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GDNF Prevents Degeneration and Promotes the Phenotype of Brain Noradrenergic Neurons In Vivo

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Summary

The locus coeruleus (LC), the main noradrenergic center in the brain, participates in many neural functions, as diverse as memory and motor output, and is severely affected in several neurodegenerative disorders of the CNS. GDNF, a neurotrophic factor intitially identified as dopaminotrophic, was found to be expressed in several targets of central noradrenergic neurons in the adult rat brain. Grafting of genetically engineered fibroblasts expressing high levels of GDNF prevented >80% of the 6-hydroxydopamine-induced degeneration of noradrenergic neurons in the LC in vivo. Moreover, GDNF induced a fasciculated sprouting and increased by 2.5-fold both tyrosine hydroxylase levels and the soma size of lesioned LC neurons. These findings reveal a novel and potent neurotrophic activity of GDNF that may have therapeutic applications in neurodegenerative disorders affecting central noradrenergic neurons, such as Alzheimer's, Parkinson's, and Huntington's diseases.

Introduction

The locus coeruleus (LC) is the most important noradrenergic nucleus in the brain and provides widespread innervation to many regions of the CNS (Loughlin and Fallon, 1985). This organization makes the LC a regulator of many essential functions, including motor output, vestibular reflexes, vigilance, attention, and memory (Barnes and Pompeiano, 1991). Furthermore, a marked loss of LC neurons and a severe impairment of some of these functions have been reported in the most important and devastating neurodegenerative disorders of the brain, including Alzheimer's (Tomlison et al., 1981; Bondareff et al., 1982; Hardy et al., 1985), Parkinson's (Hassler, 1938; Forno, 1966; Jellinger, 1991), Pick's (Arima and Akashi, 1990), and Huntington's (Zweig et al., 1988) diseases, Down's syndrome (German et al., 1992), progressive supranuclear palsy (Mann et al., 1983), and olivopontocerebellar atrophy (Tomonaga, 1983).

Neurotrophic factors are polypeptides that promote survival, differentiation, and phenotype of neurons. Neurotrophin-3 (NT-3), a member of the nerve growth factor family of neurotrophic factors, is a survival factor for LC noradrenergic neurons in vitro (Friedman et al., 1993). Furthermore, NT-3, but no other member of the neurotrophin family, has been shown to prevent the 6-hydroxydopamine (6-OHDA)–induced degeneration of LC noradrenergic neurons in the adult brain in vivo (Arenas and Persson, 1994). Surprisingly, however, homozygous mice carrying a deletion of the NT-3 gene showed no reduction in the number of neurons or in the level of tyrosine hydroxylase (TH) staining in the LC (Ernfors et al., 1994; Fariñas et al., 1994), suggesting that other neurotrophic factors may be responsible for maintaining the survival and phenotype of central noradrenergic neurons.

Glial cell line–derived neurotrophic factor (GDNF) a distant member of the transforming growth factor β superfamily, was originally isolated by virtue of its ability to induce dopamine, but not GABA or serotonin, uptake and to promote the survival of dopaminergic neurons in cultures of embryonic ventral midbrain (Lin et al., 1993). Recent findings have extended the spectrum of biological activities of this molecule to different populations of neurons in both the peripheral and central nervous systems. In the periphery, GDNF has been reported to support the survival of neurons in nodose and sympathetic ganglia, as well as small subpopulations of embryonic sensory neurons in dorsal root and trigeminal ganglia (Henderson et al., 1994; Oppenheim et al., 1995; Trupp et al., 1995). GDNF has also been shown to modify the phenotype of sympathetic neurons in culture by increasing the expression of vasoactive intestinal peptide and preprotachykinin mRNAs and inducing robust bundle-like sprouting (Trupp et al., 1995). In the CNS, high doses of GDNF have been shown to prevent the axotomy-induced death of facial or spinal cord motorneurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) and to protect adult nigral dopaminergic neurons from axotomy, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, or 6-OHDA toxicity (Beck et al., 1995; Kearns and Gash, 1995; Tomac et al., 1995). GDNF administration in the nigrostriatal system has been shown to induce sprouting from dopaminergic neurons, increase levels of dopamine, noradrenaline, and serotonin, and improve motor behavior (Tomac et al., 1995). *GDNF* mRNA expression has so far been documented only in the developing rat striatum, the major target of nigral dopaminergic innervation, and in a few other regions, including hippocampus and cortex (Schaar et al., 1993; Strömberg et al., 1993; Poulsen et al., 1994), but not in the normal adult brain.

In this study, we have assessed the distribution of *GDNF* mRNA in the adult rat brain and its neurotrophic activity on LC noradrenergic neurons. Our results show that *GDNF* mRNA is expressed at low levels throughout the adult CNS and that GDNF prevents the death of LC neurons and displays remarkable neurotrophic activities on the phenotype of central noradrenergic neurons in vivo.

Results

GDNF mRNA Is Widely Expressed in the Adult Brain

We examined *GDNF* mRNA expression in adult brain regions using a sensitive RNase protection assay (Trupp et

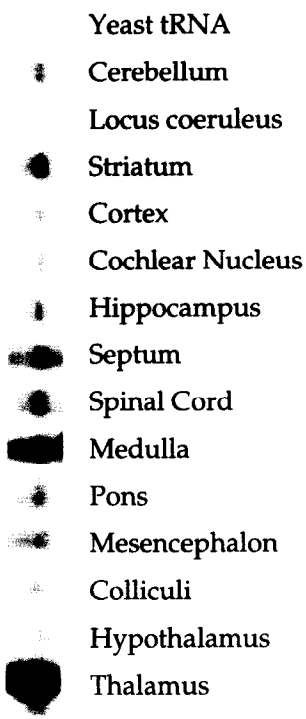

Figure 1. Expression of *GDNF* mRNA in the Adult Rat Brain

GDNF mRNA expression was analyzed by RNase protection. RNA (5 μg) from the indicated brain regions was analyzed using a specific *GDNF* riboprobe. Yeast tRNA was used as a negative control. Striatum, septum, spinal cord, medulla, and thalamus showed the highest levels of *GDNF* mRNA expression in the brain.

al., 1995). *GDNF* mRNA was found to be widely expressed in the adult rat brain, albeit at lower levels than in nonneuronal tissues (Trupp et al., 1995). *GDNF* mRNA was detected in thalamus, striatum, septum, cerebellum, spinal cord, and medulla oblongata (Figure 1). Lower levels were also found in cortex, hippocampus, cochlear nucleus, pons, mesencephalon, colliculi, and hypothalamus (Figure 1). No *GDNF* mRNA was detected in the LC (Figure 1). This widespread pattern of *GDNF* mRNA expression in the brain, including many areas innervated by noradrenergic neurons, suggested that GDNF may be a neurotrophic factor for LC noradrenergic neurons.

Characterization of a Cell Line Genetically Engineered to Express High Levels of GDNF

To assess the ability of GDNF to function as a neurotrophic factor for adult neurons in vivo, Fischer 344 rat 3T3 fibroblasts were genetically engineered to produce GDNF in high amounts. These cells, designated F3A-GDNF, expressed high levels of *GDNF* mRNA up to 1 month after their implantation in the brain (Figure 2E), and like purified recombinant GDNF protein (Trupp et al., 1995), their conditioned media promoted survival of dissociated embryonic day 10 chick sympathetic neurons (data not shown). F3A-GDNF cells produced more than 100 ng of GDNF per 10^6 cells per day, as determined in a sympathetic neuron survival bioassay. Mock-transfected 3T3 cells (designated F3A-MT) expressed very low levels of *GDNF* mRNA (Figure 2E), and conditioned media from these cells had no survival-promoting effect on dissociated sympathetic neurons (data not shown).

GDNF Prevents the 6-OHDA-Induced Degeneration of Adult Noradrenergic Neurons In Vivo

To assess the ability of GDNF to prevent the death and functional decline of neurons in the LC, noradrenergic neurons were selectively lesioned with an ipsilateral injection of 6-OHDA at the beginning of the dorsal bundle, 1 day after implantation of F3A-GDNF or F3A-MT cells (Figures 2A–2D). Seven days after surgery, brains were processed for histological analysis, and LC noradrenergic neurons were visualized using either antibodies against TH or cresyl violet staining in serial sections through the entire LC. In animals with no cells implanted, the 6-OHDA lesion caused a selective loss of TH-positive noradrenergic neurons of about 50% in the LC compared with the contralateral side in the same animals (Figures 3 and 4A). This decrease was more pronounced in the anterior part of the nucleus, near the injection site of 6-OHDA (Figure 4B). As previously demonstrated (Arenas and Persson, 1994), the numbers of TH-positive and Nissl-stained neurons were shown to decrease to the same extent in this lesion model (compare Figures 4A and 4C), indicating a true loss of noradrenergic neurons after 6-OHDA injection. In nonlesioned animals, unilateral grafting of F3A-GDNF or F3A-MT cells did not modify the number of noradrenergic neurons compared with the nontreated nucleus, indicating that the graft did not compromise the integrity of the LC. In the 6-OHDA-lesioned LC, implantation of F3A-MT cells did not prevent the decrease in the number of TH-positive and cresyl violet–stained neurons in the LC (Figures 3 and 4), indicating that the graft per se had no survival-promoting effects on noradenergic neurons. In contrast, implantation of the F3A-GDNF cells prevented the degeneration of about 80% of the lesioned neurons in the LC. The numbers of both TH-positive and cresyl violet–stained neurons were rescued to the same extent (Figures 4A and 4C), indicating that GDNF prevented the 6-OHDA-induced death of LC noradrenergic neurons. In parallel experiments, NT-3-secreting fibroblasts (F3A-NT3) were shown to prevent the degeneration of noradrenergic neurons, as previously described (Arenas and Persson, 1994). NT-3 prevented the decrease in the number of TH-positive neurons (ipsilateral, 598 ± 50; contralateral, 670 ± 19 [mean ± SEM]; n = 3) and cresyl violet–stained LC neurons (ipsilateral, 620 ± 44; contralateral, 708 ± 23 [mean ± SEM]; n = 3) after lesion to an extent similar to GDNF.

GDNF Promotes Phenotypic Changes in Adult LC Noradrenergic Neurons

F3A-GDNF, F3A-NT3, or F3A-MT cells were unilaterally grafted posterolateral to the LC (see Figures 2A–2C), and 1 day later, some of the animals received ipsilateral 6-OHDA injections rostral to the LC. Seven days after surgery, brains were processed for TH immunohistochemistry, and the phenotype of LC noradrenergic neurons was analyzed in serial sections at the level of the grafts.

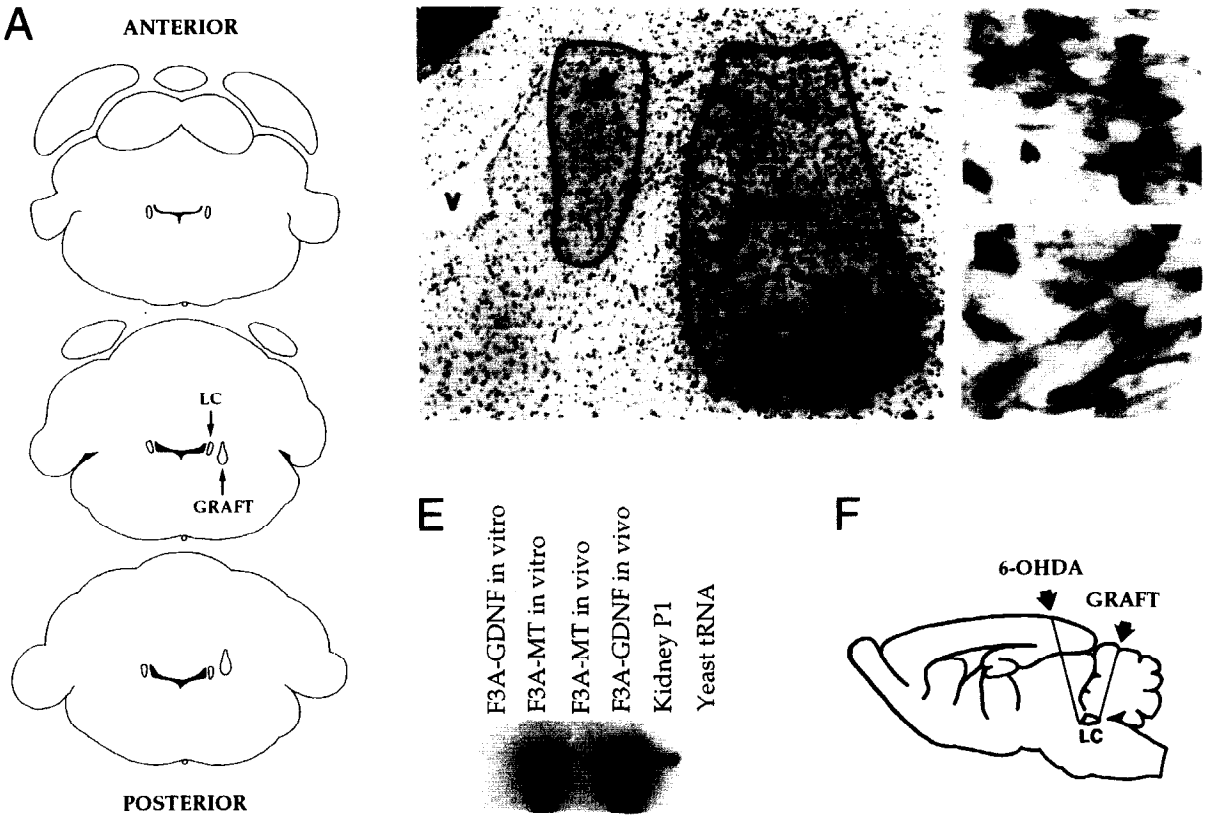

Figure 2. Characterization and Grafting of a GDNF-Producing Fibroblast Cell Line (F3A-GDNF)

(A) Camera lucida drawing of three representative cresyl violet–stained coronal sections through the anteroposterior extension of the LC. The upper drawing corresponds to a section through the anterior LC (0.4 mm caudal to interaural line), the middle drawing to the medial LC (0.7 mm caudal to interaural line), and the lower drawing to the posterior LC (1 mm caudal to interaural line).

(B) Photomicrograph of the posteromedial LC (interaural −0.8), showing the grafting site, lateral to the LC. V, fourth ventricle.

(C and D) High power photomicrographs of cresyl violet–stained grafts illustrating the survival of F3A-MT (C) and F3A-GDNF (D) fibroblasts 1 week after implantation and 6-OHDA lesion.

(E) Expression of *GDNF* mRNA in the F3A-MT and F3A-GDNF cells before and after grafting. RNA (2 μg) from GDNF-expressing cells (F3A-GDNF) or mock-transfected cells (F3A-MT) in culture (in vitro) or 1 month after implantation in the brain (in vivo) was analyzed by RNase protection assay. The levels of *GDNF* mRNA expressed by the cell lines are compared with that in 10 μg of total RNA from postnatal day 1 kidney, one of the organs with the highest levels of *GDNF* mRNA in the rat. Yeast tRNA was used as a negative control.

(F) Schematic drawing of the position of the cell lines (GRAFT) and 6-OHDA injection relative to the LC. Note that, in this paradigm, GDNF is delivered from the posterolateral part of the nuclei, and the lesion, placed anterior to the LC, induces an anterior to posterior gradient of cell loss (Arenas and Persson, 1994). Both manipulations were performed unilaterally, leaving the contralateral side intact.

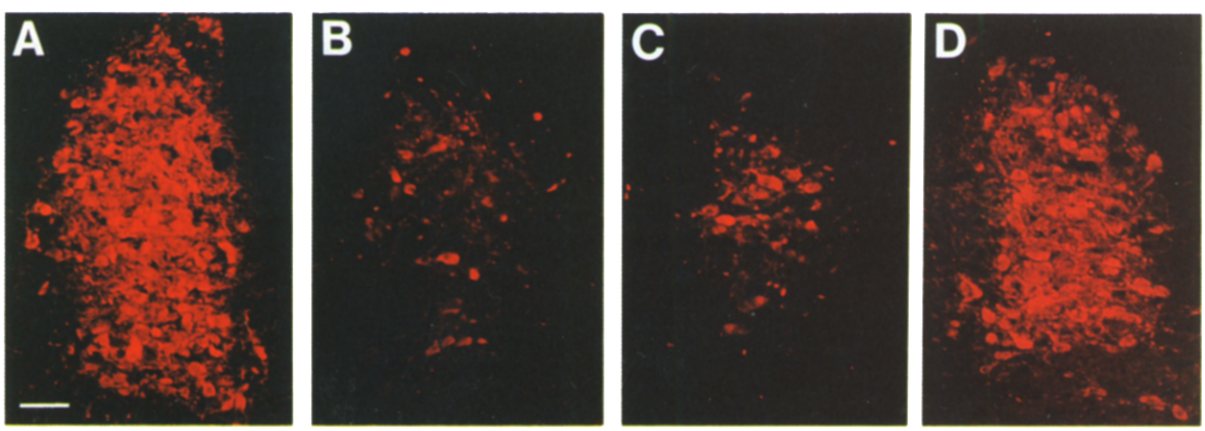

Figure 3. Effect of GDNF on Survival of Noradrenergic Neurons in the LC

Photomicrographs of sections through the anterior part of the LC stained with a monoclonal antibody against TH. (A) control side; (B) 6-OHDA lesioned side; (C) 6-OHDA lesioned LC implanted with the control cell line (F3A-MT); (D) 6-OHDA lesioned LC implanted with the GDNF-expressing cell line (F3A-GDNF), showing an almost complete prevention of 6-OHDA-induced cell death. Bar, 200 μm.

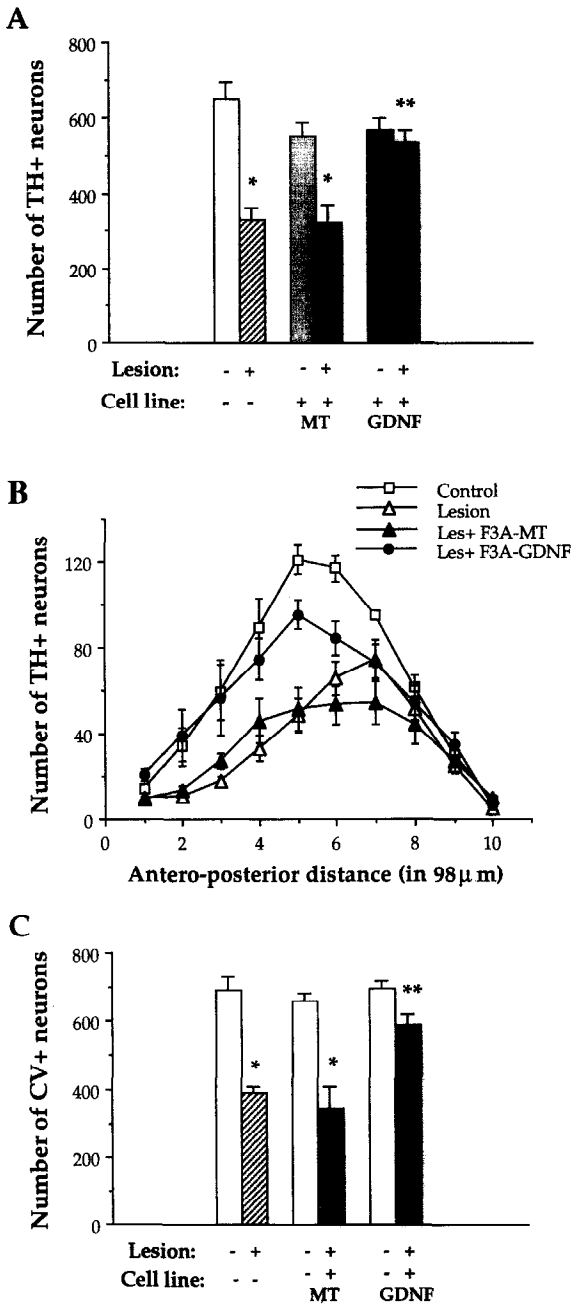

Figure 4. Effect of GDNF on the Number of Noradrenergic Neurons in the LC

(A) Number of TH-positive neurons in the LC after implantation of GDNF-expressing (F3A-GDNF) or mock-transfected (F3A-MT) cell lines in the intact or 6-OHDA lesioned LC.

(B) Anteroposterior distribution of TH-positive neurons in the LC after lesion and grafting of F3A-MT or F3A-GDNF cells.

(C) Number of cresyl violet–stained neurons in the LC after lesion and grafting of F3A-MT or F3A-GDNF cells.

Values represent mean ± SEM of the number of TH-positive cells in the LC. Asterisk, $p < .01$ versus unlesioned side; double asterisk, $p < .01$ versus lesion with or without control graft (F3A-MT) (two-tailed Student's t test).

Implantation of F3A-MT or F3A-NT3 cells had no effect on the cytoplasmic area of TH-positive neurons (see Figure 6), nor did they induce sprouting from noradrenergic neurons in the intact LC (see Figure 8), indicating that neither the cells per se nor NT-3 promotes these phenotypic parameters in LC noradrenergic neurons. In contrast, F3A-GDNF cells induced a 70% increase in the cytoplasmic area of TH-positive neurons, compared with the contralateral side (Figures 5B and 6), and a mild increase in sprouting from noradrenergic neurons in the ipsilateral LC (see Figure 8).

In nongrafted animals, 6-OHDA injections did not affect the soma size of the surviving TH-positive neurons at any level of the LC, nor did it selectively kill neurons of any particular size (Figures 5 and 6). In addition, the intensity of TH staining in the cell bodies of LC neurons was unchanged compared with the control side (Figure 7), although TH staining was increased in varicosities close to cell bodies (data not shown). Implantation of the F3A-MT or F3A-NT3 cells in lesioned animals had no effect on the size of the soma (Figures 5B and 6), the levels of TH staining (Figure 7), or sprouting (Figure 8) of LC noradrenergic neurons. In contrast, GDNF treatment induced a dramatic increase in the area of the soma of TH-positive neurons (see Figures 5B and 6) and the levels of TH staining (Figure 7) as well as promoted pronounced bundle-like sprouting (Figure 8F). Compared with the nonlesioned animals, the lesion appeared to potentiate the effects of GDNF on both hypertrophy and sprouting of LC neurons (compare Figures 8E and 8F).

Discussion

In the present study, we show that GDNF prevents the degeneration of adult central noradrenergic neurons of the LC in vivo. Although survival was comparable to that previously reported after NT-3 administration (Arenas and Persson, 1994), GDNF, but not NT-3, induced a remarkable hypertrophy as well as a dramatic increase in TH levels and sprouting of noradrenergic neurons. Thus, GDNF appears to be a very powerful neurotrophic factor for adult central noradrenergic neurons, capable of promoting both the survival and phenotype of LC neurons. Since the levels of *GDNF* mRNA expression have been found to be higher in the developing brain than in the adult (Schaar et al., 1993; Strömberg et al., 1993; Poulsen et al., 1994), our data suggest that GDNF, or a combination of GDNF and NT-3, may play a central role in the maturation and survival of developing noradrenergic neurons. Analysis of *GDNF*$^{-/-}$ mutant mice may shed light on this issue.

In the experimental paradigm used in the present study, the 6-OHDA lesion generates a gradient of cell death, being more pronounced in the anterior part of the nucleus than in the posterior part (Arenas and Persson, 1994; and Figure 4B). It is also conceivable that the graft, posterolateral to the LC, generates a caudal to rostral gradient of concentration of the factor. This gradient could be responsible for elevated effects of GDNF on hypertrophy and sprouting in the posteromedial LC (close to the graft) but

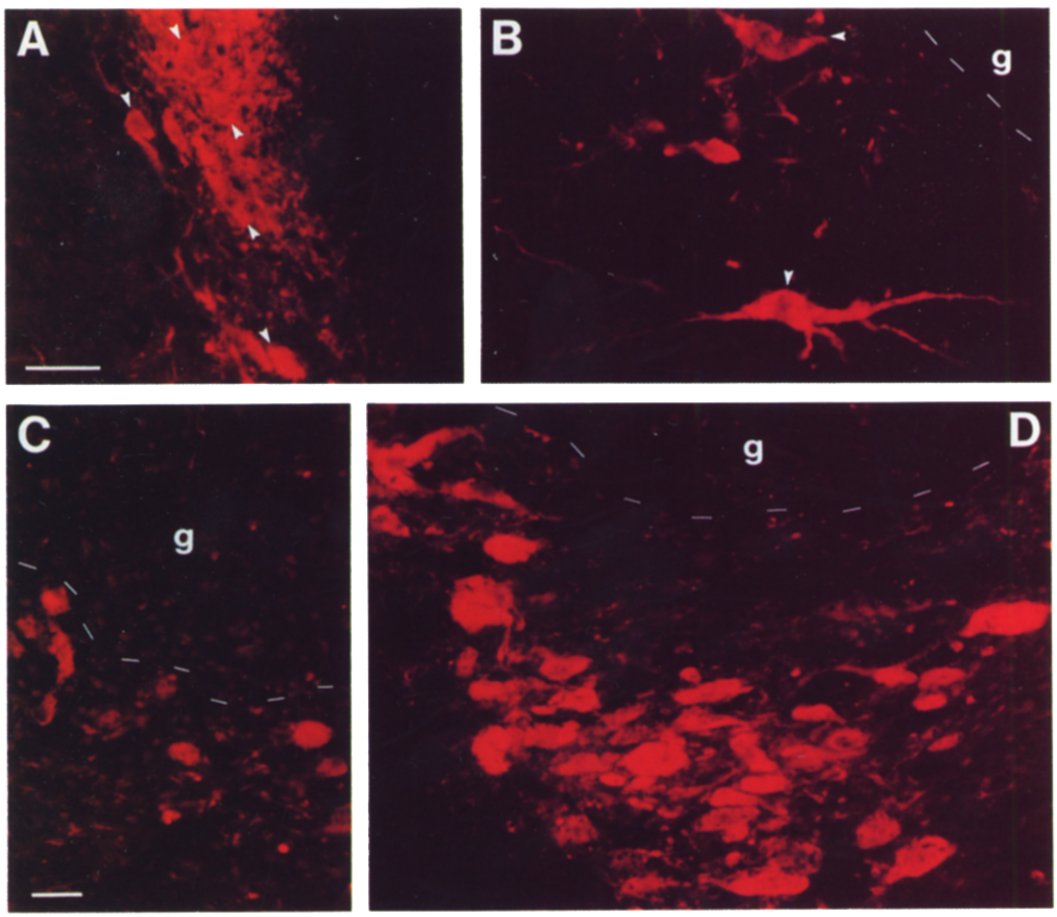

Figure 5. Effect of GDNF on the Size of the Soma of TH-Positive Neurons in the LC

In the nonlesioned LC, F3A-GDNF cells (B) induced an increase in the soma size of noradrenergic neurons (arrowheads) compared with the control side (A). In the 6-OHDA lesioned LC, the F3A-GDNF cell line (D), but not the F3A-MT cell line (C), induced a hypertrophy of noradrenergic neurons. The borders of the grafts (g) are labeled with dashed lines. Photomicrographs in (A) and (B) were taken from the ventrocaudal LC. Photomicrographs in (C) and (D) were taken from the dorsocaudal LC. Bars, 18 μm (A and B), 32 μm (C and D).

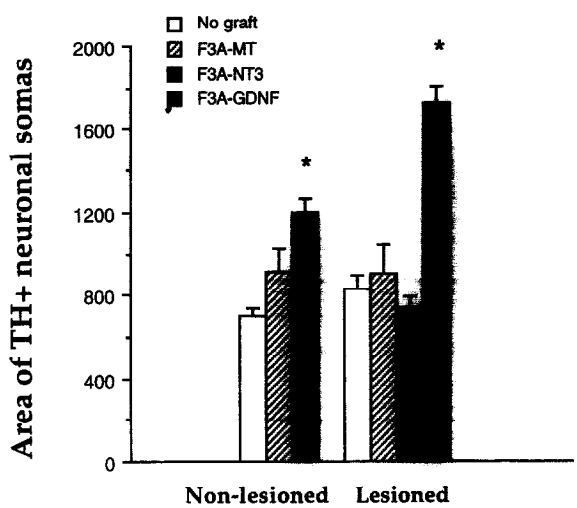

Figure 6. Effect of GDNF on the Cytoplasmic Area of Lesioned and Unlesioned TH-Positive Neurons in the LC

GDNF increased the size of noradrenergic neurons in the 6-OHDA lesioned LC to a greater extent than in nonlesioned nuclei. Values represent the mean area (in square micrometers) ± SEM (n = 3–4). Asterisk, $p < .01$ versus unlesioned side (two-tailed Student's t test).

not in the anterior part of the LC (close to the lesion site). Because complete prevention of cell death was observed in the anterior part of the LC, higher doses of GDNF may be required to promote hypertrophy and sprouting as compared with survival. In this context, it is worth noting that most of the previous in vivo studies used doses of GDNF up to two orders of magnitude higher than those used here; this may have precluded the observation of different dose requirements for distinct biological activities.

The dramatic effects of GDNF on the phenotype of noradrenergic neurons, particularly the hypertrophy, and the nearly complete protection of LC neurons from 6-OHDA-induced degeneration suggest that GDNF exhibits broader and more pronounced neurotrophic activities on noradrenergic neurons than those previously reported on dopaminergic neurons (Beck et al., 1995; Kearns and Gash, 1995; Tomac et al., 1995). In the nigrostriatal system, administration of GDNF was shown to increase the levels not only of dopamine but also of noradrenaline (Tomac et al., 1995). Interestingly, however, the increase in noradrenaline was seen only in the substantia nigra, a target of LC noradrenergic neurons, and not in the striatum, which is not inner-

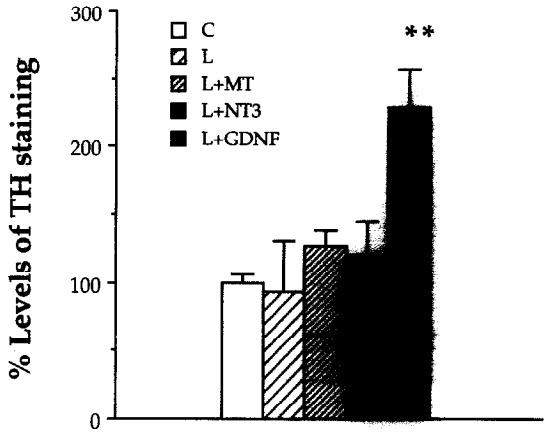

Figure 7. Effect of GDNF on the Intensity of TH Immunostaining in Neurons of the LC

The F3A-GDNF cell line, but not the F3A-NT3 or F3A-MT cell line, increased the levels of TH immunostaining in the LC at 7 days after lesion. Note that the intensity of the staining is a semiquantitative measure and is expressed in relative values. C, control locus; L, 6-OHDA lesioned LC; L + MT, lesioned LC grafted with the mock-transfected cell line; L + NT3, lesioned LC with a NT-3-producing graft (Arenas and Persson, 1994); L + GDNF, lesion plus grafting of the GDNF-transfected cell line. Double asterisks, $p < .01$ versus any of the other conditions (two-tailed Student's t test).

vated by LC neurons (Loughlin and Fallon, 1985). Our findings suggest that, in this case, GDNF may have induced a neurotrophic response on LC neurons via noradrenergic terminals in the substantia nigra, reinforcing the notion that GDNF is a neurotrophic factor for LC noradrenergic neurons.

Interestingly, GDNF elicited a more profound induction of the phenotype of noradrenergic neurons following 6-OHDA injection than in the nonlesioned LC. This result suggests that noradrenergic neurons may respond to the lesion by increasing the expression of GDNF receptors, perhaps as part of a lesion-induced mechanism to increase responsiveness to low levels of neurotrophic factor. In this context, it is worth noting that, while the lesion increased the responsiveness of LC neurons to GDNF, levels of *GDNF* mRNA in target areas of noradrenergic neurons, or in the LC itself, were unaffected by the lesion (E. A., M. T., P.Å., and C. F. I., unpublished data). On the other hand, the increased responsiveness after the lesion could also be the result of elevated levels of downstream elements in the GDNF signaling pathway. Regardless of the mechanism by which 6-OHDA induces GDNF responsiveness in the LC, the hypertrophy, induction of TH, and sprouting of nerve terminals observed in surviving noradrenergic neurons after GDNF administration point to an increased functional capacity of these cells and suggest that GDNF may be a promising candidate molecule to prevent and compensate for the degeneration of central noradrenergic neurons.

Degeneration of basal forebrain cholinergic neurons is a well-known pathological feature of Alzheimer's disease (Davies and Maloney, 1976; Whitehouse et al., 1982; Fibiger, 1991). However, other populations of neurons are also affected early, constantly, and severely by the disease, such as the noradrenergic neurons of the LC (Tomlison et al., 1981; Bondareff et al., 1982; Hardy et al., 1985). In particular, the greatest loss of noradrenergic neurons in Alzheimer's disease, as well as in Down's syndrome and Parkinson's disease with dementia, occurs within the anteromedial portion of the nucleus, corresponding to cortical projecting neurons (Marcynuik et al., 1986; Zweig et al., 1988; Chan-Palay and Asan, 1989; German et al., 1992). The 6-OHDA lesion model used in this study (Arenas and Persson, 1994) mimics those disorders not only in the rostral pattern of neuronal loss within the LC but also in the proposed mechanism of cell death, namely oxidative stress (Beal et al., 1993; Behl et al., 1994). In agreement with the role of these neurons in attention and memory (Harley, 1991), degeneration of LC neurons has been correlated with dementia in both Alzheimer's disease (German et al., 1992) and Parkinson's disease (Bondareff et al., 1982; Mann and Yates, 1983; Gaspar and Gray, 1984; Chui et al., 1986; Cash et al., 1987; Zweig et al., 1993).

It has been known for a long time that degeneration of neuromelanin-containing neurons in the brain, namely nigral dopaminergic neurons (Pakkenberg and Brody, 1965; Hornykiewicz and Kish, 1987; Hirsch et al., 1988) and LC noradrenergic neurons (Hassler, 1938; Halliday et al., 1990; Jellinger, 1991), is a prominent pathological feature of idiopathic Parkinson's disease. In this case, neuronal loss in the LC affects all levels of the nucleus to a similar extent (Chan-Palay and Asan, 1989; Chan-Palay, 1991; German et al., 1992; Zweig et al., 1993), and it is accompanied by noradrenergic denervation of several structures, including cerebellum, spinal cord, and cortical motor areas. Accordingly, degeneration of LC noradrenergic neurons has been reported to contribute to the motor alterations and to be responsible for the propioceptive deficits observed in Parkinson's disease (Schneider et al., 1987; Gaspar et al., 1991). Furthermore, loss of dopaminergic neurons, decreased dopamine content in striatum, and parkinsonian signs induced by dopaminergic lesions are potentiated by simultaneous lesioning of the LC in monkeys and mice (Mavridis et al., 1991; Marien et al., 1993). Thus, GDNF, by inducing both individual and synergistic trophic responses on central dopaminergic and noradrenergic neurons, may constitute a very powerful therapeutic candidate for Parkinson's disease.

In summary, we have found widespread expression of GDNF in the adult CNS and a novel and potent neurotrophic activity of GDNF on LC noradrenergic neurons. Although it is presently unknown why the neurons in the LC are so vulnerable and degenerate in so many diseases, the ability of GDNF to promote both the survival and phenotype of LC neurons suggests that this protein may be a physiological trophic factor for central noradrenergic neurons with potential therapeutic application in neurodegenerative disorders affecting the LC.

Experimental Procedures

RNase Protection Assay

Assays were performed using the RPAII Ribonuclease Protection Assay Kit (Ambion) following the manufacturers' recommendations. GDNF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

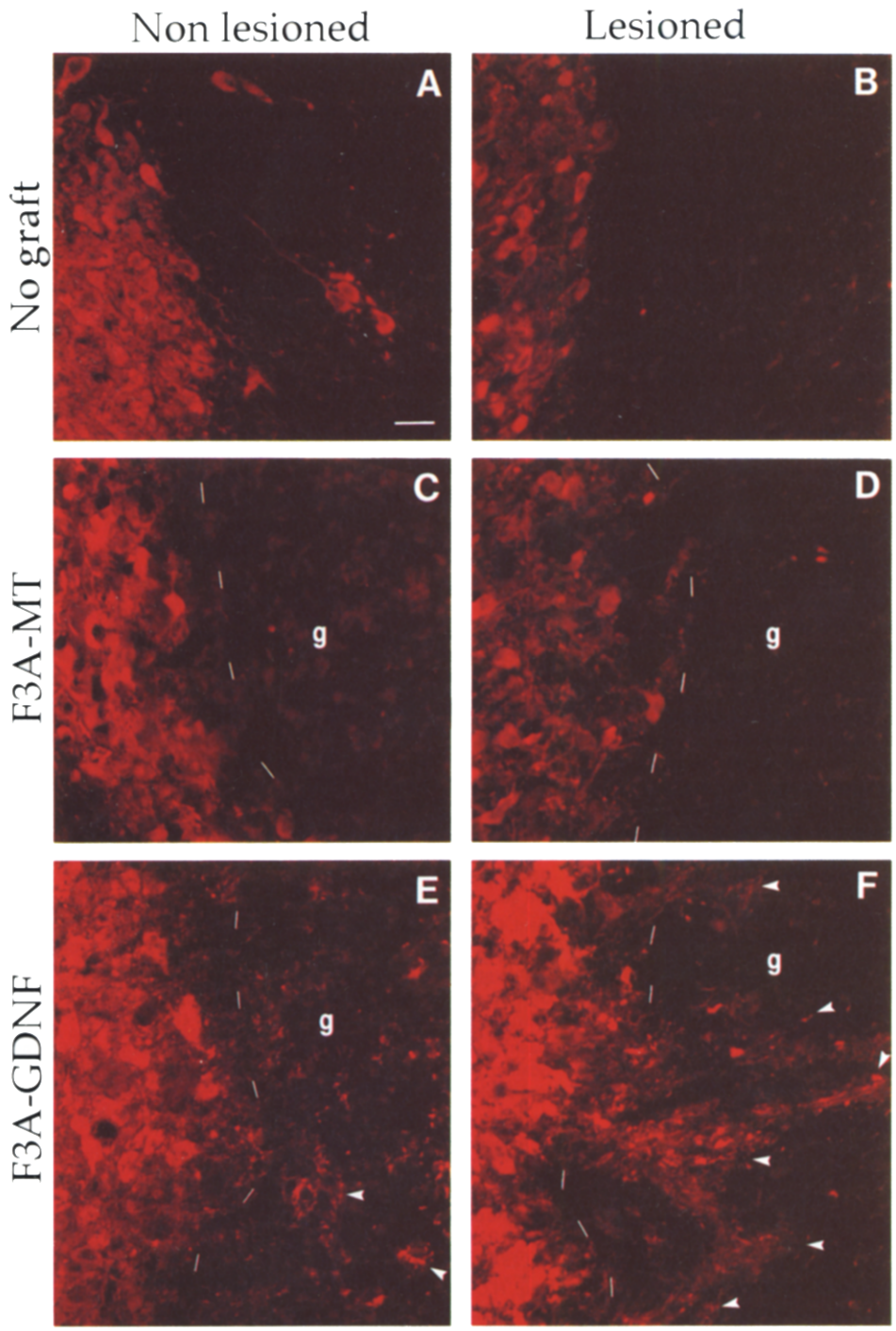

Figure 8. **Effect of GDNF on Sprouting of Noradrenergic Neurons in the LC**

Photomicrographs of sections through the posteromedial part of the LC stained with a monoclonal antibody against TH. (A) Control LC; (B) 6-OHDA lesioned LC; (C) LC implanted with the control cell line (F3A-MT); (D) grafting of the control cell line (F3A-MT) in 6-OHDA lesioned animals did not induce sprouting toward the graft; (E) LC implanted with the GDNF-expressing cell line (F3A-GDNF), showing a moderate sprouting; (F) 6-OHDA lesioned LC implanted with the GDNF-expressing cell line (F3A-GDNF). Note the prominent bundle-like sprouting (arrowheads) and the presence of strongly stained hypertrophic neurons. Note also that the exposure of the photomicrograph in (F) is adjusted to the sprouting and that the cell bodies are overexposed owing to the increase in the levels of TH staining induced by GDNF. The dashed lines delineate the border of the graft (g). Arrowheads point to sprouts entering the graft. Bar, 32 μm.

cRNA probes were labeled with [α-^{32}P]CTP by in vitro transcription. The probe was hybridized to 2–10 μg of RNA extracted from different brain regions or from cell lines in vivo or in culture (n = 2–3). Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing conditions, followed by film autoradiography, as described (Trupp et al., 1995).

GDNF Cell Line

A rat *GDNF* cDNA was cloned into the PstI site of the plasmid MRE-(4x)-OVEC (Westin et al., 1987). This plasmid was linearized and cotransfected with pBMN-Neo^r and pCH110-lacZ (Pharmacia) plasmids (in a molar ratio of 10:1:1) into Fischer 344 rat 3T3 fibroblasts, using the calcium phosphate/glycerol technique. Transfected cells were selected with the neomycin analog G-418, isolated, amplified and subsequently analyzed. Fischer rat 3T3 cell lines, F3A-MT, F3A-GDNF, and F3A-NT3, were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 1 mg/ml penicillin–streptomycin, 1 mg/ml glutamine, and 200 μg/ml G-418.

Surgery

Male Fischer 344 rats (200–300 g), housed and treated according to institutional guidelines, were anesthesized with thiobarbital. Cultured fibroblasts were collected with Cell Lifters (Costar), washed twice, counted, and resuspended in Dulbecoo's modified Eagle's medium without fetal calf serum. Subsequently, 0.75 × 10^6 cells in 3 μl of medium were sterotaxically injected dorsolateral to the LC at the following coordinates relative to lambda and the dural surface: AP = −3.1 mm, L = +1.5 mm, DV = −6mm, with the incisor bar set at −10 mm. The cells typically formed a small focal tumor of about 0.5 × 1 mm along the final tract of the injection canula, which was located not further than 100 μm lateral from the caudal end of the LC. The location chosen for the injections preserved the integrity of the nucleus and allowed the neurons to access the proteins secreted by the cell lines.

One day after the implant, some of the rats were reanesthetized, treated with 4 mg/kg of amfonelic acid (intraperitoneally), and sterotaxically injected with 8 μg of 6-OHDA (Sigma) in the dorsal bundle, 100 μm lateral and inferior to the rostral end of the locus, at the following coordinates: AP = 1.5 mm, L = 1.2 mm, DV = −7.1 mm relative to lambda and the dural surface, with the incisor bar set at 5 mm over 0.

Histology

Seven days after surgery, rats were perfused and the brains were processed for TH immunohistochemistry or cresyl violet staining, as described (Arenas and Persson, 1994). In brief, frontal serial cryostat sections (14 μm thick) were obtained covering the entire LC. Sections every 98 μm were processed for TH immunohistochemistry and cresyl violet staining. Only brains that showed a correct position of implant and lesion sites were included in the study. Neurons through all the dorsal LC showing a clearly TH-positive fusiform or multipolar cytoplasm around a nonstained nuclei were counted as positive. In cresyl violet–stained sections, neurons showing a nucleus with a clear nucleoplasm and prominent nucleolus and a stained fusiform or multipolar cytoplasm were counted as positive. Counts were performed in blind duplicate or triplicate determinations using a Nikon microphot-FXA microscope (100 × magnification). Three to four animals receiving graft alone and six to seven animals receiving lesion alone or lesion plus graft were analyzed. Positive neuron counts were taken for statistical analysis and were not corrected for split nucleoli.

The intensity of TH staining was measured with the spot photometer of the microscope from randomly chosen TH-positive stained cytoplasms in the LC. Only measures of neurons that lay in the linear range of detection were used. About 200 neurons were measured for every experimental condition, with the exception of animals receiving 6-OHDA and the F3A-NT3 cell line (100 neurons); 4 cells were measured per section (100 × magnification) in 6–8 serial sections per brain at the level of the graft, in 3–8 animals per condition. The background level of fluorescence, measured lateral to the LC, was subtracted from the values obtained in the cytoplasms of TH-positive neurons at every level.

The cytoplasmic area of TH-positive neurons was measured using a Quantimet 570 analysis system and a JVC KY F30 video camera, coupled to a Nikon FXA microscope at 100 × magnification. The level of the cutoff was set for every neuron to fit its size precisely. This setting usually varied very few units from the experimental neurons in the ipsilateral side to the control neurons in the contralateral side. About 100 clearly defined TH-positive somas in the LC were randomly selected. We measured 5 neurons on each side per level, from serial sections at 6–8 different levels per animal through the posterior and medial LC, in 3–4 animals per experimental condition.

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