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Retrograde propagation of GDNF-mediated signals in sympathetic neurons

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Glial cell line-derived neurotrophic factor (GDNF) family ligands are target-derived trophic factors for several neuronal subpopulations. They promote survival and neurite outgrowth through binding to specific members of the GDNF family receptor alpha (GFRα) and subsequent activation of the RET tyrosine kinase receptor. Using compartmentalized cultures of sympathetic neurons, we have studied the mechanism of GDNF retrograde signaling. Our results demonstrate the presence of GDNF receptors RET and GFRα1 in the two cellular compartments, cell bodies and distal axons. Addition of GDNF to either compartment initiated local signaling, including activation of RET and its downstream effectors AKT and ERK1/2. Addition of GDNF to distal axons induced a retrograde signal leading to neuronal survival and neurite outgrowth. Retrograde signaling was associated with retrograde transport of radiolabeled GDNF and GFRα1, as well as activation of RET and AKT, but not of ERK1/2, in cell bodies. No anterograde signal propagation or transport was observed. Our results suggest a general mechanism for retrograde signaling initiated at distal axons through tyrosine kinase receptors.

Introduction

During development, neurons extend axons across long distances to innervate postsynaptic target cells. Factors released by target cells support neuronal growth and survival (Barde, 1989; Levi-Montalcini, 1987; Oppenheim, 1989). The mechanisms by which the information produced at the nerve terminal is retrogradely transported to the cell body have been under considerable investigation in recent years. Most studies have utilized nerve growth factor (NGF), a member of the neurotrophin family, to address this question. NGF has been shown to be capable of activating receptors at the nerve terminal and to convey signals to the cell bodies (for review, see Ginty and Segal, 2002).

Glial cell line-derived neurotrophic factor (GDNF) family ligands are a group of polypeptides structurally related to the transforming growth factor-beta (TGF-β) superfamily, which are produced by target cells and that have been implicated in survival and differentiation of several neuronal subpopulations in the developing nervous system (reviewed in Airaksinen and Saarma, 2002; Baloh et al., 2000). The four members of this family, GDNF, Neurturin (NTN), Artemin (ART), and Persephin (PSP), utilize a multicomponent receptor system consisting of the transmembrane receptor tyrosine kinase RET and a ligand-binding coreceptor named GDNF family receptor alpha (GFRα). Different members of the GDNF ligand family bind to distinct GFRα coreceptors (GFRα 1 to 4) which confer ligand specificity (reviewed in Airaksinen and Saarma, 2002; Baloh et al., 2000). GFRα receptors can mediate GDNF binding to and activation of RET when expressed on the surface of the same cell (i.e., *cis* signaling) or when presented in soluble form or immobilized from adjacent cells (i.e., *trans* signaling) (Paratcha et al., 2001; Yu et al., 1998). Ligand-mediated activation of RET initiates many of the same signal transduction pathways elicited by other tyrosine kinase receptors, including the mitogen-activated protein kinases (MAPKs) ERK-1 and ERK-2, and the serine-threonine kinase AKT (Besset et al., 2000; Trupp et al., 1999). In addition, GDNF signaling independently of RET has also been demonstrated (Poteryaev et al., 1999; Trupp et al., 1999). In cells devoid of RET, the association of GFRα1 with the neural cell adhesion molecule NCAM in the presence of GDNF promotes activation of Fyn and FAK kinases, leading to stimulation of cell migration and neurite outgrowth (Paratcha et al., 2003). In addition, GDNF has been shown to induce tyrosine phosphorylation of the MET receptor tyrosine kinase in kidney cells lacking RET (Popsueva et al., 2003).

The presence of GDNF ligands in target cells has suggested target-derived actions of these factors, implicating mechanisms of retrograde transport from the axons to the cell bodies. In vivo experiments have demonstrated retrograde transport of GDNF family ligands in sensory, sympathetic, parasympathetic, and motor neurons (Laurikainen et al., 2000; Leitner et al., 1999). Moreover, administration of GDNF in muscle by means of an adeno-

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associated viral vector produced protective effects on spinal cord motor neurons, indicating a retrograde trophic effect (Wang et al., 2002). The receptors and molecular mechanisms involved in the retrograde transport of GDNF ligands are at present unknown.

In this study, we have utilized compartmentalized cultures of sympathetic neurons to begin addressing mechanisms of retrograde signaling in the GDNF ligand system. Compartmentalized cultures allow a physical separation of cell bodies and axons and therefore permit selective stimulation and analysis of components in either compartment.

Results

Activation of GDNF signaling in distal axons of sympathetic neurons

Downstream signaling activated by GDNF was investigated both in mass culture and compartmentalized cultures of SCG neurons isolated from E21 rats, a neuronal subpopulation that is highly responsive to GDNF (Buj-Bello et al., 1995; Trupp et al., 1995). In agreement with previous studies (Coulpier et al., 2002), stimulation of mass cultures of sympathetic neurons with GDNF produced a rapid activation of the RET receptor as monitored using a polyclonal antibody against phosphorylated RET (Fig. 1A). Downstream signaling effectors, including AKT and ERK1/2, were also strongly activated by GDNF in these cultures (Fig. 1A). We then investigated whether such a signaling could also be initiated in the axons of sympathetic neurons. To this purpose, we cultured E21 sympathetic neurons for 15 days in compartmentalized cultures allowing growth of axons to the distal compartment (see Experimental Methods). Both the short (RET9) and the long (RET51) isoforms of RET, as well as GFRα1 could be detected by Western blotting in extracts prepared from the central compartment, containing cell bodies and proximal axons (hereafter called cell bodies), and the distal compartment (hereafter called distal axons) (Fig. 1B). RET appeared enriched in cell bodies compared to distal axons, while the opposite was true for GFRα1 (Fig. 1B). Expression of GDNF receptors in distal axons prompted us to examine whether treatment of distal axons with GDNF was able to activate downstream signaling events locally within that compartment. RET was strongly activated in extracts from distal axons 10 min after stimulation, while activation of AKT and ERK1/2 was delayed with a peak at 1 h (Fig. 1C). Activation of ERK1/2 in distal axons was maintained for up to 24 h after GDNF stimulation (Fig. 1C).

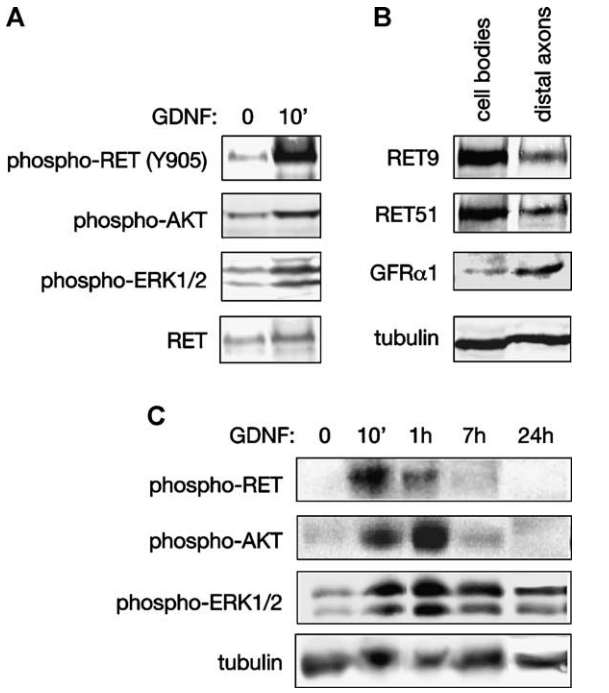

Fig. 1. Activation of GDNF signaling in distal axons of sympathetic neurons. (A) Activation of RET, AKT, and ERK1/2 in response to GDNF in mass cultures of sympathetic neurons from the E21 rat SCG. (B) Expression of GDNF receptors RET and GFRα1 in cell bodies and distal axons of sympathetic neurons grown in compartmentalized cultures. RET9 and RET51 refer to the short and long isoforms of the RET receptor, respectively. (C) Local activation of axonal pools of RET, AKT, and ERK1/2 following exposure of distal axons to GDNF. Tubulin III was used as loading control.

GDNF-mediated signals can be propagated retrogradely but not anterogradely in sympathetic neurons

To determine whether the information generated locally could be propagated retrogradely, from distal axons to cell bodies, or anterogradely, from cell bodies to distal axons, we stimulated each compartment separately with GDNF for 60 min and examined downstream signaling events in extracts prepared from either compartment. Stimulation of distal axons resulted in activation of RET and AKT in cell bodies (Figs. 2A and B), demonstrating the retrograde propagation of an intracellular GDNF-mediated signal. Interestingly, ERK1/2 kinases were not activated in cell bodies after stimulation of distal axons (Fig. 2B), although they were detected in cell bodies and could be activated when GDNF was applied directly to this compartment (Fig. 2B). In contrast, stimulation of cell bodies failed to elicit activation of either signaling component in distal axons despite these could be activated locally (Fig. 2B), indicating that GDNF-mediated signals cannot be anterogradely propagated in sympathetic neurons.

Retrograde transport of GDNF and GFRα1 in sympathetic neurons

The results presented above indicate that signaling effectors such as RET and AKT are activated in cell bodies when GDNF is applied at the periphery, but whether any proteins are physically transported from the periphery to the cell bodies remained to be established. To begin addressing this question, we applied I^{125}–GDNF to distal axons and monitored its transport to cell bodies. I^{125}–GDNF could be detected in cell bodies 7 h after its application to distal axons but not at earlier time points (Fig. 3A). The retrograde transport of I^{125}–GDNF was specific and receptor-mediated as it could be competed by addition of a 100-fold excess of unlabeled GDNF (Fig. 3A). To elucidate whether part of the receptor machinery was also retrogradely transported, we applied a soluble version of the GFRα1 receptor (GFRα1–Fc) radiolabeled as above. Several studies have confirmed the ability of soluble GFRα1 to mediate RET activation in the presence of GDNF (Paratcha et al., 2001; Yu et al., 1998), making it likely that it gets incorporated into the receptor complex upon retrograde transport. As shown in Fig. 3B, I^{125}–GFRα1 could

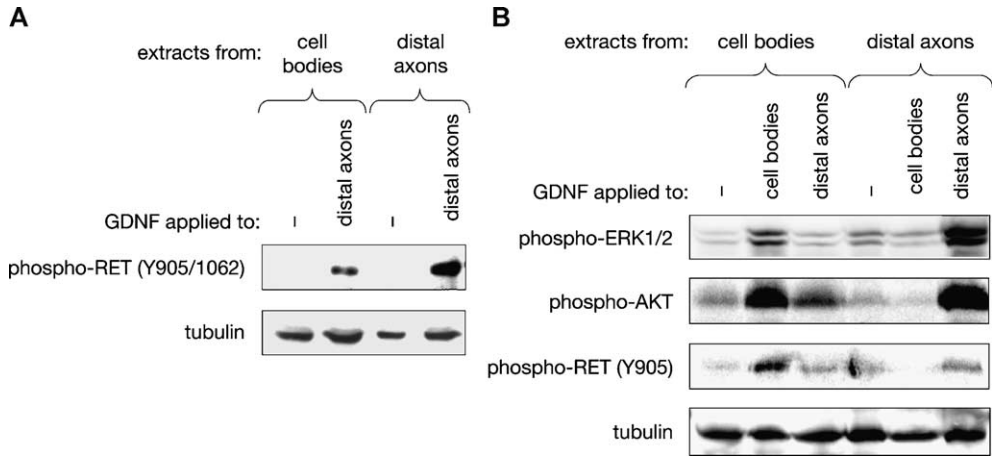

Fig. 2. GDNF-mediated signals can be propagated retrogradely but not anterogradely in sympathetic neurons. Cell bodies or distal axons were left unstimulated (–) or were stimulated with GDNF as indicated. In each condition, cellular extracts prepared from cell bodies and distal axons as indicated were immunoblotted with the indicated antibodies. Extracts from two biochemistry chambers were pooled for each lane. The experiment was performed twice using independent cultures with similar results. (A) Analysis of RET phosphorylation in Y905 and Y1062 after GDNF application in distal axons. (B) Analysis of AKT, ERK, and RET (Y905) phosphorylation after GDNF application in cell bodies or distal axons.

be detected in cell bodies 7 and 24 h after its application to distal axons together with GDNF. Importantly, no I^{125}–GFRα1 was observed in cell bodies in the absence of GDNF (Fig. 3B), indicating specific retrograde transport of soluble GFRα1 receptors from distal axons to cell bodies. In addition, application of a 100-fold molar excess of unlabeled GFRα1 prevented retrograde transport of I^{125}–GFRα1 (Fig. 3B), indicating that a receptor-mediated mechanism was at work.

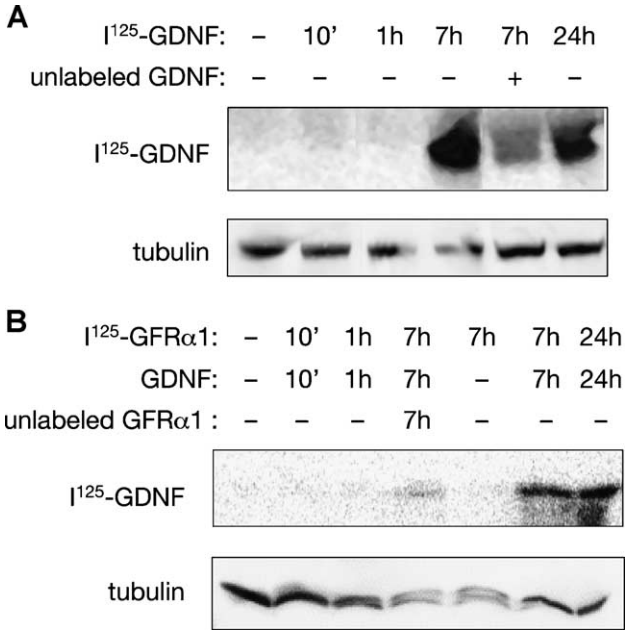

Fig. 3. Retrograde transport of GDNF and GFRα1 in sympathetic neurons. Sympathetic neurons were grown in compartmentalized chambers for 15 days before addition of I^{125}–GDNF (A) or I^{125}–GFRα1 (B) to distal axons and incubated for the indicated periods of time. A chase experiment was performed at 7 h using 100-fold excess of unlabeled GDNF or GFRα1 as indicated. For the retrograde transport of I^{125}–GFRα1, a control experiment was also performed at 7 h in absence of GDNF. The experiments were each performed twice using independent cultures with similar results.

Exposure of distal axons to GDNF promotes cell survival and neurite outgrowth

Having demonstrated the retrograde transport of GDNF and GFRα1 and the retrograde propagation of GDNF-mediated signals in sympathetic neurons, we investigated their possible biological significance by comparing cell survival and neurite outgrowth in response to GDNF applied to distal axons or to cell bodies. As expected, GDNF promoted robust neuronal survival when applied to cell bodies (Fig. 4A). A comparable response was obtained when GDNF was supplied at the distal axons (Fig. 4A), indicating that GDNF-induced signals propagating retrogradely from distal axons are capable of supporting the survival of cell bodies. In both instances, survival responses to GDNF were abolished by Ly294002, a specific inhibitor of PI3K, the upstream activator of AKT (Fig. 4B). Ly294002 applied to cell bodies also blocked GDNF-induced survival at distal axons, indicating that the PI3K/AKT pathway was playing an important role in both compartments. These observations were in agreement with the activation of AKT in cell bodies following application of GDNF to distal axons (Fig. 2), and with previous studies indicating the essential role of the PI3K/AKT pathway in the survival of sympathetic neurons mediated by GDNF (Besset et al., 2000).

Neurite outgrowth was evaluated by measuring the neurite length in distal axon compartments 2, 4, and 6 days after axotomy (see Experimental Methods). Regenerating distal axons exposed to anti-NGF antibodies halted their growth after 4 days in culture, while those that received NGF continued growing for the remaining period of the assay (Fig. 5A, compare responses at 4 and 6 days). Anti-NGF treatment of distal axons had no effect on cell survival as this was maintained by a low dose (2.5 ng/ml) of NGF supplied to the cell body compartment. Axonal growth in the presence of anti-NGF could be rescued by addition of GDNF to the distal axon compartment (Fig. 5A), indicating that GDNF acting solely on distal axons is also capable of stimulating neurite outgrowth in sympathetic neurons. Neurite density in regenerating distal axons was also restored in cultures treated with GDNF, as assessed by phase contrast microscopy (Fig. 5B) and Western blotting of tubulin in total protein extracts prepared from distal

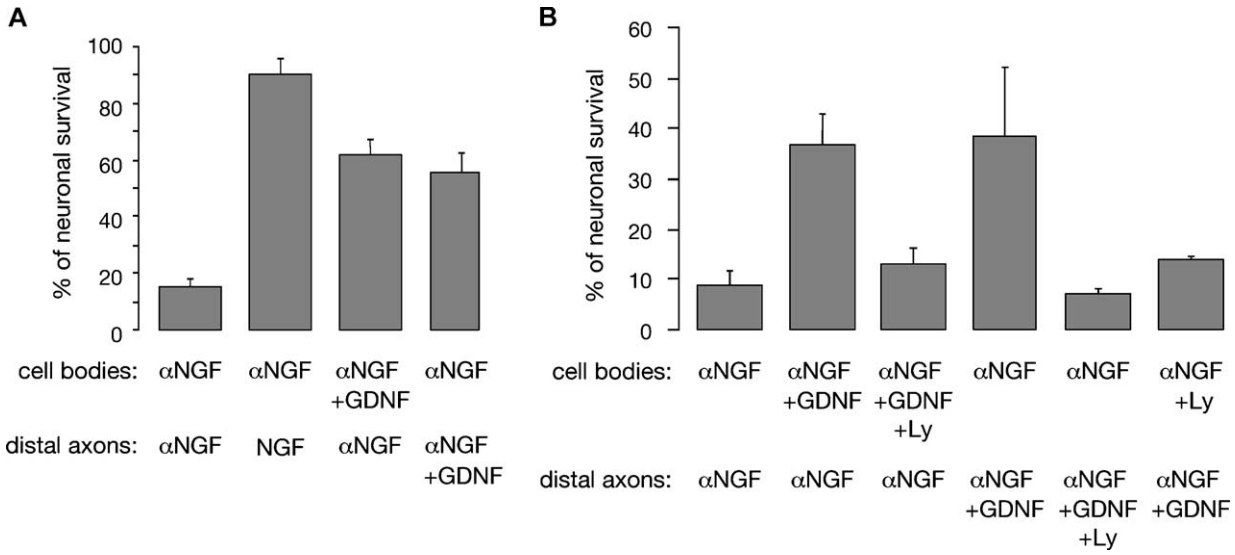

Fig. 4. Exposure of distal axons to GDNF promotes cell survival via the PI3K pathway. (A) Comparable neuronal survival after application of GDNF to either cell bodies or distal axons. (B) The PI3K inhibitor LY 294002 blocked GDNF-induced neuronal survival in either compartment. αNGF, blocking anti-NGF antibodies.

axons (Fig. 5C). The effect of GDNF on both neurite length and density could be mimicked by the GDNF family homolog Neurturin (NTN) but not by Artemin (ART) (Figs. 5A, B, and C). In agreement with this, NTN but not ART, stimulated RET tyrosine phosphorylation in mass cultures of E21 sympathetic neurons (Fig. 5D). In keeping with these observations, Andres et al.

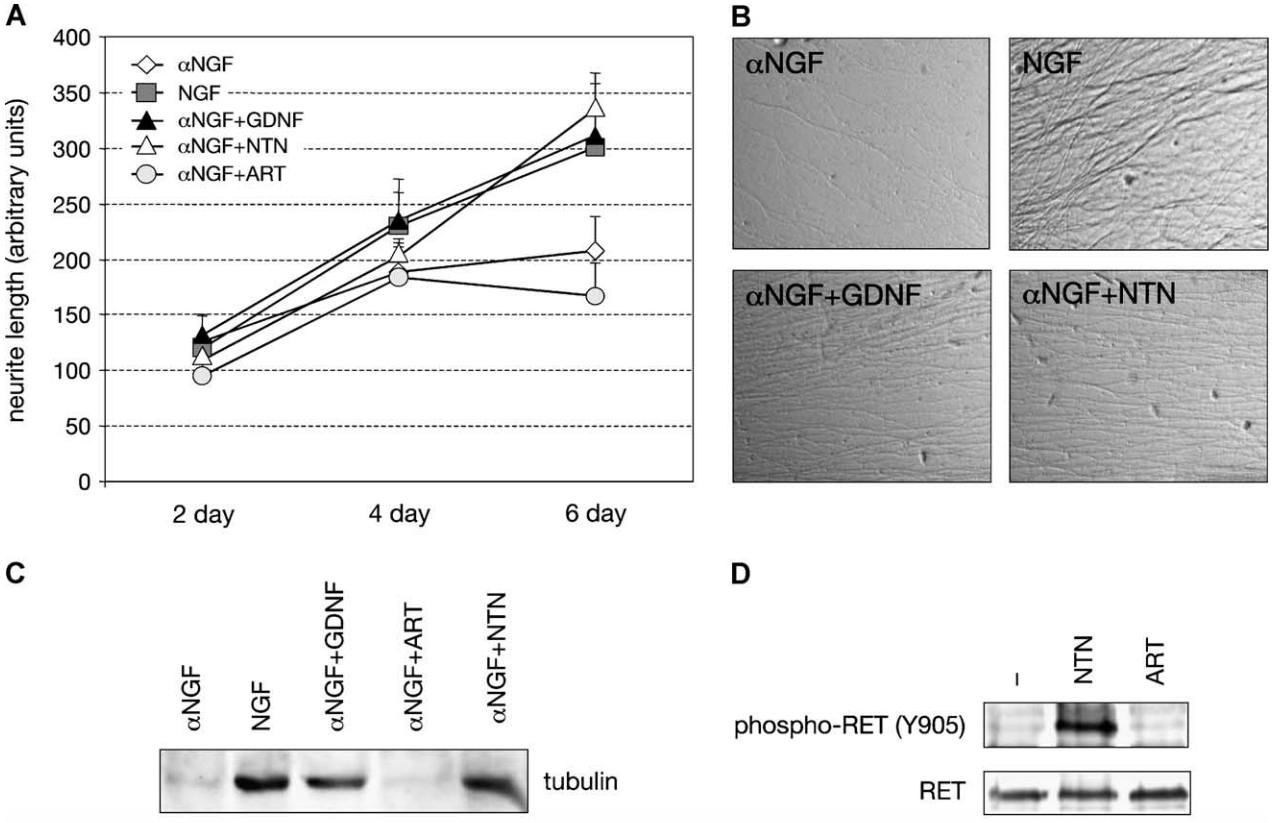

Fig. 5. Exposure of distal axons to GDNF promotes neurite outgrowth. (A) Neurite length (in arbitrary units) under the indicated conditions was evaluated every 2 days in the distal axon compartment. Results are averages of more than 50 determinations from three independent cultures. (B) Photomicrographs illustrating neurite density in the distal axons compartment after the indicated treatments. (C) Neurite density under the indicated conditions was evaluated by the levels of tubulin detected by Western blotting in total extracts of distal axons. (D) NTN but not ART stimulated RET tyrosine phosphorylation in mass cultures of E21 sympathetic neurons. αNGF, blocking anti-NGF antibodies.

(2001) have reported a biphasic developmental response of mouse SCG neurons to ART, with maximal effects at E12 and P20 but negligible effects between E16 and P8.

Discussion

The problem of how signals generated at receptors located in axons and dendrites are propagated to the neuronal cell body and nucleus has attracted the attention of neurobiologists for several decades. Through pioneering studies on the NGF/TrkA system, several models have been put forth to explain how signals are conveyed over long distances from the nerve terminal to the cell body (reviewed and discussed in Ginty and Segal, 2002; Miller and Kaplan, 2001, 2002). The "signaling endosome" hypothesis postulates that, upon ligand binding, the ligand/receptor complex is internalized by endocytosis and retrogradely transported to the cell body in vesicles from which the activated receptor elicits downstream signaling events (Delcroix et al., 2003; Grimes et al., 1996, 1997). Several elegant experiments have been devised to demonstrate that retrograde transport of NGF/TrkA complexes is required for gene transcription and survival responses (Riccio et al., 1997; Ye et al., 2003). An alternative view, the so-called "domino" model, postulates the existence of a wave of ligand-independent phosphorylation of receptors propagating from the axon to the cell body (Senger and Campenot, 1997) and that a neuronal survival signal can reach the cell bodies unaccompanied by the NGF that initiated it (MacInnis and Campenot, 2002), presumably carried by mechanisms downstream of TrkA activity (MacInnis et al., 2003). Whether any of these models may be applicable to other neurotrophic factors has so far been unknown.

GDNF family ligands are produced by target cells and tissues innervated by subpopulations of neurons that are known to depend upon their actions for survival and neurite outgrowth (Airaksinen and Saarma, 2002; Hashino et al., 2001), indicating that they function as target-derived neurotrophic factors. Previous in vivo studies have indicated that GDNF family ligands can be retrogradely transported in several adult neuronal subpopulations, including motor neurons (Leitner et al., 1999), dorsal root ganglion (DRG) and parasympathetic neurons (Laurikainen et al., 2000; Leitner et al., 1999), and dopaminergic neurons (Tomac et al., 1995). Using compartmentalized cultures, we have demonstrated that GDNF can be retrogradely transported in a receptor-mediated manner in sympathetic neurons of the newborn rat SCG, in agreement with its well-known trophic effects on these cells (Buj-Bello et al., 1995; Trupp et al., 1995). This is in contrast to results from a previous in vivo study in which neither GDNF nor NTN was found to be transported to sympathetic neurons of the adult SCG after injection into the anterior eye chamber (Leitner et al., 1999). Lack of GDNF transport by adult sympathetic neurons could have been due to very low levels of GFRα1 and GFRα2 receptors in the rodent adult SCG, as demonstrated by the results of an expression study performed in the adult mouse (Golden et al., 1998). In contrast to the adult SCG, our present results, as well as those from several previous studies, indicate that both GFRα1 and RET receptors are highly expressed in sympathetic neurons of the newborn and early postnatal rat SCG. In our cultures, RET appeared enriched in cell bodies compared to distal axons, while the opposite was true for GFRα1. Intriguingly, early in development and before target innervation, GFRα1 would appear to be less abundant in axons compared to cell bodies, at least in embryonic sensory neurons and paravertebral sympathetic neurons of the chick (Ledda et al., 2002). At these stages, high levels of GFRα1 could be detected in the target tissues of these neurons, heart and carotid body, which were found to decline later in development, concomitantly with target innervation (Ledda et al., 2002). It is thus possible that axonal expression of GFRα1 becomes elevated at later stages, such as those examined in the present study, concurrently with the decline that occurs in target cells.

As predicted by the "signaling endosome" model, we found that GFRα1 could also be retrogradely transported in sympathetic neurons via a saturable, receptor-mediated mechanism that was dependent upon the presence of GDNF. Although these experiments used a soluble form of GFRα1, we expect a similar behavior for GFRα1 molecules present in the membrane as these are known to interact with both GDNF and RET (Trupp et al., 1998) and to be internalized upon GDNF binding (Vieira et al., 2003). At present, our results cannot unequivocally establish whether the same RET molecules activated in distal axons upon ligand binding are retrogradely transported to cell bodies, as the appearance of phosphorylated RET in cell bodies is also compatible with an indirect, "domino"-like effect of signal propagation. Ligand-independent, lateral propagation of receptor activation has been demonstrated for the epidermal growth factor receptor ErbB1 using FRET techniques (Verveer et al., 2000) and has been invoked, but not proven directly as yet, to explain retrograde signaling by the TrkA receptor (MacInnis and Campenot, 2002; Senger and Campenot, 1997). A potential problem with this idea, however, is its inability to explain the unidirectional signal propagation, that is, retrograde but not anterograde, which we have observed following application of GDNF to the distal axons of sympathetic neurons. This would appear to be a general feature of neurotrophic factor signaling as only retrograde, but never anterograde, signal propagation has been observed in the NGF/TrkA system (Watson et al., 2001; Ye et al., 2003).

We have shown that exposure of distal axons to GDNF elicits a rapid and local activation of axonal pools of AKT and ERK1/2 serine-threonine kinases. Activation of axonal ERK1/2 by NGF has been shown to participate in the local regulation of neurite extension (Markus et al., 2002), while activation of the PI3K/AKT pathway has been shown to play a crucial role in the internalization and trafficking of ligand/receptor complexes (Joly et al., 1995; Sasaoka et al., 1999) and in NGF retrograde transport (Kuruvilla et al., 2000). Similar activities are likely to play a role in the local responses of distal axons to GDNF ligands.

In addition to local activation of signaling pathways, exposure of distal axons to GDNF caused the retrograde propagation of activated RET and AKT, but not ERK1/2, to cell bodies. Several studies have confirmed the importance of the PI3K/AKT pathway for neuronal survival in response to GDNF (Besset et al., 2000; Encinas et al., 2001; Soler et al., 1999). In agreement with those results, we have observed that the retrograde signals elicited by GDNF binding to receptors in distal axons are sufficient to maintain survival of sympathetic neurons at levels comparable to those obtained after application of GDNF to cell bodies. In both cases, survival could be blocked by pharmacological inhibition of the PI3K/AKT signaling cascade. The important role of the PI3K/AKT pathway in GDNF-induced survival at distal axons was further demonstrated when Ly application to cell bodies blocked survival, indicating a role for this pathway in both compartments. In contrast to these observations, activated ERK1/2 could not be

detected in cell bodies following GDNF treatment of distal axons. However, this result is in line with the lack of involvement of the Ras/ERK pathway in the survival of sympathetic neurons in the presence of GDNF (Besset et al., 2000). Using compartmentalized cultures of embryonic DRG neurons, Watson et al. (2001) found that retrograde propagation of activated ERK5, but not ERK1/2, was necessary for neuronal survival in response to NGF applied to distal axons. Although previous work in cell lines showed the ability of GDNF to activate ERK5 (Hayashi et al., 2001), in our hands, however, GDNF was unable to activate this enzyme in SCG neurons, either in compartmentalized or mass cultures (data not shown). This discrepancy may be due to differences in the cellular contexts of distinct neuronal subtypes or different developmental stages. It is also possible that a different stimulation regime may be required for activation of ERK5 in SCG neurons, as we have previously observed for activation of CDK5, which required the concomitant exposure of distal axons to GDNF and soluble GFRα1 receptors (Ledda et al., 2002).

In conclusion, our observations demonstrated that GDNF initiates a local signaling at axon terminals which is propagated to the cell body where it mediates survival responses. Using compartmentalized cultures such as those described in the present study, several aspects of GDNF signaling can now be addressed. Notably, it would be interesting to determine whether recently described RET-independent GDNF signaling mechanisms mediated by the neural cell adhesion molecule NCAM (Paratcha et al., 2003), the receptor tyrosine kinase MET (Popsueva et al., 2003), and possibly other receptors (Sariola and Saarma, 2003) may also be differentially activated in distal axons and cell bodies, and whether a retrograde signal can also be generated in those circumstances. It would also be important to investigate whether activation of RET in *trans* by soluble GFRα1 receptors generates different retrograde signals compared to activation in *cis* (Paratcha et al., 2001). Finally, as the short and long isoforms of RET would appear to function in independent signaling complexes (Tsui-Pierchala et al., 2002), it could be asked whether the retrograde signals elicited by these two types of receptors may be different in some way. Answers to these questions may also be of importance for designing strategies towards the use and delivery of GDNF family ligands for the treatment of neurodegenerative diseases and nerve damage.

Experimental methods

Primary culture of sympathetic neurons

Sympathetic neurons were isolated from the superior cervical ganglia (SCG) of embryonic day 21 Sprague–Dawley rats by enzymatic, trypsin 0.025%-collagenase 5 mg/ml (Sigma), followed by mechanical dissociation. For mass cultures, dissociated cells were plated on 35-mm dishes previously coated with polyornithine-laminin (Sigma). Cells were kept in neuronal medium composed by equal parts of Ham F12 and Dulbecco modified Eagle medium (Gibco), 2 mM glutamine, 1 mg/ml bovine serum albumin, penicillin (1 U/ml), streptomycin (1 U/ml), 10% fetal bovine serum, and 20 ng/ml NGF (Promega). Cytosine arabinoside (Sigma), 10 μM, was added 1 day after plating to avoid the proliferation of nonneuronal, mitotically active cells. Medium was changed every 2–3 days. Twelve hours before stimulation, cells were switched to serum-free medium, and stimulation was achieved using 100 ng/ml GDNF (R&D) in the presence of anti-NGF blocking antibodies (1:2000 dilution, Sigma).

Compartmentalized cultures of sympathetic neurons

Compartmentalized cultures of sympathetic neurons were established according to previously described procedures (Campenot, 1992). Briefly, 35- or 60-mm dishes were coated with rat tail collagen and air dried, and parallel scratches were made on the substratum using a pin rake (Tyler Research Instruments, Edmonton, Alberta, Canada). Teflon dividers (Tyler Research Instruments) were sealed onto the collagen tracks previously wetted with a drop of medium, using silicon vacuum grease (Dow Corning). Three different designs of chambers were used in the present study: (i) three-compartment chambers (C10), (ii) five-compartment chambers (C11), and (iii) the so-called biochemistry chambers, a larger design which provides sufficient quantity of material for Western blotting experiments (Tsui-Pierchala and Ginty, 1999). Each chamber was tested for potential leakage by an overnight incubation with medium, and defective chambers were discarded. Neurons were prepared as described above and plated in the central compartment of the chambers: 20,000 to 50,000 cells in C10 and C11 chambers, 250,000 cells in biochemistry chambers. Axons extended into adjacent compartments containing the same medium supplemented with NGF 100 ng/ml. Medium was changed every 2–3 days. Cultures were maintained in a 5% CO_2 atmosphere at 37°C.

Neuronal survival and neurite outgrowth assays

Axons were allowed to extend for 9–10 days after plating. Two to three days before stimulation, cells were incubated in serum-free medium supplemented with NGF. One day before stimulation, the culture was changed to serum-free medium without NGF. For survival assay, 2 μg/ml DiI was added to one of the lateral compartments to allow the (overnight) retrograde labeling of the projecting neurons. On the following day, cells were treated with anti-NGF (1:2000, Sigma) for 4 h in both the center and the side compartments and fluorescent cells were counted. Cells were then stimulated with 100 ng/ml NGF or 100 ng/ml GDNF together with anti-NGF. Some cultures also received GDNF in presence or absence of the phosphatidylinositol-3 kinase (PI3K) inhibitor Ly294002 (Calbiochem), and fluorescent cells were counted again 48 h after treatment. The percentage of survival was determined as the ratio of cell number before and after treatments.

For neurite outgrowth experiments, neurites were allowed to extend under the conditions described above. After 9 days in culture, the neurites in a lateral compartment were removed (axotomy) by repeated exposure to cold distilled water. Axotomy was confirmed by visual examination under phase contrast microscopy. Serum-free medium containing 2.5 ng/ml NGF was added to the central compartment. In previous studies, such a concentration of NGF has been shown to be sufficient to maintain neuronal survival but insufficient to promote neurite outgrowth. Lateral compartments were treated with 100 ng/ml GDNF, NTN, or ART together with anti-NGF antibodies and compared to cultures grown with 100 ng/ml NGF. Neurite outgrowth was evaluated every other day by measuring neurite length using a reticule with a ruler under phase contrast illumination. The same samples were then processed for Western blotting as described below.

Preparation and retrograde transport of radiolabeled GDNF and GFRα1

Radioiodination of GDNF and GFRα1–Fc (R&D) was performed as described previously, using the lactoperoxidase method (Trupp et al., 1995). Cells were grown for 15 days in compartmentalized chambers, and I^{125}–GDNF (50 ng/ml) or I^{125}–GFRα1–Fc (50 ng/ml) were added to the lateral (i.e., distal axons) compartment in the presence of serum-free medium for the indicated periods of time. Extracts from the central (i.e., cell bodies) compartment were prepared by directly adding 1× sample loading buffer into the central compartment after several washes with cold PBS. Samples were electrophoresed by SDS–PAGE and transferred onto PVDF membranes (Amersham). Retrogradely transported I^{125}–GDNF and I^{125}–GFRα1–Fc were visualized in a STORM 860 phosphorimager. Blots were subsequently probed with antitubulin III antibodies (Sigma) for protein normalization.

Western blotting and antibodies

To recover sufficient quantity of material, all biochemistry analyses in compartmentalized cultures were performed using neurons grown in larger, so-called "biochemistry" chambers. Axons were allowed to extend for 15 days. Before simulation, cells were incubated for 4 h in serum-free medium containing anti-NGF. Cells were then stimulated with 200 ng/ml GDNF together with anti-NGF either at the cell bodies or at distal axons compartments. Cell bodies and distal axons extracts were then obtained at the indicated time points by directly adding 1× sample loading buffer to the compartment. Samples were electrophoresed by SDS–PAGE and blotted onto PVDF membranes. Primary antibodies used for Western blotting analyses are as follows: (i) anti-phospho-Ret (P-RET) affinity-purified, polyclonal antibodies that specifically recognize phosphotyrosine residues at positions 905 or 1062—as indicated—in mammalian RET (Coulpier et al., 2002); (ii) anti-P-AKT (Biolabs); (iii) anti-P-ERK1/2 (Biolabs); (iv) anti-RET51 long isoform (Santa Cruz); (v) anti-RET9 short isoform (Santa Cruz); (vi) anti-GFRα1 (R&D); and (vii) anti-tubulin III (Sigma). When anti-phospho-Ret-specific antibodies were used, secondary antibody was HRP-conjugated (Amersham). Immunoblots were developed with the West Femto Detection System from Pierce. In all other cases, secondary antibodies were alkaline phosphatase-conjugated (Amersham). Immunoblots were developed with the ECF Western Detection System from Amersham and scanned in a STORM 860 fluorimager. Before reprobing, immunoblots were stripped for 90 min at room temperature in 0.1 M acetic acid, 0.15 M NaCl.

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