Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9231–9235, December 1986
Neurobiology

Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection

(medial septum/diagonal band of Broca/neuronotrophic factor/neuronal survival/osmotic minipump)

LAWRENCE R. WILLIAMS*, SILVIO VARON†, GARY M. PETERSON†, KLAS WICTORIN‡, WALTER FISCHER‡, ANDERS BJORKLUND‡, AND FRED H. GAGE*§

Departments of *Neurosciences and †Biology, School of Medicine, University of California–San Diego, La Jolla, CA 92093; and ‡Department of Histology, University of Lund, Lund, Sweden

Communicated by Jean-Pierre Changeux, July 31, 1986

ABSTRACT **Neurons in the rat medial septum (MS) and vertical limb of the diagonal band of Broca (VDB) undergo a rapid and severe cell death after transection of their dorsal projection to the hippocampus by aspiration of the ipsilateral fimbria fornix and supracallosal striae. By 2 weeks posttransection, the extent of neuronal loss was 50% of the total neurons and 70% of the cholinergic neurons in the MS and 30% of the total neurons and 40% of the cholinergic neurons in the VDB. We hypothesized that (*i*) the death was due to the loss of a hippocampus-derived neuronotrophic factor, and (*ii*) exogenous nerve growth factor (NGF) might provide trophic support to the MS/VDB cholinergic neurons, in light of recent reports that the septal diagonal band cholinergic neurons are responsive to NGF and that NGF is present and produced in the hippocampus. In the present study, we attempted to prevent the transection-induced neuronal death by continuous infusion of exogenous 7S NGF (1 μg/wk) through an intraventricular cannula device. We report here that NGF treatment significantly reduces both the total neuronal and cholinergic neuronal death found 2 weeks after fimbria fornix transection; there was a sparing of 50% of the neurons in the MS and essentially 100% of those in the VDB that otherwise would have died. We conclude that NGF also has a protective effect on noncholinergic neurons since calculations indicate that 80% of the NGF-affected neurons are noncholinergic.**

The cholinergic neurons of the basal forebrain are believed to be involved in the functional processes of learning and memory (1–5). Impaired memory is a cardinal symptom in the diagnosis of Alzheimer disease and presenile dementia of the Alzheimer type (1–6). A morphological correlate of the disease is the shrinkage and/or death of cholinergic neurons in the basal forebrain and a loss of choline acetyltransferase (ChAT) in the cortex and hippocampus (6–8). It has been speculated that the death of central neurons in Alzheimer disease, in other idiopathic neuropathologies, and after traumatic injuries may be due to either the loss, to inappropriate delivery, or to dysfunctional utilization of neuronotrophic factors (NTFs). Such NTFs may be derived from target neurons and/or supporting glial cells (9–12). To explore these mechanisms experimentally, we have developed a model in the rat to study adult central nervous system neuronal death and the prevention of neuronal death by administration of exogenous NTFs.

Neurons of the medial septum (MS) and vertical limb of the diagonal band of Broca (VDB) project topographically to the ipsilateral hippocampus primarily via a dorsal pathway through the fimbria fornix and supracallosal striae. About 50% of this projection is cholinergic, originating from dorsal, intermediate, and ventral groups (or groups Ch-1 to Ch-4) in the MS/VDB, and it provides ≈90% of the total cholinergic innervation of the hippocampus (13–16). When the axons of these neurons are transected by unilateral aspiration of the fimbria fornix, they undergo a rapid and substantial cell death (17–19). Fifty percent of the total neurons, as indicated by counts of cresyl violet-stained neurons, and nearly 70% of the cholinergic neurons die within 2 weeks posttransection (19). We hypothesize that many if not all these neurons die due to the deprivation of a retrogradely transported target (i.e., hippocampus)-derived NTF(s).

Several NTFs have now been detected or are suspected to be present in the hippocampus (20–28). Nerve growth factor (NGF) is known to be synthesized in the hippocampus and retrogradely transported specifically to neurons of the basal forebrain (29–31). Moreover, exogenous NGF has been shown *in vivo* and *in vitro* to stimulate ChAT activity of these neurons in both developing and adult animals (32, 33). In addition, NGF has been recently reported to promote the survival of MS cholinergic neurons *in vivo* after partial deafferentation (44).

In the present investigation, we have tested the ability of NGF, continuously infused through an intraventricular cannula device, to prevent or reduce the neuronal death that occurs in the MS/VDB after axotomy induced by fimbria fornix transection. The results indicate that continuous administration of NGF does in fact prevent the death of neurons that otherwise would have died.

MATERIALS AND METHODS

Cannula Device. The chronic intraventricular cannula device developed for these experiments is illustrated and briefly described in Fig. 1. Details of its construction will be provided in a separate report.

Animal Surgery. A female Sprague–Dawley (200–225 g) rat was deeply anesthetized with a mixture (4 ml/kg) of ketamine (25 mg/ml), rompun (1.3 mg/ml), and acepromazine (0.25 mg/ml) and placed in a Kopf stereotaxic apparatus. A hole was drilled in the skull at the stereotaxic coordinates: 0.8 mm anterior to bregma, 1.2 mm lateral to midline (34). A 2-mm square of the skull directly caudal to the cannula hole was also removed, leaving the dura intact, to expose the surgical area for fimbria fornix aspiration (see below).

The cannula device was filled with the experimental infusion solution (see below) and the connecting line was attached to a loaded Alzet model 2002 miniosmotic pump (flow rate, 0.5 μl/hr; Alza, Palo Alto, CA). The cannula was lowered into the

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Abbreviations: MS, medial septum; VDB, vertical limb of diagonal band of Broca; ChAT, choline acetyltransferase; NTF, neuronotrophic factor; NGF, nerve growth factor; AcChoE, acetylcholinesterase; ACSF, artificial cerebrospinal fluid; ARS, autologous rat serum.

§To whom reprint requests should be addressed at: Department of Neurosciences, M-024, University of California–San Diego, La Jolla, CA 92093.

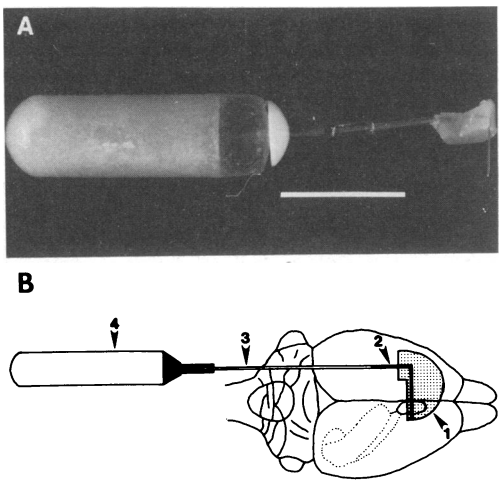

FIG. 1. Cannula device construction and placement. (*A*) A 33-gauge stainless steel tube is embedded in a dental acrylic stabilization platform, mounted in a stereotaxic apparatus using a triceps forceps, attached with a vinyl connecting line to an Alzet miniosmotic pump containing the infusion solution. The vinyl connecting line was implanted into the rat and glued to the skull with cyanoacrylate. The length of the vinyl tubing has been shortened for purposes of illustration. (Bar = 1 cm.) (*B*) Schematics of the implanted device. Arrowheads: 1, acrylic stabilization platform; 2, stainless steel cannula; 3, vinyl connecting line; 4, Alzet miniosmotic pump. The bend of the cannula to the left provides greater stabilization and permits later access to the fimbria fornix for surgical aspiration. Drawing is not to scale.

right ventricle and the stabilization platform was cemented to the skull with cyanoacrylate. The pump was placed subcutaneously in the neck of the animal, and the scalp wound was closed with wound clips. The cannula device is stable for 4 weeks and remains patent for at least 17 days postimplantation.

NGF Infusion Solution. The basic infusion vehicle was a phosphate-buffered artificial cerebrospinal fluid (ACSF): 150 mM NaCl/1.8 mM $CaCl_2$/1.2 mM $MgSO_4$/2.0 mM K_2HPO_4/10.0 mM glucose, pH 7.4. NGF (7S form) was purified according to Varon *et al.* (35). NGF biologic activity was assayed by the method of Manthorpe *et al.* (36). Preliminary experiments testing the stability of NGF in the pump revealed that only 10% of the activity was delivered by the pump when purified 7S NGF was loaded at low concentrations in saline. The loss was prevented if the vehicle also included 0.01% albumin as a carrier protein. For these experiments, therefore, the vehicle used was ACSF containing 0.1 mg of autologous rat serum (ARS) per ml, prepared from blood obtained from the same animal via cardiac puncture. When NGF dissolved in ACSF/ARS at 11 μg/ml was incubated at 37°C, maximal biologic activity was maintained in the solution for 10–14 days.

Five animals were infused with ACSF/ARS alone, 5 were infused with ACSF/ARS containing cytochrome *c* (11 μg/ml; Sigma), and 10 animals were infused with ACSF/ARS containing 7S NGF at 11 μg/ml—i.e., 2000 biological units per ml or 10,000 trophic units per ml.

Fimbria Fornix Transection. Three days after cannula implantation and continuous delivery of experimental infusion solution, all experimental animals received a complete unilateral aspirative lesion of the fimbria fornix and supracallosal striae as described (37, 38). The aspirations were conducted visually with the aid of a surgical microscope and were made on the right side of the brain ipsilateral to the implanted cannulae.

Quantitative Histology. Two weeks after the fimbria fornix transection and 17 days after the start of the continuous administration of experimental infusion solution (i.e., 17 days after cannula implantation), the animals were taken for histochemical evaluation. An additional four animals were sacrificed to represent normal unlesioned animals. All animals were prepared on the day of sacrifice according to the method of Butcher (39) for the visualization of cholinergic neuronal cell bodies. A sublethal dose of bis(1-methylethyl)-phosphofluoridate (2 mg/kg) was injected into the anesthetized animal to block, irreversibly, endogenous acetylcholinesterase (AcChoE). After a 4-hr period to allow new synthesis of AcChoE in the cell bodies, the animal was perfused with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed for 2 hr in the same fixative, and then left overnight in 10% sucrose at 4°C. Coronal sections were cut at 20 μm with a cryostat and collected throughout the MS/VDB complex. Every third section was processed for the visualization of AcChoE according to Koelle (40), using 0.1 mM prometazine as an inhibitor of nonspecific esterases and silver nitrate intensification of the reaction product (41). Alternate sections were stained with cresyl violet.

In the MS/VDB complex, 88–99% of the cholinergic neurons, identified by immunohistochemical reaction with antibody to ChAT, have been shown to be AcChoE positive, and 100% of the AcChoE-positive neurons reacted with antibody to ChAT (14, 42, 43). Thus, AcChoE histochemistry provides a reliable marker for estimates of MS/VDB cholinergic neurons. Quantification of total (cresyl violet-stained) neurons and cholinergic neurons in the ipsi- and contralateral MS and VDB was done on three coronal sections, located ≈360 μm, ≈600 μm, and ≈840 μm rostral to the decussation of the anterior commissure, areas where the neurons of the MS and VDB that project to the hippocampus are concentrated (15). The animals to be evaluated were randomly separated into three groups and counted by three individuals unaware of the animal treatment. Both total and cholinergic neuronal numbers were determined by direct count, and the MS and VDB were sampled as described, using a counting grid (0.5 × 0.5 mm) to entirely cover the areas of interest (19). Neurons were identified using size and morphological criteria. Briefly, cresyl violet-stained neurons were defined as cells having diameters >12 μm and containing both a nucleus and Nissl substance. An AcChoE-positive cell was defined as a dark brown body with a minimum diameter of 12 μm along the major axis. The vast majority of AcChoE-stained cells fell within the range of 18–29 μm. AcChoE-stained profiles <12 μm in diameter were without regular cell shape or processes and could not be distinguished as a cell.

Statistical Analysis. In animals infused with ACSF plus ARS (0.1 mg/ml), the number of neurons counted both ipsilateral and contralateral to the transection was comparable to those counted in transected noninfused animals (see ref. 19). Therefore, ARS included in the infusion vehicle as a protein carrier had no apparent toxic or sparing effect on transected neurons. Animals infused with vehicle alone (ACSF/ARS) or vehicle with cytochrome *c* did not differ from each other either in the number of total neurons or in the number of cholinergic neurons on the side contralateral to the lesion. Likewise, no differences were found between these two groups on the side ipsilateral to the lesion. Therefore, the vehicle- and cytochrome *c*-treated animals were combined to form a single statistical group, designated non-NGF-treated animals, in all subsequent analyses. The effects of the non-NGF and NGF treatments on MS/VDB neuronal death ipsilateral to the lesion were evaluated by the actual counts of total neurons and cholinergic neurons. In addition, the neuronal counts were normalized as the percentages of surviving neurons ipsilateral to the lesion compared to the neurons in the MS/VDB of the same animal contralateral to the lesion (19).

RESULTS

The average numbers of total neurons (cresyl violet-stained) and cholinergic neurons (AcChoE-positive neurons) counted in the MS and VDB of non-NGF-treated and NGF-treated animals, both ipsi- and contralateral to the lesion, are presented in Table 1. As previously reported (19), no statistical difference was found in either the total neurons or cholinergic neurons between normal unoperated animals (data not shown) and operated animals on the side contralateral to the lesion. Infusion of non-NGF- or NGF-containing vehicle also had no effect on neuronal numbers contralateral to the lesion (Table 1). Fig. 2 illustrates the AcChoE-positive cholinergic neurons present in the left MS/VDB contralateral to the transection of operated animals.

In non-NGF-treated animals, transection of the fimbria fornix resulted in a substantial loss of MS/VDB neurons, both total and cholinergic, by 2 weeks after transection ($P < 0.05$, Table 1). The loss of cholinergic neurons was conspicuous on the side of transection (Fig. 2 *A* and *B*) and the number of dead noncholinergic neurons was almost 6-fold greater than the loss of cholinergic neurons (Table 1).

Infusion of NGF significantly reduced both the total and cholinergic neuronal losses after fimbria fornix transection (Table 1). The obvious sparing of cholinergic neurons ipsilateral to the transection is illustrated in Fig. 2 *C* and *D*. The numbers of surviving neurons in the ipsilateral MS of NGF-treated animals were significantly greater than in non-NGF-treated animals ($P < 0.05$, Table 1). However, the MS cholinergic neuronal survival was significantly less than on the contralateral side ($P < 0.05$). A similarly incomplete (though not statistically verifiable) protection may have occurred with regard to total neurons (Table 1). In the VDB, on the other hand, NGF provided complete protection, since both total and cholinergic neuronal numbers ipsilateral to the transection were statistically indistinguishable from those on the contralateral side. Note that the noncholinergic neurons in the MS/VDB spared by NGF were 3- to 6-fold more numerous than the spared cholinergic neurons.

Table 1. Average number of neurons in the MS and VDB

	Total neurons		Cholinergic neurons	
	Contra-lateral	Ipsi-lateral	Contra-lateral	Ipsi-lateral
MS				
Non-NGF	817 (85)	448 (66)*	104 (14)	41 (7)*
NGF	879 (128)	721 (105)*†	123 (20)	87 (14)*†
VDB				
Non-NGF	1231 (140)	833 (100)*	224 (25)	135 (21)*
NGF	1253 (160)	1190 (133)†	246 (25)	235 (22)†

Numbers in parentheses are SEM. $n = 10$.
*$P < 0.05$ compared to contralateral side.
†$P < 0.05$ compared to ipsilateral non-NGF.

The effects of fimbria fornix transection and NGF treatment were even more explicit when neuronal numbers on the ipsilateral side were expressed as percentages of those on the contralateral side of the same animal, as shown in Fig. 3. Aspiration of the fimbria fornix resulted in the loss of 45% of the total and 60% of the cholinergic neurons in the MS, and 30% of the total and 40% of the cholinergic neurons in the VDB. In all cases, percentage losses were highly significant ($P < 0.005$). In the MS, infusion of NGF reduced these losses to 20% of the total neurons and 30% of the cholinergic neurons ($P < 0.002$), which represents a sparing of 60% and 50%, respectively. The percentages of total and cholinergic neurons surviving after NGF treatment were significantly greater than those in non-NGF-treated animals ($P < 0.002$) but were less than those in normal animals ($P < 0.005$). In the VDB, NGF treatment promoted the survival of 100% of both total and cholinergic neurons ($P < 0.002$, Fig. 3).

In all the NGF-treated animals examined 2 weeks post-transection, a large accumulation of cholinergic axons was

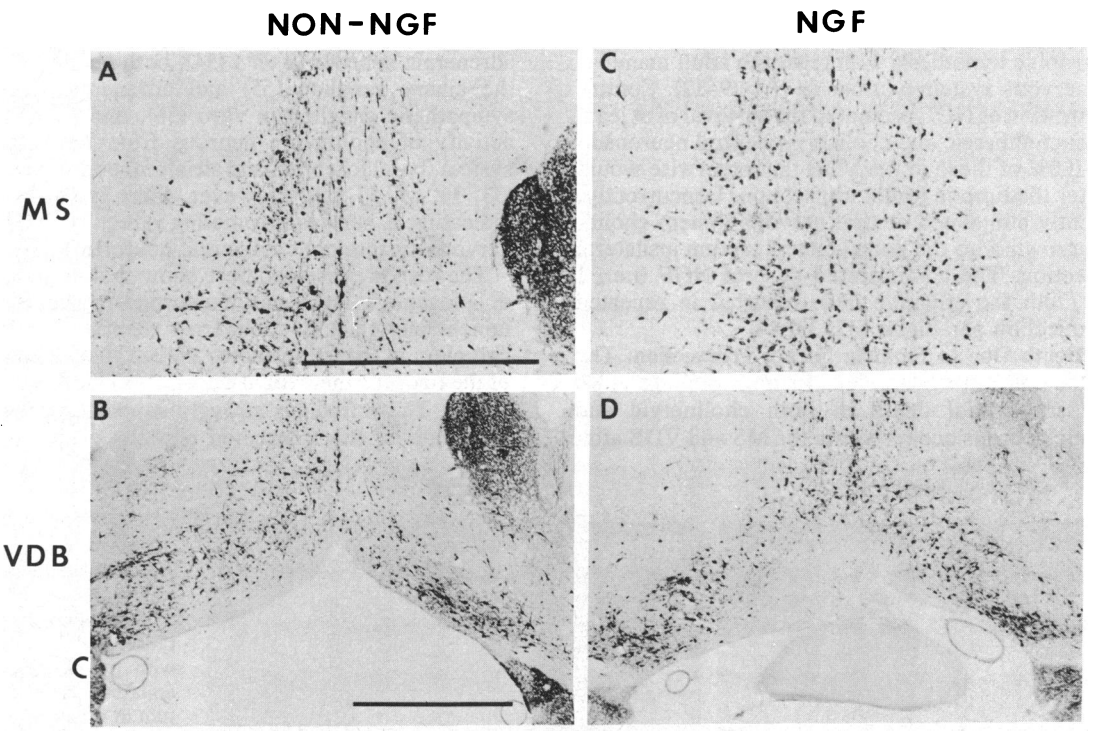

FIG. 2. Micrographs of AcChoE-stained neurons in the MS (*A* and *C*) and VDB (*B* and *D*) of animals 2 weeks after transection of the right fimbria fornix and intraventricular infusion of vehicle with cytochrome *c* (NON-NGF, *A* and *B*) or 7S NGF (*C* and *D*) for both the contralateral (left) and ipsilateral (right) sides. On the right side ipsilateral to the transection in non-NGF-treated animals, a substantial death of cholinergic neurons was observed in both the MS and VDB. In the NGF-treated animals, neuronal death was prevented. (Bars = 1 mm.)

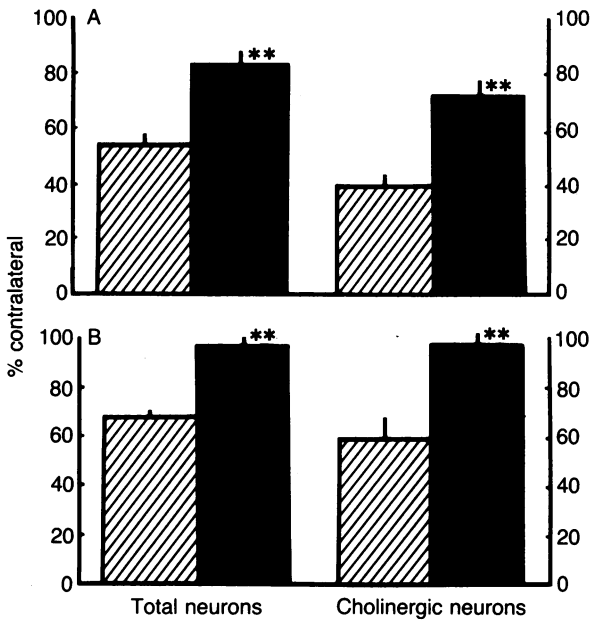

FIG. 3. The numbers of total neurons and cholinergic neurons present 2 weeks after fimbria fornix transection in the basal forebrain ipsilateral to the lesion are shown for non-NGF-treated (hatched) and NGF-treated (solid) animals as the percentage of those counted on the side contralateral to the transection. **, $P < 0.002$ compared to non-NGF treated animals. (*A*) MS; (*B*) VDB.

observed in the dorsal lateral quadrant of the septum ipsilateral to the lesion—i.e., the site of origin of the fimbria fornix connection to the ipsilateral hippocampus prior to transection (Fig. 4). Such accumulations were never observed on the side contralateral to the transection in either NGF- or non-NGF-treated animals.

DISCUSSION

The results reported here provide a quantitative validation of the neuronotrophic hypothesis with regard to adult mammalian central nervous system neurons *in vivo* (9–12). Continuous administration of NGF promoted the survival of at least half of the noncholinergic and cholinergic central neurons in the MS and 100% of those in the VDB that otherwise would have died after the fimbria fornix transection. Concurrently, NGF apparently stimulated central nervous system cholinergic axonal growth into the dorsal lateral septum ipsilateral to the transection. The administered dose of NGF (i.e., 1 μg/wk) is 1/20th the effective dose reported in repeated acute NGF injection paradigms (32, 33, 44).

Neuronal Death Due to Fimbria Fornix Transection. The present results confirm the previous findings of Gage *et al.* (19) that a substantial death of both cholinergic and noncholinergic neurons occurs within the MS and VDB after transection of the axonal projection to the ipsilateral hippocampus via the fimbria fornix and supracallosal striae. The implanted cannula and continuous infusion of vehicle or vehicle with cytochrome *c* had no detectable influence on the neuronal death, because the loss of neurons ispilateral to the lesion and implanted cannula in non-NGF-treated animals was similar to that in animals receiving the lesion alone.

The numerical decline in AcChoE-positive cells in the MS/VDB could be due, in principle, to either (*i*) a loss of the ability of living neurons to produce new and active AcChoE enzyme, or (*ii*) an actual death of AcChoE-producing (i.e., cholinergic) neurons, or both. The cresyl violet evidence for substantial neuronal death strongly supports the contention that cholinergic neurons do participate in the transection-induced neuronal death.

Of the total neurons in the MS and VDB that project to the hippocampus, ≈50% are cholinergic neurons (14, 15). Some noncholinergic neurons that project from the MS/VDB are γ-aminobutyric acid (GABA)ergic neurons (45, 46). From the areas of the MS/VDB complex sampled in the present experiments, 16% of the total MS neurons counted in normal animals were found to be cholinergic (i.e., AcChoE-positive) while 25% of the VDB neurons were cholinergic. Since about one-third of the cholinergic neurons were still observed by AcChoE histochemistry 2 weeks after transection, it is likely that fimbria fornix transection also causes the death of noncholinergic neurons. Gage *et al.* (19) estimated that at least 50% of the total neurons lost after the lesion were noncholinergic. The morphometric results of the present experiments indicate that, in the areas of the MS/VDB sampled, ≈80% of the total neurons lost were noncholinergic neurons. Indeed, Peterson *et al.* (47) have recently demonstrated significant loss of glutamic acid decarboxylase-positive neurons in both the MS and VDB after the fimbria fornix transection.

Neuronotrophic Effect of NGF. NGF is known to be a necessary NTF for developing peripheral neurons from sympathetic and dorsal root ganglion and continues to be an important factor for these neurons in their adult life, both *in vivo* and *in vitro* (48–53). NGF has also been reported to stimulate neuritic outgrowth from central nervous system adrenergic neurons *in vivo* (54), both cholinergic neurons of the ciliary ganglion (55) and adrenergic neurons of the sympathetic ganglion *in vitro* (56), and ChAT biochemical activity of cholinergic neurons from the central nervous system (basal forebrain and striatum) both *in vitro* and *in vivo* (33, 34, 45, 57, 58). However, there is so far no evidence indicating a survival-promoting effect of NGF on central nervous system noncholinergic neurons *in vitro* or *in vivo*.

The results presented here show that continuous infusion of exogenous NGF has a neuronotrophic effect on both noncholinergic and cholinergic neurons in the MS/VDB complex, in that it promotes the survival of most, if not all, of the neurons that would die after transection of the fimbria fornix. These findings strongly support the hypothesis that the death of the transected neurons is due to the loss of

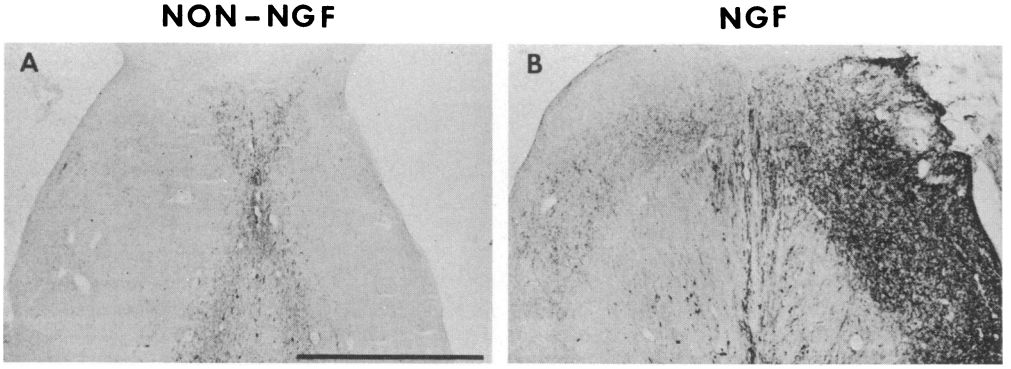

FIG. 4. Micrographs show AcChoE-stained sections of the caudal dorsal lateral septum 2 weeks after fimbria fornix aspiration and continuous infusion with cytochrome *c*. Note the apparent shrinkage of the septum in the non-NGF-treated animal (*A*) and the large accumulation of AcChoE-positive fibers only on the side of lesion and NGF treatment (*B*). (Bar = 1 mm.)

target-derived endogenous NTF(s). The present data, however, do not demonstrate that NGF need be *the* endogenous factor involved. Nevertheless, this appears to be one plausible interpretation since it is known that (*i*) NGF is synthesized in the hippocampus (31), and (*ii*) NGF can be retrogradely transported from the hippocampus specifically to afferent neurons in the MS and VDB (29, 30). It is not yet known whether this transport phenomenon is limited to a specific type of neuron in the MS or VDB.

The ability of NGF to protect noncholinergic neurons is susceptible to several interpretations related to several alternative possibilities underlying their death following fimbria fornix transection. It may be that NGF mimics an endogenous NTF(s) for noncholinergic neurons. On the other hand, NGF may act directly only on MS/VDB cholinergic neurons and, by preventing their death after transection, might limit an additional neuronal death due to (*i*) the transsynaptic degeneration of interneurons (59) or (*ii*) the release of toxic agents from otherwise dying neurons.

NGF-treated animals also displayed a striking amount of AcChoE activity in the dorsal lateral quadrant of the septum. One reasonable interpretation is that this AcChoE-positive staining was observed despite the preceding bis(1-methylethyl)phosphofluoridate treatment, only in NGF-treated animals and only ipsilateral to the lesion. We do not know at present whether this apparent cholinergic sprouting represents (*i*) regenerating axons from the spared NGF-treated neurons, (*ii*) a separate neurite-promoting effect of NGF on these neurons (cf. refs. 55 and 56), and/or (*iii*) axonal sprouts from undamaged cholinergic neurons stimulated by the NGF treatment.

Further investigation of this model will make possible the determination of the minimum effective dose of NGF, the critical time period of treatment initiation, and the duration of infusion required for the survival-promoting effect. It will also be important to determine (*i*) the mechanism(s) by which NGF prevents the death of the central nervous system cholinergic neurons; (*ii*) the effect of NGF on their transmitter-related function; (*iii*) which types of noncholinergic neurons in the MS/VDB region benefit from the NGF treatment and how they do so; (*iv*) whether NGF, in addition to its effects on neuronal survival, can stimulate collateral or regenerative axonal sprouting from the transected neurons; and (*v*) whether NGF can influence behavioral recovery from fimbria fornix lesions. In addition, experimental models of central nervous system dysfunction in aged animals, which may be related to degenerative changes in central cholinergic projection systems (60), can be used to ascertain whether administration of NGF, or other identified NTFs, via the continuous infusion cannula device can alleviate or prevent age-related functional impairments. Such studies may ultimately lead to clinical applications for treatment of neurodegenerative conditions in humans (12).

We gratefully thank Mr. H. Lee Vahlsing and Mr. Jan Berglund for their excellent technical assistance. This work was supported by National Science Foundation Grant BNS 85-01766 to S.V. and National Institute on Aging Grant AG 06088 to F.H.G.

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