

# Peripheral Expression and Biological Activities of GDNF, a New Neurotrophic Factor for Avian and Mammalian Peripheral Neurons

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**Abstract.** Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic polypeptide, distantly related to transforming growth factor- $\beta$  (TGF- $\beta$ ), originally isolated by virtue of its ability to induce dopamine uptake and cell survival in cultures of embryonic ventral midbrain dopaminergic neurons, and more recently shown to be a potent neurotrophic factor for motoneurons. The biological activities and distribution of this molecule outside the central nervous system are presently unknown. We report here on the mRNA expression, biological activities and initial receptor binding characterization of GDNF and a shorter spliced variant termed GDNF $\beta$  in different organs and peripheral neurons of the developing rat. Both GDNF mRNA forms were found to be most highly expressed in developing skin, whisker pad, kidney, stomach and testis. Lower expression was also detected in developing skeletal muscle, ovary, lung, and adrenal gland. Developing spinal cord, superior cervical ganglion (SCG) and dorsal root ganglion (DRG) also expressed low levels of GDNF mRNA. Two days after nerve transection, GDNF mRNA levels increased dramatically in the sciatic nerve. Overall, GDNF mRNA expression was sig-

nificantly higher in peripheral organs than in neuronal tissues. Expression of either GDNF mRNA isoform in insect cells resulted in the production of indistinguishable mature GDNF polypeptides. Purified recombinant GDNF promoted neurite outgrowth and survival of embryonic chick sympathetic neurons. GDNF produced robust bundle-like, fasciculated outgrowth from chick sympathetic ganglion explants. Although GDNF displayed only low activity on survival of newborn rat SCG neurons, this protein was found to increase the expression of vasoactive intestinal peptide and preprotachykinin-A mRNAs in cultured SCG neurons. GDNF also promoted survival of about half of the neurons in embryonic chick nodose ganglion and a small subpopulation of embryonic sensory neurons in chick dorsal root and rat trigeminal ganglia. Embryonic chick sympathetic neurons expressed receptors for GDNF with  $K_d$   $1-5 \times 10^{-9}$  M, as measured by saturation and displacement binding assays. Our findings indicate GDNF is a new neurotrophic factor for developing peripheral neurons and suggest possible non-neuronal roles for GDNF in the developing reproductive system.

**T**HE development and maintenance of the vertebrate nervous system requires the activity of a range of polypeptides known as neurotrophic factors. These molecules have been shown to control the generation, survival, differentiation and regeneration of neurons in the peripheral and central nervous systems (Barde, 1989; Thoenen, 1991). They include members of the neurotrophin family, including NGF and related molecules (Thoenen, 1991; Persson and Ibáñez, 1993), members of the neurokinin family, such as ciliary neurotrophic factor (CNTF),<sup>1</sup>

and leukemia inhibitory factor (LIF) (Lin et al., 1989; Stöckli et al., 1989; Yamamori et al., 1989) and other growth factors with neurotrophic activity such as FGF (Knusel et al., 1990; Cheng and Mattson, 1991; Ip et al., 1994) and insulin-like growth factors (IGFs) (Knusel et al., 1990; Near et al., 1992; Caroni et al., 1994). During development, trophic circuits formed by these molecules regu-

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1. *Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic proteins; ChAT, choline acetyltrans-

ferase; CGRP, calcitonin gene-related peptide; CNTF, ciliary neurotrophic factor; DRG, dorsal root ganglion; FGF, fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; GITC, guanidine isothiocyanate; IGF, insulin-like growth factor; KLH, keyhole limpet hemocyanin; LIF, leukemia inhibitory factor; NT-3, neurotrophin-3; PPTA, preprotachykinin-A; RPA, RNase protection assay; RT-PCR, reverse transcriptase-polymerase chain reaction; SCG, superior cervical ganglion; SOM, somatostatin; VIP, vasoactive intestinal peptide.

late the number and biochemical properties of developing neurons. For example, sympathetic neuroblasts can be induced to proliferate with FGF, while requiring neurotrophin-3 (NT-3) for survival (Birren et al., 1993; DiCicco-Bloom et al., 1993). At later stages, developing sympathetic neurons lose responsiveness to NT-3 and become dependent on NGF (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993). In addition, CNTF and LIF induce neurotransmitter phenotype changes in NGF-dependent sympathetic neurons during injury responses (Rao et al., 1993; Lewis et al., 1994). Similar multi-trophic interactions have also been described for developing sensory neurons (Wright et al., 1992) and motoneurons (Hughes et al., 1993; Mitsumoto et al., 1994).

Considerable effort has been devoted to the search for novel neurotrophic molecules, among others, factors with survival-promoting activities on dopaminergic neurons of the substantia nigra, which degenerate during Parkinson's disease (O'Malley et al., 1992; Nagata et al., 1993). One such candidate molecule has recently been purified from the conditioned medium of rat B49 glial cells on the basis of its ability to stimulate dopamine uptake in primary cultures of ventral midbrain neurons (Lin et al., 1993, 1994). This molecule, termed glial cell line-derived neurotrophic factor (GDNF), is a disulphide bridge-linked homodimer of two 134-residue-long glycosylated polypeptide chains that are released by proteolytic cleavage from a 211-residue-long precursor. GDNF has been shown to promote survival of ventral midbrain dopaminergic neurons in culture and uptake of dopamine, but not of GABA or serotonin (Lin et al., 1993), and to increase the number of tyrosine hydroxylase-immunoreactive neurons in intraocular grafts of fetal ventral mesencephalon (Strömberg et al., 1993). Recently, the ability of GDNF to rescue dopaminergic neurons has also been confirmed in situ (Tomac et al., 1995; Beck et al., 1995), supporting the notion that GDNF may ameliorate degeneration of dopaminergic neurons in patients with Parkinson's disease. The limited information available on GDNF mRNA expression supports a role for GDNF as a dopaminotrophic factor. GDNF mRNA has been detected by in situ hybridization and by reverse transcriptase-polymerase chain reaction (RT-PCR) in some brain areas rich in dopaminergic fibers and cell bodies, including early postnatal striatum and substantia nigra T1 astrocytes (Schaar et al., 1993; Strömberg et al., 1993). However, low levels of widespread GDNF mRNA expression have also been reported, including normal and kainic acid-stimulated hippocampus, cerebral cortex and cerebellum (Humpel et al., 1994; Springer et al., 1994), suggesting GDNF may also have effects on other cells in addition to mesencephalic dopaminergic neurons. In the present study, we have investigated the expression and function of GDNF outside the central nervous system. Our findings indicate GDNF is a neurotrophic factor for subpopulations of peripheral neurons with both target-derived and paracrine modes of action, and suggest possible non-neuronal roles for GDNF in the developing reproductive system.

## Materials and Methods

### DNA cloning

A cDNA fragment containing the coding region of the rat GDNF gene

(Lin et al., 1993) was amplified by RT-PCR from rat P1 brain polyA<sup>+</sup> RNA. Oligonucleotides 5'GGTCTACGGAGACCGGATCCGAGGTGC3' (from nucleotide 1 to 27 of the GDNF cDNA sequence as published) and 5'TCTCTGGAGCCAGGGTCCAGATACATC3' (from 675 to 700) were used as 5' and 3' primers, respectively. The PCR fragment was subsequently subcloned into pBS-KS<sup>+</sup> (Stratagene Corp., La Jolla, CA). The identity of the GDNF fragment isolated was confirmed by DNA sequencing. This fragment was subcloned into the baculovirus transfer vector pBacPAK1 (Clontech, Palo Alto, CA) and used to produce recombinant baculovirus for GDNF production in insect cells. A BamHI-BglII fragment from positions 15 to 383 from the rat GDNF cDNA sequence (Lin et al., 1993) was subcloned into pBS-KS<sup>+</sup> (Stratagene Corp.). The resulting plasmid was linearized and used to generate <sup>32</sup>P-labeled run off riboprobes for RNase protection analysis. This fragment spanned the  $\alpha/\beta$  isoform boundary, and it therefore allowed detection of both GDNF mRNA forms in the same sample. cDNA fragments corresponding to rat neuropeptide genes were obtained from P1 rat brain RNA by RT-PCR and subsequently subcloned into pBS-KS<sup>+</sup>. The CGRP probe consisted of a 384-bp fragment encompassing nucleotides 5–388 from the rat CGRP sequence (Amara et al., 1982). The PPTA probe consisted of a 234-bp fragment encompassing nucleotides 1 to 234 from the rat PPTA sequence (Kawaguchi et al., 1986). The vasoactive intestinal peptide (VIP) probe consisted of a 218-bp fragment encompassing nucleotides 23–240 from the rat VIP sequence (Nishizawa et al., 1985). The somatostatin (SOM) probe consisted of a 348-bp fragment encompassing nucleotides 61–408 from the rat somatostatin sequence (Goodman et al., 1983). The CCK probe consisted of a 355-bp fragment encompassing nucleotides 137–491 from the rat CCK sequence (Deschenes et al., 1984).

### Antisera

GDNF antisera were obtained by immunizing rabbits with three different synthetic peptides corresponding to amino acid residues 23–40, 51–62, and 84–97, respectively, from the mature GDNF peptide sequence coupled to keyhole limpet hemocyanin (KLH). Each peptide produced anti-sera which recognized GDNF in Western blotting (at a 1:5,000 dilution) and in immunoprecipitation.

### Production and Purification of Recombinant GDNF

Recombinant baculovirus clones used for GDNF production from insect cells were generated using a kit of reagents from Clontech according to manufacturer's instructions. Low-multiplicity, low-passage virus stocks were used to infect Sf21 cells cultured in serum-free medium (Sf-900-II; GIBCO BRL, Gaithersburg, MD) in 1 liter spinner flasks with an agitation rate of 30 rpm. Conditioned medium was harvested at 72 h after infection, adjusted to pH 7.9 with 5 N NaOH and clarified of debris. The medium was then subjected to chromatography on High performance SP-Sepharose (Pharmacia, Uppsala, Sweden) in 50 mM phosphate, pH 7.9, 5 mM EDTA, and eluted with a 0 to 1 M NaCl linear gradient. Selected fractions, identified by Western blotting, were concentrated by ultrafiltration (Amicon Corp., Beverly, MA), pooled, and chromatographed on Superdex75 (Pharmacia) in 50 mM phosphate, pH 7.4, 5 mM EDTA, 0.5 M NaCl, 10% acetonitrile. Selected fractions, identified by Western blotting, were concentrated by ultrafiltration (Amicon Corp.), pooled and adjusted to pH 3.5 with concentrated acetic acid. This sample was then applied to a reverse phase C8 HPLC column (Vydac, Hesperia, CA) and eluted with a 0 to 60% acetonitrile linear gradient in 0.1% TFA. GDNF fractions identified by Western blotting, which eluted at 42% acetonitrile, were vacuum dried, reconstituted in water, and aliquoted. Silver staining of SDS-PAGE gels and NH<sub>2</sub>-terminal automated Edman degradation sequence analysis showed the recombinant GDNF preparation had a purity greater than 90%. GDNF protein was quantified after silver staining of SDS-PAGE gels using standard curves obtained with quantified commercial samples of proteins of molecular weight similar to that of GDNF.

### RNA Preparation and RNase Protection Assay

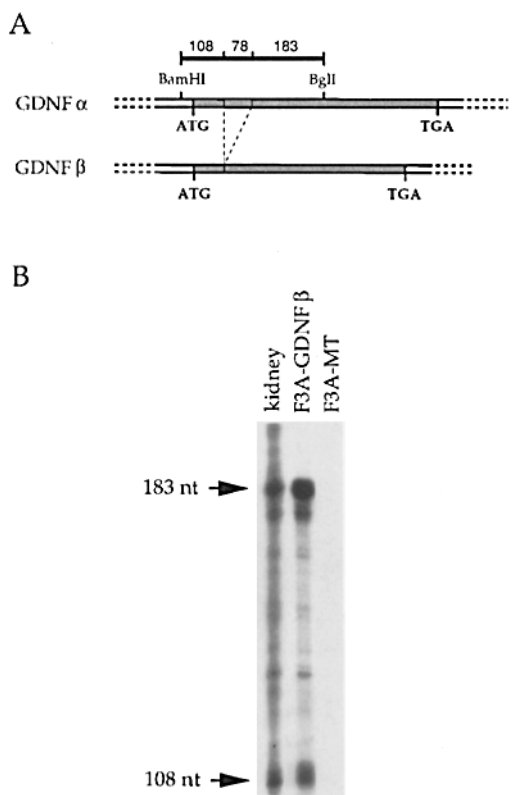
Tissue or cells were homogenized in guanidine isothiocyanate (GITC) and  $\beta$ -mercaptoethanol and RNA was isolated as previously described (Chirgwin et al., 1979). For RNA extraction from small samples ( $\leq 100$  mg tissue), 1/10 vol 2 M sodium acetate, pH 4.0, was added, followed by phenol/chloroform extraction, and ethanol precipitation. Riboprobes were generated from linearized plasmids using T3 or T7 RNA polymerase (Promega Biotech, Madison, WI) and <sup>32</sup>P- $\alpha$ -CTP. Equal amounts of total RNA (typically 10  $\mu$ g) were analyzed by RNase protection assay according to manufacturer's instructions (Ambion Inc., Austin, TX). A rat glyceraldehyde

3-phosphate dehydrogenase (GAPDH) riboprobe (kindly provided by Dr. J. M. Blanchard, Institut de Genetique Moleculaire, Montpellier, France) labeled at low specific activity was added to each sample for subsequent standardisation. A 160-nucleotide-long fragment from this probe is protected from RNase digestion. Samples were then separated in 4% polyacrylamide denaturing gels and exposed to x-ray films with intensifying screens at  $-70^{\circ}\text{C}$ . Autoradiograms were analyzed with an image analysis system (Leica); for quantification, all sample signals were standardised using the signal obtained with the GAPDH probe. To quantitatively compare signals for GDNF mRNA in strongly versus weakly expressing tissues, autoradiograms obtained after different exposure times were used for quantification. In some cases, lower amounts of RNA from highly expressing tissues were used for comparison with samples from low GDNF mRNA expressing tissues.

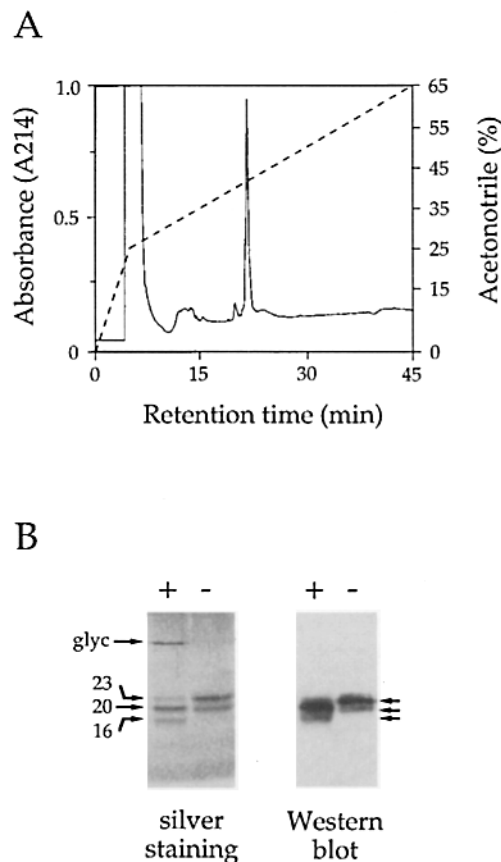
### Bioassays

For survival assays, chick sympathetic paravertebral chains or rat superior cervical ganglia (SCG) were dissected at different times of development, cell dissociates were obtained by trypsin treatment and plated on poly-L-ornithine- and laminin-coated 24-well plates at a density of 2,000–10,000 cells/well in DME/F12 (GIBCO BRL) supplemented with glutamine, pen-

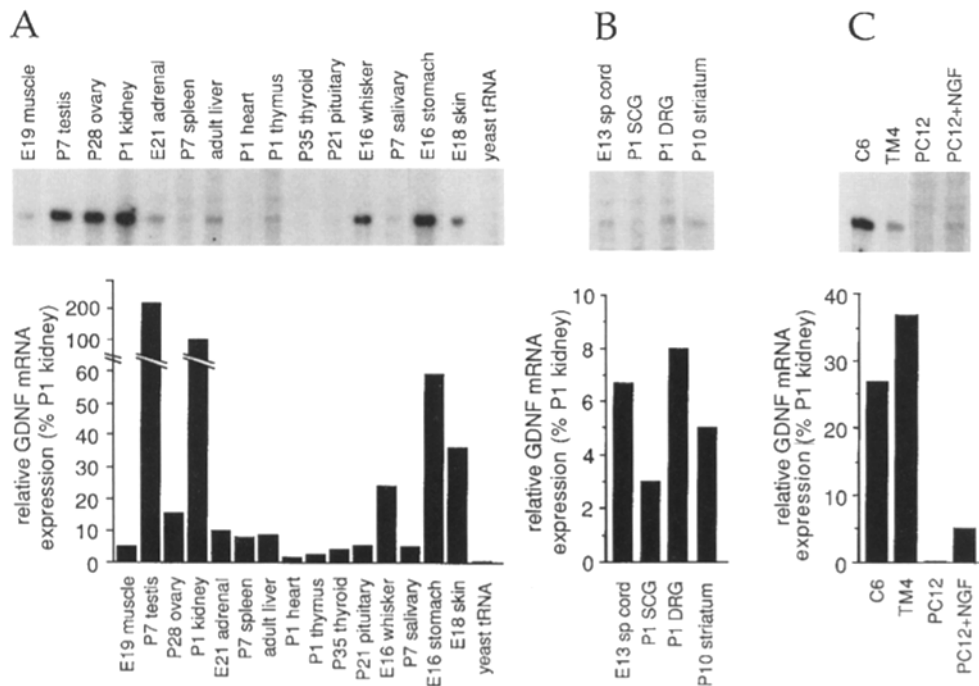
icillin, streptomycin, and BSA. Plating efficiency was 60 to 80%. E10 chick dorsal root and nodose ganglia, and E16 rat trigeminal ganglia were also dissected and dissociated in the same way, except that, after trypsinization, cells were pre-plated 2 h in uncoated dishes, where non-neuronal cells remain attached, to enrich the preparation in neuronal cells. Survival was scored by counting the number of phase-bright process-bearing cells in a defined area (corresponding to  $\sim 20\%$  of the well) after 48 h in culture, and it was expressed as percentage of surviving neurons from the total number of cells initially plated. Explants of chick sympathetic ganglia were cultured in rat tail collagen gels as previously described (Ebendal, 1989) and photographed after 48 h in culture. For the analysis of phenotypic changes in newborn rat SCG neurons, dissociated cultures were established as above in 24-well plates at a density of 50,000 cells/well in defined medium supplemented with 20 ng/ml NGF. After 24 h, the anti-mitotic agent aphidicolin was added to the cultures at 4  $\mu\text{g/ml}$ , effectively eliminating non-neuronal cells. GDNF (100 ng/ml) was added from day 2,



**Figure 1.** (A) Schematic representation of the predicted GDNF $\alpha$  and GDNF $\beta$  mRNA isoforms. Hatched bars indicate coding sequence, flanked by start (ATG) and termination (TGA) codons. The riboprobe used in RNase protection analyses, transcribed from a BamHI-BglII cDNA fragment, is indicated in the top of the figure; fragment sizes are in nucleotides. (B) RNAse protection analysis of postnatal day 1 (P1) kidney RNA and RNA from a fibroblast cell line stably transfected with the GDNF $\beta$  mRNA isoform (F3A-GDNF $\beta$ ) and from a mock-transfected control line (F3A-MT). A full-length probe fragment of 369 nucleotides, not shown in the figure, corresponding to the GDNF $\alpha$  mRNA isoform, was protected by P1 kidney RNA but not by the cell line RNA. Apart from this band, both samples protected fragments of sizes corresponding to those predicted by the structure of the GDNF $\beta$  cDNA clone (see A), confirming that GDNF $\beta$  corresponds to an authentic GDNF mRNA isoform. No bands were detected in mock-transfected cell line RNA.



**Figure 2.** (A) Reverse-phase HPLC purification of recombinant GDNF. A concentrated pool of fractions from the Superdex75 size exclusion chromatography column showing immunoreactivity with anti GDNF peptide antisera was applied to a C8 column and eluted with an acetonitrile gradient. The absorbance was monitored at 214 nm. Protein immunoreactive with GDNF anti-peptide antibodies and displaying biological activity was present in the major peak of the chromatogram eluting at 42% acetonitrile. (B) Glycanase treatment of insect cell-derived recombinant GDNF. Insect cell-derived GDNF runs as a doublet of 20 and 23K, respectively, in silver stained SDS-PAGE gels (–) (left). Glycanase treatment (+) results in a relatively higher proportion of the 20 K species and in the appearance of a 16K species, presumably representing the unglycosylated mature GDNF peptide. Molecular weights are indicated in kilo Daltons. glyc indicates the migration of the glycanase enzyme (mol wt = 52K). Western blotting analysis of a SDS-PAGE gel similar to that shown in A using a cocktail of three anti-GDNF peptide antisera (right). All bands are recognized by the GDNF antibodies, confirming they all correspond to GDNF.



**Figure 3.** Overview of the distribution of GDNF mRNA ( $\alpha$  isoform) expression in rat peripheral organs, peripheral ganglia, and cell lines. The upper panels show autoradiograms of RNase protection assays ( $\sim 10 \mu\text{g}$  of total RNA per lane), the lower panels show the quantification of the level of GDNF mRNA in each sample relative to the level of GDNF mRNA in P1 kidney, and standardised to the signal obtained with a low specific activity GAPDH riboprobe added simultaneously with the GDNF probe to each RNA sample. Autoradiograms of different exposure times and from several different experiments were used for GDNF mRNA quantification. (A) GDNF mRNA distribution at the time of maximal expression

in rat peripheral tissues. (B) Expression of GDNF mRNA in embryonic spinal cord, newborn dorsal root ganglia (DRG), superior cervical ganglia (SCG), and postnatal striatum. (C) Expression of GDNF mRNA in the astroglioma line C6, in the Sertoli cell line TM4, in the pheochromocytoma line PC12 and in PC12 cells after 24 h treatment with NGF.

and cultures were harvested by GITC lysis at day 6. Approximately  $10 \mu\text{g}$  of total RNA were obtained from six wells using the acid-phenol method (see above).  $5 \mu\text{g}$  RNA was used for analysis of neuropeptide and choline acetyltransferase (ChAT) mRNAs by RNase protection assay as described above.

### Binding Assays

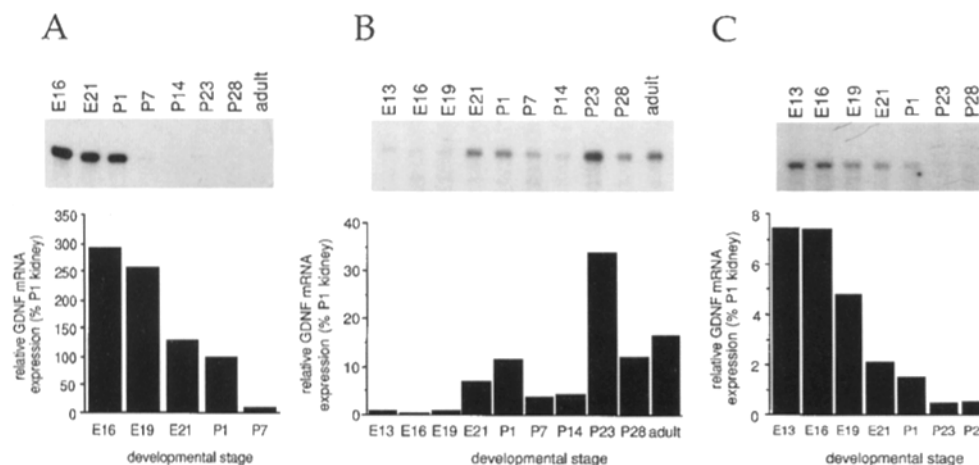
Purified recombinant GDNF was labeled with  $^{125}\text{I}$  by the chloramine-T method to an average specific activity of  $5 \times 10^7$  cpm/ $\mu\text{g}$ . Iodinated GDNF retained more than 90% of its biological activity as assessed in the sympathetic ganglion explant bioassay. Chick sympathetic neurons were isolated from E10 paravertebral sympathetic chains by trypsinization and plated for 3 h to allow receptor re-expression. The plating procedure also effectively enriched the preparation in neuronal cells, since these do not adhere to the plastic surface. Steady state saturation binding was performed in triplicate wells with 350,000 neurons/ml and serial dilutions of iodinated GDNF in Dulbecco-phosphate buffer saline supplemented with 1 mg/ml BSA and 1 mg/ml glucose. Competition binding assays were per-

formed at  $4^\circ\text{C}$  using  $0.3 \times 10^{-9}$  M iodinated factor and serial dilutions of unlabeled GDNF or NGF. All components were added at the same time and the cells were collected by centrifugation after equilibrium was reached (90–120-min incubation). Cell pellets were then counted in a gamma counter. Nonspecific binding was measured in a parallel incubation to which 300- to 500-fold molar excess of unlabeled purified GDNF was added. All results were corrected for this nonspecific binding.

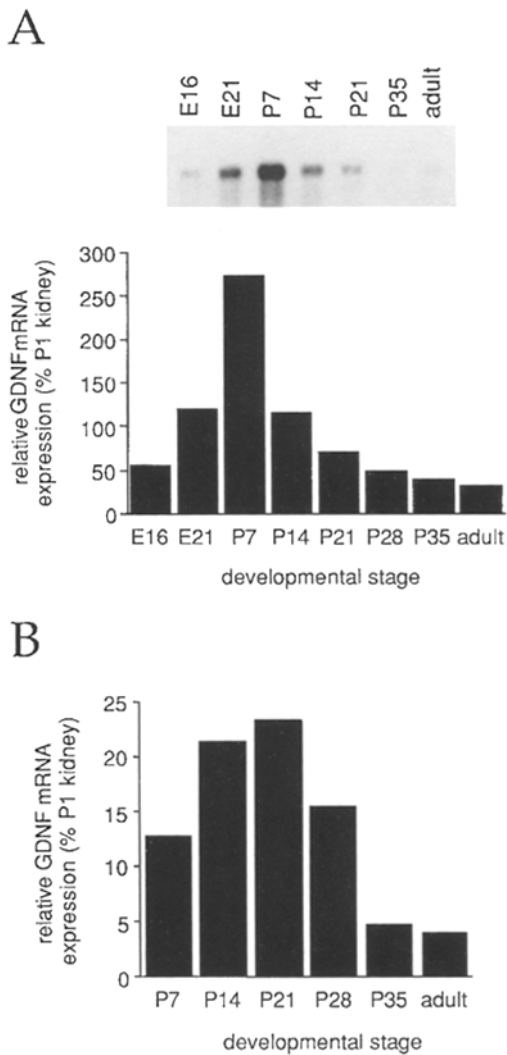
## Results

### Alternative Splicing Generates a Shorter GDNF Precursor Protein

A GDNF cDNA was cloned by RT-PCR from postnatal day 1 (P1) rat brain mRNA using primers flanking the initiation and stop codons, respectively, of the reported GDNF gene open reading frame (Lin et al., 1993). In addi-

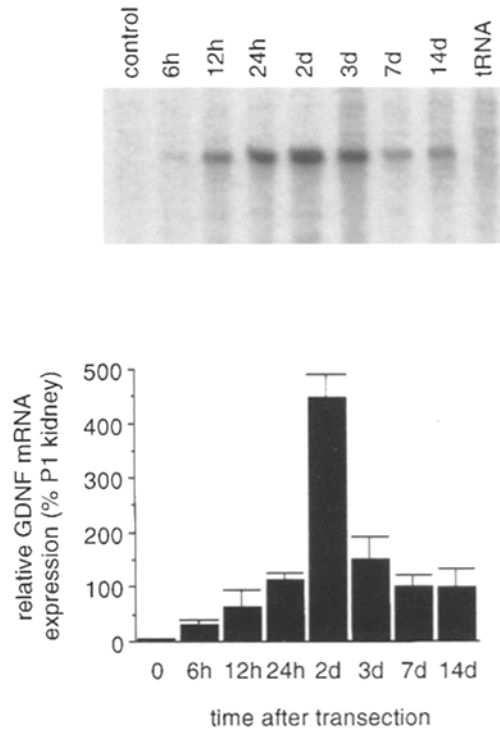


**Figure 4.** Expression of GDNF mRNA in developing rat kidney (A), lung (B), and gastrocnemius muscle (C). E13 and E16 samples in C correspond to limb bud RNA. The upper panels show autoradiograms of RNase protection assays, the lower panels show the quantification of the level of GDNF mRNA in each sample relative to the level of GDNF mRNA in P1 kidney and standardised to the signal obtained with a GAPDH riboprobe added simultaneously with the GDNF probe to each RNA sample.



**Figure 5.** Expression of GDNF mRNA in developing rat testis (A) and ovary (B). The upper panel in A shows an autoradiogram of a RNase protection assay. The lower panels show the quantification of the level of GDNF mRNA in each sample relative to the level of GDNF mRNA in P1 kidney and standardised to the signal obtained with a GAPDH riboprobe added simultaneously with the GDNF probe to each RNA sample.

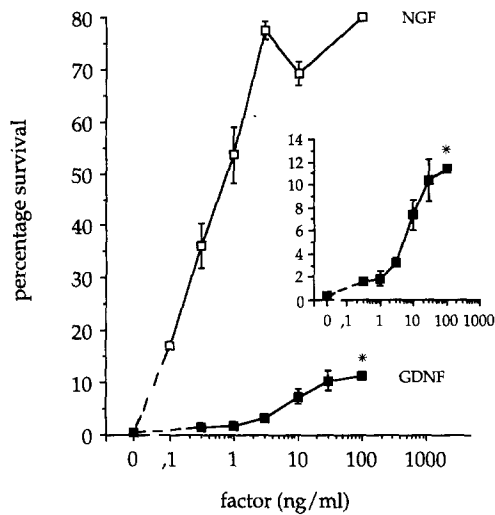
tion to the previously described GDNF cDNA, a shorter cDNA was also specifically amplified. Sequence analysis revealed this shorter fragment corresponded to a GDNF cDNA with a 78 nucleotide deletion in the prepro region of the coding sequence (Fig. 1 A). Comparison with the longer GDNF cDNA, termed GDNF $\alpha$ , indicated that the shorter form, termed GDNF $\beta$ , resulted from the use of an alternative donor splice site during GDNF mRNA processing. To verify that the amplified GDNF $\beta$  cDNA corresponded to a bone fide transcript, RNase protection analysis was performed using a riboprobe spanning the splice junction of GDNF $\alpha$  mRNA (Fig. 1 A). The pattern of protected bands in a GDNF mRNA expressing tissue (post natal day 1 [P1] kidney, see below) was compared to that in a fibroblast cell line which had been stably transfected with a GDNF $\beta$  cDNA (F3A-GDNF $\beta$ ). As expected, a fragment of 369 nucleotides, corresponding to the full-



**Figure 6.** Time course of the expression of GDNF mRNA in the rat sciatic nerve after nerve transection. The upper panel shows an autoradiogram of a RNase protection assay. Yeast tRNA was used as negative control (tRNA). The lower panel shows the quantification of the level of GDNF mRNA in each sample relative to the level of GDNF mRNA in P1 kidney and standardised to the signal obtained with a GAPDH riboprobe added simultaneously with the GDNF probe to each RNA sample. Data are expressed as mean  $\pm$  SD of two independent experiments.

length probe (Fig. 1 A), was protected by P1 kidney RNA but not by the cell line RNA (not shown, see below). In addition, two other major fragments of 183 and 108 nucleotides, corresponding to RNase cleavage of the probe at the GDNF splice junction and at a second site 78 nucleotides upstream, respectively, were protected by both RNA samples (Fig. 1 B), indicating that the GDNF $\beta$  cDNA corresponds to an authentic GDNF mRNA isoform. No protected fragments were seen using RNA from the parental untransfected cell line (Fig. 1 B).

In addition, the deletion (26 amino acids) also changes Gly25 into Ala. Although the alternative splicing does not change the reading frame of translation, it could conceivably influence the processing of the GDNF precursor. GDNF $\alpha$  and  $\beta$  cDNAs were expressed in baculovirus-infected insect cells and recombinant protein was purified to homogeneity. Insect cell-derived GDNF eluted at 42% acetonitrile in reverse phase HPLC (Fig. 2 A). NH<sub>2</sub>-terminal amino acid sequencing for 11 cycles confirmed the purity and the predicted sequence of mature GDNF; contaminants consisted of GDNF molecules lacking the first two amino acid residues from the NH<sub>2</sub> terminus. Both GDNF $\alpha$  and  $\beta$  protein isoforms gave identical NH<sub>2</sub>-terminal sequences. GDNF $\alpha$  runs as a doublet of 20 and 23K, respectively, in polyacrylamide gels under denaturing conditions (Fig. 2 B). Treatment with N-glycanase resulted in the appearance of a polypeptide band of 16K, indicating that,

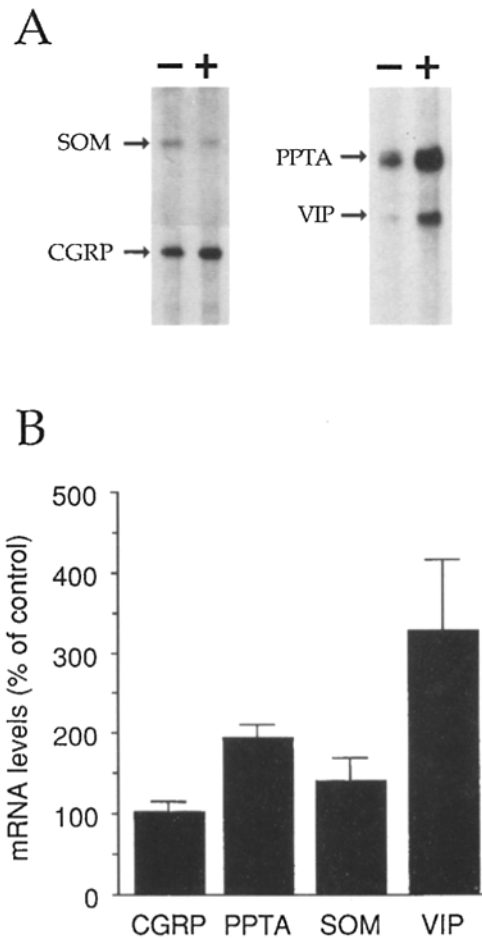


**Figure 7.** Survival of newborn rat SCG neurons with GDNF. Dissociated cultures of rat P1 SCG neurons were grown for 48 h in the presence of increasing concentrations of purified recombinant GDNF (■) or NGF (□). Results are expressed as the percentage of the total number of neurons plated that survived after 48 h in culture (triplicate determinations)  $\pm$  SD. The inset details the response to GDNF. \*,  $P < 0.001$  versus no factor added.

like native GDNF (Lin et al., 1993), insect cell-derived GDNF is N-glycosylated (Fig. 2 B). The sample was enriched in the 20 and 16K bands after N-glycanase treatment, suggesting that the 20K species is a glycosylated intermediate. Heterogeneity in the carbohydrate content of GDNF has recently been detected in preparations of native GDNF (Lin et al., 1994). All three species were recognized by anti-GDNF peptide antiserum in Western blots, confirming they all corresponded to GDNF (Fig. 2 B). No differences in mobility were observed between the mature forms of GDNF produced using either the  $\alpha$  or the  $\beta$  cDNA (data not shown).

### Expression of GDNF mRNA in Peripheral tissues

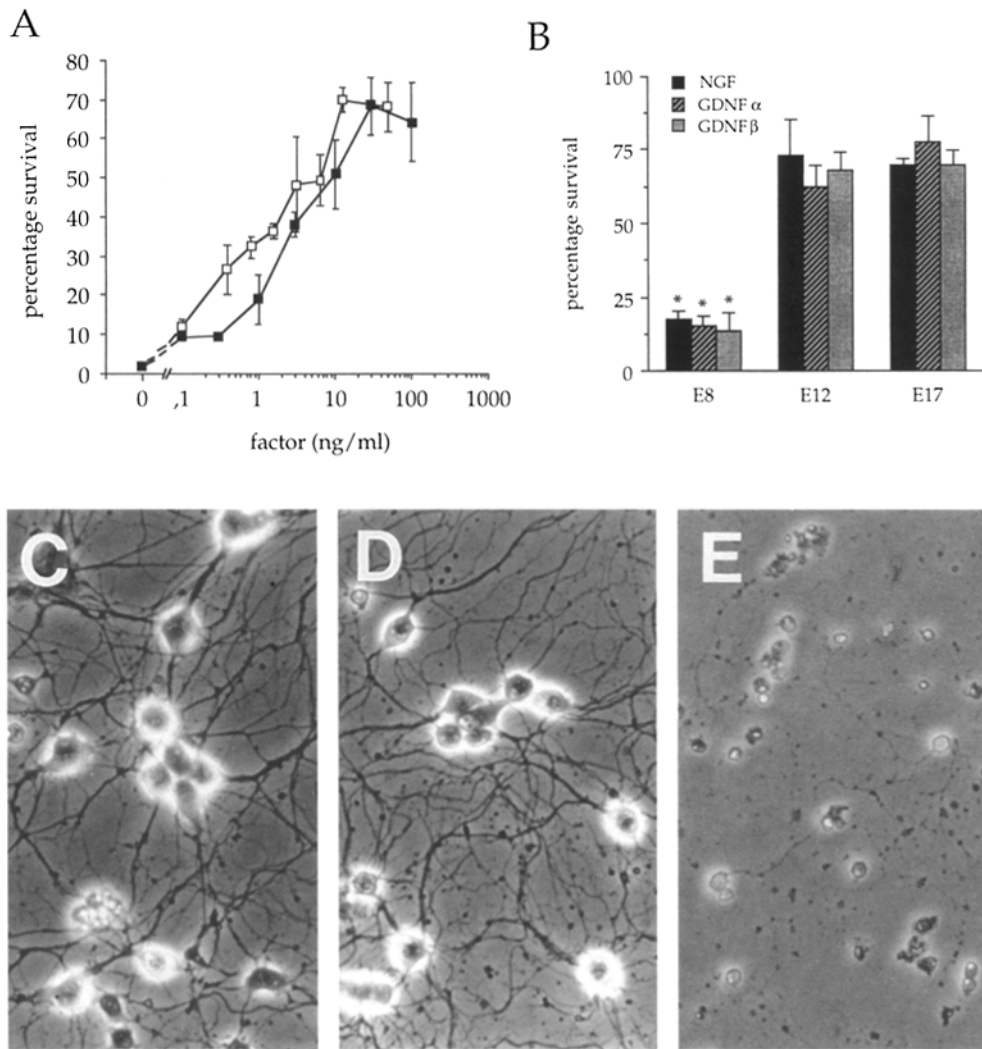
The limited information available indicates GDNF mRNA is expressed at very low levels (Schaar et al., 1993; Strömberg et al., 1993; Springer et al., 1994). We studied the expression of non-amplified GDNF mRNA in the developing rat using a sensitive RNase protection assay (RPA). Fig. 3 A shows an overview of the distribution of GDNF $\alpha$  mRNA at the time of maximal expression in different peripheral tissues. GDNF $\alpha$  mRNA was found to be expressed in several tissues of the developing rat, notably postnatal testis and kidney, and embryonic whisker pad, stomach, and skin (Fig. 3 A). Expression in whisker pad was maximal between embryonic day 13 (E13) and E16 and subsequently declined towards E18 (data not shown). Lower levels were also seen in embryonic muscle and adrenal gland and postnatal lung, liver, and ovary (Fig. 3 A). Very low levels of GDNF $\alpha$  mRNA could be detected in postnatal heart, spleen, thymus and thyroid, pituitary, and salivary glands (Fig. 3 A). Low levels of GDNF $\alpha$  mRNA were also found in early postnatal dorsal root ganglia (DRG) and SCG (Fig. 3 B), but could not be detected in the adult ganglia (not shown). GDNF $\alpha$  mRNA was also



**Figure 8.** GDNF regulates the phenotype of newborn rat SCG neurons. (A) Autoradiograms of RNase protection assays of RNA samples from cultures of P1 rat SCG neurons grown for 4 d in the absence (-) or presence (+) of 100 ng/ml recombinant GDNF. (B) Quantification of neuropeptide mRNA levels in cultures of rat SCG neurons treated with 100 ng/ml GDNF relative to the levels in untreated cultures. The values have been standardized to the signal obtained with a GAPDH riboprobe run simultaneously with the neuropeptide probes. Data are expressed as mean  $\pm$  SD of three independent experiments.

found to be expressed in embryonic spinal cord at levels comparable to those detected in P10 striatum (Fig. 3 B), the region that shows the maximal expression of GDNF $\alpha$  mRNA in the rat brain (Trupp, M., and C. F. Ibáñez, unpublished results). In general, the expression of GDNF $\beta$  mRNA paralleled that of the  $\alpha$  isoform (data not shown). Overall, the expression of GDNF mRNA was significantly higher in peripheral organs compared to neuronal tissues. Several cell lines were found to express GDNF mRNA, including the astrocytoma line C6 and the Sertoli cell line TM4 (Fig. 3 C). Expression of GDNF mRNA in TM4 cells suggests Sertoli cells may be the source of GDNF mRNA in the testis. The rat pheochromocytoma cell line PC12 did not show detectable GDNF mRNA expression, but it was found to express GDNF mRNA after a 24 h treatment with NGF (Fig. 3 C), which causes differentiation into a sympathetic neuron-like cell type.

GDNF mRNA was highly expressed throughout the



**Figure 9.** Survival of developing chick paravertebral sympathetic neurons with GDNF. (A) Dissociated cultures of E10 chick sympathetic neurons were grown for 48 h in the presence of increasing concentrations of purified NGF (□) or purified recombinant GDNF (■). Results are expressed as the percentage of the total number of neurons plated that survived after 48 h in culture (triplicate determinations)  $\pm$  SD. (B) Survival of E8, E12, and E17 chick sympathetic neurons in the presence of 20 ng/ml NGF (solid bars), GDNF $\alpha$  (hatched bars), or GDNF $\beta$  (shaded bars). Results are expressed as the percentage of the total number of neurons plated that survived after 48 h in culture (triplicate determinations)  $\pm$  SD. \*,  $P < 0.001$  compared to the cultures grown in the absence of factors. (C) Bright field micrograph of E10 chick sympathetic neurons growing in the presence of 10 ng/ml NGF. (D) Bright field micrograph of E12 chick sympathetic neurons growing in the presence of 10 ng/ml GDNF $\alpha$ . (E) Bright field micrograph of E12 chick sympathetic neurons growing in the absence of trophic factors.

embryonic development of kidney (from E13 onwards), with decreasing levels after birth (Fig. 4 A). No GDNF mRNA could be detected in adult kidney (not shown). In contrast, GDNF mRNA expression in lung increased during development and showed a biphasic profile, with a first peak around birth and a second peak at the third postnatal week (Fig. 4 B). Levels of GDNF mRNA decreased in developing gastrocnemius muscle from E19 to P23, maximal levels were seen in E13 and E16 limb bud (Fig. 4 C). No GDNF mRNA could be detected in adult muscle (not shown). GDNF mRNA expression in testis increased during development and peaked at P7 (Fig. 4 A). Subsequently, the level of GDNF mRNA in testis decreased during the second and third postnatal weeks and it was lowest in adult testis (Fig. 5 A). GDNF mRNA expression in ovary increased during early postnatal development, peaked at three weeks and subsequently decreased down to very low levels in the adult (Fig. 5 B).

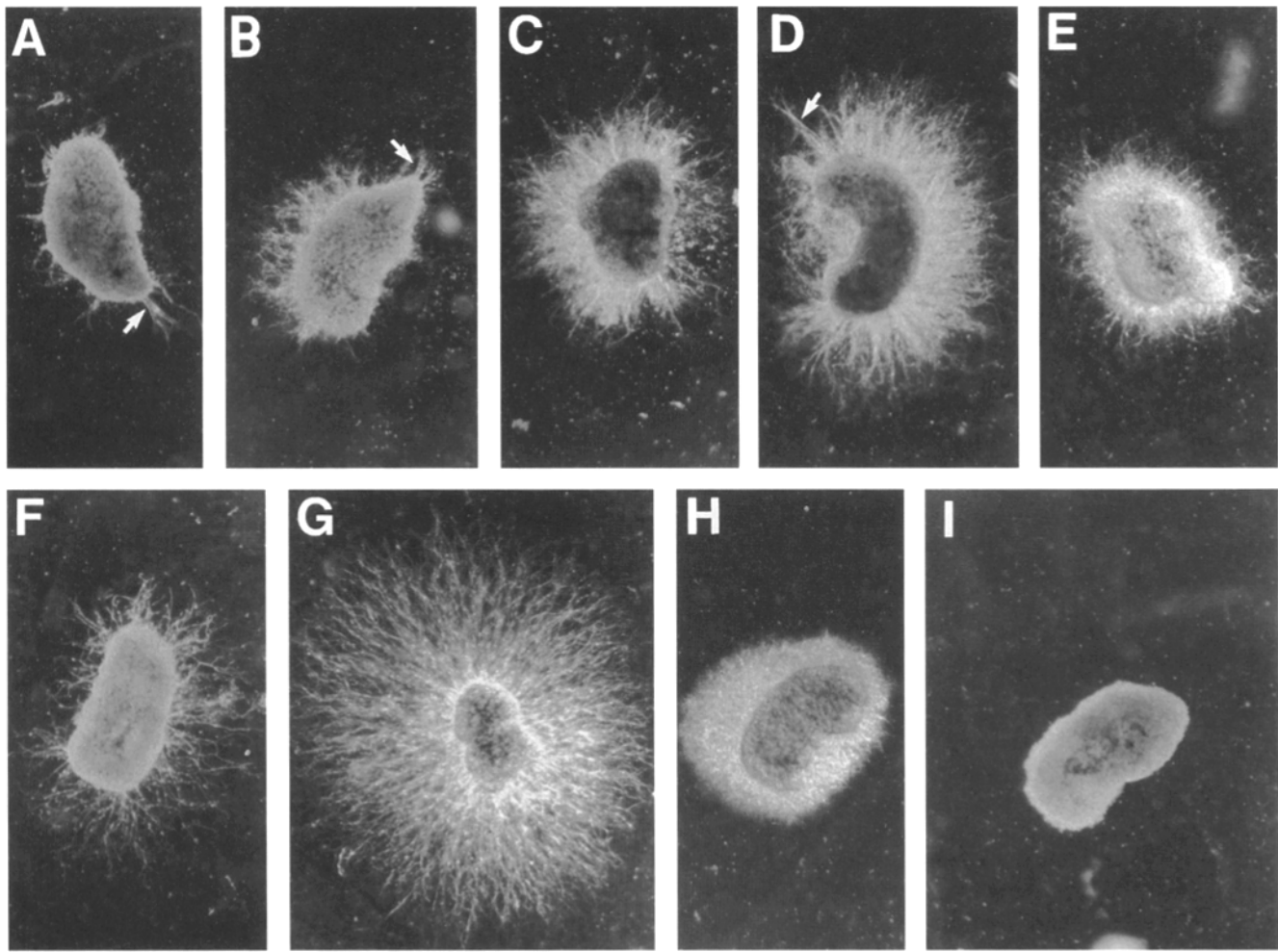
GDNF mRNA levels were very low in intact rat sciatic nerve (Fig. 6). However, a dramatic increase was observed after nerve transection (Fig. 6). GDNF mRNA levels ( $\alpha$  isoform) peaked 2 d after the lesion and decreased thereafter (Fig. 6). 14 d after transection, the latest time point

tested, GDNF mRNA was still highly expressed in the sciatic nerve.

#### **Trophic Activities of GDNF in Developing Peripheral Mammalian and Avian Neurons**

The pattern of expression of GDNF mRNAs in peripheral tissues suggested that GDNF might have biological effects on peripheral neurons. We investigated the biological activities of GDNF in sympathetic neurons from the rat SCG. GDNF (produced from the  $\alpha$  cDNA isoform) displayed low but significant activity on survival of P1 SCG neurons in culture (Fig. 7). At 100 ng/ml, GDNF rescued survival of about 10% of the P1 SCG neurons; half-maximal response was seen at 4–5 ng/ml (Fig. 7). NGF, in contrast, supported the majority of the SCG neurons, and half-maximal response was seen at 0.3–0.4 ng/ml (Fig. 7). GDNF also displayed low but measurable activity on E16 SCG neurons (data not shown) at levels comparable to NGF, which, in contrast to P1 SCG, supports only a small fraction of neurons in E16 SCG (Birren et al., 1993; DiCicco-Bloom et al., 1993).

A number of trophic factors and cytokines, including



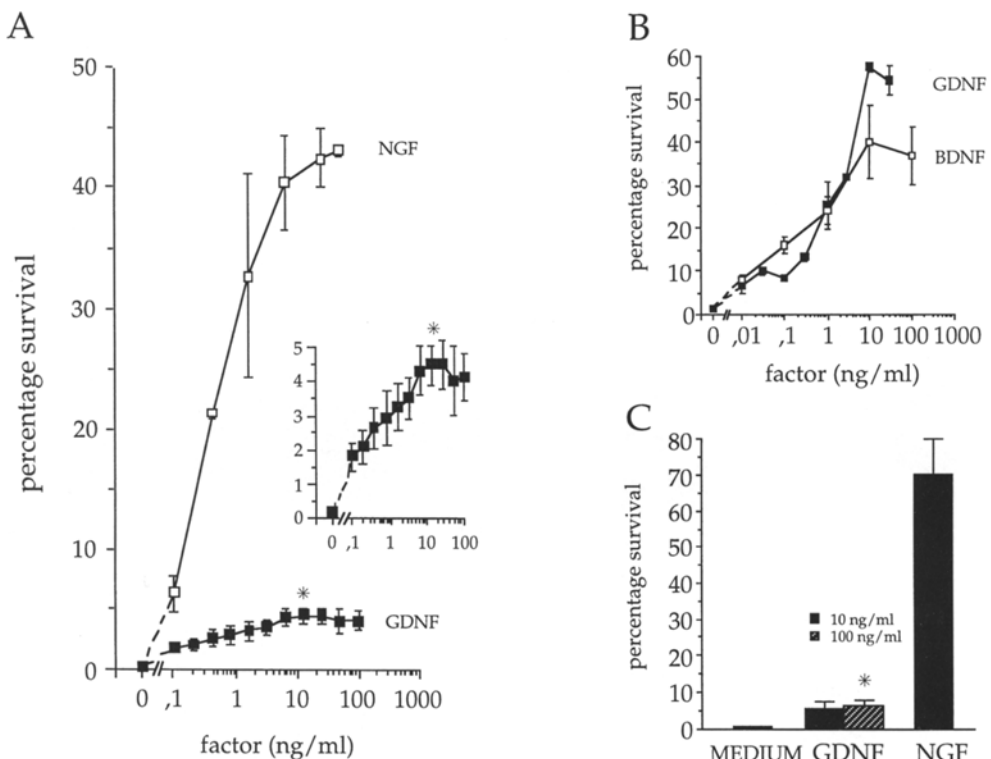
**Figure 10.** Neurite outgrowth promoted by GDNF and NGF in explanted E10 chick sympathetic paravertebral ganglia. Explants were cultured for 48 h in the presence of purified GDNF (*A*, 3 ng/ml; *B*, 10 ng/ml; *C*, 30 ng/ml; *D*, 100 ng/ml; *E*, 300 ng/ml) or purified NGF (*F*, 0.1 ng/ml; *G*, 1 ng/ml; *H*, 10 ng/ml). An explant cultured in the absence of trophic factors is shown in *I*. Arrows in *A*, *B*, and *D* show bundle-like fibers emanating from GDNF-treated explants.

members of the TGF $\beta$  superfamily, have been shown to influence the complement of neuropeptides and neurotransmitters expressed by newborn rat SCG neurons in culture (Fann and Patterson, 1994). Despite its low effects on survival of rat P1 SCG neurons, GDNF was found to regulate the phenotype of these neurons *in vitro*. Cultures of P1 rat SCG neurons were grown in the presence of NGF as a survival factor and aphidicolin as an antimetabolic agent. The expression of mRNAs coding for calcitonin gene-related peptide (CGRP), ChAT, preprotachykinin-A (PPTA), vasoactive intestinal peptide (VIP), and SOM were analyzed by RPA on RNA extracted from cultures grown for 4 d in the presence or absence of 100 ng/ml GDNF. GDNF treatment upregulated PPTA and VIP mRNA levels (2- and 3.3-fold, respectively) (Fig. 8). No significant change was seen in the levels of CGRP, SOM (Fig. 8), or ChAT (data not shown) mRNAs.

In contrast to the effects on rat SCG neurons, GDNF promoted robust survival of E10 chick paravertebral sympathetic neurons (Fig. 9 *A*). Although GDNF was able to rescue the majority of these neurons, it was somewhat less potent than NGF, with an EC<sub>50</sub> at 2ng/ml, compared to 0.8

ng/ml for NGF (Fig. 9 *A*). GDNF protein produced from the GDNF $\beta$  cDNA displayed identical potency and efficacy in E10 chick sympathetic neurons as that produced from the GDNF $\alpha$  cDNA (data not shown). GDNF responsiveness increased during embryonic chick sympathetic development from E8 to E12 (Fig. 9 *B*). Again, no difference could be observed between proteins produced from either the  $\alpha$  or  $\beta$  GDNF cDNA isoforms (Fig. 9 *B*). Responsiveness to NGF also increased between E8 and E12 (Fig. 9 *B*), in agreement with previous observations (Dechant et al., 1993). Cultures of E10 chick sympathetic neurons grown in the presence of 10 ng/ml GDNF or NGF had similar morphological appearance; like NGF, GDNF induced extensive neurite outgrowth and increase in cell body size (compare Fig. 9, *C*, *D*, and *E*). The ability of GDNF to induce neurite outgrowth was evaluated in explants of E10 chick paravertebral sympathetic chains. GDNF elicited a characteristic bundle-like, fasciculated outgrowth from sympathetic explants (Fig. 10, *A* to *E*). NGF, in contrast, produced thin, long neurites, that resulted in a dense halo with increasing concentrations (Fig. 10, *F*–*H*). As previously observed with NGF, a saturation





**Figure 11.** Survival of developing sensory neurons with GDNF. (A) Dissociated cultures of E10 chick dorsal root ganglion (DRG) neurons were grown for 48 hr in the presence of increasing concentrations of purified recombinant GDNF (■) or NGF (□). Results are expressed as the percentage of the total number of neurons plated that survived after 48 h in culture (triplicate determinations)  $\pm$  SD. The inset details the response to GDNF. \*,  $P < 0.001$  versus no factor added. (B) Dissociated cultures of E10 chick nodose ganglion neurons were grown for 48 h in the presence of increasing concentrations of purified recombinant GDNF (■) or BDNF (□). Results are expressed as the percentage of the total number of neurons plated that survived after 48 h in culture (triplicate de-

terminations)  $\pm$  SD. (C) Dissociated cultures of E16 rat trigeminal ganglion neurons were grown for 48 h in the absence (MEDIUM) or presence of 10 ng/ml GDNF, 100 ng/ml GDNF, or 10 ng/ml NGF. Results are expressed as the percentage of the total number of neurons plated that survived after 48 h in culture (triplicate determinations)  $\pm$  SD. \*,  $P < 0.01$  versus no factor added.

in the response to GDNF was seen at high concentrations, resulting in an inverted U-shaped dose-response curve. GDNF-induced neurite outgrowth was observed from 3 ng/ml, peaked at 100 ng/ml and showed saturation above 300 ng/ml (Fig. 10, A-E). Outgrowth response to NGF was observed from 0.1 ng/ml, peaked at 3 ng/ml and saturated above 10 ng/ml (Fig. 10, F-H). No outgrowth was observed in controls grown in the absence of factor (Fig. 10 I).

GDNF promoted survival of a small subpopulation (4–5%) of neurons from E10 chick DRG (Fig. 11 A). Half maximal response, however, was obtained at levels comparable to those of NGF (0.3–0.4 ng/ml). In contrast, GDNF promoted robust survival of E10 chick nodose ganglion neurons, at levels comparable, or superior, to brain-derived neurotrophic factor (BDNF) (Fig. 11 B). Half maximal response was at 1–2 ng/ml for GDNF, compared to 0.3 ng/ml for BDNF. A small proportion of neurons from E16 rat trigeminal ganglia ( $\approx$ 5%) also responded to GDNF (Fig. 11 C).

### GDNF Receptors in Embryonic Chick Sympathetic Neurons

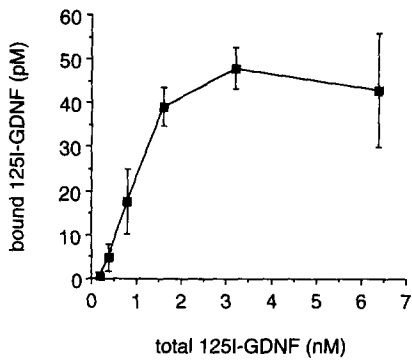
GDNF receptors in embryonic chick sympathetic neurons were examined in saturation and displacement binding assays. GDNF iodinated with chloramine T retained more than 90% of its biological activity as assessed in the sympathetic ganglion explant bioassay.  $^{125}$ I-GDNF binding to embryonic sympathetic neurons showed saturation (Fig. 12 A) with half maximal saturation at  $1 \times 10^{-9}$  M and 75,700 binding sites/cell (as calculated from the  $B_{MAX}$ ).

Unlabeled GDNF displaced iodinated GDNF from binding sites in sympathetic neurons in a dose-dependent manner (Fig. 12 B); the  $IC_{50}$  was at  $5 \times 10^{-9}$  M. NGF failed to displace iodinated GDNF from sympathetic neurons (Fig. 12 B).

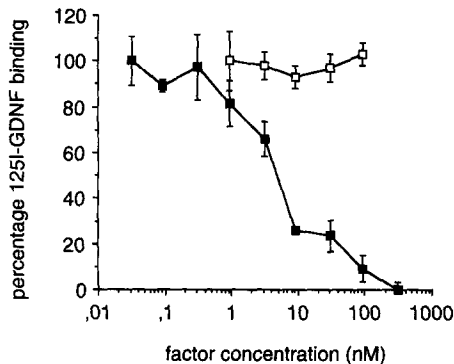
### Discussion

It has been proposed that GDNF is a highly specific and potent neurotrophic agent for dopaminergic neurons of the ventral mesencephalon (Lin et al., 1993; Tomac et al., 1995; Beck et al., 1995), which degenerate in patients with Parkinson's disease. In this study we investigated the profile of expression and biological activities of this protein in peripheral tissues and neurons of the developing rat. GDNF mRNA was expressed in various peripheral tissues as two isoforms termed  $\alpha$  and  $\beta$ , respectively. These two mRNAs are generated by alternative splicing, resulting in a 26-amino acid residue deletion in the middle of the pre-pro region of the shorter (GDNF $\beta$ ) form. We have shown that the deletion, however, does not interfere with the site of proteolytic cleavage that releases the mature GDNF form since both GDNF protein isoforms have identical  $NH_2$ -terminal sequences. Both protein isoforms appear to be glycosylated to the same extent in Sf21 insect cells, as judged by polyacrylamide gel electrophoresis and, furthermore, display indistinguishable biological activity in a sympathetic survival bioassay, suggesting that identical mature GDNF proteins are produced from the two mRNA isoforms. The significance of the existence of two GDNF iso-

A



B



**Figure 12.** GDNF receptors in embryonic chick sympathetic neurons. (A) Saturation steady-state binding of  $^{125}\text{I}$ -GDNF to E10 embryonic chick sympathetic neurons. Data are expressed as mean  $\pm$  SD of triplicate determinations. (B) Steady-state binding of  $^{125}\text{I}$ -GDNF to E10 embryonic chick sympathetic neurons competed with increasing concentrations of unlabeled GDNF (■) or unlabeled NGF (□). Data are expressed as mean  $\pm$  SD of triplicate determinations.

forms is at present not understood but could be related to differences in mRNA or prepropeptide processing or stability, which may only be manifested in cells that normally express GDNF.

GDNF mRNA was found to be expressed in some tissues receiving sympathetic innervation, such as kidney, stomach, lung and salivary gland. The predominantly postnatal expression in the latter two organs suggest GDNF may be serving a maintenance role for the sympathetic innervation of these tissues, as sympathetic neurons are known to require continuous trophic support. The earlier expression of GDNF mRNA in developing kidney may indicate a role for GDNF during the establishment of the sympathetic innervation of this tissue. It may also be possible that GDNF synthesis in kidney is unrelated to sympathetic innervation and has an exocrine function, like that of the bone morphogenetic proteins (BMPs), which, as GDNF, are also TGF $\beta$  superfamily members expressed during kidney embryonic development (Ozkaynak et al., 1991, 1992).

Although not directly innervated by the sympathetic

trunk, the reproductive organs receive sympathetic innervation from the inferior mesenteric ganglion. However, the temporally restricted, predominantly postnatal pattern of GDNF mRNA expression in testis and ovary suggests GDNF may play a non-neuronal role in these tissues. In the rat testis, postmeiotic cells appear at about 25 d of age, whereas somatic cells, including Sertoli cells, make up the bulk of the seminiferous epithelium during the early postnatal development of the testis (Bellve, 1979). The temporal expression of GDNF mRNA in testis correlates with the expansion of the Sertoli cell population, which becomes diluted at later stages by the increased number of germ cells. GDNF mRNA was expressed by the Sertoli cell line TM4, suggesting that GDNF may be produced by developing Sertoli cells *in vivo*. GDNF in the testis could have effects on the early stages of spermatogenesis, perhaps in the proliferation or maturation of spermatogonia, or on Leydig cells. It is interesting to note in this respect that TGF $\beta$  has been shown to be expressed by Sertoli cells and it appears to affect the maturation of Leydig cells and the production of extracellular matrix components in the seminiferous epithelium (Benahmed et al., 1989).

The expression of GDNF mRNA in embryonic whisker pad and skin, together with the effects of GDNF on the survival of a small subpopulation of developing trigeminal neurons suggest this protein may be a physiological factor for sensory fibers innervating skin and hair follicles. A complex and partially overlapping pattern of trophic factor expression has been reported in the rat whisker pad (Ibáñez et al., 1993), suggesting these molecules may act in concert to orchestrate the innervation of different structures by different subpopulations of trigeminal ganglion neurons. Interestingly, robust activity was seen on survival of embryonic chick nodose ganglion neurons, suggesting that other populations of placode-derived sensory neurons may also respond to the survival-promoting effects of GDNF.

The presence of GDNF mRNA in limb bud and in developing skeletal muscle may also indicate a target-derived action on sensory neurons, as GDNF was able to rescue the survival of a small subpopulation of embryonic chick DRG neurons in culture. Interestingly, the sensory neurons rescued by GDNF appeared to be of large diameter, presumably proprioceptive neurons (C. F. Ibáñez, unpublished results), although confirmation of this observation must await quantitative analysis. After submission of this paper, a study reported on the ability of newborn rat DRG neurons to retrogradely transport iodinated GDNF (Yan et al., 1995), suggesting that rat DRG sensory neurons may also be responsive to the factor. Another possible function for GDNF produced in the muscle has been suggested by recent reports on the effects of GDNF on the survival of spinal cord and facial motor neurons (Henderson et al., 1994; Yan et al., 1995; Oppenheim et al., 1995). In this context, it is worth noting the dramatic upregulation of the levels of GDNF mRNA in the rat sciatic nerve after nerve transection, suggesting that GDNF may be a physiological factor for sensory and motor nerve repair. This lesion has previously been shown to induce the expression of other motor neuron trophic factors such as BDNF (Funakoshi et al., 1993). In contrast to BDNF, however, GDNF mRNA upregulation occurs with a faster time course; after a peak

at two days post-lesion, GDNF mRNA expression in the nerve decreased and appeared to stabilize at intermediate levels. The recent demonstration by Henderson et al. (1995) of GDNF mRNA expression in cultured newborn rat Schwann cells suggests these cells may be the source of GDNF mRNA detected after transection of the sciatic nerve in the present study.

The demonstration of trophic effects of GDNF on developing sympathetic neurons adds this protein to the list of factors with trophic activities on these cells (Levi-Montalcini and Angeletti, 1968; Manthorpe et al., 1985; Unsicker et al., 1987). Although somewhat less potent than NGF, GDNF promoted survival of as many E10 chick sympathetic neurons as NGF, with  $EC_{50}$  in the order of 2 ng/ml. Neurite outgrowth stimulated by GDNF in sympathetic ganglion explants displayed characteristic bundles of relatively short, fasciculated fibers emanating from the explant, which contrasted with the thin and dense neurite halo observed in response to NGF. No morphological differences in the response to the two factors were, however, apparent in dissociated cultures. The different morphology of the outgrowth stimulated in explants could be due to differences in the sets of cell adhesion molecules or cytoskeletal elements induced by each factor. The fact that rat GDNF displayed substantial activity on chick neurons suggests the existence of a putative GDNF homologue in chicken with close structural similarities to the mammalian protein. After submission of this paper, Oppenheim et al. (1995) reported on the ability of exogenous GDNF to increase the number of sympathetic neurons in avian embryos, suggesting GDNF may regulate sympathetic cell number in vivo. Interestingly, however, our results revealed a difference in the response to GDNF between developing chick and rat sympathetic neurons. Responsiveness to GDNF increased during embryonic development of chick paravertebral sympathetic neurons, but it was low in newborn rat SCG neurons. This could be due to differences in the developmental programs of sympathetic neurons in the two species or to differences in the trophic requirements of paravertebral versus superior cervical sympathetic neurons. Differences in responsiveness to factors have previously been observed between rat and chicken sympathetic neurons (Smith et al., 1993). Despite its low effects on neuronal survival of rat SCG neurons, GDNF was able to regulate the phenotype of these cells in culture. Given the low numbers of neurons responding to the survival-promoting effects of GDNF, further analysis will be required to determine whether the elevated levels of neuropeptide mRNA expression induced by GDNF correspond to a very high increase in a small number of cells or to a moderate homogenous increase. In this respect, it is worth noting that members of the TGF $\beta$  superfamily have been shown to interact with multiple receptors with intrinsic serine-threonine kinase activity (Attisano et al., 1994; Kingsley, 1994). TGF $\beta$ , for example, has been shown to bind and promote distinct biological effects through a type II serine-threonine kinase receptor combined with either one out of three different type I receptors (Cárcamo et al., 1994). Thus, it is conceivable that GDNF may mediate survival and phenotypic changes in sympathetic neurons via related, yet distinct, subsets of receptors, which could be present in partially overlapping,

yet different, subpopulations of sympathetic neurons. Some of these receptor subunits may even be shared with other TGF $\beta$  superfamily members. In a recent study, Fann and Patterson (1994) reported the results of a systematic survey of neurotrophic cytokines and growth factors for effects on the phenotype of cultured newborn rat SCG neurons. Activin A (a member of the TGF $\beta$  superfamily) induced a pattern of neuropeptide and neurotransmitter-synthesizing enzyme mRNAs that was distinct from that induced by neurotrophic cytokines such as LIF and CNTF. In particular, activin A upregulated the expression of SOM and CGRP mRNAs, but, as opposed to LIF and CNTF, it had no effects on VIP and PPTA mRNAs (Fann and Patterson, 1994). GDNF appears to share with LIF and CNTF the ability to upregulate mRNAs for PPTA and VIP but, in contrast to the two neurotrophic cytokines, GDNF does not affect the levels of ChAT or SOM mRNAs. Moreover, unlike activin A, LIF, CNTF, and GDNF have no effect on the level of CGRP mRNA. Thus, these findings place GDNF in a distinct category among modulators of the sympathetic neuronal phenotype.

In summary, we have found widespread expression of GDNF mRNA in developing peripheral tissues and trophic activities of GDNF protein on populations of developing mammalian and avian peripheral neurons. In some cases, the pattern of GDNF mRNA expression correlated with sympathetic, sensory or motor innervation, suggesting a physiological trophic role for GDNF produced in these tissues. In addition, the endogenous production of GDNF mRNA in newborn DRG, SCG and embryonic spinal cord may indicate a paracrine or autocrine mode of action in these tissues. The expression of GDNF mRNA in reproductive organs did not correlate with innervation and could indicate a non-neuronal role for GDNF in these organs, perhaps in the differentiation of early germ cell precursors or in the maturation of accessory cells.

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