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Target-Derived GFRα1 as an Attractive Guidance Signal for Developing Sensory and Sympathetic Axons via Activation of Cdk5

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Summary

Immobilized and diffusible molecular cues regulate axon guidance during development. GFRα1, a GPI-anchored receptor for GDNF, is expressed as both membrane bound and secreted forms by accessory nerve cells and peripheral targets of developing sensory and sympathetic neurons during the period of target innervation. A relative deficit of GFRα1 in developing axons allows exogenous GFRα1 to capture GDNF and present it for recognition by axonal c-Ret receptors. Exogenous GFRα1 potentiates neurite outgrowth and acts as a long-range directional cue by creating positional information for c-Ret-expressing axons in the presence of a uniform concentration of GDNF. Soluble GFRα1 prolongs GDNF-mediated activation of cyclin-dependent kinase 5 (Cdk5), an event required for GFRα1-induced neurite outgrowth and axon guidance. Together with GDNF, target-derived GFRα1 can function in a non-cell-autonomous fashion as a chemoattractant cue with outgrowth promoting activity for peripheral neurons.

Introduction

Target-derived factors play a prominent role during the development and maintenance of the nervous system. The first step in establishing specific neuronal connections requires axons to navigate with precision to their targets. This process is controlled in part by external cues which define pathways to the target area. Such cues include extracellular matrix components, molecules present in the membrane of neighboring cells, and soluble factors. Several families of attractive and repulsive guidance molecules are involved in the targeted growth of axons, including extracellular matrix proteins, netrins, slits, semaphorins, ephrins, and neurotrophic factors such as hepatocyte growth factor (HGF), members of the transforming growth factor β (TGF-β) superfamily, and neurotrophins. Neurotrophic factors are best known for their role in regulating neuronal survival. However, work during the last years has established that these molecules can also act in a paracrine way to regulate the direction of axonal growth.

GDNF (glial cell line-derived neurotrophic factor) is a distant member of the transforming growth factor β superfamily, originally discovered by its ability to promote the survival of ventral midbrain dopaminergic neurons (Lin et al., 1993). GDNF was subsequently found to have similar effects in motorneurons (Henderson et al., 1994; Li et al., 1995; Oppenheim et al., 1995), other neuronal populations in the central nervous system (Arenas et al., 1995; Ha et al., 1996; Mount et al., 1995) and in the peripheral nervous system, where it promotes the survival of embryonic sympathetic, parasympathetic, and sensory neurons (Buj-Bello et al., 1995; Trupp et al., 1995). The physiological relevance of the in vitro effects of GDNF on the survival of peripheral neurons has been substantiated by the reduction in the number of sensory, sympathetic, and enteric neurons observed in GDNF mutant mice (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996).

Three close mammalian homologs of GDNF have been identified, Neurturin (NTN), Persephin (PSP), and Artemin (ART) (Airaksinen and Saarma, 2002). An unusual feature of the receptor complex for GDNF family ligands is the requirement of two receptor subunits, one specialized in transmembrane signaling, the c-Ret receptor tyrosine kinase (Durbec et al., 1996; Trupp et al., 1996), and another involved in ligand binding, the glycosylphosphatidyl inositol (GPI)-anchored co-receptor named GDNF family receptor α (GFRα) (Jing et al., 1996; Treanor et al., 1996). c-Ret is unable to bind GDNF family ligands on its own but can be activated in a complex with GFRαs. Different members of the GDNF ligand family utilize different GFRα co-receptors, although there is some overlap in specificity among the four known GFRαs. GDNF signals primarily via the GFRα1 receptor (Klein et al., 1997; Sanicola et al., 1997), although it is to some extent also able to use GFRα2 (Airaksinen and Saarma, 2002). Lack of GFRα1 results in a 15% loss of sensory neurons in the nodose ganglion (Cacalano et al., 1998). The fact that 40% of these cells are missing in GDNF mutant mice indicates that GDNF may be utilizing other receptors in these cells, presumably GFRα2, in addition to GFRα1. On the other hand, there appears not to be any cell loss in the superior cervical ganglion of GFRα1 mutant mice (Cacalano et al., 1998), and one group reported the detection of tyrosine hydroxylase (TH)-positive fibers associated with facial blood vessels (Enomoto et al., 1998), suggesting that at least some sympathetic fibers were able to reach this target. The paravertebral sympathetic chain has not yet been analyzed in GFRα1 knockout mice.

Although both c-Ret and GFRαs are required for responsiveness to GDNF family ligands, GFRα receptors are widely expressed in the absence of c-Ret (Yu et al., 1998), suggesting alternative roles for "ectopic" sites of GFRα expression. We have recently proposed that GFRα molecules may function in a non-cell-autonomous fashion to capture GDNF and present it to c-Ret receptors in *trans*. In particular, we found that exogenous GFRα1 supplied together with GDNF potentiates intracellular signaling and neuronal survival of developing sensory neurons (Paratcha et al., 2001). A common feature of GPI-anchored molecules is that they are found as both membrane bound and soluble forms. We have also established that GFRα1 can be released from the

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surface of neuronal and Schwann cells and from explants of sciatic nerve. Interestingly, release of GFRα1 was greatly enhanced in sciatic nerve explants taken one week after a crush lesion, suggesting a physiological role for the released receptors in the injury response (Paratcha et al., 2001). It is known that nerve regrowth and regeneration mimics many of the same cellular events and shares many of the same molecular mechanisms that regulate axonal growth and target innervation during development. It became, therefore, of interest to study possible roles of target-derived GFRα molecules in axonal growth during development.

In the work presented here, we have investigated whether the expression and release of GFRα1 molecules by peripheral target tissues during the period of innervation could represent a target-derived cue for ingrowing axons. To approach this question, we have examined the relationship between expression of GDNF receptors in peripheral target tissues and axonal growth and chemoattractant activities of GFRα1 on sensory and sympathetic axons during embryonic development. We have also investigated the signaling mechanisms underlying the activities of exogenous GFRα1 in axon growth and guidance.

Results

Developmental Expression of GFRα1 in Nodose Ganglion and Its Targets

To determine whether soluble GFRαs could represent target-derived diffusible molecules involved in axon guidance, we examined the developmental pattern of mRNA expression of GDNF receptors in nodose ganglion and in two of its peripheral targets, heart and carotid body, in chicken embryos. RT-PCR analysis revealed that both c-Ret and GFRα1 mRNAs are expressed in the nodose ganglion between embryonic day 6 (E6) and E16 without significant variation in their levels of expression (Figure 1A). No expression of c-Ret mRNA was observed in carotid body and heart at any of the embryonic stages studied (Figure 1A). On the other hand, GFRα1 mRNA expression was readily detected in both targets, with higher levels during earlier stages (E6 to E13) and lower levels at E16. A quantitative analysis using real-time PCR revealed a progressive reduction of GFRα1 mRNA expression in the two tissues between E6 and E16 (Figure 1B). GDNF itself was predominantly expressed by target cells (data not shown), in agreement with previous results (Buj-Bello et al., 1995; Erickson et al., 2001; Nosrat et al., 1996).

We next determined the spatial localization of GDNF receptors in dissociated cultures of E9 nodose and paravertebral sympathetic ganglion neurons by immunofluorescence. This analysis demonstrated that the majority of the neurons express GFRα1 and c-Ret. Intriguingly, GFRα1 was mainly localized to the cell body, with minor immunoreactivity along the neurites and in growth cones (Figure 1C and data not shown). In several cases, sensory neurons displayed a segmented pattern of GFRα1 immunoreactivity along the neurites (Figure 1C). In agreement with previous results (Worley et al., 2000), c-Ret was detected in cell bodies, along the neurites and in growth cones (data not shown). The localization of GFRα1 was confirmed by whole-mount immunofluorescence of explanted E9 nodose ganglia, which showed prominent GFRα1 expression in the ganglion proper (Figures 1Da–1Dc) and cells within it (Figures 1Dd–1Df), but little or no immunoreactivity in fibers exiting from the ganglion (Figures 1Dg–1Di). These observations suggest a relative deficit of GFRα1 molecules in developing axons and growth cones of sensory and sympathetic neurons compared to c-Ret. Thus, the expression of GDNF receptors in peripheral targets, and their subcellular localization in developing neurons, are consistent with a role for GFRα1 as a target-derived factor.

Release of Functional GFRα1 Molecules by Targets of Sensory Neurons

A prerequisite for the action of GFRα1 as a soluble mediator of GDNF responses in vivo is its release from the membrane of GFRα1-expressing cells. In order to determine whether bioactive GFRα1 was synthesized and secreted by targets tissues, we analyzed the release of this molecule ex vivo in conditioned medium of explanted heart and carotid body isolated from E9 chicken embryos. GFRα1 could be detected by Western blotting in the supernatants of both tissues (Figure 1E). GFRα1 was not detected in conditioned medium of fibroblasts that do not express this receptor (Figure 1E). In the presence of GDNF, conditioned media of heart and carotid body were able to stimulate c-Ret activation in *trans* in fibroblast cells expressing this receptor (Figure 1F), indicating the presence of biologically active soluble GFRα1. Conditioned medium did not stimulate c-Ret phosphorylation in the absence of GDNF (Figure 1F).

Soluble GFRα1 Potentiates Neurite Outgrowth during Early Development of Sensory and Sympathetic Ganglia

We next investigated whether soluble GFRα1 presented in *trans* together with GDNF could influence neurite outgrowth in E9 nodose and paravertebral sympathetic ganglia. As expected, a saturating dose of GDNF (100 ng/ml) induced the extension of neurites from both nodose and sympathetic ganglion explants (Figure 2A, see also Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/36/3/387/DC1). Addition of a soluble GFRα1-Fc fusion protein promoted a robust and dose-dependent increase in this response, exceeding that obtained with a saturating dose of GDNF (Figure 2A). In the absence of GDNF, exogenous GFRα1-Fc had no effect on neurite outgrowth (Figure 2A). The response of nodose and sympathetic ganglia at different stages of development was evaluated by measuring both neurite length and area of growth outside the explant. The effects of soluble GFRα1-Fc on both these parameters were more pronounced at earlier times of development (E6 and E9), coinciding with a very low or negligible spontaneous growth in control explants (Figure 2B). In the nodose ganglion, the effect of soluble GFRα1-Fc at E13 was modest but significant on both the area and the length of neurite outgrowth (Figure 2B). GDNF alone had no effect at this age. On the other hand, sympathetic ganglion explants still showed a robust response to GFRα1-Fc at E13 (Figure 2B). Neither nodose nor sympathetic ganglion explants responded to treatment with

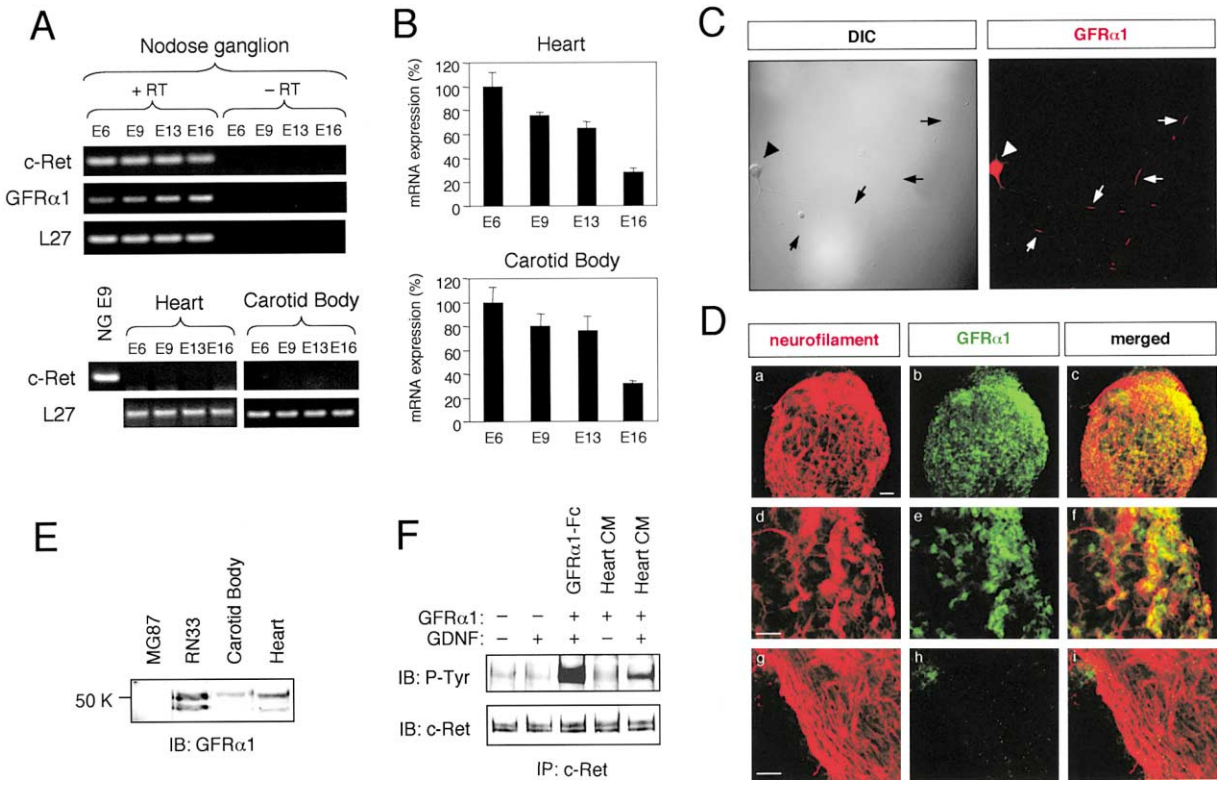


Figure 1. Developmental Expression and Release of GFRα1 in Nodose Ganglion and Its Targets

(A) Expression of GDNF receptors in chicken nodose ganglion and peripheral targets during embryonic development examined by RT-PCR. Control samples without reverse transcriptase (−RT) are also shown. Expression of the house-keeping gene L27 was used as loading control. NG E9, embryonic day 9 nodose ganglion.

(B) Quantitative analysis of developmental expression of GFRα1 mRNA by real-time PCR in chicken heart and carotid body between E6 and E16. Shown are averages ± SD of triplicate determinations.

(C) Photomicrographs of dissociated cultures of E9 chick nodose ganglion neurons visualized by DIC optics (left) and by immunofluorescence after staining with anti-GFRα1 antibodies (right). Arrowheads indicate cell bodies. Small arrows denote discontinuous regions of GFRα1 staining along sensory axons.

(D) Whole-mount immunohistochemistry of explanted E9 chick nodose ganglion and nerve fibers. Double staining for neurofilament (red) and GFRα1 (green) are shown in the left and right columns. Merged images are shown in the last column. The first row (a–c) shows a low magnification view of a nodose ganglion with overlapping neurofilament and GFRα1 staining. Scale bar, 100 μm. The middle row (d–f) shows a higher magnification image of cells within the same ganglion double-labeled for neurofilament and GFRα1. Scale bar, 50 μm. The last row (g–i) shows a close-up of fibers exiting the ganglion labeled with neurofilament but lacking GFRα1 immunoreactivity. Scale bar, 50 μm.

(E) Immunoblot of GFRα1 in conditioned medium of fibroblasts (MG87), neuronal cells (RN33B), and explanted E9 chicken carotid body and heart.

(F) Functional GFRα1 in developing heart conditioned medium. Fibroblast cells expressing only c-Ret (MG87-Ret) were treated with GDNF and purified GFRα1-Fc or soluble GFRα1 derived from heart conditioned medium as indicated. c-Ret phosphorylation was analyzed by immunoprecipitation (IP) followed by immunoblotting (IB) with anti-phosphotyrosine antibodies.

GDNF or GDNF plus GFRα1-Fc at E16 (Figure 2B). At this time, a pronounced spontaneous outgrowth could also be observed. Intriguingly, the lack of responsiveness at this later stage cannot be explained by a downregulation of GDNF receptor expression (see Figure 1A), and most likely reflects developmental changes in downstream signaling components. Together, these results indicate that exogenous GFRα1 is able to potentiate neurite outgrowth specifically at those times of development when it is maximally expressed in peripheral targets, consistent with the possibility that target-derived GFRα1 functions as an outgrowth-promoting factor. The persistent expression of neuronal c-Ret and GFRα1 after the period of target innervation (i.e., E16), together with the loss of neurite outgrowth responses to GDNF and GFRα1 at these later stages, suggest additional functions for this signaling system in the maturation or maintenance of connections.

A Local Source of GFRα1 Promotes Directional Growth of Sensory and Sympathetic Axons

We next investigated whether GDNF and GFRα1 could affect the direction of axonal growth of sensory and sympathetic neurons when presented from a localized source. To this purpose, we cultured explants in collagen gels together with agarose beads soaked in different proteins. In the first set of experiments, E9 nodose ganglion explants were cultured in medium containing brain-derived neurotrophic factor (BDNF)—as a neurite outgrowth inducer—together with beads soaked in BSA, GDNF, GFRα1-Fc, or GDNF plus GFRα1-Fc. In these conditions, only beads soaked in both GDNF and

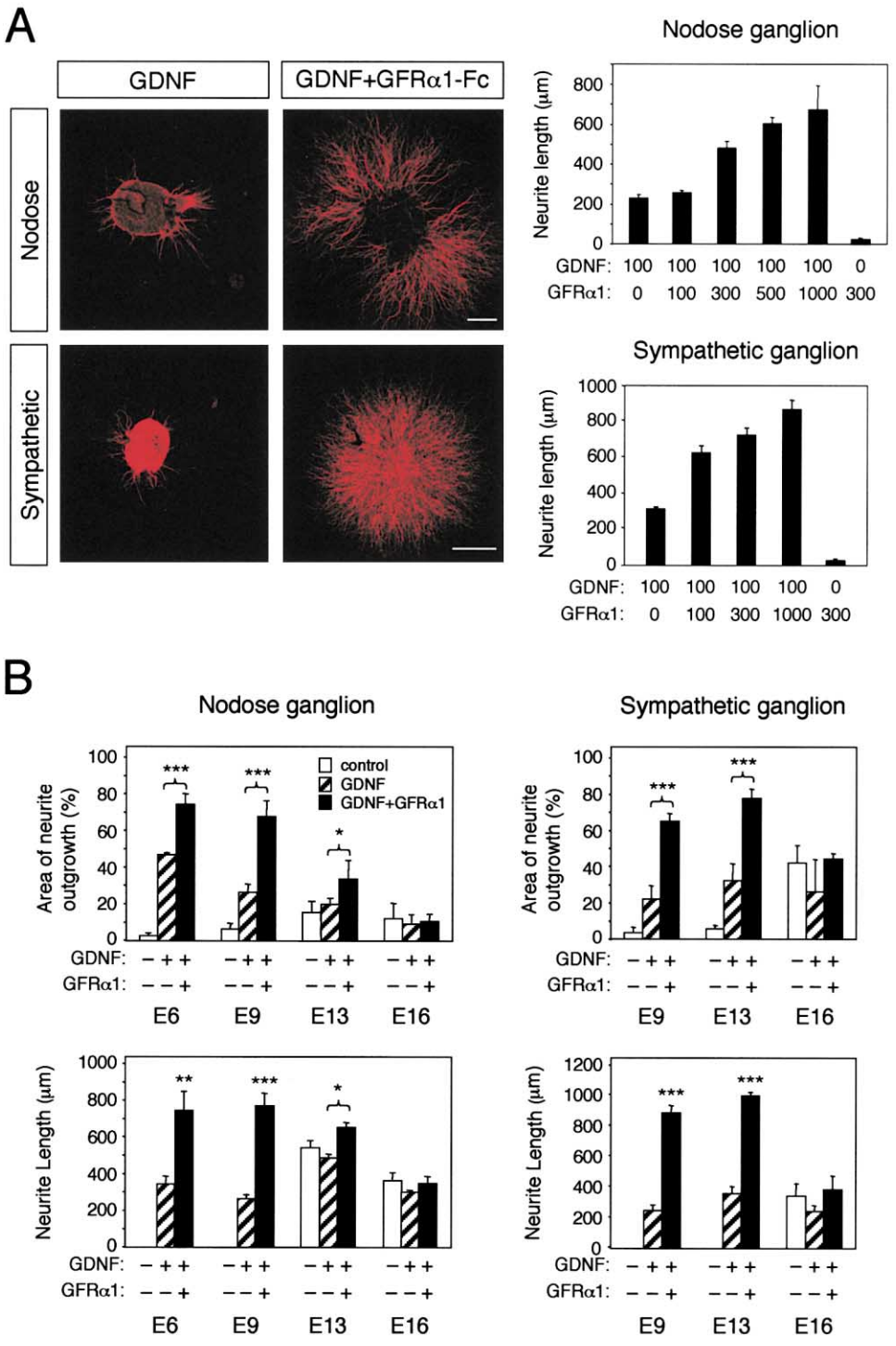
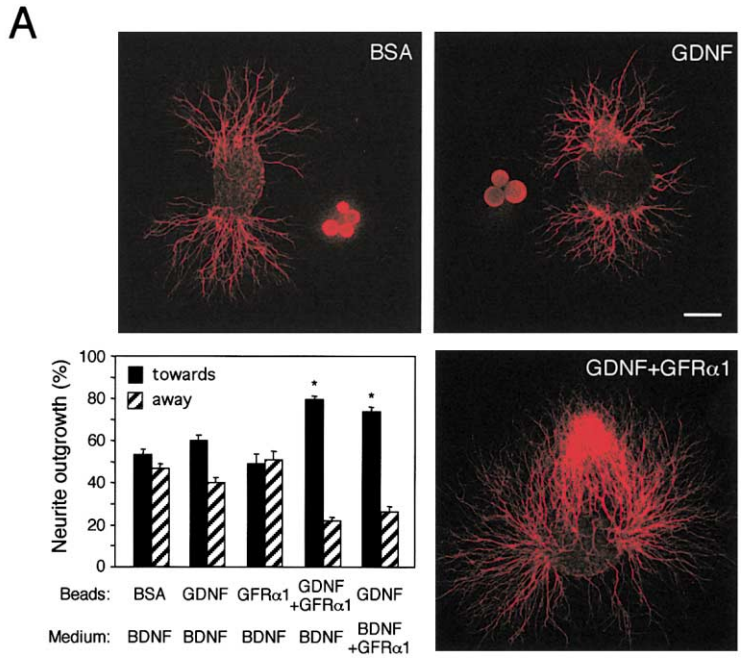


Figure 2. Soluble GFRα1 Potentiates Neurite Outgrowth in Developing Nodose and Sympathetic Ganglia

(A) Photomicrographs (left panels) of neurofilament immunofluorescence staining of explanted E9 chick nodose and sympathetic ganglia growing in collagen gel matrices in the presence of GDNF (100 ng/ml) or GDNF plus soluble GFRα1-Fc (300 ng/ml) as indicated. Scale bars, 200 μm. Quantifications (right panels) of neurite length in explants of E9 chick nodose and sympathetic ganglia grown for 72 hr with the indicated concentrations of GDNF and soluble GFRα1 (in ng/ml). Results are presented as average ± SD of two independent experiments performed in triplicate.

(B) Quantifications of neurite outgrowth area (relative to total explant area, see Experimental Procedures) and length in explants of chick nodose and sympathetic ganglia of different developmental ages grown for 72 hr with GDNF (100 ng/ml) and soluble GFRα1 (300 ng/ml) as indicated. Results are presented as average ± SEM of three independent experiments each measured in quadruplicate. ***, $p < 0.0001$; **, $p < 0.01$; *, $p < 0.05$ (ANOVA). At least 15 neurites were evaluated in each explant.



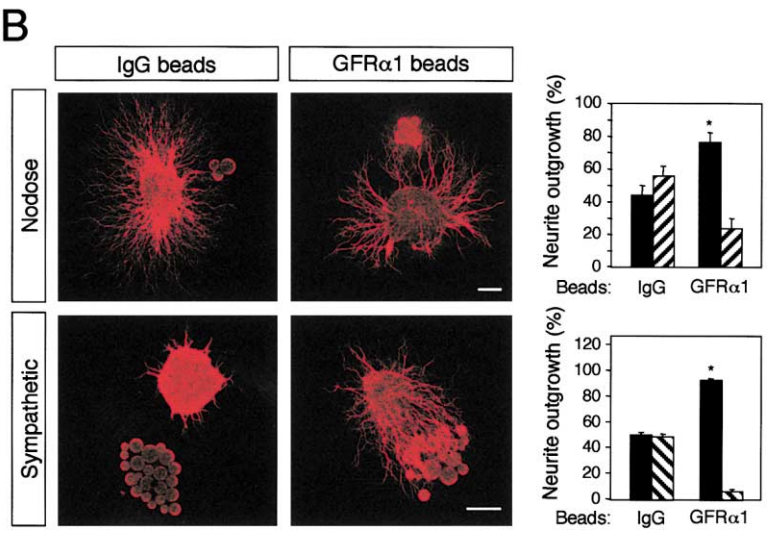


Figure 3. A Local Source of GFRα1 Induces Directional Growth of Sensory and Sympathetic Axons

(A) Nodose ganglion explants (E9) were cultured in collagen gels containing BDNF together with agarose beads soaked in BSA, GDNF, or GDNF plus GFRα1-Fc as indicated. After 72 hr, the explants were fixed and stained with anti-neurofilament antibodies. Scale bars, 200 μm. The histogram shows the quantification of neurite outgrowth in the quadrants proximal (solid bars, "toward") and distal (hatched bars, "away") to the beads. Results are presented as average ± SEM. For each condition, a total of 25–30 explants were evaluated in three independent experiments. *, $p < 0.05$ (ANOVA).

(B) Nodose and sympathetic ganglion explants (E9) were cultured in collagen gels containing a uniform concentration of GDNF (100 ng/ml) together with agarose beads soaked in IgG or in GFRα1-Fc as indicated. Results are presented as average ± SEM. For each condition, a total of 25−30 explants were evaluated in three independent experiments. ***, $p < 0.0001$ (Student's t test).

GFRα1-Fc were capable of inducing a clear and robust directional outgrowth of neurites toward the beads (Figure 3A). In the absence of GFRα1-Fc, GDNF had only a very modest effect, while GFRα1-Fc alone or BSA were completely inactive. Interestingly, however, an attractive effect of beads soaked in GDNF could be observed if soluble GFRα1 was added to the medium (Figure 3A). The requirement of exogenous GFRα1 for the effects of GDNF on the directional growth of nodose ganglion fibers prompted us to examine whether a localized source of GFRα1 could also influence the direction of neurite outgrowth in the presence of a uniform concentration of GDNF. Explants of E9 nodose and sympathetic ganglia were cultured in medium containing a saturating dose of GDNF together with beads soaked in GFRα1-Fc or in control IgG. A strong directed outgrowth could be observed toward beads soaked in GFRα1-Fc in the presence of a uniform concentration of GDNF (Figure 3B). This chemoattractive effect of GFRα1-Fc could also be observed in the presence of BDNF, for nodose ganglion explants, or nerve growth factor (NGF), for sympathetic explants, two strong inducers of neurite outgrowth in these neurons (data not shown). Together, these results indicate that exogenous GFRα1 can create positional information for c-Ret-expressing axons even in the presence of a uniform concentration of GDNF and in the presence of other outgrowth-promoting factors.

Immobilized GFRα1 Can Drive and Reorient Axonal Growth along Sites of GFRα1 Expression

GDNF and GFRα1 mRNA and protein were also detected in mesenchymal and glial cells isolated from developing sensory nerves and expanded in vitro (data not shown), indicating that they could provide local stimulation to

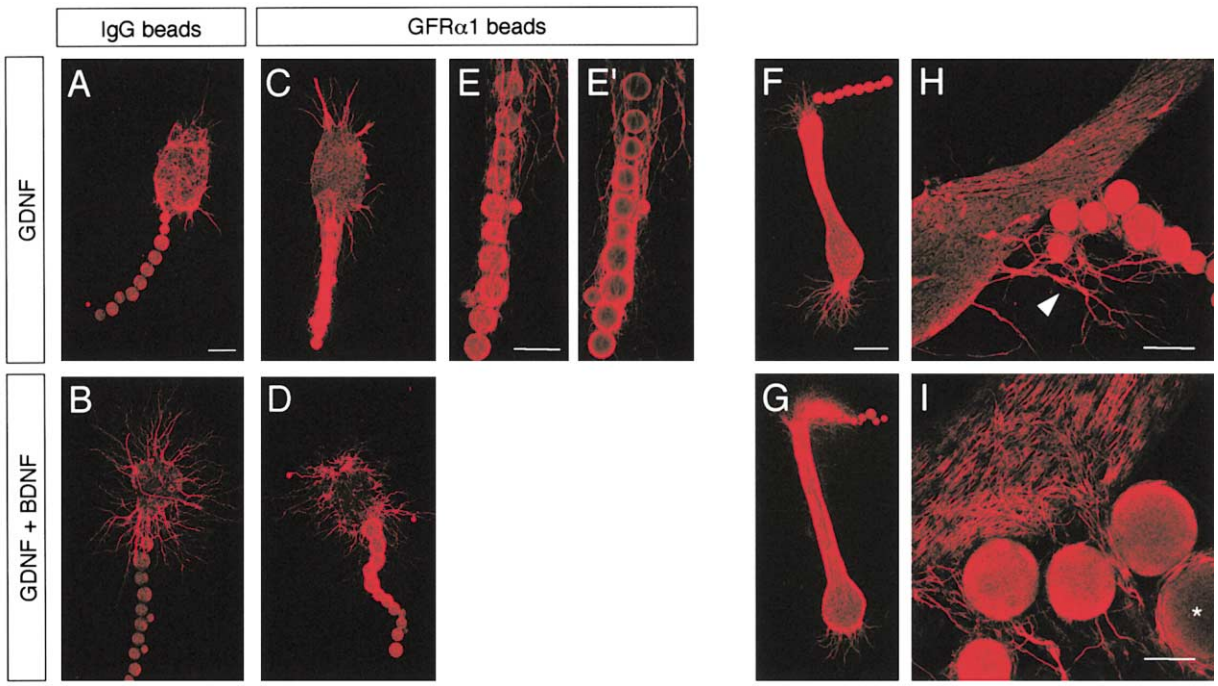


Figure 4. Immobilized GFRα1 Can Drive and Reorient Axonal Growth along Sites of GFRα1 Expression

(A–D) Photomicrographs of nodose ganglion explants stained with anti-neurofilament antibodies cultured in collagen gels containing GDNF (100 ng/ml) or GDNF plus BDNF (10 ng/ml) together with beads soaked in IgG (A and B) or GFRα1-Fc (C and D). Scale bar, 200 μm.

(E and E′) High magnification images (from panel C) of nodose ganglion neurites growing on agarose beads soaked in GFRα1-Fc. (E) and (E′) represent two different confocal planes of the same region. Scale bar, 200 μm.

(F and G) Nodose ganglia explanted with a piece of nerve growing in collagen gels containing a uniform concentration of GDNF and BDNF, together with beads soaked in IgG (F) or GFRα1-Fc (G) placed at a right angle over the tip of the nerve. Scale bar, 400 μm.

(H and I) Explants of nodose ganglia plus nerve growing in a uniform concentration of GDNF together with beads soaked in GFRα1-Fc placed at a right angle in the middle portion of the nerve. Arrowhead in (F) indicates neurites emanating from the ganglion toward the beads. Scale bar, 150 μm. A higher magnification is shown in (I). One of the beads (asterisk) appears encircled in neurites. Scale bar, 60 μm.

growing sensory fibers during target innervation and regeneration. Since GFRα1 is initially synthesized as a membrane-anchored molecule, we investigated localized effects of GFRα1 presented on rows of agarose beads directly in contact with ganglion explants cultured in a uniform concentration of GDNF, thereby mimicking a feature of the molecular environment encountered by growing axons in developing nerves and targets. Neurites induced in the presence of GDNF, or a combination of GDNF and BDNF, ignored beads soaked in IgG and extended in all directions from the explant (Figures 4A and 4B). In stark contrast, beads soaked in GFRα1-Fc promoted a robust growth of neurites that followed the path demarcated by the beads (15 of 15 explants examined) (Figure 4C). Even the additional neuritic growth stimulated by BDNF was oriented to converge and grow over the beads containing GFRα1-Fc (Figure 4D). At higher magnification, a dense web of neurites could be observed ensheathing the beads soaked in GFRα1-Fc (Figures 4E and 4E′). These results indicated that, in the presence of GDNF, a spatially confined source of GFRα1 can act as a local directional cue for growing sensory axons, orienting and stabilizing them along sites of GFRα1 expression.

The robust ability of exogenous GFRα1 to promote and direct de novo axonal growth in the presence of GDNF prompted us to examine its effects on the pathfinding behavior of pre-existing axons. To this purpose, we cultured explants of E9 nodose ganglia together with a piece of nerve in collagen gel matrices, and placed rows of agarose beads soaked in GFRα1-Fc at a right angle over the tip of the nerve. A combination of GDNF and BDNF was applied in the culture medium over a period of three days. Control beads soaked in IgG had no effect on the direction of growth of axons emerging from the explanted nerve (Figure 4F). In contrast, beads soaked in GFRα1-Fc produced a dramatic change in the pathfinding behavior of sensory axons, which turned at a right angle to grow over the path delineated by the GFRα1-containing beads (15 of 15 explants examined) (Figure 4G). Importantly, beads coated with GFRα1-Fc had no effect on the direction of axon growth if GDNF was omitted from the medium (see Supplemental Figure S2 on the *Neuron* website).

We also asked whether GFRα1 could alter the normal trajectory of axons within a nerve. For this purpose, we placed rows of beads soaked in GFRα1-Fc at a right angle in the middle portion of explanted nodose ganglion nerves. After three days in culture in the presence of GDNF, we observed extensive outgrowth of neurites emanating from the nerve and extending over the beads containing GFRα1 (Figure 4H); in some cases, axons were seen completely encircling the beads (10 of 10 explants examined) (Figure 4I). Together, these localized

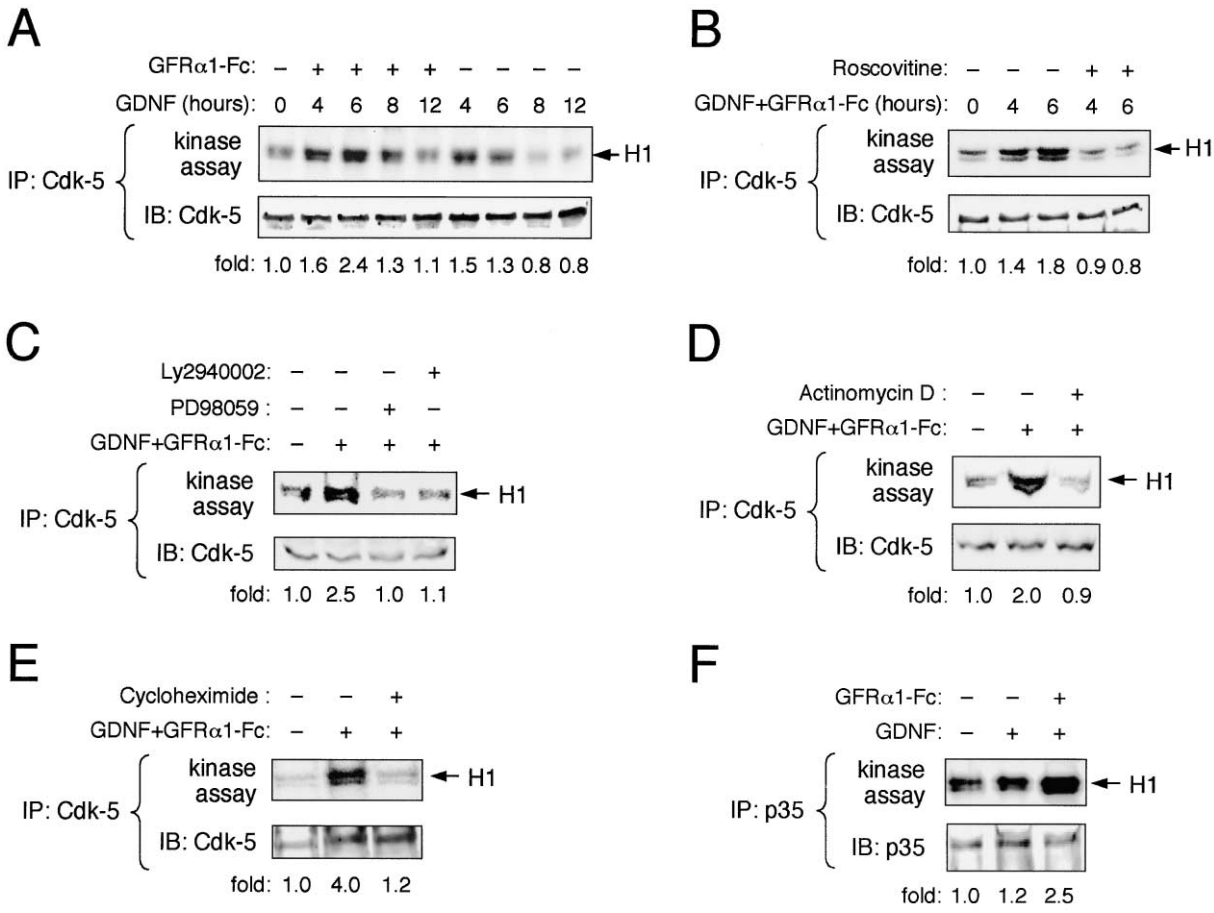


Figure 5. Increased and Sustained Activation of Cyclin-Dependent Kinase 5 (Cdk5) by GDNF in the Presence of Exogenous GFRα1

(A) Cdk5 kinases assay in MN1 cells treated with GDNF (100 ng/ml) or GDNF plus GFRα1 (300 ng/ml) for the indicated times. Immunoprecipitation of Cdk5 was followed by in vitro kinase assay using histone H1 as substrate and autoradiography. Reprobing of the same blot with Cdk5 antibodies is shown below.

(B) Inhibition of Cdk5 activity in MN1 cells stimulated with GDNF and soluble GFRα1 by the Cdk5 inhibitor Roscovitine (50 μM). The same concentration of Roscovitine had no effect on MAP kinase activity (not shown).

(C) Inhibition of Cdk5 activity in MN1 cells stimulated with GDNF and soluble GFRα1 (6 hr) by the MEK inhibitor PD98059 and the PI3K inhibitor Ly294002 (each at 50 μM).

(D and E) Inhibition of Cdk5 activity in MN1 cells stimulated with GDNF and soluble GFRα1 by 30 min pre-incubation with 2 μg/ml actinomycin D (D) or 25 μM cycloheximide (E).

(F) Induction of kinase activity associated to the Cdk5 regulator p35 in MN1 cells after 6 hr stimulation with GDNF and soluble GFRα1. Reprobing with anti-p35 antibodies shows that treatment with GDNF and GFRα1 had no major effect on p35 levels in these cells.

effects of exogenous GFRα1 on the direction of growth of sensory neurites document the ability of this protein to influence axon guidance.

Increased and Sustained Activation of Cyclin-Dependent Kinase 5 (Cdk5) by GDNF in the Presence of Exogenous GFRα1

In our previous work, we demonstrated that exogenous GFRα1 can potentiate and prolong downstream signaling in response to GDNF (Paratcha et al., 2001). In particular, stimulation with GDNF and soluble GFRα1-Fc resulted in a more sustained phosphorylation of Erk and Akt kinases compared to treatment with GDNF alone in developing sensory and sympathetic neurons and in the motor neuron cell line MN1 (Paratcha et al., 2001). Sustained activation of signaling pathways, in particular Erk kinases, has been associated with neuronal differentiation (York et al., 1998). However, the pathways linking sustained activation of Erk kinases to neuronal differentiation are only beginning to emerge. One such link may be represented by the cyclin-dependent kinase 5 (Cdk5) (Harada et al., 2001). Cdk5 is an atypical member of the serine/threonine cyclin-dependent kinase family that is highly expressed in the nervous system (reviewed by Dhavan and Tsai, 2001). Although a role for Cdk5 in cell cycle regulation has not yet been identified, this kinase has been implicated in neuronal cell migration and axon guidance (Dhavan and Tsai, 2001). Activation of Cdk5 requires its association with either one of two cyclin-like proteins, p35 and p39, as well as phosphorylation (Dhavan and Tsai, 2001).

We investigated the participation of Cdk5 in GDNF signaling, and its possible role in neurite outgrowth and axon guidance induced by exogenous GFRα1. In the

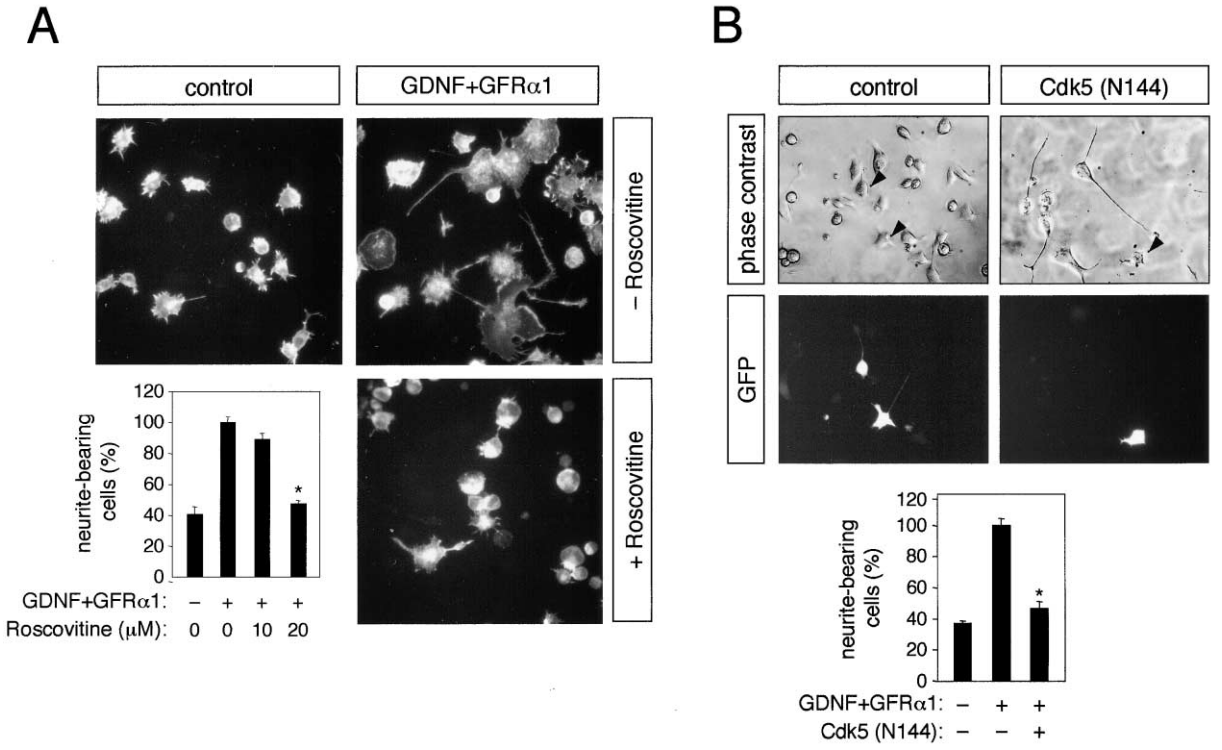


Figure 6. Cdk5 Activity Is Required for Neuronal Differentiation of MN1 Cells Mediated by GDNF and Soluble GFRα1

(A) MN1 cell differentiation mediated by GDNF (100 ng/ml) and soluble GFRα1 (300 ng/ml) is inhibited by Roscovitine. Photomicrographs show MN1 cells stained with Phalloidin, which reveals polymerized actin filaments. The histogram shows the quantification of the relative number of cells bearing neurites longer than 1.5 cell diameters in different conditions. The results are averages ± SD of a representative experiment performed in triplicate. *, $p < 0.0001$ versus GDNF + GFRα1 alone (ANOVA).

(B) Inhibition of MN1 cell differentiation by expression of a dominant-negative Cdk5 mutant. GFP was transfected in MN1 cells in the presence or absence of the Cdk5 (N144) dominant-negative construct. The histogram shows the quantification of the relative number of neurite-bearing cells in each condition. The results are averages ± SD of a representative experiment performed in triplicate. *, $p < 0.0001$ versus GDNF + GFRα1 alone (ANOVA).

first set of experiments, we used the motor neuron cell line MN1 stimulated with either GDNF alone or a combination of GDNF plus GFRα1-Fc. Using an in vitro kinase assay with Histone 1 (H1) as exogenous substrate, a modest stimulation of Cdk5 kinase activity could be detected after 4 hr treatment with GDNF (Figure 5A). Addition of GFRα1-Fc together with GDNF resulted in a more pronounced and sustained activation of Cdk5 which lasted for at least 8 hr (Figure 5A). Kinase activity in Cdk5 immunoprecipitates could be blocked by the specific Cdk5 inhibitor Roscovitine (Figure 5B). Importantly, Roscovitine did not affect Erk phosphorylation under the same conditions (data not shown). Activation of Cdk5 could be blocked by the MEK inhibitor PD98059 and by the PI3K inhibitor Ly2940002 (Figure 5C), indicating the participation of both these pathways in Cdk5 activation by GDNF and GFRα1. In contrast, the specific Src-family kinase inhibitor PP2 had no effect on the regulation of Cdk5 activity by GDNF (data not shown). In agreement with the slow kinetics of stimulation of Cdk5 activity in response to GDNF and GFRα1, both actinomycin D (Figure 5D) and cycloheximide (Figure 5E) inhibited Cdk5 activation by these factors, indicating the requirement of transcription and translation for this signaling event. Finally, GDNF and GFRα1 also stimulated kinase activity in p35 immunoprecipitates without inducing appreciable changes in p35 levels (Figure 5F). Using real time PCR, we could independently verify that treatment with GDNF and GFRα1 had no effects on the total levels of p35 or p39 mRNAs in MN1 cells (data not shown). Together, these results indicate that the combination of GDNF and exogenous GFRα1 can promote sustained activation of Cdk5 in MN1 cells via the MEK/Erk and PI3K pathways, and suggest the participation of the Cdk5 activator p35. However, although Cdk5 activation by GDNF and GFRα1 requires de novo transcription and translation, the mechanism of kinase activation does not appear to involve upregulation of p35 or p39.

Cdk5 Activity Is Required for Neuronal Differentiation of MN1 Cells Mediated by GDNF and Soluble GFRα1

In the presence of GDNF and soluble GFRα1, MN1 cells adopt a flattened morphology (i.e., cell spreading) and develop long neuritic processes (Figure 6A and Paratcha et al., 2001). This morphological differentiation of MN1 cells could be blocked by Roscovitine (Figure 6A), suggesting the requirement of Cdk5 activity. The effect of the inhibitor was verified using a dominant-negative

form of Cdk5 (Nikolic et al., 1996). MN1 cells were transfected with a GFP expression plasmid in the absence or presence of the Cdk5 dominant-negative construct and stimulated with GDNF and soluble GFRα1. Blockade of Cdk5 activity resulted in a complete inhibition of the effects of GDNF and GFRα1 on MN1 cell differentiation and neurite outgrowth as assessed by examination of the morphology of GFP-positive cells (Figure 6B). Similar results were obtained in PC12 cells co-transfected with c-Ret and dominant-negative Cdk5 treated with GDNF and GFRα1 (data not shown). These results demonstrate the requirement of Cdk5 for the effects of GDNF and GFRα1 on neuronal differentiation and neurite outgrowth.

Requirement of Cdk5 Activity for GFRα1-Mediated Neurite Outgrowth and Axon Guidance in Sympathetic Neurons

In agreement with the results obtained in MN1 cells, GDNF and soluble GFRα1 also induced increased Cdk5 kinase activity in dissociated cultures of E9 chicken sympathetic neurons with a delayed kinetics (Figure 7A). This increase in Cdk5 activation could also be blocked by Roscovitine (Figure 7A). To determine the importance of exogenous GFRα1 in the activation of c-Ret receptors in distal axons, we utilized compartmentalized cultures of sympathetic neurons. Stimulation of distal axons with GDNF and GFRα1 induced activation of Cdk5, while treatment with GDNF alone had no effect (Figure 7B), indicating that exogenous GFRα1 is required for activation of Cdk5 by a source of GDNF locally applied to axon terminals.

We then examined the requirement of Cdk5 activation for the neurite outgrowth induced by GDNF and soluble GFRα1-Fc in sympathetic ganglion explants. In the presence of Roscovitine, neurite outgrowth stimulated by GDNF and GFRα1 was severely impaired (Figure 8A). Interestingly, low doses of Roscovitine (10 μM) affected mainly the potentiated response obtained in the presence of soluble GFRα1 (primarily its effects on the area of neurite outgrowth), while sparing outgrowth responses to treatment with GDNF alone (Figure 8A). At higher doses (25 μM), Roscovitine had pronounced inhibitory effects on the area of neurite outgrowth and the neurite length in response to both GDNF and GDNF plus soluble GFRα1 (Figure 8A). Similar effects were also observed in explants of E9 nodose ganglia (data not shown). At neither dose did Roscovitine affect neuronal survival, as demonstrated in dissociated neuronal cultures grown in the presence of GDNF and GFRα1. These cultures also revealed the inhibitory effects of Roscovitine on neurite outgrowth (Figure 8B). The participation of Cdk5 signaling in neurite outgrowth promoted by GDNF and GFRα1 could also be demonstrated with the dominant-negative Cdk5 construct N144. In this experiment, expression of the dominant negative caused a marked reduction in neurite outgrowth responses to GDNF + GFRα1 in sympathetic neurons (Figure 8C).

Lastly, we investigated the role of Cdk5 in the ability of a localized source of GFRα1 to promote directional growth of sympathetic axons. To this purpose, we cultured explants of E9 sympathetic ganglia in collagen gels together with beads soaked in GFRα1-Fc in a medium containing GDNF and NGF. Neurites induced in the presence of GDNF and NGF were directed toward the beads containing GFRα1 (Figure 7D). In the presence of low concentrations of Roscovitine (12.5 μM), however, directed outgrowth of sympathetic neurites toward GFRα1-containing beads was inhibited (Figure 7D). Importantly, under the conditions used in this experiment (i.e., low concentration of Roscovitine and NGF in the medium), the drug treatment had no effect on the overall neurite outgrowth response of the explants, i.e., axons

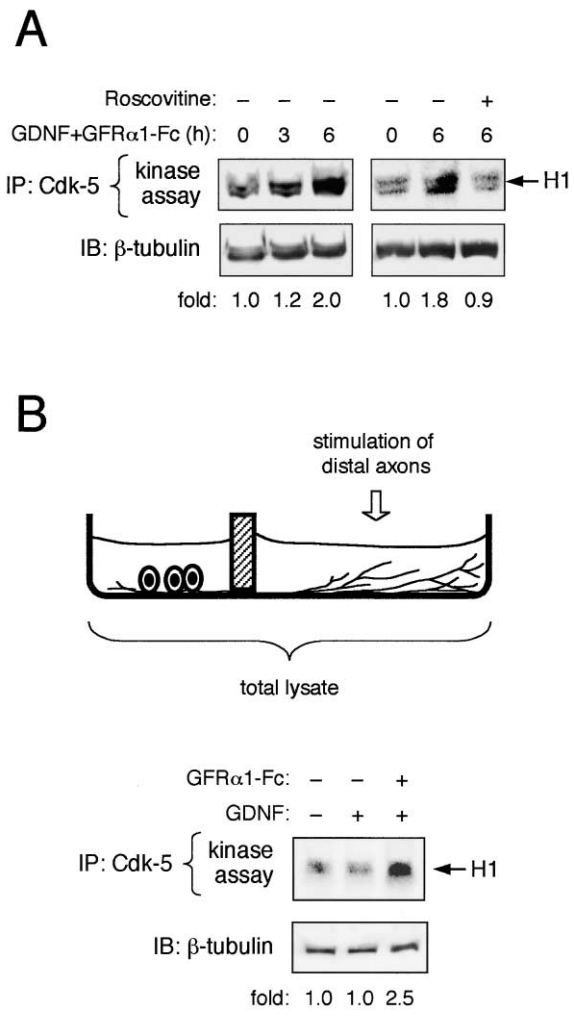


Figure 7. Requirement of Exogenous GFRα1 in Distal Axons for GDNF-Mediated Activation of Cdk5 in Sympathetic Neurons

(A) Cdk5 kinase activity in dissociated cultures of E9 chick sympathetic neurons. Cultures were treated with GDNF and soluble GFRα1 for the indicated times in the presence or absence of Roscovitine (50 μM). Reprobing control was done with antibodies against chick β-tubulin.

(B) Cdk5 kinase activity in dissociated cultures of E21 rat superior cervical ganglion neurons grown in compartmentalized chambers. The distal axon compartment was stimulated for 6 hr with GDNF in the presence or absence of soluble GFRα1. Total cell lysates were prepared from the pooled compartments and subjected to immunoprecipitation (IP) with anti-Cdk5 antibodies. Immunoprecipitates were used for kinase assay as above. Reprobing control was done with antibodies against β-tubulin. The experiment was repeated two times with similar results.

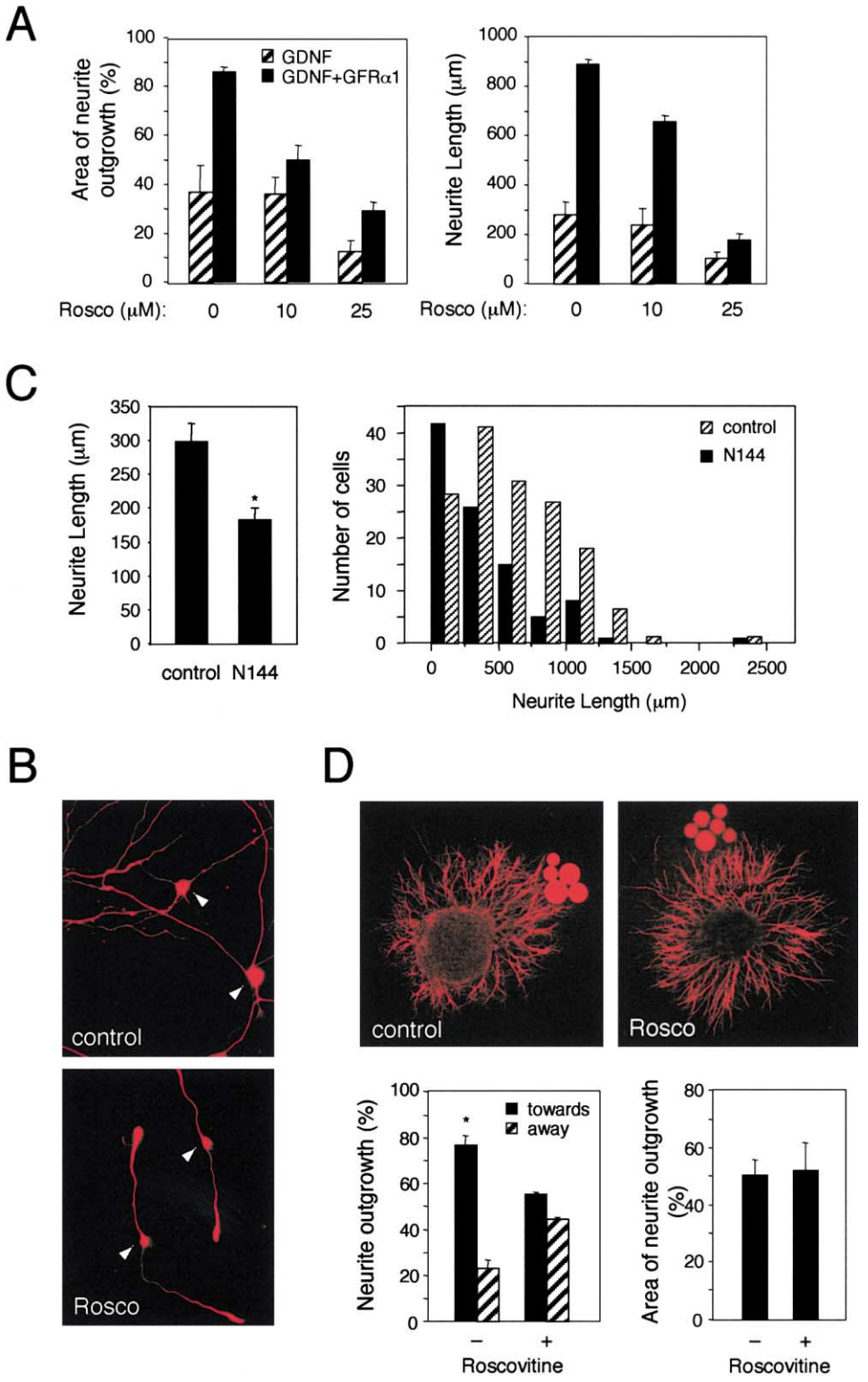


Figure 8. Requirement of Cdk5 Activity for GFRα1-Mediated Neurite Outgrowth and Axon Guidance in Sympathetic Neuron

(A) Roscovitine blocks neurite outgrowth stimulated by GDNF and soluble GFRα1 in explanted sympathetic ganglia. Both neurite outgrowth area and length are shown. The results are averages ± SEM of a representative experiment.

(B) Dissociated sympathetic neurons grown in culture in the presence of GDNF plus GFRα1 in the presence or absence of Roscovitine (25 μM). After 48 hr in culture, the cells were fixed and stained with anti-neurofilament antibodies. Arrowheads indicate neuronal cell bodies.

(C) Inhibition of neurite outgrowth in sympathetic neurons by expression of the dominant-negative Cdk5 mutant N144. GFP was transfected in E9 chick sympathetic neurons in the presence (N144) or absence (control) of the Cdk5 dominant-negative construct. Cultures were then stimulated with GDNF and soluble GFRα1. The histogram to the left shows the quantification of the neurite length in the two conditions. The

extended normally but were not attracted by the beads soaked in GFRα1 (Figure 7D). Together, these results demonstrate that activation of Cdk5 is not only required for neurite outgrowth induced by GDNF and GFRα1, but also for the ability of exogenous GFRα1 to promote axon guidance when presented from a localized source.

Discussion

Examination of the expression patterns of GDNF family ligands and their receptors have pointed to additional functions for this group of molecules in the developing and adult nervous system (Fundin et al., 1999; Naveilhan et al., 1997; Trupp et al., 1997; Yu et al., 1998). The wide expression of GFRα molecules in the absence of c-Ret, together with their ability to activate this receptor in *trans* when presented in soluble form in the presence of GDNF ligands, have suggested non-cell-autonomous roles for GFRα proteins during nervous system development and regeneration. In many cases, these "ectopic" sites of GFRα expression are associated with targets of c-Ret-expressing neurons, as in the trigeminal, hippocamposeptal, thalamocortical, and retinotectal pathways, or in close juxtaposition to c-Ret-expressing cells (Fundin et al., 1999; Trupp et al., 1997; Yu et al., 1998). The expression of GFRα1 by accessory cells in developing peripheral nerves and their targets suggests a physiological role for this molecule in the development of axonal projections, target invasion and the refinement of synaptic contacts with targets. In this work, we present evidence supporting a role for target-derived GFRα1 in the growth and guidance of developing sensory and sympathetic axons.

GFRα Proteins as Molecular Cues for Axonal Growth and Guidance

The cellular and molecular mechanisms that participate in axonal growth and guidance during development and regeneration are still not fully understood. Our results indicate a novel, non-cell-autonomous role for GFRα proteins in these processes. Although GFRα1 is coexpressed with c-Ret in sensory and sympathetic neurons, we find that soluble GFRα1 is able to potentiate neurite outgrowth induced by GDNF. Moreover, our results demonstrate striking localized effects of exogenous GFRα1 on the direction of growth of new and preexisting axons which were not observed using GDNF alone. This is in agreement with the inability of beads soaked in just GDNF to promote directional outgrowth in explanted mouse superior cervical ganglia and lumbar dorsal root ganglia (Young et al., 2001). We can think of at least two complementary explanations for the effects of exogenous GFRα1 on neurite outgrowth. On the one hand, stimulation of c-Ret in *trans* by exogenous GFRα molecules could be eliciting qualitatively or quantitatively distinct signaling responses compared to activation in *cis*. Previous work from ours and other laboratories has demonstrated that activation of c-Ret in *trans* results in a different kinetics of activation of this receptor, consisting of a first phase of activation outside lipid rafts followed by a delayed but sustained recruitment to this membrane compartment (Paratcha et al., 2001; Tansey et al., 2000). This results in a prolonged activation of c-Ret and downstream pathways, including the serine-threonine kinases Erk and Akt (Paratcha et al., 2001). Although it is not known at present whether activation of c-Ret in *cis* and in *trans* differ in some of their downstream targets, these results suggest that there could be differences in signaling between the two activation regimes. On the other hand, the effects of exogenous GFRα1 on axonal growth and guidance could also be explained by our observation of a relative deficit of GFRα1 molecules in axons compared to cell bodies of developing sensory and sympathetic neurons. In contrast, c-Ret immunoreactivity is observed in both neurites and cell bodies, suggesting that the relatively low abundance of endogenous GFRα1 in axons and growth cones may be limiting for c-Ret activation by GDNF in these compartments. In agreement with this, we find that although GDNF is on its own able to stimulate Cdk5 when supplied to whole-cell cultures of sympathetic neurons, it requires GFRα1 when administered specifically to the distal axons of these cells. This localized action could explain the remarkable effects of exogenous GFRα1 on axon guidance and branching, particularly when presented locally in close proximity to axons and axon terminals. Intriguingly, expression of GFRα1 is developmentally downregulated in heart and carotid body, two targets of nodose ganglion sensory neurons, in temporal coincidence with the completion of target innervation and the loss of neuronal responsiveness to exogenous GFRα1, suggesting a physiological significance for the differential subcellular localization of GDNF receptors in developing neurons. In direct support of this notion, a recent study of the distribution of GDNF receptors in cutaneous targets of trigeminal ganglion neurons identified a close juxtaposition between sensory nerve endings expressing c-Ret, but lacking GFRα1, and terminal Schwann cells expressing GFRα1, suggesting local interactions that may be essential for the formation of sensory terminals (Fundin et al., 1999).

Gradients of long-range guidance cues constitute an important mechanism for the specification of neuronal connections. Several classes of neurotrophic factors,

results are averages ± SEM of a representative experiment performed in triplicate. *, $p < 0.001$ (Student's t test). The experiment was repeated three times with the same results. The histogram to the right shows the frequency of neurons found carrying neurites in different length categories after transfection with Cdk5 N144 (solid bars) or GFP alone (control, stippled bars). Note the marked shift to the left of the distribution of neurons that received the dominant-negative construct.

(D) Roscovitine inhibits the chemotropic activity of exogenous GFRα1 on sympathetic axons. Sympathetic ganglion explants were cultured for 48 hr in collagen gels containing GDNF (100 ng/ml) and NGF (2 ng/ml) together with agarose beads soaked in GFRα1-Fc in the presence or absence of Roscovitine (12.5 μM). The first histogram shows the quantification of relative neurite outgrowth area toward (solid bars) and away (hatched bars) from GFRα1 beads. For each condition, a total of 15 explants were evaluated. Results are presented as mean ± SD of two independent experiments. *, $p < 0.01$ (Student's t test). The second histogram shows the quantifications of total neurite outgrowth area (relative to total explant area) from the same experiments.

including members of the neurotrophin (Tucker et al., 2001) and GDNF ligand families, are known to contribute to axonal growth during development. GDNF and the related factor NTN have recently been proposed as target-derived signals for developing cilliary ganglion neurons (Hashino et al., 2001); and ART, another member of the GDNF ligand family, is an important regulator of the growth and guidance of sympathetic axons from the developing superior cervical ganglion (Enomoto et al., 2001). Until now, however, target-derived neurotrophic receptors were not known to have non-cell-autonomous effects on axonal growth and guidance. A feature shared by GPI-anchored proteins is that they can be found in either membrane bound or soluble forms. Secretion of biological, active GFRα1 by known targets of c-Ret expressing neurons may form diffusible gradients of this protein that can promote long-range effects on the growth and guidance of developing sensory and sympathetic axons. On the other hand, our previous results have demonstrated that GPI-anchored GFRα1 molecules on stand-by cells can also capture and present GDNF in *trans* to c-Ret receptors on adjacent cells (Paratcha et al., 2001), supporting the ability of exogenous GFRα1 to produce localized effects. Our present results using microspheres coated with GFRα1 indicate that activation of c-Ret in *trans* by cell-cell contact may play a role in stabilizing axonal trajectories along sites of GFRα1 expression. Thus, GFRα1 has the capacity to elicit both long-range and localized guidance effects by creating positional information for c-Ret-expressing axons even in the presence of a uniform concentration of GDNF. GFRα1 can thus rearrange GDNF into spatial patterns that may be different from the patterns of GDNF gene expression.

Clearly, a number of target-derived molecules, in addition to GDNF and GFRα1, are likely to contribute to axonal growth and guidance of peripheral projections in vivo, a fact that may complicate the analysis of specific loss-of-function animal models. In addition to the neurotrophins and other neurotrophic factors, members of other families of guidance molecules, such as semaphorins, netrins, and slits, are known to contribute to these processes. Functional redundancy is also likely to occur within the GDNF signaling system itself, as indicated by our observation that GFRα2, a GFRα1 homolog that can also act as a receptor for GDNF (Sanicola et al., 1997; Trupp et al., 1998), is also expressed by target cells of sensory neurons and has effects on axonal growth and guidance that are comparable to those reported here for GFRα1 (F.L., unpublished observations). Thus, complex, developmentally regulated signaling networks are likely to contribute to the development and maturation of peripheral projections.

Role of Cdk5 in Axon Guidance by Exogenous GFRα1

Cdk5 is the only member of the Cdk family which is not known to have a role in cell cycle regulation. Most studies on this kinase indicate pleiotropic cellular functions for Cdk5 in cell migration, membrane transport, synaptic function, and axon guidance (Dhavan and Tsai, 2001). The role of Cdk5 in axon guidance appears to be highly conserved during evolution, as alterations in the activity of *Drosophila* Cdk5 result in errors in axonal pathfinding and target recognition by abdominal motor neurons (Connell-Crowley et al., 2000). p35, also known as neuronal Cdk5 activator (NCK5a), has been identified as an indispensable Cdk5 activator in neurons (Tsai et al., 1994). p35 and the related protein p39, also known as NCK5a isoform (NCK5ai), are small cyclin-like proteins that are capable of activating Cdk5 by direct association. A recent study has demonstrated that sustained Erk activation in response to NGF was sufficient for induction of p35 expression leading to Cdk5 activation and neuronal differentiation of PC12 cells (Harada et al., 2001). In addition, BDNF has also been shown to induce Cdk5 kinase activity in primary cerebellar cells in culture (Harada et al., 2001). In our previous work, we demonstrated that activation of c-Ret in *trans* by GDNF and soluble GFRα1 induces strong and sustained activation of MAP kinases Erk1 and 2 as well as Akt (Paratcha et al., 2001). Our present results suggest that the prolonged activation of these pathways leads to sustained Cdk5 activity, which is in turn required for the neurite outgrowth and guidance activities of exogenous GFRα1. Although our data showed that treatment with GDNF and GFRα1 stimulates Cdk5 activity in complex with p35, and indicated the requirement of gene transcription and protein translation, we have not detected any changes in the levels of expression of either p35 or p39 in response to GDNF and GFRα1. This suggests the existence of alternative mechanisms of Cdk5 activation in these cells, perhaps mediated by Cdk5 phosphorylation. A role for Cdk5 in neurite outgrowth mediated by exogenous GFRα1 was demonstrated using the specific inhibitor Roscovitine and the N144 dominant-negative Cdk5 mutant. Intriguingly, dose-response experiments indicated that lower doses of the inhibitor preferentially blocked the effects of exogenous GFRα1 on axonal growth. Moreover, low concentrations of Roscovitine effectively blocked the attractive effect of a localized source of GFRα1 on axons growing in a combination of GDNF and NGF without affecting overall neuritic growth. This experimental setup allowed us to dissociate the effects of Cdk5 on neurite outgrowth and axon guidance. The fact that one but not the other could be inhibited with low doses of Roscovitine underlies the specific involvement of Cdk5 in the biological activities of exogenous GFRα1, and suggests that axon guidance mediated by this receptor requires a higher threshold of Cdk5 activity.

In conclusion, our observations suggest a model in which GDNF, together with soluble and membrane bound GFRα1 produced by target and accessory nerve cells, interact with c-Ret at axon terminals and initiate local signaling cascades that propagate from distal axons to the cell nucleus where they activate the expression of factors critical for Cdk5 activation, ultimately leading to enhanced axonal growth.

Experimental Procedures

Cell Lines and Recombinant Proteins

RN33 is a conditionally immortalized neuronal precursor cell line isolated from embryonic raphe nucleus (Trupp et al., 1999; White et al., 1994). MG87-Ret lines were derived from MG87 fibroblasts by stable transfection with c-Ret. MN1 is an immortalized motorneu-

ron cell line that is responsive to GDNF (Trupp et al., 1999). GDNF and GFRα1-Fc were purchased from R&D. NGF was purchased from Promega, and BDNF was kindly provided by Regeneron Pharmaceuticals.

Cell Transfection, Plasmids, and Pharmacological Treatments

Transient transfection of MN-1 cells were performed in 24 well plates (1 μg total DNA/well) in 0.5 ml of DMEM with 1% serum using the Fugene-6 reagent (Roche). Transfection of primary sympathetic neurons was performed by the same method in DMEM:F12 serum-free medium. Plasmid cDNA encoding Cdk5DN (N144) was kindly provided by Dr Li-Hue Tsai (Department of Biological Sciences, University of South Carolina). Plasmid cDNA encoding GFP was from Clontech. The Cdk5 inhibitor, Roscovitine, was purchased from Calbiochem and used at the specified concentrations. The protein translation inhibitor cycloheximide (Sigma) was used at 25 μg/ml, and the transcription inhibitor actinomycin D (Sigma) at 2 μg/ml. MEK and PI3K inhibitors PD98059 and Ly294002 (Calbiochem) were used at 50 μM. Src inhibitor PP2 (Alexis) was used at 1 μM.

Immunoprecipitation, Western Blotting, and Kinase Assay

For total cell lysates, cells were lysed at 4°C in buffer containing 0.5% Triton X-100, 1% β-octylglucoside plus protease and phosphatase inhibitors. Protein lysates were clarified and analyzed by immunoprecipitation and Western blotting as previously described (Paratcha et al., 2001). All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics), and quantifications were done with ImageQuant software (Molecular Dynamics). Numbers below the lanes indicate fold of induction relative to control normalized to total levels of target protein.

The antibodies were obtained from various sources as follows: anti GFRα1 was kindly provided by Michael Sanicola, Biogen; anti-phosphotyrosine, anti-Ret, anti-p35, and anti-Cdk5 were from Santa Cruz Biotechnology (Santa Cruz, California). For kinase assay, endogenous Cdk5 and p35 were immunoprecipitated from cell lysates by incubation with 1.5 μg anti-Cdk5 or anti-p35 antibodies for 16 hr at 4°C and then 2 hr with protein G Sepharose beads (Pharmacia). The beads were precipitated, washed, and then mixed with 10 μg of histone H1 in kinase reaction buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1 mM sodium vanadate, 20 mM sodium fluoride, and protease inhibitors) with 100 μM ($\gamma-^{32}$P)ATP (2 μCi). The reaction was performed at room temperature during 20 min and stopped by addition of sample buffer. After SDS-PAGE, radioactivity was analyzed in a Storm 840 phosphorimager using ImageQuant software (Molecular Dynamics).

Compartmentalized Cultures

Compartmentalized cultures were performed as described by Campenot (1992). Briefly, 35 mm culture dishes were coated with rat tail collagen, and parallel tracks in the substrate were made by scratching the culture dish surface with a pin-rake (Tyler Research Instruments, Canada). Subsequently, a two chambered Teflon divider (Tyler Research Instruments) was seated on top of the tracks as described previously (Campenot, 1992). Dissociated E21 rat superior cervical ganglion neurons were plated in one compartment of a two-compartment chamber and maintained for 2 days in DMEM:F12 supplemented with 2 mM glutamine, 1 mg/ml BSA, 10 μM cytosine arabinoside (Sigma), and 100 ng/ml of NGF. After 2 days, the medium was replaced for DMEM:F12 containing 20 ng/ml of NGF in the cell body compartment and 100 ng/ml of NGF in the side (i.e., distal-axon) compartment. The medium was changed every 3 days, and after 10 days the cells were starved by incubation with anti-NGF blocking antibodies and then treated with GDNF or GDNF + GFRα1 in the side compartment. Following 6 hr of treatment, the cells were lysed and assayed for kinase activity as above.

Neuronal Differentiation and Neurite Outgrowth Assay

For MN1 cell differentiation assays, the number of cells with neurites longer than 1.5 cell diameter were counted in random fields of three different wells in each experiment. GDNF was used at a saturating dose (100 ng/ml) and soluble GFRFα1-Fc at a sub-saturating dose (300 ng/ml).

For neurite outgrowth assay, chick nodose and paravertebral sympathetic ganglia were dissected at different times of embryonic (E) development (E6, E9, E13, E16). The explants were cultured in rat tail collagen gels as previously described (Ebendal, 1989) in the presence of the specified neurotrophic factors. For directed neurite outgrowth assays, 10 μl of agarose beads (Sigma Chemical, Co) were washed in PBS and incubated with 2 μg of BSA, IgG, GDNF, or GFRα1-Fc over night at 4°C in a total volume of 50 μl. In some cases, 25 μl of beads coated with GFRα1-Fc were incubated with GDNF (2 μg) for 4 hr at 4°C, washed again, and inserted in collagen gel matrices. After 3 days in culture, the explants were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained as indicated below.

Immunofluorescence and Microscopy

GFRα1 staining was done on non-permeabilized, paraformaldehyde-fixed dissociated neuronal cultures (or ganglion whole mounts) by overnight incubation with an affinity-purified anti-GFRα1 antiserum, kindly provided by M. Sanicola, used at 1:400 dilution. Neurofilament staining was done after permeabilization with 0.3% Triton X-100 at room temperature and incubated at 4°C overnight with an anti-neurofilament antibody (Zymed) at 1:1500 dilution. TRITC-conjugated secondary antibodies (Jackson Lab) were used at 1:200 dilutions. Alexa-conjugated Phalloidin was from Molecular Probes. After immunostaining, confocal microscopy was performed in a Zeiss LSM 510 confocal microscope with 5× and 25× objective lenses using laser excitation wavelengths of 543 nm and 488 nm.

Neurite outgrowth from peripheral ganglion explants was quantified using the Axiovision software (Zeiss) version 2.01. The areas covered by the entire explant (ganglion plus neurites) and the neurites alone (from the outer perimeter of the ganglion) were measured and used to calculate the percentage of neurite outgrowth as the area occupied by neurites, divided by the total area of the explant, multiplied by 100. This value automatically normalizes neurite outgrowth measurements among explanted ganglia of different sizes and reflects both the length and the number of neurites (Wang et al., 1996). In addition, neurite length was directly quantified by measuring the distance between the tip of the neurite and the outer border of the ganglion. For each explant, the lengths of the 15 longest neurites were measured and averaged. In each experiment, measurements were taken in three to four different explants as indicated in the figure legends.

To quantify directional axon growth, the field was divided into four orthogonal quadrants and the areas covered by neurofilament-stained neurites (i.e., excluding the ganglion) were measured in the distal (away) and proximal (toward) quadrants with respect to the position of the agarose beads (see also Alcantara et al., 2000). The results are presented as percentage of neurite outgrowth toward or away from the beads relative to the sum of the outgrowth in both quadrants. Statistical analyses were performed by ANOVA and Student t test using the program Statview.

RT-PCR

The expression of c-Ret, GFRα1, GDNF, and L27 was analyzed by RT-PCR. Total RNA was isolated from nodose and sympathetic ganglia, carotid body, and the superior area of the heart (close to the point of entry of the great vessels) using SNAP columns (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized using Multiscribe reverse transcriptase and random hexamers (Perkin Elmer). The cDNA was amplified using the following primer sets: c-Ret: forward, 5′ ATC CAG CTA TTT GCC CCC AG 3′; reverse, 5′ CCA AGG TTT CCA GTG CCT TTC 3′. L27: forward, 5′ GAT GCC CAC CCG GTA TTC T 3′; reverse, 5′AGC AGG GTC CCT GAA CAC AT 3′. GFRα1: forward, 5′ AGT GTC ATA AGG CCC TCC GG 3′; reverse, 5′ CAG GAG CAG AAG AGC ATC CC 3′. Real-time PCR was performed using a LightCycler rapid thermal cycler system (PE) according to the manufacturer's instructions. Reactions were performed in 25 μl volume. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler–DNA Master SYBR Green I mix (PE).

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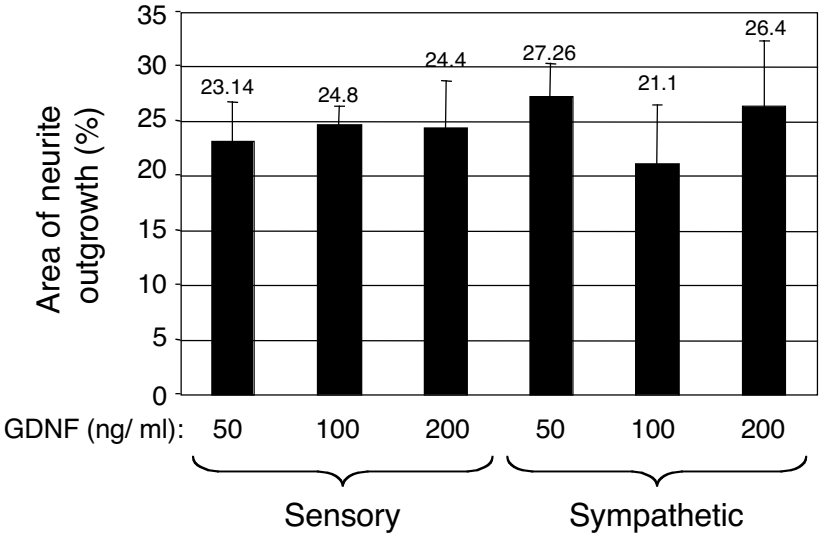
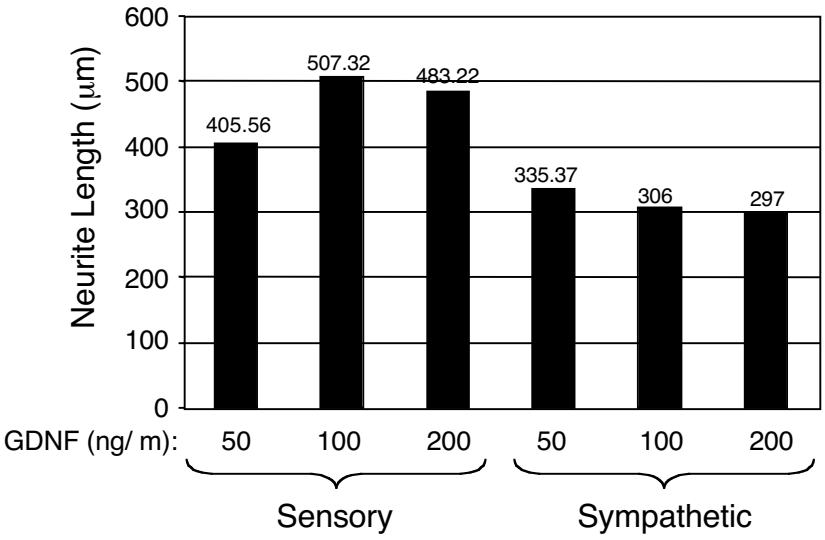
Supplementary Figure S1





Supplementary Figure S2