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Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult

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ABSTRACT **The recently cloned, distant member of the transforming growth factor β (TGF-β) family, glial cell line-derived neurotrophic factor (GDNF), has potent trophic actions on fetal mesencephalic dopamine neurons. GDNF also has protective and restorative activity on adult mesencephalic dopaminergic neurons and potently protects motoneurons from axotomy-induced cell death. However, evidence for a role for endogenous GDNF as a target-derived trophic factor in adult midbrain dopaminergic circuits requires documentation of specific transport from the sites of synthesis in the target areas to the nerve cell bodies themselves. Here, we demonstrate that GDNF is retrogradely transported by mesencephalic dopamine neurons of the nigrostriatal pathway. The pattern of retrograde transport following intrastriatal injections indicates that there may be subpopulations of neurons that are GDNF responsive. Retrograde axonal transport of biologically active ^{125}I-labeled GDNF was inhibited by an excess of unlabeled GDNF but not by an excess of cytochrome *c*. Specificity was further documented by demonstrating that another TGF-β family member, TGF-β1, did not appear to affect retrograde transport. Retrograde transport was also demonstrated by immunohistochemistry by using intrastriatal injections of unlabeled GDNF. GDNF immunoreactivity was found specifically in dopamine nerve cell bodies of the substantia nigra pars compacta distributed in granules in the soma and proximal dendrites. Our data implicate a specific receptor-mediated uptake mechanism operating in the adult. Taken together, the present findings suggest that GDNF acts endogenously as a target-derived physiological survival/maintenance factor for dopaminergic neurons.**

Glial cell line-derived neurotrophic factor (GDNF) was initially cloned from a glial cell line and shown to exert trophic effects on fetal mesencephalic dopamine neurons in culture (1). We have subsequently shown that it stimulates transplants of fetal mesencephalic dopamine neurons, resulting in larger grafts, larger numbers of dopamine nerve cell bodies, and an increased amount of tyrosine hydroxylase (TH)-positive nerve fibers (2). Recently, *in vivo* effects of GDNF have been demonstrated in the 6-hydroxydopamine (6-OHDA)-lesioned (3), the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned (4), and the mechanically lesioned (5) nigrostriatal system in rodents.

It is apparent from both *in situ* hybridization data (2, 6) and reverse transcription–PCR detection of GDNF mRNA (7–9) that GDNF is widely distributed in the central nervous system and should have effects in several systems other than the nigrostriatal dopamine system. Indeed, motoneurons appear to be another GDNF-sensitive target (refs. 10–13; K. Trok, B.J.H., and L.O., unpublished data).

In situ hybridization has revealed expression of GDNF mRNA in the developing striatum, consistent with a role for GDNF during development of the nigrostriatal dopamine system (2). Induction of limbic motor status epilepticus by pilocarpine leads to the appearance of GDNF mRNA signals in a subset of striatal neurons (15), showing that the adult striatum is also capable of GDNF production. Pilocarpine (15), as well as pentylenetetrazol-induced excitation (16), also leads to up-regulation of GDNF mRNA in hippocampus and cortex.

Demonstration of specific retrograde transport from dopaminergic targets to nigral neurons is critical to establishing a role for endogenous GDNF as a target-derived trophic factor in maintenance of midbrain dopamine circuits. We have injected ^{125}I-labeled or unlabeled GDNF into the striatum to study the possible uptake and retrograde transport of GDNF in the dopaminergic system. We now report neuron-specific retrograde transport all the way to nigral TH-positive cell bodies and dendrites.

MATERIALS AND METHODS

Preparation and Bioactivity of ^{125}I-Labeled GDNF (^{125}I-GDNF). GDNF protein (68 μg) in 30 μl of phosphate-buffered saline (PBS; 0.1 M sodium phosphate/0.14 M NaCl/2.6 mM KCl, pH 7.5) was placed in the dried 4 mCi (1 Ci = 37 GBq) of ^{125}I-labeled Bolton–Hunter reagent, and the reaction mixture was incubated at 4°C. After 1 h, 20 μl of PBS was added, and the incubation was continued for another 10 h at 4°C. A gel filtration system (PD10; Pharmacia), which had been preequilibrated with PBS containing 0.1% gelatin, was used to separate ^{125}I-GDNF from the glycine conjugate and hydrolysis products. The ^{125}I-GDNF was eluted with PBS containing 0.1% gelatin, and peak fractions were pooled. Thus prepared, ^{125}I-GDNF has a specific activity of 161 μCi/nmol. Labeled GDNF was bioassayed for stimulation of neurite outgrowth from explanted E9 chicken sympathetic ganglia (17) cultured in collagen gels (18). Iodinated and unlabeled GDNF was added in different concentrations (0–200 ng/ml). Ganglia were scored for neurite outgrowth 24 h later by using a scale from 0 to 10, where 0 = no neurite formation and 10 = maximal neurite outgrowth.

^{125}I-GDNF Injections. Adult (150–200 g) outbred Sprague–Dawley rats (B & K International, Sollentuna, Sweden) were housed three per cage, given food and water ad libitum, and kept on a 12:12-h light:dark cycle. Animals were anesthetized with halothane and solutions of ^{125}I-GDNF, unlabeled GDNF, or other proteins were injected stereotaxically in a volume of

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Abbreviations: GDNF, glial cell line-derived neurotrophic factor; TGF-β, transforming growth factor β; TH, tyrosine hydroxylase; NGF, nerve growth factor.

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4 μl into the right striatum (coordinates from bregma in mm: AP, 0.5; ML, 3.0; and DV 5.2) with a Hamilton syringe (1 μl/min). Animals were killed 18–20 h later by transcardial perfusion under deep barbiturate anesthesia with heparinized saline (50 ml) followed by buffered 4% (wt/vol) paraformaldehyde solution (400 ml). Portions of the central nervous tissue were used for γ counting; the remainder was sectioned for autoradiography.

Recombinant GDNF. Human recombinant GDNF was produced by expressing the gene in *Escherichia coli*. Purified protein was renatured to restore biological activity. The production and purification of recombinant protein have been described (1).

Emulsion Autoradiography. Coronal cryostat sections (10 μm) of substantia nigra were mounted on gelatin-treated slides. Sections were dehydrated, dried, dipped in emulsion (Kodak NTB-2), and exposed for 5–6 weeks. Slides were subsequently developed (Kodak D19 developer) and counterstained with thionin blue.

Immunohistochemistry. Animals were killed by transcardial perfusion of fixative [4% (vol/vol) formalin/0.4% picric acid in PBS] under deep barbiturate anesthesia 6, 18, or 24 h and 3 days after injection of either 10 μg GDNF (n = 6) or cytochrome c (n = 2) into striatum. Brains were dissected, postfixed for 1.5–2 h in the same fixative, and rinsed thoroughly in 10% sucrose in PBS. Tissue pieces were then frozen, and 14-μm cryostat sections were treated with primary rabbit polyclonal antibodies against GDNF. These antibodies have been shown to function in ELISAs (1). Following incubation with primary serum diluted 1:1000 in PBS containing 0.3% Triton X-100 for 48 h at 4°C, sections were rinsed three times for 10 min and incubated with secondary goat anti-rabbit fluorescein isothiocyanate-labeled antibodies (Boehringer Mannheim). The sections were then rinsed and mounted in 90% glycerol in PBS containing 0.1% p-phenylenediamine to diminish fading. To further document the identity of GDNF-positive cell bodies, selected sections were double-labeled by using a monoclonal antibody raised against TH (Boehringer Mannheim) and visualized by using Cy-3-labeled anti-mouse IgG secondary antibodies (The Jackson Laboratory). Positive controls included studying immunoreactivity at the injection site; negative controls included studies of immunoreactivity following injection of vehicle or omitting the primary antibody. Slides were analyzed by epifluorescence microscopy (Nikon Microphot-FXA).

RESULTS

Bioactivity and Stability of ^{125}I-GDNF. ^{125}I-GDNF and the nonradioactive parent GDNF protein were compared for biological activity by using the chick embryo sympathetic ganglion bioassay. Both ^{125}I-GDNF and the parent compound elicited a dose-dependent neurite outgrowth from ganglia, while there was no neurite formation in control cultures without addition of GDNF. As illustrated in Fig. 1, iodination did not impair the bioactivity of GDNF. Mean neurite outgrowth scores were identical for ^{125}I-GDNF and unlabeled GDNF over a concentration range from 12 to 200 ng/ml.

^{125}I-GDNF was also stable over time. Retests of two different batches carried out 2 weeks or 2.5 months and 1 month after iodination, respectively, demonstrated unchanged bioactivity. Thus, it appears that iodination does not change the stability of the molecule and that the current labeling procedure has not caused any significant radiolysis of GDNF.

Retrograde Transport of ^{125}I-GDNF in the Nigrostriatal System. Following stereotaxic injection of 25 or 100 ng of ^{125}I-GDNF in 4 μl into the center of the right striatum, autoradiography revealed labeling precisely of the ipsilateral substantia nigra pars compacta area 20–24 h later. As illustrated in Fig. 2 *A* and *D*, we did not detect any radioactivity in any other area of the brain visible in the same sections as substantia nigra. Although the γ emission from ^{125}I limits the autoradiographic resolution, detailed studies of the labeled area strongly suggest that the label is present exclusively in the dopamine nerve cell bodies (Fig. 2*C*).

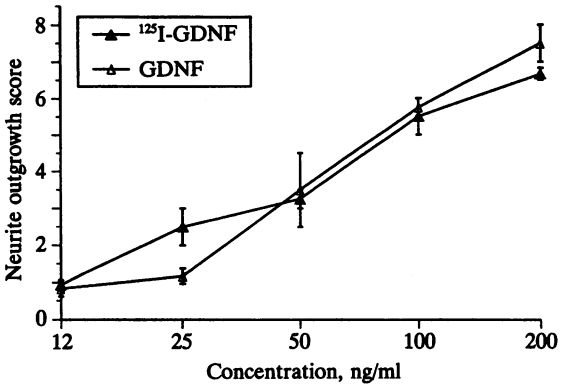

FIG. 1. Bioassay of ^{125}I-GDNF and unlabeled GDNF. Recombinant human ^{125}I-GDNF and unlabeled GDNF are equally potent in promoting neurite outgrowth from E9 chick sympathetic ganglia explants. Neurite outgrowth scores of E9 chick sympathetic ganglia are shown on the ordinate.

Retrograde transport of GDNF to the substantia nigra area was also quantified. As shown in Fig. 3, significant amounts of radioactivity were only found in the ipsilateral substantia nigra area when ^{125}I-GDNF or ^{125}I-GDNF and cytochrome c had been injected into striatum. Radioactivity in the contralateral substantia nigra area was at background levels.

Specificity of Retrograde Transport. Several protocols were undertaken to investigate the degree of specificity of the observed transport of ^{125}I-GDNF. Most important, we found no detectable radioactivity in the ipsilateral substantia nigra if the same amount of ^{125}I-GDNF was delivered together with an excess of unlabeled GDNF (Figs. 2*B* and 3). To control for a possible general effect of an excess amount of protein, ^{125}I-GDNF was also mixed with an excess amount of cytochrome c. In contrast to unlabeled GDNF, the presence of cytochrome c did not significantly change retrograde transport of ^{125}I-GDNF (Figs. 2*D* and 3). There was, however, a tendency toward increased radioactivity in the nigra area when cytochrome c was added, possibly indicating that an excess amount of protein decreased non-specific binding of the ^{125}I-GDNF to injection devices or tissues.

An excess amount of another member of the transforming growth factor β (TGF-β) family, TGF-β1 (2.5 μg, 100-fold excess, 3 animals), had no effect on retrograde transport of ^{125}I-GDNF, strongly suggesting that the two compounds were not competing for the same receptor. As a further control, we also found that an excess amount of nerve growth factor (NGF) did not influence retrograde transport of ^{125}I-GDNF.

Retrograde Transport of Unlabeled GDNF. To provide a second and independent demonstration of retrograde transport and to obtain better cellular resolution of transport, we also injected unlabeled human recombinant GDNF into striatum and subsequently localized the molecule by using immunohistochemistry. Injection of 10 μg of GDNF in 2 μl was readily detected in striatum 6 and 18 h later (Fig. 4 *A* and *B*). After 18 h, GDNF immunoreactivity was found to have spread to most of the ipsilateral striatum, while the fluorescence intensity was somewhat lower than at 6 h. Twenty-four hours after GDNF injection, the distribution and fluorescence intensity of striatal GDNF immunoreactivity was similar to that seen after 18 h. GDNF immunoreactivity was still present in low amounts in striatum after 3 days. Immunoreactivity was found as a diffuse staining of striatal neuropil and, in addition, as a strong and distinct staining of a subpopulation of nerve

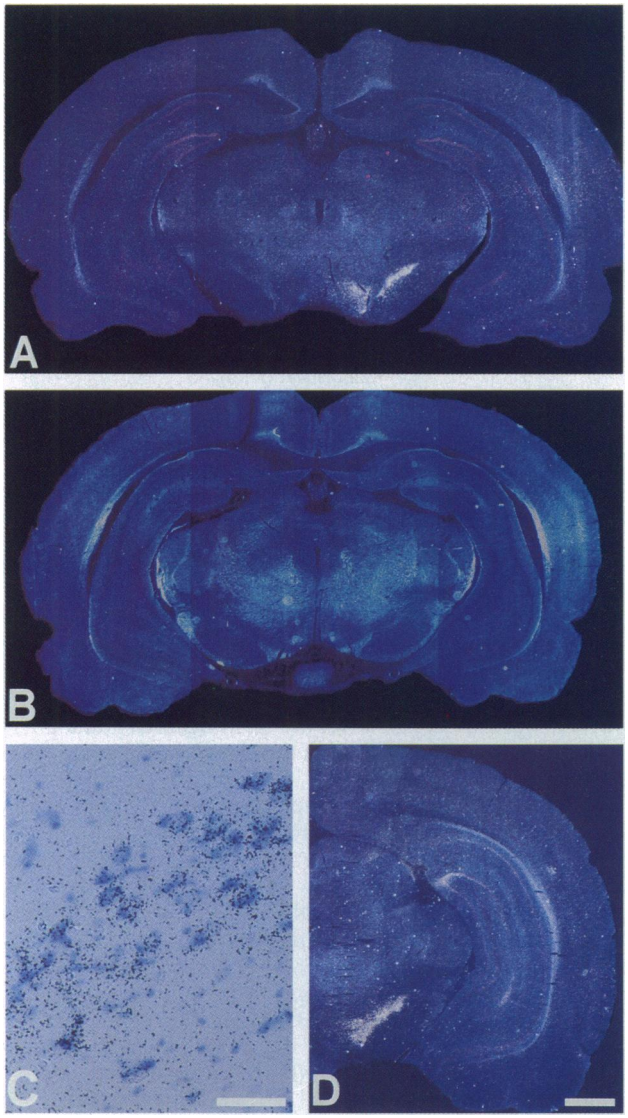

FIG. 2. Retrograde transport of ^{125}I-GDNF from striatum to substantia nigra in the adult rat. One hundred nanograms of ^{125}I-GDNF was injected into the right striatum, and animals were killed 24 h later. Emulsion-coated sections (10 μm) were exposed for 5.5 weeks. Coronal sections of adult rat brain revealing retrograde transport of ^{125}I-GDNF from striatum to the midbrain dopaminergic neurons of the ipsilateral (right) substantia nigra. (*A*) Following injection of ^{125}I-GDNF into striatum, a strong radioactive signal is found over the ipsilateral substantia nigra area. (*B*) When the ^{125}I-GDNF is mixed with a 100-fold excess of unlabeled GDNF, no radioactivity is found in the area of the substantia nigra. (*C*) Close-up of labeled substantia nigra area illustrated in *A*. Silver grains cluster over nerve cell bodies. (*D*) When the ^{125}I-GDNF is mixed with a 100-fold excess of cytochrome *c*, a similar amount of radioactivity is found in the ipsilateral substantia nigra area, as in *A*. [Scale bars: *A*, *B*, and *D* (bar in *D*), 1 mm; *C*, 100 μm.]

fibers in the striatal fiber bundles at 6, 18, and 24 h (Fig. 4*A*, *Inset*).

Immunohistochemistry also revealed GDNF-immunoreactive neuronal cell bodies in the ipsilateral substantia nigra pars compacta. Such cell bodies were more frequently seen and more strongly fluorescent at 6 h than at either 18 or 24 h after injection. Immunoreactivity was confined to granules in the perinuclear cytoplasm, as well as in proximal dendrites (Fig. 4). Three days after injection, GDNF-immunoreactive cells were no longer seen. Injections of similar amounts of cytochrome *c* did not cause any GDNF-like immunoreactivity.

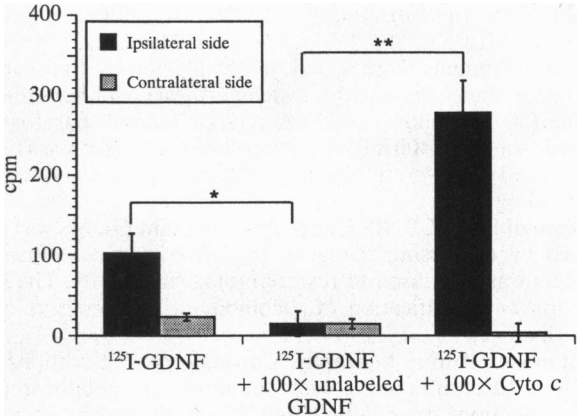

FIG. 3. Quantification and specificity of ^{125}I-GDNF retrograde transport in the adult nigrostriatal system. Four microliters of ^{125}I-GDNF was injected into the right striatum with PBS or (in a competitive manner) with a 100-fold excess of either unlabeled GDNF or cytochrome *c* (Cyto *c*). After 18 h, animals were killed, tissues were dissected, and radioactivity was counted with a γ counter. Results are expressed as mean ± SD of 8, 3, and 2 animals, respectively. *, $P < 0.05$; **$P < 0.07$ (analysis of variance using Scheffe's correction). Similar data were obtained in two other independent experiments.

TH and GDNF Double Labeling. The use of polyclonal antibodies against GDNF and monoclonal antibodies against TH enabled us to carry out co-localization studies. We found that all cell bodies in the substantia nigra area that were GDNF immunoreactive were also TH immunoreactive. Additionally, there were many TH-immunoreactive neurons in which we could not detect GDNF immunoreactivity (Fig. 4 *C* and *E*). There were no striking differences in either morphology or intensity of TH immunoreactivity between GDNF-positive and GDNF-negative TH-positive neurons.

DISCUSSION

The present experiments used two independent methods, based on autoradiographic or γ counting detection of ^{125}I-GDNF and immunohistochemical detection of unlabeled GDNF, respectively, to demonstrate specific retrograde transport of GDNF from striatal dopamine nerve terminals to cell bodies in substantia nigra. The fact that ^{125}I-GDNF, with a biological activity identical to unlabeled GDNF in the ganglion assay (17), is transported in the presence of a 100-fold excess of cytochrome *c*, NGF, or TGF-β1 but not in the presence of a similar excess of unlabeled GDNF suggests that transport must be preceded by binding to specific receptors and that these receptors are not common to TGF-β1. The presence of GDNF binding, uptake, and transport mechanisms suggests that the adult nigrostriatal dopamine system is continuously supported by low levels of GDNF generated by the striatal target tissue. This postulate is also in line with recent findings of positive effects of GDNF on both lesioned (3–5, 19) and intact (20) rodent dopamine neurons. The hypothesis is also supported by the presence of GDNF mRNA as determined by reverse transcription–PCR in adult striatum (9).

Injections of unlabeled GDNF followed by immunohistochemical detection provided a second independent demonstration of retrograde transport. Additionally, this method permitted cellular localization of the transported protein and demonstrated its presence in granules throughout nigral neural cytoplasm and proximal dendrites. This distribution of a neurotrophic factor following retrograde transport is very similar to the distribution of brain-derived neurotrophic factor (BDNF) immunoreactivity in cholinergic neurons of the medial septum and diagonal band, also believed to be the result of retrograde transport from target areas (21). The localization

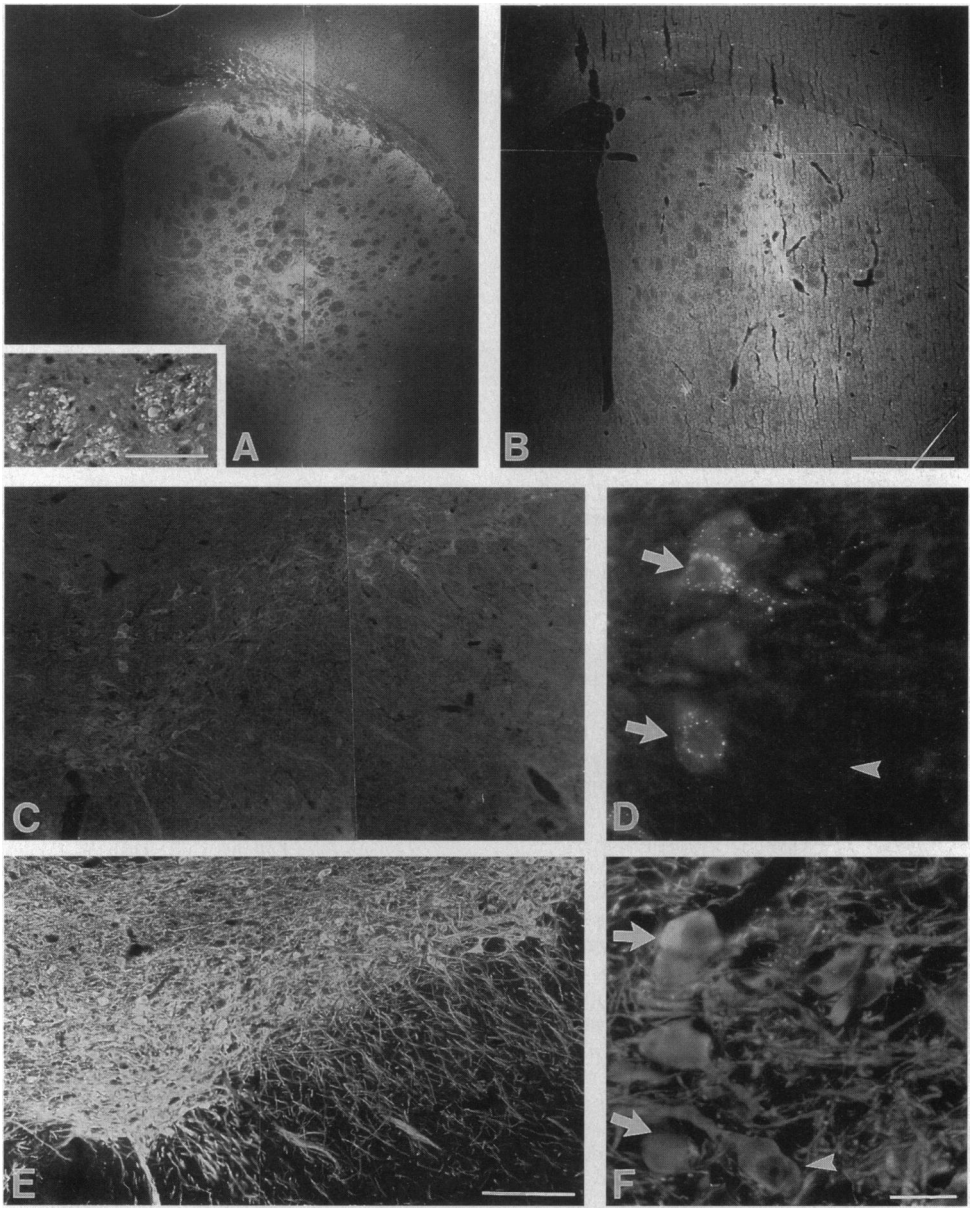

FIG. 4. Immunohistochemical localization of GDNF in striatum and in mesencephalic dopamine neurons following injection of GDNF into striatum. (*A*) Strong GDNF-like immunofluorescence is found in striatum 6 h after injection. (*Inset*) In addition to a diffuse fluorescence in the neuropil, selected axons in the axon fiber bundles are also fluorescent. (*B*) GDNF immunoreactivity is found in practically the entire body of the striatum 18 h after injection into striatum. The fluorescence intensity is lower than at 6 h. (*C–E*) Substantia nigra area 6 h after a striatal injection of GDNF. (*C* and *E*) Section double-labeled for GDNF (*C*) and TH (*E*). Many nerve cell bodies are GDNF positive in *C*. These cells constitute a subpopulation of the TH-positive cells shown in *E*. (*D* and *F*) Close-up of the double-labeled section illustrated in *C* and *E*. (*D*) GDNF immunofluorescence in several cells (from an area in the left half of figure *C*). Note that immunoreactivity is punctate in cell bodies and proximal processes. (*F*) TH immunoreactivity in the same area as shown in *D*. By comparing *D* and *F*, it is evident that all GDNF-positive cells (two of them indicated by arrows in *D*) are also TH positive (arrows in *F*). There are also GDNF-negative cells (arrowhead in *D*) that are TH positive (arrowhead in *F*). [Scale bars: *Inset* in *A*, 25 μm; *A* and *B*, 500 μm (bar in *B*); *C* and *E*, 100 μm (bar in *E*); *D* and *F*, 25 μm (bar in *F*).]

is different from the distribution of BDNF in BDNF-synthesizing neurons, in which case immunoreactivity is found evenly distributed in the cytoplasm (21).

Although the immunohistochemical data suggested diffuse and widespread distribution of GDNF injected into striatum, only a subset of the substantia nigra neurons became immunoreactive. We estimate the number of labeled neurons to be less than 50%. While there are several possible explanations for this finding, one alternative is that only a subset of nigrostriatal dopamine neurons is able to take up and transport GDNF. If this is the case, we can speculate that striatum synthesizes additional dopaminotrophic factors, perhaps related to GDNF, to support those neurons which remain unlabeled in the present retrograde tracing experiments. Receptors for such a factor may not bind and internalize GDNF.

In situ hybridization data suggest that GDNF mRNA is preferentially found in the patches and marginal zone of the developing striatum (A.T., E. Lindqvist, C. Humpel, I. Strömberg, B.J.H., and L.O., unpublished data). Thus, one possibility is that GDNF preferentially supports the dopamine neurons projecting to patches and the marginal zone. Neurons projecting to the patches are calcium-binding-protein-negative and located in the ventral aspects of pars compacta (22). Recent studies involving intraocular transplantation of fetal ventral mesencephalic tissue and GDNF treatments have also suggested preferential effects on calcium-binding-protein-negative

dopamine neurons (23), further supporting the possibility that GDNF might act specifically on the subpopulation of ventral mesencephalic neurons that project to the patches and marginal zone. Developmentally, these areas are the first to become innervated by dopamine nerve terminals (24, 25). This structural subdivision of striatum persists in the adult in the form of striosomes (26–29).

It remains to be determined which cells in striatum normally synthesize GDNF. Although it appears as if a subset of neurons can make GDNF following pilocarpine-induced status epilepticus (15), a neuronal localization of synthesis could not be determined in the developing striatum (2). However, GDNF was cloned from a glial cell line (1), and it has been demonstrated that striosomes contain specific subpopulations of glial cells characterized by their content of 5′-nucleotidase (30). It is therefore possible that such a subset of glial cells synthesize GDNF to attract and maintain GDNF-sensitive dopaminergic nerve terminals.

Taken together, our results have further established a role for GDNF as an endogenous trophic factor for nigrostriatal dopamine neurons by showing specific transport from nerve terminals and accumulation in nerve cell bodies. Studies with GDNF in experimental models of Parkinson disease (3–5, 19) suggest that GDNF might be beneficial as both a protective and a reparative treatment (4). In animal experiments, injections of GDNF have been carried out both into the substantia nigra and into the striatum (3, 4). In patients, local treatment in the substantia nigra area would appear more difficult than infusion of a trophic factor into striatum. However, as suggested by the present experiments, an intrastriatal injection might lead to retrograde transport of GDNF in humans and, thus, be able to exert a trophic influence at multiple levels of the dopamine pathway from a single site of delivery. We have previously demonstrated that another trophic factor, NGF, can be safely delivered into the human striatum of patients with Parkinson disease to support intraputaminal adrenal medullary autografts (14, 31). Further experimental studies documenting specificity and duration of effects, as well as possible negative side effects or toxic actions of GDNF, are needed before clinical trials of GDNF treatment in Parkinson disease should be considered.

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