REFERENCES AND NOTES

1. K. Morihara and J. Y. Homma, in *Bacterial Enzymes and Virulence*, I. A. Holder, Ed. (CRC Press, Boca Raton, FL, 1985), pp. 49–79.
2. B. H. Iglewski and D. Kabat, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2284 (1975).
3. B. H. Iglewski, J. C. Sadoff, M. J. Bjorn, E. S. Maxwell, *ibid.* **75**, 3211 (1978).
4. R. A. Bever and B. H. Iglewski, *J. Bacteriol.* **170**, 4309 (1988).
5. E. Kessler and M. Saffrin, *ibid.*, p. 5241.
6. G. Doring, J. Obernesser, K. Botzenhart, *Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A* **249**, 89 (1981).
7. L. W. Heck *et al.*, *J. Immunol.* **6**, 2253 (1990).
8. L. W. Heck, K. Morihara, W. B. McRae, E. J. Miller, *Infect. Immun.* **51**, 115 (1986).
9. K. Morihara, H. Tsuzuki, K. Oda, *ibid.* **24**, 188 (1979).
10. D. R. Schultz and K. D. Miller, *ibid.* **10**, 128 (1974).
11. M. J. Gambello and B. H. Iglewski, *J. Bacteriol.* **173**, 3000 (1991). After publication of this paper, we were informed that the pMG designation was in conflict with other constructs listed in a plasmid data bank. To avoid confusion, we have renamed pMG1.7 [as originally referenced in the Gambello and Iglewski paper] as pMJG1.7.
12. M. J. Gambello, S. A. Kaye, B. H. Iglewski, *Infect. Immun.* **61**, 1180 (1983).
13. D. S. Toder, M. J. Gambello, B. H. Iglewski, *Mol. Microbiol.* **5**, 2003 (1991).
14. M. J. Gambello, thesis, University of Rochester (1991).
15. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977); the GenBank–European Molecular Biology Laboratory accession number for the sequence is L04681.
16. S. E. West and B. H. Iglewski, *Nucleic Acids Res.* **16**, 9323 (1988).
17. J. Devereaux, P. Haeberli, O. Smithies, *ibid.* **12**, 387 (1984).
18. J. M. Cook and B. H. Iglewski, unpublished results.
19. E. A. Meighen, *Microbiol. Rev.* **55**, 123 (1991).
20. A. Eberhard, *J. Bacteriol.* **109**, 1101 (1972).
21. A. Eberhard *et al.*, *Biochemistry* **20**, 2444 (1981).
22. T. Beppu, *Gene* **115**, 159 (1992).
23. H. B. Kaplan and E. P. Greenberg, *J. Bacteriol.* **163**, 1210 (1985).
24. S. Henikoff, J. C. Wallace, J. P. Brown, *Methods Enzymol.* **183**, 111 (1990).
25. G. S. Shadel, R. Yound, T. O. Baldwin, *J. Bacteriol.* **172**, 3980 (1990); J. Schlock, D. vanRiet, D. Kolibachuk, E. P. Greenberg, *ibid.*, p. 3974.
26. A. Sinai and B. H. Iglewski, unpublished results. To construct pTS400-1.7, we subcloned the 1.7-kb fragment of pMJG1.7 carrying the *lasR* gene into the unique Tth 111I site downstream of *lacZ*.
27. J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972).
28. We subcloned an Eco RV–Sal I fragment from pJMC30 into pBluescriptII SK+ to produce pLPL100. We then subcloned a 1.1-kb Bam HI–Sfi I fragment from pMJG1.7 that extends from the Bam HI site in the multiple cloning site of pBluescriptII SK+ up to the Sfi I site just before the end of the *lasR* coding sequence into pLPL100 to construct pLPL104. This plasmid carries the entire reconstructed *lasR* gene and the entire *lasI* coding sequence. A 2.4-kb Bam HI–Xho I fragment from pLPL104 that carries both *lasR* and *lasI* was then subcloned into Bam HI– and Sal I–digested pSW200 (*11*) or into the Tth 111I site downstream of *lacZ* on pTS400 to construct pLPL106, which contains the *lasB::lacZ* translational fusion in addition to *lasR* and *lasI*.
29. G. Ralling, S. Bodrug, T. Linn, *Mol. Gen. Genet.* **201**, 379 (1985).
30. Gibco–Bethesda Research Laboratories Life Technologies, *Focus* **6**, 4 (1984).
31. The plasmid pLASI-1 carries the *lasI* gene as a 989-bp fragment that extends from an Nla IV site 90 bp upstream of the start of *lasI* up to a Sal I site distal to the gene. We constructed it by subcloning a 300-bp Nla IV–Eco RI fragment from pMJG1.7 (*11*) and a 689-bp Eco RI–Sal I fragment from pJMC31 into Sma I– and Sal I–digested pBluescriptII SK+. Plasmid pLASI-2 consists of the same fragment containing the *lasI* gene used in pLASI-1 cloned onto pSW200, which carries the pRO1600 replicon, which allows replication in *P. aeruginosa* (*11*).
32. L. Passador and B. H. Iglewski, unpublished results.
33. T. F. Smith and M. S. Waterman, *Adv. Appl. Math.* **2**, 482 (1981).

34. We thank P. Greenberg and K. Tucker for discussions, T. Linn for the gift of *E. coli* MG4, and J. Nezezon for technical assistance. Supported by PHS grant AI33713 and by NIH grants T32GM07356 (to M.J.G.) and T32AI07362 (to L.R.).

16 October 1992; accepted 12 February 1993

GDNF: A Glial Cell Line–Derived Neurotrophic Factor for Midbrain Dopaminergic Neurons

Leu-Fen H. Lin, Daniel H. Doherty, Jack D. Lile, Susan Bektesh, Frank Collins*

A potent neurotrophic factor that enhances survival of midbrain dopaminergic neurons was purified and cloned. Glial cell line–derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor–β superfamily. In embryonic midbrain cultures, recombinant human GDNF promoted the survival and morphological differentiation of dopaminergic neurons and increased their high-affinity dopamine uptake. These effects were relatively specific; GDNF did not increase total neuron or astrocyte numbers nor did it increase transmitter uptake by γ-aminobutyric–containing and serotonergic neurons. GDNF may have utility in the treatment of Parkinson's disease, which is marked by progressive degeneration of midbrain dopaminergic neurons.

Parkinson's disease is characterized by degeneration of dopaminergic neurons in the midbrain that innervate the striatum (*1*). Current treatments are aimed at pharmacologically augmenting striatal dopamine but do not prevent continued neuron degeneration. Neurotrophic factors that specifically prevent this degeneration and increase the functional activity of the remaining dopaminergic neurons are therefore of substantial clinical interest.

The search for such neurotrophic factors has focused on dissociated cultures of embryonic midbrain, where high-affinity dopamine uptake and expression of tyrosine hydroxylase (TH) can be used as markers for dopaminergic neuron survival and differentiation (*2*). These bioassays have established the existence of dopaminergic neurotrophic activity in conditioned media derived from primary glial cells (*3*) and from several cell lines with the properties of glia (*4*). However, these factors have not been characterized.

Here we report the characterization of a specific dopaminergic neurotrophic factor secreted by one of these glial cell lines, rat B49 (*5*). This factor, termed GDNF (for glial cell line–derived neurotrophic factor), was purified to apparent homogeneity (*6*) on the basis of its ability to promote dopamine uptake in midbrain cultures (*7*). Purified GDNF produced a single peak on reversed-phase high-performance liquid chromatography (RP-HPLC) but a broad smear on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), a property suggestive of glycosylation (Fig. 1). The presence of *N*-linked glycosylation was confirmed by treatment with *N*-glycanase, which decreased the apparent molecular mass of GDNF from ~20 kD to ~15 kD (Fig. 1). GDNF behaved like a disulfide-bonded dimer; its apparent molecular mass on nonreducing SDS gels was 32 to 42 kD compared with 18 to 22 kD on reducing gels (Fig. 1).

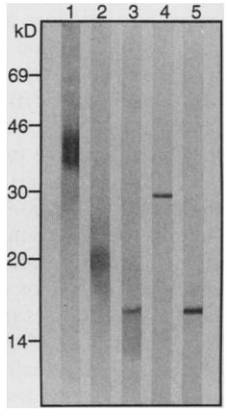

Fig. 1. SDS-PAGE of GDNF. Samples were heated to 100°C for 10 min with or without reducing agent (200 mM dithiothreitol) or after treatment with *N*-glycanase to remove Asn-linked sugars. The positions of molecular weight markers are indicated on the left. Lane 1, purified B49 cell GDNF (25 ng), detected by silver staining; lane 2, purified B49 cell GDNF (25 ng) after reduction of disulfide bonds, detected in an immunoblot with antibodies to rhGDNF; lane 3, purified B49 cell GDNF (25 ng) after treatment with *N*-glycanase, detected in an immunoblot with antibodies to rhGDNF; lane 4, purified rhGDNF (2 μg), detected by Coomassie brilliant blue staining; lane 5, purified rhGDNF (2 μg) after reduction of disulfide bonds, detected by Coomassie brilliant blue staining.

Synergen, Inc., 1885 33rd Street, Boulder, CO 80301.

*To whom correspondence should be addressed.

The rat and human genes for GDNF were cloned by means of probes that were based on the amino-terminal sequence of purified GDNF (8). The inferred amino acid sequences of rat and human GDNF are 93% identical (Fig. 2A). The sequence data suggest that GDNF is synthesized as a precursor that is processed and secreted as a mature protein of 134 amino acids. There are two potential *N*-linked glycosylation sites in the mature protein. Interestingly, GDNF contains the seven conserved Cys residues in the same relative spacing found in all members of the transforming growth factor–β (TGF-β) superfamily (Fig. 2B). Other members of the TGF-β superfamily also appear to be synthesized as precursors that are processed into mature disulfide-bonded dimers (9).

The TGF-β superfamily has been divided into five subfamilies within which the mature proteins share 42 to 92% amino acid sequence homology (*10*). Mature GDNF shares less than 20% homology with any of these subfamilies and thus may represent a new subfamily. There are no other homologies between GDNF and sequences in the databases.

A nucleotide sequence coding for the mature human GDNF was expressed in *Escherichia coli* (*11*). Recombinant human GDNF (rhGDNF) was refolded to restore biological activity and purified to apparent homogeneity (*11*). The rhGDNF is a disulfide-bonded homodimer (Fig. 1) that is not glycosylated (*11*) but exhibits the full biological activity of the native protein.

To determine the activity of GDNF in midbrain cultures, we identified dopaminergic neurons by immunohistochemistry (*12*) with antibody to TH. The TH^+ neurons did not react with antibody to dopamine β-hydroxylase (DβH), supporting their identification as dopaminergic, rather than noradrenergic, neurons (*12*). Consistent with this finding, dopamine uptake in midbrain cultures was specifically inhibited by antagonists of dopaminergic, but not noradrenergic, monoamine uptake (*7*). Total neurons were identified with antibody to neuron-specific enolase (NSE). Astrocytes were identified with antibody to glial fibrillary acidic protein (GFAP).

GDNF specifically promoted the survival of dopaminergic neurons in dissociated rat embryo midbrain cultures. In control cultures, the number of dopaminergic (TH^+) and total (NSE^+) neurons decreased by ~70% between days 2 and 21 (Fig. 3). In rhGDNF-treated cultures, the number of total neurons also decreased by ~70%, but the number of dopaminergic neurons did not decrease significantly (Fig. 3). In five separate experiments carried out to day 21, there were 2.7 ± 0.5 times more dopaminergic neurons in cultures treated with rhGDNF (1 ng/ml) than in control cultures ($P < 0.001$ Mann-Whitney U test).

High-affinity uptake of dopamine, a biochemical marker of dopaminergic neuron function, was also enhanced by GDNF (Fig. 3). In 20 independent determinations, GDNF caused a 2.5- to 3-fold increase in dopamine uptake per TH^+ neuron, with an average half-maximal effective concentration (EC_{50}) of 1.2 pM or 36 pg/ml. In contrast, GDNF had no effect on high-affinity uptake of γ-aminobutyric acid (GABA) or serotonin by GABAergic and serotonergic neurons in midbrain cultures at concentrations that were ~30,000-fold higher than the EC_{50} for increasing dopamine uptake (Fig. 3).

GDNF also dramatically increased the morphological differentiation of TH^+ neurons, resulting in more extensive neurite out-

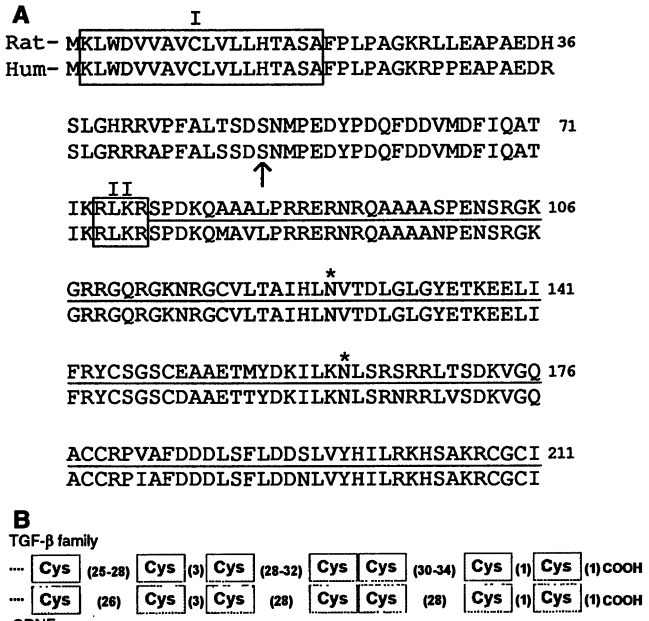

Fig. 2. (**A**) Sequences of rat and human GDNF. The initial Met at position 1 is followed by a potential secretion signal (boxed area I) predicted to be cleaved between Ala^{19} and Phe^{20} (*18*). The sequence Arg-Leu-Lys-Arg^{77} (boxed area II), which matches the consensus sequence for proteolytic processing in the constitutive secretion pathway (*19*), is the presumed cleavage site for release of mature GDNF. Consistent with this hypothesis, the amino-terminal sequence of purified B49 cell GDNF begins with the Ser^{78} immediately after this potential cleavage site. The predicted amino acid sequence of mature GDNF is underlined; the *N*-linked glycosylation sites are indicated by asterisks; and the position of an intron within the human GDNF gene is denoted by an arrow. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Alignment of the seven Cys residues in GDNF with those conserved in members of the TGF-β superfamily (*10*). The number of amino acids between each conserved Cys is indicated in parentheses. There is only one amino acid between the final Cys and the COOH-terminus of the mature protein.

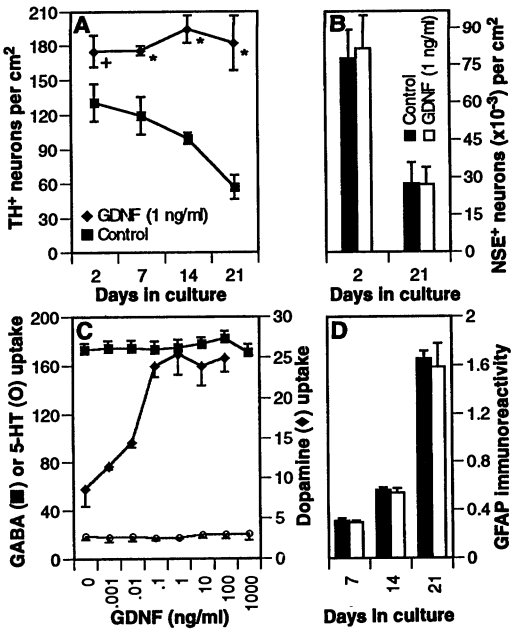

Fig. 3. (**A** and **B**) Enhanced survival of dopaminergic neurons in rat embryo midbrain cultures treated with GDNF. Cultures in defined medium (*7*) were untreated (■ and filled bars) or exposed to rhGDNF (1 ng/ml) (◆ and open bars) added every 3 days starting on day 0. After 2 to 21 days, dopaminergic (TH^+, left) or total (NSE^+, right) neurons were counted (*12*). Neuron counts are the average ± SD of four determinations. Significance of the difference between control and GDNF was $P < 0.01$ (+) or $P < 0.001$ (*) in the Mann-Whitney U test. (**C**) Increased dopamine uptake in midbrain cultures treated with GDNF. Dopamine, serotonin (5-HT), or GABA uptake (*7*) was determined after 12 days of treatment with the indicated concentrations of rhGDNF every 3 days from day 0. Uptake values (expressed as femptomoles per minute per 10^5 cells plated) are the average ± SD of three determinations. (**D**) GDNF does not affect GFAP immunoreactivity. Cultures were untreated (filled bars) or exposed to rhGDNF (1 ng/ml) (open bars) added every 3 days starting on day 0. GFAP immunoreactivity (*12*) is plotted in optical density units in the ELISA (*12*) as the average ± SD of four determinations.

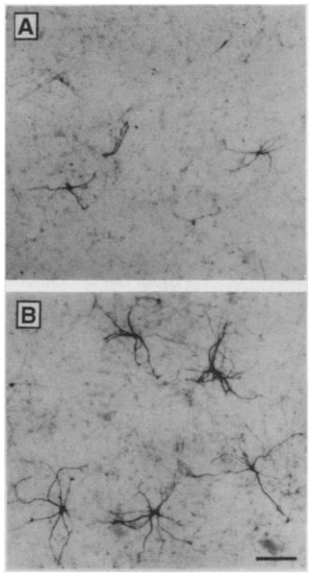

Fig. 4. Effect of GDNF on the morphology of TH^+ neurons. Dissociated midbrain cultures were incubated as in Fig. 3 for 21 days without (**A**) or with (**B**) rhGDNF (1 ng/ml) and processed for TH immunohistochemistry (*12*). The bar represents 100 μm.

growth and increased cell body size (Fig. 4). Such morphological differences between GDNF-treated and control cultures were obvious by day 7 and increased with time. After 21 days, the average cell body area of TH^+ neurons in control cultures was 207 ± 15 μm^2 compared with 340 ± 32 μm^2 ($P < 0.001$) in cultures treated with rhGDNF (1 ng/ml).

GDNF did not increase the density of astrocytes nor their expression of GFAP in the midbrain cultures. In four separate experiments, GFAP immunoreactivity (*12*) was not significantly affected out to day 21 (Fig. 3).

GDNF is both more specific and more potent than other factors that promote dopamine uptake or TH^+ neuron survival in embryonic midbrain cultures. Insulin-like growth factors (IGF-I and -II), epidermal growth factor (EGF), fibroblast growth factors (a- and b-FGF), and brain-derived neurotrophic factor (BDNF) all increase GABA or serotonin uptake in addition to their effects on dopaminergic neurons (*13*, *14*). Furthermore, IGFs, EGF, and FGFs are glial mitogens in midbrain cultures (*13*, *15*). All of these factors exhibit an EC_{50} in the 1 to 100 ng/ml range (*15*, *16*), which is 25 to 2500 times higher than the EC_{50} of GDNF.

The effect of GDNF on midbrain dopaminergic neurons in vitro has been confirmed in intact adult animals (*17*). Our work provides the basis for defining at the molecular level the physiological role of GDNF and for exploring its potential utility as an alternative approach to the treatment of Parkinson's disease, a neurodegenerative disorder of midbrain dopaminergic neurons.

REFERENCES AND NOTES

1. O. Hornykiewicz and S. J. Fish, *Adv. Neurol.* **45**, 19 (1986); D. C. German *et al.*, *Ann. N.Y. Acad. Sci.* **648**, 42 (1992).
2. A. Prochiantz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5387 (1979).
3. G. Gaul and H. Lubbert, *Proc. R. Soc. London Ser. B* **249**, 57 (1992); E. K. O'Malley *et al.*, *Brain Res.* **582**, 65 (1992).
4. J. Engele, D. Schubert, M. C. Bohn, *J. Neurosci. Res.* **30**, 359 (1991).
5. D. Schubert *et al.*, *Nature* **249**, 224 (1974).
6. Serum-free conditioned medium (CM) was prepared from confluent B49 cells (*5*), concentrated, and applied to a heparin-Sepharose column (Pharmacia) developed with a linear gradient of 0.15 to 1.5 M NaCl in 50 mM Na-phosphate (pH 8). Bioactive fractions were concentrated and further purified by fast protein liquid chromatography (FPLC) on a Superose 12 sizing column (HR10/30, Pharmacia) developed in 0.5 M NaCl and 50 mM Na-phosphate (pH 7.4). Bioactive fractions were applied to a C-8 RP-HPLC column (Aquapore RP-300, Applied Biosystems, San Jose, CA) developed with a linear gradient of 5 to 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Finally, the bioactive fractions were subjected to preparative SDS-PAGE (*20*), followed by RP-HPLC, as described above. Edman degradation of purified GDNF gave the following amino-terminal amino acid sequence: SPDKQAAALPRRERN?QAAAASPDN.
7. Rostral mesencephalic tegmenta of E16 Sprague-Dawley rat embryos were dissociated mechanically in Ca^{2+}- and Mg^{2+}-free saline. Dissociated cells (2×10^5) were plated in 16-mm polyornithine-coated culture wells (Falcon, Lincoln Park, NJ) filled with serum-containing culture medium (*21*) or serum-free defined medium (*13*), which was then changed weekly. Uptake of dopamine, GABA, and serotonin was determined as previously described (*13*). Specific neuronal uptake, determined by means of appropriate inhibitors (0.1 μM GBR 12909 for dopaminergic, 10 μM NO-711 for GABAergic, and 10 μM quipazine for serotonergic neurons) was 85 to 95% of total uptake. Desipramine, a preferential inhibitor of monoamine uptake by noradrenergic neurons (*22*), was required in at least 300-fold higher concentrations than GBR 12909 to decrease dopamine uptake.
8. For cloning of rat and human GDNF genes, we used polyadenylated RNA from B49 cells to generate a cDNA library in lambda-ZapII (Stratagene, La Jolla, CA) (*23*). The library was screened with an oligonucleotide [5′-CCIGAYAARCARGCIGCIGC-3′ (I = inosine; Y = C or T; R = A or G) derived from the amino acid sequence PDKQAAA near the amino terminus of B49 cell GDNF. The human GDNF gene was cloned from a lambda-FixII human genomic library (Stratagene) by screening with a fragment of B49 cDNA corresponding to amino acids 79 to 163 (Fig. 2) at reduced stringency (42°C, 30% formamide). Sequencing of the human gene revealed an intron at Ser^{51}. To clone the upstream exon, we screened the human genomic library with a probe corresponding to amino acids 1 to 41. The nucleic acid sequences for rat and human GDNF have been deposited in GenBank (accession numbers L15305 and L15306, respectively).
9. A. B. Roberts *et al.*, *Biochemistry* **22**, 5692 (1983); K. Miyamoto *et al.*, *Biochem. Biophys. Res. Commun.* **129**, 396 (1985); N. Ling *et al.*, *Nature* **321**, 779 (1986); D. Schubert *et al.*, *ibid.* **344**, 868 (1990); E. A. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2220 (1990).
10. D. W. Burt, *Biochem. Biophys. Res. Commun.* **184**, 590 (1992).
11. The DNA sequence encoding mature human GDNF was expressed in *E. coli* by methods previously described (*24*). The rhGDNF, localized in the insoluble fraction after cell lysis, was solubilized by homogenization in 20 mM Na-phosphate (pH 7.4), 8 M urea, 100 mM Na-sulfite, and 10 mM Na-tetrathionate. It was then applied to a Q-Sepharose column (Pharmacia) in 10 mM tris (pH 8.0) containing 4 M urea, and the column developed with a linear gradient of 0 to 0.5 M NaCl in this buffer. Pooled fractions (~3 mg/ml rhGDNF) were diluted with 19 volumes of refold buffer (100 mM Na_2HPO_4, brought to pH 8.3 with tris containing 4 M urea; 5% polyethylene glycol 300; and 3 mM cysteine) and held under argon at 5°C for 3 days. The refold mix was concentrated and applied to an S-Sepharose column (Pharmacia) in 50 mM tris (pH 8.0) developed with a linear gradient of 0 to 1 M NaCl in this buffer. Fractions containing rhGDNF were concentrated and applied to a hydroxyapatite column (American International Chemical, Natick, MA) in 25 mM Na-phosphate (pH 6.8) developed with a linear gradient of 25 to 450 mM Na-phosphate (pH 6.8). Purified rhGDNF contained <0.01% bacterial proteins and <5 pg/mg bacterial endotoxin. Edman degradation and mass spectroscopy confirmed the identity of rhGDNF and demonstrated the absence of glycosylation.
12. Cultures were prepared for immunohistochemistry with rabbit antibody to TH (TE-101, Eugene Tech International, Ramsey, NJ), rabbit antibody to DβH (TE-103, Eugene Tech), rabbit antibody to NSE (16625, Polysciences, Warrington, PA), or mouse antibody to GFAP (6400, Labsystems, Helsinki, Finland) and biotinylated secondary antibodies, with staining performed according to the manufacturer's instructions (ABC kits, Vector Labs, Burlingame, CA). Positive control cultures of dissociated sympathetic ganglia (*25*) were used to demonstrate that antibody to DβH, a marker of noradrenergic neurons, was active, despite the absence of $D\beta H^+$ neurons in midbrain cultures. Enzyme-linked immunosorbent assays (ELISA) were performed on fixed and permeabilized midbrain cultures with antibody to GFAP, as previously described (*26*).
13. D. Casper, C. Mytilineou, M. Blum, *J. Neurosci. Res.* **30**, 372 (1991).
14. G. Ferrari *et al.*, *Dev. Biol.* **133**, 140 (1989); G. Ferrari *et al.*, *J. Neurosci. Res.* **30**, 493 (1991); C. Hyman *et al.*, *Soc. Neurosci. Abstr.* **17**, 908 (1991).
15. B. Knusel *et al.*, *J. Neurosci.* **10**, 558 (1990).
16. J. Engele and M. C. Bohn, *ibid.* **11**, 3070 (1991); B. Knusel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 961 (1991).
17. B. J. Hoffer *et al.*, in preparation. A single intracranial injection of 1 or 10 μg of rhGDNF in the area adjacent to the substantia nigra of adult rats elicited an increase in TH immunoreactivity and dopamine turnover.
18. G. von Heijne, *Nucleic Acids Res.* **14**, 4683 (1986).
19. M. Hosaka *et al.*, *J. Biol. Chem.* **266**, 12127 (1991).
20. L.-F. H. Lin, L. G. Armes, A. Sommer, D. J. Smith, F. Collins, *ibid.* **265**, 8942 (1990).
21. L. Friedman and C. Mytilineou, *Neurosci. Lett.* **79**, 65 (1987).
22. L. Hetey and B. Zimmermann, *Biomed. Biochim. Acta* **45**, 1203 (1986).
23. P. Chomcyznski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987).
24. J. R. McDonald, C. Ko, D. Mismer, D. J. Smith, F. Collins, *Biochim. Biophys. Acta* **1090**, 70 (1991).
25. F. Collins and J. D. Lile, *Dev. Biol.* **502**, 99 (1989).
26. J. E. Coligan *et al.*, Eds., *Current Protocols in Immunology* (Wiley, New York, 1991), pp. 13 and 14.

27. We thank L. Armes, J. Hunt, T. Malmstrom, H. Musa, D. Pratt, B. Rosenzweig, J. Ross, D. Smith, B. Weaver, and T. J. Zhang for their invaluable contributions; J. Cox, G. Gerhardt, B. J. Hoffer, D. Martin, and J. Vannice for critical reading of the manuscript; and C. Mytilineou, T. Park, and J. Shen for guidance with midbrain cultures.

2 March 1993; accepted 16 April 1993