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Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat

(neuroprotection/substantia nigra)

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ABSTRACT **The trophism of brain-derived neurotrophic factor (BDNF) for dopaminergic cells in culture has led to significant interest in the role of BDNF in the etiology and potential treatment of Parkinson disease. Previous *in vivo* investigation of BDNF delivery to axotomized substantia nigra dopaminergic neurons in the adult rat has shown no protective effect. In this study, we produced nigral degeneration by infusing 1-methyl-4-phenylpyridinium (MPP^+), a mitochondrial complex I inhibitor and the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), into the rat striatum. The subsequent loss of nigral neurons was presumably due to mitochondrial toxicity after MPP^+ uptake and retrograde transport to the substantia nigra. We engineered immortalized rat fibroblasts to secrete human BDNF and implanted these cells near the substantia nigra 7 days before striatal MPP^+ infusion. We found that BDNF-secreting fibroblasts markedly increased nigral dopaminergic neuronal survival when compared to control fibroblast implants. The observation that BDNF prevents MPTP-induced dopaminergic neuronal degeneration in the adult brain has significance for the treatment of neurodegenerative disorders, which may involve mitochondrial dysfunction, such as Parkinson disease.**

The range of neuroprotective effects for any putative neurotrophic molecule must be determined in *in vivo* models of neurodegeneration (1–8) before practical application of neurotrophic factor neuroprotection can be contemplated (9). Of particular interest are neurotrophic factors found to protect *in vivo* against neuronal death caused by mechanisms similar to those thought to occur in human neurodegenerative disorders, such as nerve growth factor (NGF) protection against Huntington disease-like lesions of the striatum (7, 8) or ciliary neurotrophic factor protection in a mouse model of motoneuron degeneration (10). Brain-derived neurotrophic factor (BDNF) (11, 12) promotes the survival of fetal mesencephalic dopaminergic cells in culture (13). *In vivo*, BDNF protects cholinergic neurons from degeneration after fimbria–fornix lesions (4) and motoneurons from degeneration after axotomy (5, 6); however, BDNF does not rescue axotomized substantia nigra compacta (SNC) dopaminergic neurons after axotomy (4).

However, the cellular insults that cause some human neurodegenerative diseases may be only partially modeled by axotomy-induced cell death (14–16). Other insults to cellular integrity, such as impaired energy metabolism, may more accurately model the degeneration seen in Huntington disease or Parkinson disease (17–20). Indeed, evidence for abnormalities in mitochondrial function has been found in patients with both of these disorders (21, 22). A well described animal model of Parkinson disease is produced by the systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into mice or primates (23). MPTP, which is metabolized to the mitochondrial complex I inhibitor 1-methyl-4-phenylpyridinium (MPP^+), causes dopaminergic cell death in the SNC, presumably due to mitochondrial impairment (for review, see ref. 24). BDNF has been shown to protect dopaminergic neurons against MPP^+ toxicity in culture (13). In view of the findings of a lack of *in vivo* BDNF dopaminergic protection after axotomy (4), we used a different model of SNC dopaminergic neuronal degeneration based on MPP^+-mediated mitochondrial impairment to test whether BDNF can protect dopaminergic neurons against such insults *in vivo*.

MATERIALS AND METHODS

Genetic Engineering and Selection of a BDNF-Secreting Fibroblast Cell Line. Cells used for implantation were generated by infection of an immortalized rat fibroblast cell line (Rat I, initially provided by M. Rosenberg, University of California, San Diego) with a retrovirus vector carrying a human cDNA encoding a full-length preproBDNF precursor identified by sequence analysis (provided by G. Walz and B. Seed, Massachusetts General Hospital) as described (25) and as shown in Fig. 1*A*. Rat I BDNF subclones were selected under G418 and then screened for their level of BDNF mRNA production by Northern blot analysis and for their level of BDNF secretion as determined by an *in vitro* rat dorsal root ganglion neurite outgrowth bioassay, as described (26, 27).

Coculture of BDNF-Secreting Fibroblasts with Fetal Ventral Mesencephalic Neurons. To fully confirm the secretion of BDNF by the selected putative BDNF-secreting Rat I cell line (Rat I-BDNF no. 7), ventral mesencephalic neurons were dissected and plated on confluent cultures of Rat I-BDNF no. 7 cells as described (13). After 5 days, cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) (PBS) for 15 min and then immunostained for tyrosine hydroxylase (TH) (Fig. 1*B*) exactly as described below for tissue sections. Cells per plate were counted in a uniform square centered in the plate (to avoid edge artifact) of ≈256 mm^2. Plates containing areas of nonconfluence were ex-

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Abbreviations: BDNF, brain-derived neurotrophic factor; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGF, nerve growth factor; SNC, substantia nigra compacta; TH, tyrosine hydroxylase.

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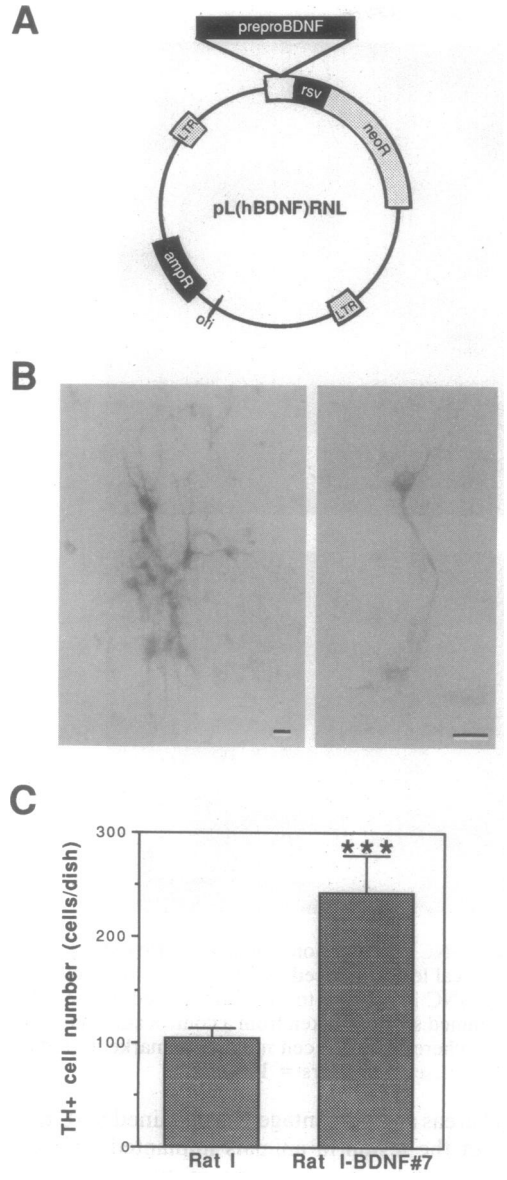

FIG. 1. Engineering of BDNF-secreting fibroblasts for transplantation. (*A*) Schematic of the pL(BDNF)RNL retroviral plasmid used for transfection of the ψ-2 producer cells. PreproBDNF indicates the DNA fragment containing a full-length human BDNF cDNA inserted into the polylinker region of the plasmid; rsv, Rous sarcoma virus internal promoter; neoR, neomycin-resistance gene; LTR, Moloney murine leukemia (5′) or sarcoma (3′) virus long terminal repeat; ampR, ampicillin-resistance gene; ori, *Escherichia coli* origin of replication. Plasmid is not drawn to scale. (*B*) Photomicrographs of coculture of TH-immunopositive fetal ventral mesencephalic neurons with confluent plating of fibroblasts. (*Left*) Low-power view of cluster of TH-immunopositive cells plated on confluent Rat I-BDNF no. 7 cells. (*Right*) Higher-power view of individual TH-immunopositive neuron plated on Rat I-BDNF no. 7 cells. (Bars = 20 μm.) (*C*) Bar graph of effects of coculture with Rat I fibroblasts or Rat I-BDNF no. 7 fibroblasts on TH-immunopositive fetal mesencephalic neurons after 5 days *in vitro*. Error bars represent SD. ***, Significant to $P < 0.001$ by *t* test.

cluded from cell counting analysis. TH-positive cell counts were averaged from three Rat I confluent plates as control and from seven Rat I-BDNF no. 7 confluent plates and are expressed as means ± SD. These two conditions were compared by *t* test to confirm the bioactivity of the secreted BDNF (Fig. 1*C*).

Cell Preparation and Implantation in Substantia Nigra Degeneration Model. Cells were harvested by trypsin treatment and injected as a suspension in PBS with $CaCl_2$ (1.0 mg/liter)/$MgCl_2$ (1.0 mg/liter)/0.1% glucose. Cell count and viability were assessed by trypan blue dye exclusion before intracerebral injection into rat hosts. Rat I cells not altered by retroviral infection were injected into control animals. Under pentobarbital anesthesia (65 mg/kg), eight male Sprague–Dawley rats (300–350 g) were grafted with Rat I fibroblast cells (BDNF[−]) and eight rats were grafted with Rat I-BDNF no. 7 cells (BDNF[+]). Using a Kopf rat stereotactic frame, burr holes for grafting into the dorsal tegmentum of the mesencephalon were made at coordinates calculated from bregma as AP −5.3, L −2.4. Injections were made using a 10-μl Hamilton syringe at V −6.0 calculated from the dura. A total of 2×10^5 cells were infused over 2 min in a vol of 2 μl; the needle was withdrawn after a 1-min pause. Seven days after cell implantation, animals received MPP^+ infusions into the striatum (28). In a manner identical to that described above, burr holes for injection were calculated from bregma and made at AP +1.0, L −2.5; over 1 min, 100 nmol of MPP^+ in 1 μl was infused at V −4.5 calculated from the dura. A single animal in the BDNF[−] group did not survive the second surgical procedure. Animals were perfusion fixed 7 days after lesioning while under deep barbiturate anesthesia. After standard preparation of the brain tissue, serial sections were stained with cresyl violet (Nissl) or immunohistochemically against TH (East Acres Biologicals, Southbridge, MA) at a primary antibody dilution of 1:3000.

Morphometric Procedures. The maximal cross-sectional area of the striatal lesion, as seen by characteristic neuronal loss after cresyl violet staining, was determined for each animal and measured as described (7). SNC neurons were visually counted bilaterally both on Nissl-stained sections and on TH-immunostained sections. Since all SNC cell counts are expressed as a percentage of surviving cells relative to the control cell number on the non-MPP^+-injected side, no correction factors were used. The TH-immunopositive SNC cell counts were independently confirmed by two observers, one of whom was blinded to the treatment regime.

RESULTS

BDNF-Secreting Fibroblasts. Immortalized fibroblasts were engineered to secrete BDNF using techniques previously employed for constructing NGF-secreting fibroblasts (25). As described (26), transgenic BDNF mRNA synthesis was screened by Northern blotting of total RNA from Rat I BDNF cells followed by hybridization to a radiolabeled human preproBDNF antisense cRNA probe. This analysis revealed an mRNA species of similar size to previously described preproNGF-neoR (neomycin resistance) mRNA transcribed in the identical vector backbone (29).

BDNF secretion by the engineered cells was initially screened by testing Rat I BDNF conditioned medium in a neurite outgrowth assay using neonatal rat dorsal root ganglion explants. The presence of BDNF secretion by the highest producing selected subclone was fully confirmed by coculture of Rat I-BDNF no. 7 cells with TH-immunopositive fetal ventral mesencephalic neurons (Fig. 1*B*), a specific property of BDNF bioactivity, as described (13).

Striatal Infusion of MPP^+ and SNC Degeneration. The presence of significant striatal lesions due to infusion of 100 nmol of MPP^+ was confirmed in every animal by characteristic neuronal loss after cresyl violet staining (Fig. 2*B*). The lesion size was quantifiable by selection and measurement of a maximal cross-sectional lesion area for each animal, as has been performed previously for excitotoxic lesions in the striatum (7, 8). This analysis revealed that there was no difference between the size of MPP^+-induced striatal lesions in the BDNF[−] (5.46 ± 2.61 mm^2; mean ± SD) and BDNF[+] (5.57 ± 2.02 mm^2) groups, indicating that the

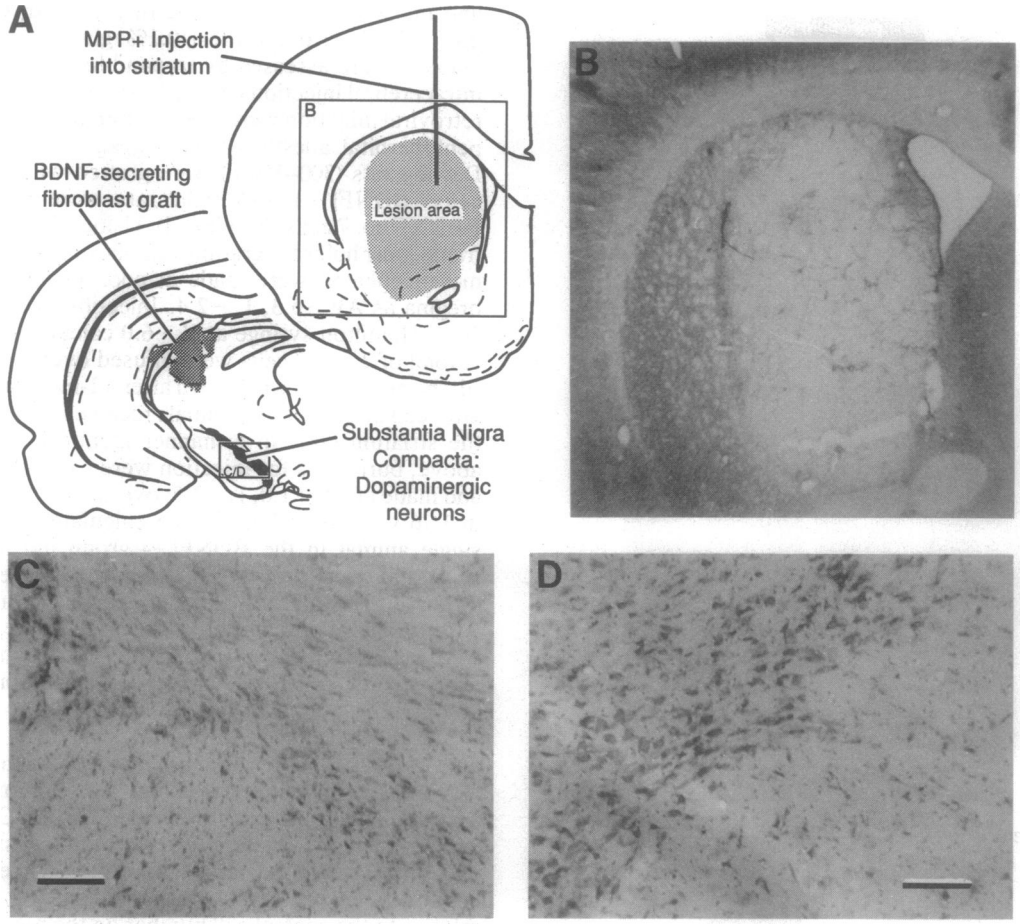

FIG. 2. SNC degeneration after striatal MPP^+ infusion. (*A*) Schematic overview of SNC degeneration model used. Boxed area corresponds to that shown in *B*; a cresyl violet-stained section through the striatum showing a typical lesion induced by MPP^+ infusion. Lower section of *A* shows an example of fibroblast graft placement and highlights the location of the SNC in relation to the graft. Boxed area on this section represents the approximate areas shown in *C* and *D*. (*C* and *D*) Cresyl violet (Nissl)-stained sections taken from a control animal implanted with a nonsecreting (BDNF[−]) graft. (*C*) SNC ipsilateral to the striatal MPP^+ infusion where neuronal cell number is markedly reduced when compared to *D*. (*D*) Nonlesioned contralateral SNC from the same cresyl violet-stained section. (Bars = 100 μm.)

infused MPP^+ had the same potential toxicity in both treatment groups.

Effects of BDNF-Secreting Grafts on SNC Cell Survival. Graft placement was confirmed on Nissl-stained sections, although both the BDNF[−] and BDNF[+] grafts tended to extend out from the injection tract in the mesencephalic tegmentum and into the dorsal and ventral paramesencephalic cistern on the side of the implantation (Fig. 2*A*). For this reason, it was impossible to obtain an accurate estimate of graft size in every animal; however, no obvious differences were noted between the overall amount of surviving BDNF[−] and BDNF[+] fibroblasts at the time of sacrifice. In particular, all animals seemed to have a significant number of implanted fibroblasts either within the mesencephalon or adjacent to the midbrain in the paramesencephalic cistern. This placed grafted cells within 1–2 mm of the SNC on the grafted side in all animals.

Cell counting of Nissl-stained neurons within the lesioned (Fig. 2*C*) and nonlesioned (Fig. 2*D*) SNC of animals grafted with BDNF[−] cells showed a 53% decrease in neurons on the lesioned side, to 47% ± 11% of the nonlesioned side, consistent with previous observations of SNC degeneration in this model (30). There was a significantly greater reduction in TH-immunopositive cells than in Nissl-stained neurons ($P < 0.017$) in the SNC on the side of the striatal lesion in BDNF[−] implanted animals, to 35% ± 15% (Fig. 3). Particularly noteworthy is a very high inverse correlation ($R = 0.921$; $F = 27.957$; $P < 0.003$) between striatal lesion cross-sectional areas and percentage Nissl-stained SNC neurons on the side of the lesion in animals implanted with BDNF[−] grafts. This emphasizes the utility of this model for studying MPP^+-mediated SNC degeneration. The correlation between striatal lesion sizes and TH-immunopositive SNC cells approached significance ($R = 0.709$; $F = 5.053$; $P < 0.075$).

Placement of BDNF[+] grafts near the SNC on the lesioned side resulted in a significant ($P < 0.001$) increase in the percentage of surviving Nissl-stained neurons to 86% ± 18% and rendered the correlation between striatal lesion size and SNC cell survival not significant ($R = 0.394$; $F = 0.919$; $P = 0.382$). The effect of the BDNF-secreting grafts on TH cell survival was also dramatic ($P < 0.001$) in that BDNF[+] grafts promoted the survival of 83% ± 22% of the TH-immunopositive SNC cells on the side of the lesion (Fig. 3*B*). The magnitude of these changes is shown graphically in Fig. 3*D*. As observed for the counts of Nissl-stained neurons, BDNF[+] grafts reduced the inverse correlation between striatal lesion size and TH-immunostained surviving cells to $R = 0.321$ from $R = 0.709$ in the BDNF[−] implanted animals.

DISCUSSION

We have constructed immortalized rat fibroblasts capable of synthesizing and secreting transgenic human BDNF in amounts adequate to support explanted dorsal root ganglion neurite extension and dopaminergic neurons in culture. Im-

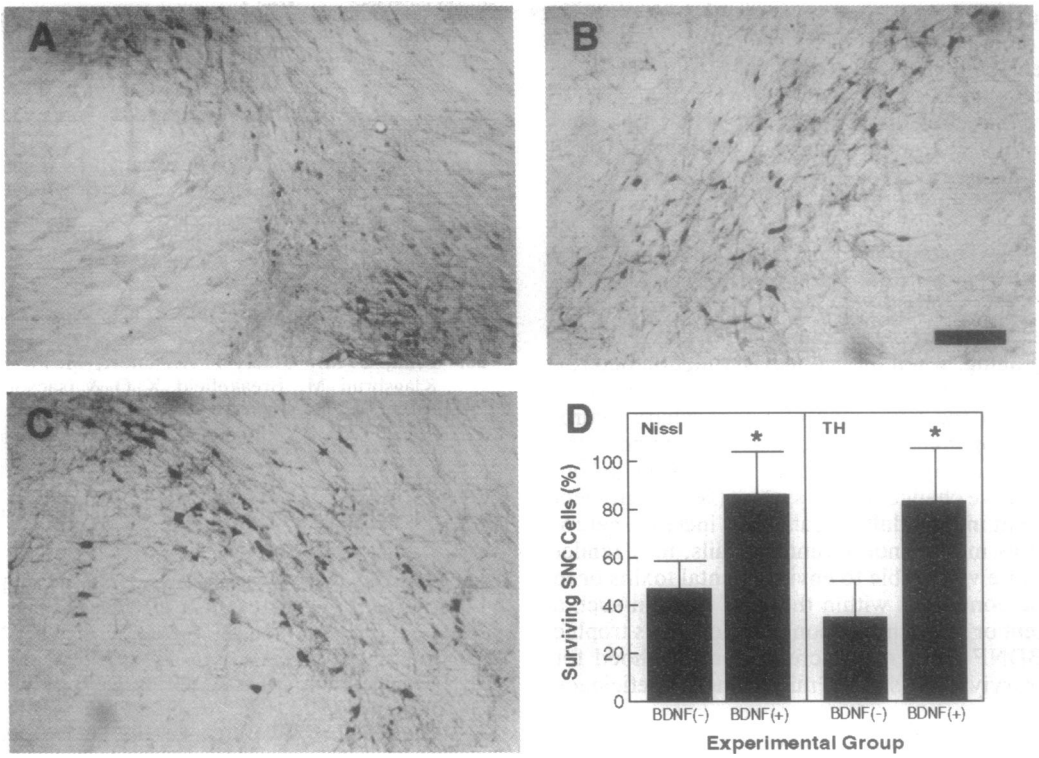

FIG. 3. Effects of BDNF-secreting fibroblast implants on dopaminergic cell degeneration in the SNC. TH-immunostained cells found in the SNC ipsilateral to striatal MPP^+ infusion with a BDNF[−] graft (*A*), contralateral to striatal MPP^+ infusion with a BDNF[+] graft (*B*), and ipsilateral to MPP^+ infusion in a BDNF[+] implanted animal (*C*) are shown. There is a marked reduction in TH-immunopositive cells after MPP^+ infusion in the BDNF[−] implanted animals (*A*) when compared to a section at a similar level in the SNC contralateral to a MPP^+ infusion (*B*). Relative to the BDNF[−] animal (*A*), the number of SNC TH-immunopositive cells spared ipsilateral to the striatal MPP^+ infusion in the BDNF[+] implanted animal (*C*) is markedly increased and seems to approach the corresponding nonlesioned SNC in *B*. *B* and *C* are taken from the same histological section in a BDNF[+] implanted animal so as to facilitate comparison between control SNC and BDNF sparing. (*D*) Graphic representation of the effects of the BDNF[−] and BDNF[+] grafts on cell sparing in the SNC, both for cresyl violet (Nissl)-stained neurons and for TH-immunostained neurons (means ± SD). Cell survival is expressed as percentage of the number of SNC neurons found contralateral to MPP^+ infusion. *, Difference significant to $P < 0.001$. (Bar = 100 μm.) (*A*, *B*, and *C* are the same magnification.)

plantation of these fibroblasts near the substantia nigra in rats 7 days before striatal infusion of MPP^+ significantly protected against subsequent SNC TH-immunopositive neuronal death. Nissl-stained neurons were significantly spared in BDNF[+] animals when compared to BDNF[−] controls.

In addition to a local lesion, infusion of MPP^+ into rat striatum causes a profound loss of dopaminergic SNC neurons (28, 30). This is presumably due to the uptake of MPP^+ by dopaminergic terminals in the striatum and retrograde transport to the SNC (31). In the present study, the loss of substantia nigra neurons was highly correlated with striatal lesion size. This correlation between striatal lesion size and SNC cell loss implies a similar cause for both striatal and SNC neuronal death. Since other insults that cause massive striatal excitotoxic lesions, such as toxic levels of *N*-methyl-D-aspartate receptor agonists, do not cause appreciable SNC cell loss (32, 33), the cause of the SNC degeneration would likely be direct MPP^+ effects on SNC neurons, not an indirect effect from the striatal neuronal death. The neuronal death in the substantia nigra is likely caused by mitochondrial blockade after retrograde transport of MPP^+ from striatum to SNC and therefore acutely models the dopaminergic cell loss seen in Parkinson disease. Interestingly, patients with Parkinson disease can manifest mitochondrial defects (22, 34) or, more specifically, a deficiency in mitochondrial complex I activity (35, 36).

The protective effects of BDNF on survival of dopaminergic neurons in this model probably reflect a trophic influence on neuronal function, perhaps through increased mitochondrial efficiency or by induction of cellular protective mechanisms. Mechanisms of neurotrophin-mediated neuroprotection in the adult brain are poorly understood. NGF, the most extensively investigated neurotrophin, is known to modulate a number of intracellular events, including changes in intracellular Ca^{2+} stores (37), Na^+/K^+-ATPase activity (38), Cl^- flux (39), and induction of the peroxidative enzyme catalase, both in culture (40) and *in vivo* (41). BDNF protects cultured neuroblastoma cells against MPP^+ and 6-hydroxydopamine toxicity and induces a 100% increase in the activity of glutathione reductase, a protective enzyme (42). However, BDNF had no effect on catalase activity in that system.

NGF and BDNF share significant homology (43); both are able to bind to a low-affinity neurotrophin receptor (44), and both bind with high affinity to specific members of the trk family of receptors (45–47). It seems possible that BDNF may also mediate intracellular changes similar to those described for NGF. There also exists a level of specificity in neuronal populations for neurotrophin activity; for example, NGF does not protect cultured dopaminergic neurons against MPP^+ toxicity (13). Basic fibroblast growth factor (bFGF), on the other hand, does have some protective effects against MPTP toxicity in the mouse (48) but does not share homology with BDNF. The suggestion has been made that bFGF may cause glial cells to secrete neurotrophic factors, which in turn affect neuronal protection (49). These observations imply either multiple, complementary protective pathways for neurotrophic support or a final common pathway, which can be activated through a number of receptors.

In vivo, BDNF protects against cholinergic neuronal degeneration after fibria–fornix lesioning (4) and ameliorates

motoneuron death after axotomy (5, 6). However, previous investigations of *in vivo* effects of BDNF failed to show any protective effects on SNC neurons after medial forebrain bundle transection (13). Our paradigm investigated BDNF amelioration of mitochondrial impairment in neurons, perhaps a more limited neurodegenerative insult than proximal axotomy. The cellular response to axotomy is complex (14–16) and may not model the neuronal loss seen in neurodegenerative conditions.

Our observations that BDNF can protect dopaminergic neurons in the SNC against mitochondrial blockade indicate that adult SNC neurons still respond to neurotrophic input and perhaps require neurotrophic support for survival, particularly during aging, when mitochondrial function deteriorates (20). A recent report made available to us after this work was completed, on the protective effect of ciliary neurotrophic factor on dopaminergic neurons *in vivo*, supports the idea of neurotrophic input as a means to prevent lesion-induced degenerative changes in this system (50). Insufficient neurotrophic input in the adult, or failure to increase neurotrophic support as mitochondrial function fails, may render SNC neurons more vulnerable to environmental toxins or to potentially toxic conditions within the SNC (51). In such a case, replacement or supplementation of endogenous trophic support with BDNF input may increase the likelihood for SNC neuronal survival in a state of mitochondrial deficiency or oxidative stress.

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