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Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons

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ABSTRACT **Neurturin (NTN) is a neurotrophic factor that shares homology with glial cell line-derived neurotrophic factor (GDNF). Recently, a receptor complex has been identified for GDNF that includes the Ret tyrosine kinase receptor and a glycosylphosphatidylinositol-linked protein termed "GDNFRα." However, differences in the phenotype of Ret and GDNF knockout animals suggest that Ret has at least one additional ligand. In this report, we demonstrate that NTN induces Ret phosphorylation in primary cultures of rat superior cervical ganglion (SCG) neurons. NTN also caused Ret phosphorylation in fibroblasts that were transfected stably with Ret and GDNFRα but not in cells expressing Ret alone. A glycosylphosphatidylinositol-linked protein also was important for NTN and GDNF signaling in SCG neurons; phosphatidylinositol-specific phospholipase C treatment of SCG cultures reduced the ability of NTN to phosphorylate Ret and the ability of NTN or GDNF to activate the mitogen-activated protein kinase pathway. NTN and GDNF also caused sustained activation of Ret and the mitogen-activated protein kinase pathway in SCG neurons. Finally, both NTN and GDNF activated the phosphatidylinositol 3-kinase pathway in SCG neurons, which may be important for the ability of NTN and GDNF to promote neuronal survival. These data indicate that NTN is a physiologically relevant ligand for the Ret receptor and suggest that NTN may have a critical role in the development of many neuronal populations.**

Neurturin (NTN) is a neurotrophic factor that was purified and cloned based on its ability to support the survival of SCG (SCG) neurons *in vitro* (1). The amino acid sequence of NTN is 42% identical to mature glial cell line-derived neurotrophic factor (GDNF), and, together, NTN and GDNF compose a subfamily of the transforming growth factor β superfamily. In this regard, we propose to name this family of neurotrophic factors the "TRN family" (transforming growth factor β-related neurotrophic factor family). In addition to sequence similarity, NTN and GDNF exhibit similar biologic activities. For example, both factors support comparable numbers of SCG neurons, nodose neurons, and dorsal root ganglion neurons *in vitro* (1).

Because GDNF is a member of the transforming growth factor β superfamily, it would seem that the GDNF receptor would be a transmembrane serine/threonine kinase. Recently, however, the Ret tyrosine kinase protein was identified as the signal transducing component of the GDNF receptor complex (2–6). The association between Ret and GDNF was deduced in part from the phenotypic similarities of mice with null mutations for either the ligand or the receptor. Transgenic animals lacking Ret (7) or GDNF (8–10) die soon after birth, and a null mutation in either gene results in dysplastic kidneys and a failure of the enteric nervous system to develop properly. In addition to Ret, another protein, termed "GDNF receptor α" (GDNFRα), was cloned based on its ability to bind GDNF (3, 4). GDNFRα attaches to the membrane through a glycosylphosphatidylinositol (GPI) linkage and facilitates the activation of Ret (3, 4). GDNFRα, whether attached to the membrane or in soluble form, binds GDNF and leads to Ret activation (3, 4).

Although the similarities between the Ret and the GDNF null mutations provided evidence for a link between these proteins, the knockout animals also suggested that GDNF may not be the sole ligand for Ret. The Ret $-/-$ and GDNF $-/-$ animals have distinct differences. For example, in the GDNF knockout, $\approx$35% of the cells in the SCG are lost (10) whereas in the Ret knockout, the SCG is completely absent (11). Given these discrepancies in the phenotypes of knockout animals and the striking similarities between NTN and GDNF activities, we investigated whether NTN might also serve as a physiologically relevant ligand for Ret by analyzing the signal transduction pathways activated by NTN and GDNF in primary sympathetic neurons.

MATERIALS AND METHODS

Cell Culture. Neuronal cultures were prepared from the SCG of postnatal day 1 rats (12). The neurons were plated on collagen-coated dishes and maintained at 37°C in a humidified atmosphere (5% CO_2 in air) in medium comprised of minimal essential medium (Earle's salts, Life Technologies, Gaithersburg, MD) containing 2 nM nerve growth factor (NGF), 10% fetal bovine serum (HyClone), 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, and 10 μM aphidicolin. After 7 days, the neuronal cultures were deprived of NGF by switching to medium lacking NGF and containing an anti-NGF antibody (13). This medium was replaced after 2–4 h with medium containing NTN, GDNF, or NGF. To terminate the incubation, the medium was aspirated, and the neurons were rinsed once in cold PBS (2.7 mM KCl/140 mM NaCl/1.5 mM KH_2PO_4/10 mM Na_2HPO_4, pH 7.4) and then lysed in either 500 μl of immunoprecipitation buffer [1 mM EDTA/1 mM EGTA/0.2 mM $NaVO_3$/1 mM Pefabloc (Boehringer Mannheim)/1 μM pepstatin A/10 μg/ml leupeptin/2 μg/ml aprotinin/1% Triton X-100/0.5% Nonidet P-40/150 mM NaCl in 10 mM Tris, pH 7.4] or 200 μl of SDS sample buffer. The lysates were stored at -70°C until analyzed on Western blots.

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Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GDNFRα, GDNF receptor α; GPI, glycosylphosphatidylinositol; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; NTN, neurturin; PI-3-K assay, phosphatidylinositol 3-kinase assay; PI-PLC, phosphatidylinositol-specific phospholipase C; SCG, superior cervical ganglion.

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NIH 3T3 cells were grown in DMEM plus 10% fetal bovine serum (HyClone). Stable transfectants expressing Ret were selected in medium containing 1 mg/ml G418 after calcium phosphate precipitation of the full length human Ret cDNA subcloned into the pCMV-Neo vector (14). The human Ret cDNA was the generous gift of H. Donnis-Keller, Washington University School of Medicine. Positive clones were assayed for Ret expression on immunoblots probed with a Ret antibody (Santa Cruz Biotechnology). A clonal line of the Ret-expressing 3T3 cells was used to generate Ret- and GDNFRα-expressing fibroblasts. The GDNFRα cDNA was obtained from a rat postnatal day 1 library using klentaq long and accurate PCR (15) with the following primers: 5′-GCG GTA CCA TGT TCC TAG CCA CTC TGT ACT TCG C-3′ and 5′-GCT CTA GAC TAC GAC GTT TCT GCC AAC GAT ACA G-3′. The amplified product was cloned into the *Eco*RV site of pBluescript KS (Stratagene) and sequenced. The cDNA was subsequently subcloned into the *Hin*dIII and *Bam*HI sites of pCMV-Neo. The GDNFRα–pCMV-Neo plasmid was co-transfected with SV2-HisD into a line of the Ret-expressing 3T3 cells, and a double transfectant was selected in 2 mM histidinol. Expression of GDNFRα was assayed by Northern blots. SV2-HisD was the generous gift of R. Mulligan, Massachusetts Institute of Technology.

Preparation of NTN and GDNF. The GDNF expression plasmid was prepared by cloning the rat GDNF cDNA sequence into pET30a(+). The GDNF coding sequence was obtained from an embryonic day 21 rat kidney cDNA library by PCR using primers 5′-CAG CAT ATG TCA CCA GAT AAA CAA GCG GCG GCA CT-3′ and 5′-CAG GGA TCC GGG TCA GAT ACA TCC ACA CCG TTT AGC-3′ and subcloned into pET30a(+) using the *Nde*I and *Sal*I sites. A six-His tag and enterokinase site was placed at the amino terminus of GDNF sequence by inserting oligonucleotide linkers A (5′-TAT GCA CCA TCA TCA TCA TCA TGA CGA CGA CGA CAA GGC-3′) and B (5′-TAG CCT TGT CGT CGT CGT CAT GAT GAT GAT GAT GGT GCA-3′) into the *Nde*I site.

A synthetic gene was prepared for the mouse NTN coding sequence using the *Escherichia coli* codon preferences. Four partially overlapping oligonucleotides (oligos 1–4 below) were gel purified and annealed for 10 min at 68°C and then 30 min at 22°C. Primer extension was used to extend the annealed oligonucletides, and the resultant fragment was ligated into pBluescript KS plasmid. The authenticity of the NTN fragment was verified by nucleotide sequencing and transferred to the *Nde*I and *Bam*HI sites of pET30a(+). A six-His tag and enterokinase site was added to the amino terminus of the NTN sequence at the *Nde*I site as above. The oligonucleotide sequences were as follows: oligo 1, 5′-GCA TAT GCC GGG TGC TCG TCC GTG CGG CCT GCG TGC AAC TGG AAG TTC GTG TTT CTG AAC TGG GTC TGG GTT ACA CTT CTG ACG AAA CTG T-3′; oligo 2, GCT GAC GCA GAC GAC GCA GAC CCA GGT CGT AGA TAC GGA TAG CAG CTT CGC ATG CAC CAG CGC AGT AAC GGA ACA GAA CAG TTT CGT-3′; oligo 3, 5′-CTG CGT CAG CGT CGT CGT GTT CGT CGT GAA CGT GCT CGT GCT CAC CCG TGC TGC CGT CCG ACT GCT TAC GAA GAC GAA GTT TCT TTC-3′); and oligo 4 (5′-CGG ATC CTT AAA CGC AAG CGC ATT CAC GAG CAG ACA GTT CCT GCA GAG TGT GGT AAC GAG AGT GAA CGT CCA GGA AAG AAA CTT CG-3′).

The NTN and GDNF pET30a(+) constructs were sequenced to confirm their authenticity and then transformed into *E. coli* strain BL21/DE3. The bacteria were grown at 37°C in 2× YT medium (30 μg/ml kanamycin) with vigorous shaking. For GDNF production, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1.0 mM to induce expression of the protein after the culture reached OD_{600} = 0.7. Incubation was continued for an additional 2 h. Bacteria containing the NTN plasmid were grown for 24 h without IPTG. Cells were harvested by centrifugation at 4000 × *g* for 20 min, solubilized with buffer A (6 M guanidine HCl/0.1 M sodium phosphate/10 mM Tris·HCl, pH 8.0) at 1/10 the volume of original culture volume and rocked overnight. Lysate was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was exposed to 4 ml of nickel-NTA (nitrilotriacetic acid; Qiagen, Chatsworth, CA) resin per 1 liter of original culture and washed with 10–20 column volumes of buffer A, 10 column volumes of buffer B (8 M urea/0.1 M sodium phosphate/10 mM Tris·HCl, pH 8.0), and 5–10 volumes of buffer C (8 M urea/0.1 M sodium phosphate/10 mM Tris·HCl, pH 6.3) until the A_{280} was <0.01. The recombinant protein was eluted with 10–20 ml of buffer E (8 M urea/0.1 M sodium phosphate/10 mM Tris·HCl, pH 4.5). Fractions were collected and analyzed by SDS/PAGE. The eluate was immediately diluted to 50 ng/μl in buffer B and dialyzed against 4 M renaturation buffer (4 M urea/5 mM cysteine/0.02% Tween-20/10% glycerol/10 mM Tris·HCl/150 mM NaCl/100 mM sodium phosphate, pH 8.3, under argon) at 4°C overnight and then against 2 M renaturation buffer (as above except 2 M urea) for 2–3 days with changes every 24 h.

Immunoprecipitations and Western Blotting. Cultures were lysed by adding 500 μl of immunoprecipitation buffer, described above, for 20 min at 4°C. A 50-μl fraction was removed for blots of total protein, and the remaining cell lysate was immunoprecipitated with 20 μl of anti-phosphotyrosine antibodies conjugated to agarose (Oncogene Research Products, Cambridge, MA) for 1 h at 4°C. The antibody–agarose conjugate was washed three times with immunoprecipitation buffer and then solubilized by boiling for 5 min in SDS sample buffer.

Samples were subjected to SDS/PAGE in either 7.5 or 10% gels. After electrophoresis, the gels were transferred to poly(vinylidene difluoride) membranes (Millipore). The blots were blocked for 1 h at 25°C in solution W (Tris-buffered saline: 150 mM NaCl in 50 mM Tris, pH 7.4) containing 0.1% Tween-20 and 5.0% wt/vol nonfat dry milk and then incubated for 24 h at 4°C in primary antibody diluted in Tris-buffered saline plus 0.05% Tween-20 and 5% BSA. The membranes were then washed in solution W (3 × 15 min) and incubated for 1 h in solution W containing a 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit antibody (New England Biolabs). This was followed by three washes (15 min/wash) in solution W, two washes (10 min/wash) in Tris-buffered saline plus 0.1% Tween-20, and two washes (10 min/wash) in 1 mM $MgCl_2$, 10 mM NaCl, and 10 mM Tris·HCl (pH 9.5). The blot was then incubated for 5 min in CDP-Star (Tropix, Bedford, MA) and exposed to x-ray film.

To insure equal loading of samples for the mitogen-activated protein kinase (MAPK) blots, the blots were stripped and re-probed with an antibody that detects total MAPK (New England Biolabs). The membranes were stripped by incubating for 1 h at 25°C in 1.0% Tween-20, 0.1% SDS, and 0.2 M glycine (pH 2.2). To verify that the blots were completely stripped, they were washed as described above and developed by using the CDP-Star reagent. The blots were then returned to solution W for 1 h and probed as described above with the total MAPK antibody diluted 1:1000.

Phosphatidylinositol 3-Kinase (PI-3-K) Assay. PI-3-K assays were performed as described by Newberry and Pike (16). Neuronal cultures were washed once with PBS and then lysed in PI-3-K lysis buffer (1 mM $MgCl_2$/1 mM $CaCl_2$/0.4 mM $NaVO_3$/1 mM Pefabloc/1 μg/ml leupeptin/1 μg/ml aprotinin/1 mM DTT/10% glycerol/1% Triton X-100/137 mM NaCl in 20 mM Tris, pH 8.0). The samples were immunoprecipitated with phosphotyrosine antibody conjugated to agarose as described above. Immune complexes were isolated by centrifugation and washed twice with PBS plus 1% Triton X-100, twice with 0.5 mM LiCl in 10 mM Tris (pH 7.5), and

twice with 100 mM NaCl and 1 mM EDTA in 10 mM Tris (pH 7.5). PI-3-K activity was assayed in an *in vitro* kinase reaction, and the products were separated by thin-layer chromatography (17). Phosphatidylinositol 3-phosphate was identified based on its migration relative to that of a radiolabeled phosphatidylinositol marker (18), which was the generous gift of L. Pike, Washington University School of Medicine. Radioactivity was visualized and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Materials. NGF was prepared from male mouse submaxillary glands by the method of Bocchini and Angeletti (19). The anti-Ret antibody was from Santa Cruz Biotechnology, the phospho-specific MAPK antibody was from New England Biolabs, and recombinant phosphatidylinositol-specific phospholipase C was obtained from Oxford Glycosystems (Rosedale, NY). All other reagents were obtained from Sigma. Each figure presented is representative of similar results obtained from at least three independent experiments.

RESULTS

The purification and cloning of NTN was achieved based on the ability of NTN to promote SCG neuronal survival (1). To determine whether Ret is involved in NTN signaling, SCG neurons were assessed for Ret phosphorylation after treatment with NTN. Cultures of SCG neurons were plated and maintained in NGF for 7 days and then deprived of NGF for 4 h before the addition of NTN, NGF, or vehicle. Lysates were prepared after 10 min and assayed for Ret tyrosine phosphorylation. A fraction of each sample was saved to determine the amount of Ret in total lysates (Fig. 1, lanes 1–3); the remainder of the lysate was immunoprecipitated with an anti-phosphotyrosine antibody (Fig. 1; lanes 4–6). SCG neurons treated with NTN (Fig. 1, lane 5), but not NGF (Fig. 1, lane 6), contained tyrosine-phosphorylated Ret protein. Thus, as has been reported for GDNF, NTN activates the Ret receptor.

GDNF activation of Ret depends on the presence of GDNFRα, a GPI-linked protein that serves as a high affinity binding site for GDNF (3, 4). To assess whether NTN also requires this accessory protein to activate Ret, mouse 3T3 fibroblasts were transfected with a Ret expression vector and subsequently with a plasmid expressing GDNFRα. Expression of Ret and GDNFRα was confirmed by protein or RNA blot analysis (data not shown). Ret Westerns of total cell lysates from the transfected fibroblasts revealed a doublet (Fig. 2) whereas the neurons contained a single band (Fig. 1) that comigrated with the higher molecular weight species in the fibroblasts. This higher molecular weight protein represents

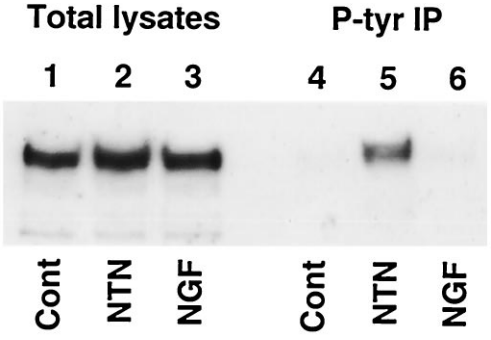

FIG. 1. NTN induces Ret tyrosine-phosphorylation in SCG neurons. SCG cultures were deprived of NGF for 4 h then treated with medium containing no additions [Control (Cont), lanes 1 and 4], 50 ng/ml NTN (lanes 2 and 5), or 50 ng/ml NGF (lanes 3 and 6). After 10 min, the cultures were lysed. Protein immunoblots were probed with an anti-Ret antibody; lanes 1–3 contain total lysates, and lanes 4–6 contain phosphotyrosine immunoprecipitates of the corresponding samples.

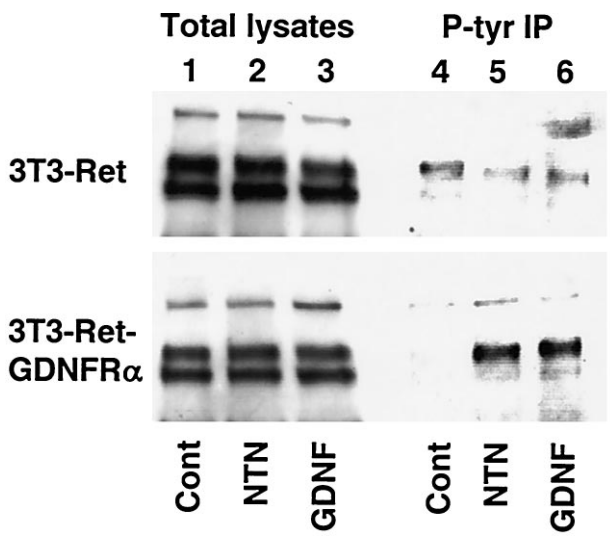

FIG. 2. NTN induces Ret phosphorylation in fibroblasts expressing Ret and GDNFRα but not in those expressing Ret alone. NIH 3T3 fibroblasts expressing Ret alone (*Upper*) or Ret and GDNFRα (*Lower*) were treated with medium containing no additions [Control (Cont), lanes 1 and 4], 50 ng/ml NTN (lanes 2 and 5), or 50 ng/ml GDNF (lanes 3 and 6). Western blots of total protein (lanes 1–3) or phosphotyrosine immunoprecipitates (lanes 4–6) were probed with an anti-Ret antibody.

mature Ret protein (20), and only this band is phosphorylated upon Ret activation (see below). In the fibroblasts expressing Ret alone, neither NTN nor GDNF induced Ret phosphorylation (Fig. 2, *Upper*). However, in fibroblasts expressing GDNFRα in addition to Ret, either NTN or GDNF treatment induced Ret phosphorylation (Fig. 2, *Lower*).

A similar requirement for a GPI-linked accessory protein was found in SCG neurons treated with NTN. SCG cultures were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) to cleave GPI-linked proteins from the membrane. This treatment reduces the survival-promoting ability of GDNF on several neuronal populations including SCG neurons (3). For these experiments, the neurons were switched to serum-free N2 medium to maximize the activity of PI-PLC. Treatment of SCG neurons with 1 unit/ml PI-PLC before the addition of NTN decreased the ability of NTN to induce Ret phosphorylation (Fig. 3*A*). The PI-PLC-treated cultures contained little detectable Ret in the phosphotyrosine immunoprecipitates (Fig. 3*A*, lane 6). Thus, as with GDNF (3), PI-PLC treatment interfered with the ability of NTN to induce Ret phosphorylation.

We also assessed the effect of PI-PLC treatment on MAPK activation to determine whether the level of Ret phosphorylation correlated with downstream signaling events. Either NTN or GDNF causes an increase in MAPK activation in SCG neurons (1). Western blots of total cell lysates were probed with a phospho-specific MAPK antibody to assess MAPK activity. As with Ret phosphorylation, PI-PLC treatment reduced the ability of NTN (Fig. 3*B*, lanes 3 and 4) or GDNF (Fig. 3*B*, lanes 5 and 6) to stimulate MAPK phosphorylation. In contrast, NGF-induced MAPK phosphorylation was not affected by PI-PLC treatment (Fig. 3*B*, lanes 7 and 8), indicating that PI-PLC treatment is specifically inhibiting NTN and GDNF signaling.

Because NTN promotes SCG survival equivalent to NGF (1), we were interested in examining the signal transduction cascades activated by NTN and GDNF. For example, NGF causes a sustained activation of the Trk receptor (21, 22) and the MAPK pathway (23–26). To determine whether NTN or GDNF also promotes long term activation, neurons were treated with NTN or GDNF for up to 30 h before assaying for

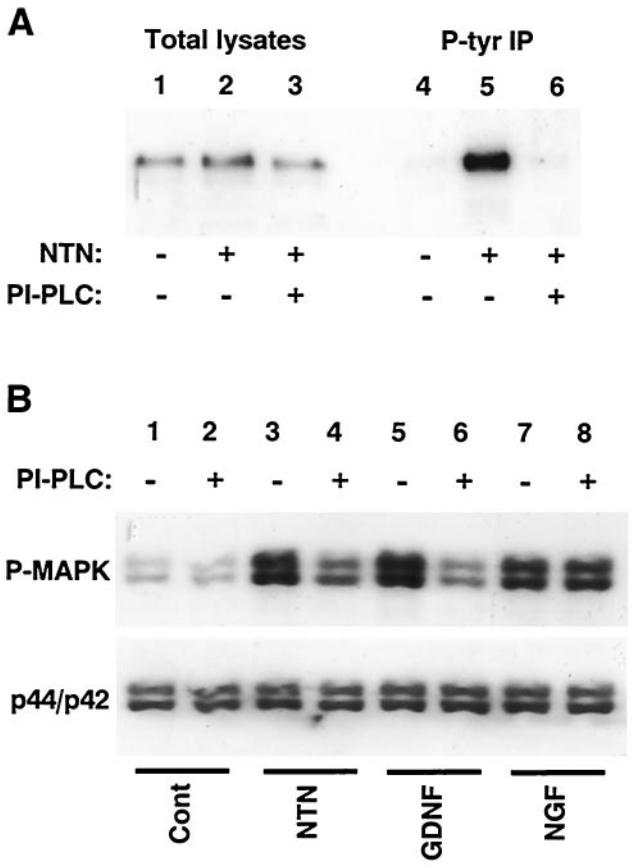

FIG. 3. Cleavage of GPI linkages reduces the ability of NTN to induce Ret phosphorylation or MAPK activation. (*A*) Before the addition of 20 ng/ml NTN for 10 min, SCG cultures were treated for 2 h with or without PI-PLC to cleave GPI-linked proteins from the cell membrane. Western blots of total lysates (lanes 1–3) and corresponding phosphotyrosine immunoprecipitates (lanes 4–6) were probed with an anti-Ret antibody. (*B*) SCG neurons treated with or without PI-PLC for 2 h were then treated for 10 min with medium containing no additions (lanes 1 and 2), 20 ng/ml NTN (lanes 3 and 4), 20 ng/ml GDNF (lanes 5 and 6), or 20 ng/ml NGF (lanes 7 and 8). A protein blot of total cell lysates was first probed with a phospho-specific MAPK antibody (P-MAPK; *Upper*); the same blot was then re-probed with an antibody that recognizes phosphorylated and nonphosphorylated MAPK (p44/p42; *Lower*).

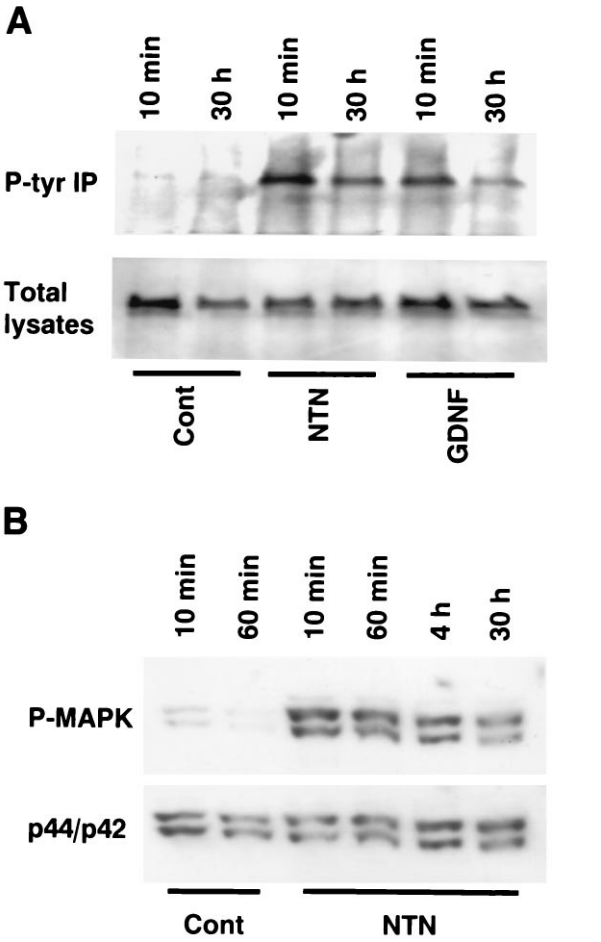

FIG. 4. NTN causes sustained activation of Ret and MAPK. (*A*) SCG cultures were treated with control medium (Cont), NTN, or GDNF and lysed either 10 min or 30 h after treatment. Western blots of phosphotyrosine immunoprecipitates (*Upper*) and corresponding total lysates (*Lower*) were probed with an anti-Ret antibody. (*B*) SCG cultures were deprived of NGF for 2 h then treated with control medium (Cont) or NTN for 10 min, 60 min, 4 h, or 30 h. A protein blot of total cell lysates was first probed with a phospho-specific MAPK antibody (P-MAPK; *Upper*) and then re-probed with an antibody that recognizes phosphorylated and nonphosphorylated MAPK (p44/p42; *Lower*).

Ret phosphorylation and MAPK activation. Either NTN or GDNF caused sustained Ret phosphorylation (Fig. 4*A*), similar to the sustained activation of Trk in the presence of NGF. NTN (Fig. 4*B*) or GDNF (data not shown) also caused a sustained activation the MAPK pathway.

PI-3-K also is activated by NGF (27) and has been implicated in the survival of PC12 cells (28) and cerebellar granule cells (29, 30). To assess the ability of NTN or GDNF to activate PI-3-K, neuronal cultures were treated for 10 min with NTN, GDNF, NGF, or vehicle, then lysed, and phosphotyrosine immunoprecipitates were assayed for PI-3-K activity. Like NGF, treatment with either NTN or GDNF stimulated PI-3-K (Fig. 5), indicating that all three of these neurotrophic factors share this intracellular signaling pathway.

DISCUSSION

We have established that NTN can activate the Ret receptor and that NTN and GDNF activate the MAPK and PI-3-K signaling pathways in primary neurons. These data suggest that NTN acts as a physiologically relevant ligand for Ret *in vivo*. Multiple ligands for Ret would be consistent with the differences between the Ret and GDNF knockout animals. Although both null mutations lack a distal enteric nervous system and have dysplastic kidneys, in the Ret knockout, the SCG is completely absent (11) whereas in the GDNF knockout, the SCG has only a 35% loss (10).

The loss of the SCG in the Ret knockout mouse occurs well before the critical period of naturally occurring cell death in the SCG during the first postnatal week (31), when these neurons are dependent on NGF (32). At embryonic day 10.5, the SCG anlage appear normal in Ret-deficient animals, but 48 h later little trace is seen of a presumptive ganglion in the region of the dorsal aorta where the SCG forms (11). This suggests that Ret activation is important for maintaining the survival of rostral sympathetic neuroblasts or for stimulating the proliferation of these cells during this critical time (11). The failure of the GDNF knockout to show complete loss of the SCG raises the possibility that NTN may also be a critical Ret ligand during this stage of development. These observations with Ret are somewhat reminiscent of those obtained with Trk C and neurotrophin-3. Trk C, together with its ligand neurotrophin-3, contributes to the formation of the SCG but, again, not in the context of naturally occurring cell death. Null mutants for neurotrophin-3 have 50% fewer SCG neurons at the time of birth (33).

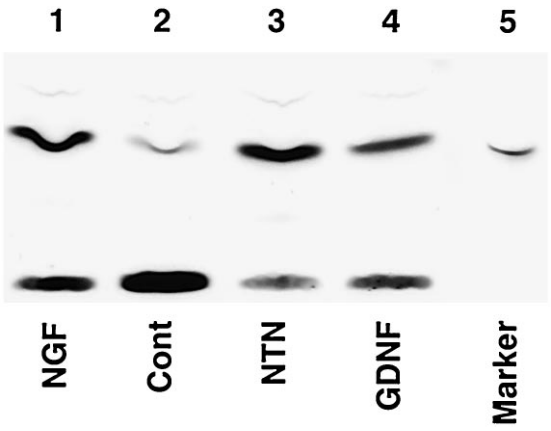

FIG. 5. NTN and GDNF activate PI-3-K. SCG cultures were deprived of NGF for 4 h and then treated with medium containing 50 ng/ml NGF (lane 1), control medium (Cont; lane 2), 50 ng/ml NTN (lane 3), or 50 ng/ml GDNF (lane 4) for 30 min. The cultures were lysed, and phosphotyrosine immunoprecipitates were assayed for PI-3-K activity. Lane 5 contains a ^{32}P-radiolabeled phosphatidylinositol-3-phosphate marker.

NTN did not cause Ret phosphorylation in 3T3 cells expressing Ret alone, similar to what has been observed with GDNF (3, 4). Moreover, the fact that coexpression of GDNFRα conferred the ability of NTN to induce Ret phosphorylation rapidly provides evidence that NTN also interacts with GDNFRα. This is consistent with the extensive overlap of NTN and GDNF activity (1) and with our finding that a GPI-linked protein was important for NTN and GDNF signaling in SCG neurons.

Several other treatments are capable of maintaining SCG neurons *in vitro*, including cAMP (34–36), potassium depolarization (22), and ciliary neurotrophic factor or leukemia inhibitory factor (37). Although adequate survival-promoting agents, none of these treatments promotes the overall growth of the neurons as does NGF. This is likely because of differences in the intracellular pathways activated by different survival-promoting agents. Whereas NGF promotes a sustained activation of the MAPK pathway in SCG neurons (25, 26), cAMP fails to activate MAPK (25, 26), and ciliary neurotrophic factor causes only a transient activation (25). Potassium depolarization does activate MAPK, but the level of activation is considerably less than that obtained with NGF (unpublished observations). In PC12 cells, NGF-induced MAPK activation is essential for neurite outgrowth (38). In SCG neurons, these differences in the ability to stimulate MAPK may, therefore, account for the differences in growth. This correlation is strengthened by our results with NTN and GDNF, which, through stimulation of Ret, also induce sustained activation of MAPK and promote the growth of SCG neurons. Of interest, EGF-Ret chimeric receptors treated with EGF also display persistent receptor phosphorylation compared with the EGF receptor (39).

NTN and GDNF, like NGF, also stimulated PI-3-K activity. The PI-3-K activity is of particular interest because this pathway has been implicated in survival promotion whereas inhibition of MEK, the upstream activator of MAPK, does not affect survival (26, 40). Inhibition of PI-3-K blocks the NGF-mediated survival of PC12 cells (28), and this pathway is important in the survival of cerebellar granule neurons maintained by potassium depolarization, insulin-like growth factor I (29), or insulin (30). PI-3-K activity can also block c-myc-induced cell death (41), and inhibition of PI-3-K causes apoptosis in oligodendrocytes (42). Of the known targets of PI-3-K, the serine/threonine protein kinase AKT/PTB is important for promoting survival (30, 41). The similarities among NTN, GDNF, and NGF signaling raise the possibility that these factors may all promote neuronal survival by activating PI-3-K.

The finding that NTN can activate Ret may help explain why some neuronal populations appear unperturbed in the GDNF knockout mice. For instance, because GDNF was identified based on its ability to sustain tyrosine hydroxylase-positive neurons from central nervous system cultures (43), it seems surprising that these neurons are not decreased in the substantia nigra of GDNF knockout animals. One possible explanation for this finding is that the deficits might appear later in development, but the GDNF knockout mice die shortly after birth (8–10). Another possibility is that an additional Ret ligand may provide redundant Ret activation to insure survival of these neurons. The identification of NTN as a physiologically relevant ligand for Ret in primary neurons may help resolve some of these apparent discrepancies.

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