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GDNF: A Potent Survival Factor for Motoneurons Present in Peripheral Nerve and Muscle

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For survival, embryonic motoneurons in vertebrates depend on as yet undefined neurotrophic factors present in the limb bud. Members of the neurotrophin family are currently the best candidates for such neurotrophic factors, but inactivation of their receptor genes leads to only partial loss of motoneurons, which suggests that other factors are involved. Glial cell line–derived neurotrophic factor (GDNF), originally identified as a trophic factor specific for dopaminergic neurons, was found to be 75-fold more potent than the neurotrophins in supporting the survival of purified embryonic rat motoneurons in culture. GDNF messenger RNA was found in the immediate vicinity of motoneurons during the period of cell death in development. In vivo, GDNF rescues and prevents the atrophy of facial motoneurons that have been deprived of target-derived survival factors by axotomy. GDNF may therefore be a physiological trophic factor for spinal motoneurons. Its potency and specificity in vitro and in vivo also make it a good candidate for treatment of motoneuron disease.

The survival of developing motoneurons depends on trophic factors derived from their target, the limb bud, and from in the central nervous system (*1*). The best candidates for physiological motoneuron trophic factors are currently the neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5): These molecules are the most potent survival factors known for motoneurons in vitro and in vivo, are expressed in muscle during naturally occurring motoneuron cell death, and can be retrogradely transported by motoneurons (*2–4*). Moreover, mice in which TrkB and TrkC (the high-affinity receptors for these factors) are inactivated show a 30% loss of spinal motoneurons and 70% loss of facial motoneurons (*5*) at the end of fetal development, which suggests that at least a subpopulation of motoneurons requires neurotrophins to survive. However, the incomplete motoneuron loss in the null-mutant mice suggests that some motoneurons have access to other survival-promoting molecules. We therefore searched for additional factors that might act specifically on motoneurons. GDNF, a distant member of the transforming growth factor–β family, was recently purified from the supernatant of the B49 glial cell line. It has potent effects on the survival and maturation of cultured dopaminergic neurons from rat embryonic midbrain and is expressed in the embryonic striatum and other neuronal structures (*6*).

To determine the effect of GDNF on motoneurons, we prepared enriched cultures from embryonic day 14 (E14) rat spinal cord by a combination of metrizamide density gradient centrifugation and "immunopanning" (adhesion to plates coated with antibodies) with a specific antibody to $p75^{NGFR}$ (where NGFR is the nerve growth factor receptor) (*4*, *7*). In these cultures, 93 ± 3% (mean ± SEM; n = 4) of the cells were typical motoneurons. They were large and multipolar and were stained by antibodies to the motoneuron-specific homeoprotein Islet-1 (*8*) (Fig. 1A). The remaining cells (~7%) were smaller, did not stain for Islet-1, and were excluded from the cell counts. As reported (*4*, *9*), a subpopulation of Islet-1–positive motoneurons (33.4 ± 0.6%; n = 5) does not require trophic support other than that provided by basal culture medium. However, most motoneurons (~67%) are dependent on trophic factors and require muscle extract for survival (*4*). We tested the ability of recombinant rat GDNF to support the muscle-dependent motoneuron population. Purified motoneurons were seeded at low density in the presence of serial dilutions of GDNF (Fig. 1B). After 55 hours in culture, motoneuron survival increased in a dose-dependent manner with a median effective concentration (EC_{50}) of 0.2 ± 0.1 pg/ml (n = 3), equivalent to 7 fM GDNF dimer. Concentrations above 10 pg/ml were saturating, and 88.8 ± 4.1% (n = 4) of viable motoneurons were maintained (*10*).

We compared the potency of GDNF to that of other factors with motoneuron survival activity. Dose-response analysis demonstrated that in this in vitro assay, GDNF is 75-, 650-, and 2500-fold more potent than recombinant rat BDNF, rat ciliary neurotrophic factor (CNTF), and human cholinergic differentiation factor–leukemia inhibitory factor (CDF-LIF), respectively. The EC_{50} values for the other factors (BDNF, CNTF, and CDF-LIF) were similar to those reported (*4*, *9*, *11*); the rank order of potency presented here is thus not a function of the culture system used. GDNF also had the highest efficacy in supporting motoneuron survival. Expressed relative to the value of the optimal concentration of BDNF (defined as 100%), GDNF caused 115 ± 8% (n = 5), CNTF 51 ± 7% (n = 4), and CDF-LIF 44 ± 6% (n = 4) of the motoneurons to survive. Furthermore, no additive effect was apparent, even when all factors were combined. These findings demonstrate that GDNF is the most potent survival factor for motoneurons identified so far and suggest that GDNF and BDNF each support essentially all motoneurons purified by immunopanning, whereas CNTF and CDF-LIF act on only approximately half of the same population (*9*, *11*).

In contrast to the neurotrophins, GDNF had no effect on the survival of peripheral sensory neurons from the E15 nodose, E15 trigeminal, or E18 trigeminal ganglia or of E18 sympathetic neurons. Among those, the only responsive neurons were from a subpopulation of the E18 nodose neurons that also respond to BDNF (Table 1). The neurotrophic effects of GDNF at the periphery are therefore specific.

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Given the potent in vitro action of GDNF on motoneurons purified in the early phase of developmental cell death, we looked for evidence of GDNF synthesis in the environment of the motoneuron at this stage in vivo. Using reverse transcription–polymerase chain reaction (RT-PCR), we detected mRNA for GDNF in primary cultures of embryonic myotubes, E15 limb bud, and primary cultures of neonatal Schwann cells but not in embryonic (E15) spinal cord or neonatal [postnatal day 4 (P4)] skin (Fig. 2A). Further evidence for the availability of GDNF to motoneurons in vivo was provided by in situ hybridization. In E14.5 rats, GDNF transcripts were detected in ventral roots, in association with peripheral nerve, and in some muscles (Fig. 2, B through F). Identification of these structures was aided by staining with antibodies to p75, the low-affinity NGF receptor (which at this developmental stage stain Schwann cells and muscle), antibodies to neurofilaments (which stain the axons in the ventral root), and antibodies to myosin (which stain muscle cells) (*12*). The sites of synthesis of GDNF were clearly different from those of the neurotrophins. No GDNF was detected in the embryonic spinal cord, whereas both BDNF and NT-3 mRNAs are present (*4*). All muscles in the limb bud stain for NT-3, whereas GDNF is found only in a distinct subpopulation of muscles, in Schwann cells, and in the vicinity of peripheral nerve. In addition, an intense signal is present in the ventral root adjacent to the spinal cord (Fig. 2E) in the nerve sheath region, which is covered by fibroblastlike cells that are not present in the peripheral nerve in the limb (*13*). The distinct tissue distributions of GDNF and the neurotrophins support the idea that although in vitro all spinal motoneurons can respond to multiple factors (as reflected by the lack of additive effect on survival), in vivo GDNF and the neurotrophins are each available only to a subpopulation of these cells.

Although GDNF is a potent and specific survival factor for motoneurons in culture, its full physiological importance can be evaluated only in vivo. We determined whether exogenously supplied GDNF can support the survival of neonatal rat facial motoneurons that were deprived of target-derived factors by axotomy. The facial motoneurons of newborn rats were disconnected from the facial musculature by a unilateral transection of the facial nerve at the level of the stylomastoid foramen, and GDNF (20 μg) or vehicle was applied to the proximal nerve stump in a piece of gel foam. The animals

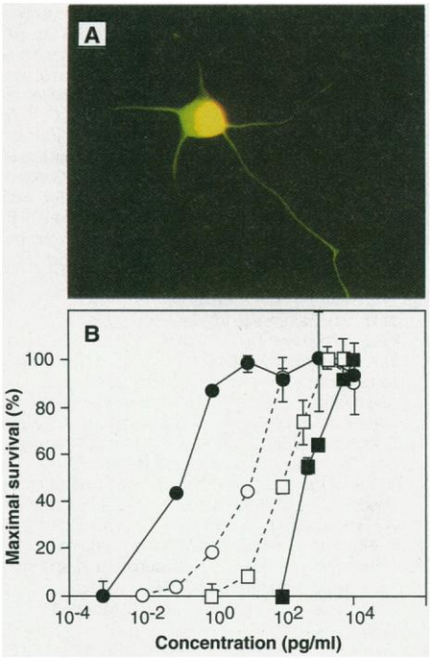

Fig. 1. Effects of GDNF and other neurotrophic factors on the survival of cultured motoneurons. (**A**) Double-labeling of a cultured motoneuron with antibodies to Islet-1 (yellow) and neurofilament (green) (*10*). The diameter of the cell body is approximately 35 μm. (**B**) Comparative dose-response analysis of GDNF (●), BDNF (○), CNTF (□), and CDF-LIF (■). Dose-response curves were normalized to survival in basal medium as 0%, and the highest survival rate shown in each curve was considered 100%. Absolute maximal survival values differed between factors. Values are mean ± range of duplicate dishes; where not shown, error bars are smaller than the symbols.

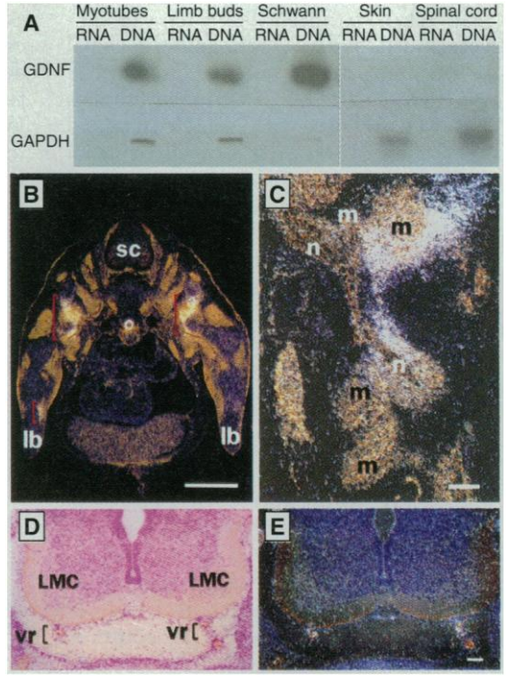

Fig. 2. Expression of GDNF mRNA in the area of the motoneurons. (**A**) RT-PCR analysis (*15*) of GDNF expression in primary E16 rat myotube cultures, limb buds from E15 rat embryos, primary cultures of enriched neonatal Schwann cells, skin from 4-day-old rats, and spinal cord from E15 rat embryos. RNA lanes: PCR reactions performed on RNA samples without reverse transcription. DNA lanes: PCR reactions performed on corresponding cDNAs. To compensate for the higher signal in Schwann cells, we loaded less PCR product (note the relative strength of the GAPDH band). (**B** through **E**) In situ hybridization to sections of an E14.5 rat embryo with the use of specific probes to rat GDNF. In (B) is a dark-field image of a transverse section through the cervical region of the embryo stained with antibody to p75 and reacted by in situ hybridization for GDNF. The brown color is indicative of immunoreactivity for p75. White regions (marked by brackets) in both proximal and distal regions of the limb bud (lb), but not in spinal cord (sc), represent in situ hybridization for GDNF. In (C) is a higher power view of a proximal region in the limb bud, showing labeling in muscle (m) and nerve (n). In (D) and (E) is shown GDNF expression in the ventral root (vr) of an E14.5 embryo. Lateral motor column, LMC. Scale bars: (B), 1 mm; (C) and (E), 50 μm.

Table 1. GDNF does not support the survival of most peripheral neurons. Low-density dissociated cultures of mouse nodose, trigeminal, and superior cervical ganglion (SCG) neurons were prepared in defined medium as described (*16*). The number of neurons attached within a standard area of the dishes was counted 6 to 12 hours after plating, and the number of neurons surviving in the same area 48 hours after plating is expressed as a percentage of this initial number. Not determined, ND.

Neuronal type	Percent surviving neurons after addition of neurotrophic factor (10 ng/ml)				
	No factor	GDNF	NGF	BDNF	BDNF + GDNF
E15 nodose	0	7 ± 1	ND	70 ± 3	ND
E18 nodose	0	43 ± 2	ND	72 ± 5	73 ± 4
E15 trigeminal	0	1 ± 1	89 ± 8	ND	ND
E18 trigeminal	0	3 ± 1	65 ± 6	ND	ND
E18 SCG	0	0	83 ± 5	ND	ND

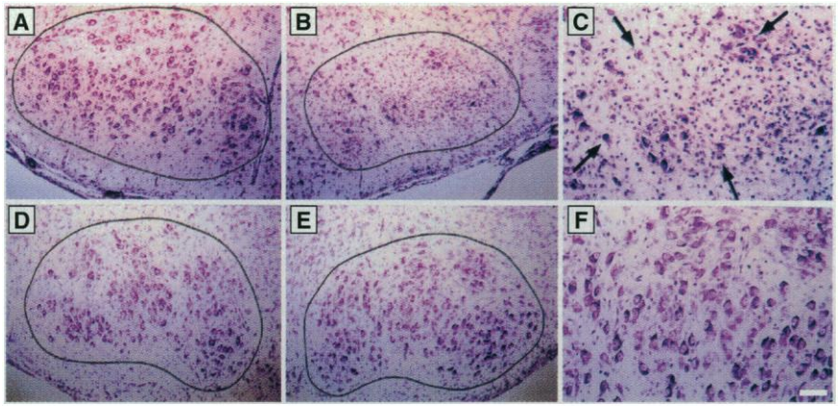

Fig. 3. Photomicrographs of facial nuclei with unilateral transections of the facial nerve and subsequent vehicle or GDNF treatment. (**A**) Nontransected facial nerve. (**B** and **C**) The facial nerve contralateral to that shown in (A) transected and treated with vehicle (shrunken motoneurons are marked by arrows). (**D**) Nontransected facial nerve. (**E** and **F**) The facial nerve contralateral to that shown in (D) transected and treated with GDNF. Scale bar: (A), (B), (D), and (E): 120 μm; (C) and (F): 60 μm.

were killed 6 days later, and the facial motoneurons in the lesioned and control side were counted (*3*). Lesioned animals that were treated with vehicle retained only 29.2 ± 1.9% (n = 6) of their facial motoneurons compared to the unlesioned side, and pronounced infiltration with glial cells was observed (Fig. 3, B and C). In contrast, the lesioned facial nucleus that received GDNF retained almost all facial motoneurons (92.4 ± 1.2%; n = 6) (Fig. 3, E and F). Neuronal perikarya retained good basophilia and Nissl structure, nuclei were centrally located, and infiltration by microglial cells was minimal. The difference in neuronal survival between the vehicle- and GDNF-treated groups was statistically significant (t = 25.6; P < 0.001). The average volume of the surviving motoneurons in facial nuclei that received GDNF was 28.5 ± 2.3 μm^3 (n = 3), close to that in the control nucleus (35.6 ± 3.3 μm^3; n = 3). In contrast, although BDNF and NT-4/5 both enhanced motoneuron survival to a similar degree as did GDNF (*2*, *3*), neither of them prevented the axotomy-induced shrinkage of facial motoneurons. In BDNF-treated nuclei, the volume of surviving motoneurons was only 20.2 ± 1.8 μm^3 (n = 3), and in NT-4/5–treated nuclei it was 16.7 ± 2.1 μm^3 (n = 3) (*14*). Thus, GDNF can prevent both death and atrophy of axotomized neonatal motoneurons for at least 6 days in vivo, which suggests that it can effectively replace survival factors of peripheral origin for motoneurons.

GDNF is the most potent survival factor yet described for motoneurons in vitro and is accessible to motoneurons during the period of developmental cell death. In vivo, not only does GDNF keep all axotomized facial motoneurons alive, but it is also the only factor known to prevent axotomy-induced motoneuron atrophy. These observations support the idea that GDNF is a physiological trophic factor for spinal motoneurons and an excellent candidate to slow the loss of function, degeneration, and death of motoneurons in diseases such as amyotrophic lateral sclerosis (ALS) and the spinal muscular atrophies (SMA).

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10. Purified motoneurons were seeded at nominal densities of 1 to 2 cells per square millimeter. The 100% value for survival was defined as the number of large neurite-bearing cells in a predetermined area after 15 hours in optimal conditions, and this represented 51% of all cells seeded. For immunohistochemistry, fixed motoneurons were permeabilized, then incubated with 2D6 antibody to Islet-1 (1:2) or rabbit antiserum to neurofilament (Sigma; 1:2000). Motoneuron abundance was evaluated by expressing the number of Islet-1–positive nuclei in at least 15 microscopic fields as a percentage of the total number of neurons with neurites larger than two cell diameters in length (*4*). Rat GDNF was cloned from the B49 cell line with the use of published sequences. Mature GDNF was expressed in *Escherichia coli* by inserting the coding sequence downstream of the *trp* promoter and immediately upstream of the ATG start codon of the tetracycline resistance gene in the pBR322-derived vector pHGH207-1 [D. G. Yansura and D. J. Henner, *Methods Enzymol.* **185**, 54 (1990)]. The insoluble fraction after lysis of *E. coli* cells was solubilized in 8 M urea buffer at pH 7.4, then treated with sodium sulfite and sodium tetrathionate to sulfonate the cysteine residues. The monomeric GDNF was partially purified by anion exchange chromatography and then refolded. The resulting dimeric GDNF was purified by anion exchange, cation exchange, size exclusion, and hydroxylapatite chromatography.
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14. Cell volumes were calculated stereologically according to the concept of isotropic rotator [E. B. Vedel Jensen and H. J. G. Gundersen, *J. Microsc.* **170** (no. 1), 35 (1993)] with an Olympus BH-2 microscope with a motorized stage coupled by means of a microencoder (a detector of positional information) to a color video monitor-camera and an Amiga 2000 computer [V. E. Koliatsos, W. L. Price, C. A. Pardo, D. L. Price, *J. Comp. Neurol.* **342**, 35 (1994)]. Volumes of GDNF-treated facial motoneurons were compared with those of BDNF- and NT4/5-treated motoneurons from tissues used for cell counts in previous studies (*3*).
15. Primary E16 myotube cultures enriched by differential adhesion of fibroblasts were prepared as described [G. E. Jones, S. J. Murphy, D. J. Watt, *J. Cell Sci.* **97**, 659 (1990)]. Schwann cells were prepared from sciatic nerves of 2-day-old rats, and cytosine arabinoside (10^{-5} M) was added to reduce contamination by fibroblasts [J. P. Brockes, K. L. Fields, M. C. Raff, *Brain Res.* **165**, 105 (1979)]. Total RNA (50 μg) was incubated with ribonuclease-free deoxyribonuclease I and 5 μg was used for reverse transcription. Complementary DNA (cDNA) (<0.1 ng) was amplified with primers for rat GDNF (35 cycles) (nucleotides 205 to 222 and 457 to 475; GenBank accession number L15305) or rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (26 cycles) (nucleotides 132 to 149 and 414 to 431; GenBank accession number M17701). Samples (5 μl) were run on 5% acrylamide gels, treated with NaOH, and transferred to Hybond-N+. Blots were hybridized with probes corresponding to nucleotides 15 to 338 of the rat GDNF sequence and nucleotides 3 to 177 of the rat GAPDH sequence, washed three times in 0.5× saline sodium citrate and 0.1% SDS at 55°C, and exposed for autoradiography. The signal was proportional to the cDNA dilution in this range. In situ hybridization was performed as described [H. S. Phillips, J. M. Hains, G. R. Laramee, A. Rosenthal, J. W. Winslow, *Science* **250**, 290 (1990)] with a combination of two overlapping probes labeled with [^{33}P]uridine triphosphate and spanning the entire coding region of GDNF.
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