6.3. La supervivencia de las motoneuronas espinales de pollo mantenidas con GFLs está mediada por PI 3-K

The Journal of Neuroscience, November 1, 1999, *19*(21):9160–9169

Receptors of the Glial Cell Line-Derived Neurotrophic Factor Family of Neurotrophic Factors Signal Cell Survival through the Phosphatidylinositol 3-Kinase Pathway in Spinal Cord Motoneurons

Rosa M. Soler, Xavier Dolcet, Mario Encinas, Joaquim Egea, Jose R. Bayascas, and Joan X. Comella

Grup de Neurobiologia Molecular, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, 25198 Lleida, Catalonia, Spain

The members of the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors (GDNF, neurturin, persephin, and artemin) are able to promote *in vivo* and *in vitro* survival of different neuronal populations, including spinal cord motoneurons. These factors signal via multicomponent receptors that consist of the Ret receptor tyrosine kinase plus a member of the GDNF family receptor α (GRFα) family of glycosylphosphatidylinositol-linked coreceptors. Activation of the receptor induces Ret phosphorylation that leads the survival-promoting effects. Ret phosphorylation causes the activation of several intracellular pathways, but the biological effects caused by the activation of each of these pathways are still unknown. In the present work, we describe the ability of the GDNF family members to promote chicken motoneuron survival in culture. We show the presence of Ret and GFRα-1, GFRα-2, and GFRα-4 in chicken motoneurons using *in situ* hybridization and reverse transcription-PCR techniques. By Western blot analysis and kinase assays, we demonstrate the ability of these factors to induce the phosphatidylinositol 3 kinase (PI 3-kinase) and the extracellular regulated kinase (ERK)–mitogen-activated protein (MAP) kinase pathways activation. To characterize the involvement of these pathways in the survival effect, we used the PI 3-kinase inhibitor LY 294002 and the MAP kinase and ERK kinase (MEK) inhibitor PD 98059. We demonstrate that LY 294002, but not PD 98059, prevents GDNF-, neurturin-, and persephin-induced motoneuron survival, suggesting that PI 3-kinase intracellular pathway is responsible in mediating the neurotrophic effect.

Key words: GDNF; persephin; neurturin; artemin; motoneuron; neurotrophic factor; GFRα receptors; chicken; intracellular signaling pathway

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), persephin (PSP), and artemin (ART) are the members of a new family of neurotrophic factors distantly related to the members of the TGFβ family (Lin et al., 1993; Buj-Bello et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Maxwell et al., 1996; Klein et al., 1997; Baloh et al., 1998; Horger et al., 1998; Milbrandt et al., 1998). All of them can support the survival of a wide variety of neuronal populations in both the peripheral nervous system and CNS, including motoneurons (MTN) (Henderson et al., 1994; Oppenheim et al., 1995; Klein et al., 1997; Milbrandt et al., 1998). GDNF family members signal via multicomponent receptors that consist of the Ret receptor tyrosine kinase plus a glycosylphosphatidylinositol (GPI)-linked coreceptor named GDNF family receptor α (GFRα), which gives binding specificity. GDNF binds preferentially to GFRα-1, NTN shows binding preference to GFRα-2, PSP to GFRα-4, and ART to GFRα-3 (Buj-Bello et al., 1997; Klein et al., 1997; Baloh et al., 1998; Enokido et al., 1998). Recently, deficient mice for the GDNF family members GDNF (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) and NTN (Heuckeroth et al., 1999), for their cognate receptors GFRα-1 (Cacalano et al., 1998; Enomoto et al., 1998) and GFRα-2 (Rossi et al., 1999) or for Ret (Schuchardt et al., 1994), have been generated. Analysis of the trigeminal motor nucleus and the spinal cord show moderate loss of MTNs in GDNF (Moore et al., 1996; Sanchez et al., 1996) and GFRα-1 (Cacalano et al., 1998) mutants. However, the facial nucleus does not show significant variation in the number of MTNs in the same animals.

Binding of GDNF family of neurotrophic factors to Ret and GFRα family members induce Ret phosphorylation (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). After phosphorylation, Ret induces the activation of several intracellular pathways, among which the extracellular regulated kinase (ERK)–mitogen-activated protein (MAP) kinase and the phosphatidylinositol 3 kinase (PI 3-kinase) are of particular interest (Kotzbauer et al., 1996; Creedon et al., 1997). PI 3-kinase has been implicated in the survival-promoting mechanisms (Yao and Cooper, 1995; Dudek et al., 1997; Miller et al., 1997; Crowder and Freeman, 1998; Dolcet et al., 1999) and ERK–MAP kinase seems to be involved in differentiation processes (Qiu and Green, 1992; Cowley et al., 1994; Fukuda et al., 1995; Pang et al., 1995). To understand the mechanisms implicated in the GDNF family-

Received April 15, 1999; revised July 19, 1999; accepted Aug. 11, 1999.

This work was funded by Spanish Govern Comisión Interministerial de Ciencia y Tecnologia through the Plan Nacional de Salud y Farmacia (Contract 97–0094), Telemarató de TV3 (edició 1997: Malalties Degeneratives Hereditàries), European Union Biotech Program (Contract BIO4-CT96–0433), and Ajuntament de Lleida. We thank Isu Sanchez for her technical support. We also thank Eugene M. Johnson and Jeff Milbrandt (Washington University, St. Louis, MO) for supplying the neurotrophic factors and Malú G. Tansey (Washington University, St. Louis, MO) for her scientific comments and help. GFRα plasmids used for the *in situ* hybridization were a generous gift of Alun M. Davies (University of St. Andrews, Fife, Scotland). M.E. and J.E. are predoctoral fellows funded by Generalitat de Catalunya, and X.D. is a predoctoral fellow funded by the Spanish Government. J.R.B. is a postdoctoral fellow funded by European Union Biotech Contract BIO4-CT96–0433.

Drs. Soler and Dolcet contributed equally this work.

Correspondence should be addressed to Joan X. Comella, Grup de Neurobiologia Molecular, Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, Rovira Roure, 44, 25198 Lleida, Spain.

mediated MTN survival and the intracellular pathways involved in this process, we have investigated the ability of GDNF, NTN, and PSP to activate PI 3-kinase and ERK–MAP kinase pathways. We provide evidence showing that all these neurotrophic factors are able to activate both pathways in our culture system, and the PI 3-kinase pathway has an important role on MTN survival, whereas the Ras/MAP kinase does not have a relevant contribution in this process.

MATERIALS AND METHODS

MTN isolation, survival evaluation, and cell death characterization. MTNs were purified from embryonic day 5.5 (E5.5) chick embryos according to Comella et al. (1994) with minor modifications described by Dolcet et al. (1999). Survival evaluation was performed as described by Soler et al. (1998).

Neurotrophic factors were obtained from E. M. Johnson and J. Milbrandt (Washington University, St. Louis, MO) and were prepared as described by Creedon et al. (1997).

To evaluate the cell death process, cultures were stained with the Hoechst 33258 dye (Sigma, Madrid, Spain). MTNs (2×10^5) were grown in 35 mm culture dishes for 48 hr in the presence of muscle extract (MEX), and then cells were washed and grown for an additional 15 hr with culture medium containing different supplements or drugs. At that time, medium was removed, and cells were washed once with PBS and fixed with 4% (w/v) paraformaldehyde (Fluka, Buchs, Switzerland) in PBS for 30 min. Neurons were stained for 30 min with 0.5 μg/ml Hoechst 33258 and were mounted with glass coverslips using Fluoprep (Biomerieux, Marcy-l'Etoile, France). Stained cells were observed and counted with a vertical microscope equipped with epifluorescence and UV filters. Results are expressed as the percentage of apoptotic cells with respect to the total cell number counted in each condition (1000 cells) and show the mean ± SEM of the percentages for three independent experiments. Where applicable, statistical analysis was performed with the Student's *t* test.

To assess the enzymatic activity of caspases in dying MTNs, cultures were grown in the presence of MEX during 48 hr. After this period of time, cells were washed and treated in the indicated culture media. At the adequate time, cells were rinsed with ice-cold PBS and lysed in a buffer containing 100 mM HEPES, pH 7.4, 5 mM DTT, 5 mM EGTA, 0.04% NP-40, and 20% glycerol. Extracts were then centrifuged at $5000 \times g$ for 10 min, and protein concentrations were determined by Bradford assay (Bradford, 1976). Eight micrograms of cell extracts were diluted in 50 μl of reaction buffer (100 mM HEPES, pH 7.4, 5 mM DTT, 5 mM EGTA, 0.04% NP-40, and 20% glycerol) and incubated with 100 μM fluorescent substrate Z-DEVD-AFC (Enzyme System Products, Livermore, CA) at 37°C for 1 hr. The fluorescent signals were determined with a spectrofluorometer (Bio-Tek Instruments, Winooski, Vermont) at an excitation wavelength of 360 nm and an emission wavelength of 530 nm. Protease activity was expressed as the amount of cleaved substrate per microgram of protein per minute (pmol of $AFC \cdot min^{-1} \cdot \mu g$ of protein).

In situ *hybridization.* Probes were generated by transcribing linearized plasmids containing the cDNA for the desired chicken gene, obtained from Alun M. Davies (University of St. Andrews, Fife, Scotland). The GFRα-1-, GFRα-2-, and GFRα-3-containing plasmids were linearized with *Pst*I, *Sph*I, and *Sac*II, respectively, and transcribed with T7, T3, and SP6 RNA polymerases to generate 686, 626, and 800 bp antisense probes, respectively. The Ret probe was synthesized from total RNAs of MTNs using specific primers corresponding to positions 983–1007 for the forward direction and 2035–2059 for the reverse direction of the published sequence (Robertson and Mason, 1995). The amplified fragment gives a product of 1076 bp that was subcloned in pBluescript SK (Stratagene, La Jolla, CA). Antisense probes were obtained by digesting the plasmid with *Bam*HI and transcribed with T7.

For *in situ* hybridization, chicken embryos were immersion-fixed for 12–16 hr at 4°C in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH7.4, cryoprotected overnight in 30% sucrose, embedded in Tissue-Tek(Miles, Inc, Elkhart, IN), and frozen. Fourteen micrometers of transversal sections were cut using a cryostat, collected onto 3-aminopropyltriethoxysilane-coated slides, and stored at −80° until they were used. Sections were air dried for 2–3 hr before use, treated during 30 min with 1% Triton X-100 in PBS, post-fixed with 4% paraformaldehyde for 10 min, and acetylated in 0.25% acetic anhydride and 0.1 M triethanolamine HCl for 10 min. Sections were prehybridized 1 hr at room temperature in a solution containing 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim, Barcelona, Spain), 1 mM EDTA, 1 mg/ml tRNA, 0.5 mg/ml Herring sperm DNA, 0.1% CHAPS, and 0.1% Tween 20. Hybridization was performed in the same solution containing 500 ng/ml the respective denatured probe for 16–20 hr at 60°C and subsequently washed three times for 15 min in 0.2× SSC at the hybridization temperature. Sections were blocked with 10% fetal calf serum (Life Technologies, Barcelona, Spain) and 2 mg/ml BSA in 0.1 M Tris, pH7.5, and 0.150 M NaCl 1 hr at room temperature. Digoxigenin-labeled nucleotides were detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody. The antibody was developed with 4-nitro blue tetrazolium chloride and 5-brom-4-chlor-indolyl-phosphate.

Reverse transcription-PCR. A semiquantitative reverse transcription (RT)-PCR assay was used to compare the expression levels for Ret, GFRα-1, GFRα-2, and GFRα-4 in E7.5 chicken spinal cord and 48 hr cultured MTNs. One microgram of total RNA (Chomczynski and Sacchi, 1987) was digested with 2 U of DNase I RNase-free (Amersham Pharmacia Biotech, Uppsala, Sweden) and was reverse transcribed using 1 nmol of random hexamers (Boehringer Mannheim) and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) for 1 hr at 37°C. Ten nanograms of cDNA were used to perform a multiplex PCR amplification, with 200 nM each Ret, GFRα-1, GFRα-2, and GFRα-4 set of primers and 20 nM each housekeeping L27 ribosomal protein as an internal control. Samples were subjected to 27–30 cycles of 94°C for 20 sec (60°C for Ret and GFRα-4, 50°C for GFRα-1, and 53°C for GFRα-2) and 72°C for 30 sec on a Perkin-Elmer (Emeryville, CA) thermal cycler, with hot start at 94°C. All products were analyzed on 3% ethidium bromide-stained gels. Care was taken to arrest the amplification in the linear phase that was determined in pilot experiments. To achieve this, the amount of product was plotted against number of cycles and amount of starting sample.

Primer sequences were as follows: Ret forward, TCGCTACCACAAGAATTCTCCAAAG; Ret reverse, GATGGGATATGACTGGGCTGGGCGC; GFRα-1 forward, ACCTGAGAAGGAGGATGG; GFRα-1 reverse, TGACATCCTTGATAATCT; GFRα-2 forward, CCTTTGTGGATCAGAAGGC; GFRα-2 reverse, AGCTTCAGCAGCACAATGG; GFRα-4 forward, ACCATCGTTCCGGCCTGCTCC; GFRα-4 reverse, GCATAACGCGACCTACAGACG; L27 forward, AGCTGTCATCGTGAAGAACAT; and L27 reverse, CTTGGCGATCTTCTTCTTGCC.

Primers for GFRα-1 and GFRα-2 were described previously by Thompson et al. (1998) and those for L27 by Allsopp et al. (1993). The amplified products for Ret, GFRα-1, GFRα-2, GFRα-4, and L27 were 82, 99, 82, 101, and 128, respectively.

Immunoprecipitation, Western blot analysis, and kinase assays. Ret phosphorylation assay was performed as described by Creedon et al. (1997) with minor modifications. Briefly, 100 μg of protein was immunoprecipitated with 1 μg of 4G10 anti-phospho-tyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Immunocomplexes were collected with a mixture of Protein A and Protein G (Sigma) and resolved by Western blot as described below. Before the immunoprecipitation, 10 μg of protein was removed and blotted to assess equal loading of the lanes.

For detection of the phosphorylated forms of MAP kinase and ERK kinase (MEK), ERK2, and Akt in total cell lysates, 10 μg of total protein was resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Bedford, MA) using an Amersham Pharmacia Biotech semidry TransBlot according to the manufacturer's instructions. The membranes were blotted with specific anti-phospho-MEK (anti-P-MEK), anti-phospho-ERK (anti-P-ERK), and anti-phospho-Akt (anti-P-Akt), antibodies (New England Biolabs, Beverly, MA) following the instructions of the provider. To control the content of the specific protein per lane, membranes were stripped with 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl, pH 6.8, for 30 min at 70°C and reprobed with a rabbit anti-pan-MEK antibody (New England Biolabs), a mouse monoclonal anti-pan-ERK antibody (Transduction Laboratories, Lexington, KY), or a goat anti-pan-Akt antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described by the providers. Ret was detected with a similar protocol, but antibodies specific for this protein were used (Santa Cruz Biotechnology). Blots were developed using the Super Signal Chemiluminescent Substrate (Pierce, Rockford, IL).

ERK–MAP kinase activity assay was performed as described by Dolcet et al. (1999). Kinase activity was assayed on whole-cell lysates using

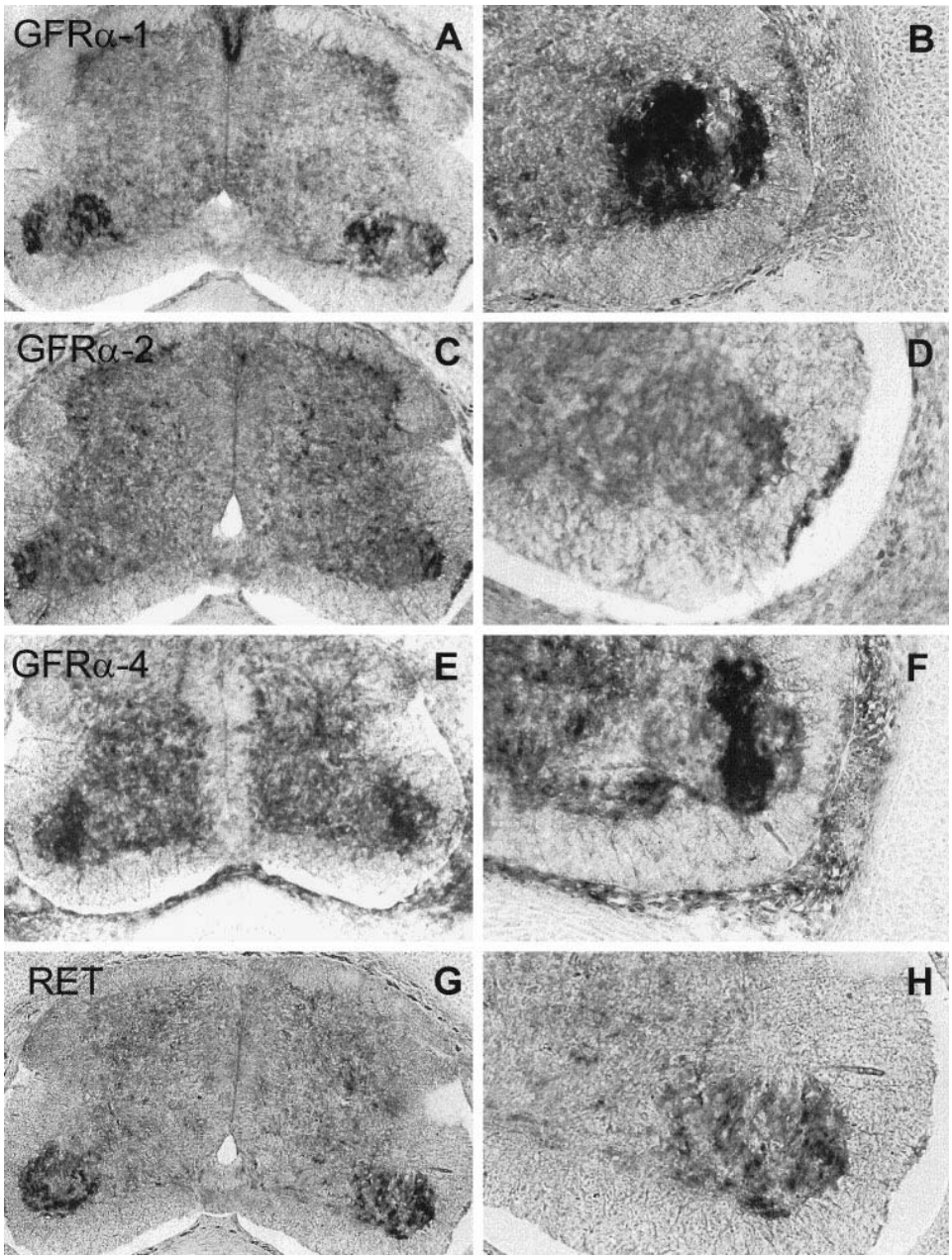


Figure 1. Ret and GFRα receptors are expressed in embryonic chick spinal cord. *In situ* hybridization analysis of GRFα-1 (*A*, *B*), GRFα-2 (*C*, *D*), GRFα-4 (*E*, *F*), and Ret (*G*, *H*) expression in E7.5 chicken. Serial transversal sections through the spinal cord were hybridized to antisense riboprobes against the four receptors. *B*, *D*, *F*, and *H* are amplifications of the lateral motor column showing the expression in MTNs of GRFα-1, GRFα-2, GRFα-4, and Ret, respectively. Control sense probe did not show any specific patterns of hybridization signal (data not shown).

as substrates [γ^{32}P]ATP (Amersham Pharmacia Biotech) and a specific peptide provided by the BIOTRAK p42/p44 MAP kinase enzyme assay (Amersham Pharmacia Biotech) following the manufacturer's instructions. Incorporation of radioactivity was measured in a scintillation counter. Results were obtained as picomoles of inorganic phosphate incorporated per minute per microgram of protein extract and are expressed in the figures as the fold induction over the activity found in nonstimulated cultures.

Akt activity was performed essentially as described by Khwaja et al. (1998). Briefly, 150 μg of total protein was immunoprecipitated with 1 μg of anti-Akt1 antibody (C-20) (Santa Cruz Biotechnology) for 1 hr at 4°C. Immunocomplexes were collected with protein G (Sigma), washed, and incubated with 3 μCi of [γ^{32}P]ATP (Amersham Pharmacia Biotech) and histone H2B (Boehringer Mannheim) as substrates. Reaction was performed for 30 min at room temperature and stopped by adding sample buffer and boiling for 5 min. Immunocomplexes were resolved by SDS-PAGE. Quantification of H2B phosphorylation was performed by PhosphorImager Analysis (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Ret and GFRα receptors are expressed in embryonic chicken MTNs *in vivo* and *in vitro*

GDNF family of neurotrophic factors mediate neuronal survival through a receptor complex composed of Ret and a member of the GFRα family of GPI-linked receptors (GFRα-1, GFRα-2, and GFRα-4) (Jing et al., 1996; Buj-Bello et al., 1997; Enokido et al., 1998; Trupp et al., 1998). Because we wanted to study whether this family of neurotrophic factors promoted cell survival in our culture system, we were first interested to know whether the functional receptors were present in the corresponding embryonic day in chicken spinal cord MTNs. Thus, *in situ* hybridization analysis using antisense riboprobes specific for each of the receptors were performed in E7.5 chicken sections. In the spinal cord, low-to-moderate levels of Ret and moderate-to-high levels of

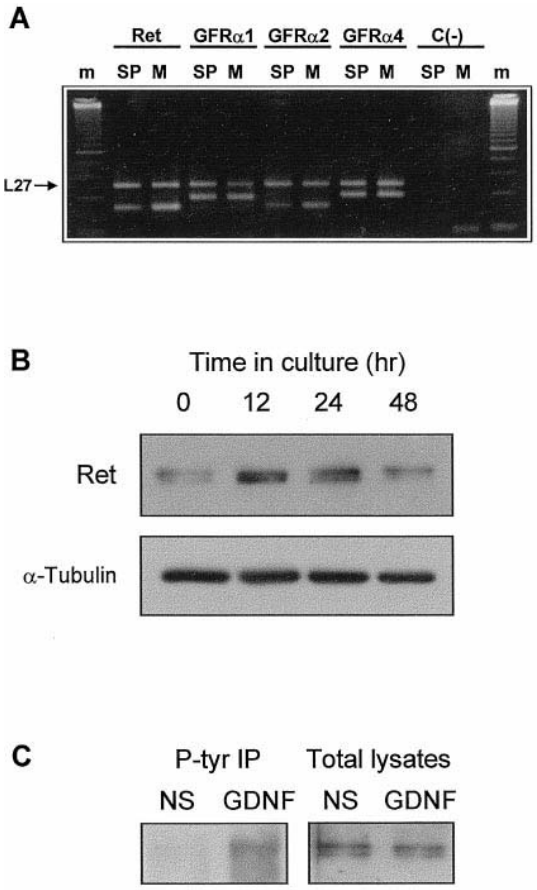


Figure 2. Ret and GFRα receptors are expressed in cultured MTNs. *A*, Agarose gel showing the RT-PCR products for Ret, GFRα-1, GFRα-2, and GFRα-4 in E7.5 spinal cord (*SP*) and 48 hr cultured MTNs (*M*). Coamplification of the L27 mRNA (*top band* in each *lane*) serves as an internal control. All RNAs were present at similar levels in spinal cord and cultured MTNs, and for all, except GFRα-4, there was an enrichment of these transcripts in cultured MTNs. Control reactions performed without reverse transcriptase synthesis [*C*(−)] show that there was no detectable contamination by genomic DNA. *m* corresponds to the 50 bp molecular size marker (Amersham Pharmacia Biotech). The same results were obtained without L27 amplification (data not shown). *B*, Western blot analysis of Ret expression in 0, 12, 24, and 48 hr MEX-cultured MTNs. After these periods of time in culture, cells were lysed, and protein extracts were analyzed by Western blot with an anti-c-Ret antibody. To assess the protein content in each lane, membranes were stripped and reprobed with an anti-β-tubulin antibody. *C*, GDNF induces Ret tyrosine phosphorylation in MTNs. Cultures were deprived of MEX for 5 hr and then treated with medium containing no additions (*NS*) or 100 ng/ml GDNF. After 7 min, cultures were lysed. Western blot of phospho-tyrosine immunoprecipitates (*left*) or total lysates (*right*) were probed with an anti-Ret antibody.

GFRα-1, GFRα-2, and GFRα-4 expression were observed in the ventral horn in which MTN cell bodies are located (Fig. 1). The GFRα-1 and GFRα-4 expression patterns were found to be complementary, and GFRα-2-positive cells were found in the peripheral area of the ventral horn. This expression pattern suggests that the GFR family receptors could be expressed in different MTN subpopulations located in the ventral horn, whereas Ret expression was homogeneously distributed in this spinal cord area (Fig. 1).

To investigate further the expression of these receptors in our system, MTNs were cultured in the presence of MEX for 48 hr. After this period of time, we used semiquantitative RT-PCR with specific primers to detect Ret, GFRα-1, GFRα-2, and GFRα-4 mRNA. Figure 2*A* shows that the four transcripts were present in cultured MTNs with levels similar to those detected in the E7.5 chicken dissociated spinal cord, suggesting that, after 48 hr in culture, MTNs are still expressing all these genes. Moreover, we could also detect an enrichment of Ret, GFRα-1, and GFRα-2 mRNA levels in the cultured MTN with respect to the entire spinal cord. This is probably caused by an enrichment of cells expressing these genes during the MTN purification process. Furthermore, we were able to detect the expression of Ret protein in cultured MTNs by Western blot analysis. The expression increased with time in culture, and the maximum levels were observed after 24 hr (Fig. 2*B*). Thereafter, the levels tend to slightly decrease (Fig. 2*B*). Moreover, we also investigated the ability of GDNF to induce Ret tyrosine phosphorylation. Thus, after GDNF stimulation, cells were lysated and immunoprecipitated with an anti-phospho-tyrosine antibody. Figure 2*C* shows that cultures treated with GDNF contained tyrosine-phosphorylated Ret protein, whereas nonstimulated control cultures did not. We can conclude that GFRα family of receptors and Ret are expressed in chicken spinal cord MTNs both *in vivo* and *in vitro* and that GDNF treatment is able to activate Ret in cultured MTNs.

GDNF, NTN, and PSP promote chick MTN survival in culture

To analyze the effect of GDNF family of neurotrophic factors on chicken spinal cord MTN survival in culture, MTNs were isolated from E5.5 embryos essentially as described by Comella et al. (1994). Isolated MTNs are able to survive in culture in the presence of a saturating concentration (300 μg/ml) of muscle extract (MEX) (Comella et al., 1994). However, when MTN were cultured in the absence of MEX, <30% of the cells initially plated remained alive after 36 hr in culture (data not shown). To study the ability of GDNF, NTN, and PSP to promote MTN survival, MTNs were cultured in the presence of MEX for 2 d. Afterward, the culture medium was replaced and the different conditions were established. On readdition of a medium containing 10 ng/ml GDNF, NTN, or PSP, ~90% of the MTNs remained alive after an additional 24 hr of culture (Fig. 3*A*). These survival percentages were comparable with those observed in cultures supplemented with MEX (94.7 ± 6.2). However, when they were deprived of MEX and maintained in the basal medium (no extract medium, NE), significantly lower percentages of MTNs (~60%) survived. The survival-promoting effect of GDNF family of neurotrophic factors was clearly dose-dependent (Fig. 3*B*). Concentrations above 100 pg/ml were saturating, and ~90% of viable MTNs were maintained. These results showed that GDNF family members are able to maintain the survival of chicken cultured MTNs to approximately the same level of that observed with MEX. No major morphological differences were observed between MTNs treated with MEX and those treated with any of the different trophic factors assayed.

In these survival experiments, we found that saturating concentrations of a single factor rescued ~90% of the cultured MTNs. Because the expression of the GFRα receptors is distributed in different neuronal subpopulations in the spinal cord, we were interested to know whether combinations of factors used at 10 pg/ml would improve MTN survival. Figure 3*C* shows that all of the combinations of factors studied did an additive effect on cell survival. This result suggests that, at high concentrations, a

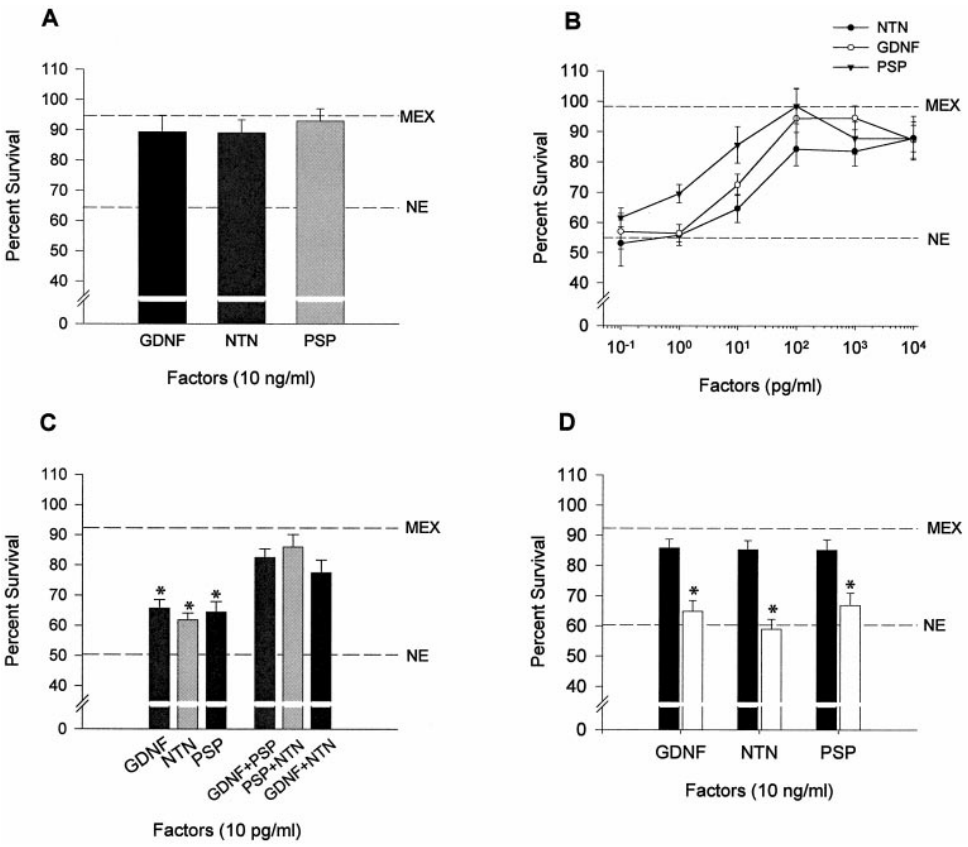


Figure 3. GDNF family of neurotrophic factors promote MTN survival in culture. *A*, Percentages of MTN survival after 24 hr in the presence of 10 ng/ml GDNF, NTN, or PSP. *B*, Dose-dependence curves of MTN survival after 24 hr in the presence of different doses of GDNF, NTN, or PSP. *C*, In the *left half*, bars show the percentage of MTN survival after 24 hr in the presence of 10 pg/ml GDNF, NTN, or PSP. In the *right half*, bars show the percentage of survival in cultures treated during 24 hr with different combinations of factors, all of them added at doses of 10 pg/ml. *D*, Percentage of MTN survival after 24 hr in 10 ng/ml GDNF-, NTN-, or PSP-treated cultures in the absence (*filled bars*) or presence (*open bars*) of 1 U/ml PIPLC. *Broken lines* in *A–D* show survival of cells in sibling control cultures maintained in the presence (*top line*, *MEX*) or absence (*bottom line*, *NE*) of MEX culture medium for the same culture period. Values are the mean ± SEM of eight wells from a representative experiment that was repeated twice more with results comparable with those presented. *Asterisk* in *C* indicates that the values are significantly different ($p < 0.01$) between cultures treated with a single factor and cultures treated with a combination of factors as determined by the Student's *t* test. *Asterisk* in *D* indicates survival values significantly different ($p < 0.01$) when comparing cultures treated with PIPLC or not as determined by the Student's *t* test.

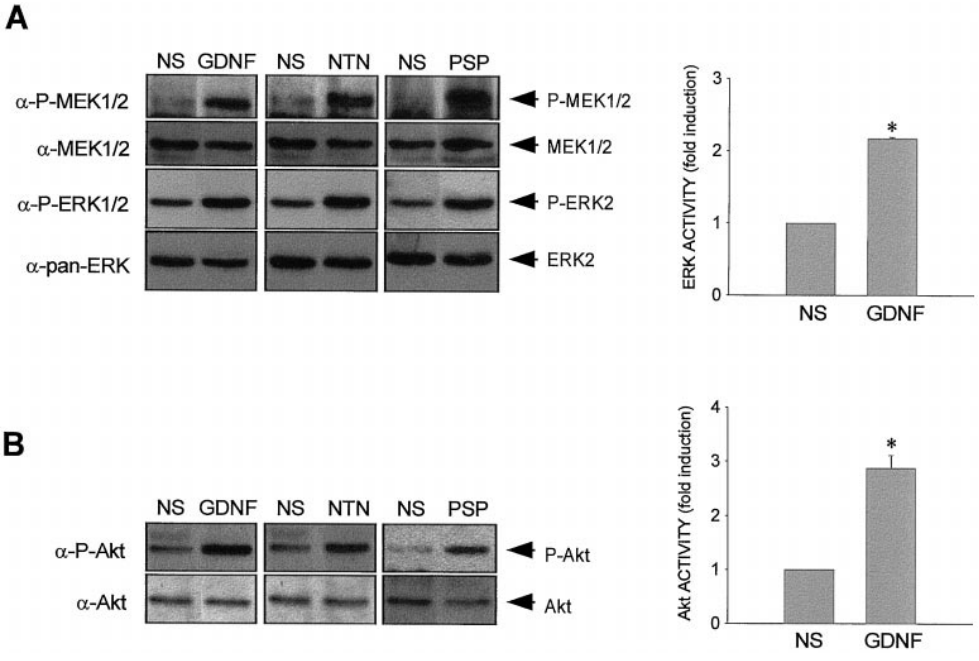


Figure 4. Effect of GDNF family of neurotrophic factors treatment on the activation of MEK, ERK, and Akt. MTNs were cultured for 48 hr in the presence of MEX, deprived of MEX for 5 hr, and then stimulated as indicated for 7 min with 50 ng/ml (for Western blot analysis) or 100 ng/ml (for kinase activity) GDNF, NTN, or PSP. After treatment, cells were lysed, and protein extracts were obtained. *A*, Protein extracts were analyzed by Western blot with an anti-P-MEK1/2 antibody (*α-P-MEK1/2*) or with an anti-P-ERK1/2 antibody (*α-P-ERK1/2*) and stripped and reprobed with an anti-MEK1/2 antibody (*α-MEK1/2*) or an anti-pan-ERK antibody (*α-pan-ERK*). *NS* indicates nonstimulated control cultures. *Arrows* labeled *P-MEK1/2*, *MEK1/2*, *P-ERK2*, and *ERK2* indicate the position of the phosphorylated and nonphosphorylated forms of MEK1/2 and ERK2 proteins, respectively. Protein extracts were subjected to an ERK–MAP kinase assay. Results are expressed as fold induction over basal activity. *Asterisk* indicates survival values significantly different ($p <$ 0.01) when comparing cultures treated with GDNF or not as determined by the Student's *t* test. *B*, Protein extracts were analyzed by Western blot with an anti-P-Akt antibody (*α-P-Akt*) and stripped and reprobed with an anti-Akt antibody (*α-Akt*). *NS* indicates nonstimulated control cultures. *Arrows* labeled *P-Akt* and *Akt* show the position of the phosphorylated and nonphosphorylated forms of Akt protein, respectively. Protein extracts were subjected to immunoprecipitation with an anti-Akt antibody, and the immunoprecipitates were tested by an Akt kinase assay. Results are expressed as fold induction over basal activity. *Asterisk* indicates survival values significantly different ($p <$ 0.01) when comparing cultures treated with GDNF or not as determined by the Student's *t* test.

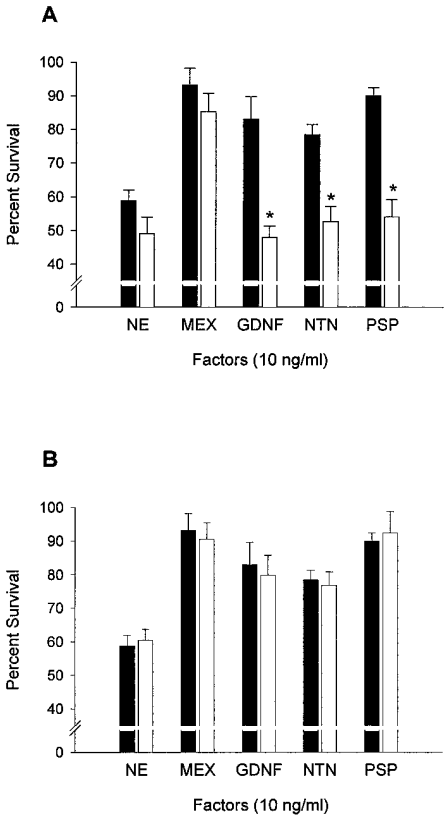


Figure 5. Effects of MEK and PI 3-kinase inhibitors on MTN survival mediated by GDNF family of neurotrophic factors. *A*, MTN survival in cultures treated during 24 hr in basal medium (NE), MEX, or 10 ng/ml GDNF, NTN, or PSP medium in the absence (*filled bars*) or presence (*open bars*) of 50 μM LY 294002. *Asterisk* indicates survival values significantly different ($p < 0.01$) when comparing cultures treated with LY 294002 or not as determined by the Student's *t* test. *B*, MTN survival in cultures treated during 24 hr in basal medium (NE), MEX, or 10 ng/ml GDNF, NTN, or PSP medium in the absence (*filled bars*) or presence (*open bars*) of 20 μM PD 98059. No significant differences in cell survival were found after this MEK inhibitor treatment.

single factor could also promote survival acting through any of the GFRα receptors.

To know whether GPI-linked proteins are normally required for the survival response of MTNs to GDNF, NTN, and PSP, we treated 48 hr cultured MTNs with phosphoinositide-specific phospholipase C (PIPLC), an enzyme that specifically cleaves GPI linkages (Koke et al., 1991). PIPLC treatment reduced the number of surviving neurons in the presence of GDNF, NTN, or PSP to the level observed in basal medium (NE) (~60%) (Fig. 3*D*). However, addition of PIPLC to NE-, MEX-, or BDNF-supplemented cultures did not have any effect on MTN survival (data not shown). All of these results suggest that PIPLC treatment eliminated the specific survival response of cultured MTNs to GDNF family of neurotrophic factors, indicating the involvement of the GPI-linked receptors in this process.

GDNF family of neurotrophic factors activates PI 3-kinase and ERK–MAP kinase pathways in cultured MTNs

Neurotrophic factors, such as neurotrophins, induce tyrosine phosphorylation of Trk receptors and the activation of several intracellular pathways in several neuronal populations, including chicken MTNs (Dolcet et al., 1999). Two of these pathways, ERK–MAP kinase and PI 3-kinase, are the best characterized signaling pathways activated by Trk receptors, and they have been related in neuronal differentiation and survival (Kaplan and Miller, 1997). To know whether GDNF family of neurotrophic factors induces activation of ERK–MAP kinase and PI 3-kinase intracellular signaling pathways, MTNs were cultured in the presence of MEX during 48 hr. Then, cells were washed and stimulated with 50 ng/ml GDNF, NTN, and PSP for 7 min. MTNs were lysed, and cell lysates were analyzed by immunoblotting using anti-P-MEK, anti-P-ERK antibodies (two kinases of the MAP kinase pathway), and anti-P-Akt antibodies (a well known downstream effector of the PI 3-kinase). Figure 4 shows an increase of tyrosine phosphorylation of MEK, ERK, and Akt after GDNF, NTN, and PSP treatment compared with the basal level observed in nonstimulated cultures. When the same filters were stripped and reprobed with anti-pan-MEK, anti-pan-ERK, and anti-pan-Akt antibodies, bands with similar intensities appeared in nonstimulated and stimulated cultures.

Moreover ERK–MAP kinase and Akt kinase assays were performed in cell lysates of MTNs stimulated with 100 ng/ml GDNF for 7 min. Results in Figure 4 show that GDNF stimulation caused an induction of Akt (2.8-fold induction) and ERK–MAP kinase (2.1-fold induction) activity when we compared it with the level of kinase activity in nonstimulated cells.

Taking together all of these observations, we conclude that both ERK–MAP kinase and PI 3-kinase pathways could be activated by GDNF, NTN, and PSP, suggesting the possibility that they could transduce the survival effect of these neurotrophic factors on MTNs.

Inhibition of PI 3-kinase, but not ERK–MAP kinase, reverts GDNF family of neurotrophic factors-promoted MTN survival

To further investigate the role of PI 3-kinase and ERK–MAP kinase intracellular pathways mediating the survival-promoting effect of GDNF family members, we analyzed the effect of the PI 3-kinase inhibitor LY 294002 and the MEK inhibitor PD 98059 on cultured MTNs. Cells were cultured for 2 d in the presence of MEX, washed with L15H, and switched to culture medium supplemented with neurotrophic factors (10 ng/ml) in the presence or absence of LY 294002 (Fig. 5*A*) or PD 98059 (Fig. 5*B*). After 24 hr, evaluation of MTN survival showed that LY 204002, but not PD 98059, reverted the survival-promoting effect mediated by GDNF, NTN, and PSP (Fig. 5). The effect of LY 294002 was dose-dependent (data not shown) and, at 50 μM, the number of surviving neurons was comparable with the one obtained in control cultures maintained in basal medium (NE). The same dose of LY 294002 was also able to prevent the increase in the Akt phosphorylation levels after GDNF, NTN, or PSP stimulation, demonstrating the functional blockade of PI 3-kinase activity (Fig. 6*B*).

On the other hand, Western blot analysis of 20 μM PD 98059-treated cultures showed that ERK phosphorylation was prevented after neurotrophic factor treatment (Fig. 6*A*). At the same dose of PD 98059, the level of MTN survival was not affected, and the number of surviving neurons after 24 hr of treatment was the same as the one obtained in control cultures (MEX, GDNF, NTN, or PSP) (Fig. 5*B*). Moreover, the 20 μM dose of PD 98059 was unable to alter the level of phosphorylation of Akt after GDNF, NTN, or PSP stimulation (data not shown). Together, these results suggest that the downstream elements activated by ERK–MAP kinases are not necessary for GDNF-, NTN-, and

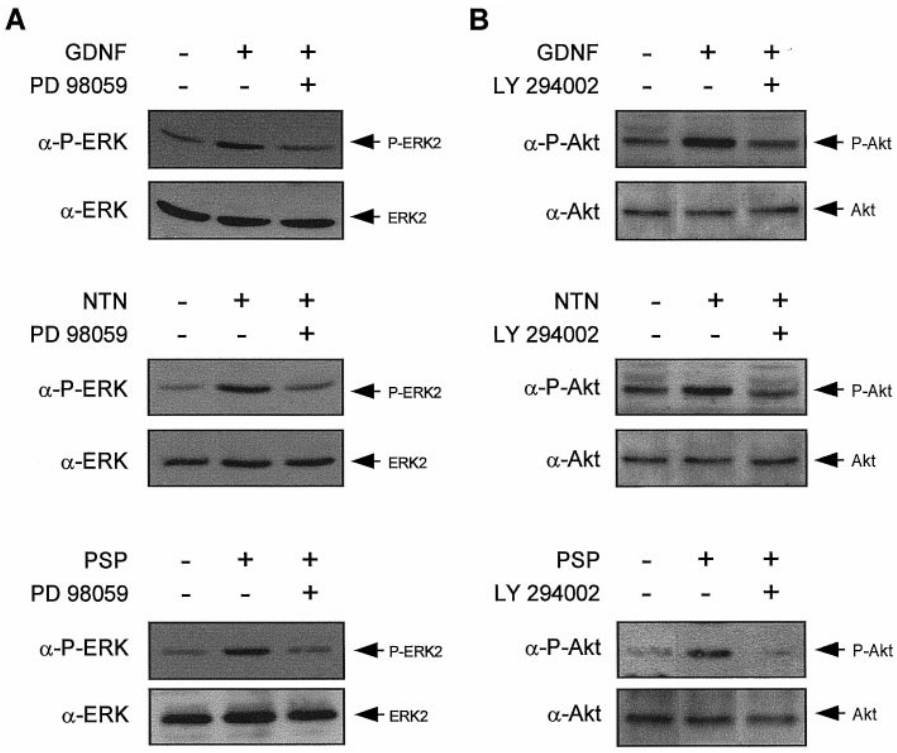


Figure 6. Effect of MEK and PI 3-kinase inhibitors on the activation of ERK and Akt, respectively. MTNs were cultured for 48 hr in the presence of MEX, were MEX-starved for 5 hr, pretreated (+) or not (−) for 30 min with 50 μM PD 98059 (*A*) or LY 294002 (*B*), and then stimulated (+) or not (−) for 7 min with 50 ng/ml GDNF, NTN, or PSP. After treatment, cells were lysed, and protein extracts were analyzed by Western blot with an anti-phospho-ERK1/2 antibody (α-*P-ERK1/2*) or an anti-phospho-Akt antibody (α-*P-Akt*) and stripped and reprobed with an anti-pan-ERK antibody (α-*pan-ERK*) or with an anti-Akt antibody (α-*Akt*). *Arrows* labeled *P-ERK2*, *ERK2*, *P-Akt*, and *Akt* indicate the position of the phosphorylated and nonphosphorylated forms of ERK2 and Akt, respectively.

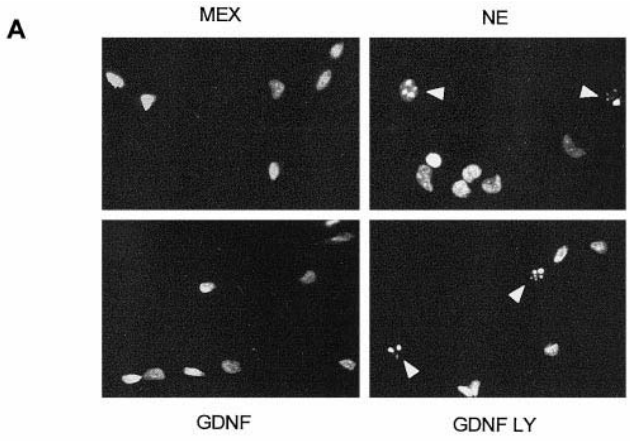
PSP-mediated survival; however, the PI 3-kinase pathway is one of the key elements involved in the survival process induced by the GDNF family of neurotrophic factors.

To asses whether LY 294002 suppresses the survival effects of these neurotrophic factors inducing the same kind of neuronal cell death as that observed after trophic factor deprivation (i.e., MEX withdrawal), we have quantified the percentage of apoptotic MTNs after LY 294002 treatment. Experiments were performed with the Hoechst 33258 dye, which binds specifically to the chromatinic DNA. Apoptotic cells display a highly condensed DNA that is normally fragmented in two or more chromatin aggregates (Fig. 7*A*). In cultures grown in the presence of MEX, GDNF, NTN, or PSP, the percentage of cells displaying this morphological feature was found to be <6%. However, after 15 hr of MEX deprivation or 50 μM LY 294002 treatment, the percentage of apoptotic cells greatly increased, and the percentage of apoptotic cells was found to be doubled when compared with their LY 294002 untreated counterparts (Fig. 7*B*). These results suggest that LY 294002 reverted the trophic effect promoted by GDNF, NTN, or PSP, inducing an apoptotic cell death process at the same dose that blocked Akt phosphorylation induced by these neurotrophic factors.

It has been reported previously that apoptotic cell death is accompanied by the enzymatic activity of a family of proteases referred as to caspases (for review, see Cryns and Yuan, 1998). This activity has been established as a good criteria to characterize the apoptotic cell death in chicken spinal cord MTNs after trophic factor deprivation (Li et al., 1998; Dolcet et al., 1999). To analyze caspase activity in our culture system, we performed an *in vitro* assay using the fluorogenic substrate DEVD-AFC (Talanian et al., 1997). Once DEVD-AFC is cleaved by caspases, the AFC group is released from the whole molecule, becoming fluorescent (Nicholson et al., 1995). Using this approach, we observed an induction of caspase activity in MEX-deprived cells (NE) when we compared it with the level of caspase activity found in MEX-treated (2.3-fold induction), GDNF-treated (2.6-fold induction), NTN-treated (2-fold induction), or PSP-treated (1.7-fold induction) cultures (Fig. 7*C*). To know whether LY 294002 treatment on GDNF family-maintained cells induced the same effect on caspase activity as deprived cultures, MTN were treated with GDNF, NTN, or PSP (10 ng/ml) in the presence or absence of 50 μM LY 294002. Figure 7*C* shows that the level of caspase activity in LY 294002-treated cultures was similar to that obtained in basal medium (NE)-treated cultures and higher ($p < 0.01$) than that obtained in GDNF-, NTN-, or PSP-treated cells. These results suggest that GDNF family of neurotrophic factors inhibit the activation of caspases through a PI 3-kinase-dependent pathway.

DISCUSSION

We have shown in the present study that the members of GDNF family of neurotrophic factors promote MTN survival through the activation of the PI 3-kinase intracellular signaling pathway. These neurotrophic factors are potent survival factors for different neuronal populations, including spinal cord MTNs (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995; Klein et al., 1997; Horger et al., 1998; Milbrandt et al., 1998). In our culture system, GDNF, NTN, and PSP promoted the survival of MTNs deprived of MEX. The effect was dose-dependent, and it was reverted by adding PIPLC in the culture medium. This enzyme specifically cleaves the GPI linkage of the GFRα to the cell membrane, thus precluding the activation of Ret (Koke et al., 1991). Our results indicated that these neurotrophic factors need to bind to a GPI-linked receptor to do their survival effect on cultured MTNs. It has been described previously that GDNF, NTN, and PSP act through their binding to the GPI-linked coreceptors GFRα-1, GFRα-2, and GFRα-4, respectively (Buj-Bello et al., 1997; Klein et al., 1997; Enokido et al., 1998) (for



B

LY294002	-	+
	% of apoptotic cells	
GDNF	5,9 ± 1	9,8 ± 1,3
NTN	5,3 ± 0,6	10,5 ± 1,8
PSP	3,5 ± 0,6	6,7 ± 0,3

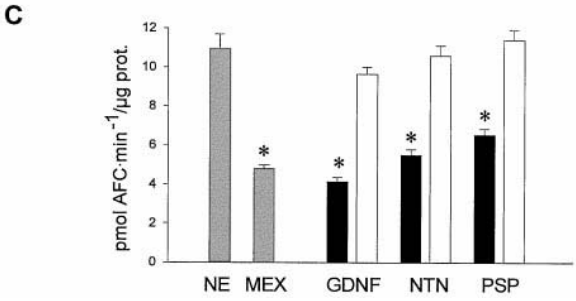


Figure 7. Cell death induced by LY 294002 shared apoptotic features with that observed after MEX deprivation. *A*, MTNs were maintained in culture in the presence of MEX for 48 hr. Then, cells were grown for 15 hr in different culture conditions: basal medium without any trophic support (NE); supplemented with MEX; or supplemented with 10 ng/ml GDNF, NTN, or PSP in the presence or absence of 50 μM LY 294002. After this time, MTN nuclei were stained with Hoechst. Representative photomicrographs of cultures treated with basal medium (NE), MEX, GDNF, or GDNF plus LY 294002. *B*, Percentage of apoptotic cells in each condition. Quantification of apoptotic nuclei was performed as described in Materials and Methods. The percentages in control cultures were 8.5 ± 2.7 for NE and 5.9 ± 1.3 for MEX. *C*, Assay of caspase activity performed in cultures treated for 8 hr with 10 ng/ml GDNF, NTN, or PSP in the presence (*open bars*) or absence (*filled bars*) of 50 μM LY 294002, MEX, or basal medium (NE). At the end of the treatment, cells were lysed, and protein extracts were assayed for its caspase activity as described in Materials and Methods. Results are expressed as pmol of AFC · min^{-1} · μg of protein. *Asterisk* indicates significant differences ($p < 0.01$) on caspase activity between GDNF-, NTN-, or PSP-treated cultures and NE- or GDNF, NTN, or PSP plus LY 294002-treated cultures.

review, see Ibáñez, 1998). In the present work, we also demonstrated the presence of Ret and GFRα family receptors in embryonic chicken spinal cord MTNs both *in vivo* and *in vitro* using *in situ* hybridization and RT-PCR techniques. The presence of GRFα-1 and GFRα-2, but not GFRα-3, receptors in the ventral horn of rat embryos spinal cord has been demonstrated previously (Yu et al., 1998). Moreover, GFRα-4 is expressed in embryonic chicken spinal cord MTNs (Thompson et al., 1998). However, our results demonstrated the presence of these receptors in chicken spinal cord MTNs by both methods. We also show that the distinct GFRα receptors are located on different MTN subpopulations. This situation could not be compatible with the fact that, in the survival experiments, high doses of a single factor were able to promote ~100% survival of purified MTNs, but at subsaturating concentrations, the combinations of factors always had an additive effect on the survival of these cells. This situation could be explained by the possibility that high doses of any of these factors could also bind to another member of the GFRα family receptors. This cross-talk between the members of both families, neurotrophic factors and receptors, has been described previously by different authors, especially when neuronal survival was assessed by culture methods (Baloh et al., 1998; Enokido et al., 1998; Ibañez, 1998; Trupp et al., 1998). Thus, the studies performed in mutant mice lacking GDNF, NTN, or GFRα-1 receptors show moderate or no loss of MTNs number in the trigeminal motor nucleus, the spinal cord, or the facial nucleus (Moore et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Heuckeroth et al., 1999). These results, together with the *in vitro* observations, suggest that it may possible that this cross-talk also exists *in vivo*. Experiments with double mutant mice lacking more than one of the members of family receptors or of the neurotrophic factors will answer this question.

Little is known about the intracellular signaling pathways triggered by the GDNF family of neurotrophic factors when they activate Ret. In the present work, we show that GDNF, NTN, and PSP increased the phosphorylation of MEK, ERK, and Akt, indicating that the ERK–MAP kinase and the PI 3-kinase intracellular signaling pathways were activated. Moreover, we present evidence that only the blockade of the PI 3-kinase pathway using the PI 3-kinase-specific inhibitor LY 294002 was able to revert the survival effect induced by GDNF, NTN, and PSP. This effect was found at the same doses that prevent the activation of the PI 3-kinase. Moreover, by morphological criteria, MEX deprivation and LY 294002 treatment induce a comparable apoptosis, suggesting that this pathway plays a central role in the survival-promoting effects elicited by GDNF, NTN, or PSP. Furthermore, these results were reinforced when a specific biochemical parameter, i.e., caspase activity, was measured. We have found that MEX deprivation and LY 294002 treatment increased the level of DEVD-specific caspase activity. On the contrary, when cells were treated with the MEK inhibitor PD 98059, a drug that inhibits the phosphorylation and the ERK–MAP kinase activity, it does not affect MTN survival. It has been demonstrated previously that GDNF and NTN were able to stimulate the activation of ERK–MAP kinase and the PI 3-kinase intracellular signaling in sympathetic neurons (Kotzbauer et al., 1996; Creedon et al., 1997). However, in these reports, no evidence about the role of these pathways in mediating survival effects were provided. Our study expands the initial findings in GDNF and NTN to PSP. Moreover, we report that MTN behaves similarly to sympathetic neurons with regard to the stimulation of these intracellular pathways. Therefore, we demonstrate that PI 3-kinase pathway seems to mediate the survival-promoting effects induced by this family of neurotrophic factors. The role of the PI 3-kinase pathway as a mediator of the trophic effects of several trophic factors has been described previously in BDNF-mediated survival of cultured

cerebellar granule neurons (Nonomura et al., 1996; Shimoke et al., 1997) or spinal cord MTNs (Dolcet et al., 1999), in NGF maintained PC12 or SGC cells (Yao and Cooper, 1995; Crowder and Freeman, 1998), and in cerebellar granule neurons maintained with IGF-1 (D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997). However, the present work is the first demonstration of the involvement of the PI 3-kinase pathway in mediating the survival-promoting effects of the GDNF family of neurotrophic factors.

The activation of the ERK–MAP kinase pathway after trophic factor stimulation has been described previously in different neuronal populations, such as MTNs (Becker et al., 1998), cerebellar granule cells (Nonomura et al., 1996), and hippocampal pyramidal neurons (Marsh and Palfrey, 1996). In our culture system, the stimulation by GDNF, NTN, or PSP also increases ERK and MEK phosphorylation and, consequently, the activation of this intracellular pathway. However, the inhibition of this pathway was unable to block the survival-promoting effect caused by GDNF family of neurotrophic factors. The present results are in accordance with those reported by other authors using different approaches in several neuronal systems (Creedon et al., 1996; Marsh and Palfrey, 1996; Virdee and Tolkovsky, 1996; Miller et al., 1997; Soler et al., 1998). These studies suggest that the activation of the ERK–MAP kinase pathway is not involved in the cellular events directly related with cell survival. However, the activation of this pathway could be an important step in mediating neuronal differentiation. In that sense, it has been reported the involvement of this pathway mediating neurite outgrowth, which is one of the indicators for neuronal differentiation (Qiu and Green, 1992; Cowley et al., 1994; Fukuda et al., 1995; Pang et al., 1995; Creedon et al., 1996).

In conclusion, our work demonstrated the involvement of the PI 3-kinase intracellular pathway mediating neuronal survival after GDNF family of neurotrophic factors stimulation. It seems that this will be a general process in signaling transduction mechanisms of different neurotrophic factors on many neuronal populations. The involvement of other pathways mediating different processes others than neuronal survival could allow us to understand the mechanisms by which neurotrophic factors regulate diverse neuronal responses.

REFERENCES

Allsopp TE, Robinson M, Wyatt S, Davies AM (1993) Ectopic trkA expression mediates a NGF survival response in NGF-independent sensory neurons but not in parasympathetic neurons. J Cell Biol 123:1555–1566.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson Jr EM, Milbrandt J (1998) Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRα3-Ret receptor. Neuron 21:1291–1302.

Becker E, Soler RM, Yuste VJ, Gine E, Sanz-Rodriguez C, Egea J, Martin-Zanca D, Comella JX (1998) Development of survival responsiveness to brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5, but not to nerve growth factor, in cultured motoneurons from chick embryo spinal cord. J Neurosci 18:7903–7911.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248–254.

Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron 15:821–828.

Buj-Bello A, Adu J, Piñon LGP, Horton T, Thompson J, Rosenthal A, Chinchetru M, Buchman VL, Davies AM (1997) Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature 387:721–724.

Cacalano G, Fariñas I, Wang LC, Hagler K, Forgie A, Moore M, Armanini M, Phillips H, Ryan AM, Reichardt LF, Hynes M, Davies A, Rosenthal A (1998) GFRα-1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron 21:53–62.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol-chloroform extraction. Anal Biochem 162:156–159.

Comella JX, Sanz-Rodriguez C, Aldea M, Esquerda JE (1994) Skeletal muscle-derived trophic factors prevent motoneurons from entering an active cell death program *in vitro*. J Neurosci 14:2674–2686.

Cowley S, Paterson H, Kemp P, Marshall CJ (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3t3 cells. Cell 77:841–852.

Creedon DJ, Johnson EM, Lawrence JC (1996) Mitogen-activated protein kinase-independent pathways mediate the effects of nerve growth factor and cAMP on neuronal survival. J Biol Chem 271:20713–20718.

Creedon DJ, Tansey MG, Baloh RH, Osborne PA; Lampe PA, Fahrner T, Heuckeroth RO, Milbrandt J, Johnson Jr EM (1997) Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. Proc Natl Acad Sci USA 94:7018–7023.

Crowder RJ, Freeman RS (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18:2933–2943.

Cryns V, Yuan J (1998) Proteases to die for. Genes Dev 12:1551–1571.

D'Mello SR, Borodezt K, Soltoff SP (1997) Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 17:1548–1560.

Dolcet X, Egea J, Soler RM, Martin-Zanca D, Comella JX (1999) Activation of PI 3-kinase, but not ERK MAP kinases, is necessary to mediates BDNF-induced motoneuron survival. J Neurochem 73:521–531.

Dudek H, Datta SR, Franke TF, Bimbaum MJ, Yao M, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665.

Durbec P, Marcos-Gutierrez CV, Kilkenny C, Grigoriou M, Wartiowaara K, Suvantro P, Smith D, Ponder B, Costantini F, Saarma M, Sariola H, Pachnis V (1996) GDNF signalling through the Ret receptor tyrosine kinase. Nature 381:789–793.

Enokido Y, de Sauvage F, Hongo JA, Ninkita N, Rosenthal A, Buchman VL, Davies AM (1998) GFRα-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr Biol 8:1019–1022.

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson Jr EM, Milbrandt J (1998) GRFα-1 deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21:317–324.

Fukuda M, Gotoh Y, Tachibana T, Dell K, Hattori S, Yoneda Y, Nishida E (1995) Induction of neurite outgrowth by MAP kinase in PC12 cells. Oncogene 11:239–244.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simpson LC, Moffet B, Vandlen RA, Koliatsos VE, Rosenthal A (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266:1062–1064.

Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson Jr EM, Milbrandt J (1999) Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. Neuron 22:253–263.

Horger BA, Nishimura MC, Armanini MP, Wang LC, Poulsen, KT, Rosenblad C, Kirik D, Moffat B, Simmons L, Johnson Jr E, Milbrandt J, Rosenthal A, Bjorklund A, Vandlen RA, Hynes MA, Phillips H (1998) Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. J Neurosci 18:4929–4937.

Ibáñez CF (1998) Emerging themes in structural biology of neurotrophic factors. Trends Neurosci 21:438–444.

Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. Cell 85:1113–1124.

Kaplan DR, Miller FD (1997) Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 9:213–221.

Khwaja A, Lehman K, Marte BM, Downward J (1998) Phosphoinositide

3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. J Biol Chem 273:18793–18801.

Klein RD, Sherman D, Ho WH, Stone D, Bennett GL, Moffat B, Vandlen R, Simmons L, Gu Q, Hongo JA, Devaux B, Poulsen K, Armanini M, Nozaki C, Asai N, Goddard A, Phillips H, Henderson CE, Takahashi M, Rosenthal A (1997) A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387:717–721.

Koke JA, Yang M, Henner DJ, Volwerk JJ, Griffin OH (1991) High-level expression in *Escherichia coli* and rapid purification of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*. Protein Expr Purif 2:51–58.

Kotzbauer PT, Lampe PA, Heuckeroth, RO, Golden JP, Creedon DJ, Johnson Jr EM, Milbrandt J (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384:467–470.

Li L, Prevette D, Oppenheim RW, Milligan CE (1998) Involvement of specific caspases in motoneuron cell death *in vivo* and *in vitro* following trophic factor deprivation. Mol Cell Neurosci 12:157–167.

Lin LF, Doherty DH, Lile, JD, Bektesh S, Collins F (1993) GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132.

Marsh HN, Palfrey HC (1996) Neurotrophin-3 and brain-derived neurotrophic factor activate multiple signal transduction events but are not survival factors for hippocampal pyramidal neurons. J Neurochem 67:952–963.

Maxwell GD, Reid K, Elefanty A, Bartlett PF, Murphy M (1996) Glial cell line-derived neurotrophic factor promotes the development of adrenergic neurons in mouse neural crest cultures. Proc Natl Acad Sci USA 93:13274–13279.

Mildrandt J, de Sauvage FJ, Farçhrner TJ, Baloh RH, Leitner ML, Tansey MG, Lampe PA, Heuckeroth RO, Kotzbauer, PT, Simburger KS, Golden J, Davies JA, Vejsada R, Kato AC, Hynes M, Sherman D, Nishimura M, Wang LC, Vandlen R, Moffat B, Klein RD, Poulsen K, Gray C, Garces A, Henderson CE, Phillips H, Johnson Jr EM (1998) Persephin a novel neurotrophic factor related to GDNF and Neurturin. Neuron 20:245–253.

Miller TM, Tansey MG, Johnson EM, Creedon DJ (1997) Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization- and insulin-like growth factor I-mediated survival of cerebellar granule cells. J Biol Chem 272:9847–9853.

Moore MW, Klein RT, Fariñas I, Sauer H, Armanini N, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382:76–79.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL, Miller DK (1995) Identification and inhibition of ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43.

Nonomura T, Kubo T, Oka T, Shimoke K, Yamada M, Enokido Y, Hatanaka H (1996) Signaling pathways and survival effects of BDNF and NT-3 on cultured cerebellar granule cells. Dev Brain Res 97:42–50.

Oppenheim RW, Houenou LJ, Johnson JE, Lin LF, Li L, Lo A, Newsome AL, Prevette D, Wang S (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature 373:344–346.

Pang L, Sawada T, Decker SJ, Saltiel AR (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 270:13585–13588.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382:73–76.

Qiu MS, Green SH (1992) PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. Neuron 9:705–717.

Robertson K, Mason I (1995) Expression of ret in the chicken embryo suggests roles in regionalisation of the vagal neural tube and somites and in development of multiple neural crest and placodal lineages. Mech Dev 53:329–344.

Rossi J, Luukko K, Poteryaev D, Laurikainen A, Sun YF, Laakso T, Eerikainen S, Tuominen R, Lakso M, Rauvala H, Arumae U, Pasternack M, Saarma M, Airaksinen MS (1999) Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFRα2, a functional neurturin receptor. Neuron 22:243–252.

Sanchez MP, Silos-Santiago I, Frisén J, He B, Lira SA, Barbacid M (1996) Renal agenesis and the absence of the enteric neurons in mice lacking GDNF. Nature 382:70–73.

Schuchardt A, D'Agati V, Larsson-Blomberg L, Constantini A, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor ret. Nature 367:380–383.

Shimoke K, Kubo T, Numakawa T, Abiru Y, Enokido Y, Takei N, Ikeuchi T, Hatanaka H (1997) Involvement of phosphatidylinositol-3 kinase in prevention of low K^+-induced apoptosis of cerebellar granule neurons. Dev Brain Res 101:197–206.

Soler RM, Egea J, Mintenig GM, Sanz-Rodriguez C, Iglesias M, Comella JX (1998) Calmodulin is involved in membrane depolarization-mediated survival of motoneurons by phosphatidylinositol-3 kinase- and MAPK-independent pathways. J Neurosci 18:1230–1239.

Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD, Wong WW (1997) Substrate specifities of caspase family proteases. J Biol Chem 272:9677–9682.

Thompson J, Doxakis E, Piñon LGP, Strachan P, Buj-Bello A, Wyatt S, Buchman VL, Davies AM (1998) GRFα-4, a new GDNF family receptor. Mol Cell Neurosci 11:117–126.

Treanor JJS, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips H, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A (1996) Characterization of a multicomponent receptor for GDNF. Nature 382:80–83.

Trupp M, Rydén M, Jörnvall H, Funakoshi H, Timmusk T, Arenas E, Ibàñez C (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130:137–148.

Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, Grigoriou M, Kilkenny C, Salazar-Grueso E, Pachnis V, Arumäe U, Sariola H, Saarma M, Ibañez CF (1996) Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature 381:785–789.

Trupp M, Raynoschek C, Belluardo N, Ibáñez CF (1998) Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. Mol Cell Neurosci 11:47–63.

Virdee K, Tolkovsky AM (1996) Inhibition of p42 and p44 mitogen-activated protein kinase activity by PD48059 does not suppress nerve growth factor- induced survival of sympathetic neurones. J Neurochem 67:1801–1805.

Yan Q, Matheson C, Lopez OT (1995) *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature 373:341–344.

Yao RJ, Cooper GM (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003–2006.

Yu T, Scully S, Yu Y, Fox GM, Jing S, Zhou R (1998) Expression of GDNF family receptor components during development: implications in the mechanisms of interaction. J Neurosci 18:4684–4696.

6.4. c-Src media la supervivencia inducida por GFLs a través de un mecanismo depediente de PI 3-K

The Journal of Neuroscience, March 1, 2001, *21*(5):1464–1472

c-Src Is Required for Glial Cell Line-Derived Neurotrophic Factor (GDNF) Family Ligand-Mediated Neuronal Survival via a Phosphatidylinositol-3 Kinase (PI-3K)-Dependent Pathway

Mario Encinas,[1,2] Malú G. Tansey,[2] Brian A. Tsui-Pierchala,[2] Joan X. Comella,[1] Jeffrey Milbrandt,[3] and Eugene M. Johnson Jr[2]

[1]*Grup de Neurobiologia Molecular, Departament de Ciències Mèdiques Basiques, Facultat de Medicina, Universitat de Lleida, 25198 Lleida, Spain, and* [2]*Departments of Neurology and Molecular Biology and Pharmacology, and* [3]*Department of Pathology and Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110*

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), consisting of GDNF, neurturin, persephin, and artemin, signal via a multicomponent complex composed of Ret tyrosine kinase and the glycosyl-phosphatidylinositol (GPI)-anchored coreceptors GFRα1–α4. In previous work we have demonstrated that the localization of Ret to membrane microdomains known as lipid rafts is essential for GDNF-induced downstream signaling, differentiation, and neuronal survival. Moreover, we have found that Ret interacts with members of the Src family kinases (SFK) only when it is localized to these microdomains. In the present work we show by pharmacological and genetic approaches that Src activity was necessary to elicit optimal GDNF-mediated signaling, neurite outgrowth, and survival. In particular, p60Src, but not the other ubiquitous SFKs, Fyn and Yes, was responsible for the observed effects. Moreover, Src appeared to promote neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway because the PI-3K inhibitor LY294002 prevented GFL-mediated neuronal survival and prevented activated Src-mediated neuronal survival. In contrast, the inhibition of Src activity had no effects on NGF-mediated survival, indicating that the requirement for Src was selective for GFL-mediated neuronal survival. These data confirm the importance of protein–protein interactions between Ret and raft-associated proteins in the signaling pathways elicited by GDNF, and the data implicate Src as one of the major signaling molecules involved in GDNF-mediated bioactivity.

Key words: Ret; GDNF family ligands (GFLs); Src family kinases (SFKs); lipid rafts; phosphatidylinositol-3 kinase (PI-3K); cerebellar granule neurons

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) constitute a group of structurally related neurotrophic factors: GDNF (Lin et al., 1993), neurturin (NRTN; Kotzbauer et al., 1996), persephin (PSPN; Milbrandt et al., 1998), and artemin (ARTN; Baloh et al., 1998). The GFLs can support neuronal populations in the CNS and, except for PSPN, a variety of peripheral neuronal populations (Lin et al., 1993; Henderson et al., 1994; Buj-Bello et al., 1995; Ebendal et al., 1995; Oppenheim et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Baloh et al., 1998; Cacalano et al., 1998; Heuckeroth et al., 1998; Horger et al., 1998; Milbrandt et al., 1998). Analysis of mice deficient for some of the GFLs as well as their receptors (see below) has identified neuronal populations that require GFLs during neural development (for review, see Airaksinen et al., 1999; Baloh et al., 2000).

GFLs signal via a multicomponent receptor system consisting of the transmembrane receptor tyrosine kinase (Ret), which does not bind GFLs directly, and a high-affinity ligand-binding glycosyl-phosphatidylinositol (GPI)-linked coreceptor (GFRα). Four coreceptors have been characterized (GFRα1–α4) that interact with preferred GFLs to provide ligand specificity, although some degree of promiscuity exists. GDNF interacts mainly with GFRα1, whereas NRTN interacts with GFRα2, ARTN with GFRα3 (Airaksinen et al., 1999; Baloh et al., 2000), and PSPN with GFRα4 (Enokido et al., 1998; Lindahl et al., 2000).

One notable feature of GFRαs is their targeting to the outer leaflet of the plasma membrane via a GPI anchor (Jing et al., 1996; Treanor et al., 1996). This property predicts that these coreceptors will partition to detergent-insoluble, sphingolipid-rich, and cholesterol-rich membrane microdomains that exist as rafts in the plasma membrane (Simons and Ikonen, 1997; Brown and London, 1998, 2000). The enrichment of these microdomains in signaling proteins such as Src family kinases (SFKs) has led to the hypothesis that these rafts may function as specialized signaling organelles (Anderson, 1998).

We have shown recently that, to achieve efficient downstream signaling and maximal levels of GDNF-mediated bioactivity, Ret must be recruited to lipid rafts by GFRα1. Moreover, activated Ret interacts with SFKs only when Ret is recruited to lipid rafts, although the Src-SH2 docking site on Ret is generated after GDNF stimulation irrespective of the localization of the receptor (Tansey et al., 2000). Thus, these data suggest that SFK may represent proximal signaling elements specifically compartmentalized into lipid rafts that are necessary for maximal downstream signaling of the optimal biological effects of GFLs.

Received Sept. 13, 2000; revised Nov. 16, 2000; accepted Dec. 11, 2000.

This work was supported by National Institutes of Health Grants AG-13729 (E.M.J.) and AG-13730 (J.M.) and by the Commission for Cultural, Educational, and Scientific Exchange between the United States of America and Spain. M.E. is a predoctoral fellow of the Generalitat de Catalunya. We thank our colleagues from the Johnson, Milbrandt, and Comella laboratories for many discussions and a critical reading of this manuscript. In addition, we thank Krista Moulder and Charles Harris for their assistance in the dissection and primary culture of cerebellar granule cells and Montse Iglesias and Robert H. Baloh for many helpful discussions.

M.E. and M.G.T. contributed equally to this work.

Correspondence should be addressed to Dr. Eugene M. Johnson, Jr. or Dr. Jeffrey Milbrandt, Washington University School of Medicine, 4566 Scott Avenue, Box 8103, St. Louis, MO 62110. E-mail: ejohnson@pcg.wustl.edu or jeff@pathbox.wustl.edu.

 0270-6474/01/211464-09$15.00/0

In the present work we investigated the relevance of the Ret–SFK association in GDNF-induced downstream signaling. We found that Src activity was necessary for optimal GDNF-mediated Akt and mitogen-activated protein kinase (MAPK) phosphorylation and for neuronal survival and neurite outgrowth. Specifically, p60Src, but not the other ubiquitous SFKs Fyn and Yes, appeared to be the major target for activated Ret. PI-3K activity was necessary for GFL- and activated Src-mediated, but not NGF-mediated, neuronal survival. These data suggest that p60Src was a key proximal element in the signaling cascades initiated by GFLs.

MATERIALS AND METHODS

Neuroblastoma differentiation. Neuro2a neuroblastoma cells, which express Ret, but not GFRαs, were plated at 70,000 cells/well in a 12-well plate. Cells were transfected 24 hr after plating by using Superfect transfection reagent (Qiagen, Valencia, CA) with an enhanced green fluorescent protein (EGFP) expression plasmid (Clontech, Palo Alto, CA) and either GPI-GFRα1 or transmembrane (TM)-GFRα1 expression plasmids (Tansey et al., 2000). When indicated, the cells were transfected with either a dominant-negative mutant of Src (catalog number 21-154; Upstate Biotechnology, Lake Placid, NY) or the corresponding empty vector (Upstate Biotechnology). After 12–16 hr of incubation with the DNA (1.5 μg total) the cells were rinsed with standard growth medium (MEM with Earle's salts, 10% fetal bovine serum, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin) and were incubated for an additional 24 hr in growth medium. Then the cells were switched to 1% FBS-containing medium supplemented with the indicated amounts of GDNF with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2, 1 μM) or 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3, 1 μM; Calbiochem-Novabiochem, San Diego, CA). After 4–5 d, differentiation was scored by counting cells with neurites longer than two cell bodies present in 20 random fields in duplicate cultures. Counting was conducted by a naïve observer. The data shown are representative of three independent experiments.

Cerebellar granule cell survival. Rat cerebellar granule cell dissection and cultures were performed at postnatal day 7 (P7), as originally described by D'Mello et al. (1993) and as modified by Miller and Johnson (1996). Granule cell transfection of EGFP with the indicated constructs was performed by using a calcium phosphate protocol (Xia et al., 1996) modified by Moulder et al. (1999). To quantitate transfection results, we counted the number of initial EGFP-positive cells in designated fields (minimum of 150 cells) of two to four wells in a four-well dish (Nunc, Naperville, IL) per condition at 24 hr after transfection. Cultures were rinsed twice in DMEM and switched to high potassium plus serum (K25+S) medium, low potassium without serum medium (K5−S), or K5−S supplemented with the indicated factors for 48 hr. After this period the number of EGFP-positive cells remaining in the same fields was scored in a blinded manner to obtain the percentage of neuronal survival. As for neuronal differentiation, PP2 and PP3 were used at 1 μM. Solvent concentrations (DMSO) never exceeded 0.1%.

Immunoprecipitation. Neuro2a cells transfected with GPI-GFRα1 or TM-GFRα1 were stimulated, as were sympathetic neurons, with 30–50 ng/ml GDNF for 10 min. Cells were lysed on ice in cold coimmunoprecipitation buffer [containing (in mM) 50 Tris-HCl, pH 7.5, 1% Brij 96 or 1% NP-40, 150 NaCl, 1 EDTA, 1 EGTA, 10 NaF, 2 Pafabloc, and 1 Na_2VO_4 plus 1 μg/ml leupeptin and 1 μg/ml aprotinin]. Cleared lysates were immunoprecipitated with 10 μl of a goat anti-Ret antibody (C-19G for CGCs and Neuro2a cells or C-20 for SCGs; Santa Cruz, Santa Cruz, CA) and a mix of Protein A/Protein G agarose conjugates (Life Technologies, Gaithersburg, MD). Immunoblot analyses of Ret immune complexes with a second Ret antibody (C-19G for CGGs and Neuro2a cells or C-20 for SCGs; Santa Cruz) confirmed quantitative immunoprecipitation of Ret. Ret-associated Src family kinase immunoreactivity was detected with specific antibodies against anti-p60Src (N-16; Santa Cruz), anti-Fyn, or anti-Yes (Transduction, Lexington, KY). For detection of Ret autophosphorylation the cells were lysed and immunoprecipitated as above with a goat anti-Ret antibody, and immune complexes were probed with an anti-phospho-tyrosine antibody (4G10, Upstate Biotechnology).

Western blotting. Neuro2a cells transfected with GFRα1 or SH-SY5Y neuroblastoma cells treated with retinoic acid for 3 d were stimulated for 10 min with 30 ng/ml GDNF. Cerebellar granule cells were maintained for 7 d in K25+S medium, deprived for 3 hr in K5−S, and then stimulated with K25+S for 15 min. When required, a 30 min preincubation step with the indicated doses of PP2 or PP3 was included before stimulation. Cells then were resuspended in 2× SDS-Laemmli sample buffer, boiled, and analyzed by immunoblot with an anti-phospho-p42/p44 MAPK antibody (T202/Y204) or anti-phospho-Akt (Ser-473) (1:1000; New England Biolabs, Beverly, MA). Blots were stripped in 2% SDS and 125 mM Tris, pH 6.8, for 30 min at 65°C and reprobed with an antibody against total MAPK to confirm equal protein loading. Activation of p60Src was monitored with a phospho-specific antibody to Tyr-418 on p60Src (Biosource, Camarillo, CA) that is autophosphorylated when Src is activated.

Sympathetic neuronal cultures and treatments. The superior cervical ganglia from P1 Sprague Dawley rats were dissected, dissociated, and seeded onto collagen-coated 35 mm culture dishes or two-well glass chamber slides (Nunc) as previously described (Martin et al., 1992). Cultures were maintained *in vitro* (DIV) in medium (90% MEM, 10% fetal bovine serum, 2 mM glutamine, 20 μM uridine, 20 μM fluorodeoxyuridine, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing NGF (50 ng/ml, AM50). After 5 d the cultures were washed twice with medium without NGF (AM0). AM50, anti-NGF (goat polyclonal, 1:10,000 dilution), NGF in the presence of LY294002 or PP2 (50 and 1 μM, respectively), NRTN (50 ng/ml) in the presence of anti-NGF, or NTRN in the presence of anti-NGF with LY294002 or PP2 then was added to the cultures. The neurons were maintained in this medium for 3 d, with one medium replacement after the second day, before fixation and processing for survival assays. Because NRTN supports a higher percentage of survival of SCG neurons, probably reflective of coreceptor expression, NRTN was used rather than GDNF in these survival assays.

Sympathetic neuron survival assays. After the indicated treatments the cultures were washed with ice-cold PBS and fixed for 2 d with 4% paraformaldehyde at 4°C. Then the cultures were washed with deionized water, stained for 45–60 sec in toluidine blue O (1 gm/l), and incubated for an additional 60 sec in deionized water. The cells were dehydrated by using successive 2 min washes in deionized water containing increasing concentrations of ethanol to reach 100%, after which the slides were washed in toluene, and the coverslips were mounted with Permount (Sigma, St. Louis, MO). Neurons displaying smooth cell bodies that were Nissl-stained were considered alive and were counted. Survival counts were obtained in a blinded manner from duplicate wells from three independent cultures.

RESULTS

Src activity is necessary for GDNF-mediated neurite outgrowth

To test the functional relevance of SFKs in GDNF-induced neurite outgrowth, we used the selective inhibitor PP2, which inhibits all members of the Src family kinases in the nanomolar range *in vitro* (Hanke et al., 1996). The structurally related but inactive analog PP3 was used as a negative control in all of these experiments. Neuro2a neuroblastoma cells, which express endogenous Ret but not GFRα1, were cotransfected with either wild-type GFRα1 (GPI-GFRα1) or with a chimeric receptor consisting of the extracellular domain of this coreceptor and the transmembrane and cytoplasmic tail of HLA-B44. This transmembrane version of the coreceptor (TM-GFRα1) lacks the GPI anchor and therefore does not localize to lipid rafts (Tansey et al., 2000). At 2 d after transfection the cells were switched to GDNF-containing medium in the presence or absence of PP2, and the number of neurite-bearing cells was counted after a period of 4–5 d. In agreement with our previous results, GDNF elicited a potent neuritogenic response in GPI-GFRα1-transfected cells. The effect was dose-dependent and, at saturating concentrations (30 ng/ml), GDNF supported a four- to sixfold increase in the number of cells with neurites (Fig. 1*A*). Moreover, the length of neurites was significantly longer in GDNF-treated than in control cells (data not shown). This effect was inhibited significantly by 1 μM PP2, whereas PP3 had no effect (Fig. 1*A*). Consistent with our previous work, GFRα-TM-expressing cells displayed attenuated

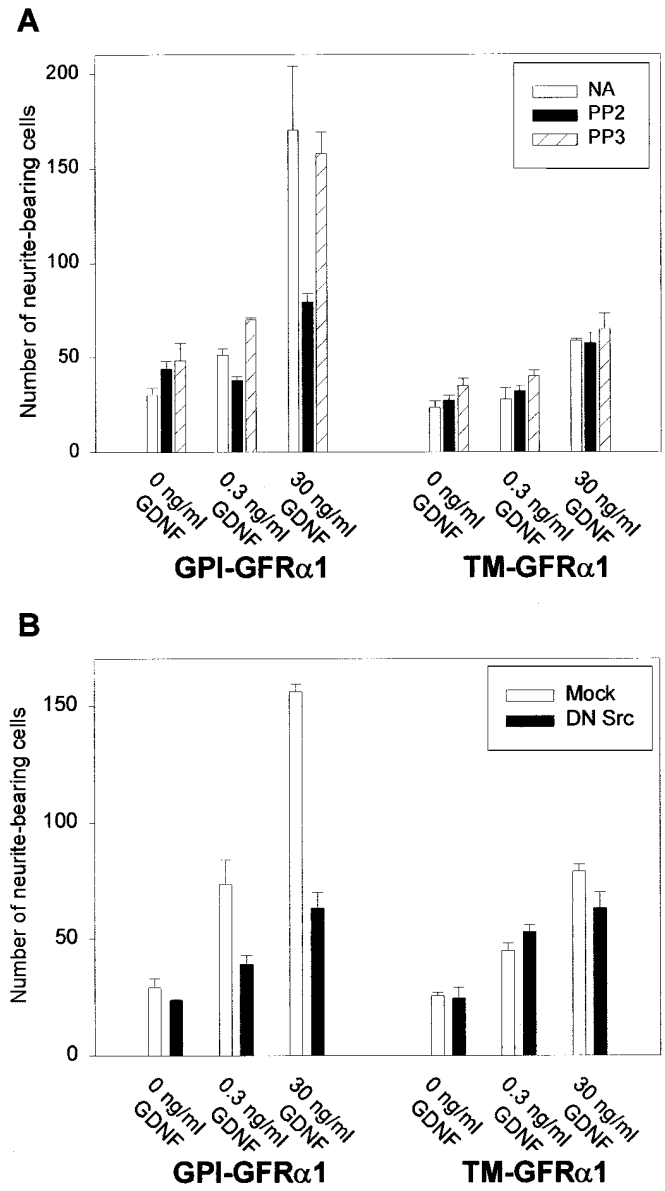


Figure 1. Src activity is necessary for GDNF-mediated neurite outgrowth. *A*, Neuro2a neuroblastoma cells transfected either with the GPI-linked or the transmembrane (*TM*) version of GFRα1 were treated with GDNF in the presence of the SFK inhibitor PP2 (1 μM), the structurally related but inactive analog PP3 (1 μM), or neither (*NA*). After 4 DIV the cells with neurites longer than two cell bodies were counted as described in Materials and Methods. *B*, Neuro2a cells were cotransfected with either the GPI-linked or the transmembrane (*TM*) version of GFRα1 and either a plasmid coding for a dominant-negative mutant of Src (*DN Src*) or the empty expression vector (*Mock*). Cells were treated with the indicated concentrations of GDNF, and neurite outgrowth was assessed as in *A*. Each condition was performed in triplicate. The results shown represent the means ± SEM ($n = 3$).

neurite outgrowth in response to GDNF; however, PP2 did not affect the GDNF-induced neurite outgrowth in these cells.

To test further the hypothesis that SFKs are important in GDNF-induced responses, we cotransfected Neuro2a cells with a dominant-negative mutant of Src and either the GPI or the TM version of GFRα1. This dominant-negative Src most likely acts as a pan-Src inhibitor because it encodes both K296R and Y528F mutations and, therefore, is able to bind to phosphotyrosine docking sites, but it is unable to phosphorylate downstream targets (Courtneidge et al., 1993). Again, only the GPI-GFRα1-expressing cells showed a robust neuritogenic response to GDNF, whereas this biological effect was attenuated in TM-GFRα1-transfected cells. Consistent with the results obtained with PP2, inhibition of SFK activity by the dominant-negative construct resulted in a significant reduction in the number of neurite-bearing cells as compared with the mock-transfected cells (Fig. 1*B*). This reduction was not observed in the TM-expressing cells, consistent with the fact that Ret is not able to interact with SFK when activated by the transmembrane version of GFRα1 (Tansey et al., 2000). Taken together, these data indicate that SFK activity was necessary for GDNF-mediated neurite outgrowth via wild-type Ret-GFRα1.

SFK activity is necessary for GDNF-mediated neuronal survival

We also investigated whether SFK activity was necessary for another Ret-mediated function, neuronal survival. To test this hypothesis, we used cerebellar granule cells (CGCs), which survive in a medium containing high potassium (25 mM) plus serum (K25+S) but undergo apoptosis in low potassium (5 mM) medium without serum (K5−S) (D'Mello et al., 1993). Cultured cerebellar granule cells do not express either Ret or any of the GFRα coreceptors, allowing for reconstitution of this receptor system by transfection with defined (wild-type or mutant) components (M. Tansey and E. M. Johnson, unpublished observations). As expected, cotransfection of Ret and GFRα1 is sufficient to elicit a dose-dependent increase in GDNF-mediated cell survival (Tansey et al., 2000). Concentrations as low as 0.3 ng/ml of GDNF in K5−S medium supported ~30% maximum survival; the effect was maximal at 30 ng/ml, reaching levels comparable with those obtained in K25+S medium (Fig. 2*A*). However, the addition of PP2, but not PP3, to the GDNF-containing medium significantly inhibited the survival-promoting effect. In contrast, PP2 did not have any effect on the survival elicited by K25+S medium, indicating that the cell death observed in the presence of this compound was not caused by nonspecific toxicity. Furthermore, SFK activity was not required for the survival-promoting activity of depolarization and serum (Fig. 2*A*). Consistent with this finding, depolarization-induced MAPK (data not shown) and Akt phosphorylation were unaffected by the addition of PP2 (Fig. 2*C*), although they were affected by LY294002 treatment. Similar effects on survival were obtained when a plasmid coding for a dominant-negative form of Src was used instead of PP2 (Fig. 2*B*). Moreover, PP2 also inhibited GDNF-mediated survival in rat sympathetic neurons, which express both Ret and GFRα1 (see below).

p60Src, but not Fyn or Yes, interacts with activated Ret

We and others have demonstrated previously that activated Ret interacts with a member or members of the SFK (Melillo et al., 1999; Tansey et al., 2000). In particular, we have detected pan-Src immunoreactivity in Ret immunoprecipitates only when the activated Ret complex is associated with lipid rafts. Moreover, a Src-SH2 probe is able to precipitate Ret in GDNF-stimulated, but not control, Neuro2a lysates (Tansey et al., 2000). A critical question was to determine which members of the SFKs interact with Ret under these conditions. To address this, we performed Ret coimmunoprecipitation experiments in Neuro2a cells transfected either with the GPI-GFRα1 or the TM-GFRα1 and stim-

ulated with GDNF. Then the immune complexes were resolved by SDS-PAGE and probed with specific antibodies against p60Src, Fyn, and Yes, the three ubiquitous SFKs (Thomas and Brugge, 1997). As shown in Figure 3*A*, p60Src was found to be associated with Ret only in GDNF-stimulated cells expressing GPI-GFRα1. However, neither Fyn nor Yes was associated significantly with Ret under any of the experimental conditions (Fig. 3*B,C*). Only small amounts of Fyn and Yes immunoreactivity were detected after longer exposures of the film but were negligible when compared with the amount of these proteins in total extracts, indicating that only a minor fraction of these proteins was associated with the activated receptor complex (Fig. 3*B,C*). To test whether an association between Ret and p60Src also occurred in rat sympathetic neurons, we stimulated SCG neurons with GDNF, immunoprecipitated Ret, and subjected the immune complexes to p60Src immunoblotting. Similar to Neuro2a cells, p60Src coimmunoprecipitated with Ret only in GDNF-stimulated extracts from SCG neurons, suggesting that this association was phosphotyrosine-dependent. In contrast, neither Fyn nor Yes coimmunoprecipitated with Ret (Fig. 3*D*) despite the fact that significant levels of these proteins were detected in total lysates from these neurons. Thus, the effects of PP2 and dominant-negative mutants of Src on neuronal differentiation and survival were attributable primarily to the inhibition of p60Src, but not Fyn or Yes, activity.

p60Src becomes activated after GDNF stimulation

The ability of the SH2 domain of Src to precipitate Ret in a phosphotyrosine-dependent manner (Tansey et al., 2000) suggests that the interaction of Ret with Src may activate Src kinase activity. Intramolecular interactions between the SH2 domain and the phosphorylated tail of SFKs maintain their inactive or "closed" conformation. Displacement of this interaction by SH2-binding phosphotyrosines in activated receptors leads to SFK activation (Thomas and Brugge, 1997; Abram and Courtneidge, 2000). To address whether GDNF can activate Src tyrosine kinase activity, we immunoprecipitated Ret from GPI- or TM-GFRα-expressing Neuro2a cells, and we probed immune complexes with an antibody that specifically recognizes Src only when it is phosphorylated in tyrosine 418. This residue, located in the Src tyrosine kinase domain, is autophosphorylated when Src is activated (Abram and Courtneidge, 2000). As shown in Figure 4, Src became phosphorylated on Tyr-418 only in GDNF-stimulated, wild-type GFRα1-expressing cells, indicating that only in this condition was Src activated. Thus, as expected, the interaction of Ret with Src led to the activation of the tyrosine kinase activity of Src.

The SFK inhibitor PP2 blocks GDNF-mediated Akt and MAPK phosphorylation

The decreased ability of nonraft-associated Ret to promote differentiation and survival of a mislocalized Ret correlates with diminished downstream signaling, as measured by Akt and MAPK phosphorylation (Tansey et al., 2000). Therefore, we investigated whether Src inhibition also resulted in decreased phosphorylation of these signaling molecules. Neuro2a cells transfected with wild-type GFRα1 were stimulated with GDNF in the presence of PP2 or PP3, and the amount of phosphorylated Akt and MAPK was assessed by using phospho-specific antibodies. PP2, but not PP3, inhibited both Akt and MAPK phosphorylation in GPI-GFRα1-transfected cells (Fig. 5*A*). Note that 1 μM PP2 produced significant, but not complete, inhibition of GDNF-induced Akt phosphorylation (see below). As expected, the induction of phosphorylation of both Akt and MAPK in TM-

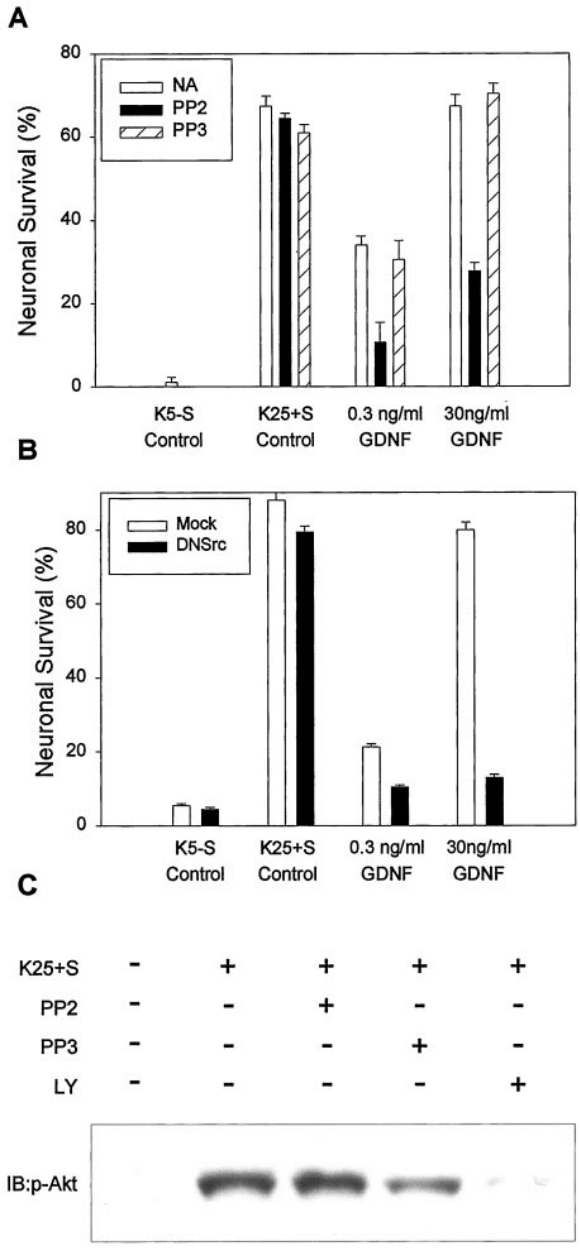


Figure 2. Inhibition of Src activity results in the blockade of GDNF-mediated survival. Cerebellar granule cells (CGCs) were transfected with Ret and wild-type GFRα1. At 2 d after transfection the cells were switched to K5−S medium containing increasing concentrations of GDNF or vehicle alone (*NA*), PP2 (1 μM), or PP3 (1 μM). Neuronal survival was evaluated after 48 hr in culture as described in Materials and Methods. Each condition was performed in duplicate. The results shown represent the means ± SEM (n = 4). *B*, CGCs were cotransfected with Ret and wild-type GFRα1 and either a dominant-negative Src mutant (*DN Src*) or the corresponding empty expression plasmid (*Mock*). After 48 hr in culture, cell survival was evaluated as in *A*. *C*, Depolarization-induced Akt phosphorylation in CGC is unaffected by PP2. Cerebellar granule cells were maintained in K25+S medium for 7 d, deprived for 3 hr in K5−S, and then stimulated for 15 min with K25+S. Where indicated, a preincubation step with either PP2 or PP3 (both at 1 μM) or LY294002 (30 μM) was included before stimulation. Whole-cell lysates were probed with anti-phospho-Akt antibodies as described in Materials and Methods. Equal amounts of protein were loaded in each condition. The data shown are representative of two independent experiments.

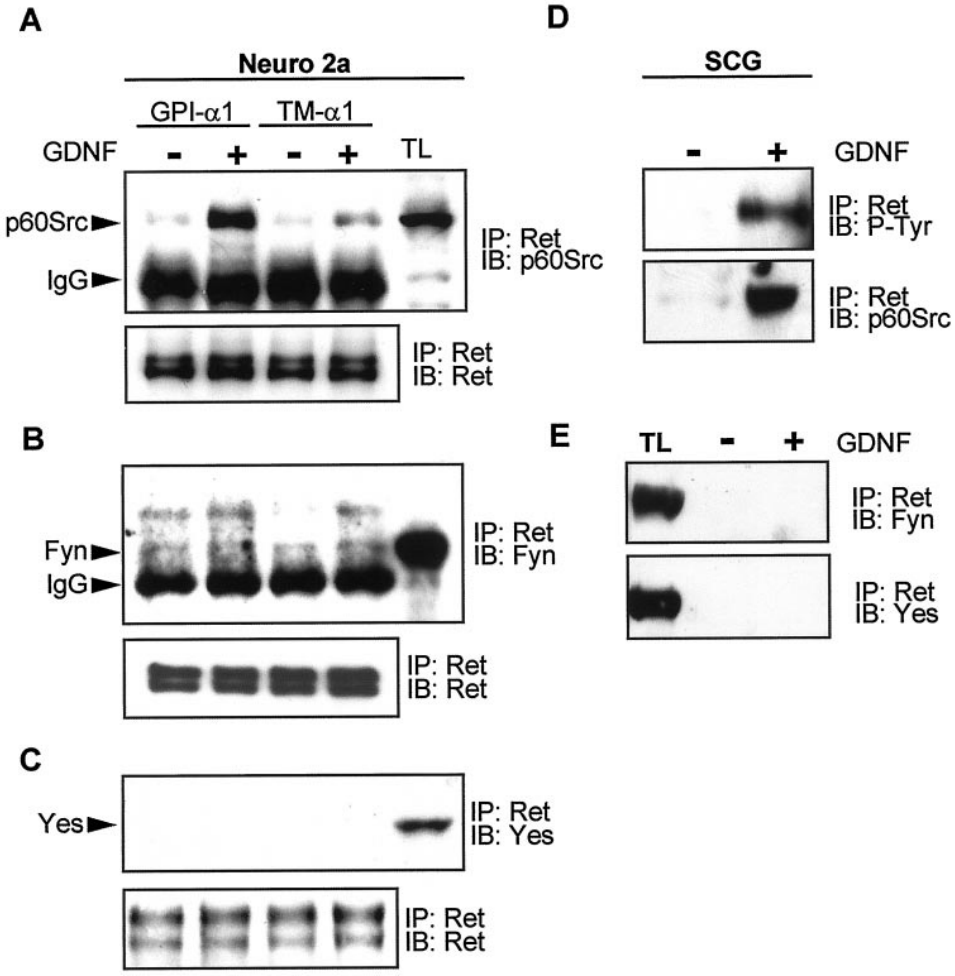


Figure 3. p60Src, but not Fyn or Yes, associates with activated Ret. *A*, Neuro2a cells transfected with either the GPI-GFRα1 or the TM-GFRα1 were stimulated with 30 ng/ml GDNF, Ret was immunoprecipitated, and immune complexes were probed with a specific anti-p60Src antibody (*top panel*). The TL lane (*TL*) corresponds to an aliquot of total lysate removed before immunoprecipitation, representing 10% of the protein that was immunoprecipitated. The *bottom panel* shows an immunoblot against Ret from the same membrane to assess the quantitative immunoprecipitation of the receptor. *B*, Lysates treated as in *A* were probed with an anti-Fyn antibody. *C*, Lysates treated as in *A* were probed with an anti-Yes antibody. *D*, p60Src coimmunoprecipitates with phosphorylated Ret in sympathetic neurons. Superior cervical ganglion cells were stimulated with 30 ng/ml GDNF, and Ret was immunoprecipitated. Ret phosphorylation was determined by probing immune complexes with an anti-phospho-tyrosine antibody (*P-Tyr*; *top*); whether Src coimmunoprecipitates was determined by probing these immune complexes with an anti-p60Src antibody (*bottom*). *E*, Superior ganglion cells treated as in *D*, but Ret immunoprecipitates were probed with either anti-Fyn or anti-Yes antibodies. A whole-cell lysate (*TL*) was included to show the migration of these species. *IP*, Immunoprecipitation; *IB*, immunoblot.

expressed cells was attenuated but was not affected by PP2 or PP3 (data not shown), consistent with the fact that the activated Ret complex in GFRα1-TM-expressing cells is not in lipid rafts and, therefore, does not interact with SFKs (Tansey et al., 2000). We also tested the effect of PP2 in the SH-SY5Y neuroblastoma cell line, which expresses Ret and GFRα1 after exposure to retinoic acid. As shown in Figure 5*B*, PP2 caused a dose-dependent decrease in the state of phosphorylation of both Akt and MAPK, suggesting that the involvement of SFKs in GDNF-mediated signaling was a generalized event.

Because PP2 is able to inhibit EGFR directly, although with a 100-fold higher IC_{50} than SFKs (Hanke et al., 1996), we tested whether PP2 was eliciting the observed effects via SFK inhibition or via a direct inhibition of the kinase activity of Ret. Neuro2a cells transfected with the GPI version of GFRα1 were stimulated with GDNF in the presence of 1 μM PP2 or PP3, Ret was immunoprecipitated, and the level of tyrosine phosphorylation was analyzed by Western blotting. The addition of PP2 had a negligible effect on the autophosphorylation of Ret (Fig. 5*C*), which was not likely to account for the inhibitory effects of PP2 on downstream signaling and bioactivity. Higher concentrations (5 μM) of PP2, however, significantly inhibited Ret phosphorylation (see below).

GDNF-dependent survival of granule neurons requires PI-3K and Src activities

The PI-3K/Akt pathway has a pivotal role in cerebellar granule neuron survival (D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997). To test whether PI-3K activity was necessary for GDNF-mediated survival, we maintained Ret- and GFRα1-expressing granule cells in the presence of the PI-3K-selective

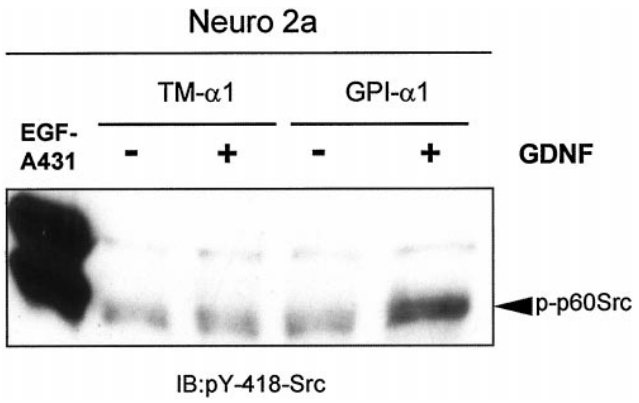


Figure 4. Src becomes activated after GDNF stimulation in GPI-GFRα1-expressing, but not TM-GFRα1-expressing, cells. Neuro2a cells were transfected with the indicated constructs and stimulated with GDNF (30 ng/ml; 10 min). Ret was immunoprecipitated, and immune complexes were probed with a phospho-specific antibody against the Tyr-418 of Src. This residue is the major autophosphorylation tyrosine in Src, and its phosphorylation correlates with the kinase activity. As a positive control a lysate from A431 cells stimulated with EGF was included (*left lane*). The data shown are representative of two independent experiments.

inhibitor LY294002 (30 μM). This inhibitor completely prevented the survival-promoting activity of GDNF, indicating that PI-3K activity was necessary for GDNF-induced survival of these cells (Fig. 6). To investigate further whether Src was promoting cell survival via a PI-3K-dependent pathway or whether both PI-3K and Src contributed to this biological effect via independent mechanisms, we transfected granule cells with a constitutively active mutant of Src (CA Src). As shown in Figure 6, this mutant was able to promote a partial rescue in the absence of any trophic

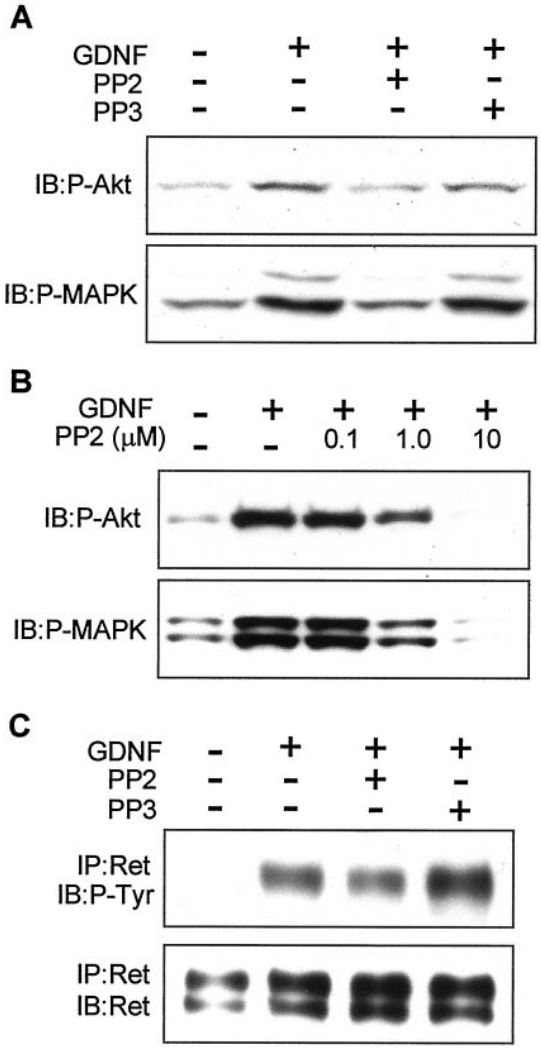


Figure 5. The SFK inhibitor PP2 blocks distal, but not proximal, GDNF-mediated signaling. *A*, Neuro2a cells transfected with GFRα1 were stimulated with 30 ng/ml GDNF for 10 min. When indicated, the cells were preincubated with either PP2 or PP3 (1 μM) for 30 min. Then total lysates were resolved by SDS-PAGE and probed with phospho-specific antibodies against Akt and MAPKs. *B*, SH-SY5Y cells, which endogenously express both Ret and GFRα1 after retinoic acid treatment, were stimulated with GDNF and increasing concentrations of PP2. Total lysates were resolved by SDS-PAGE and probed with phospho-specific antibodies against Akt and MAPKs. The data shown are representative of two to three independent experiments. *C*, Neuro2a cells transfected with wild-type GFRα1 were stimulated with 30 ng/ml GDNF for 10 min. When indicated, the cells were incubated with PP2 or PP3 (1 μM) for 30 min before stimulation. Ret was immunoprecipitated, and the level of autophosphorylation was assessed by Western blot (*top*). The filter was stripped and reprobed with anti-Ret antibody to assess equal protein loading of the lanes (*bottom*). *IP*, Immunoprecipitation; *IB*, immunoblot.

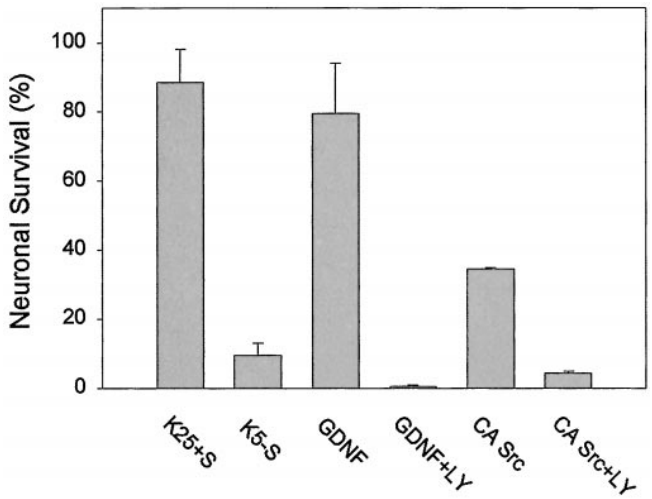


Figure 6. Src promotes CGC survival via a PI-3K-dependent mechanism. CGCs either were cotransfected with Ret and GFRα1 or were transfected with a constitutively active mutant of Src alone (*CA Src*). In the first case the cells were maintained with K25+S, K5−S, or K5−S containing 30 ng/ml GDNF alone or in combination with 30 μM LY294002. Cells transfected with CA Src were maintained in K5−S medium alone or K5−S containing 30 μM LY294002. After 2 d in culture, cell survival was assessed as described in Materials and Methods. Each condition was performed in duplicate. The results shown represent the means ± SEM ($n = 3$).

support, but this rescue was prevented completely by LY294002. The inability of CA Src to promote survival as efficiently as GDNF may reflect the lack of scaffolding and membrane localization provided by association with the Ret signaling complex, or it may be attributable to the requirement of additional GDNF-activated signaling molecules upstream of PI-3K. This observation, together with the reduction of Akt phosphorylation by PP2 (see Fig. 5), suggests that Src maintained cell survival by modulating the activation of the PI-3K/Akt pathway.

Src activity is necessary for GFL-mediated, but not NGF-mediated, survival of granule and sympathetic neurons

We next investigated whether the requirement for SFK activity in survival promotion was specific to Ret-mediated survival or also was involved in TrkA-mediated survival. Granule cells transfected with TrkA did survive in K5−S medium supplemented with NGF. However, PP2 did not affect NGF/TrkA-mediated survival (Fig. 7*A*), suggesting that SFKs do not have a major role in survival promotion by this neurotrophic factor.

To determine whether primary neurons that normally express Ret and GFRαs required Src and PI-3K activities for GFL-dependent survival, we examined the NRTN-mediated survival of sympathetic neurons from the superior cervical ganglion. After being maintained in NGF for 5 DIV, sympathetic neurons were switched to medium containing NRTN in the presence or absence of LY294002 or PP2. Compared with NGF, NRTN saved ~50% of the neurons after 3 d, in contrast to neurons that had been switched to medium containing no factors (Fig. 7*B*). Inhibition of PI-3K with LY294002 completely blocked the NRTN-dependent survival. In contrast, PI-3K inhibition had only modest effects on NGF-mediated survival. In fact, NRTN-maintained neurons treated with LY294002 died with the same time course as NGF deprivation, suggesting that PI-3K completely accounted for the survival-promoting ability of NRTN on sympathetic neurons.

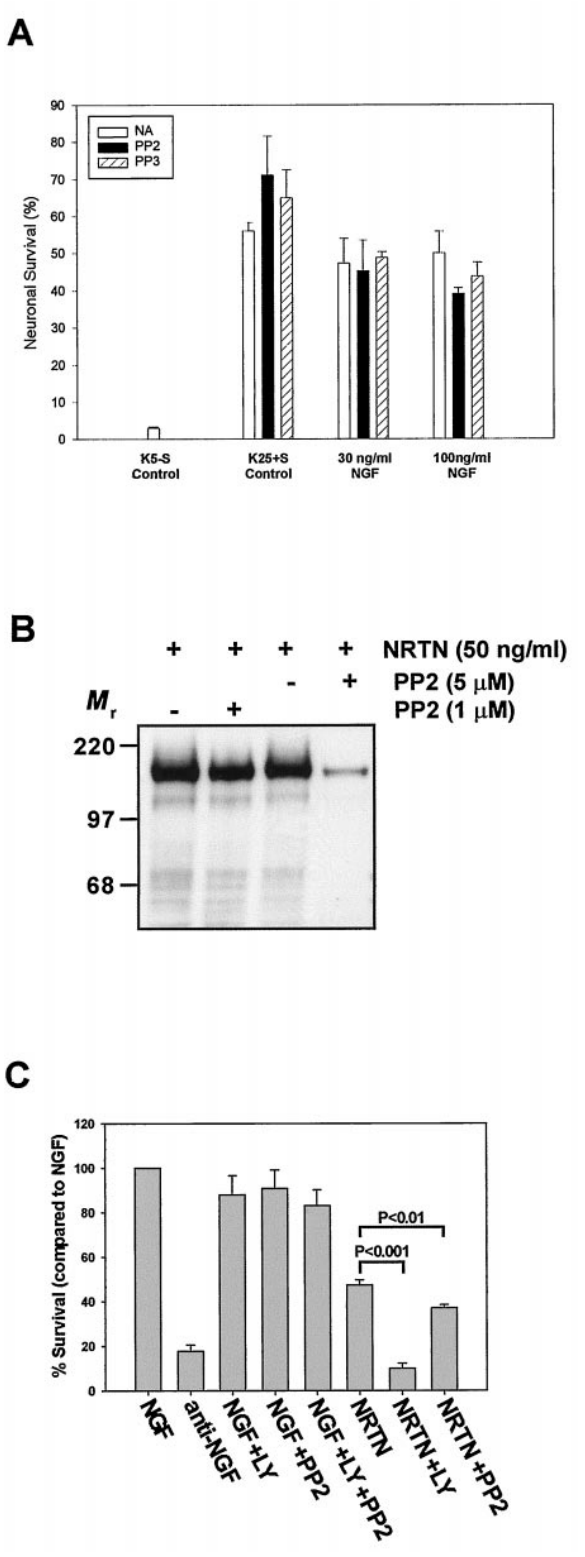


Figure 7. The SFK inhibitor PP2 blocks GFL-mediated, but not NGF-mediated, neuronal survival. *A*, Cerebellar granule cells transfected with TrkA were maintained in K5−S medium containing increasing concentrations of NGF in the presence or the absence of either PP2 or PP3 (1 μM). Neuronal survival was assessed after 48 hr in culture as described in Materials and Methods. Each condition was performed in duplicate. The results shown represent the means ± SEM ($n = 3$). *NA* designates no addition of PP2 or PP3. *B*, Sympathetic neurons (5 DIV) were deprived of NGF for 7 hr and then treated with medium containing NRTN (50 ng/ml) or medium containing NRTN in the presence of 1 or 5 μM PP2. Neurons treated with 1 or 5 μM PP2 and NRTN also were pretreated with that same concentration of PP2 for 30 min before the NRTN addition. The cultures were washed, Ret was immunoprecipitated from each condition, and the immunoprecipitates were subjected to phosphotyrosine immunoblotting. *C*, Sympathetic neurons (5 DIV) were deprived of NGF, maintained in NGF alone or in the presence of LY294002 (50 μM), PP2 (1 μM), or both, or switched to medium containing NRTN (50 ng/ml) alone or in the presence of LY294002 or PP2. The sympathetic neurons were maintained for an additional 3 d, with one medium change, after which they were fixed and toluidine blue O-stained; the number of surviving neurons was determined in a blinded manner.

Src inhibition with 1 μM PP2 also decreased NRTN-mediated survival ($p < 0.01$), in contrast to NGF-dependent survival in which PP2 had no effect (Fig. 7*B*). As in granule neurons, treatment with PP2 (1 μM) had little effect on Ret phosphorylation (Fig. 7*C*), although higher doses of PP2 (5 μM) were more effective in blocking Ret activation. Because sympathetic neurons were treated with only 1 μM PP2 to avoid substantial inhibition of Ret itself, Src activity was not inhibited completely (data not shown) at this concentration in sympathetic neurons, similar to SY5Y cells (see Fig. 5*B*), leading to less death than was observed in granule neurons. In this regard, 1 μM PP2 did not prevent GDNF-mediated survival in CGCs completely as compared with the dominant-negative mutant of Src (see Fig. 2*A,B*), indicating that the inhibitor did not block Src activity completely. In conclusion, consistent with data from granule neurons, GFLs promoted survival in sympathetic neurons via activation of PI-3K in a, at least partially, Src-dependent manner.

DISCUSSION

In a previous study we demonstrate that localization of Ret to lipid rafts is necessary for optimal GFL-mediated differentiation and survival responses as well as for maximal downstream signaling. One suggested explanation for this observation is the requirement of important protein–protein interactions between Ret and raft-associated signaling molecules to elicit full GFL-mediated responses. In particular, SFKs interact with Ret only when the receptor is localized in these membrane microdomains (Tansey et al., 2000). In the present work we showed that SFK activity was necessary for both differentiation and survival events elicited by GFLs, indicating that this interaction was biologically important. Moreover, the reduced bioactivity observed after SFK inhibition (achieved by two independent approaches) correlated with a decrease in the state of activation of both Akt and MAPK pathways, providing a possible mechanism by which Src could be driving these biological effects. We identified p60Src as the specific member of the SFK that interacted with activated Ret. Interaction with Ret correlated with the activation of p60Src, as measured by phosphorylation of Src at Tyr-418. The survival-promoting effects of Src were mediated via a PI-3K-dependent mechanism, because LY294002 blocked GFL-activated and activated Src-dependent cell survival in multiple neuronal types. Finally, Src was involved in GFL-mediated, but not NGF-mediated, neuronal survival in two different primary cultures, suggesting that the families of factors use different pathways to promote neuronal survival. In summary, these data indicate that Src was a key proximal-signaling molecule in the transduction cascades initiated by GFLs and provide an explanation as to why compartmentalized signaling in lipid rafts was necessary to achieve efficient neuronal survival and neurite outgrowth.

Role of Src in cellular differentiation

The first biological effect modulated by Src activity that we measured was neurite outgrowth. The addition of PP2 or transfection with a dominant-negative form of p60Src markedly reduced the number of neurite-bearing cells. Inhibition of Src did not affect TM-driven differentiation, indicating that the role of Src was to enhance rather than to mediate entirely the neurite-promoting effect of GDNF. Thus, one hypothesis was that the differences between GPI- and TM-induced differentiation were attributed almost exclusively to the presence or the absence of Src activity, i.e., in the localization, or not, of the activated Ret/GFRα complex to lipid rafts.

The role of Src in cellular differentiation has been investigated primarily by infection with the Rous sarcoma virus (RSV), which expresses the oncogenic variant *v-Src*. These studies reveal that the function of Src on differentiation depends mainly on the cellular context. In many cell types such as avian myoblasts, retinoblasts, or chondroblasts, expression of *v-Src* results in the abrogation of differentiation responses (Muto et al., 1977; Yoshimura et al., 1981; Crisanti-Combes et al., 1982; Alema and Tato, 1987). In some neuronal cell types, however, expression of *v-Src* induces differentiation. Avian sympathetic neuroblasts infected with RSV stop proliferating and differentiate by extending neurites and expressing neuronal markers (Haltmeier and Rohrer, 1990). In PC12 pheochromocytoma cells the expression of *v-Src* causes morphological differentiation that mimics the effects of NGF by different criteria (Alema et al., 1985; Thomas et al., 1991). Thus, the effects of overexpression of the viral form of Src may not necessarily reflect an involvement of its cellular counterpart *c-Src* in neuronal differentiation.

Src promotes neuronal survival via a PI-3K-dependent pathway

In contrast to the well established roles of Src in cell cycle control, cell movement and adhesion, and cell differentiation (Thomas and Brugge, 1997), the role of Src in survival has remained elusive. Early experiments performed with *v-Src* reveal that its expression in certain cell types rescues these cells from apoptosis that is induced by cytokine withdrawal (Anderson et al., 1990; McCubrey et al., 1993) and the loss of extracellular matrix adhesion (Frisch and Francis, 1994). The role of the cellular homolog *c-Src*, however, is less well documented. Perhaps the most compelling evidence for the involvement of *c-Src* in cell survival comes from a recent report by Wong et al. (1999). In this work osteoclasts derived from $Src^{-/-}$ mice show an impaired TRANCE-mediated survival response with respect to wild-type osteoclasts. Interestingly, this reduction in survival correlates with a decreased ability of TRANCE to activate the Ser/Thr kinase Akt, the anti-apoptotic function of which is well established (for review, see Datta et al., 1999). This finding extended to IL-1- and LPS-induced Akt activation, suggesting that the mechanism may represent a general pathway in cytokine-mediated survival (Wong et al., 1999). Thus, Src may activate PI-3K that, in turn, activates Akt and ultimately promotes cell survival (see Schlessinger, 2000). Consistent with this hypothesis, our results show that Src inhibition resulted in a blockade of Akt phosphorylation and marked attenuation of neuronal survival induced by GFLs. Accordingly, in our model the PI-3K inhibitor LY294002 abolished GDNF- and NRTN-mediated survival in granule and sympathetic neurons, respectively. Therefore, we speculate that the survival-promoting effects of GFLs likely were mediated through p60Src via the PI3K pathway. Consistent with this model, we found that a constitutively active mutant of Src partially rescued CGCs from death in K5−S medium and that this effect was blocked completely by the PI-3K inhibitor LY294002, suggesting that the survival-promoting effects of Src were mediated principally by PI-3K. The relevance of the PI-3K/Akt pathway in GDNF-mediated survival occurs in other neuronal models such as spinal cord motor neurons (Soler et al., 1999). Consistent with this, sympathetic neurons were completely dependent on PI-3K for the survival-promoting effects of NRTN. This is in contrast to NGF-mediated survival of sympathetic neurons, which rely on additional signaling pathways in conjunction with PI-3K for survival (Philpott et al., 1997; Virdee et al., 1999; Tsui-Pierchala et al., 2000). Thus, signaling pathways mediating survival are not only cell type-specific, but in neurons they may also be growth factor- and receptor-specific.

Specificity of Ret–SFK interactions

The selectivity of the interaction between Ret and p60Src, but not Fyn or Yes, perhaps was somewhat surprising, given the high degree of functional redundancy that analysis of mice deficient in these proteins has revealed (for review, see Lowell and Soriano, 1996). However, the specificity of protein–protein interactions between a given member of the SFK and certain receptors is not unprecedented. For example, Lyn interacts specifically with FcϵRI in basophils (Sheets et al., 1999), and LAT (linker of activation of T cells) specifically binds to the T cell receptor (TCR) (Harder and Simons, 1999; Janes et al., 1999). Moreover, similar cellular functions may be mediated by different SFKs depending on cell type, as is the case for Fyn, but not Src or Yes, in the morphological differentiation of oligodendrocytes (Osterhout et al., 1999). Thus, although p60Src was the major target for activated Ret in sympathetic neurons and Neuro2a neuroblastoma cells, other SFKs might have interacted with Ret, depending on the cellular context. Finally, our work supports a model in which the requirement for SFK activity is specific for GFL/Ret-mediated neuronal survival and suggests that SFK members are unlikely to have a central function in survival responses elicited by NGF/TrkA signaling.

REFERENCES

Abram CL, Courtneidge SA (2000) Src family tyrosine kinases and growth factor signaling. Exp Cell Res 254:1–13.

Airaksinen MS, Titievsky A, Saarma M (1999) GDNF family neurotrophic factor signaling: four masters, one servant? Mol Cell Neurosci 13:313–325.

Alema S, Tato F (1987) Interaction of retroviral oncogenes with the differentiation program of myogenic cells. Adv Cancer Res 49:1–28.

Alema S, Cassalbore P, Agostini E, Tato F (1985) Differentiation of PC12 pheochromocytoma cells induced by *v-src* oncogene. Nature 316:557–559.

Anderson RG (1998) The caveolae membrane system. Annu Rev Biochem 67:199–225.

Anderson SM, Carroll PM, Lee FD (1990) Abrogation of IL-3-dependent growth requires a functional *v-src* gene product: evidence for an autocrine growth cycle. Oncogene 5:317–325.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson Jr EM, Milbrandt J (1998) Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRα3–RET receptor complex. Neuron 21:1291–1302.

Baloh RH, Enomoto H, Johnson Jr EM, Milbrandt J (2000) The GDNF family ligands and receptors—implications for neural development. Curr Opin Neurobiol 10:103–110.

Brown DA, London E (1998) Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 14:111–136.

Brown DA, London E (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem 275:17221–17224.

Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron 15:821–828.

Cacalano G, Fariñas I, Wang L-C, Hagler K, Forgie A, Moore M, Armanini M, Phillips H, Ryan AM, Reichardt LF (1998) GFRα1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron 21:53–62.

Courtneidge SA, Fumagalli S, Koegl M, Superti-Furga G, Twamley-Stein GM (1993) The Src family of protein tyrosine kinases: regulation and functions. Dev Suppl 1993:57–64.

Crisanti-Combes P, Lorient AM, Girard A, Pessac B, Wasseff M, Calothy G (1982) Expression of neuronal markers in chick and quail embryo neuroretina cultures infected with Rous sarcoma virus. Cell Differ 11:45–54.

Datta SR, Brunet A, Greenberg ME (1999) Cellular survival: a play in three Akts. Genes Dev 13:2905–2927.

D'Mello SR, Galli C, Ciotti T, Calissano P (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci USA 90:10989–10993.

D'Mello SR, Borodezt K, Soltoff SP (1997) Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI-3 kinase in IGF-1 signaling. J Neurosci 17:1548–1560.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine–threonine protein kinase Akt. Science 275:661–665.

Ebendal T, Tomac A, Hoffer BJ, Olson L (1995) Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. J Neurosci Res 40:276–284.

Enokido Y, de Sauvage F, Hongo J-A, Ninkina N, Rosenthal A, Buchman VL, Davies AM (1998) GFRα4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr Biol 8:1019–1022.

Frisch SM, Francis H (1994) Disruption of epithelial cell–matrix interactions induces apoptosis. J Cell Biol 124:619–626.

Haltmeier H, Rohrer H (1990) Distinct and different effects of the oncogenes *v-myc* and *v-src* on avian sympathetic neurons: retroviral transfer of *v-myc* stimulates neuronal proliferation whereas *v-src* transfer enhances neuronal differentiation. J Cell Biol 110:2087–2098.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollock BA, Connelly PA (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. J Biol Chem 271:695–701.

Harder T, Simons K (1999) Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. Eur J Neurosci 29:556–562.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simpson LC, Moffet B, Vandlen RA, Koliatsos VE, Rosenthal A (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266:1062–1064.

Heuckeroth RO, Lampe PA, Johnson Jr EM, Milbrandt J (1998) Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors *in vitro*. Dev Biol 200:116–129.

Horger BA, Nishimura MC, Armanini MP, Wang LC, Poulsen KT, Rosenblad C, Kirik D, Moffat B, Simmons L, Johnson Jr EM, Milbrandt J, Rosenthal A, Bjorklund A, Vandlen RA, Hynes MA, Phillips HS (1998) Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. J Neurosci 18:4929–4937.

Janes PW, Ley SC, Magee AI (1999) Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J Cell Biol 147:447–461.

Jing S, Wen DZ, Yu YB, Holst PL, LuoY, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. Cell 85:1113–1124.

Kotzbauer PT, Lampe PA, Heuckeroth RO, Golden JP, Creedon DJ, Johnson Jr EM, Milbrandt J (1996) Neurturin, a relative of glial cell line-derived neurotrophic factor. Nature 384:467–470.

Lin L-FH, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132.

Lindahl M, Timmusk T, Rossi J, Saarma M, Airaksinen MS (2000) Expression and alternative splicing of mouse Gfrα4 suggest roles in endocrine cell development. Mol Cell Neurosci 15:522–533.

Lowell CA, Soriano P (1996) Knock-outs of Src family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev 10:1845–1857.

Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson Jr EM (1992) Biochemical characterization of programmed cell death in NGF-deprived sympathetic neurons. J Neurobiol 23:1205–1220.

McCubrey JA, Smith SR, Algate PA, Devente JE, White MK, Steelman LS (1993) Retroviral infection can abrogate the factor dependency of hematopoietic cells by autocrine and non-autocrine mechanisms depending on the presence of a functional viral oncogene. Oncogene 8:2905–2915.

Melillo RM, Barone MV, Lupoli G, Cirafici AM, Carlomagno F, Visconti R, Matoskova B, Di Fiore PP, Vecchio G, Fusco A, Santoro M (1999) Ret-mediated mitogenesis requires Src kinase activity. Cancer Res 59:1120–1126.

Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, Tansey MG, Lampe PA, Heuckeroth RO, Kotzbauer PT, Simburger KS, Golden JP, Davies JA, Vejsada R, Kato AC, Hynes M, Sherman D, Nishimura M, Wang LC, Vandlen R, Moffat B, Klein RD, Poulsen K, Gray C, Garces A, Henderson CE, Phillips HS, Johnson Jr EM (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 20:245–253.

Miller TM, Johnson Jr EM (1996) Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. J Neurosci 16:7487–7495.

Miller TM, Tansey MG, Johnson Jr EM, Creedon DJ (1997) Inhibition of phosphatidylinositol-3 kinase activity blocks depolarization- and insulin-like growth factor I-mediated survival of cerebellar granule cells. J Biol Chem 272:9847–9853.

Moulder KL, Onodera O, Burke JR, Strittmatter WJ, Johnson Jr EM (1999) Generation of neuronal intranuclear inclusions by polyglutamine-GFP: analysis of inclusion clearance and toxicity as a function of polyglutamine length. J Neurosci 19:705–715.

Muto M, Yoshimura M, Okayama M, Kaji A (1977) Cellular transformation and differentiation. Effect of Rous sarcoma virus on sulfated proteoglycan synthesis by chicken chondrocytes. Proc Natl Acad Sci USA 74:4173–4177.

Oppenheim RW, Houenou LJ, Johnson JE, Lin LFH, Li LX, Lo AC, Newsome AL, Prevette DM, Wang SW (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature 373:344–346.

Osterhout DJ, Wolven A, Wolf RM, Resh MD, Chao MV (1999) Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. J Cell Biol 145:1209–1218.

Philpott KL, McCarthy MJ, Klippel A, Rubin LL (1997) Activated phosphatidylinositol-3 kinase and Akt kinase promote survival of superior cervical neurons. J Cell Biol 139:809–815.

Schlessinger J (2000) New roles for Src kinases in control of cell survival and angiogenesis. Cell 100:293–296.

Sheets ED, Holowka D, Baird B (1999) Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcεRI and their association with detergent-resistant membranes. J Cell Biol 145:877–887.

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572.

Soler RM, Dolcet X, Encinas M, Egea J, Bayascas JR, Comella JX (1999) Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol-3 kinase pathway in spinal cord motoneurons. J Neurosci 19:9160–9169.

Tansey MG, Baloh RH, Milbrandt J, Johnson Jr EM (2000) GFRα-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron 25:611–623.

Thomas SM, Brugge JS (1997) Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol 13:513–609.

Thomas SM, Hayes M, D'Arcangelo G, Armstrong RC, Meyer BE (1991) Induction of neurite outgrowth by *v-src* mimics critical aspects of nerve growth factor-induced differentiation. Mol Cell Biol 11:4739–4750.

Treanor JJS, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A (1996) Characterization of a multicomponent receptor for GDNF. Nature 382:80–83.

Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, Arenas E, Ibañez CF (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130:137–148.

Tsui-Pierchala BA, Putcha GV, Johnson Jr EM 2000 Phosphatidylinositol-3 kinase is required for the trophic, but not the survival-promoting, actions of NGF on sympathetic neurons. J Neurosci 20:7228–7237.

Virdee K, Xue L, Hemmings BA, Goemans C, Heumann R, Tolkovsky AM (1999) Nerve growth factor-induced PKB/Akt activity is sustained by phosphoinositide-3 kinase-dependent and independent signals in sympathetic neurons. Brain Res 837:127–142.

Wong BR, Besser D, Kim N, Arron JR, Vologodskaia M, Hanafusa H, Choi Y (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol Cell 4:1041–1049.

Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci 16:5425–5436.

Yoshimura M, Iwasaki Y, Kaji A (1981) *In vitro* differentiation of chicken embryo skin cells transformed by Rous sarcoma virus. J Cell Physiol 109:373–385.

DISCUSIÓN

7.1. Aparición de TrkB y TrkC funcionales en células SH-SY5Y pretratadas con ácido retinoico

Dada la falta de líneas celulares expresoras de TrkB en condiciones basales, nuestro punto de partida para analizar las vías de señalización mediadas por TrkB fue un trabajo realizado por Kaplan y colaboradores, en el que se mostraba cómo el tratamiento con ácido retinoico (RA) inducía la expresión del RNA mensajero de *trkB* en la línea de neuroblastoma humano SH-SY5Y (Kaplan et al., 1993). Nuestros resultados demuestran que, al margen de la expresión de TrkB, RA induce la aparición de TrkC funcional en la superficie de estas células. Aunque en el anterior trabajo se muestra que tanto NT-3 como BDNF o NT-4/5 inducen el mismo grado de autofosforilación en inmunoprecipitados pan-Trk, se concluye que esta neurotrofina está actuando sobre TrkB, dado que inicialmente se describió que NT-3 inducía la fosforilación de TrkB con la misma potencia que BDNF (Soppet et al., 1991). Sin embargo, un estudio posterior demostró que la afinidad de NT-3 por TrkB se reducía dos órdenes de magnitud respecto a BDNF cuando los receptores se expresaban en un contexto neuronal en lugar de en fibroblastos (Ip et al., 1993). En nuestro caso, tanto NT-3 como BDNF fosforilan Trk con un perfil dosis dependiente muy similar, detectándose fosforilación a concentraciones de 0.2 nM ($\approx$ 5 ng/ml). Ello sugiere que NT-3 está actuando a través de su receptor "preferido", es decir, de TrkC. Este resultado se confirmó al analizar inmunoprecipitados con anticuerpos específicos para TrkB o TrkC, demostrándose que, en nuestro sistema, TrkB se fosforila en respuesta a BDNF o NT-4/5, pero no NT-3, y a la inversa, sólo NT-3 es capaz de inducir la autofosforilación de TrkC.

Tanto BDNF como NT-3 y NT-4/5 son capaces de estimular la supervivencia y el crecimiento neurítico en células pretratadas con RA, demostrándose que los receptores inducidos por RA son capaces de mediar efectos biológicos bien conocidos de las neurotrofinas. En este sentido, se ha descrito que el tratamiento con RA es capaz de inducir la expresión de TrkA en neuroblastos simpáticos de pollo, haciéndolos sensibles a NGF (Rodríguez-Tebar and Rohrer, 1991; VonHolst et al., 1997). Por el contrario, el RA inhibe tanto el incremento del mRNA de TrkA como el descenso en los niveles de mRNA de TrkC en neuroblastos simpáticos de ratón (Wyatt et al., 1999).

Estudios realizados con anterioridad revelan, además, que el RA y las neurotrofinas promueven la supervivencia y diferenciación de células pluripotentes de la cresta neural en neuronas maduras (Sieber-Blum, 1991). Por último, un reciente trabajo indica que las células madre derivadas del hipocampo regulan al alza la expresión de receptores de neurotrofinas tras ser tratadas con RA, y que la posterior adición de BDNF o NT-3 a éstas causa un incremento en el número de neuronas maduras generadas (Takahasi et al., 1999). Así pues, existen evidencias que apuntan a que el RA, un morfógeno clásico, está involucrado en la maduración de las células derivadas de cresta neural hacia un linaje neuronal, y que la acción conjunta de RA y neurotrofinas puede ser uno de los pasos necesarios para la adquisición de un fenotipo neuronal maduro. En nuestro caso, las células diferenciadas con RA y BDNF expresan diversos marcadores neuronales, como neurofilamentos, MAP-2 y Tau-1. Además, el patrón regionalizado de estas dos últimas proteínas sugiere que estas células están polarizadas, dado que MAP-2 se distribuye en el dominio somatodendrítico, y Tau lo hace en el dominio axonal, como ocurre en neuronas primarias (Goedert et al., 1991).

7.2. Efectos del RA y el suero sobre el fenotipo de las células SH-SY5Y

Al margen de la expresión de TrkB y TrkC, el tratamiento con RA provoca un modesto crecimiento neurítico en células SH-SY5Y. Sin embargo, la respuesta de la población celular no es uniforme, detectándose, a medida que se incrementaba el tiempo en cultivo, la aparición de células con fenotipo "adherente a substrato" o "tipo S", que acababan siendo mayoritarias. Las células SH-SY5Y derivan de la línea SK-N-SH, que se estableció a partir de una biopsia de médula ósea de un paciente con neuroblastoma (Biedler et al., 190.73). Ésta línea celular, como la mayoría de las derivadas de neuroblastoma, contiene células de fenotipo neuroblástico ("tipo N") y de fenotipo epitelioide o adherente a substrato ("tipo S"), que aparentemente pueden "transdiferenciarse", o interconvertirse entre ellas (Ross et al., 1983). Se cree que este fenómeno refleja la naturaleza pluripotencial de las células de cresta neural de donde derivan los neuroblastomas, y que la predominancia de un determinado fenotipo sobre otro refleja una disminución de la tasa de

interconversión entre ellos, no una pérdida de la capacidad de transdiferenciarse (Sadee et al., 1997). Pese a derivar de un subclón neuroblástico de las SK-N-SH, las células SH-SY5Y presentan una pequeña proporción de células tipo S, cuyo número incrementa a lo largo de los días en cultivo con RA. Esta observación ha sido también documentada por otros autores en esta misma línea celular (Jensen, 1987; Hill and Robertson, 1997; Arcangeli et al., 1999), y en otras líneas de neuroblastoma (Matsushima and Bogenmann, 1992). La explicación más sencilla de este fenómeno pasa por la observación de que durante los sucesivos pases las células de tipo S sería seleccionadas en contra, debido a su mayor adherencia al substrato (Jensen, 1987). Así, cuando estas células se mantienen durante periodos largos sin subcultivar, se evidenciaría la tendencia espontánea de estas células a transdiferenciarse. Este hecho se vería agravado por la aparente resistencia de las células tipo S a los efectos inhibitorios del RA sobre la proliferación celular. Sin embargo, no se puede desestimar la posibilidad de que el RA ejerza efectos duales sobre la diferenciación de las SH-SY5Y hacia los dos fenotipos, como de hecho ocurre con cultivos de células de cresta neural, en los que el tratamiento con RA induce la aparición de células adrenérgicas y melanocitos (Dupin and Lendouarin, 1995).

La retirada del RA del medio de cultivo y adición simultánea de BDNF induce un crecimiento neurítico robusto e uniforme. Sin embargo, la aparición de células tipo S sólo se evita si se retiraba el suero en el momento de aplicar el BDNF. Una posible explicación para este comportamiento es que las células tipo S no expresen TrkB tras ser tratadas con RA y por ello la retirada de los factores tróficos del suero resulte en su muerte. Esta hipótesis está de acuerdo con la observación de que las células de tipo S no expresan marcadores neuronales (Sadee et al., 1987; Ciccarone et al; 1989) y que las células SH-EP, un subclón de tipo S de las SK-N-SH, no pueden sobrevivir en ausencia de suero (ver Leventhal et al., 1995).

7.3. MAPK y supervivencia mediada por TrkB

Nuestros resultados indican que la activación de las MAPK no es necesaria para la supervivencia inducida por BDNF en células SH-SY5Y

pretratadas con ácido retinoico. Pese a que parece claro que las MAPK no son necesarias para la supervivencia mediada por NGF (Creedon et al., 1996; Virdee and Tolkovsky, 1996; Klesse et al., 1999), u otros agentes promotores de supervivencia neuronal como la despolarización de membrana (Soler et al., 1998) o el cAMP (Creedon et al., 1996; Virdee and Tolkovsky,1996), existen trabajos contradictorios sobre el papel de las MAPK en la supervivencia inducida por TrkB. En un primer estudio, se evidenció que el inhibidor farmacológico de la MEK PD98059 no ejercía ningún efecto sobre la supervivencia mediada por BDNF o insulina en cultivos de neuronas granulares del cerebelo provenientes de ratas P6-7, pese a que ambos factores son capaces de inducir una activación sostenida de las MAPK (Gunn-Moore et al., 1997). Este mismo inhibidor es incapaz también de bloquear la supervivencia mediada por BDNF en motoneuronas espinales de pollo (Dolcet et al., 1999). Además, en neuronas corticales de rata, la activación de las MAPK no es necesaria ni suficiente para mediar el efecto antiapoptótico del BDNF frente a la deprivación sérica (Hetman et al., 1999). En este mismo estudio, sin embargo, se demuestra que tanto la actividad MAPK como la actividad Akt contribuyen en la protección frente a camptotecina mediada por BDNF. Existen además trabajos adicionales en los que se demuestra que las MAPK median de forma importante los efectos neuroprotectores del BDNF frente a diferentes condiciones de estrés, como por ejemplo en modelos de hipoxia-isquemia (Hee Han et al., 1999), axotomía (Klöcker et al., 2000) o estrés oxidativo (Skaper et al., 1998). Además, un trabajo reciente muestra cómo la vía MEK/ERK es importante para la protección frente a arabinósido de citosina, pero no frente a deprivación de NGF, en neuronas simpáticas (Xue et al., 2000). Así, estos resultados parecen indicar que las MAPK serían relevantes en la neuroprotección frente a estrés, mientras que no lo serían en procesos de deprivación trófica. Sin embargo, dos trabajos recientes indican que estas proteínas pueden mediar parcialmente la supervivencia debido a BDNF, ya que la inhibición de la vía MEK/MAPK bloquea en un 50% aproximadamente la supervivencia mediada por BDNF en neuronas simpáticas transfectadas con TrkB (Atwal et al., 2000) y un 20% en neuronas granulares del cerebelo (Bonni et al., 1999). Los resultados de este último trabajo difieren de los obtenidos por Gunn-Moore y colaboradores (1997), quizá debido al empleo de condiciones de

cultivo diferentes, a la distinta edad embrionaria de las neuronas o al método utilizado para evaluar la supervivencia. En cualquier caso, es probable que la implicación de esta vía en la supervivencia neuronal pueda depender del tipo celular, así como del factor trófico en cuestión.

7.4. MAPK y diferenciación neuronal mediada por TrkB

Como se ha mencionado anteriormente, la mayoría de estudios centrados en los efectos neuritogénicos de las neurotrofinas se han realizado en la línea de feocromocitoma de rata PC12, que expresa niveles altos de TrkA. En estos estudios se evidenció, mediante el uso de dominantes negativos de MEK (Cowley et al., 1995) o del inhibidor PD98059 (Pang et al., 1995), que la activación de las MAPK era suficiente para el crecimiento neurítico mediado por NGF. En nuestro caso, la incubación con PD98059 produce una reducción dosis dependiente en el número de células que emiten neuritas tras añadir BDNF al medio de cultivo. La reducción correlaciona, además, con el grado de inhibición de las MAPK. Asimismo, la expresión de GAP-43, una proteína relacionada con el crecimiento neurítico, se induce tras la incubación con BDNF mediante un mecanismo sensible a PD98059. La sobrexpresión de GAP-43 en ratones transgénicos induce crecimiento axonal (Aigner et al., 1995), mientras que su deplección correlaciona con un colapso del cono de crecimiento y un crecimiento axonal anormal (Aigner and Caroni, 1995; Strittmatter et al., 1995). En células PC12, además, la sobrexpresión de GAP-43 potencia el crecimiento neurítico mediado por NGF (Yankner et al., 1990). Nuestros resultados concuerdan con los obtenidos por Feldman y colaboradores, en los que se demuestra que el crecimiento neurítico y la expresión de GAP-43 mediados por IGF-I requieren de la actividad MEK en células SH-SY5Y (Kim et al., 1997). De acuerdo con el concepto de que la vía MEK/MAPK estaría involucrada en la diferenciación neuronal por BDNF, la expresión de neuropéptido Y, un neurotransmisor que se incluye entre los genes de inducción tardía por NGF, necesitaría de la actividad MEK en PC12 transfectadas con TrkB e incubadas en presencia de BDNF (Williams et al., 1998).

Existen pocos trabajos, sin embargo, en los que se muestre la implicación de las MAPK en el crecimiento neurítico inducido por BDNF. Entre

ellos, destaca un reciente estudio en el que se evalúa la participación de las vías MEK/MAPK y PI 3-K/Akt en el crecimiento axonal *local* mediado por BDNF en neuronas simpáticas transfectadas con TrkB (Atwal et al., 2000). En este trabajo se utilizan cámaras de Campenot, en las que mantienen los cuerpos celulares en presencia de NGF, mientras que las neuritas se incuban en presencia de BDNF más inhibidores de la MEK y la PI 3-K, llegándose a la conclusión de que el crecimiento axonal local inducido por BDNF utiliza ambas vías de señalización. De manera importante, se establece que el residuo crítico de TrkB para este fenómeno es el lugar de unión a Shc, mientras que ni el sitio de unión PLCγ ni los residuos yuxtamembrana KFG, responsables de la unión a SNT, lo regulan.

7.5. PI 3-K y supervivencia neuronal mediada por TrkB

Nuestros resultados apuntan a que la vía PI 3-K/Akt es la utilizada preferentemente por BDNF para inducir la supervivencia de células SH-SY5Y pretratadas con RA. El uso del inhibidor específico de la PI 3-K LY294002 revierte de manera dosis dependiente este efecto, a dosis que previenen de manera efectiva tanto la actividad PI 3-K como la fosforilación de Akt. Como se ha comentado en la introducción, esta vía ha sido ampliamente involucrada en fenómenos de supervivencia neuronal en diversos sistemas (revisado por Kaplan and Miller, 2000). Algunos de estos resultados, no obstante, han de ser revisados cuidadosamente, como es el caso de las neuronas simpáticas mantenidas con NGF. En dos estudios en que se afirma que la inhibición de la PI 3-K es suficiente para revertir los efectos del NGF, las dosis de LY294002 utilizadas son inusualmente altas (100 μM) (Crowder and Freeman, 1998; Mazzoni et al., 1999). En otros dos estudios en que se utilizan dosis más bajas (50 μM y 30 μM respectivamente), se halla que este inhibidor tiene efectos modestos sobre la supervivencia, pese a que a esas dosis la fosforilación de Akt está totalmente inhibida (Philpott et al., 1997; Tsui-Pierchala et al., 2000). En nuestro caso, concentraciones de 10 μM son capaces de revertir totalmente la supervivencia mediada por BDNF, así como de reducir a niveles basales la fosforilación de Akt y la actividad PI 3-K, mostrándose así una buena

correlación entre el estado de activación de estos enzimas y el bloqueo de la supervivencia por BDNF.

Nuevamente, la información de la que se dispone sobre el papel de la PI 3-K/Akt en la supervivencia mediada por BDNF es limitada. Estudios iniciales en neuronas granulares de cerebelo demuestran que la Wortmanina, otro inhibidor de la actividad Pi 3-K, bloquea la supervivencia por BDNF (Nonomura et al., 1996; Shimoke et al., 1997). Además, estudios realizados en nuestro propio laboratorio muestran que esta vía es necesaria para la supervivencia mediada por BDNF en motoneuronas espinales de pollo (Dolcet et al., 1999). En un estudio *in vivo*, sin embargo, se halló que los ratones "knock in" para la tirosina responsable de la unión de Shc a TrkB no muestran pérdidas severas de neuronas dependientes de BDNF, aunque sí de NT-4/5 (Minichiello et al., 1998). En este trabajo no se analiza la actividad PI 3-K de neuronas derivadas de ratones $TrkB^{shc/shc}$, aunque es presumible que, como en el caso de TrkA, esta actividad dependa en gran medida de esta tirosina (revisado por Kaplan and Miller, 1995). Tanto el comportamiento diferencial de las poblaciones dependientes de BDNF o NT-4/5 respecto a esta mutación puntual, como la falta de pérdida graves en las poblaciones que responden a BDNF, son resultados que requieren de futuras aproximaciones para poder ser explicados satisfactoriamente en el contexto actual. En contraposición, existe un trabajo reciente en el que se demuestra que la tirosina encargada de la unión a Shc, pero no así la de PLCγ, media casi completamente los efectos de supervivencia del BDNF en neuronas simpáticas transfectadas con TrkB, a través de un mecanismo que utiliza tanto la actividad PI 3-K como la actividad MEK (Atwal et al., 2000).

7.6. Efectos del tratamiento con RA y BDNF sobre el ciclo celular

El tratamiento combinado con RA y BDNF induce una salida progresiva del ciclo celular, como ocurre con los neuroblastos que se diferencian terminalmente (revisado por Ross, 1996). Además, la retirada de BDNF induce un intento de las células de re-entrar en fase S del ciclo celular, a juzgar por el aumento de la incorporación de BrdU y la fosforilación de pRb observados. Existen numerosos trabajos que implican a la maquinaria de ciclo celular en la

inducción de muerte apoptótica en neuronas y otros tipos celulares (revisado por Kasten and Giordano, 1998; King and Cidlowsky, 1998). En primer lugar, la retirada de NGF induce la expresión selectiva de ciclina D1 en neuronas simpáticas (Freeman et al., 1994), mientras que la sobrexpresión de este gen causa la muerte apoptótica en la línea de neuroblastoma N1E-115 (Kranenburg et al., 1996). En segundo lugar, la muerte de neuronas granulares del cerebelo se acompaña de un aumento de la expresión de ciclina D y de la incorporación de BrdU en ratones *staggerer* y *lurcher*, en los que estas neuronas mueren por pérdida de sus dianas de proyección (Herrup and Busser, 1995). En tercer y último lugar, la inhibición de la actividad cdk por diferentes medios bloquea la muerte de neuronas simpáticas por retirada de NGF (Park et al., 1997) y la de neuronas granulares del cerebelo por deprivación de potasio y suero (Padmanabham et al., 1999). Los mecanismos exactos mediante los que la maquinaria de ciclo interactúa con la de muerte celular se desconocen, pero se cree que la liberación no regulada de E2F-1 de los complejos pRb/E2F es la causa de esta muerte (revisado por Kasten and Giordano, 1998; King and Cidlowsky, 1998). Así por ejemplo, en ratones $RB^{-/-}$, se observa muerte apoptótica, acompañada de un intento de entrar en fase S del ciclo, de aquellas células que por su localización deberían estar abandonando el ciclo celular para convertirse en postmitóticas (Lee et al., 1994; Morgenbesser et al., 1994). De acuerdo con el anterior modelo, esta muerte apoptótica en ratones $RB^{-/-}$ se elimina si se delecciona E2F-1 (Tsai et al., 1998). Además, la expresión de genes víricos que compiten por la unión de E2F-1 a pRb también induce apoptosis (Howes et al., 1994; Pan and Griep, 1994, 1995), mientras que la mera sobrexpresión de E2F-1 causa apoptosis en fibroblastos justo después de que éstos entren en fase S (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). Por último, recientemente se ha demostrado que E2F-1 es necesario y suficiente para la muerte apoptótica de neuronas corticales de ratón (Hou et al., 2000). Pese a todas estas evidencias, la manera como E2F-1 induce apoptosis, y particularmente en neuronas postmitóticas, continúa siendo desconocida, aunque sin duda el esclarecimiento de esos mecanismos es de gran importancia para el diseño de estrategias terapéuticas contra, por ejemplo, enfermedades neurodegenerativas, en las que también se han demostrado

alteraciones de genes de ciclo celular (Vincent et al., 1997; Nagy et al., 1997; Busser et al., 1998).

7.7. Ret y supervivencia

Nuestros resultados establecen la primera demostración funcional de que la vía PI 3-K/Akt media la supervivencia neuronal por GFLs. Previamente se había demostrado que tanto GDNF como NTRN eran capaces de activar la actividad PI 3-K en neuronas simpáticas (Creedon et al, 1997), pero este hecho no se había correlacionado con la supervivencia mediada por estos factores. Contrariamente, los únicos trabajos en los que se mostraba un efecto biológico de la activación por GDNF de la vía PI 3-K, apuntaban a que esta estaba relacionada con diferenciación neuronal. Así, van Weering y colaboradores demuestran que la formación de lamelipodios inducida por GDNF en células de neuroblastoma SK-N-MC depende de la activación de PI 3-K (van Weering et al., 1997), mientras que en otro trabajo se sugiere que este enzima regula la diferenciación morfológica de neuronas dopaminérgicas (Pong et al., 1998). Sin embargo, en este último estudio resulta difícil discernir si los efectos observados (disminución de la captación de dopamina) son debidos indirectamente a una reducción de la supervivencia mediada por GDNF. Recientemente, nuestros resultados se han corroborado en neuronas simpáticas del ganglio cervical superior, en las que se ha demostrado que el inhibidor LY294002 revierte de forma dosis dependiente (60% de reducción a 10 μM y 80% a 50 μM) la supervivencia mediada por GDNF (Besset et al., 2000). Además, en este trabajo se muestra que la supervivencia inducida por GDNF no está mediada por Ras, dado que un anticuerpo que bloquea su función no es capaz de revertirla. Este último resultado estaría de acuerdo con nuestras observaciones de que la vía MEK/MAPK no está implicada en supervivencia mediada por GFLs, dado que Ras es uno de los activadores más potentes de la misma, aunque no se puede descartar que los GFLs puedan activar las MAPK por mecanismos independientes de Ras. Por último, nuestro modelo de trabajo hace difícil establecer si esta vía se halla implicada en procesos de crecimiento neurítico, dado que en el momento de tratarse con GDNF, las células ya han alcanzado un alto grado de diferenciación.

7.8. Src y supervivencia

Nuestros resultados indican que la supervivencia mediada por GDNF en neuronas granulares del cerebelo transfectadas con Ret y GFRα1, y por NRTN en neuronas simpáticas, está mediada por Src. Además, Src es necesario para la activación de las vías MEK/MAPK y PI 3-K/Akt por GDNF, siendo esta última vía necesaria para la supervivencia mediada tanto por GDNF como por NRTN en ambos tipos neuronales. Clásicamente se ha implicado a Src en el control de funciones celulares tales como la proliferación, la movilidad o la adhesión (revisado por Thomas and Brugge, 1997). Sin embargo, el papel de esta proteína en el control de la supervivencia celular está poco documentado. Experimentos realizados utilizando la forma vírica v-src demostraron que ésta podía proteger contra la apoptosis debida a deprivación de citoquinas (Anderson et al., 1990; McCubrey et al., 1993) o pérdida de adhesión a la matriz extracelular (Frisch and Francis, 1994). Un trabajo más reciente, en el que se utilizan fibroblastos Rat-1 transfectados con un mutante termosensible de v-Src, muestra como éste es capaz de rescatar la muerte por deprivación sérica (Johnson et al., 2000). A temperatura restrictiva, la muerte celular se acompaña con una activación de p38 y JNK, junto con un aumento de la actividad caspasa y una disminución de la activación de las vías PI 3-K/Akt y MEK/MAPK. El rescate que se produce a temperatura permisiva es revertido por LY294002 pero no por PD98059, sugiriendo que la supervivencia debida a v-Src es mediada por la vía PI 3-K/Akt y no por la vía MEK/MAPK (Johnson et al., 2000). En cualquier caso, cabe destacar que los efectos de v-Src no tienen por que ser directamente comparables con los de su homólogo celular c-Src, de los que existen muy pocos estudios centrados en su papel sobre la supervivencia. En este sentido, quizá la evidencia más concluyente del papel de c-Src en el control de la supervivencia proviene de un estudio en el que se demuestra una sustancial reducción en la supervivencia mediada por TRANCE en osteoclastos derivados de ratones $Src^{-/-}$. Además, se demuestra que Src es necesario para la activación de la vía PI 3-K/Akt, y que la supervivencia mediada por TRANCE depende de la activación de esta vía (Wong et al., 1999). Así pues, como en nuestro caso, se establece un vínculo Src-PI 3-K/Akt

y supervivencia celular, demostrándose además que Src actúa por encima de PI 3-K.