolic mechanisms through the tyrosine

residues in the cytoplasmic domain of TrkA is required for stimulation of neurite growth by NGF (Inagaki et al., 1995).

To test the chemotropic responses, a microscopic gradient of NGF was created near the growth cones of isolated *Xenopus* spinal neurons in 14–20 hr cultures by repetitive pulsatile application of picoliters of NGF-containing saline (50 μg/ml in the pipette) (Lohof et al., 1992; Ming et al., 1997a, 1997b). The tip of the micropipette was positioned at a distance of 100 μm from the growth cone and at an angle of 45° with respect to the original direction of neurite extension. The final direction and total length of neurite extension were measured 1 hr after the onset of the gradient. Control growth cones from TrkA⁻ neurons exhibited no apparent bias in the direction of extension, with the final growth cone positions at the end of the 1 hr period being scattered symmetrically around the original direction of neurite extension (Figures 2A–2C). However, in the same gradient of NGF, growth cones of TrkA⁺ neurons showed a marked chemotropic turning response toward the source of NGF (Figures 2D–2F). The behavior of a population of neurons is shown by superimposing traces of trajectories of individual neurites over the 1 hr period for a random sample of 15 neurons (Figures 2C and 2F).

Reduction of extracellular Ca^{2+} ($[Ca^{2+}]_o$) from 1 mM to 1 μ M completely abolished the turning responses of $TrkA^+$ growth cones in an NGF gradient (Figure 2G). In addition, when a PKA inhibitor, Rp-cAMPS (10 μ M) (Rothermel and Parker Botelho, 1988), was added to the culture medium, the same NGF gradient induced repulsive turning responses of $TrkA^+$ growth cones (Figure 2G). This dependence on Ca^{2+} and cAMP was identical to that of the turning response of $TrkA^-$ growth cones induced by a gradient of BDNF or netrin-1 (Ming et al., 1997b; Song et al., 1997b). Thus, NGF-induced turning responses may share the same signaling pathways as those induced by netrin-1 and BDNF, two of the group I guidance cues.

Involvement of PLC- γ and PI3-Kinase Pathways in NGF-Induced Chemoattraction

Previous studies have identified a number of downstream effectors that are activated through specific tyrosine residues in the cytoplasmic domain of TrkA (Obermeier et al., 1993, 1995). We found that neurons expressing the F8 mutant of TrkA did not exhibit any significant chemotropic responses toward NGF (Figure 2G), indicating that NGF-induced chemoattraction requires signaling through tyrosine residues of the cytoplasmic domain of TrkA. The role of specific downstream effectors in conferring the turning response was examined by expressing mutated forms of TrkA with specific substitution of tyrosine residues in the cytoplasmic domain. Expression of mutant TrkA proteins in *Xenopus* spinal neurons was confirmed by immunostaining (data not shown). First, we examined the effects of expression of mutations with a single tyrosine to phenylalanine substitution at position 499, 760, or 794, which were proposed to be responsible for activation of the downstream effectors Shc, PI3-kinase, or PLC- γ , respectively (Obermeier et al., 1993, 1995). We found that attractive turning responses of the growth cones were absent in either $TrkA$ -Y794F⁺ or $TrkA$ -Y760F⁺ neurons (Figures 3A and 3B), suggesting that activation of both PLC- γ and PI3-kinase pathways is necessary for the attractive turning response toward NGF. In contrast, growth cones of $TrkA$ -Y499F⁺ neurons still exhibited chemoattraction toward NGF (Figure 3C), suggesting that the Shc pathway is not required for the turning response.

Is activation of the PLC- γ and PI3-kinase pathways through Y760 and Y794 sufficient to trigger the turning response? We addressed this question by examining neurons expressing mutant proteins in which the tyrosine residues 760 and 794 had been restored either singly or in combination in a $TrkA$ -F8 mutant background (Inagaki et al., 1995). We found that restoring the tyrosine residue at either position 760 or 794 alone in $TrkA$ -F8 ($TrkA$ -Re760Y or $TrkA$ -Re794Y) was not sufficient to confer a turning response toward NGF (Figures 3A and 3B). However, when tyrosine residues at position 760 and 794 were both restored in the $TrkA$ -F8 mutant ($TrkA$ -Re760/794Y), clear attractive turning was observed (Figure 3C). Thus, coactivation of PI3-kinase and PLC- γ pathways appears to be essential for triggering NGF-induced turning. Since PI3-kinase and PLC- γ pathways are immediately downstream of the receptors, a gradient of activities of these two enzymes may be the earliest cytoplasmic signal following receptor activation induced by a gradient of NGF.

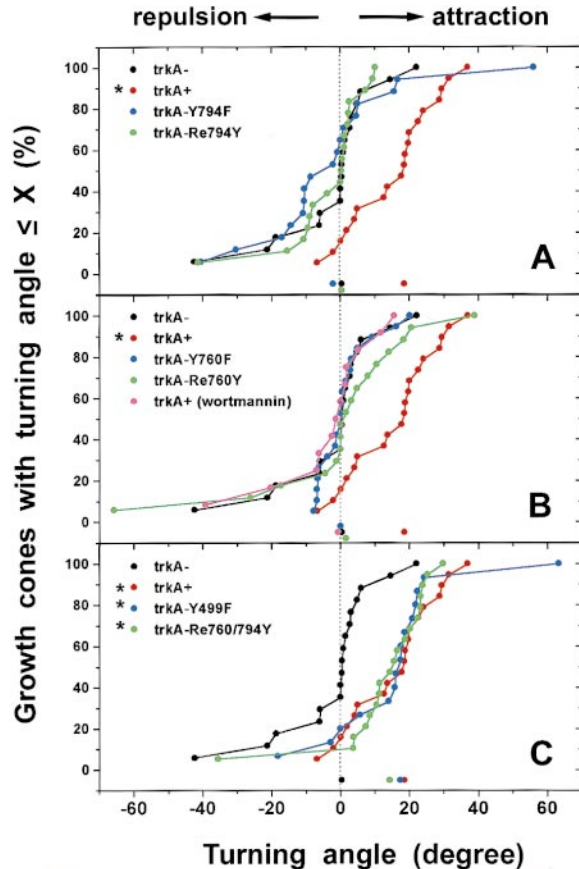


Figure 3. NGF-Induced Attraction in Neurons Expressing Different Mutant Forms of TrkA

Distribution of turning angles in an NGF gradient (50 μ g/ml in the pipette) for neurons that expressed different TrkA mutants, which affect activation of PLC- γ (A), PI3-kinase (B), or Shc (C) pathways, respectively. Also included are data for $TrkA^+$ neurons treated with wortmannin (50 nM) as well as those for $TrkA^-$ neurons as the control group. An asterisk indicates significant difference from the control group ($p < 0.01$, Kolmogorov-Smirnov test).

Further experiments using pharmacological inhibitors of these two enzymes were carried out. A specific inhibitor of PI3-kinase, wortmannin (50 nM) (Arcaro and Wyman, 1993), completely abolished the chemoattraction of $TrkA^+$ growth cones toward NGF (Figure 3B), without any significant effect on the rate of neurite extension (data not shown). A pharmacological inhibitor of PLC- γ , U-73122 (Smith et al., 1990), caused collapse of these growth cones at all concentrations tested (0.1–5 μ M), suggesting involvement of PLC- γ activity in growth cone motility. This collapsing response also prevented the assay of the turning responses after treatment with this drug.

Cross-Desensitization of Turning Responses among Various Guidance Cues

The above results indicated that NGF-induced turning responses of growth cones of $TrkA^+$ neurons depend on $[Ca^{2+}]_o$ and cAMP-dependent pathways, similar to that of the turning of $TrkA^-$ neurons induced by a gradient of BDNF or netrin-1 (Ming et al., 1997b; Song et al., 1997b), suggesting that they may share the signaling pathways in these neurons. As shown in Figure 4A, we

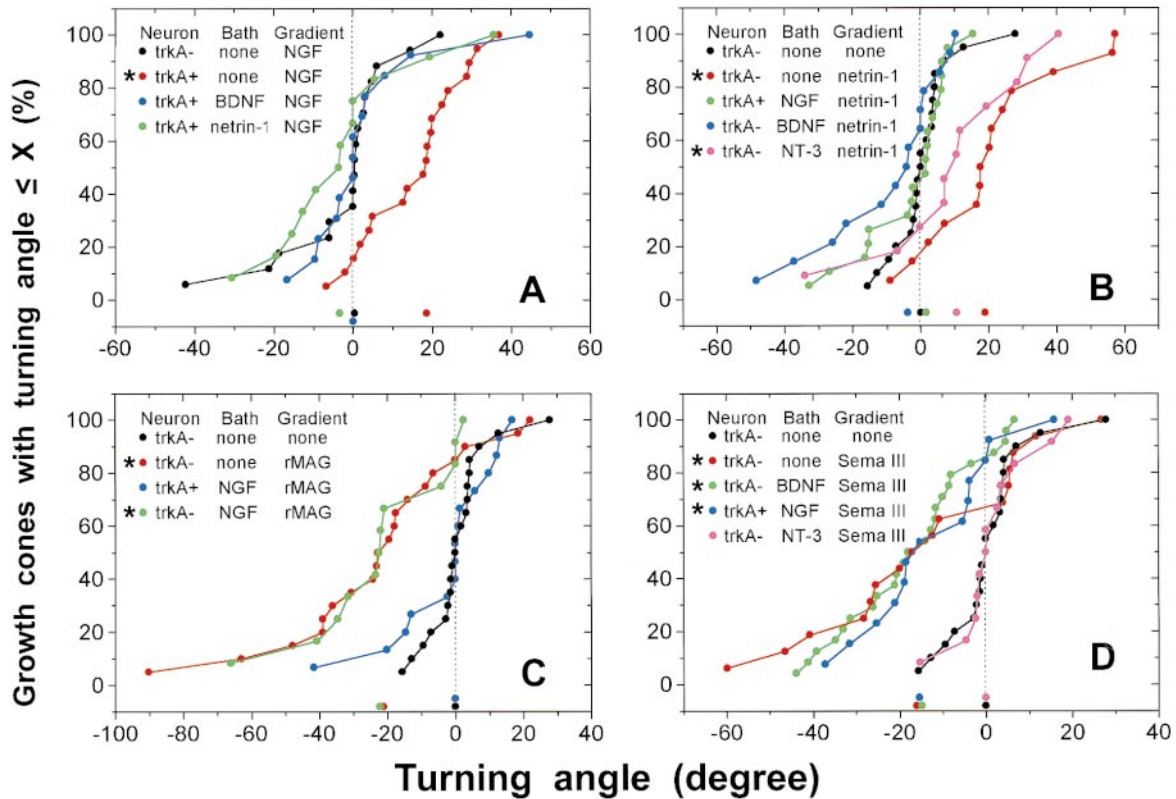


Figure 4. Cross-Desensitization among Various Guidance Cues

(A) Turning responses of TrkA⁺ growth cones induced by a gradient of NGF (50 $\mu\text{g/ml}$ in the pipette) in the uniform presence of netrin-1 (10 ng/ml) or BDNF (100 ng/ml) in the bath, respectively. TrkA⁻ neurons in the same NGF gradient in the absence of any guidance cues served as controls.

(B–D) Turning responses induced by a gradient of netrin-1 (5 $\mu\text{g/ml}$ in the pipette), rMAG (150 $\mu\text{g/ml}$ in the pipette), or Sema III (50 $\mu\text{g/ml}$ in the pipette) in the presence of uniform NGF (100 ng/ml), BDNF (100 ng/ml), or NT-3 (100 ng/ml) in the bath, respectively. TrkA⁻ neurons in the absence of any guidance cues served as controls. An asterisk indicates significant difference from the control group ($p < 0.05$, Kolmogorov-Smirnov test).

found that the uniform presence of BDNF (100 ng/ml) or netrin-1 (10 ng/ml) in the culture medium abolished the attractive turning of TrkA⁺ growth cones induced by a gradient of NGF. Uniform activation of downstream effectors of BDNF and netrin-1 apparently had led to a cross-desensitization of the response of the growth cone to an NGF gradient. Conversely, chemoattraction of TrkA⁺ growth cones in a gradient of netrin-1 (5 $\mu\text{g/ml}$ in the pipette) was abolished by the uniform presence of NGF (100 ng/ml) in the bath (Figure 4B). This effect of NGF was not due to overexpression of exogenous proteins in these neurons, since uniform presence of BDNF (100 ng/ml) in the bath had a similar effect in abolishing netrin-1-induced attraction in TrkA⁻ neurons. This cross-desensitization effect was also specific for group I guidance cues since in the uniform presence of NT-3 (100 ng/ml), a group II cue, a netrin-1 gradient still induced significant attractive response (Figure 4B). The effect of NGF cannot be accounted for only by p75, the low-affinity NGF receptor (Segal and Greenberg, 1996), as it required specifically the cytoplasmic domain of TrkA: uniform presence of NGF had no effect on netrin-1-induced chemoattraction in TrkA-F8⁺ neurons (Figure 5A).

We next examined potential cross-desensitization between cytosolic signals induced by NGF and by MAG

and Sema III, which are known to induce repulsive turning responses of these *Xenopus* neurons (Song et al., 1998). In the uniform presence of NGF (100 ng/ml) in the bath, the repulsive turning of TrkA⁺ growth cones induced by a gradient of recombinant MAG (rMAG, 150 $\mu\text{g/ml}$ in the pipette) was abolished (Figure 4C), an effect that was not observed when TrkA⁻ neurons were examined. In contrast to rMAG-induced turning, repulsion of TrkA⁺ growth cones induced by Sema III (50 $\mu\text{g/ml}$ in the pipette) was not affected by the uniform presence of NGF (Figure 4D). Similarly, uniform exposure to BDNF had no effect on Sema III-induced repulsion of TrkA⁻ growth cones, while a uniform presence of NT-3 blocks the repulsive response in a Sema III gradient. These results on cross-desensitization by other guidance cues are consistent with the notion of shared cytosolic signals within the same group of guidance cues, but not between group I and group II cues.

Cross-Desensitization of Netrin-1-Induced Chemoattraction

To further delineate the interaction of TrkA-mediated signaling with that induced by netrin-1, we carried out more extensive studies on neurons expressing various

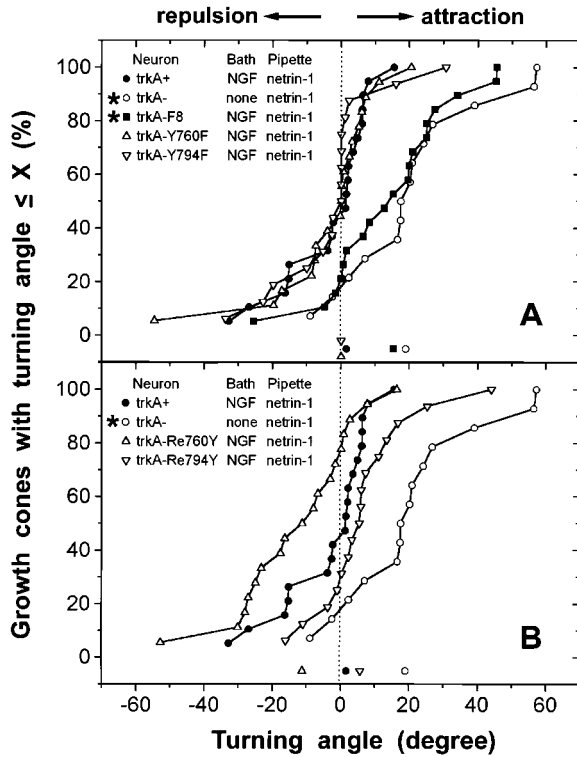


Figure 5. Cross-Desensitization of Netrin-1-Induced Turning Responses by NGF

Data shown are turning responses in a netrin-1 gradient (5 $\mu\text{g/ml}$ in the pipette) observed for neurons expressing different mutant forms of TrkA in the presence of NGF (100 ng/ml) in the medium. TrkA⁺ neurons in a netrin-1 gradient, with NGF (100 ng/ml) in the bath, served as controls. An asterisk indicates significant difference from the control group ($p < 0.01$, Kolmogorov-Smirnov test).

forms of TrkA in which putative tyrosine residues responsible for activation of PI3-kinase and PLC- γ had been mutated singly or in combination. As shown in Figure 5A, in neurons expressing TrkA mutants in which activation of either one of the two enzymes (TrkA-Y760F or TrkA-Y794F) was affected, uniform exposure to NGF (100 ng/ml) was fully effective in abolishing attractive turning of growth cones in a netrin-1 gradient. Thus, uniform activation of either the PI3-kinase or the PLC- γ -dependent pathways alone appears to be sufficient in abolishing chemoattraction induced by netrin-1. This notion is further supported by the findings that in neurons expressing TrkA mutants with restoration of a single tyrosine in the TrkA-F8—namely, TrkA-Re794Y or TrkA-Re760Y—uniform presence of NGF was fully effective in abolishing chemoattraction induced by a netrin-1 gradient (Figure 5B). Interestingly, neurons expressing TrkA-Re760Y appeared to exhibit some repulsive responses to netrin-1, although the difference between this and control group (TrkA⁺) was not statistically significant ($p = 0.1$, Kolmogorov-Smirnov test). These results are consistent with the notion that netrin-1-induced attraction requires cytoplasmic gradients of activated PI3-kinase and PLC- γ pathways; disruption of either one of these two gradients by uniform activation of these pathways results in a loss of netrin-1-induced attraction.

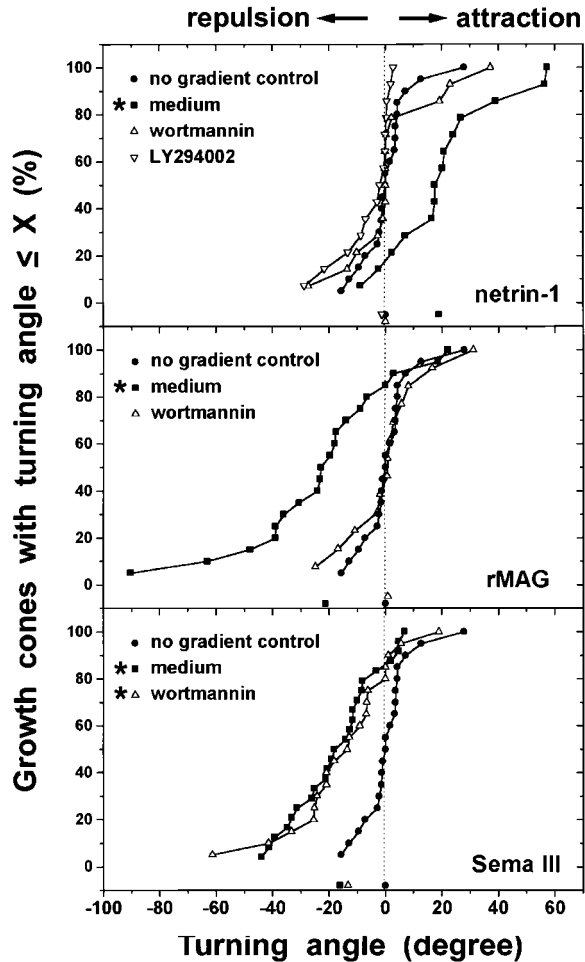


Figure 6. Effects of Pharmacological Inhibition of PI3-Kinase

The turning responses of TrkA⁻ growth cones induced by a gradient of netrin-1 ([A], 5 $\mu\text{g/ml}$ in the pipette), rMAG ([B], 150 $\mu\text{g/ml}$ in the pipette), or Sema III ([C], 50 $\mu\text{g/ml}$ in the pipette), respectively, were examined in the absence and presence of an inhibitor of PI3-kinase, wortmannin (50 nM) or LY294002 (10 μM). TrkA⁻ neurons in the absence of any gradient or inhibitors served as controls. An asterisk indicates significant difference from the control group ($p < 0.01$, Kolmogorov-Smirnov test).

Our cross-desensitization experiments suggest that PI3-kinase and PLC- γ -dependent pathways may be involved in the turning responses induced by netrin-1 as well as other group I guidance cues. Further pharmacological tests were performed to examine the involvement of PI3-kinase. As shown in Figure 6, netrin-1-induced attractive turning responses in normal TrkA⁻ control neurons were abolished in the presence of wortmannin (50 nM) (Arcaro and Wymann, 1993) or LY294002 (10 μM) (Vlahos et al., 1994), two specific inhibitors for PI3-kinase. Similarly, we found that the repulsive turning response of these neurons induced by a gradient of rMAG was abolished by wortmannin (50 nM), while that induced by Sema III was not. These results are consistent with the involvement of PI3-kinase in all group I but not group II guidance cues. Pharmacological tests of the involvement of the PLC- γ pathway in the turning

response were not feasible because of the collapse of growth cones induced by the inhibitor of PLC- γ .

Discussion

The apparent lack of TrkA in *Xenopus* spinal neurons (Ming et al., 1997a) and the reliable transient expression of exogenous genes in early *Xenopus* embryos (Alder et al., 1995; Song et al., 1997a) had allowed us to dissect TrkA-mediated signal transduction underlying the growth cone turning response, which turned out to be shared by other guidance cues. The expression of exogenous TrkA in *Xenopus* spinal neurons is demonstrated by immunocytochemical staining of the neurons with TrkA antibodies and by a significant increase in neuronal survival in cultures treated with NGF. Our findings indicate that tyrosine residues at positions 760 and 794 in the cytoplasmic domain of rat TrkA are essential to initiate the turning responses induced by an NGF gradient. The interpretation of these results depends on previous findings on the TrkA signal transduction from assays of in vitro binding and NGF-induced neuronal differentiation in PC12 cells (Obermeier et al., 1993, 1995; Loeb et al., 1994; Stephens et al., 1994). Our results are consistent with the notion that coactivation of PI3-kinase and PLC- γ at the growth cone is essential for the turning response. Whether activation of PI3-kinase is mediated by Y760 is controversial. In vitro binding studies have identified the association site for PI3-kinase at Y760 (Obermeier et al., 1993, 1995), although there is evidence in PC12 cells that PI3-kinase activity might be affected by mutation of the Shc-binding site on TrkA at Y499 (Baxter et al., 1995). Our findings that NGF-induced turning was not affected in TrkA-Y499F⁺ neurons, but was abolished in TrkA-Y760F⁺ neurons, suggest that in these neurons PI3-kinase activation is independent of association and activation of Shc through Y499. That PI3-kinase activation is essential for the turning responses was also confirmed by results of pharmacological studies using an inhibitor of PI3-kinase. The discrepancy regarding the interaction between PI3-kinase and TrkA may reflect differences between PC12 cells and neurons in primary cultures (see also Aibel et al., 1998; Ganju et al., 1998).

It is of interest to note that the turning response may have a requirement for cytosolic signaling different from that of neurite extension. In the absence of NGF-TrkA signaling, these *Xenopus* spinal neurons exhibit a basal growth rate. Our results on the effect of pharmacological inhibitors suggest that basal activity of PLC- γ is required for neurite extension, whereas that of PI3-kinase is not. Other pathways besides PI3-kinase and PLC- γ may also contribute to the rate of neurite extension, as suggested by previous findings on NGF-dependent stimulation of neurite outgrowth in PC12 cells (Inagaki et al., 1995). Previous assays of NGF-dependent induction of neurite outgrowth involved prolonged treatments of the cells with NGF and presumably activation of specific programs of immediate-early and delayed-response genes (Segal and Greenberg, 1996). Here, we focused on acute actions of TrkA activation, processes that lead to rapid cytoskeletal reorganization at the growth cone. Our conclusion that coactivation of PI3-kinase and PLC- γ at the

growth cone is essential for the turning responses does not exclude the involvement of other pathways, such as the Shc-Ras-MAP kinase pathway, in triggering more long-term effects of NGF, including an increase in the rate of neurite extension. Cell lines (PC12nnr5) expressing Trk receptor mutated in the PLC- γ interaction site showed no defects in NGF-induced neurite outgrowth (Loeb et al., 1994), but mutations at both Shc and PLC- γ interaction sites abolished NGF-dependent neuritogenesis (Stephens et al., 1994). Recently, Qian et al. (1998) have identified two proteins, rAPS and SH2-B, in developing neurons that can bind to TrkA-F8 and mediate neurite outgrowth in PC12nnr5 cells. Whether these two proteins are also involved in the growth cone turning responses remains unknown.

TrkA-mediated growth cone attraction in an NGF gradient showed a similar dependence on the levels of extracellular Ca²⁺ and cytosolic cAMP as those of other group I guidance cues, including netrin-1, BDNF, and MAG. The dependence on extracellular Ca²⁺ may reflect a requirement for Ca²⁺ influx or for release of Ca²⁺ from internal stores in response to the guidance cue. In the latter scenario, reduction of [Ca²⁺]_o may have led to the depletion of internal Ca²⁺ stores, resulting in a lack of Ca²⁺ release upon reception of the guidance cue. Our finding of the dependence of TrkA-mediated turning on PLC- γ is consistent with the role of internal release of Ca²⁺ in mediating the turning response. Activated PLC- γ cleaves phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositoltrisphosphate (IP₃). IP₃ binds to its receptor and triggers the release of Ca²⁺ from internal stores (Berridge, 1998). A gradient of activation of PLC- γ thus can be converted into a gradient of elevation of cytoplasmic Ca²⁺. Gradients of Ca²⁺ at the growth cone created by an extracellular gradient of Ca²⁺ ionophore (Gundersen and Barrett, 1980) or by ACh (Zheng et al., 1994) are sufficient for inducing turning responses of growth cones. The dependence on PI3-kinase of turning responses induced by group I guidance cues suggests that generation of a gradient of 3-phosphorylated inositol lipids in the membrane or other phosphorylated signaling proteins (Fruman et al., 1998) is necessary for the turning responses. Furthermore, the PI3-kinase pathway may regulate PLC- γ -mediated Ca²⁺ signaling (Rameh et al., 1998) and it may operate in concert with other inputs to control PKC (Chou et al., 1998; Le Good et al., 1998).

The experiments on cross-desensitization yielded two important pieces of information concerning signal transduction of various guidance cues. First, the finding that uniform presence of one guidance cue completely abolishes the turning response of another guidance cue of the same group strongly supports the notion of shared signaling mechanisms among members of the same group of guidance cues. Second, the lack of interference of uniform group I guidance cues on the turning responses induced by group II cues, and vice versa, confirms the existence of two independent pathways that can trigger turning responses. In these cross-desensitization experiments, relatively high concentrations of guidance molecules were added in the bath in order to induce a saturated uniform activation of downstream effectors. High-level background activation of common effectors may diminish the effectiveness of the gradient

of activated effectors induced by the second cue. Our results are fully consistent with these expectations: complete cross-desensitization of turning responses was observed for each pair of group I guidance cues tested, either between attractive cues or between attractive and repulsive cues. It is of interest to note that two different transduction pathways were also found to be involved in growth cone collapse induced by myelin and Sema III (Kuhn et al., 1999).

In principle, cross-desensitization of the turning response could be attributed to sharing of effectors at any step along the signal transduction pathways activated by the two guidance cues. Since PLC- γ and PI3-kinase have been identified as the immediate downstream effectors activated by TrkA, it is of interest to know whether these two enzymes are also immediate effectors for other group I guidance cues. Pharmacological experiments using specific inhibitors of PI3-kinase showed that this enzyme is likely to be an effector mediating all group I guidance cues. While PLC- γ may be also directly involved in all other group I guidance cues, as suggested by mutational studies, it remains possible that cross-desensitization of the response to other group I cues is due to shared signaling molecules more downstream from PLC- γ , in addition to PI3-kinase. During pathfinding in developing embryos, the axon may encounter an environment that imposes several factors sharing the same downstream effectors. Cross-desensitization between two factors may affect the guidance behavior of the axon.

It is generally believed that specific pathfinding behaviors of different types of neurons in the nervous system are coded by the presence of various guidance cues within the tissue and by differential expression of their membrane receptors on the neuronal surface. The present results point to the importance of PI3-kinase and PLC- γ pathways in the signal transduction triggered by group I guidance cues that include NGF, BDNF, netrin-1, and MAG. Furthermore, our data strongly support the notion that an independent pathway underlies the signal transduction induced by group II guidance cues that include Sema III and NT-3. The intracellular signal mechanisms that lead to the turning behavior eventually must converge onto a set of common cytoplasmic effectors to generate asymmetric cytoskeletal rearrangement leading to an oriented neurite extension. Identification of PLC- γ and PI3-kinase provides a basis for further elucidation of intervening steps between signaling at the membrane receptor and cytoskeletal responses.

Experimental Procedures

Embryo Injection of mRNA Encoding TrkA and Mutants

Mutants of rat TrkA were constructed as described previously (Inagaki et al., 1995). Wild type and mutants of TrkA were subcloned into pBluescript II KS (Stratagene) at the XhoI site. Capped mRNA for TrkA and mutants were prepared from pBS-TrkA linearized with EcoRI and transcribed in vitro using T3 RNA polymerase (mMESAGE mMACHINE, Ambion). Messenger RNA was suspended in RNase-free H₂O mixed with FITC-conjugated dextran (25 mg/ml, Molecular Probe). The final concentration of mRNA is about 2 μ g/ μ l. About 2 ng mRNA was injected into one of the two blastomeres of *Xenopus* embryos at the 2-cell stage using a picospritzer (General Valve, NJ), as described previously (Alder et al., 1995; Song et al., 1997a). The embryos were kept at room temperature (20°C–22°C) for 24 hr prior to culture preparation.

Culture Preparation

Cultures of *Xenopus* spinal neurons were prepared from the neural tube tissue of 1-day-old *Xenopus* embryos by methods previously described (Tabti et al., 1998). The tissue was dissociated in Ca²⁺/Mg²⁺-free solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, and 0.5 mM EDTA [pH 7.4]) for 20 min and plated on the surface of clean glass coverslips. The cultures were used between 14 and 20 hr after plating at room temperature (20°C–22°C) for the experiment. The culture medium consisted of 49% (v/v) Leibovitz medium (GIBCO, Gaithersburg, MD), 1% (v/v) fetal bovine serum (HyClone, Logan, UT), and 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM CaCl₂, 2.5 mM KCl, and 10 mM HEPES [pH 7.4]). The experiments were carried out at room temperature in modified Ringer's solution (140 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES [pH 7.4]). For bath application experiments, chemicals were added to the culture medium at least 30 min before and were present during the experiments. Saline of 1 μ M [Ca²⁺]_o was modified Ringer's solution containing 140 mM NaCl, 1 mM MgCl₂, 0.9 mM CaCl₂, 5 mM KCl, 4 mM EGTA, and 10 mM HEPES (pH 7.4).

In experiments on neuronal survival, NGF (100 ng/ml) was added to the culture at the time of cell plating. The assay for neuronal survival followed that as described previously (Ming et al., 1997a). The neurite-bearing cells were counted daily for 6 days after plating. A cell was considered a neuron only if it had a neurite process of uniform diameter that was at least 20 μ m.

Immunocytochemistry

For immunostaining of cells in culture, cultures were washed with phosphate buffered saline (PBS) and fixed with 4% formaldehyde in PBS. They were blocked with 10% donkey serum in TBS (10 mM Tris [pH 7.0] and 150 mM NaCl) for 1 hr at room temperature and incubated with polyclonal antibodies raised against the extracellular domain of rat TrkA (rabbit, 1:1000) in blocking solution overnight at 4°C on a shaker. Cultures were subsequently washed with TBS and incubated with anti-rabbit antibodies conjugated to Cy3 (1:1000) in blocking solution for 1 hr at room temperature. Following washes of these cultures in TBS, photographs were taken on a Nikon microscope at 20 \times .

Production of Microscopic Gradients of Guidance Cues

Microscopic gradients of chemicals were produced by methods previously described (Lohof et al., 1992; Song et al., 1997b). Briefly, repetitive pressure injection of picoliter volumes of solutions containing the guidance cue was applied through a micropipette with a tip opening of about 1 μ m. The pressure was applied with an electrically gated pressure application system (Picospritzer, General Valve, Fairfield, NJ). Standard pressure pulses of 3 psi in amplitude and 20 ms in duration were applied to the pipette at a frequency of 2 Hz, using a pulse generator (SD9, Grass Instruments, Quincy, MA). By measuring the size of the droplet in mineral oil after 50 pulses of repetitive ejection with the same pressure pulse parameters, the average volume of ejected solution per pulse was estimated to be about 1.5 pl. Theoretical analysis (Lohof et al., 1992) and direct measurements of the gradient using fluorescent dyes (Zheng et al., 1994) have shown that, using standard pulsing parameters, the average concentration of the chemical is about 10³-fold lower at the growth cone than that in the pipette. The concentration gradient across a typical growth cone (10 μ m in width) at a distance of 100 μ m from the pipette tip was estimated to be in the range of 5%–10%. The total volume of the saline in the culture dish during the experiment was 5 ml. The final bath concentration of factors ejected from the pipette at the end of the 1 hr experiment was estimated to be about 10⁵-fold lower than that in the pipette. Recombinant BDNF, NT-3, and purified murine NGF were purchased from Promega. Recombinant netrin-1, Sema III, and rMAG were produced as previously described (Song et al., 1998).

Measurements of Neurite Extension and Growth Cone Turning

Phase-contrast inverted microscopes (Nikon Diaphot or TMS) were used to observe the neurite growth. Microscopic images of neurites were recorded into a computer with a CCD camera (Toshiba IK-541RA) attached to the microscope. To determine the total length of neurite extension, the entire trajectory of the neurite at the end

of the 1 hr period was measured with a digitizer. For assaying growth cone turning, the tip of the pipette containing the chemical was placed 100 μm away from the center of the growth cone and at an angle of 45° with respect to the initial direction of neurite extension. The initial direction of the neurite was determined by the last 15 μm segment of the neurite. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the 1 hr period. Only growth cones with net extension of >5 μm over the 1 hr period were included for analysis.

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