Mouse Nerve Growth Factor Prevents Degeneration of Axotomized Basal Forebrain Cholinergic Neurons in the Monkey

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NGF, a trophic polypeptide, is necessary for the normal development and survival of certain populations of neurons in the CNS and PNS. In the CNS, cholinergic neurons of the basal forebrain magnocellular complex (BFMC) are prominent targets of NGF. During rat development, NGF increases the activity of ChAT in these neurons. In adult rats with experimental injury of axons in the fimbria–fornix, NGF prevents degenerative changes in axotomized cholinergic BFMC neurons in the medial septal nucleus (MSN). Because the amino acid sequences of NGF and its receptor (NGF-R) are highly conserved across species, we hypothesized that mouse NGF would also prevent degeneration of cholinergic BFMC neurons in nonhuman primates. Therefore, the present study was designed to test whether fimbria–fornix lesions result in retrograde degenerative changes in basal forebrain cholinergic neurons in macaques, whether these changes are prevented by mouse NGF, and whether the protective effect of NGF is selective for cholinergic neurons of the basal forebrain. Following unilateral complete transection of the fornix, animals were allowed to survive for 2 weeks, during which time half of the subjects received intraventricular NGF in vehicle and the other half received vehicle alone. In animals receiving vehicle alone, there was a 55% reduction in the number of ChAT-immunoreactive cell bodies within the MSN ipsilateral to the lesion; loss of immunoreactive somata was more severe in caudal planes of the MSN. Remaining immunoreactive neurons appeared smaller than those in control, unoperated animals. In Nissl stains, there was no apparent loss of basophilic profiles in the MSN, but cells showed reduced size and intensity of basophilia. Treatment with NGF almost completely prevented reductions in the number and size of cholinergic neurons and had a significant general effect in preventing atrophy of basophilic magnocellular neurons of the MSN, though some basophilic neurons in the MSN did not appear to respond to NGF. Adjacent 7-μm-thick sections stained with ChAT and NGF-R immunocytochemistry revealed that these markers are strictly co-localized in individual neurons in the MSN in controls and in both groups of experimental animals. Thus, mouse NGF profoundly influences the process of axotomy-induced retrograde degeneration in cholinergic BFMC neurons in primates. The in vivo effectiveness of mouse NGF on primate BFMC neurons suggests that mouse or human recombinant NGF may be useful in ameliorating the ACh-dependent, age-associated memory impairments that occur in nonhuman primates. Such experiments will prove essential for the design of strategies for use of trophic factors in human diseases associated with degeneration of basal forebrain cholinergic neurons.

In the CNS and PNS, certain populations of neurons are dependent for their normal development and survival on NGF, a 13-kDa peptide (Korsching et al., 1985; Thoenen et al., 1987; Whittemore and Seiger, 1987; Mobley et al., 1989). Projection targets of these neurons express NGF mRNA and protein (Korsching et al., 1985; Shelton and Reichardt, 1986; Ayer-LeLievre et al., 1988). At target fields, NGF is taken up by high-affinity NGF receptors (NGF-R) on nerve terminals (Greene and Shooter, 1980; Taniuchi et al., 1986; Stach and Perez-Polo, 1987), and the complex of NGF with its receptor is transported retrogradely to neuronal cell bodies (Seiler and Schwab, 1984, Johnson et al., 1987), where it has a number of actions, including apparent enhancement of cell viability (Hefti et al., 1988). In the CNS, cholinergic neurons of the basal forebrain magnocellular complex (BFMC) are the main targets of NGF. During development, NGF increases levels of ChAT activity in rat BFMC neurons (Gahn et al., 1983; Hefti et al., 1985; Mobley et al., 1986; Gathwiler et al., 1987; Johnston et al., 1987; Martinez et al., 1987) and the expression of a variety of genes, including those coding for the prion protein and the amyloid precursor protein (Mobley et al., 1988). In adult rats with fimbria–fornix lesions, NGF treatment prevents the axotomy-induced degenerative changes that occur in BFMC cells (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Gage et al., 1988; Rosenberg et al., 1988; Whittemore et al., 1989).

To date, there has been no direct examination of the actions of NGF in nonhuman primates. However, several indirect lines
of evidence suggest that BFMC neurons of primates are capable of responding to NGF. The amino acid sequence of NGF, including 1 hydrophilic domain around residue 33 (glycine) implicated in the binding of NGF to its receptor, is highly conserved across species (Angeletti and Bradshaw, 1971; Dunbar et al., 1984; Meier et al., 1986). NGF-R, also highly conserved (Johnson et al., 1986), is expressed in BFMC neurons of primates (Hefti et al., 1986; Schatteman et al., 1988). Because of the conservation of NGF and NGF-R, it is likely that mouse NGF can act upon primate neurons that express NGF-R. Consistent with this prediction is the preliminary observation that developing neurons of the human dorsal root ganglia respond to mouse NGF (Caviedes and Rapoport, 1988).

To test the potential for NGF to ameliorate the effects of BFMC cell injury in primates, we used a well-established, simple model: transection of axons of BFMC neurons in the septohippocampal pathway. These axons originate in cholinergic and other neurons of the medial septal nucleus (MSN) and nucleus of the diagonal band of Broca (DBB) and course to hippocampal targets predominantly in the fornix (Swanson and Cowan, 1979), a dorsally coursing discrete bundle that is easily accessible to experimental manipulations (Fig. 1A). In the rat, following transection of the fornix, neurons of the MSN show reductions in cell size, decrements in cholinergic markers (AChE histochemical activity and ChAT immunoreactivity), and alterations in elements of the neuronal cytoskeleton (Daitz and Powell, 1954; McLaray, 1955; Sofroniew et al., 1983, 1987; Pearson et al., 1984; Gage et al., 1986; Hefti, 1986; Armstrong et al., 1987; Koliatsos et al., 1989a). Eventually, there is evidence of cell loss (Gage et al., 1986; Armstrong et al., 1987; Applegate et al., 1989; Koliatsos et al., 1989a; O'Brien et al., 1990; Tuszynski et al., 1990). The present study demonstrates that similar events occur in macaque monkeys following transection of BFMC axons in the fornix, and that mouse NGF prevents the axotomy-induced degenerative changes induced in these cholinergic BFMC neurons.

Preliminary data from this study have been presented in abstract form (Koliatsos et al., 1989b).

Materials and Methods

Surgery. Cynomolgus monkeys (Macaca fascicularis; n = 10; weight, 3–7 kg) were used as subjects in this study. Seven animals were anesthetized with halothane, intubated, and artificially ventilated, 3 animals recovered without complications.

NGF administration. Mouse NGF was prepared by ion-exchange chromatography and characterized by gel electrophoresis and by a chick dorsal root ganglion bioassay as described previously (Mobley et al., 1986). Prior to use, NGF was passed through a 0.2-μm filter (Uniflo, Schchueler and Schueler, Keene, NH) and stored at 200 μg/ml in 0.2% acetic acid at −20°C. NGF was lyophilized and resuspended in acidified cerebrospinal fluid (CSF; see below) immediately prior to the intraventricular injection.

Following surgery, NGF dissolved in vehicle (n = 3), or vehicle alone (n = 3), was injected immediately and then every second day under aseptic conditions into the Silastic reservoir of the ventricular access device (625 μg per injection for a total of 8 injections, resulting in a total dose of 5 mg). Vehicle consisted of roughly 300 μl acetic acid-acidified artificial CSF. pH 7.4, followed by roughly 200 μl of a 0.1 M solution of 5% 5-bromo-4′-chloro-3-indolyl-β-D-galactoside (X-Gal). This device (625 ILR per injection for a total of 8 injections, resulting in a total dose of 5 mg). NGF-R immunocytochemistry (40 μm), using the monoclonal antibody 6-17, and the concentration of mouse peroxidase-antiperoxidase was 1:20, and the concentration of mouse peroxidase-antiperoxidase was 1:20. The purpose of this dual immunocytochemical protocol was to characterize the lesion, sections through the fornix stained with Cresyl violet and with immunocytochemistry for the phosphorylated neurofilament epitope 6-17. Sections through the hippocampus were processed for CHAT immunocytochemistry or AChE histochemistry using a silver intensification of the Tsuji reaction (Tsuji, 1974). In selected animals from all 3 groups, pairs of adjacent 7-μm-thick sections (200 μm apart; on average, 10 pairs per monkey brain) were processed for NGF-R and CHAT immunocytochemistry on slides. Procedures were essentially the same as with the flocculating sections, with the following exceptions: incubations were done at room temperature, concentrations of primary antibodies were 1:25 for CHAT and 1:1000 for NGF-R, the concentration of linking antibody (goat anti-mouse) was 1:20, and the concentration of mouse peroxidase-antiperoxidase was 1:100. The purpose of this dual immunocytochemical protocol was to examine the degree of concomitant expression of CHAT and NGF-R immunoreactivity in single neurons of the MSN under normal conditions and following axotomy with or without NGF treatment.
Figure 1. Diagram of location and type of lesion and implantation of infusion device. A. The anatomy of the septohippocampal system and fornix in the monkey is depicted. The lesion was placed in the body of the fornix (arrow). The number at the top of the arrow represents the distance (in mm) from the interaural line. B. A lateral callosotomy permits access to the right half of the body of the fornix (panel 1), which is subsequently transected at the coronal plane together with perforating fornical branches in the corpus callosum (panel 2, shaded area). Subsequently, the cannula of the vehicular access device is implanted in the ipsilateral lateral ventricle through the window of the callosotomy (panel 3). C. Three-dimensional rendering of the callosotomy-fornical lesion; see text and B, panels 1 and 2.
Figure 2. Standard coronal planes through MSN, where quantitative cell data were obtained. In all planes, a solid line indicates the medial plane and outlines various components of the BFMC. Scale bars, 1 mm. a-d. ac, anterior commissure; BNST, bed nucleus of stria terminalis; fo, fornix (precommissural); LS, lateral septum; MSN, medial septal nucleus; NA, nucleus accumbens; NBM, nucleus basalis of Meynert; NDBB, nucleus of DBB; OT, olfactory tubercle; PA, preoptic hypothalamic area; VP, ventral pallidum. a. In this rostralmost plane, the MSN is continuous with the nucleus of the DBB. The horizontal dashed line, which passes through the border of the middle with the lower third of the nucleus accumbens, demarcates the MSN from the nucleus of the DBB. b. In this plane, the MSN is readily separated from the nucleus of the DBB (horizontal dashed line). c. In this plane, immediately rostral to the commissural decussation, the MSN is entirely separate from the nucleus of the DBB. The rectangle outlined with the dashed line denotes the area quantitated in Nissl sections (same method was followed for all planes, a–d). d. Plane midlevel through the decussation of the anterior commissure, representing the caudalmost plane at which quantitative MSN data were collected.
Figure 3. Efficacy of lesion. A profound reduction in AChE staining in the hippocampus ipsilateral to the lesion (a) as compared to the control side (b) is shown. The comparison confirms the cholinergic deafferentation of the hippocampus and, accordingly, the completeness of the transection of cholinergic hippocampopetal axons originating in the MSN and nucleus of the DBB. Some persistence of AChE staining in the dentate granule cell layer represents perikaryal reaction. AChE-positive terminal axons of the BFMC are depleted in the molecular and polymorph layers. Moderate AChE histochemical staining in the prosubiculum is believed to derive from cholinergic innervation of the hippocampus via a ventral BFMC efferent pathway (see text). Scale bars, 1 mm, a and b. CA1–CA4, Lorente de No's fields of Ammon's horn; Pus, parasubiculum; PreS, presubiculum; Pros, prosubiculum; S, subiculum.

Abercrombie's adjustment for split-cell error (Abercrombie, 1946). This correction was chosen after considering several more recent stereological methods that are more appropriately applied to less complex systems (Gundersen et al., 1988a,b). Mean neuronal area was calculated independently and compared to neuronal area at the corresponding level in control, unlesioned animals. For neuronal number, a repeated-measured analysis of variance (ANOVA; BMDP 2V program) was applied with surgical procedure (lesion/vehicle or lesion/NGF) as the main factor and level of section as the repeated measure. Duncan's multiple range test was used for post hoc analysis of group differences.

To calculate the percentage of dual-labeled (ChAT and NGF-R) profiles in the MSN in representative cases of vehicle- and NGF-treated animals and controls, initial maps of ChAT- and NGF-R-immunoreactive profiles of the MSN in adjacent sections were generated with the aid of a neuroanatomical mapping system (software provided by Dr. Mark E. Molliver, The Johns Hopkins University School of Medicine); 5 (7-μm-thick) pairs of sections were used per animal. Subsequently, mapped ChAT-immunostained sections were superimposed on corresponding adjacent sections stained for NGF-R by overlaying the respective glass slides and carefully matching outlines of sections under the microscope. Sections were studied under 20× magnification. Using visual clues provided by vessels and spatial arrangement of cell groups, dual-labeled cells were identified and marked on maps of cholinergic and NGF-R-containing neurons generated from the same sections. Dual-labeled neurons were expressed as percentages of the total number of ChAT- and NGF-R-immunoreactive MSN neurons from all 5 pairs of sections analyzed per animal.

Results

Evaluation of NGF treatment

In CSF samples collected from NGF-treated animals throughout the period of treatment, NGF was detected at CSF concentrations ranging from 2 to 150 μg/ml. In pretreatment samples, the concentration of NGF was below level of detectability by the assay (100 ng/ml). Samples taken at the end of the treatment period tended to have higher concentrations of NGF. NGF concentration was roughly proportional to the length of treatment. Because the ventricular access devices were cleared with CSF at the end of each individual treatment, the upward trend in NGF concentration suggested that NGF levels within the ventricular system increased over time, though the rate and degree of CSF clearance might have been variable.

Efficacy of the lesion

In all monkeys, there was a profound reduction in levels of AChE histochemical activity and ChAT immunoreactivity in all hippocampal sectors ipsilateral to the lesion throughout the anteroposterior extent of the hippocampal formation (Fig. 3). The prosubiculum showed moderate levels of AChE and ChAT, but, as reported elsewhere (Kitt et al., 1987), this region is innervated, besides the fornix, by a ventral pathway originating in the nucleus basalis and coursing in the ansa peduncularis; this pathway was not damaged by our manipulations. Our immunocytochemical and histochemical preparations of the hippocampus confirmed the efficacy of the transections, and tissues from all these subjects were taken for quantitative analysis of retrograde changes in neurons of the MSN.

Retrograde changes in the MSN

The septohippocampal system is topographically organized along both the mediolateral and the rostrocaudal axes, with MSN neurons utilizing the fornix exclusively for their hippocampal projections, whereas neurons in the nucleus of the DBB partially project through ventral routes (Swanson, 1976; Kitt et al., 1987; Koliatsos et al., 1988). Therefore, following lesions of the fornix, we focused on retrograde changes in the MSN and effects of NGF on these cholinergic neurons of the BFMC.

In monkeys treated with vehicle, numbers of ChAT- and NGF-R-immunostained cells ipsilateral to the lesion were re-
Produced to roughly 55% of neurons in the contralateral MSN. Remaining immunoreactive neurons were, on average, smaller than cholinergic MSN neurons from control animals (Figs. 4, 6). In Nissl stains, MSN neurons on the lesioned side showed reduced basophilia and a 10% reduction in size (Figs. 5, 7). All of these abnormalities were more severe in caudal planes of the MSN. Based on Nissl stains, there was no apparent loss of neurons on the lesioned side. In fact, morphometric analysis showed a statistically insignificant trend towards increased numbers of neurons. Because the calculated increase in basophilic profiles on the lesioned side (~10%) was equal to the average cell shrinkage on the same side, this unexpected difference was...
considered to be caused by reduction in the total area of the MSN on the lesioned side. Some magnocellular MSN neurons contralateral to the lesion also showed evidence of mild atrophy.

In monkeys treated with NGF, there was a dramatic amelioration of the retrograde changes described above. No significant reductions were noted in the number of ChAT- and NGF-R-immunoreactive perikarya on the side of the axotomy. These cells had a normal shape, and their size was, on average, 30%
Figure 6. Number of ChAT-immunoreactive MSN neurons ipsilateral to lesion expressed as percentage of contralateral (unlesioned) side for vehicle- and NGF-treated groups, per plane of section and overall (A). When all quantitated cells on the lesioned side in the vehicle- and NGF-treated groups were analyzed, the difference was statistically significant by ANOVA (p = 0.0124). When the size of the total number of ChAT-immunoreactive neurons was analyzed by ANOVA (B), the size of ChAT-positive cells in the control and vehicle- and NGF-treated groups differed significantly (p = 0.0209), but no significant stepwise differences could be detected by Duncan’s multiple range test. Per-plane analysis indicated that cholinergic MSN cells in the NGF-treated group were significantly larger than cells in the vehicle-treated group, both rostrally (p = 0.01843) and caudally (p = 0.03554), with Duncan’s post-hoc analysis. C, control; V, vehicle-treated; NGF, NGF-treated. Vertical bars on columns indicate SEM.

Figure 7. Number of basophilic profiles on side ipsilateral to lesion expressed as percentage of contralateral (unlesioned) side for vehicle- and NGF-treated groups, per plane of section and overall (A). When all quantitated cells on the lesioned side of the vehicle- and NGF-treated groups were analyzed, no statistical significance was shown. When the size of basophilic profiles in these sections was considered per plane (B, left), the only significant difference was present between cells in the caudal plane of the control and vehicle-treated subjects (p = 0.02275, Duncan’s multiple range test). When all levels are grouped and considered with ANOVA (B, right), the size of basophilic profiles in the control and vehicle- and NGF-treated groups differed significantly (p = 0.0475), but no significant stepwise differences could be detected by Duncan’s multiple range test. C, control; V, vehicle-treated; NGF, NGF-treated. Vertical bars on columns indicate SEM.
Figure 8. Nissl-stained section (10 μm thick), taken from plane c of Figure 2 in lesioned/NGF-treated monkey. Asterisk indicates lesioned side. Although all stained perikarya on the unlesioned side appear healthy, only a subpopulation of cells on the side of the lesion have normal shape and size (arrows). A number of cells on the lesioned side are atrophic (circles). Scale bar, 60 μm.

Figure 9. Two adjacent 7-μm sections, taken through plane c of Figure 2, are illustrated. One section was immunostained with ChAT antibodies (a), and the other was stained with antibodies directed against NGF-R (b). All cholinergic perikarya (a) are also immunoreactive for NGF-R (b; examples are indicated by arrows). Note that only cholinergic cells contain the receptor. Asterisks indicate vessels. Scale bar, 60 μm.

Discussion

Our results indicate that mouse NGF has significant biological effects on primate CNS neurons in vivo and can effectively prevent the progressive degenerative changes that occur in BFMC cholinergic neurons following transection of their axons in the fornix. The significance of the NGF effect on primate neurons is 3-fold: heterologous (mouse) NGF is effective on BFMC cholinergic neurons in the fornix, the same patterns of NGF-mediated trophic influences appear to exist in species with a much more complex forebrain than the rat, and similar NGF therapy may have benefits for animal and human disorders that show degeneration of cholinergic cells of the BFMC.

The septohippocampal system—a term mainly used in the literature dealing with rodents—is a component of the basal forebrain—telencephalic projection that originates predominantly from neurons in the MSN and projects primarily via the fornix to hippocampus (Swanson et al., 1987). In the monkey, the system is organized in a similar fashion: axons arise from neurons of the BFMC, situated mostly in the MSN and nucleus of the DBB, and project via the fornix and the fimbria to hippocampal targets (Fig. 1A). Approximately 70% of these neurons are cholinergic, whereas the majority of other cells presumably contain GABA (Koliatsos et al., 1988). In the rat, BFMC axons can reach the hippocampus by routes outside the fimbria—fornix, including the dorsal fornix (Wyss et al., 1980), the cingulate bundle/supracallosal striae (Swanson and Cowan, 1979; Milner et al., 1983), and a less well-defined ventral pathway, containing roughly 10% of septohippocampal axons (Gage et al., 1984; Milner and Amaral, 1984). In primates, as in rats, there is a ventral pathway, but the majority of these fibers originate in the nucleus basalis and nucleus of the DBB, rather than the MSN (Kitt et al., 1987; Koliatsos et al., 1988). Dorsal pathways outside the fornix do not contribute significantly to the cholinergic innervation of hippocampus in the monkey (Rosene and Van Hoesen, 1987); the monkey does not have a distinct dorsal fornix (Rosene and Van Hoesen, 1977), and it is unlikely that the supracallosal striae, sometimes termed the dorsal fornix...
Figure 10. In lesioned/NGF-treated animals, some MSN perikarya contain phosphorylated neurofilaments. Both perikarya depicted in this illustration belong to the anterior MSN. One MSN neuron is stained with antibody 6-17 (a), and the other MSN cell reacts with the neurofilament antibody 7-05 (b). In b, note intense immunoreactivity in a dendrite and perikaryodendritic junction (arrows). Scale bar, 20 μm.
animals and in both experimental groups. Although nerve cells that respond to NGF are bound to express NGF-R, it is conceivable that low levels of NGF-R expression may prevent immunocytochemical detection of all NGF-R-containing neurons. However, in view of the fact that NGF upregulates the expression of NGF-R in cholinergic neurons of the BFMC (Higgins et al., 1989), the correspondence of ChAT and NGF-R immunoreactivity, especially in the NGF-treated group of animals, suggests strongly that only cholinergic MSN neurons bear the NGF-R and respond to NGF.

As discussed above, the majority of noncholinergic cells of the MSN and nucleus of the DBB contain GABA. GABAergic cells comprise at least 30% of the BFMC cells projecting to the hippocampus (Köhler et al., 1984), and their axons selectively contact inhibitory interneurons in the hippocampus (Freund and Antal, 1988). The magnitude and target specificity of this inhibitory component of the septohippocampal projection suggest that GABAergic septal neurons have a major functional significance in this system (Freund and Antal, 1988). There is disagreement as to whether GABAergic neurons of the BFMC, identified with immunocytochemistry for GABA or glutamic acid decarboxylase, have NGF-R and respond to NGF. Although Dreyfus and co-workers (Dreyfus et al., 1989) have indicated that these nerve cells bear the NGF-R in vitro, in vivo studies did not show sepal GABAergic neurons to respond to NGF (Montero and Hefth, 1988). Definitive conclusions on patterns of retrograde degeneration and effects of NGF on GABAergic septal neurons, especially in the monkey, may require in vivo hybridization histochemistry for glutamic acid decarboxylase transcripts (Walker et al., 1989).

NGF treatment of animals with lesions of the septohippocampal system may partially restore innervation of deafferented terminal fields (Haroutunian et al., 1986) and may, at least transiently, ameliorate behavioral deficits related to hippocampal denervation (Will and Hefth, 1985). Moreover, NGF has been reported to have effects on age-associated deficits in behaviors dependent on the septohippocampal circuit (Fischer et al., 1987), perhaps by ameliorating degenerative age-related alterations that occur in BFMC neurons. A beneficial effect of NGF on behavior suggests its use in future experiments involving aged, memory-impaired monkeys (Bartus et al., 1979, 1980; Davis, 1985; Presty et al., 1987; Phelps et al., 1989a, b; Bachevalier et al., 1991). This view is further supported by the fact that NGF can upregulate the expression of NGF-R (Higgins et al., 1989), a phenomenon that could serve to enhance further the responsiveness of injured cells to the exogenously supplied trophic factor. If NGF proves effective and nontoxic when chronically administered to nonhuman primates, all of the conditions (Phelps et al., 1989a, b) will have been met for consideration of a carefully designed trial of NGF therapy in individuals with Alzheimer’s disease, a disorder in which there is consistent degeneration of BFMC cholinergic neurons (Bown et al., 1976; Perry et al., 1977, 1982; Davies, 1979; Whitehouse et al., 1982; Arendt et al., 1983; Francis et al., 1985; Price, 1986).

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