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Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons *in vivo*

(neuronal death/axotomy/trophic factor/tyrosine hydroxylase/Parkinson disease)

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ABSTRACT **We have investigated the neuroprotective effects of recombinant human ciliary neurotrophic factor (CNTF) for injured dopaminergic neurons of the adult rat substantia nigra compacta. Fourteen days after a unilateral transection of the nigrostriatal pathway two-thirds of the neurons (identified by retrograde labeling) had degenerated. In sharp contrast, 73% (a few cases, >90%) of this cell loss was prevented by continuous infusion of CNTF close to the injured neurons. However, CNTF did not prevent the disappearance of the transmitter-synthesizing enzyme tyrosine hydroxylase. Thus, CNTF has potent neurotrophic effects for injured adult rat dopaminergic substantia nigra neurons, whose degeneration plays a major causative role in Parkinson disease.**

During development of the peripheral nervous system, neurons become dependent for their survival and/or function on neurotrophic factors, specialized proteins that can be supplied by the innervation territory (1, 2). The importance of such factors for the maintenance of mature as well as injured adult neurons of the central nervous system (CNS) has also been recognized, in particular the relationship between nerve growth factor (NGF) and cholinergic basal forebrain neurons (3, 4). Injury of the CNS often causes degeneration of the axotomized neurons, resulting in their atrophy or death. It is still unresolved whether such degenerative changes are caused primarily by the interruption of target-derived trophic support or the injury itself (5), but they can largely be prevented by administration (substitution) of exogenous trophic factors (3, 4).

Another type of trophic factor, named ciliary neurotrophic factor (CNTF), was shown *in vitro* to have several biological activities for neurons and glial cells, including promotion of survival by several types of developing peripheral nervous system and CNS neurons (6–11). CNTF has recently been purified, cloned, and sequenced and the human gene has been expressed in bacteria and insect cells to produce recombinant protein in large enough quantities for *in vivo* investigations (12–17). On the basis of its amino acid sequence, predicted tertiary structure, and type of receptors, CNTF is now viewed as a member of the α-helical cytokine superfamily, which includes leukemia inhibitory factor, interleukin 6, granulocyte colony-stimulating factor, and oncostatin M (18, 19). *In vivo*, purified CNTF can protect spinal motor neurons of developing chickens against developmental neuronal death (20, 21) and facial motor neurons of the neonatal rat after their axotomy (22). We have recently shown in the adult rat *in vivo* that CNTF can almost completely prevent the axotomy-induced degeneration of cholinergic as well as noncholinergic medial septum neurons (23). However, although CNTF prevented reduction of cell body size and loss of the low-affinity NGF receptors in these cholinergic neurons, no protective effect was seen for their transmitter enzyme, choline acetyltransferase (ChAT). In the present study, we investigated in adult rats whether CNTF could also have protective effects for injured and degenerating dopaminergic neurons of the substantia nigra compacta.

METHODS

Nigrostriatal Transection. Female Sprague–Dawley rats (200 g; Bantin & Kingman, Fremont, CA; housed and cared for according to National Institutes of Health and U.S. Department of Agriculture guidelines) (n = 30) were anesthetized with a mixture of ketamine (62.5 mg/kg), xylazine (3.25 mg/kg), and acepromazine (0.62 mg/kg) and received a complete, but discrete, transection of their right nigrostriatal pathway about 1 mm from the rostral tip of the substantia nigra (Fig. 1 *A* and *B*). The transection was performed by stereotactically lowering a 0.3-mm-wide guidance needle containing two thin wire knifes (a in Fig. 1*A*), to coordinates medio-lateral 1.7 mm, rostro-caudal −3.6 mm, and dorso-ventral −9.0 mm, all from Bregma with the tooth bar set at −3.3 mm. After extrusion of the wires in the medio-lateral plane, a 3-mm-wide, 3-mm-high transection slit (±100 μm thick in rostro-caudal extent) (hatched area in Fig. 1*B*) was made through the nigrostriatal pathway by moving the microknife dorsally. The wires were then drawn back into the guidance needle and both were withdrawn from the brain, leaving beside the transection, only a cannulation tract. To establish an initial time course of neuronal loss, some transected animals were allowed to survive for 7 (n = 3), 14 (n = 4), or 28 (n = 3) days postlesion.

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) Retrograde Labeling. In 10 of the remaining rats, substantia nigra neurons projecting to the neostriatum were retrogradely labeled 12 days before unilateral nigrostriatal transection by injecting bilaterally the fluorescent tracer DiI (Molecular Probes) with a 200-μm tip glass needle placed on a Hamilton syringe into six stereotactically defined tracts per striatum (0.3% in 10% ethanol/phosphate-buffered saline; 2 μl each tract). DiI labeling has been shown not to affect survival of neurons *in vitro* and *in vivo* and remains robustly detectable for up to 9 months *in vivo* without leakage to other cells (24, 25). Its retrograde transport capability is based in large part on passive diffusion through association with lipids, including cellular membranes, and results in the "filling" or labeling of the neuronal cell body and all processes.

CNTF Infusion. In these 10 rats, immediately after transection, a 0.3-mm-wide metal infusion cannula was stereotactically implanted and its tip was positioned 0.5 mm caudal to the transection, directly into the brain parenchyma and just

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Abbreviations: ChAT, choline acetyltransferase; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; NGF, nerve growth factor; TH, tyrosine hydroxylase.

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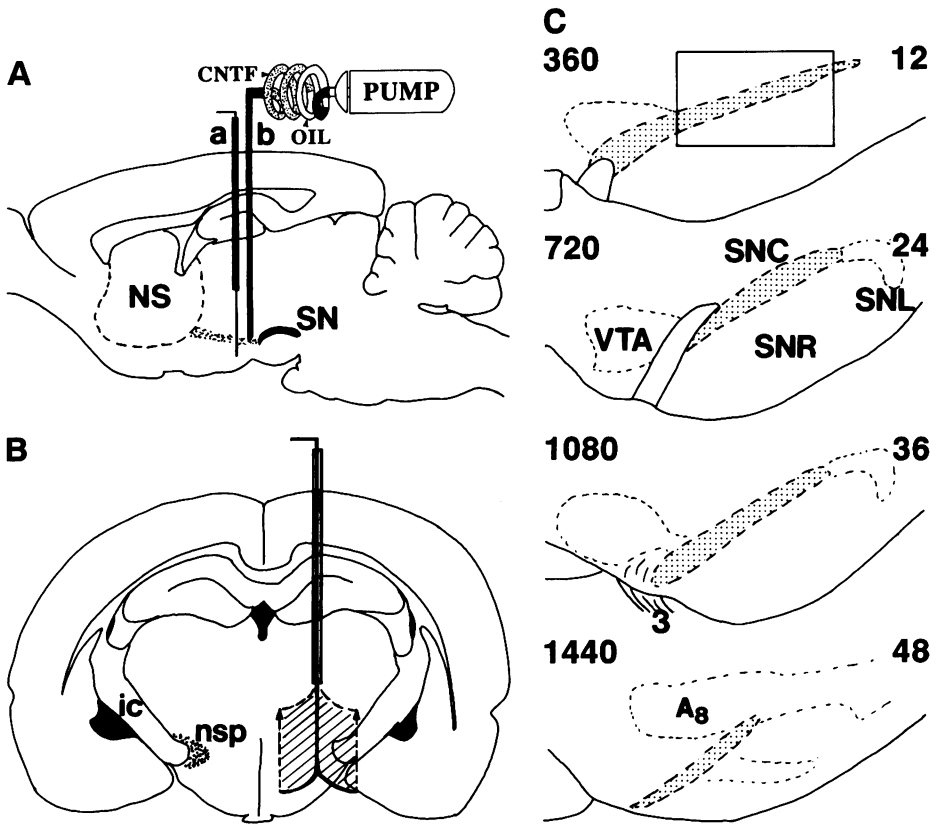

FIG. 1. Sagittal (*A*) and coronal (*B*) views of the adult rat brain illustrating the extent of the nigrostriatal transection and the position of the infusion cannula (b in *A*). SN, substantia nigra; NS, neostriatum; ic, internal capsule; nsp, nigrostriatal pathway. (*C*) Illustration of the borders used to define the substantia nigra compacta (SNC: neuron group A9; shaded and outlined by broken lines; left side is medial). VTA, ventral tegmental area, group A10; SNR, substantia nigra reticulata; SNL, lateralis; 3, oculomotor nerve. Numbers on the left = distance from rostral pole of substantia nigra; numbers on the right = section number. Boxed area in section 12 = Fig. 2.

rostral to the substantia nigra (Fig. 1*A*). Each cannula was connected to an Alzet 2002 mini-osmotic pump (Alza; partly coated with paraffin to reduce the flow rate from 0.5 to 0.2 μl/hr) by a coiled reservoir (26). The reservoir was filled with vehicle (artificial cerebrospinal fluid with 1 mg of rat serum albumin per ml; Sigma A-6272) (n = 4) or vehicle containing recombinant human CNTF (5×10^3 Trophic Units/day or 1.5 μg/day) (n = 6) for a 14-day infusion. Two different batches of CNTF were used (n = 3 each) and were produced, purified, characterized, and assayed for biological activity as described in detail elsewhere (23). In a pilot study, rats without DiI labeling had received 14-day infusions with vehicle (n = 5) or CNTF (n = 5). In addition, 3 normal animals were used.

Histology and Analysis. After their respective survival times the animals were anesthetized again and transcardially perfused with 75 ml of cold phosphate-buffered saline and 250 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and their brains were postfixed for 16 hr and then cryoprotected in 30% sucrose for 16–24 hr. Symmetrical 30-μm-thick coronal sections were cut on a freezing microtome from the brain immobilized in a standardized position in the plane of the Paxinos and Watson atlas (27). The section containing the rostral pole of the substantia nigra compacta (neuron group A9) was carefully determined (named section 0) and was ≈4.0 mm caudal to Bregma, a level corresponding to figure 76 in ref. 28. The most caudal section analyzed (assigned no. 54, ≈1.6 mm from section 0) corresponded to level P6.0, figure 91 in ref. 28. Every sixth section through the nigral complex was mounted in water-based Fluoromount-G (Fisher Scientific) on uncoated glass slides, analyzed for the number of DiI-labeled substantia nigra neurons, and photographed. The sections were then carefully removed from the slides, washed, and immunostained with a polyclonal antibody against tyrosine hydroxylase (TH) (Eugene Tech, Eugene, OR). Sections were incubated free floating at room temperature, unless otherwise indicated, for 3 × 10 min with Tris-buffered saline (TBS) containing 0.25% Triton X-100, 30 min with 5% goat serum in TBS, 16 hr with primary antibody (1:30,000) in TBS/serum/Triton X-100, 1.5 hr with goat anti-rabbit IgG (Vector Laboratories) in TBS/serum, 1.5 hr with avidin biotin complex (ABC Elite, Vector Laboratories) in TBS, and, finally, 10 min with a mixture of 0.04% 3'3-diaminobenzidine tetrahydrochloride/0.06% $NiCl_2$/0.02% H_2O_2 in Tris·HCl. In between steps, sections were washed for 3 × 10 min in TBS. Stained sections were mounted on gelatin-subbed glass slides, air-dried, incubated for 30 sec in 0.005% OsO_4, dehydrated in ethanol, cleared in xylene, and coverslipped in Permount. After analysis and photography, these sections were rehydrated and stained with cresyl violet (Nissl staining). For each animal the completeness of the lesion was evidenced in sections rostral to the transection by the absence of TH-positive fibers in the region of the ipsilateral nigrostriatal tract, medial forebrain bundle, and neostriatum. For the counting of substantia nigra neurons on both sides of the brain, the compacta regions were defined by the distribution of the TH- or DiI-positive neurons and a set of clear anatomical landmarks/boundaries (ref. 28; Fig. 1*C*). The number of TH-immunopositive or DiI-labeled compacta neurons having a maximal cell body diameter ≥10 μm was determined by counting at 250×. In a few cases the number of cresyl violet-stained neurons was determined in adjacent sections stained exclusively for cresyl violet and defined by their size and the presence of a nucleolus.

RESULTS

Normal TH- and DiI-Positive Compacta Neurons. On the nonlesioned side of animals with a unilateral nigrostriatal transection the total number of TH-positive neurons in the substantia nigra compacta was not significantly different from that in normal animals {3546 ± 88 (mean ± SEM) vs. 3813 ± 84; 19.3-μm neuron diameter; Mann–Whitney U test; totals corrected according to Abercrombie and used for calculations of the percentages in the text: total neurons = neurons counted × [section thickness ÷ (section thickness + average neuron diameter)] × section interval; ref. 29}. The

number of TH-positive neurons was 91% of the number of cresyl violet-stained neurons and essentially the same as the number of DiI-labeled ones (see Fig. 3*A*). Thus, the DiI- and TH-positive neurons very likely represent the same neuronal population—i.e., >90% of the substantia nigra.

Axotomy Causes Loss of TH- and DiI-Positive Nigral Neurons. After the nigrostriatal transection (no DiI labeling, no infusion) the number of TH-positive neurons of the ipsilateral substantia nigra compacta decreased gradually to 71% ± 7%, 40% ± 7%, and 29% ± 7% of the initial level by 7, 14, and 28 days, respectively (neuron diameter = 18.8, 17.1, and 16.4 μm). This reduction was accompanied by a similar reduction in the number of cresyl violet-stained neurons and occurred over the entire length of the compacta region (data not shown). In the DiI-labeled, vehicle infused animals, the transection resulted in a similar loss of TH-positive compacta neurons on the lesioned side (34% ± 3%; 17.1-μm neuron diameter) (Figs. 2*C* and 3 *A* and *B*). The number of DiI-labeled neurons in the same sections had decreased to 25% ± 4% (17.2-μm diameter) of the contralateral side (20.0-μm diameter) (Figs. 2*D* and 3 *A* and *C*). Moreover, intermingled with the labeled neurons of the lesioned side, many intensely luminous DiI "deposits" with a diameter of approximately 4–6 μm diameter (Figs. 2*D* and 3*A*) were detectable. These DiI "deposits" could represent the remains of dead substantia nigra compacta neurons or living nonneuronal (microglial) cells that had taken up the DiI of dead neurons (30, 31). Dopaminergic neurons in the ipsilateral ventral tegmentum (A10 area), which also project through the transected brain region, underwent degenerative changes similar to those in the compacta region (data not shown).

CNTF Prevents Loss of (DiI-Positive) Neurons But Not of TH Staining. Infusion with CNTF for 2 weeks reduced only slightly the loss of TH-positive compacta neurons (44% ± 3% vs. 34% ± 3% with vehicle; $P \leq 0.05$; neuron diameter, both = 17.1 μm) (Figs. 2*E* and 3 *A* and *B*). However, many more cresyl violet-stained but TH-negative compacta neurons were detectable than in the normal or vehicle-infused lesioned animal, suggesting the preservation by CNTF of the neurons but not of their TH content. In fact, the CNTF infusion almost completely prevented the reduction in the number of DiI-labeled substantia nigra compacta neurons (82% ± 2%; 20.0-μm neuron diameter; in two of six animals >95% of the DiI-labeled neurons were detectable in the rostral half of the compacta) (Figs. 2*F* and 3 *A* and *C*). The retained DiI-labeled neuronal cell bodies subjectively appeared slightly smaller and rounder than normal (same 20-μm cell body diameter), since labeling of the processes was not evident, potentially due to interrupted transport of DiI in the processes or a loss of the processes. With the CNTF infusion, far fewer DiI deposits (indicators of dead neurons, see above)

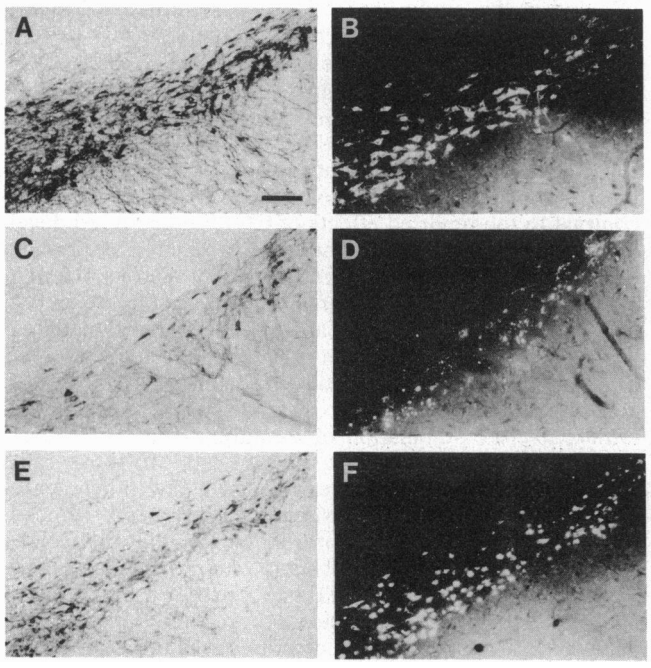

FIG. 2. CNTF prevents axotomy-induced degeneration of adult rat substantia nigra compacta neurons but not their reductions in TH. (*A*, *C*, and *E*) Coronal sections (box in Fig. 1, section 12) through the substantia nigra immunostained for TH. Left side is medial. (Bar = 100 μm.) (*B*, *D*, and *F*) Same sections photographed under epifluorescence for presence of DiI labeling before immunostaining. *A* and *B*, contralateral; *C* and *D*, vehicle-infused lesioned side; *E* and *F*, CNTF-infused lesioned side.

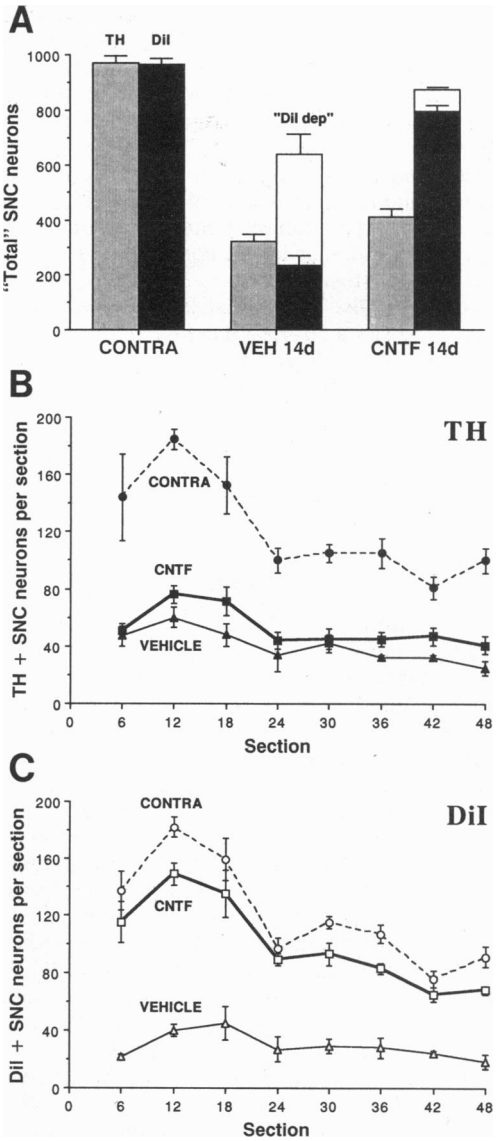

FIG. 3. Quantitative evidence that CNTF almost completely prevents the degeneration of axotomized substantia nigra compacta (SNC) dopaminergic neurons but not their reductions in TH. (*A*) "Total" number ± SEM of TH-positive or DiI-labeled compacta neurons and DiI deposits (DiI dep) *counted* in all eight sections through the nigral complex (not Abercrombie corrected). (*B*) Number of TH-positive neurons per single section counted on the nonlesioned contralateral (contra) and vehicle- or CNTF-infused lesioned sides. Section 0 = rostral pole of the nigral complex. (*C*) Same sections as in *B* but for the number of DiI-labeled compacta neurons.

were seen between the labeled compacta neurons (Figs. 2F and 3A). The protective effects of CNTF were evident across the entire length of the substantia nigra compacta (Fig. 3C) and also in the ventral tegmentum (A10 area). In the transected animals *without* DiI labeling but with a 14-day infusion with vehicle ($n = 5$) or CNTF ($n = 5$), analysis of TH-stained and cresyl violet-stained sections provided results very similar to those described for the DiI animals. Their quantitative results are not presented here since cresyl violet counts, especially under lesion conditions, are less reliable and thus less conclusive.

Nonneuronal Cells. The nigrostriatal transection and vehicle infusion caused a 50–75% increase in the number of cell bodies smaller than 10 μm detectable in the cresyl violet-stained compacta region ≈1 mm from the infusion. CNTF had no measurable effects on this number of small cells. In addition, the lesion or the vehicle or CNTF infusion had no effects on the size of a major population of small (5.7–5.9 μm) and densely cresyl violet-stained cells, which correspond to glial fibrillary acidic protein-positive astrocytes.

DISCUSSION

The present study provides clear evidence that treatment with CNTF can largely (and, in a few cases, completely) prevent axotomy-induced degeneration of substantia nigra dopaminergic neurons in the adult rat. Other factors (epidermal growth factor, basic fibroblast growth factor) and substances (GM1 ganglioside, nicotine, Deprenyl) have been tested in adult animal models of substantia nigra degeneration with varying success, but the extent of protection against neuronal death (evidenced by cell body analysis) seen here has not been reported (32–37). The present finding extends the spectrum of lesioned adult CNS neurons whose degeneration is preventable by CNTF treatment, such as the cholinergic and noncholinergic neurons of the medial septum (23) and those of the anterior thalamic nucleus (38). Yet to be resolved is the question of whether CNTF is indefinitely needed for survival of these injured neurons or could be withdrawn after an initial "rescue" period.

These results in experimentally lesioned animals raise the question of whether CNTF has a physiological role for neurons of the normal adult septum and substantia nigra. Such a relationship could exist because (*i*) although these neurons were not among those reported to have a high level of expression for CNTF α-receptor mRNA (39), they may carry sufficient numbers of signal-transducing CNTF α-receptors or up-regulate them after injury; (*ii*) in the hippocampal formation and striatum, the innervation territories of these neurons, CNTF protein and mRNA are detectable at much higher levels than those of NGF (which is known to have a physiological role) (40); and (*iii*) these neurons are protected by CNTF after their injury. On the other hand, it has been suggested that CNTF be viewed as a general "lesion" factor to be released only upon cell lysis/leakage after brain injury (22, 40, 41). The argument was largely based on (*i*) the fact that CNTF cDNA predicts a protein lacking the consensus hydrophobic signal sequence necessary for vesicular secretion and (*ii*) the findings that it is not released from several types of cultured cells (including astrocytes) (42, 43). However, other proteins (basic fibroblast growth factor, interleukin 1α) that also lack such a signal sequence are known to be secreted, possibly through "unconventional" mechanisms, which may include phosphorylation, insertion into the membrane, and extracellular proteolytic cleavage (44–47). Moreover, signal-transducing CNTF α-receptors have very recently been shown to be expressed by several populations of adult CNS neurons (39).

No effects of the infused CNTF on the nonneuronal cell population (number, size) were seen by gross analysis of cresyl violet-stained sections. It remains to be determined, however, whether the protective effects of CNTF were the result of a direct action on the lesioned neurons or on the neighboring intrinsic or infiltrating cells. Another question is whether the infused CNTF acts as a pharmacological agent *or* whether it mimics and supplements a natural mechanism that otherwise fails to protect injured neurons because of inadequate endogenous CNTF levels. Several recent findings favor the latter possibility. (*i*) Astrocytes of the adult brain are known to produce and contain CNTF (40, 48), and brain injury leads to increases in CNTF activity in the wound area that correlate in time with the appearance of reactive astrocytes (48, 49). (*ii*) Axotomy-induced death of facial motor neurons in the neonate rat, in which normal CNTF levels in the facial nerve are still very low, can be prevented by application of high doses of exogenous CNTF to the nerve stump (22). (*iii*) Such neuronal loss can also be prevented by systemic treatment with the anti-Parkinson agent Deprenyl (50), which promotes the expression of CNTF mRNA in cultured astrocytes (N. A. Seniuk, W. G. Tatton, and J. Henderson, personal communication). (*iv*) Likewise, axotomized adult facial motor neurons may not degenerate because of the high levels of endogenous CNTF in the adult nerve (22) and/or the increased expression of CNTF mRNA in the facial nucleus seen after nerve lesions (51).

The protective effect of CNTF for the dopaminergic neurons extended only slightly to the cellular content of TH. This finding is very similar to the one we have reported before (23) for the axotomized medial septum cholinergic neurons of the adult rat, where CNTF prevented their degeneration and loss of low-affinity NGF receptors but not the reductions in ChAT stainability. In contrast, NGF at lower doses can prevent degeneration and promote expression of ChAT of the axotomized septal cholinergic neurons. In addition, we have not found changes in ChAT staining of these cholinergic neurons at a 3-fold higher CNTF concentration than used previously (23) and in this study (unpublished observations). Thus, it seems unlikely that doses higher than the very high dose of CNTF used here would cause increases in TH. Apparently, CNTF by itself does not regulate the "functional" performances of the adult septal and substantia nigra neurons *in vivo*, since TH and ChAT are the rate-limiting enzymes for synthesis of the neurotransmitters dopamine and acetylcholine, respectively. These *in vivo* results might seem to be in contrast to the reported effects of CNTF on cultured developing peripheral and central neurons, where it may promote or sustain cholinergic or GABAergic properties (GABA, γ-aminobutyric acid) (7, 8, 10). However, in cultured embryonic brainstem noradrenergic and mesencephalic dopaminergic CNS neurons, promotion of TH expression by CNTF reportedly depends on the concurrent presentation of noradrenaline and dopamine (or their agonists), through autocrine/paracrine stimulation of α2 adrenoreceptors and D2 dopamine receptors, respectively (52, 53). In those same studies, noradrenaline, dopamine, and a few other neurotransmitters by themselves stimulated TH expression, although to a lesser extent. It is conceivable that the CNTF-rescued axotomized adult substantia nigra neurons still undergo reductions in levels of TH because they have a reduced capacity to produce and release dopamine for autocrine stimulation, to express D2 receptors, or to respond to neurotransmitter stimulation in general. Whether CNTF has survival-promoting effects for the dopaminergic neurons in other substantia nigra degeneration models (MPTP neurotoxin, weaver mutant mouse) and whether these have intact TH-regulating mechanisms or would also require concurrent treatments with TH-regulating agents have yet to be investigated. Such studies would further resolve CNTF's potential to be used in the treatment of Parkinson disease, which is

characterized and largely caused by the degeneration of nigral dopaminergic neurons (54, 55).

It also remains to be tested whether brain-derived neurotrophic factor, a neurotrophin family member of NGF, would have survival and transmitter-regulating effects on the adult substantia nigra neurons *in vivo* as it has for developing ones *in vitro* (56, 57). In a recent study, supranigral infusion of brain-derived neurotrophic factor into normal nonlesioned animals augmented amphetamine-induced rotation behavior and stimulated dopaminergic parameters in the neostriatum but did not promote striatal TH activity (58).

In conclusion, CNTF has potent and general neuroprotective effects—i.e., it can almost completely prevent the degeneration of several types of neurons in the adult rat CNS. The finding here that CNTF has trophic effects for the dopaminergic neurons of the substantia nigra opens up new opportunities for a better understanding and manipulation of the survival and function of these neurons, whose degeneration underlies Parkinson disease.

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