FINAL TECHNICAL REPORT

1991

N00014-90-J-1116

The Involvement of the Immune System in Neuronal Plasticity

The two projects described below comprise the major efforts during the one year duration of this contract.

I. *Cholinergic Sprouting in the Hippocampus Following Perforant Path Damage: A Role for Interleukin-1.* Fagan, A.M, and Gage, F.H.

Damage to the entorhinal afferents to the hippocampal dentate gyrus (i.e. perforant path) leads to sprouting of the remaining intact septal cholinergic afferents within the denervated outer molecular layer. To investigate the cellular and molecular events which contribute to this sprouting response we describe the temporal sequence of cellular changes in the denervated zone prior to the observed neural reorganization. Rats were given perforant path (PP) transections and sacrificed at various time points following the lesion, on Days (D) 1,2,3,4,5,6, and 30. Coronal sections at the level of the dorsal hippocampus were immunostained to localize microglia (OX-42), interleukin-1 (IL-1) and astrocytes (GFAP). We observed a rapid increase in the number of microglia in the denervated molecular layer within the first day following PP transection. Adjacent sections show a concomitant increase in the number of IL-1- positive cells. Reactive changes in GFAP-positive astrocytes are not observed Until D4. This time course of events suggests a role of microglia in astrocyte activation in vivo via production of IL-1, and supports a proposed hypothesis postulating a cascade of glial events which lead to cholinergic sprouting following PP transection.

II. *Fibroblast Growth Factors Stimulate Nerve Growth Factor Synthesis and Secretion by Astrocytes.* Yoshida, K., and Gage, F.H.

Nerve growth factor (NGF) is produced and secreted by astrocytes and fibroblasts, but not by microglia, in a primary non-neuronal cell culture derived from newborn rat brains under a standard culture

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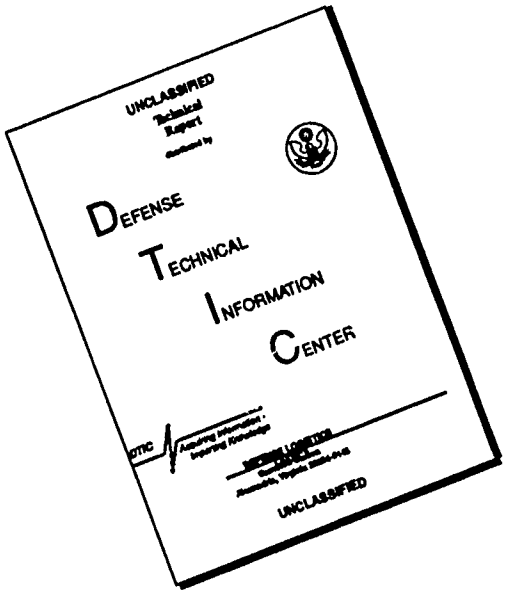

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condition. NGF secretion by astrocytes was highest just after passage and then gradually decreased. There is no significant difference in NGF secretion by astrocytes from five sites of origin tested: cerebral cortex, striatum, hippocampus, septum, and cerebellum. Acidic and basic fibroblast growth factors (aFGF and bFGF) significantly increased NGF secretion by astrocytes. The effect of aFGF was greater than that of bFGF, and the effect of both FGFs was not additive at the maximum concentration. The peak of NGF secretion stimulated by aFGF occurred 3-12 h after the addition of aFGF. On the other hand, the dramatic increase in cell numbers was observed 12-48 h after stimulation, and the morphological change became significant 24 h after aFGF stimulation. NGF synthesized by astrocytes is rapidly secreted into the culture medium and aFGF enhances NGF secretion from the transcription level, because cycloheximide and actinomycin-D completely inhibited NGF secretion by astrocytes in the presence or absence of aFGF. Epidermal growth factor (EGF), interleukin-1β (IL-1β), and tumor necrosis factor-a (TNF-a) also increased NGF secretion by astrocytes to a certain extent. NGF secretion by astrocytes in the presence of a maximum dose of aFGF was enhanced by the addition of IL-1β or TNF-a, but not EGF. However, platelet-derived growth factor, interleukin-3, and interleukin-6 had no significant effects. FGFs also enhanced NGF secretion by fibroblasts derived from meninges, but not by microglia.

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Fagan, A.M. and Gage, F.H. Cholinergic sprouting in the hippocampus: A proposed role for IL-1. *Experimental Neurology* 110: 105-120, 1990.

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Cholinergic Sprouting in the Hippocampus: A Proposed Role for IL-1

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Damage to the entorhinal afferents (i.e., perforant path) to the hippocampal dentate gyrus leads to sprouting of the remaining intact septal cholinergic afferents within the denervated outer molecular layer. To investigate the cellular and molecular events which may contribute to this sprouting response, we describe the temporal sequence of cellular changes in the denervated zone prior to the observed neural reorganization. Rats were given perforant path (PP) transections and sacrificed at various time points following the lesion, on Days (D) 1, 2, 3, 4, 5, 6, and 30. Coronal sections at the level of the dorsal hippocampus were immunostained to localize microglia (OX-42), interleukin-1 (IL-1), and astrocytes (GFAP). We observed a rapid increase in the number of immunoreactive microglia in the denervated molecular layer within the first day following PP transection. Parallel sections show a concomitant increase in the number of IL-1-positive cells. Maximal reactive changes (i.e., hypertrophy and increase in number) in GFAP-positive astrocytes are not observed until D5. This time course of events suggests a role of microglia in astrocyte activation *in vivo* via production of IL-1 and offers support for a proposed hypothesis postulating a cascade of glial events which may lead to cholinergic sprouting following PP transection. © 1990 Academic Press, Inc.

INTRODUCTION

Damage to the adult CNS initiates a variety of morphological changes in neural organization. Controversy exists, however, as to whether these "plastic" responses result in a restoration of normal circuitry leading to functional recovery or represent a disorganized rearrangement of neural inputs. Disorganized or aberrant reinnervation could theoretically play a compensatory role in recovery of function or, conversely, may exacerbate damage-induced dysfunction. In either case, the mechanisms responsible for neural reorganization are not understood. To investigate the cellular and molecular events associated with damage-induced reorganization, we have used the rat hippocampus as a model system.

Damage to the entorhinal inputs (i.e., perforant path) to the hippocampal dentate gyrus leads to robust and well-characterized sprouting responses by the remaining intact afferents: (i) The commissural and associational projections, which normally innervate the inner molecular layer (ML) of the dentate gyrus, expand their terminal fields into the denervated outer ML after entorhinal lesions (1, 7, 46); (ii) the crossed temporoammonic tract, which sends a minor projection to the outer ML of the dentate gyrus (27), increases in density after entorhinal damage (63); and (iii) there is an increase in acetylcholinesterase (AChE) content (64) and staining intensity in the normally sparse septal afferents terminating in the outer ML, suggesting an increase in the number of septal fibers or terminals (6, 44, 61). Ultrastructural studies have confirmed an increase in density of terminals over time in the deafferented zone following entorhinal damage (37, 48, 49). We have focused on cholinergic sprouting in the outer ML.

In addition to neuronal events, changes in glial cell populations have been observed in the outer molecular zone during degeneration of the perforant path (PP) and the subsequent sprouting of the septal cholinergic fibers following damage. Nissl stains have shown an increase in the number of glial cells in this area over time (45), with the increased incidence of cells at the early time points being attributed to the proliferation of the local microglial population (19, 70). In addition, others have reported astrocyte hypertrophy (45, 58) and increase in number (18) following deafferentation. We speculate that glial cell activation is necessary for the cholinergic sprouting responses observed after PP damage. These cellular events may furthermore provide clues as to the identity of the specific molecules involved in neural reorganization.

Trophic factors have been postulated to play a role in cholinergic sprouting. Recent evidence has shown that nerve growth factor (NGF)-like activity is increased in the dentate gyrus following PP lesions (8), and NGF-receptor-positive fibers show patterns of lesion-induced reorganization similar to those of the sprouting septal fibers visualized with AChE histochemistry (28). Therefore, we suggest that the cholinergic sprouting observed in the outer ML may be elicited by an increase in NGF activity in this zone. Astrocytes have been reported to produce NGF as well as other neurotrophic and neurite-promoting factors *in vitro* (14, 17, 30, 38, 40, 51, 69, 71). Others have shown that IL-1 regulates the synthesis of

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TABLE 1

Description and Immunostaining Parameters of the Primary and Secondary Antibodies

Antibody	Source	Type	Known specificity	Dilution and incubation
1° GFAP	Gift from L. F. Eng	Polyclonal rabbit IgG against GFA protein from human MS plaques	Recognizes human and rat GFAP	1:1000, 24 h at 4°C
2°	Vector Labs	Biotinylated goat anti-rabbit IgG	Recognizes rabbit IgG	1:200, 1 h at RT
1° OX-42	Serotec	Monoclonal mouse IgG against membrane polypeptide of rat resident peritoneal macrophages	Recognizes complement receptor CR-3 on most rat macrophages, including resident peritoneal and activated macrophages, Kupffer cells, dendritic cells, granulocytes, and brain microglia	1:100, 48 h at 4°C
2°	Vector Labs	Biotinylated horse anti-mouse IgG	Recognizes mouse IgG	1:150, 1 h at RT
1° IL-1	Genzyme	Monoclonal mouse IgM against naturally derived human IL-1	Recognizes human sinus macrophages and B cells, and murine keratinocytes and monocytes	1:100, 48 h at 4°C
2°	Vector Labs	Biotinylated goat anti-mouse IgM	Recognizes mouse IgM (μ chain specific)	1:150, 1 h at RT
1° NGF-r	Gift from E. Johnson	Monoclonal mouse 192-IgG against plasma membrane proteins of PC12 cells	Recognizes rat NGF-receptor	1:100, 24 h at RT
2°	Vector Labs	Biotinylated horse anti-mouse IgG	Recognizes mouse IgG	1:150, 1 h at RT

NGF in nonneuronal cells of the damaged rat sciatic nerve (39) and that IL-1 is most likely secreted from activated macrophages in the vicinity of the damaged sciatic nerve (32). Activated microglia from the CNS produce IL-1 *in vitro* (23, 31), and IL-1 stimulates astrocyte proliferation *in vitro* (21, 23, 24) and *in vivo* (25). More recently, fibroblast growth factors and IL-1 have been shown to enhance production of NGF by rat astrocytes *in vitro* (K. Yoshida and F. Gage, unpublished observations).

Taken together, these results demonstrate (i) glial activation in the deafferented outer molecular zone after PP damage, (ii) a role for IL-1 in the proliferation of astrocytes, (iii) that activated microglia and macrophages can secrete IL-1, and (iv) that IL-1 can activate NGF synthesis in astrocytes and other nonneuronal cells. On the basis of these findings we make the following suggestion for the course of events that leads to the cholinergic sprouting response in the dentate gyrus following deafferentation (18): Perforant pathway damage induces terminal degeneration from the cells that are transected in the entorhinal cortex. This terminal degeneration activates microglia to phagocytize in the restricted deafferented zone. Activated microglia produce and subsequently release IL-1 into the surrounding environment, which in turn induces the activation of astrocytes. The activated astrocytes then secrete NGF into this denervated region, which results in the attraction of NGF-responsive cholinergic fibers. As initial support for this working hypothesis, the present study outlines the temporal relations between glial cell activation after PP lesions and proposes a role for IL-1 in the damaged CNS.

METHODS

Subjects. Seventy-two adult female Sprague–Dawley rats weighing between 225 and 275 g were used. Animals were deeply anesthetized with a mixture of ketamine (75 mg/kg), Rompun (4.0 mg/kg), and acepromazine (5.6 mg/kg) and placed in a Kopf stereotaxic apparatus. Each rat received a unilateral aspirative lesion of the retrosplenial cortex, which resulted in the transection of the perforant path. Animals were sacrificed at 1, 2, 3, 4, 5, 6, and 30 days (D1–D6, D30) following the lesion. At least four animals were used in each of the time points. Four additional animals were used as unoperated controls.

Histology. Rats were deeply anesthetized and perfused through the heart with 100 ml of 0.9% saline followed by 250 ml of cold 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB; pH 7.4). The brains were removed from the skull, postfixed overnight, and then immersed in 30% sucrose in PB for 2 days. Tissue sections were cut at 35 μm in the coronal plane on a freezing sliding microtome and stored in cryoprotectant (glycerol, ethlyene glycol, and PB) until processing. For each animal, adjacent sections through the level of the dorsal hippocampus were processed for AChE histochemistry according to a modified Karnovsky–Roots method or stained with cresyl violet to visualize cell bodies or with

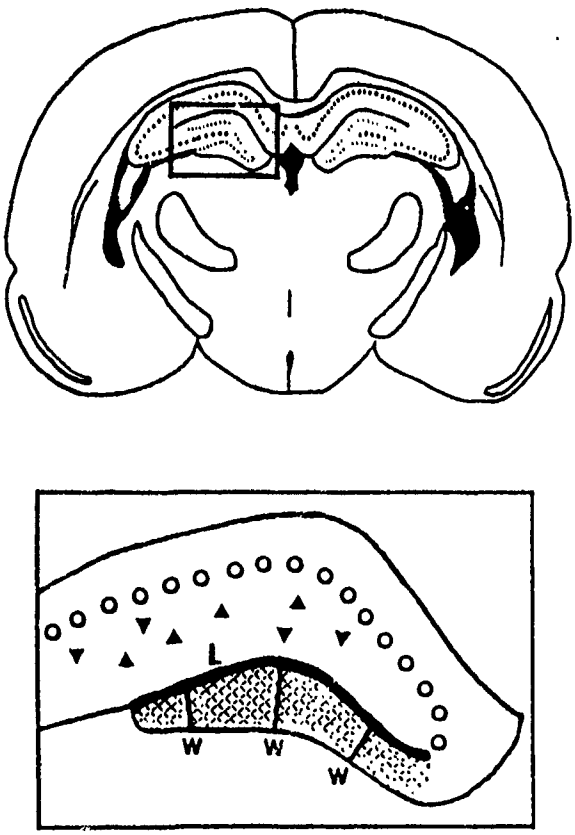

FIG. 1. Schematic illustration of the hippocampus at the level of analysis and the ventral leaf of the dentate gyrus (DG) (inset) in which all measurements were obtained. Gross dimensional measurements were obtained from one representative coronal section stained with cresyl violet from each animal. Length (L) corresponds to the medial lateral length (in μm) of the dentate granule cell layer providing the dorsal boundary of the examined molecular layer (ML). Width (W) corresponds to the dorsal ventral width of the ML (in μm). Mean width values were calculated from three positions along the ventral leaf of the DG. Area (crosshatch) corresponds to the area of the ML in the coronal plane (in μm^2). This area provides the boundaries within which all cellular analyses were performed.

antibodies recognizing glial fibrillary acidic protein (GFAP), microglia (OX-42), interleukin-1 (IL-1), or NGF-receptor (NGF-r). Procedures for peroxidase immunohistochemistry were as follows (i) 2 × 10 min rinse in 0.1 *M* Tris-buffered saline (TBS, pH 7.4) to remove any cryoprotectant; (ii) 30-min incubation in 0.6% H_2O_2 in TBS to block endogenous peroxidase activity; (iii) 2 × 10 min rinse in TBS containing 0.25% Triton X-100 detergent (TBS-X); (iv) incubation in primary antibody in TBS-X with 3% secondary host serum for appropriate time (Table 1); (v) 2 × 10 min rinse in TBS-X with 1% secondary host serum (S); (vi) incubation in biotinylated secondary antibody in TBS-X-S for 1 h at room temperature; (vii) 2 × 10 min rinse in TBS-X-S; (viii) incubation in avidin D-peroxidase complex (1:100, Vector Labs) for 1 h at room temperature; (ix) 2 × 10 min rinse in TBS; and (x) chromagen development in 0.025% 3-3-diaminobenzidine tetrachloride (DAB) with 0.4% $NiCl_2$ and 0.09% H_2O_2 in TBS for 5–15 min. After processing, sections were rinsed, mounted onto gelatin-coated slides, dried overnight, dehydrated through graded alcohols, and coverslipped. Controls for antibody staining specificity included incubation in primary antisera without the subsequent secondary and incubation in the secondary antibody without the prior primary.

Quantification. Rats from each time point were chosen for quantitative analysis on the basis of our ability to detect immunolabeling reproducibly between stained sections. The few brains that showed perfusion or sectioning artifacts were not included in the quantitative analyses, but were evaluated qualitatively along with the rest. Quantitative analyses were carried out on a single representative section for each animal at comparable levels of the dorsal hippocampus for the different staining procedures, so comparisons between stains and sections between animals can be made. PP transection denervates the dorsal hippocampus throughout its rostral-caudal extent, so a single coronal section is representative of reactive changes occurring throughout the denervated area. Lesioned hippocampal sides were evaluated, and sections from unoperated controls were evaluated bilaterally.

A Cue-2 image analysis system (Olympus Corp.) was used to enhance the image display to facilitate manual counting of immunopositive cells and determine gross dimensional measurements of the ML. Statistical evaluations of all measurements were made with ANOVAs, and post-hoc Dunnett comparisons were performed to assess individual group differences.

Cresyl violet. For sections stained with cresyl violet, the total number of cells in the ML of the ventral leaf of the dentate gyrus (DG) were calculated (Fig. 1). Average width measurements of the ML (dorsal–ventral dimension) were calculated from three positions along the ventral leaf of the DG for each animal. In addition, estimates of the length of the dentate granule cell (GC) layer bordering the ML (medial–lateral dimension) and the area of the ML (in μm^2) were obtained.

OX-42, anti-IL-1, and anti-GFAP. Values for immunostained tissue are reported as the total number of immunopositive cells in the ML of the ventral leaf of the DG.

RESULTS

PP lesion. Sections stained with cresyl violet and for AChE were examined to evaluate the extent of the lesion (Fig. 2). The lesion destroyed parts of the dorsal portions of the perirhinal cortex and transected the PP. In some cases parts of the visual cortex and parietal cortex were damaged. In all cases the corpus callosum

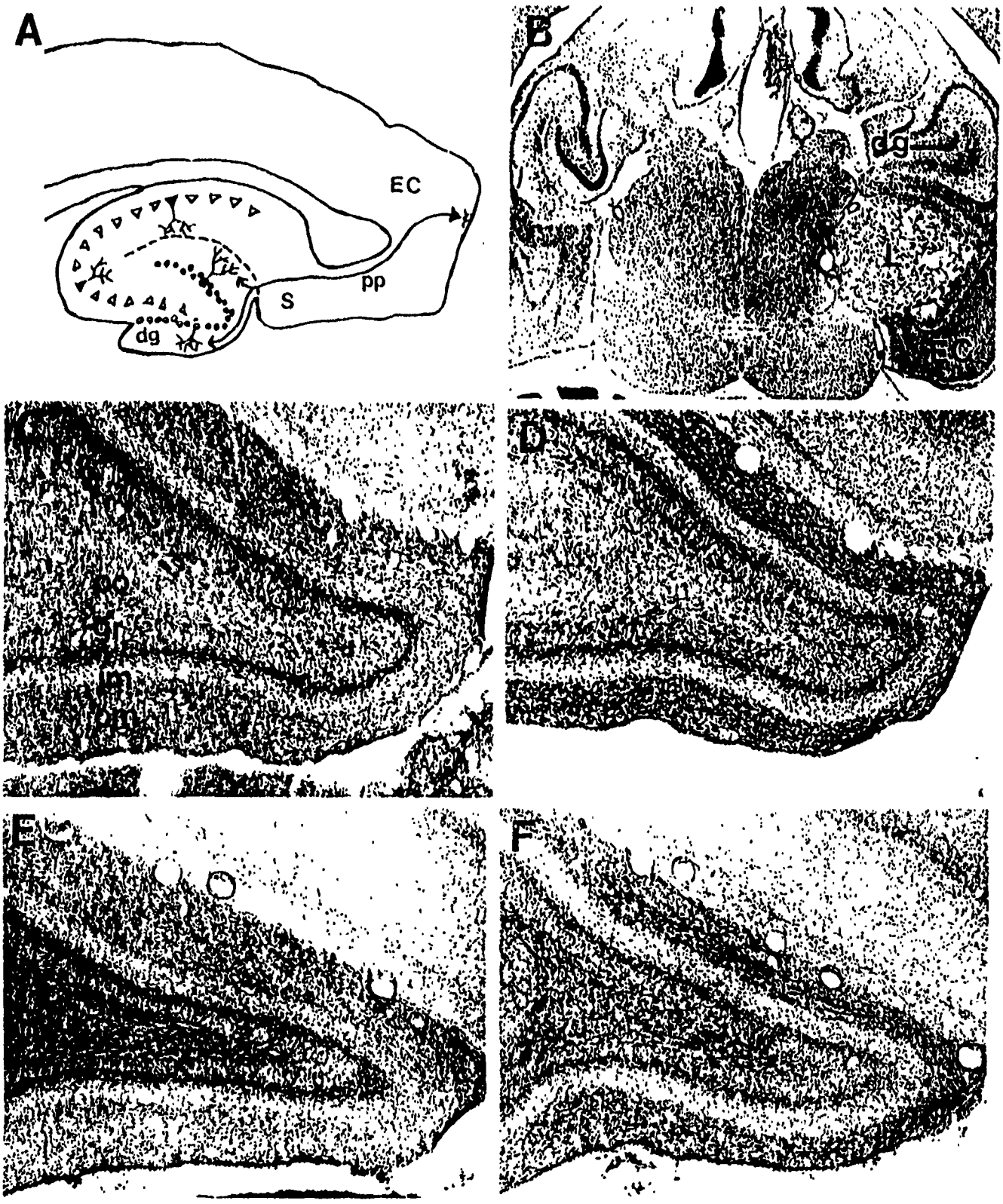

FIG. 2. Photomicrographs of the lesion employed to transect the perforant path and patterns of AChE (C, D) and NGF-receptor (E, F) fiber staining in the normal (C, E) and denervated (D, F) hippocampus. (A) Schematic illustration of a horizontal section through the hippocampus (left = rostral) showing orientations of pyramidal cells of the CA fields and granule cells of the dentate gyrus. Cells of the entorhinal cortex project to the dentate gyrus through the subiculum via the perforant path. (B) Photomicrograph of a horizontal section stained with cresyl violet showing a unilateral lesion of the perforant path. (D) An increase in density of AChE-positive fibers in the molecular layer of the dentate gyrus is observed 30 days following the lesion as compared to the normal pattern (C). NGF-receptor immunoreactivity in the molecular layer at Day 30 (F) is also increased relative to control (E). Abbreviations: dg, dentate gyrus; EC, entorhinal cortex; gr, granular

overlying the medial portions of the hippocampus was destroyed. The effectiveness of the lesion was verified by observing an increase in AChE staining in the outer ML of the DG 30 days following the lesion (Fig. 2). Patterns of NGF-r-positive fibers in the ML following deafferentation closely resemble those of AChE, supporting the idea that this sprouting response is supported by NGF.

Gross morphology. Gross morphological changes in the dentate gyrus following deafferentation were assessed on tissue stained with cresyl violet (CV; Table 2). Area measurements (in μm^2) of the ML of the ventral leaf of the DG were not statistically different from unoperated control values. Measures of the medial-lateral length of the granule cell (GC) layer providing the dorsal boundary of the examined ML were not different from controls at any time point. Measurements of the dorsal-ventral width of the deafferented ML, however, were different from control values by 4 days postlesion ($F = 7.05$, $P < 0.01$). This significant decrease in width represents a shrinkage of 15% of normal width ($F = 7.05$, $P < 0.01$), comparable to other published reports (1, 4, 7, 45). By 30 days postlesion, the differences in width represented a shrinkage of 29%. To avoid possible misinterpretation due to this tissue shrinkage over time, quantitative changes in cell populations are reported as the total number of cells rather than the cell density.

Cresyl violet. In normal, unlesioned tissue, the ML of the DG is relatively homogeneous in terms of cell types. With the exception of scattered interneurons, the ML is neuron-free. Smaller, denser staining glial cell bodies are distributed evenly throughout the ML. Within 1 week following deafferentation of the ML by PP transection, the total number of glial cells increases as a function of postoperative time ($F = 11.14$, $P < 0.01$; Fig. 3A, Fig. 4). By 30 days postlesion, the total number of cells has returned to normal.

OX-42. In the unlesioned adult rat brain, cells immunolabeled with OX-42 are sparsely scattered throughout the ML of the DG. Staining profiles show small cells extending one or two thin processes. Image-enhanced conditions are usually required for reliable identification and quantification under high power magnification. After PP transection, most OX-42-positive cells in the deafferented ML take on a very different appearance and differ in their distribution within the ML. Labeled processes are thicker, and the number of stained processes per cell increases dramatically (Fig. 5). The staining pattern often resembles a halo of processes surrounding a small cell body. This hypertrophic profile is not observed in unlesioned tissue and is less representative of cells at later time points (e.g., D5 and D6). In addition, these reactive microglial cells ipsilateral to the lesion are most often selectively distributed within the deafferented, outer portions of the ML. Quantitatively, there is a rapid increase in the total number of OX-42-positive cells in the ML within the week following PP transection ($F = 5.48$, $P < 0.01$). This increase is apparent within 24 h following the lesion, and the cell number remains elevated throughout the time points observed (Fig. 3B). By 30 days postlesion, the number of OX-42-positive cells in the deafferented ML has returned to normal control values.

IL-1. Cells stained for IL-1 exhibit cytoplasmic staining profiles, with the reaction product within the perikarya. The few labeled cells in the normal rat brain DG are typically small and scattered very sparsely throughout the ML. After deafferentation, immunopositive cells in the ML appear larger and often more darkly stained, although staining intensity was not systematically evaluated (Fig. 6). In a few cases at the later time points (e.g., D3–D6), a pattern of diffuse immunoreactivity restricted to the outer ML was observed, suggesting extracellular localization of IL-1 (data not shown). Following PP transection, the total number of cells immunopositive for IL-1 increases in the outer ML compared to normal control values ($F = 17.80$, $P < 0.01$). This increase in cell number is apparent within 24 h following the lesion, but the number returns to normal control values by Day 5 (Fig. 3C).

GFAP. GFAP-positive astrocytes are distributed evenly throughout the ML in the normal rat brain. Immunostaining is observed within the soma and thin processes of the cells. Following PP transection, GFAP-positive cells in the denervated ML become hypertrophic. Labeled processes become thicker, and the cell soma appears to swell (Fig. 7). At the longer time points (e.g., D5 and D6), individual processes often cannot be distinguished. In addition to hypertrophic changes, the number of GFAP-positive cells in the denervated area increases within the week following the lesion ($F = 7.32$, $P < 0.01$; Fig. 3D). By 30 days postlesion, values have returned to those of normal controls. One normal control had an extraordinary number of GFAP-positive cells in the ML. Since the value was two standard deviations higher than the group mean, this animal was excluded from further analysis.

DISCUSSION

The present study was undertaken to determine the sequence of events preceding septal cholinergic sprouting in the outer molecular layer of the dentate gyrus following perforant path transection. Our observations

layer; im, inner molecular; L, lesion; om, outer molecular, po, polymorphic area, pp, perforant path; S, subiculum. Magnification: (B) ×5; (C–F) ×40.6.

TABLE 2

Gross Dimensional Measurements of the Molecular Layer of the Ventral Leaf of the Dentate Gyrus at Various Time Points (Days 1-6, 30) following Perforant Path Transection

Day	Area ($\mu m^2 \times 100$)	Length ($\mu m \times 10$)	Width (μm)	% Normal width
0 ($n = 8$)*	2872 ± 130.4	1509 ± 66.9	225 ± 3.6	100.3 ± 0.02
1 ($n = 6$)	2775 ± 133.1	1543 ± 58.7	222 ± 3.4	98.7 ± 0.02
2 ($n = 7$)	2699 ± 230.5	1488 ± 63.5	202 ± 11.3	94.7 ± 0.02
3 ($n = 8$)	3014 ± 137.3	1534 ± 66.2	206 ± 8.3	91.9 ± 0.04
4 ($n = 6$)	2851 ± 163.6	1595 ± 72.1	191 ± 8.6*	85.0 ± 0.04*
5 ($n = 4$)	2486 ± 117.3	1477 ± 58.4	191 ± 4.9*	85.0 ± 0.02*
6 ($n = 4$)	2395 ± 89.6	1398 ± 41.4	184 ± 5.9**	81.8 ± 0.03**
30 ($n = 5$)	2298 ± 136.7	1437 ± 58.3	160 ± 8.2**	71.5 ± 0.04**

Note Values are obtained from one representative section stained with cresyl violet from each animal.
* 0 indicates the unoperated control group, n indicates the number of animals in each group, and values are reported as the mean ± SEM.
* ** Statistical differences from control values according to Dunnett post-hoc comparisons at the $P < 0.05$ and $P < 0.01$ levels, respectively.

suggest a rapid microglial activation and concomitant IL-1 production, followed by hypertrophy and increase in number of GFAP-positive astrocytes in the denervated outer molecular zone. We propose that these events reflect specific denervation-related phenomena in that the observed changes in glial cell populations are most often spatially restricted to the deafferented zone and temporally coincide with the known degeneration and subsequent regeneration patterns following deafferentation.

OX-42

OX-42 recognizes the complement receptor CR-3, the rat homologue to the human iC3b receptor, a membrane-bound antigen common to a subset of macrophages (57). OX-42 immunoreactivity reveals cells conforming to the traditional descriptions of microglia in the brain. These cells typically have small, elongated cell bodies with small, dense nuclei. One or two thin cytoplasmic processes extend chiefly from both poles in the undamaged adult rat brain. Similar staining profiles attributed to microglia have been observed in the rodent brain stained with silver stains (10, 20, 34, 59), ML-1 lectin (68), lectin GSA I-B4-HRP (65), and the macrophage-specific murine antibody F4/80 (55).

In the undamaged adult brain microglia are considered functionally dormant or quiescent. In response to damage, these cells transform into "reactive" or "activated" microglia (42). Morphologically, activated microglia become rounded via retraction of their processes, and functionally, they exhibit motile, proliferative, and phagocytic properties. This functional transformation with morphological change has been demonstrated *in vitro*, with transformation of rounded, ameboid microglia into ramified, process-bearing microglia associated with the loss of proliferative and phagocytic capabilities (21, 52).

In the present study, we define microglia as "activated" by observing changes in cellular morphology demonstrated by OX-42 immunolabeling profiles. We have not yet determined if these cells are phagocytizing or proliferating. Morphologically, OX-42-positive cells appear to increase their process arborization in response to damage, not retract their processes as described in the classic early literature (10, 53). Whether this change represents a true, albeit paradoxical, morphological transformation or, alternatively, reflects an upregulation of the CR-3 antigen is not certain. Little is known about how microglial activation and transformation are regulated *in situ*.

What Is the Origin of OX-42-Immunoreactive Cells in the Denervated ML following PP Transection?

Controversy exists as to whether the "reactive" microglia which appear in response to damage are derived from a population of cells intrinsic to the CNS or are monocytic macrophages of hematogenous origin, invading the damaged area from the bloodstream. Studies report differences in the origin of brain macrophages depending on the nature of the injury (2, 21, 54, 62). Reports demonstrate an extrinsic, hematogenous origin of brain macrophages in traumatic wounds in which the blood–brain barrier (BBB) is severely disrupted, as in a stab wound (9, 33, 35, 36, 56), and an endogenous origin in areas of Wallerian and retrograde degeneration (16, 41, 60, 66, 67). Our data from tissue stained with OX-42 are consistent with this distinction. In the present paradigm, the BBB is disrupted by the lesion, but the analyses are carried out at distances up to 2 mm distal from the lesion. In the wound area we often observe round cells immunopositive for OX-42, which are most likely

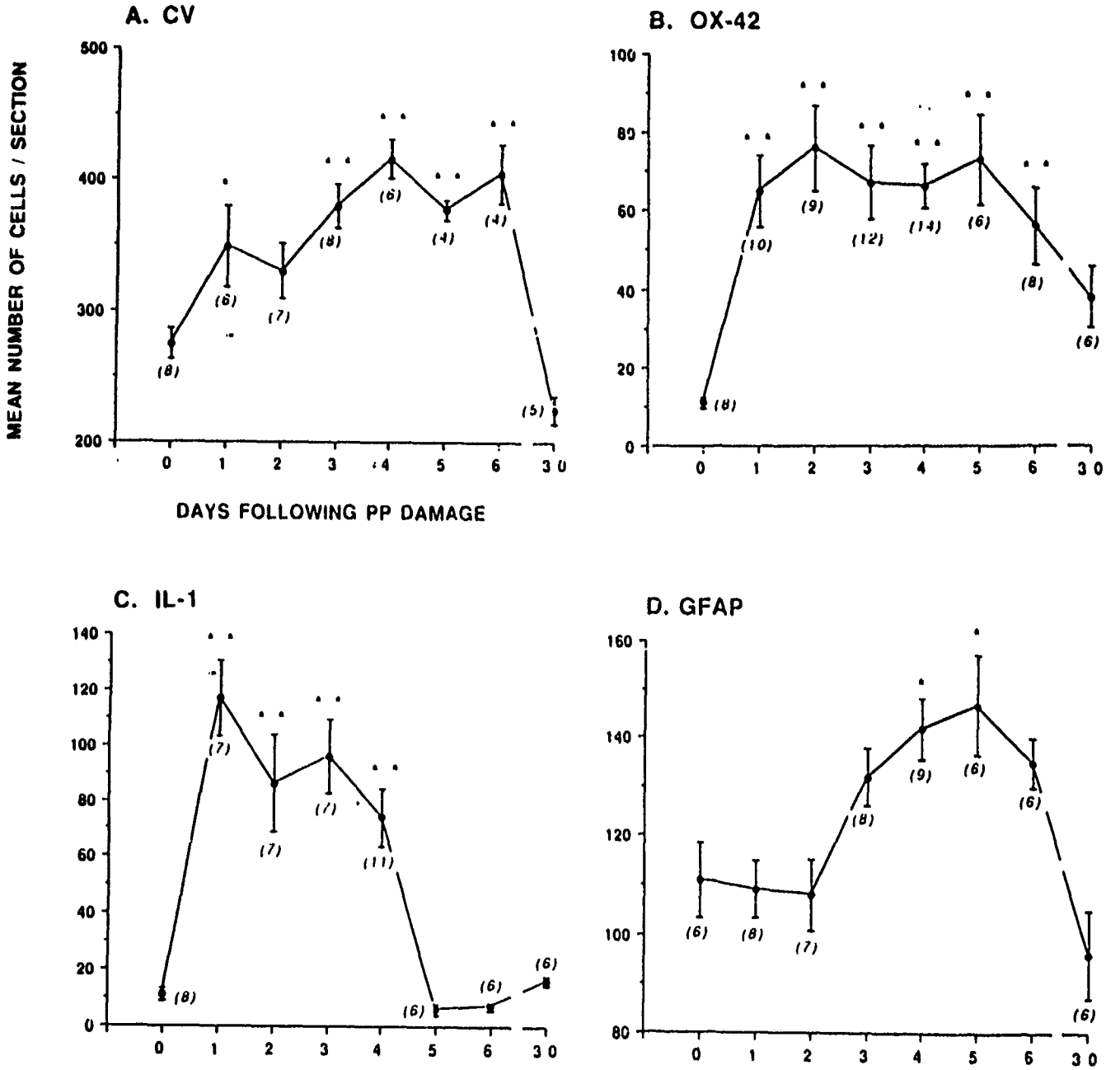

FIG. 3. Line graphs showing the number of cells stained with cresyl violet (CV), OX-42, anti-IL-1, and anti-GFAP in the molecular layer of the dentate gyrus as a function of the number of days following perforant path transection. Values are obtained from a representative coronal section for each animal and are reported as the mean number of cells. Error bars indicate SEM. Numbers in parentheses correspond to the number of animals in each group. *,**Indicates statistical differences from control values according to Dunnett post-hoc comparisons at the $P < .05$ and $P < .01$ levels, respectively.

macrophages infiltrating from the bloodstream (data not shown). Cells with this morphology are not seen in the deafferented ML. Instead, we observe in the ML OX-42-positive cells with the morphological characteristics of microglia. These observations are consistent with reports of morphologically distinct forms of macrophages in the damaged CNS, but do not directly address their origin.

Cells immunolabeled with OX-42 observed in the ML may arise from cells intrinsic to the CNS which have somehow been "activated" and thereby upregulate the CR-3 receptor. Alternatively, macrophages from the bloodstream may traverse the capillary endothelium in areas of CNS degeneration and transform to a more branched morphology. Either scenario could account for the pattern of OX-42-positive cells in the deafler-

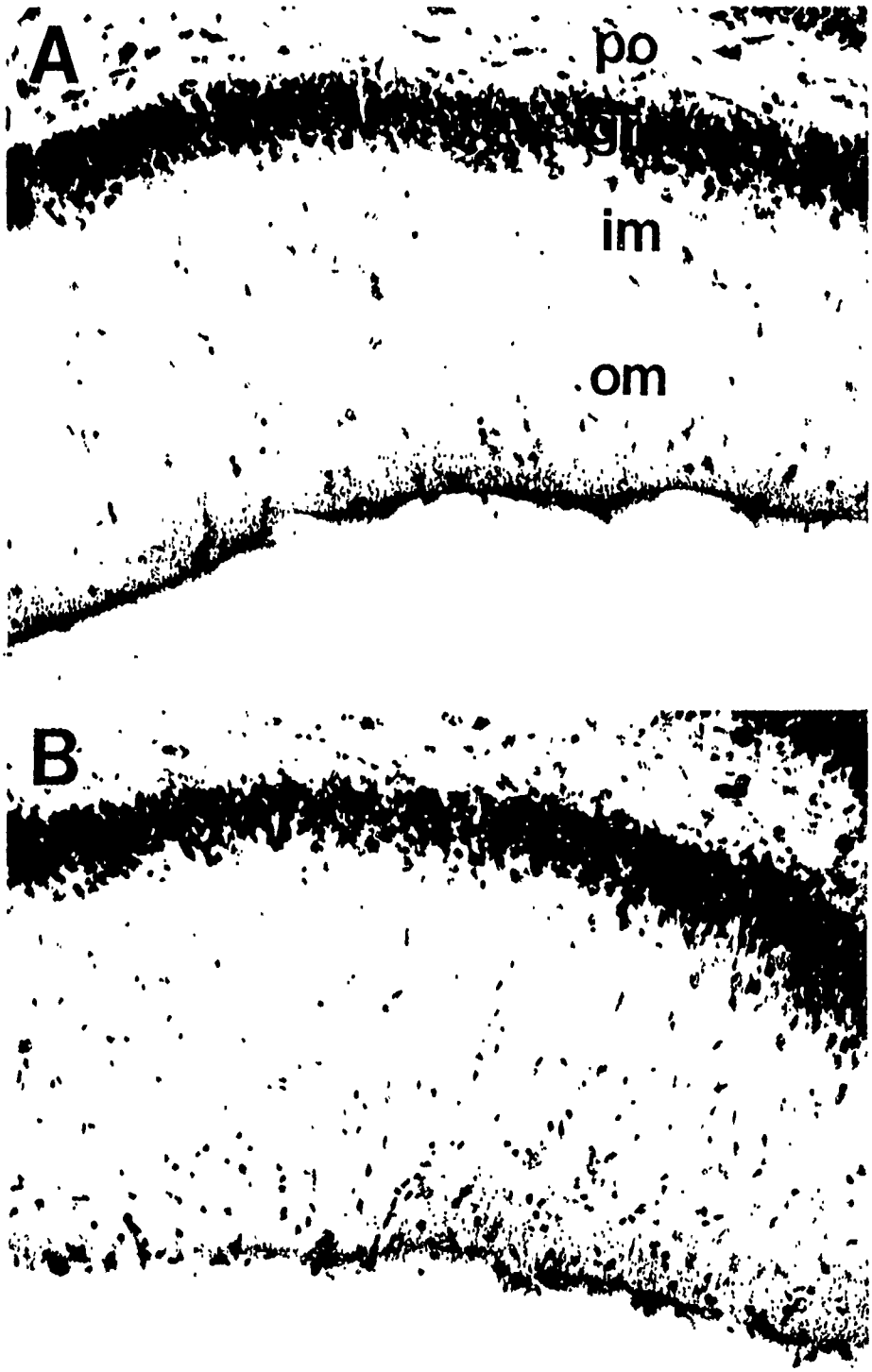

FIG. 4. High-power photomicrographs showing the molecular layer of the dentate gyrus stained with cresyl violet from a normal control (A) and 4 days following perforant path transection (B). Abbreviations: gr, granular layer; im, inner molecular; om, outer molecular; po, polymorphic area. Magnification, ×116.

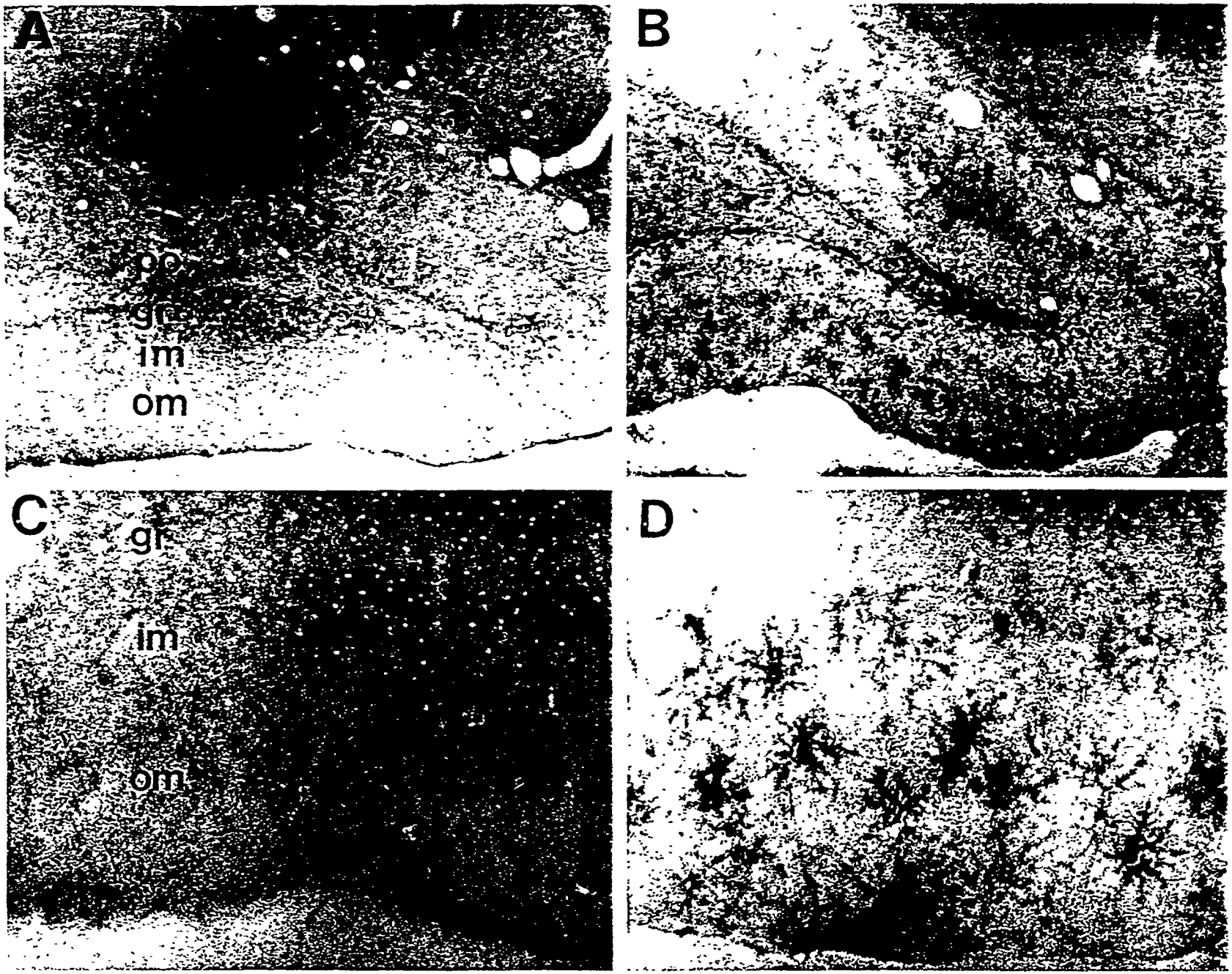

FIG. 5. Low-power (A, B) and high-power (C, D) photomicrographs showing sections from the dentate gyrus immunostained with OX-42 from a normal control (A, C) and 2 days following perforant path transection (B, D). Abbreviations: gr, granular layer; im, inner molecular, om, outer molecular; po, polymorphic area. Magnification: (A, B) ×52; (C, D) ×209

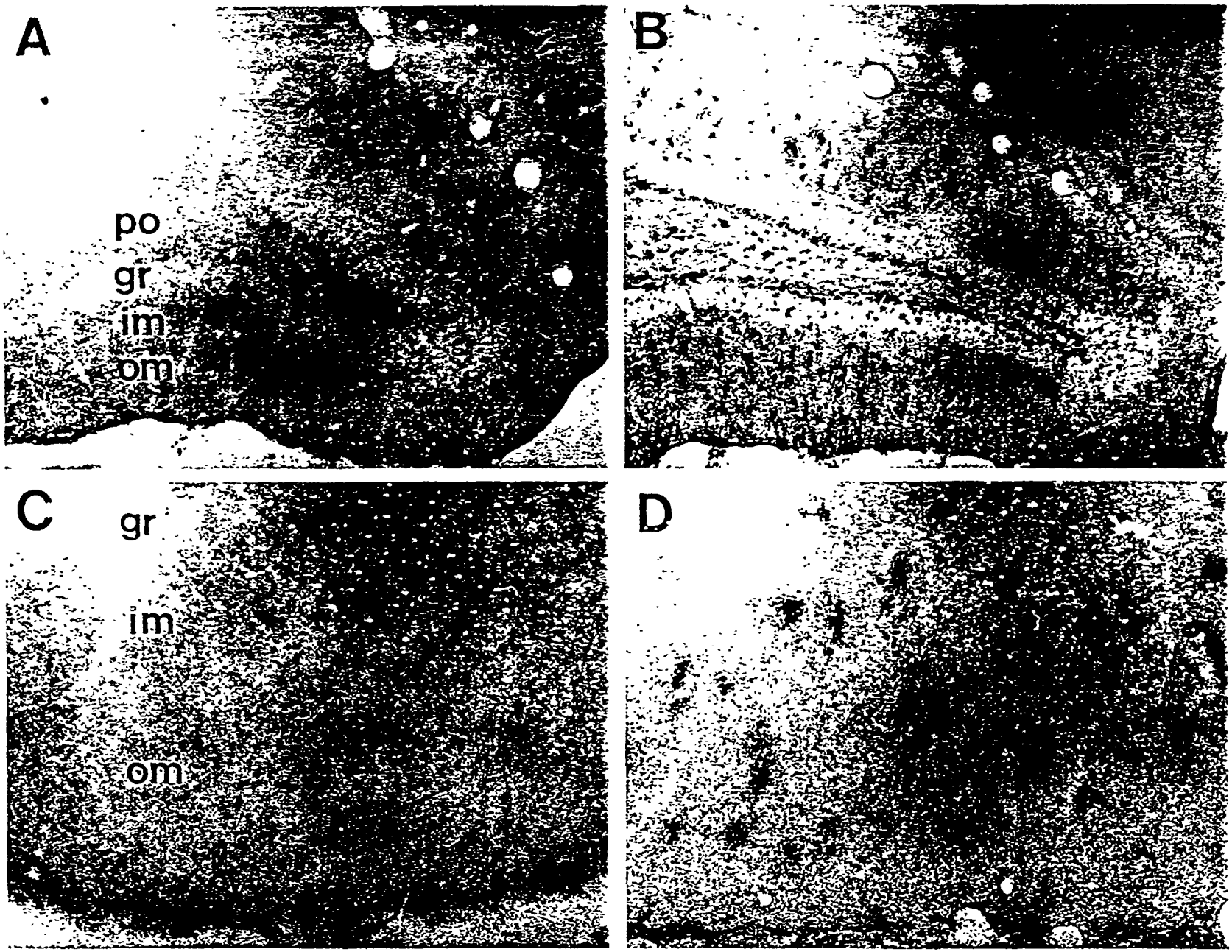

FIG. 6. Low-power (A, B) and high-power (C, D) photomicrographs showing sections from the dentate gyrus immunostained for interleukin-1 (IL-1) from a normal control (A, C) and 3 days following perforant path transection (B, D). Abbreviations: gr, granular layer, im, inner molecular; om, outer molecular; po, polymorphic area. Magnification: (A, B) ×52; (C, D) ×209.

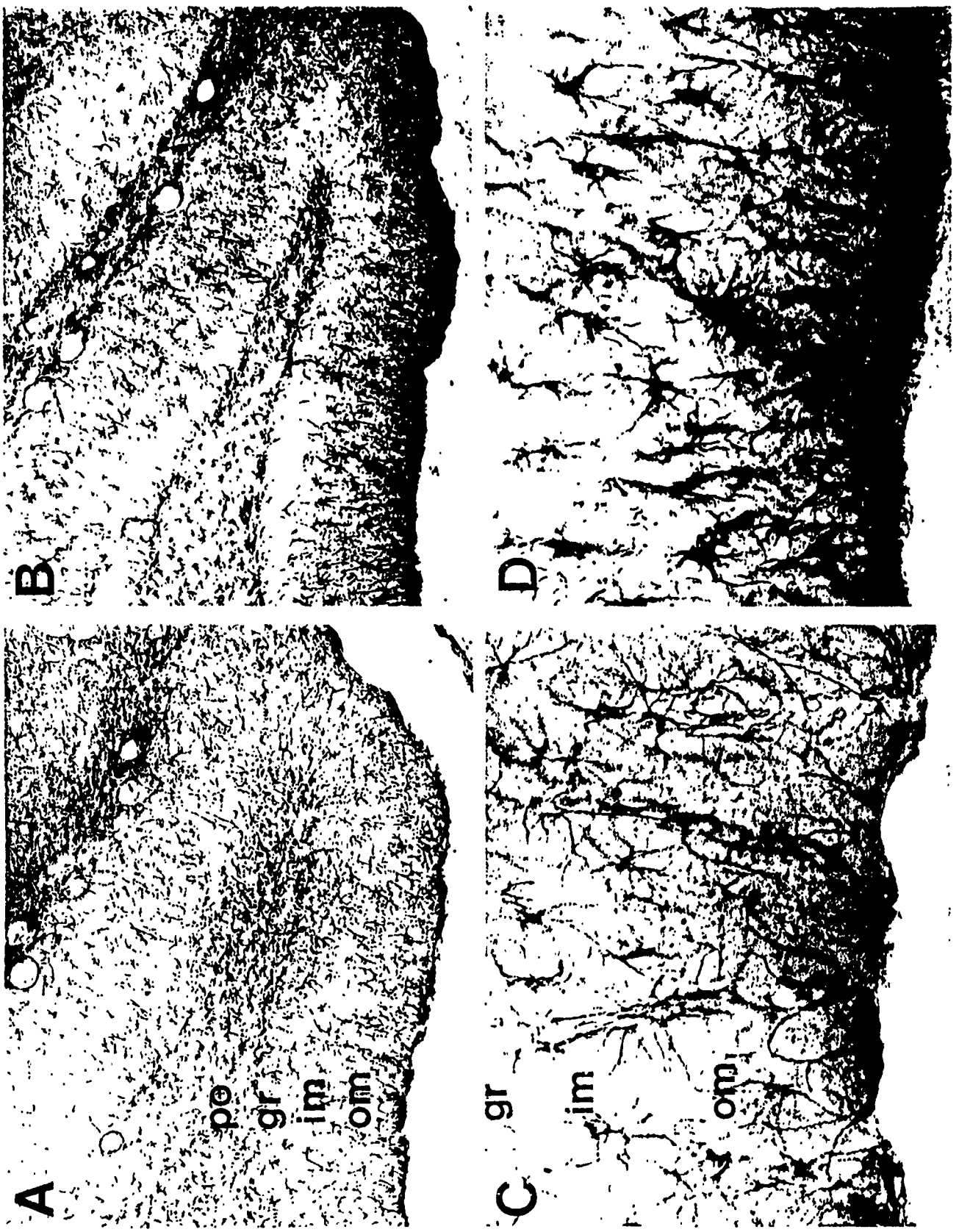

FIG. 7. Low-power (A, B) and high-power (C, D) photomicrographs showing sections from the dentate gyrus immunostained for glial fibrillary acidic protein (GFAP) from a normal control (A, C) and 4 days following perforant path transection (B, D). Abbreviations: gr, granular layer; im, inner molecular; om, outer molecular; po, polymorphic area. Magnification: (A, B) ×52; (C, D) ×209.

ented ML without verifying their origin. More direct studies investigating the origin of these cells are in progress.

IL-1

We do not observe IL-1-immunoreactive fibers in hypothalamic areas, as reported by Saper and colleagues (3) for human tissue. In fact, very few cells in the normal adult rat brain show positive staining for IL-1. Antibody species specificity may contribute to this discrepancy. However, within 24 h following PP transection, many IL-1-immunopositive cells are seen in the deafferented ML. This pattern is consistent with biochemical data demonstrating no measurable IL-1 activity in normal adult rat brain (25) and increased levels in extracts of injured brain (24).

Two distinct but related forms of human IL-1 (α and β) have been isolated (47). Both share biological activity; mediating the induction of IL-2 synthesis by thymocytes, stimulating rat astrocyte proliferation *in vitro* (21, 26), and regulating the synthesis of NGF in nonneuronal cells of the damaged rat sciatic nerve (39). Recently, cDNAs for rat IL-1α and β have been cloned (50) and exhibit 73% and 77% sequence identity to the human forms, respectively. The monoclonal antibody used in the present study is directed against naturally derived human IL-1 protein. This antibody neutralizes both α and β forms of the human protein, as well as murine IL-1, so the present data do not address possible distinctions between the roles of α and β forms of IL-1 in this paradigm. Specific staining with polyclonal antibodies directed against the α and β forms of the human protein (Genzyme, Boston, MA) could not be demonstrated. Antibodies to the rat forms are unavailable at the present time.

To demonstrate the staining specificity of the monoclonal antibody to human IL-1, we attempted to block immunostaining by preadsorbing the antibody with IL-1 protein. We were unable to block staining convincingly since we could not obtain sufficient titers of the purified protein within budget limits (anti-human IL-1 = 42 mg/ml (Genzyme), purified human IL-1 = 2000 pg/ml (Endogen)). However, standard control sections in which the primary or secondary antibodies were omitted showed no positive staining, demonstrating antibody-specific staining profiles (data not shown).

Do Microglia Produce IL-1 in Response to PP Transection?

IL-1 is a macrophage-derived cytokine that mediates a number of immunological and inflammatory responses (13). Mononuclear phagocytes are considered the most potent secretors of IL-1 (12). Microglia, considered phagocytes of the brain, produce IL-1 *in vitro* (22, 23, 31) and are the hypothesized source of IL-1 *in vivo* during CNS development in the rat (21, 26) and following penetrating brain injury in the adult (24, 25). A recent report demonstrates IL-1 immunoreactivity localized to astrocytes and microglial-like cells in brain tissue from patients with Alzheimer's disease and Down's syndrome (29). Enriched astrocyte cultures have been shown to be capable of producing IL-1, but to a much lesser extent (15, 23).

In the present paradigm, microglial activation, as defined by changes in OX-42-immunostaining profiles, spatially and temporally coincides with degenerative changes in the transected entorhinal afferents (5, 37, 43, 45, 64). The presence of degenerative debris may provide a signal for microglia in the area to phagocytize and, in the process, produce IL-1. Only microglia stimulated to phagocytize produce IL-1 *in vitro* (22, 23, 31). The rapid and concurrent appearance in parallel sections of OX-42-positive microglia and cells immunolabeled for IL-1 is consistent with the idea that reactive microglia produce IL-1 *in vivo* in response to PP transection. Demonstration of colocalization of OX-42 and IL-1 immunoreactivity in the same cell is necessary to provide definitive support for this hypothesis. In addition, studies investigating *in situ* localization of rat IL-1 mRNA in this model are in progress.

IL-1 immunoreactivity returns to normal control values by D5, the time at which the number of astrocytes is maximal. In a few cases at these later time points we observe a diffuse pattern of IL-1 immunoreactivity restricted to the outer ML, suggestive of extracellular localization. The lag between the immediate appearance of cells immunopositive for IL-1 and subsequent astroglial hypertrophy and increase in number suggests that astrocytes are not the primary cellular source of IL-1 in this model. Furthermore, the sequential pattern is compatible with the proposed role of IL-1 in mediating astrocyte proliferation.

GFAP

GFAP is an intermediate filament protein specific to astrocytes in the CNS. GFAP-positive astrocytes are normally distributed throughout the dentate gyrus in the adult rat brain and undergo reactive changes in the ML following PP transection. Immunolabeled processes become thicker and the cell soma appears to get larger. These hypertrophic changes can be observed within the first few days following deafferentation, becoming more robust with time, consistent with other published reports (45, 58). Astrocytes in the ML also increase in number by 23% of their normal value by 5 days following the lesion, a few days after the microglial response. This sequential pattern is in agreement with other reports of astrocyte activation following microglial activation in damaged tissue (11, 19, 65, 66, 70). Furthermore, this sequence of events is consistent with

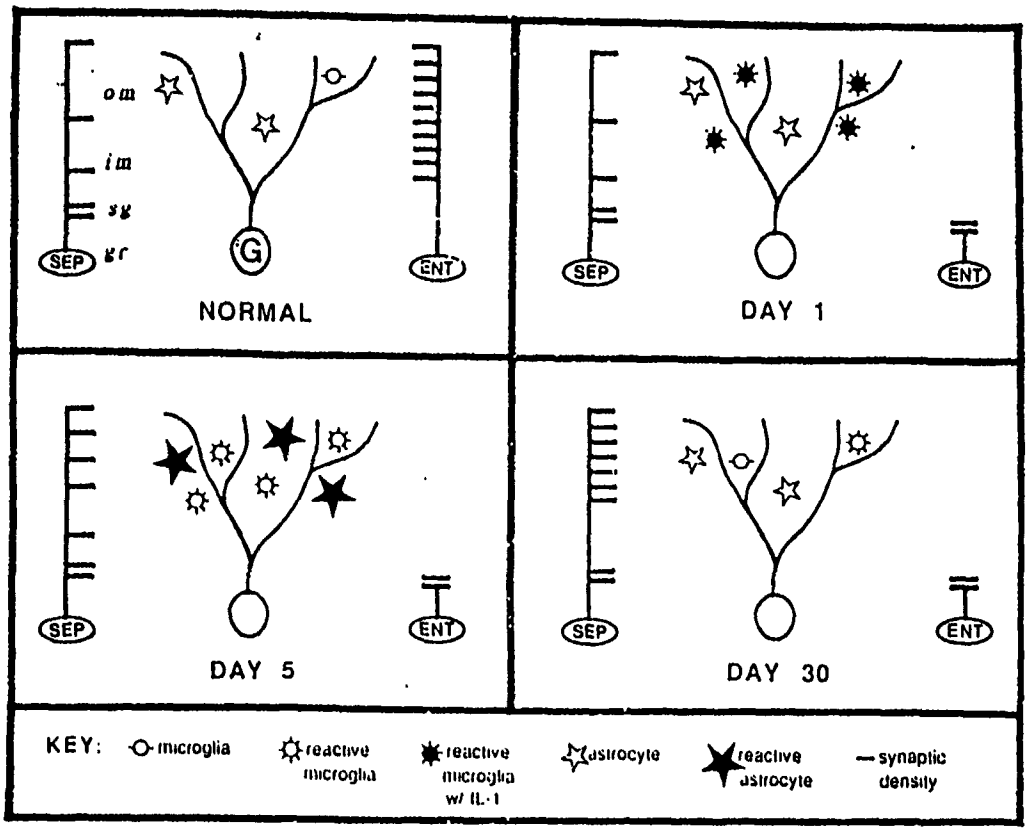

FIG. 8. Schematic illustration depicting the proposed sequence of events in the molecular layer of the dentate gyrus on representative days following perforant path transection. G indicates a granule cell and its basic dendritic arbor. SEP and ENT represent afferents to the granule cell from the septum and ipsilateral entorhinal cortex (via the perforant path), respectively. Horizontal lines illustrate topography within the molecular layer as well as relative synaptic densities of each afferent projection. Abbreviations: gr, granular layer; sg, supragranular layer; im, inner molecular; om, outer molecular.

the working hypothesis postulating rapid microglial activation and IL-1 production followed by astrocyte activation in response to PP transection.

Are Glial Cells Proliferating in Response to PP Transection?

Since the ML is relatively free of neurons, glial cells account for the majority of cells stained with cresyl violet in this area. Within a week following PP transection we observe an increase in total cell number over time in accordance with other published reports (45). Figure 3A depicts a rapid 33% increase in the number of cells within 24 h after the lesion followed by a slower rise prior to reaching asymptotic levels at D4 or D5. Although cresyl violet cell counts do not discriminate between specific glial subtypes, this time course correlates with the rapid OX-42 changes observed at D1 and the subsequent monotonic rise in the number of GFAP-positive cells. Furthermore, an increase in the number of cells in the entire ML suggests that there is an addition of cells following damage, not merely a migration of cells from the inner ML to the outer ML as proposed by others (58). By 30 days following the lesion, the total number of cells in the ML returns to normal control values, consistent with other reports (45). This decline from the previous increases observed within a week postlesion may reflect a death of cells or a migration out of the observed molecular zone.

The coincidental early and rapid increase in the number of cells stained with cresyl violet and those with OX-42 suggests that they represent the same cell population. Interpretation of the increase in number of cells immunoreactive for OX-42 is ambiguous. This increase could reflect an upregulation of the CR-3 receptor on cells intrinsic to the CNS. In this case, a fixed number of total cells stained with cresyl violet would be predicted. Alternatively, the increase in number of OX-42-positive cells could result from the proliferation of the small number of intrinsic immunoreactive microglia or an invasion of hematogenously derived cells expressing the antigen. The increased number of cells observed in sections stained with cresyl violet suggests an addition of cells, be it due to proliferation or invasion from the pe-

riphery. Studies employing [^{3}H]thymidine to label proliferating cells *in vivo* are in progress.

The 23% increase in the number of cells immunostained for GFAP observed in the present study suggests astrocyte proliferation in the ML in response to deafferentation. With respect to the proposed working hypothesis, this suggestion is consistent with the known role of IL-1 in promoting astrocyte proliferation *in vitro*. Previous studies of this model system report astrocyte hypertrophy without proliferation (45, 58). Gall and colleagues (19) concluded that microglia, identified according to morphological criteria, are the most common proliferative element within the hippocampus following deafferentation of the ML, although it was noted that astrocytes were sometimes observed to contain radioactive label as well. A previous study from this laboratory reported a 61% increase in astrocyte number in the ML by 4 days postlesion (18). The present study differs from the previous one in both histological and quantification methods, so the magnitude of the effect cannot be directly compared.

As a final point, a close inspection of Fig. 3 indicates that the combined total number of cells stained with OX-42 and anti-GFAP is less than the number stained with cresyl violet at all time points observed. This discrepancy may reflect immunostaining of a subpopulation of microglia and/or astrocytes or alternatively may indicate the presence of other cell types, e.g., oligodendrocytes, or lymphocytes, e.g., T or B cells, which have infiltrated from the periphery in response to deafferentation of the ML.

CONCLUSIONS

The results of the present study describe a sequence of events preceding septal cholinergic sprouting in the outer ML of the DG following PP transection (Fig. 8). At the cellular level, microglia become activated prior to reactive changes in the astrocyte population. An increase in the number of OX-42-positive microglia is observed within 24 h following the lesion, whereas astrocyte hypertrophy and increase in number becomes maximal 4 days later. At the molecular level, production of IL-1 precedes the astrocyte response. The appearance of cells immunolabeled for IL-1 parallels that of OX-42-positive microglia, but IL-1 immunoreactivity returns to normal control values by D5, the time at which changes in GFAP-positive astrocytes become pronounced. Together these observations are consistent with the hypothesis that terminal degeneration rapidly activates microglia to phagocytize and, in so doing, produce IL-1. IL-1 is then released into the surrounding environment and in turn induces activation of astrocytes. Further studies will investigate the role of astrocyte-derived trophic and/or tropic factors (e.g., NGF, laminin, basic FGF) as the next hypothesized step in the sequence of molecular events responsible for eliciting and perhaps maintaining the reorganization of cholinergic afferents to the ML following PP transection.

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Basal forebrain cell loss following fimbria/fornix transection

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Following fimbria/fornix transection, cells in the medial septum appear to undergo retrograde degeneration as shown by Nissl and acetylcholine esterase (AChE) staining. Recent studies using immunocytochemical techniques have also demonstrated loss of choline acetyltransferase (ChAT) and nerve growth factor receptor (NGFr) labeling of neurons in this region. Whether the apparent loss of ChAT- and NGFr-positive neurons is the result of the actual death of these neurons, or is instead a loss of ChAT enzyme or NGFr expression below levels detectable by immunocytochemical methods, remains an unresolved issue. In order to address this question, rhodamine-labeled fluorescent latex microspheres were injected into the hippocampus where they were retrogradely transported to the cell bodies of the medial septum. Five days later these animals received either unilateral or bilateral fimbria/fornix lesions and were allowed to survive an additional 4 weeks. Compared to unlesioned control animals, unilaterally lesioned animals showed a 91% loss of fluorescently labeled cells and bilaterally lesioned animals showed a 93% loss. The inability to detect the fluorescent microspheres in the medial septum suggests that the majority of medial septal cells die after fimbria/fornix transection. ChAT and NGFr immunohistochemical staining were also performed. Cells stained for ChAT were reduced in number by 92% in animals with unilateral lesions and by 75% in animals with bilateral lesions, while NGFr-stained cells were reduced in number by 75% in animals with unilateral lesions and by 68% in animals with bilateral lesions. Of note, several cells were double-labeled for both fluorescent microspheres and either ChAT or NGFr after both unilateral or bilateral lesions, suggesting that not all hippocampally projecting cholinergic cells of the medial septum die after fimbria/fornix transection. Issues concerning cell dysfunction and death are discussed.

INTRODUCTION

Cholinergic cells of the medial septum project to the dorsal hippocampus through the fimbria/fornix (FF) basal forebrain fiber bundle[8,20,25,39]. Either partial or complete lesions of this projection result in retrograde degeneration of cholinergic cell bodies of the medial septum as shown by choline acetyltransferase (ChAT) immunocytochemistry, acetylcholine esterase (AChE) staining, and generalized loss of large diameter neurons visualized with Nissl stains[2,10,29]. The degeneration of these septal neurons results in loss of cholinergic input to the dorsal hippocampus.

Recently, it has been suggested that cell dysfunction rather than cell death may occur in the septum following FF lesions[14], and that loss of AChE staining or ChAT labeling represents loss of transmitter enzyme expression in the dysfunctional cells. The latter suggestion, however, has not been supported by recent investigations of Montero and Hefti[26]. Studies in other transmitter systems have indicated that loss of transmitter-related enzymes indeed can occur despite cell survival after a lesion[19,33,41]. Thus, whether septal cholinergic cells that project to the hippocampus become *dysfunctional* or actually *die* after axotomy remains controversial. The resolution of this issue is of broad relevance since the septohippocampal projection serves as a major conduit of subcortical input to the hippocampal formation[1], possesses unique electrophysiological properties[32,42], is uniquely dependent upon a continuous supply of nerve growth factor (NGF) for normal function in the adult animal[11,15,45], and provides a useful model of memory function[27,28]. For example, it has been demonstrated that intracerebroventricular infusion of NGF following FF lesions will prevent degeneration of septal cells as assessed by AChE staining and ChAT labeling[11,15,18,45]. However, if septal cell dysfunction rather than cell death occurs, then transient infusions of NGF to save septal cells might be unnecessary. Instead, reconstruction of the septohippocampal projection might be facilitated by methods that promote axonal regeneration and restitution of NGF retrogradely transported from the natural target source, the hippocampus.

To determine whether cell dysfunction or death results after FF lesions, we have used rhodamine-labeled fluorescent latex microspheres to label cells prior to FF transection. Rhodamine microspheres are retrogradely transported across damaged axon terminals into the cell body, where they remain detectable in the cytoplasm for

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TABLE I

Injections of fluorescent microspheres at given coordinates relative to bregma (in mM)

Injection	*AP*	*ML*	*VD*
1	-2.55	-1.4	3.4
2	-3.14	-1.0	3.6
3	-3.60	-2.4	3.2
4	-4.16	-2.0	3.2

periods of at least 10 weeks in vivo and 1 year after fixation[16]. They possess hydrophobic surfaces that adhere to cell membranes, are non-toxic to the cell, and appear to remain inert within the cytoplasm[16]. Fluorescence is lost upon exposure to lipid-soluble substances (as during some tissue-fixation procedures) and upon cell lysis occurring with death. Thus, if cells survive the FF lesion, then they should remain labeled with microspheres; if cells die, the label should disappear. In the current experiment animals were injected with fluorescent microspheres and underwent FF lesions 5 days later; histological analysis was performed after an additional 4-week survival period.

MATERIALS AND METHODS

Female Sprague–Dawley rats (200–220 g) were used throughout this study, and were housed in standard laboratory cages with free access to food and water. The rats were divided into one of 3 groups: a control group (n = 9) without FF lesions, a unilateral FF lesion group (n = 9), and a bilateral lesion group (n = 9). All animals received injections of fluorescent microspheres as detailed in Table I.

Microsphere injection

Injections were placed into the dorsal hippocampus to maximally label neurons projecting through the FF[1]. Under deep anesthesia, the rat was mounted in a Kopf stereotaxic apparatus. A midline incision was made of the scalp, the periosteum was scraped and the skull was cleaned with 5% H_2O_2. A hole was drilled at each of the 4 coordinates given in Table I[30]. Next 100 nl of rhodamine fluorescent microspheres (Lumaflor Inc., New City, NY) were injected into each site through a 1 μl Hamilton syringe. Microspheres were injected over 10 min, followed by a 5-min wait and then slow withdrawal of the syringe. The wound was closed with wound clips.

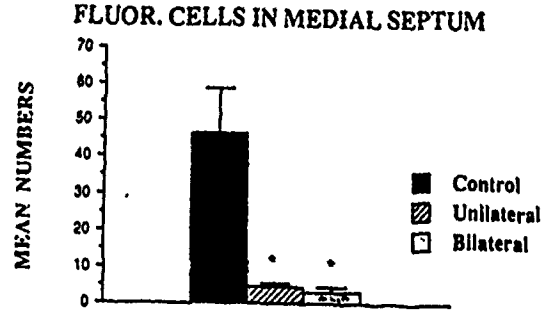

Fig. 1. Loss of fluorescence after fimbria-fornix (FF) lesions. Graph demonstrates a 91% decrease in fluorescently labeled medial septal cells in animals after unilateral FF lesions compared to control animals, and a 93% decrease in bilaterally lesioned animals. * denotes significance of difference = $P < 0.0001$.

Fimbria/fornix lesions

Five days after microsphere injections, animals receiving FF lesions were reanesthetized and the scalp wound re-opened. A 2-mm square piece of skull was removed just lateral to midline and caudal to bregma. A unilateral or bilateral aspirative lesion of the FF and supracallosal striae was made as described previously[8]. The wound was reclosed, and animals were sacrificed 4 weeks later.

Histology

Each animal was perfused with 100 ml cold 0.1 M phosphate-buffered saline (PBS) followed by 300 ml 4% formalin in PBS. The brains were removed, postfixed overnight in the same fixative, and then left for 3 days in phosphate buffer containing 30% sucrose at 4 °C. The brains were frozen with dry ice and coronal sections were cut at 40 μm with a sliding microtome and collected in 24-well tissue culture

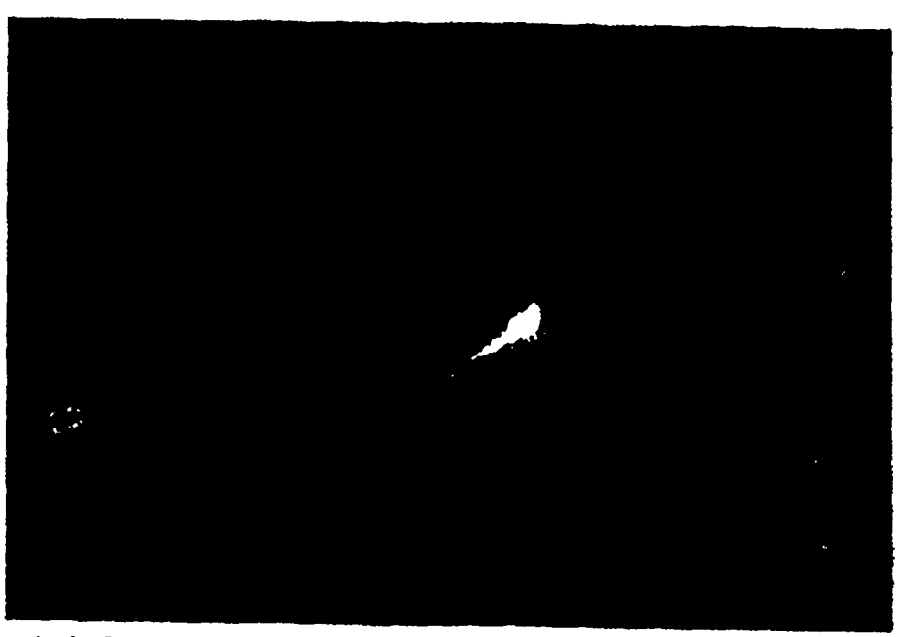

Fig. 2. Photomicrograph of a fluorescently labeled cell (blue-green ultraviolet filter; 200×).

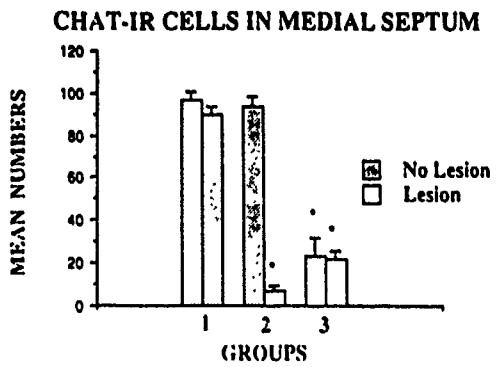

Fig. 3. Reduction of ChAT staining after FF lesions. Graph demonstrates a 92% decrease in ChAT-positive cells in unilaterally lesioned animals compared to control animals, and a 75% decrease in bilaterally lesioned animals. Group 1 = control animals (no lesions); group 2 = unilateral FF lesions; group 3 = bilateral FF lesions. * denotes significance of difference between lesioned sides relative to unlesioned controls ($P < 0.0001$).

plates (Flacon) containing cryoprotectant (glycerol/ethylene-glycol/phosphate buffer). Sections were taken rostrally from the medial septum/vertical limb diagonal band (MS/VDB) complex and caudally through the extent of the dorsal hippocampus. Every fourth section was alternately processed for fluorescence, Cresyl violet, and ChAT or NGFr immunocytochemistry.

Sections to be studied for fluorescence were mounted onto gelatin-coated slides after 3 rinses in PBS. These sections were briefly dipped in PBS before analysis to enhance contrast. It was found that fluorescence was best appreciated when sections were not exposed to alcohol rinses and were not coverslipped.

Fluorescently labeled tissue sections were examined both to verify the accuracy of injection sites in the hippocampus and to count retrogradely labeled cells in the MS and VDB. Fluorescently labeled cells were counted through 8 sections that included MS/VDB. The sections were examined under 20× magnification using a counting grid (0.5 × 0.5 mm) that entirely covered the areas of interest.

ChAT immunocytochemistry was conducted as reported previously[2]. Briefly, the procedure consisted of: (1) overnight incubation of a polyclonal antibody against ChAT[5] (courtesy of L. Hersh) of verified specificity after 1:1500 dilution with 0.1 M Tris-saline containing 1% goat serum and 0.25% Triton X-100; (2) incubation for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 with Tris-saline containing 1% goat serum; (3) 1-h incubation with avidin-biotinylated peroxidase complex (Vector Laboratories) diluted 1:1000 with Tris-saline containing 1% goat serum; and (4) treatment for 15 min with 0.05% solution of 3,3'-diaminobenzidine, 0.01% H_2O_2, and 0.04% nickel chloride in 0.1 M Tris-buffer. Immunolabeled tissue sections were mounted onto gelatin-coated glass slides, air-dried, and covered with Permount and glass coverslips.

Nerve growth factor receptor (NGFr) immunocytochemistry was conducted similarly to ChAT immunocytochemistry. Primary NGFr monoclonal antibody (192-IgG) (courtesy E. Johnson) specific for NGFr[40] was obtained from a hybridoma cell culture supernatant. Primary NGFr antibody was used at 1:100 dilution in a 24-h incubation, diluted in horse serum rather than goat serum.

Counting of ChAT or NGFr-positive cells in the medial septum was performed under light microscopy employing 3 40 μm-thick sections

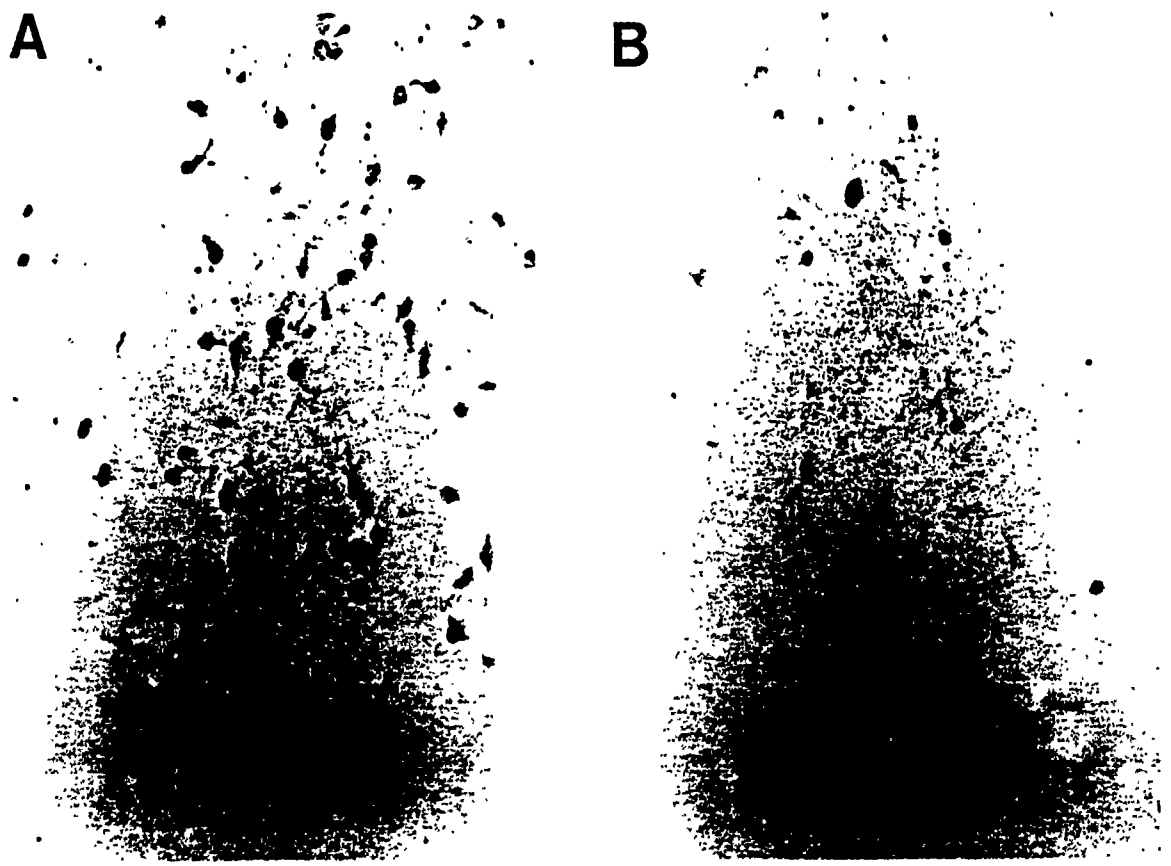

Fig. 4. ChAT immunohistochemical staining in medial septal region. A: medial septal region contralateral to side of FF lesion in unilaterally lesioned animal, demonstrating normal appearance of ChAT-immunoreactive cell bodies and processes. B: medial septal region ipsilateral to side of FF lesion. Degeneration of cholinergic cell bodies and processes is evident. 100×.

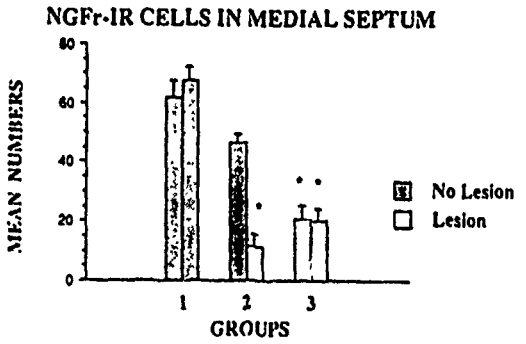

Fig. 5. Reduction of NGFr staining after FF lesions. Graph demonstrates a 75% decrease in NGFr-positive cells after unilateral FF lesions and a 70% decrease after bilateral lesions, compared to unlesioned animals. Groups 1–3 as in Fig. 3. * denotes significance of difference between lesioned sides relative to unlesioned controls ($P < 0.0001$).

located approximately 160, 320 and 480 μm rostral to the decussation of the anterior commissure. The sections were analyzed with a 10× objective using a 0.5 × 0.5 mm counting grid. Cells labeled positively with peroxidase reaction product and possessing either: (1) a cell body with emerging fiber; or (2) a cell body with well-defined nucleus were counted. Results were expressed as percentage of control animals in both unilaterally and bilaterally lesioned animals.

Sections stained for ChAT or NGFr were also examined under fluorescence to detect double-labeling with microspheres. Fluorescence was impaired by immunohistochemical processing as noted previously[34], but occasional double-labeled cells were seen and quantified.

Statistical analysis

Group differences were assessed by a one-way ANOVA. Post-Neuman–Keuls was used to assess individual group differences between control, lesioned and bilaterally lesioned animals.

RESULTS

Two animals, one from the unilateral lesion group and one from the bilateral lesion group, died before the end of the experiment. An additional animal was excluded from the fluorescence study based upon poorly placed dye injections. Nissl staining on the remaining animals (8 per group) revealed that all lesioned animals had complete transections of the FF, including all midline fibers.

As reported previously[16], the bulk of a microsphere injection remained well-localized to the injection site. Following unilateral FF lesions, a 91% decrease in the number of septal cells labeled by microspheres occurred when compared to unlesioned animals ($P < 0.0001$; Figs. 1 and 2). A 93% loss occurred on the side of the fluorescent injection in bilaterally lesioned animals, compared to unlesioned animals. Approximately 10% of labeled cells were found contralateral to the side of

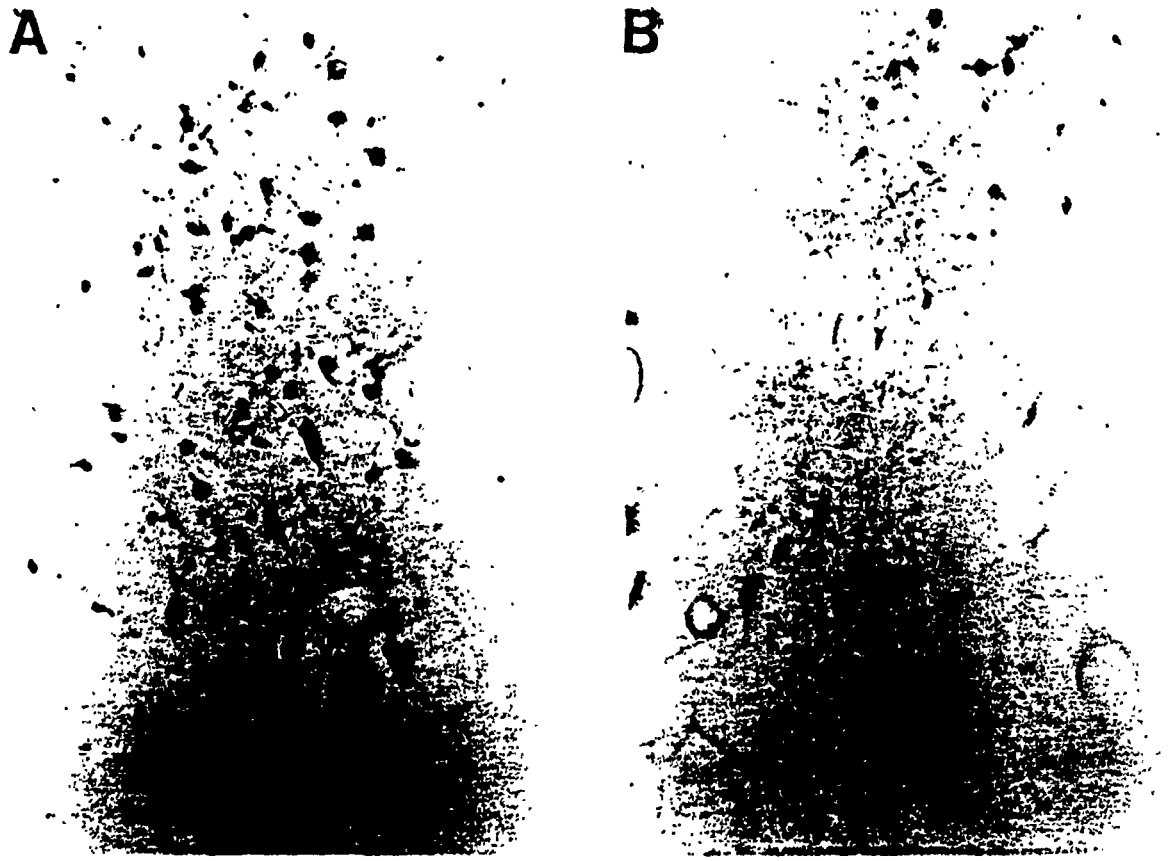

Fig. 6. NGFr immunohistochemical staining in medial septal region. A: medial septal region in control animal without FF lesion, demonstrating normal appearance of NGFr-immunoreactive cell bodies and processes. B: medial septal region in animal following bilateral FF lesion. Degeneration of NGFr-positive cell bodies and processes is evident.

injection in unlesioned animals, confirming previous reports of limited bilateral hippocampal projections of septal fibers[1].

ChAT staining showed a 92% decrease in labeled cells ipsilateral to the FF lesion, corresponding to previous reports[2], and a 75% decrease in bilaterally lesioned animals (Figs. 3 and 4). Despite the limitations on detection of cells labeled simultaneously for ChAT and fluorescent microspheres, double-labeled cells were detected both in control and lesioned animals. In 3 animals with bilateral FF lesions, 3 cells were double-labeled in the VDB (a structure that projects to the dorsal hippocampus in a manner similar to that of the medial septum)[1,9,25].

NGFr staining showed a 75% decrease in the number of labeled cells ipsilateral to the lesion in unilaterally lesioned animals, and a 70% decrease in bilaterally lesioned animals (Figs. 5 and 6), corresponding to a previous report[26]. Double-labeling for NGFr and microspheres suffered from limitations similar to those encountered with ChAT immunocytochemistry, yet a total of 4 MS cells and 14 VDB cells were double labeled in FF-lesioned animals (3 MS cells and 6 VDB cells were double-labeled among 6 unilaterally lesioned animals, and one MS cell and 8 VDB cells were double-labeled among 3 bilaterally lesioned animals).

DISCUSSION

To determine whether cholinergic cells of the MS survive in a dysfunctional state after FF lesions, or whether they actually degenerate and die, retrogradely transported fluorescent rhodamine microspheres were injected into the hippocampus prior to FF lesions. The results demonstrate a substantial loss of fluorescently labeled neurons after FF lesions, suggesting that the cells die following the lesion. Parallel results have recently been obtained by another group of workers employing the septohippocampal lesion model and another fluorescent cell marker, True blue (O'Brien et al., following article).

Rhodamine-labeled fluorescent microspheres were chosen for this experiment because they remain inert within the cytoplasm after retrograde transport, maintain fluorescence for extended periods, and show little passive diffusion from the site of injection[16]. Although changes in intracellular pH or state of lipid solubility are potentially capable of destroying the fluorescent signal, it is most likely that changes in the intracellular environment sufficient to disrupt fluorescence would result in cell death. This is supported by observations in the current experiment and in previous unpublished observations that retrogradely labeled cells of the septum examined at various time points after axotomy show release of fluorescent microspheres into the cellular interstitium in close proximity to the cell body after cell disruption. Since fluorescence in the interstitium is detected *after* cell disruption, the fluorescent markers appear to be more resistant than the cell itself to an environment hostile to cell survival. Another possibility is that the fluorescent markers are somehow extruded from dysfunctional but surviving neurons. However, rhodamine microspheres are relatively large (0.02–0.2 μm diameter) so it is unlikely that they would leak through holes in dysfunctional cell membranes unless these holes were so large on a molecular scale that membrane functions vital for cell survival were disturbed (e.g. massive Ca^{2+} influx could occur). It is also possible that dysfunctional cells would exocytose the inert intracytoplasmic microspheres, although this seems unlikely. Thus, the possibility that fluorescence is lost artifactually has not been excluded with certainty, but it would appear very likely that the loss of these markers correlates with cell death.

The death of septal cholinergic cells after FF transection might be anticipated for several reasons. First, loss of Nissl-stained neurons is detected in the MS after FF axotomy[6,10,15,22,29,45]. Since Nissl substance consists of acidic cytoplasmic structures such as DNA, RNA and rough endoplasmic reticulum, and these structures are prerequisite for cell survival, one might expect diminishment or loss of Nissl staining as cells die. In contrast, studies in the nucleus basalis of Meynert (NBM), hypoglossal and vagal systems after axotomy show long-term persistence of Nissl staining that correlates with long-term cell survival assessed by other means (e.g. fluorescence)[19,35,38]. Although Nissl staining does not reveal specifically if cells lost in the septum are cholinergic, GABAergic[31], substance P-containing[43], or galanin-positive[23], it does suggest that a population of MS cells *die* after FF lesion. That some of these Nissl-lost neurons in the septum are cholinergic is suggested by the fact that: (1) approximately 30–35% of septal fibers projecting through the FF are cholinergic, a proportion roughly equal to that of projecting GABAergic fibers[1,2,4,10,17,24,31,44]; and (2) only 30% of glutamic acid dehydrogenase (GAD)-positive cells of the septum project to the hippocampus[17], while the majority of septal cholinergic cells project to the hippocampus[1,44].

That septal cholinergic neurons die after axotomy is also suggested by the fact that septal cholinergic cells projecting to the hippocampus are thought to possess few extra-hippocampal axon collaterals[36]. Since neurons with few axon collaterals are less likely to survive axotomy than neurons with several collaterals[7], one would predict that septal cells would be severely injured after axotomy.

A recent study reported recovery of MS ChAT labeling

when NGF was infused into the lateral ventricles of rats 1–3 weeks after FF lesions, suggesting that loss of ChAT activity in a subset of neurons represents cell dysfunction (i.e. transmitter enzyme suppression) rather than death[14]. A second study, however, failed to save septal cholinergic neurons when NGF was infused beginning 2 weeks after the FF lesion[26]. The present study supports the findings of the latter study. Factors that might account for the discrepancy between these studies include the following. (1) Variations might exist in the extent and/or completeness of the FF lesions, and in surgical technique. For example, axotomy close to the cell body causes greater cell damage than distal axotomy[7,13,21,41], and could result in cell death after a proximal lesion, but in cell dysfunction after a distal lesion[37]. (2) Intensity of ChAT-immunoreactivity as a result of variations in immunocytochemical protocols and/or differences in titers of anti-ChAT antibodies could result in differing interpretations of cell survival. (3) Differences in NGF activity might exist between the two studies. While the study by Hagg et al.[14] infused 7S NGF, the second study used 2.5S NGF[26]. (4) Variations in the mode of administration of NGF may account for differing results. For example, twice-weekly NGF delivery, used in the study that failed to show ChAT recovery, might be insufficient to induce recovery while continuous infusion for 2 weeks could support recovery. However, the degree of sparing of ChAT-positive cells with biweekly injections of NGF[15] has been comparable to that seen after continuous infusion in previous, non-delayed NGF studies[11,18,45]. (5) In the present study, rhodamine microspheres might be toxic to cells, although previous experience with these markers indicate no evidence of this[16] and control animals did not appear to show cell loss in the current study. (6) Loss of fluorescence might occur without cell death, although this appears to be unlikely as pointed out above. Artifactual loss of fluorescence would not explain the discrepancy between the two NGF studies.

It should also be noted that the study of Hagg et al.[14], while showing ChAT recovery after delayed NGF infusion, *also* indicates a progressive *non-recoverable* loss of ChAT-positivity at later time points. Indeed, the degree of recovery after a 3-week delay to NGF treatment falls by 63% compared to no delay. This may, therefore, merely indicate a more protracted time course for cell death than for loss of ChAT staining. Since the present study employs a 4-week time point to study cell survival, it is possible that a 4-week delay following NGF treatment would also result in fewer cells saved.

Although the present study suggests that the majority of cholinergic cells of the MS degenerate after FF lesions, 10–30% of cells still survive[2]. That some of these surviving ChAT-positive cells originally projected to the hippocampus is demonstrated by the presence of double-labeling for fluorescent microspheres and ChAT. Additionally, the presence of double-labeling for NGFr and fluorescent microspheres also indicates that cells have survived which originally projected to the hippocampus, and which expressed NGFr. Since cholinergic MS cells appear to require hippocampally derived NGF for normal function[11,15,18,45], these findings suggest that this *subpopulation* of surviving cells may: (1) *not* require NGF for survival, despite the fact that they are cholinergic and project to the hippocampus: or (2) derive NGF support from a source other than the ipsilateral dorsal hippocampus. Possible alternative sources of NGF include the ipsilateral HPC through axon collaterals that travel in the ventral pathway and terminate in the ventral HPC[1,9,25], the contralateral HPC through collateral projections across the septal midline, cortical projections to cholinergic cells, or sprouting after the lesion into regions that possess NGF, such as the dorsolateral quadrant of the septum. Previous studies employing AChE staining and ChAT labeling have supported the latter possibility, showing sprouting of cholinergic fibers into the dorsolateral quadrant after FF lesions alone[10,12].

This study documents a loss of NGFr staining paralleling that of ChAT loss after FF lesions. This finding is not unexpected in light of the fact that a double-labeling experiment of ChAT and NGFr cells of the MS/VBD complex revealed a 90% overlap of these populations[3]. The slightly lesser degree of NGFr loss compared to ChAT loss could be attributed to: (1) the existence of NGF-dependent populations of septal cells besides the known cholinergic hippocampal projection; (2) the existence of a subset of septal NGF-dependent cells that project not only to the hippocampus but also to other regions of the CNS by means of axon collaterals, thereby obtaining NGF; or (3) variations in staining technique.

The presence of fluorescent labeling in septal cells contralateral to the side of injection suggests a 10% rate of contralateral or bilateral projections of septal-hippocampal fibers, in agreement with previous reports[1].

Conclusion

The present study supports the assumption that a population of cholinergic cells of the MS undergo atrophy and death after FF lesions, although the time course of cell loss may extend over several weeks. In addition, the survival of some hippocampally projecting septal cholinergic cells and NGFr-positive cells after FF lesions suggests the existence of an NGF source for these projecting neurons other than the ipsilateral dorsal hippocampus.

This study and previous work points out the need for caution in describing the results of lesion studies. The

term 'cell death' should be used carefully, and should not be based solely upon a loss of transmitter-related staining after a lesion. Nissl staining, fluorescent markers, and immunocytochemistry are powerful tools when used *collectively* to describe the fate of damaged neuron systems.

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CHAPTER 17

Gene therapy in the CNS: intracerebral grafting of genetically modified cells

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Grafting cells to the CNS has been suggested and applied as a potential approach to CNS therapy through the selective replacement of cells lost as a result of disease or damage. Independently, studies aimed at direct genetic therapy in model systems have recently begun to suggest conceptually new approaches to the treatment of several kinds of human genetic disease, especially those caused by single gene enzyme deficiencies. We suggest that a combination of these two approaches, namely the graftment into the CNS of genetically modified cells, may provide a new approach toward the restoration of some functions in the damaged or diseased CNS. We present evidence for the feasibility of this approach, inclucing a description of some current techniques for mammalian cell gene transfer and CNS grafting, and several possible approaches to clinical applications. Specifically, we report that fibroblasts, genetically modified to secrete NGF by infection with a retroviral vector and implanted into the brains of rats with a surgical lesion of the fimbria-fornix, prevented the degeneration of cholinergic neurons that would die without treatment.

Introduction

During the past decade techniques aimed at a conceptually new view of disease therapy based on the direct correction of a genetic deficit have begun to be developed. The extension of this approach to whole animals, that is, the correction of a disease phenotype in vivo through the use of the functional genes as a pharmacological agent, has come to be called gene therapy (Friedmann, 1983). Such a therapy is based on the assumption that the correction of a disease phenotype can be accomplished either by modification of the expression of a resident mutant gene or by the introduction of new genetic information into defective cells or organs in vivo.

At present, techniques for the ideal type of gene therapy, that is through site-specific gene sequence correction or replacement, are just beginning to be conceived, but are not well developed. Therefore, most current models of gene therapy are really genetic augmentation rather than replacement models, and rely on the development of efficient gene transfer systems to introduce functional wild-type genetic information into genetically defective cell in vitro and in vivo.

We have recently proposed that a combination of gene transfer into cultured cells followed by intracerebral grafting of the genetically modified cells may constitute an effective approach to some disorders of CNS function (Gage et al., 1987; Fig. 1). This approach relies on the development of methods to introduce the foreign genes (transgenes) into appropriate neuronal and other target cells efficiently and functionally in vitro, as well as on the long-term survival of these cells and continued stable gene expression following intracerebral grafting. The conceptual and methodological development of these general objectives depends on the solutions to a variety of questions and problems including:

(1) What is the susceptibility of the variety of

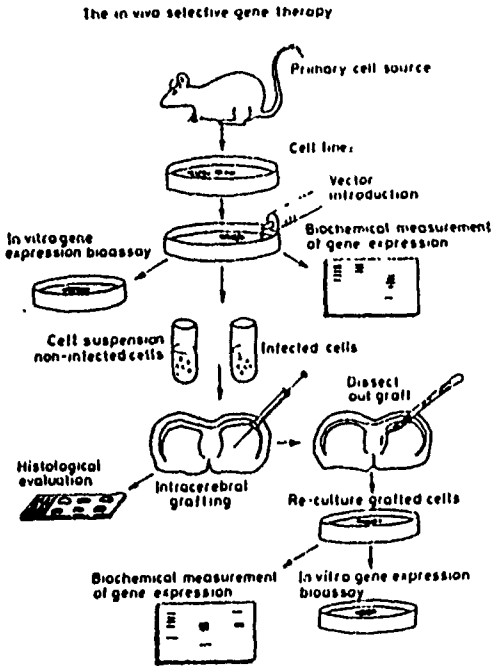

Fig. 1. Grafting of genetically modified cells to the CNS. Established cell lines or primary cell cultures are infected in vitro with retroviral vectors containing "reporter" genes encoding proteins whose activity can be easily assayed and/or selected for. The cells are then grown in selective media so that only infected cells survive. Expression of the genes in the recipient cells in vitro is characterized. The cells are injected intracerebrally, and uninfected control cells are injected contralaterally. Subsequently, the brains are examined histologically to evaluate cell survival. Alternatively, the grafted regions are dissected and expression of the reporter genes in vivo is measured. In addition, portions of the graft can be recultured and the continued presence and expression of the transgenes can be determined.

appropriate target CNS cells to the introduction of transgenes? What vectors are most suitable?

(2) Does the introduction of foreign sequences cause metabolic or genetic damage to recipient cells, or to the organism as a whole?

(3) Are the transgenes structurally and functionally stable? Are they efficiently expressed?

(4) What is the immunological response of the animal to the presence in the brain of genetically modified autologous or heterologous cells, or to the expressed gene product?

(5) Does a foreign gene product provide a needed and physiologically useful new function that can lead to correction of a particular disease phenotype?

We have addressed some of these questions and present data to test the potential applications of this approach (Gage et al., 1987). We have identified some of the technical and conceptual problems inherent in the techniques, and have suggested some of the human disease models for which this approach may be applicable and the issues that must be addressed before this approach can be considered for clinical use. We have also presented data based on the use of several prototype marker vectors expressing: (1) the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) cDNA; (2) the transposon Tn5 neomycin-resistance gene (*neoR*); and (3) the firefly luciferase cDNA. These reporter genes were chosen for their availability and the availability of sensitive assays to measure the gene products.

Choice of vector

While several procedures exist for inserting DNA into cells, (see Table I) murine retroviral vectors offer at present the most efficient, useful, and best characterized means of introducing and expressing foreign genes in mammalian cells. Briefly, these vectors have very broad host and cell type ranges, integrate by reasonably well understood mechanisms into random sites in the host genome, ex-

TABLE I

Methods of gene insertion

(A)	Microinject DNA in nucleus
(B)	Electric field/ electroporation
(C)	Calcium phosphate transfection
(D)	Liposomal transfection (lipofection)
(E)	Retroviral infection
(F)	Viral infection

press genes stably and efficiently, and under most conditions do not kill or obviously damage their host cells. Because retroviral vectors integrate at predominantly random sites in the cellular genome, insertional mutagenesis is a theoretical, and in fact a well documented, occasional consequence of viral infection (Hayward et al., 1981; Lohler et al., 1984), and can occur either by interruption of an essential cellular gene or by insertion of regulatory sequences such as promoters and enhancers near cellular genes with resulting inappropriate regulation of expression of such genes.

The methods of preparation of retroviral vectors have been reviewed extensively elsewhere (Mann et al., 1983; Anderson, 1984; Miller et al., 1985; Constantini et al., 1986; Gilboa et al., 1986; Mason et al., 1986; Readhead et al., 1987) and are now in common use in many laboratories. In principle, the aim is to design a vector in which the transgene is brought under the control of either the viral long terminal repeat (LTR) promoter-enhancer signals, or of a powerful internal promoter, and further, in which retained signals within the retroviral LTR can still bring about efficient integration of the vector into the host cell genome. To prepare transmissible virus, recombinant DNA molecules of such defective vectors are transfected into "producer" cell lines that contain a provirus expressing all of the retroviral functions required for packaging of viral transcripts into transmissible virus particles, but lacking the crucial packaging signal. Because of this deletion, transcripts from the helper cannot be packaged into viral particles. However, an integrated defective vector introduced into the same cell by means of calcium-phosphate-mediated transfection produces transcripts that can be packaged in trans since they do contain the packaging sequence. Ideally, the result is the production of infectious particles carrying the transgene completely free of replication-competent wild-type helper virus. In most models of gene therapy, the production of helper virus is probably undesirable since it may lead to spreading infection and possibly proliferative disease in lymphoid or other tissue in the host animal. An example of the prototype retrovirus and the reconstructed retroviral vector is shown in Fig. 2.

Choice of donor cells

The choice of donor cells for implantation depends heavily on the nature of the expressed gene, characteristics of the vector, and the desired phenotypic result. Because retroviral vectors are thought to require cell division and DNA synthesis for efficient infection, integration and gene expression (Varmus and Swanstrom, 1982) ~~Gage and Bjorklund, 1986)~~ our present model may be restricted to actively growing cells such as primary fibroblast cultures or established cell lines, replicating embryonic neuronal cells or replicating adult neuronal cells in selected areas such as the olfactory mucosa and possibly developing or reactive glia. The eventual use of other viral vectors, or of methods to induce a state of susceptibility in stationary, non-replicating target cells may make many other cell types suitable targets for viral transduction. For instance, we have recently developed methods that have permitted the successful retroviral vector infection of primary cultures of adult rat hepatocytes, ordinarily refrac-

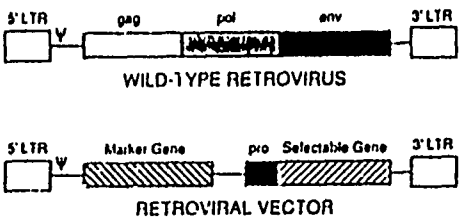

Fig. 2. Structure of integrated vectors. LTR, long terminal repeat. Helper functions required for the production of transmissible virus particles include the GAG and ENV genes which encode the capsid proteins as well as the reverse transcriptase (POL). The wild type virus also contains the packaging (psi) sequence essential for the encapsidation of RNA transcripts of the provirus into the mature virus particles. The prototype transmissible retrovirus is prepared by constructing a plasmid in which the GAG, POL and ENV genes have been replaced by the reporter gene (marker gene), and selector gene which is controlled by an internal promotor separate from the intact retroviral LTR which controls the marker gene.

tory to infection with such vectors, and similar methods may be helpful for a number of other cells (Wolff et al., 1987). The development of other kinds of vectors derived from herpes, vaccinia, or other viruses, as well as the use of efficient, non-viral methods for introducing DNA into donor cells such as the recently developed electroporation technique (Toneguzzo et al., 1986), may allow successful gene transfer into many other cells presently not susceptible to retroviral vector infection.

One can envision several mechanisms by which one can introduce a new function into target cells in a phenotypically useful way (see Fig. 3). The most direct approach, which would bypass the need for cellular grafting entirely, would be the introduction of a transgene directly into the cells in which that function is aberrant as a consequence of a genetic defect; i.e., neuronal cells in the case of Tay Sachs disease, possibly Lesch-Nyhan disease, and probably Parkinson's disease. Alternatively, one could express a new function in such defective target cells by introducing a genetically modified donor cell that could establish tight juction or other contacts with the target cell. Some such contacts are known to permit the efficient diffusion of metabolically important small molecules from one cell to another, leading to phenotypic changes in the recipient cell (Loewenstein, 1979). This process has been called "metabolic cooperation" and is known to occur between fibroblasts and glial cells (Gruber et al., 1985), although it has not yet been demonstrated conclusively in neurons. Still other donor cells could express and secrete a diffusible gene product that can be taken up and used by nearby defective target cells. The donor cells may be genetically modified in vitro or, alternatively they may be directly infected in vivo. Finally, an introduced donor cell infected with not only replication-defective vector but also replication-competent helper virus, could produce locally high titers of progeny virus that might in turn infect nearby target cells to provide a functional new transgene.

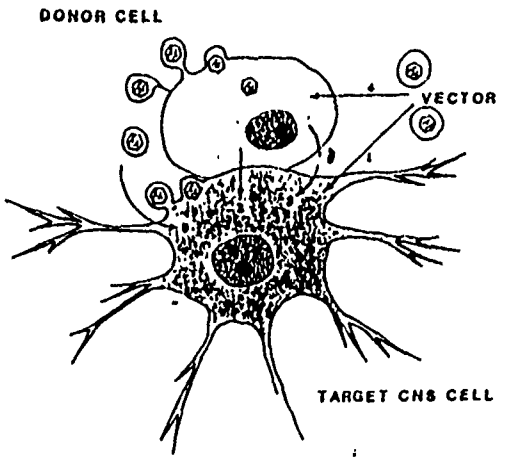

Fig. 3. Introduction of new function into target cells. In the most straightforward approach, transgenes would be introduced into the target cells directly (1). Alternatively, donor cells may be used to introduce the new function into the target cells through tight junctions (2), or through secretion and uptake of the gene products (3). The donor cells may express the new function naturally or may be genetically modified to express the function (4), either in vitro or in vivo. Finally, donor cells genetically engineered to produce transmissible retrovirus (see Fig. 1) could be used to produce virus that can, in turn, infect target cells (5).

Marker genes

Initial studies have provided support for the concept that a combination of gene transfer and neural grafting can be used as a new approach toward the restoration of some functions in the damaged or diseased brain (Gage et al. 1987). An essential requirement for the development of this approach is the availability of a reporter gene which can be easily and accurately detected by histochemical and/or biochemical methods.

Retrovirus-mediated transfer of the *Escherichia coli* (*E. coli*) β-galactosidase gene (*lacZ*), combined with histochemical staining for β-galactosidase activity (Sanes et al., 1986; Price et al., 1987; Turner et al., 1987), has recently been used to mark cell lineages in the CNS. These studies are based on the assumption that exogenous *E. coli* β-galactosidase can be distinguished from the endogenous lysosomal β-galactosidase that is widely distributed in mammalian cells (Furth and Robinson, 1965;

Dannenberg and Suga, 1981; Mann et al., 1983), taking advantage of the widely different pH optima for the two enzymes. The pH optimum for *E. coli* β-galactosidase is approximately 7.3 and that of rat brain is approximately 3.5.

Based on these important features, we chose *lacZ* as a reporter gene for the grafting of retrovirus-infected cultured cells to the rat brain. We have observed *E. coli* β-galactosidase-positive fibroblasts, stained at pH 7.5, in the brains of rats several weeks after grafting. However, some β-galactosidase-positive cells were also observed in control grafts of cells which had not been infected with the *lacZ* gene. Subsequent studies revealed that this "false" staining was due to endogenous lysosomal activity associated with macrophage infiltration induced by the damage associated with grafting. By using an antibody specific for *E. coli* β-galactosidase we have been able to distinguish between the *E. coli* enzyme and the endogenous mammalian β-galactosidase. We conclude here that *lacZ* is a useful reporter gene for intracerebral grafting studies if appropriate caution is taken for false positives. Our next objective was to test this approach with a gene which we could use to evaluate the consequences of the therapeutic intervention.

The functional gene product (NGF)

NGF is currently the best characterized neurotrophic factor (NTF) (Cohen and Levi-Montalcini, 1957; Greene and Shooter, 1980; Thoenen and Barde, 1980; Barde et al., 1983; Ullrich et al., 1983; Berg, 1984). NGF supports the survival and axonal growth in vitro and in vivo of sensory and sympathetic neurons from the peripheral nervous system (PNS) (Gundersen and Barrett, 1980; Campenot, 1982). Furthermore, NGF can attract and guide regenerating axons, whether provided to the neuron in a soluble or immobilized form (Gundersen and Barrett, 1980; Levi-Montalcini, 1982), and may stimulate the growth rate even of axons whose neurons do not require NGF for survival (Collins and Dawson, 1983). A number of studies have shown that NGF occurs in, an is produced by, the central nervous system (CNS). For example, mammalian CNS tissues have been shown to contain NGF messenger RNA by in situ hybridization (Korshing et al., 1985; Sheldon and Reichardt, 1986), NGF antigen by immunohistochemical and radioimmune assays (Greene and Shooter, 1980; Ayer LeLievre et al., 1983), NGF receptors by autoradiography (Richardson et al., 1986) and NGF by biological assays (Scott et al., 1981; Manthorpe et al., 1983; Nieto-Sampedro et al., 1983; Collins and Crutcher, 1985). The greatest NGF levels in CNS tissues appear within the target areas of the cholinoceptive basal forebrain systems (Scott et al., 1981), and NGF administered into rat brain raises choline acetyltransferase (ChAT) levels in the hippocampus and striatum (Hefti et al., 1984; Mobley et al., 1985). Radiolabeled NGF injected into target regions is taken up and retrogradely accumulated by the cholinergic neurons innervating them, such as the septal/diagonal band neurons for the hippocampus and nucleus basalis neurons for the neocortex (Schwab et al., 1979; Gnahn et al., 1983; Seiler and Schwab, 1984). NGF is also produced by purified cultures of cerebral astroglial cells (Rudge et al., 1985) and accumulates in fluids surrounding rat brain lesions (Manthorpe et al., 1983; Nieto-Sampedro et al., 1983; Assouline et al., 1985). More recently NGF has been shown to increase ChAT activity in CNS neuronal cultures (Honegger and Lenoir, 1982; Hefti et al., 1985; Martinez et al., 1985; Mobley et al., 1985).

The concept that neuronal survival in vivo may depend on the continued presence of an adequate supply of NTFs in general, and NGF in particular, is supported by the existence of such phenomena as: (1) "developmental neuronal death," in which the excessive number of neurons produced during development is decreased to accommodate the limited target cell number (Landmeser and Pilar, 1978; Cunningham, 1982; Hamburger and Oppenheim, 1982; Cowan et al., 1984; Fawcett et al., 1984); (2) "retrograde neuronal degeneration," in which axotomized neurons cut off from their in-

nervation target and surrounding glial cells undergo degeneration or even death (Grafstein, 1977; Feringa et al., 1983; Pearson et al., 1983; Grady et al., 1984); and (3) "pathological neuronal death," in which specific populations of neurons degenerate and die (Terry and Davies, 1980; Appel, 1981; Bartus et al., 1982; Bondareff et al., 1982; Whitehouse et al., 1982). One explanation commonly put forth for such neuronal death-inducing situations is that neurons normally depend for their continued health upon NTFs supplied by their target and associated glial cells, and that disruption in this trophic supply causes their death.

Model system: "retrograde neuronal degeneration"

The cholinergic projection from the adult rat septum and diagonal band to the ipsilateral hippocampus has been a useful model for examining CNS plasticity (Chun and Patterson, 1977; Gnahn et al., 1983; Amaral and Kurz, 1985; Armstrong et al., 1987; Springer et al., 1987). Neurons of the medial septum and the vertical limb of the diagonal band project dorsally to the hippocampus mainly through the fimbria-fornix (Lewis et al., 1967; Armstrong et al., 1983; Gage et al., 1983). About 50% of the septal/diagonal band neurons sending fibers through the fimbria-fornix are cholinergic (Amaral and Kurz, 1985; Wainer et al., 1985) and provide the hippocampus with about 90% of its total cholinergic innervation (Storm-Mathisen, 1974). The cholinergic neurons, axons and terminals can be visualized by acetylcholinesterase (AChE) (Butcher, 1983; Hedreen et al., 1985) and ChAT immunocytochemical analysis (Armstrong et al., 1983; Wainer et al., 1985), and the terminal fields within the hippocampal formation can be quantified biochemically by measuring extracted ChAT activity (Fonnum, 1969, 1984).

Complete transection of the fimbria-fornix pathway in adult rats results in a rapid and consistent retrograde degeneration and death of many of the septum/diagonal band neurons (including the cholinergic ~~ones~~ neurons) that originally contributed axons through this pathway (Cunningham, 1982; Grady et al., 1984; Kromer and Cornbrooks, 1984; Wainer et al., 1985; Hefti, 1986). One explanation for this axotomy-induced cell death is that the septal neurons become deprived of a critical supply of NTF provided possibly by the post-synaptic neurons or glial cells in the axonal and/or synaptic vicinity within the hippocampal innervation territory (Gnahn et al., 1983; Nieto-Sampedro et al., 1983; Heacock et al., 1984; Collins and Crutcher, 1985; Gage and Bjorklund, 1986). That this hippocampal NTF might be NGF or NGF-like is supported by the previously listed studies from several laboratories reporting an NGF presence within the septo-hippocampal system.

These studies raise the question whether exogenous administration of NGF to axotomized septal neurons might rescue them from the ensuing retrograde degeneration and death and thus allow them to regenerate their already cut axons, or even grow new ones, back to the hippocampal formation. Recently Hefti (1986) and Williams et al. (1986) have independently reported that the intraventricular administration of purified NGF into adult rats from the time of fimbria-fornix transection onward prevents the death of most of the axotomized cholinergic septum/diagonal band neurons. Furthermore, it appears that, as a result of the transection even non-cholinergic septal neurons (Panula et al., 1984) are destined to die (Gage et al., 1986) and may be saved by NGF administration (Williams et al., 1986). NGF administration also seems to prevent the degeneration of the cut septal cholinergic axons and/or stimulate their regrowth, since a large number of AChE-positive fibers appear to form a neuroma-like structure proximal to the transection site (Gage et al., 1986).

Using the above described model system we have recently tested the possible functional effects of ~~the approach suggested in the proposal~~. Specifically, we demonstrated that cultured rat fibroblasts, genetically modified to produce and secrete NGF,

genetically modified cells grafted to the brain.

and then grafted to the cavity formed in creating a fimbria-fornix lesion, prevented retrograde cholinergic degeneration and induced axonal sprouting, thereby demonstrating a functional response (Edwards et al., 1986) (Rosenberg et al 1988).

A prototypical retroviral vector was constructed that contains NGF cDNA corresponding to the shorter transcript that predominates in mouse tissue receiving sympathetic innervation (Edwards et al., 1986; Fig. 4). This transcript is believed to encode the precursor to NGF that is constitutively secreted. The vecto also includes a dominant selectable marker, the transposon Tn5 neomycin-resistance gene. Transmissible retrovirus was generated and used to infect the established rat fibroblast cell line 208F. Individual neomycin-resistant colonies, selected in medium containing the neomycin analog G418, were expanded and tested for NGF production and secretion using a two-site enzyme immunoassay (Korsching and Thoenen, 1983). The clone producing the greatest levels of NGF had 1.7 ng NGF/mg total cellular protein and secreted NGF into the medium at a rate of 50 pg/h/10^5 cells. The NGF secreted by this clone was biologically active, as determined by its ability to induce neurite outgrowth from PC12 rat pheochromocytoma cells. Uninfected 208F cells, in contrast, did not produce detectable levels of NGF in either assay.

Fimbria-fornix (FF) lesions were made in 16 rats; 8 rats received grafts of infected cells while the remaining eight received uninfected control cells. After 2 weeks, all animals were sacrificed and

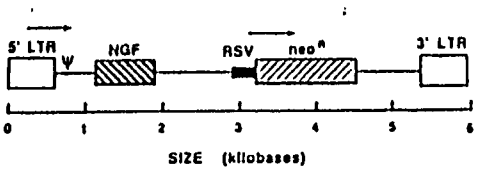

Fig. 4. NGF retroviral vector. Contructed from the Moloney murine leukemia virus, containing the 777-bp HgaI-PstI fragment of the NGF cDNA under the control of the viral 5' LTR. The vector also includes the dominant selector marker encoding the neomycin resistance function of transposon Tn5 under the control of an internal Rous sarcoma virus promotor.

processed for immunohistochemistry. Staining for fibronectin, a fibroblast-specific marker, revealed robust graft survival, which was comparable in both the NGF-secreting cells and the control cells. Sections stained for choline acetyltransferase (ChAT) to evaluate the survival of cholinergic cell bodies indicated a greater number of remaining neurons on the lesioned side of the medial septum in animals that had received grafts of infected cells than in animals that had received control grafts. Neuronal survival was quantitated and, when expressed as a percentage of the remaining cholinergic cells in the septum ipsilateral to the lesion relative to the intact contralateral septum, was shown to be 92% in animals grafted with NGF-secreting cells, but only 49% in animals grafted with control cells (Rosenberg et al., 1988). The latter results from the control group are comparable to our previous observations in lesioned animals that had received no grafts (Williams et al., 1986).

The 208F fibroblasts used in the above described study were chosen because they were immortalized, and were deficient in the HPRT gene, a feature that is useful as an in vitro model of Lesch-Nyhan disease. However, upon careful examination we found that these cells do not survive as well in vivo as the parental line, Rat1, and thus the NGF retroviral vector was used to infect the established rat fibroblast cell line Rat1 cells. As before, individual neomycin-resistant colonies, selected in medium containing the neomycin analog G418, were expanded and tested for NGF production and secretion by two-site enzyme immunoassay. This time the highest producing clone, Rat1-N.8-8, contained 66 pg NGF/10^5 cells and secreted NGF into the medium at a rate of 77 pg/h/10^5 cells. The secreted NGF was biologically active, as determined by its ability to induce neurite outgrowth from PC12 rat pheochromocytoma cells. Uninfected Rat1 cells, in contrast, did not produce detectable levels of NGF.

Again unilateral aspirative cavities were made through the cingulate cortex of Sprague-Dawley rats, completely transecting the fimbria-fornix. Retrovirus-infected (NGF-secreting) and control

uninfected fibroblasts were suspended in PBS at $8 \cdot 10^4$ cells/μl, and 4 μl aliquots were injected free-hand into the lesion cavity and lateral ventricle ipsilateral to the cavity. In this experiment, animals were sacrificed after 2 or 8 weeks and processed for immunohistochemistry and for in situ hybridization of mRNA for NGF. Staining for fibronectin, a fibroblast-specific marker, revealed robust graft survival that was comparable in both infected and control groups. Staining for choline acetyltransferase, to evaluate the survival of cholinergic neurons, indicated a significantly greater number of remaining neurons on the lesioned side of the medial septum in animals that had received grafts on infected cells than in animals that had received uninfected control grafts (see Fig. 5*A*, *B*). We have further examined these animals for their immunoreactivity to NGR receptor (IGg 192). With this antibody we observed that in the animals with control grafts there was a dramatic decrease in the number and intensity of cell staining in the medial septal area ispilateral to

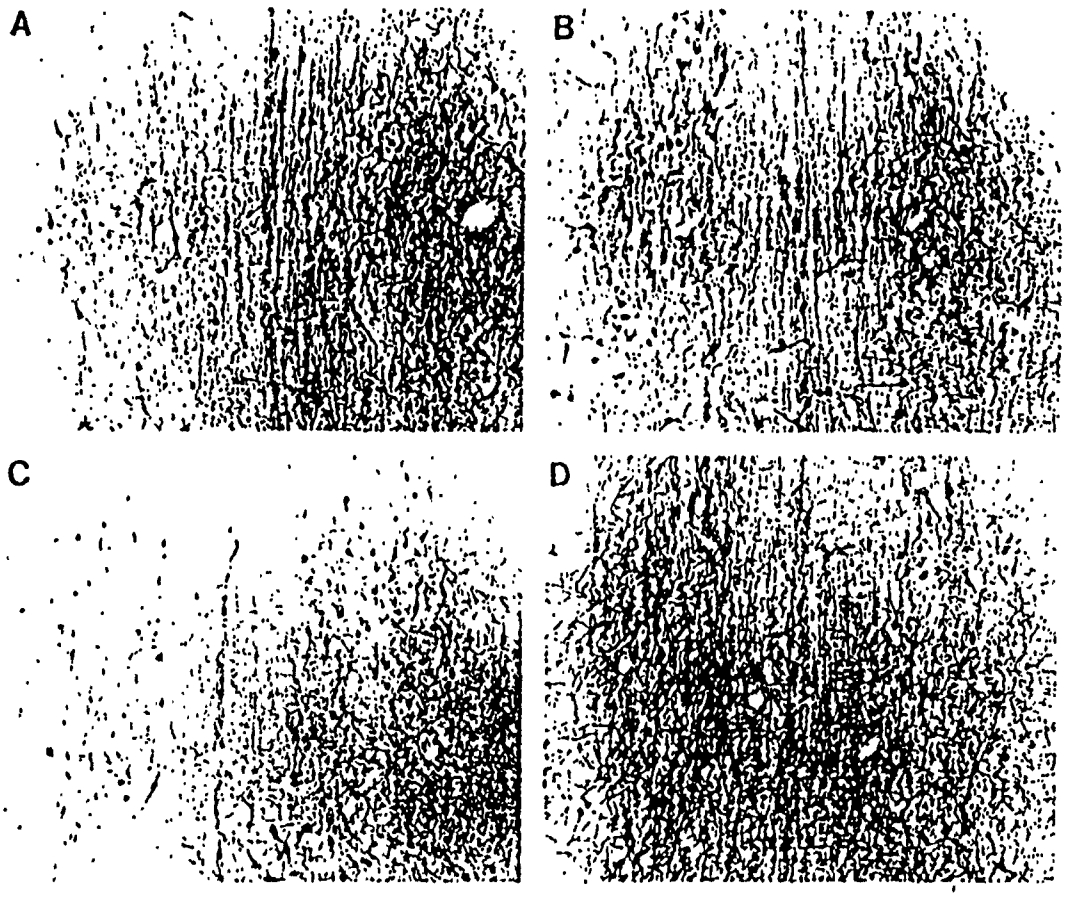

Fig. 5. Photomicrographs of immunohistochemical staining for choline acetyltransferease (ChAT) and nerve growth factor receptor (NGFr). *A*, *B*. Coronal section through medial septum of tissue stained for ChAT. *C*, *D*. Coronal section through medial septum of tissue stained for NGFr. *A*, *C*. Animal with graft of retrovirus-infected cells. *B*, *D*. Animal with graft of control cells.

Lesions were placed on the ~~right~~ left side.

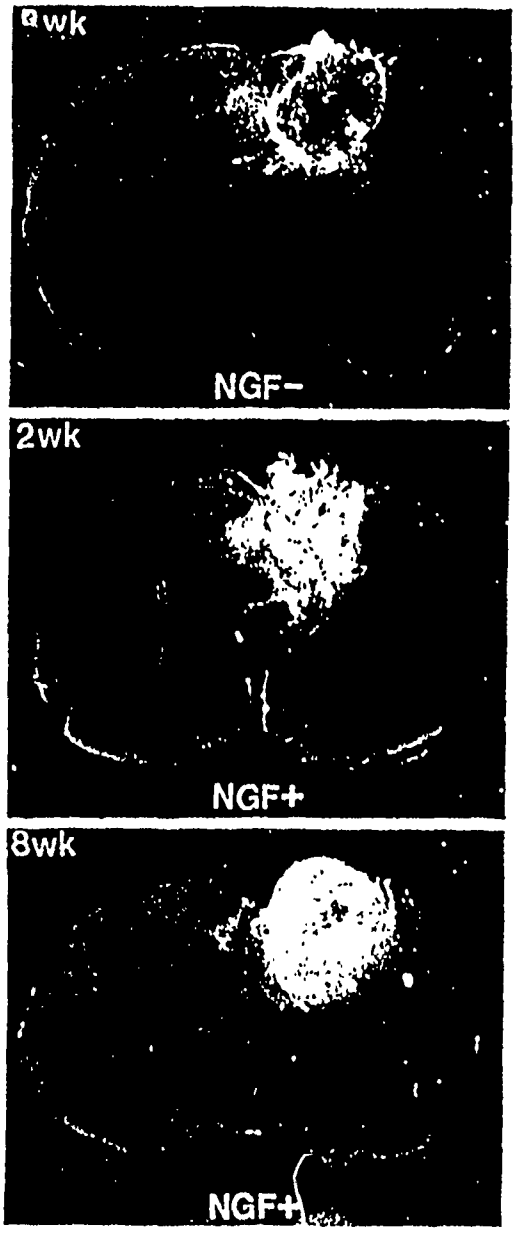

Fig. 6. Photomicrographs of sections prepared for in situ hybridization for NGF mRNA. They are coronal sections taken through the middle of the grafts in the fimbria fornix cavity.

the fimbria fornix lesion. In stark contrast, the immunoreactivity for this same antibody was intense in the ipsilateral septum for those animals that received grafts of NGF secreting fibroblasts (see Fig. 5C, D). Staining for parvalbumin, to evaluate the survival of GABAergic neurons, which also degenerate following transection of the fimbria-fornix by 8 weeks (Peterson et al., 1987), indicated no increased survival in response to NGF-secreting fibroblasts, indicating the specificity of these grafts for cholinergic neurons. It is interesting to note that uninfected control grafts resulted in 50% survival of cholinergic neurons, whereas previous studies have shown 10 – 50% survival in untreated control animals (Hefti, 1986; Williams et al., 1986). This suggests the possibility that the fibroblasts produce other as yet unknown factors that can affect cholinergic survival. Alternatively, this could reflect the variablity in the location of the lesion with respect to the cholinergic cell bodies. In our previous study with 208F fibroblasts as well as the study described here, we observed that the animals that received NGF-secreting fibroblasts showed an increase in acetylcholinesterase-positive fiber and cell staining, with a robust sprouting response in the dorsal lateral quadrant of the septum, and the most intense staining abutting the cavity containing the graft. We had previously observed this sprouting in response to chronic NGF infusion (Gage et al., 1988), but not to the extent seen in the 208F study, even though the fibroblasts secrete NGF at a rate significantly lower than administered in the infusion studies. This observation may reflect the fact that the biological activity of the NGF in the pump decreases with time, while the NGF constantly being synthesized in the cells remains more or less constant. Still other alternatives are conceivable.

Recently we have examined the grafts for their ability to express detectable levels of NGF mRNA in parallel sections of brains which had been taken for histochemical evaluation. Individual sections were processed for in situ hybridization. At 2 weeks following implantation, retroviral-infected

fibroblast grafts expressed high levels of NGF mRNA in the graft relative to the endogenous levels in the brain, though message was detected in the expected areas of the hippocampus and cortex. Non-infected grafts showed much less specific hybridization. There was, however, clear hybridization around the edges of the cavity in which the graft was implanted. This may be attributed to the increased expression of NGF mRNA in the reactive astrocytes around the wound, but at present we do not know this to be true. In 3 animals with NGF-producing grafts processed for in situ hybridization at 8 weeks there was continued, though slightly decreased expression of NGF mRNA in the graft, compared to those processed at 2 weeks following grafting (see Fig. 6).

Discussion

Our studies have demonstrated that genetically modified fibroblasts can survive intracerebral grafting and continue to express transgenes for at least 2 months. Furthermore, the grafts continue to produce and secrete sufficient NGF to have a biological effect during this period. There are several prerequisites for a neurological disease to be a candidate for this approach:

(1) The pathogenesis of the disease must be sufficiently well understood to allow identification of the relevant lacking function.

(2) The relevant gene must be available as a well-characterized cDNA clone.

(3) The affected region of the CNS must be known and sufficiently localized to permit implantation into the appropriate area(s).

(4) Restoration of normal function must not, at present, require synaptic contact with target cells. Instead, the donor cells must produce a factor that has a mechanism for release by the cells and uptake by neurons. In the example of NGF, these conditions are satisfied because the NGF precursor protein includes a signal sequence for secretion, and the secreted NGF binds to receptors on the target cholinergic neurons. In other systems the gene product may require active transport into storage vesicles or simply diffuse through the cell membrane. Alternatively, the donor cells can act as a toxin "sink" by expressing an enzyme that metabolizes a neurotoxin. This, of course, requires that the neurotoxin has a mechanism for leaving the neuron and entering the donor cell.

(5) Ideally, an animal model should be available.

We conclude that the intracerebral grafting of genetically modified cells could eventually provide a means for CNS therapy. Together with traditional neuronal grafting and the upcoming vectors for direct genetic modification of neurons in vivo, this approach should permit the treatment of numerous CNS disorders that cannot be treated by standard drug therapies.

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Nerve Growth Factor Function in the Central Nervous System*

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1 Introduction

A complete understanding of NGF function in the CNS does not exist. Instead, there is a collection of observations based on experiments conducted in the PNS and CNS, although more definitive results have come from experiments in the PNS. The conclusion that NGF is a neurotropic factor in the PNS biased experimental design and interpretation of results when a function for NGF in the CNS was sought. An example of design and interpretation bias is the very

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significant paper by SCHWAB et al. (1979), showing for the first time retrograde transport of NGF in cholinergic neurons. These authors entitled the manuscript "Nerve growth factor (NGF) in the CNS: absence of specific retrograde transport and tyrosine hydroxylase induction in locus coeruleus and substantia nigra", documenting the *expectation* of retrograde transport in catecholamine neurons of the CNS, as in the PNS. Subsequent experiments, reviewed here, support a neurotrophic role for NGF in the CNS, although in the *cholinergic* rather than catecholaminergic system of CNS neurons.

During development more neurons are generated than are needed in the mature CNS. Some neurons undergo cell death at times when their growing axons compete for target territories, reducing the final number of neurons to that found in the adult (COWAN et al. 1984). The extent of neuronal cell death during development can be affected by experimentally manipulating the target area of the developing neurons to add or subtract target tissue (LANDMESSER and PILAR 1978; HAMBURGER and OPPENHEIMER 1982). These observations have led several investigators to propose that developmental cell death and survival are regulated by proteins presumably supplied by the target territory, which have been called *neurotrophic factors* (NTF) (THOENEN and BARD 1980). "Trophic" refers to the ability of one tissue, cell, or protein to support and/or nourish another: thus a neurotrophic factor is a chemical or molecule that is made in any cell that supports the survival of, or nourishes, neurons. *Trophic* differs markedly from *tropic*; the latter refers to the influence of one cell or tissue on the direction of movement or outgrowth of another. Thus a *neurotropic factor* is a chemical or molecule that can influence the direction and/or growth of an axon.

The neurotrophic hypothesis postulates that: (1) adult CNS neurons are supported and regulated by their respective NTFs, (2) proper maintenance of these neurons depends on adequate supply and utilization of the NTFs in situ, (3) an interference with NTF support, or "neurotrophic deficit", will result in defective performance or even degeneration and death of the target neurons, and (4) such trophic deficits may be the basis of degenerative central nervous system diseases (e.g., Parkinson's, motor neuron, or Alzheimer's disease) or normal aging (APPEL 1981).

The neurotrophic hypothesis is supported in the CNS by: (1) "developmental neuronal death", in which the excessive number of neurons produced during development is decreased to accommodate the limited target cell number (COWAN et al. 1984); (2) "retrograde neuronal degeneration", in which axotomized neurons cut off from their innervation target and surrounding glial cells undergo degeneration or even death (PEARSON et al. 1983); and (3) "pathological neuronal death", where specific populations of neurons degenerate and die (APPEL 1981; BARTUS et al. 1982). Among several explanations currently being tested, one that is commonly put forth for such neuronal death-inducing situations is that neurons normally depend for their continued health upon NTFs, supplied by their target and associated glial cells, and that disruption in this trophic supply causes their death.

Nerve growth factor (NGF) is currently the best characterized NTF (BARDE et al. 1983; BERG 1984; THOENEN and BARDE 1980; ULLRICH et al. 1983). NGF supports the survival in vitro and in vivo of sensory and sympathetic neurons from the PNS (GUNDERSON and BARRETT 1980) (neurotrophic). Furthermore NGF can attract and guide developing and regenerating axons, whether provided to the neuron in a soluble or immobilized form (GUNDERSON 1980), and may even guide axons whose neurons do not require NGF for survival (COLLINS and DAWSON 1983) (neurotropic).

2 NGF Function on CNS Neurons In Vitro

NGF can support the survival of embryonic and adult cells, induce and influence the direction of fiber outgrowth, and induce the synthesis of specific enzymes in vitro. The biological actions of NGF have been well-studied in neurons of the PNS and have recently been examined in the CNS.

Sympathetic and dorsal root ganglion cells have been extensively studied in culture. Cell death is found to occur in these systems (COWAN et al. 1984), perhaps as a result of competition for a limited supply of peripherally derived trophic factors. Sympathetic ganglion neurons derived from embryonic animals require NGF for their survival (BERG 1984; CHUN and PATTERSON 1977a, and b; GREENE 1977a; LEVI-MONTALCINI and ANGELETTI 1963). NGF is also able to promote the survival of dorsal root ganglion cells (CRAIN 1975; CRAIN and PETERSON 1974; GREENE 1977b; LEVI-MONTALCINI and ANGELETTI 1963) and can induce neurite extension in these cells (GREENE 1977b; LEVI-MONTALCINI and ANGELETTI 1963). Experiments demonstrating that the direction of growth of the neurites from NGF-responsive ganglion cells in culture is influenced by a NGF concentration gradient provided evidence for a tropic effect of NGF (CAMPENOT 1977; CHARLWOOD et al. 1972; EBENDAL and JACOBSON 1977; GUNDERSON 1985; GUNDERSON and BARRETT 1979, 1980; LETOURNEAU 1978). CAMPENOT (1977) showed that NGF can influence the direction of neurite outgrowth and support the survival of cells via uptake by a cell's peripheral projections without involvement of the cell soma. Cocultures of sympathetic ganglia and NGF rich tissue results in the innervation of the tissue by fibers from the ganglion cells (BURNSTOCK 1974; CHAMLEY et al. 1973; JOHNSON et al. 1972). NGF can also increase the activity of specific enzymes in neurons. The application of NGF to rat superior cervical ganglion cells in vitro increases tyrosine hydroxylase and dopamine beta hydroxylase activity in these cells (MACDONNELL et al. 1977; MAX et al. 1978; OTTEN et al. 1977). NGF is also able to induce choline acetyltransferase (ChAT) activity in sympathetic neurons (see THOENEN and BARDE 1980 for a review).

There is increasing evidence for a role for NGF in the CNS. Unlike catecholaminergic neurons in the PNS, catecholaminergic neurons in the CNS

are unresponsive to NGF (DREYFUS et al. 1980; OLSON et al. 1979). In the CNS, *cholinergic* neurons are responsive to NGF. WAINER et al. (1986) and MARTINEZ et al. (1987) demonstrated that cultures of central cholinergic neurons require NGF for their survival, and HARTIKKA and HEFTI (1988) found that NGF affects the survival of cultures of low plating density but not of high plating density. Although cholinergic cell cultures of high plating density do not require exogenous NGF for their survival, NGF can stimulate fiber growth of septal cholinergic neurons (HARTIKKA and HEFTI 1988). Additionally, the direction of axonal outgrowth from cultured septal cells can be influenced by NGF. GAHWILER et al. (1987) showed that NGF can influence the growth of septal axons to cocultured hippocampal slices. NGF also has been shown to increase ChAT activity in cultures of neonatal and fetal basal forebrain cells (GNAHN et al. 1983; HARTIKKA and HEFTI 1988; HATANAKA and TSUKUI 1986; HEFTI et al. 1985; HONEGGER et al. 1986; HONEGGER and LENOIR 1982; MARTINEZ et al 1987) and striatal cells (MARTINEZ et al. 1985). Antibodies to NGF can block the observed increase in ChAT activity in basal forebrain cultures (HARTIKKA and HEFTI 1988; HEFTI et al. 1985) and reduce the number of acetylcholinesterase (AChE) positive cells in low plating density cultures (HARTIKKA and HEFTI 1988). Additionally, cultures of hippocampal cells, which are the targets of the cholinergic neurons in the septum, were shown to contain NGF-like activity that could be blocked by antibodies to NGF (CRUTCHER and COLLINS 1982).

NGF is also produced by cultures of Schwann cells in the PNS and astrocytes in the CNS (LINDSAY 1979; NORRGREN et al. 1980; RUDGE et al. 1985). The conditioned media from cultures of astrocytes can support the survival of (LINDSAY 1979) and stimulate neurite outgrowth from dorsal root ganglion cells (ASSOULINE et al. 1987). Additionally, cultures of embryonic Schwann cells express the receptor for NGF (NGFr) (HOSANG and SHOOTER 1985; ROHRER 1985; ROHRER and SOMMER 1983; ZIMMERMANN and SUTTER 1983). Thus, the evidence suggests a role for glial cells in the NGF response.

3 NGF Function on CNS Neurons In Vivo

A number of studies have shown that NGF occurs in and is produced by both the developing and adult CNS. In support of results obtained from in vitro developmental studies, NGF infusion to neonatal rats increased ChAT content in the basal forebrain and septum (MOBLEY et al. 1985, 1986; JOHNSON et al. 1987). NGF also appears to play a prominent role in the development of the septohippocampal cholinergic projection. The septohippocampal projection in the rat develops from embryonic day 20 to postnatal day 14 (MILNER et al. 1983), providing the source of all extrinsic cholinergic innervation to the hippocampal formation. Total NGF content and NGF mRNA in the hippocampal formation rise just prior to developmental increases in ChAT activity during development.

however, suggesting a role for NGF in the guidance and support of approaching septal cholinergic fibers (LARGE et al. 1986; WHITTEMORE et al. 1986). NGF receptor (NGFr) content (total protein) (ECKENSTEIN 1988; YAN and JOHNSON 1988), NGFr mRNA (BUCK et al. 1987), and total NGF content, but not NGF mRNA (WHITTEMORE et al. 1986), rise during embryonic and neonatal basal forebrain development, suggesting that these regions might be preparing to contact a source of, and retrogradely transport, NGF.

In vivo data also support in vitro data regarding the role of NGF in the development of cholinergic neurons of the nucleus basalis of Meynert (nucleus basalis). NGF administration to developing neurons in this region results in increases in ChAT activity (MOBLEY et al. 1986; JOHNSTON et al. 1987), although of a lesser degree than that seen in the septal region.

In the adult, mammalian CNS tissues have been shown to contain: NGF mRNA by northern (RNA) blotting and in situ hybridization (AYER-LELIEVRE et al. 1983; WHITTEMORE et al. 1986; LARGE et al. 1986; SHELTON and REICHARDT 1986; LARKFORS et al. 1987); NGF antigen by immunohistochemical and radioimmunoassays (AYER-LELIEVRE et al. 1983; GREENE and SHOOTER 1980); NGFr by autoradiography (RICHARDSON et al. 1986); and NGF by biological assays (COLLINS and DAWSON 1983; SCOTT et al. 1981). The highest NGF levels in CNS tissue appear within the target areas of the cholinoceptive basal forebrain systems (SHELTON and REICHARDT 1986), including the hippocampus and cortex, and NGF administered into rat brain raises ChAT levels in the hippocampus and septum (HEFTI et al. 1984; MOBLEY et al. 1985). Radiolabeled NGF injected into target regions is taken up and retrogradely transported by cholinergic neurons, including the septal/diagonal band neurons for the hippocampus and nucleus basalis neurons for the neocortex (SCHWAB et al. 1979; SEILER and SCHWAB 1984). Assays of NGF activity, mRNA synthesis, or NGFr positivity have also revealed NGF influence in adult olfactory bulb (FRIEDMAN et al. 1988; GOMEZ-PINILLA et al. 1988), posterior lobe of the pituitary gland (YAN et al. 1988), and neonatal and perhaps adult cerebellum (ECKENSTEIN 1988; TANIUCHI et al. 1986; PIORO et al. 1988).

Trophic factors, possibly including NGF also accumulate in fluids surrounding rat brain lesions (CRUTCHER 1987; COLLINS and CRUTCHER 1985; GAGE et al. 1984; NIETO-SAMPEDRO et al. 1983). Following lesions of the fimbria/fornix in the adult rat, total NGF content in the hippocampus rises by approximately 50%, although NGF mRNA does not rise (WHITTEMORE et al. 1986; KORSCHING et al. 1986). The latter finding suggests a buildup of NGF in the hippocampus from supplies of NGF that would normally have been retrogradely transported to cells of the medial septum. In the neonate, however, fimbria/fornix transections *are* associated with an elevation in hippocampal NGF mRNA (WHITTEMORE et al. 1986). The accumulation of NGF in the hippocampus after denervation may also result in the sprouting of another population of NGF-dependent processes into this region: axons of sympathetic neurons located in the superior cervical ganglion (CRUTCHER 1987). Cholinergic neurons of the adult striatum may also respond to NGF, primarily under conditions of trauma or stress (see below).

4 NGF Function on Damaged Adult Neurons: The Model

The cholinergic projection from the adult rat septum and diagonal band to the ipsilateral hippocampus has been a useful model for examining CNS plasticity (Fig. 1). Neurons of the medial septum and the vertical limb of the diagonal band project dorsally to the hippocampus mainly through the fimbria/fornix (GAGE et al. 1983; LEWIS et al. 1967; ARMSTRONG et al. 1987). About 50% of the septal/diagonal band neurons sending fibers through the fimbria/fornix are cholinergic (AMARAL and KURZ 1985; WAINER et al. 1985) and provide the hippocampus with about 90% of its total cholinergic innervation (STORM-MATHISEN 1974).

Complete transection of the fimbria/fornix pathway in adult rats results in the retrograde degeneration and death of many of the septal/diagonal band neurons that originally contributed axons through this pathway (ARMSTRONG et al. 1987; CUNNINGHAM 1982; GRADY et al. 1984; HEFTI 1986; WAINER et al. 1985). Markers of cell survival, including retrogradely transported fluorescent dyes and Nissl stains (GAGE et al. 1986; TUSZYNSKI et al. 1988), transmitter enzyme expression (AChE, ChAT) (GAGE et al. 1986; ARMSTRONG et al. 1987), and NGFr expression on cells of the medial septum (SPRINGER et al. 1987; MONTERO and HEFTI 1988; TUSZYNSKI et al. 1988), demonstrate a loss of 70%–90% of cells in this region. One recent study suggested that some of these cells may persist for extended periods in a dysfunctional state following lesions of the fimbria/fornix (HAGG et al. 1988), although several studies suggest that many of these cells actually die (MONTERO and HEFTI 1988; TUSZYNSKI et al. 1988; ARIMATSU et al. 1988; MV Sofroniew, personal communication).

One explanation for this axotomy-induced cell dysfunction or death is that the septal neurons become deprived of a critical supply of NTF possibly provided

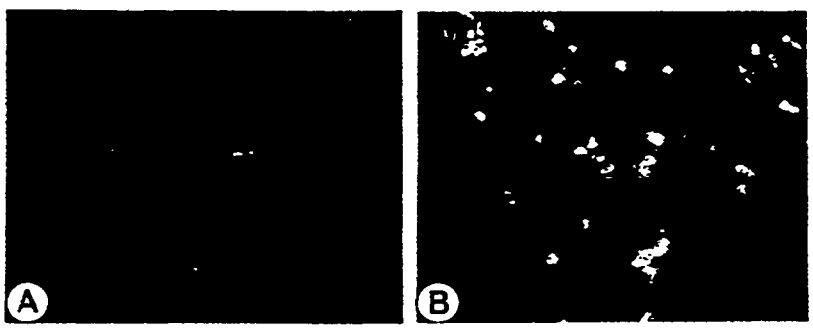

Fig. 1 A, B. NGF induction of NGF receptor mRNA within nucleus basalis neurons of the adult rat brain. Dark field photomicrographs show in situ hybridization of NGFr mRNA using a ^{35}S labeled RNA probe in a coronal tissue section. A Non-infused side and B NGF-infused side of the same tissue section. Magnification is the same in both photomicrographs (from HIGGINS et al. 1989) × 200

by the postsynaptic neurons or glial cells in the target areas of the hippocampus (COLLINS and CRUTCHER 1985; GAGE et al. 1986; GNAHN et al. 1983; NIETO-SAMPEDRO et al. 1983). That this hippocampal NTF might be NGF or NGF-like is supported by the previously listed studies from several laboratories reporting an NGF presence within the septohippocampal system.

Another model for the study of NGF function in the damaged adult brain is the nucleus basalis cholinergic projection. The nucleus basalis consists of a collection of magnocellular cholinergic neurons in the basal forebrain that provide diffuse, predominantly ipsilateral projections to most of the cerebral cortex (JOHNSTON et al. 1981, 1987). Following cortical destructive lesions, the cholinergic cell bodies of this region undergo atrophy as shown by ChAT immunoreactivity (SOFRONIEW et al. 1983, 1987) and decrements in ChAT activity (STEPHENS et al. 1985). Death of the cholinergic cell bodies in this region does not occur, however. That this system may also be influenced by NGF is suggested by the presence of high levels of NGF in the cortex (COLLINS and DAWSON 1983; WHITTEMORE et al. 1986; SHELTON and REICHARDT 1986; LARKFORS et al. 1987; SCOTT et al. 1984), the demonstration of retrograde transport of ^{125}I-NGF from cortex to nucleus basalis (SEILER and SCHWAB 1984), and the demonstration of NGFr on nucleus basalis neurons using immunocytochemistry and ^{125}I autoradiography (RICHARDSON et al. 1986; RAIVICH and KREUTZBERG 1987; SPRINGER et al. 1987; TANIUCHI and JOHNSON 1985; TANIUCHI et al. 1986; BATCHELOR et al. 1989; RIOPELLE et al. 1987).

5 NGF Function on Damaged Adult Neurons: The Effect

The above observations raise the question whether exogenous administration of NGF to neuronal populations in the adult showing NGF regulation might prevent lesion-induced neuronal degeneration and atrophy.

Within the septohippocampal circuit, for example, NGF administration to axotomized septal neurons might prevent retrograde cell degeneration and death. This rescue by NGF might then allow the axotomized neurons to regenerate their cut axons or extend new axons back to the hippocampal formation. Recently, several groups (HEFTI 1986; KROMER 1987; WILLIAMS et al. 1986; GAGE et al. 1988a) have independently reported that intraventricular administration of purified NGF into adult rats from the time of fimbria/fornix transection onward prevents the loss of most of the axotomized cholinergic septal/diagonal band neurons. Complete unilateral fimbria/fornix lesions usually result in a loss of 65%–90% of cholinergic cell bodies (GAGE et al. 1984; ARMSTRONG et al. 1987) compared to the contralateral, unlesioned side, but NGF infusion can rescue 90%–100% of the cell population (HEFTI 1986; KROMER 1987; WILLIAMS et al. 1986; GAGE et al. 1988a). Although various lesion and NGF infusion paradigms have been used in these studies, the results are consistent and

comparable. NGF has typically been infused continuously into the lateral ventricle, ipsilateral to the fimbria/fornix lesion, for at least a 2-week period through an indwelling cannula that is connected to an osmotic minipump placed subcutaneously in the animal's back (WILLIAMS et al. 1986). Another experimental paradigm using twice weekly NGF injection into the ventricle of a partially lesioned animal will also prevent retrograde neuronal degeneration in the medial septum (HEFTI 1986), and unilateral NGF infusion after bilateral fimbria/fornix lesions will spare cholinergic neurons on *both* sides of the medial septum (KROMER et al. 1981). Both the 7S and purified 2.5S form of NGF have shown efficacy in these studies. A dose-response curve in this model system has not been determined, but efficacious doses employed empirically in the above studies have ranged from a few ug/ml to greater than 100 ug/ml (HEFTI 1986; KROMER 1987; WILLIAMS et al. 1986; GAGE et al. 1988a).

Recently, *transient* infusions of NGF have been shown to be insufficient to *permanently* rescue septal cholinergic neurons (MONTERO and HEFTI 1988). Thus work is currently directed toward inducing the regeneration of lesioned axons from rescued septal cells back to their hippocampal target in an attempt to restore an endogenous, continuous supply of NGF. NGF infusions could then be discontinued when axons reconnected with their target, thus permanently rescuing the projecting septal neurons (see below). The ability of NGF to induce axonal regeneration in the adult CNS in vivo has already been evidenced by the presence of robust sprouting of cholinergic fibers into the dorsolateral quadrant of the septum 2 weeks following fimbria/fornix lesions (GAGE et al. 1985, 1988a).

Intraventricular injections of NGF after partial bilateral fimbria fornix lesions can ameliorate behavioral deficits. Following bilateral fimbria fornix lesions, rats are impaired in tests of learning and mamory (OLTON et al. 1978). After chronic NGF treatment, lesioned rats are able to learn a radial arm maze as rapidly as control nonlesioned rats following an initial, immediate postoperative impairment (WILL and HEFTI 1985). In the same study an increase in ChAT activity is observed in the septum and occurs at the same time as the behavioral recovery. Also, the behavioral changes are not permanent; the behavioral deficits reappear after cessation of NGF treatment.

NGF is also capable of ameliorating some lesion-induced effects in the nucleus basalis model. Lesion-induced, retrograde atrophic changes in the nucleus basalis can be prevented by infusion of NGF into the ventricular system, resulting in restoration of nucleus basalis ChAT activity and augmentation of remaining ChAT activity in areas of cortex spared by the lesion (HAROUTUNIAN et al. 1986). Infusion of the monosialoganglioside GM1 will also restore nucleus basalis and cortical ChAT levels, but the combined infusion of both substances will augment the response further (CUELLO et al. 1989; GAROFALO et al. 1988). These authors suggested that GM1 may act as a modulator of NGF function.

NGF effects on cells other than cholinergic neurons in the adult CNS have received less attention. GABAergic cells in addition to cholinergic cells of the medial septum degenerate after fimbria/fornix lesions (PETERSON et al. 1987), and one recent study reports a failure of NGF to save these GABAergic neurons

(MONTERO and HEFTI 1988). The identification of NGF-responsive cell populations in the CNS depends in part upon the visualization of NGFr with immunocytochemical methods. However, since immunocytochemistry currently employs antibodies that appear to identify only the low affinity NGFr (TANIUCHI and JOHNSON 1985), future studies may require methods that detect the active, high affinity receptor as well (e.g., autoradiography).

6 NGF Receptor Expression is Regulated by NGF

The ability of neurons to respond to NGF seems to depend on the presence of cell surface receptors, which in the PNS mediate the binding, internalization, and transport of NGF from the terminals to the parent cell bodies (see THOENEN and BARDE 1980 for review). Such NGFr have also been demonstrated on NGF-responsive cholinergic neurons in the CNS both during development and in the adult animal (TANIUCHI et al. 1986; RICHARDSON et al. 1986).

The cholinergic neurons of the striatum appear to represent a special case. In contrast to the neurons in the septal diagonal band area and the nucleus basalis, these neurons possess very low or undemonstrable levels of NGFr in the adult, and their responsiveness to NGF has been reported to decline dramatically during postnatal development (MARTINEZ et al. 1985; MOBLEY et al. 1985; JOHNSTON et al. 1987). Nevertheless, our previous findings showed that chronic infusion of NGF into the lateral ventricle of adult animals following fimbria/fornix lesion not only spared the medial septal neurons from degeneration, but also resulted in hypertrophy of the cholinergic neurons of the ipsilateral striatum (GAGE et al. 1988b). Similarly, chronic infusions of NGF into the lateral ventricle of aged rats ameliorated the age-related atrophy of the cholinergic neurons of the striatum as well as of the basal forebrain (FISCHER et al. 1987). The in vivo effects of NGF on the striatal cholinergic neurons are in apparent contradiction with the lack of demonstrable NGFr immunoreactivity in these neurons normally. However, TANIUCHI et al. (1986, 1988) have recently reported that peripheral nerve damage will induce the expression of NGFr on Schwann cells within the denervated distal portion of the nerve, raising the possibility that the ability of striatal neurons to respond to NGF depends on the upregulation of NGFr and that this upregulation is induced by the tissue damage. In a recent experiment we tested this hypothesis. Chronic NGF infusion into the adult neostriatum resulted in re-expression of NGFr such that many cholinergic interneurons became immunoreactive for NGFr. This effect was seen also after striatal damage induced by infusion of vehicle alone, whereas infusion of anti-NGF serum partially inhibited the receptor's re-expression. Infusion of NGF, but not vehicle alone, dramatically increased the size and ChAT immunoreactivity of these same cholinergic neurons (BATCHELOR et al. 1989).

These findings indicate that the central cholinergic neurons which lose their NGFr during postnatal development will resume their NGF responsiveness

Fig. 2A–E. Medial septal region after fimbria/fornix transection. A Appearance of medial septal cholinergic cells after unilateral fimbria/fornix transection. Lesion on *left* side, intact septum on *right*. Note the loss of total cell number and intensity of ChAT staining on the side of the lesion (ChAT immunocytochemistry) × 40. B Appearance of medial septal region after NGF infusion. Lesion on *left* side. Extensive cholinergic cell savings are evident. *Arrow* in upper left corner indicates sprouting of axons into dorsolateral quadrant of septum (ChAT) × 40. C Higher power view of sprouting into dorsolateral quadrant × 100. D Normal morphology of ChAT positive fibers in the medial septal region after unilateral fimbria/fornix lesions and NGF infusion. Lesion on *left* side, intact septum on *right* (ChAT) × 100. E High power view showing normal morphology of NGF-treated neurons (ChAT) × 1000

when the tissue is damaged. Such a damage-induced mechanism may act to enhance both the action of trophic factors, including NGF, released at the site of injury and the responsiveness of damaged CNS neurons to exogenously administered trophic factors.

Although damage produces an emergence of NGFr protein immunoreactivity in adult striatal cholinergic neurons, in situ hybridization of NGFr mRNA

shows that vehicle infusion by itself will not increase the number of NGFr mRNA positive neurons in the striatum as compared to treated animals (GAGE et al. 1989a). Thus, only NGF treatment, but not vehicle, induces NGFr gene expression within striatal neurons, suggesting the possibility that lesion-induced damage increases NGFr levels through non-transcriptional mechanisms, such as the "unmasking" of existing stores of NGFr protein. Additionally, these findings are in agreement with our recent in situ hybridization studies which showed that chronic NGF administration, but not vehicle infusions, induce NGFr gene expression within those basal forebrain cholinergic neuronal populations that normally express NGFr protein in adult rat brain (HIGGINS et al. 1989). NGF not only produced neuronal hypertrophy, as we demonstrated in earlier studies (FISCHER et al. 1987), but also increased NGFr mRNA abundance per cell and induced the expression of NGFr message within basal forebrain neurons that had previously not contained detectable levels of NGFr mRNA (Fig. 2). These findings provide strong evidence that NGF can induce expression of its own receptor within the CNS.

In vitro evidence also supports the concept that NGF can induce the expression of its own receptor. When NGF is applied to cultures of septal neurons, striatal neurons, or PC12 cells (BERND and GREENE 1984; HEFTI and GAHWILER 1988), the number of NGFr on the cells increases. In addition, the exposure of cultured chick sensory neurons to long-term NGF treatment will prevent the normal disappearance of receptors on these cells (ROHRER and BARDE 1982).

7 NGF Function in the Aged Brain

In addition to the lesion-induced degeneration described above, several laboratories have demonstrated that, in aged animals and humans, the cholinergic neurons of the basal forebrain are compromised (BARTUS et al. 1982; WHITEHOUSE et al. 1982). This compromise is reflected in cell shrinkage and, in some cases, loss of cholinergically marked neurons in the basal forebrain region, which seems to correlate with a decrease in cognitive ability in animals (FISCHER et al. 1987) and humans (COYLE et al. 1983). RNA blotting and protein data suggest a reduction of NGF levels in the hippocampus of aged rats compared to the levels in adult rats (LARKFORS et al. 1987).

We have attempted to ameliorate the functional deficits and reverse the morphological changes observed in aged animals by im, .nting fetal basal forebrain cells or by infusing NGF chronically into the aged brain. We initially reported and subsequently confirmed and extended the findings that transplantation of fetal cholinergic neurons to the hippocampal formation of aged rats prescreened for cognitive impairments could result in substantial improvement in the previously impaired animal's behavior and that this was in part mediated via the cholinergic system (GAGE et al. 1983; GAGE and BJORKLUND 1986b).

Table 1. AChE positive cell body size and spatial memory retention (modified from FISCHER et al. 1987)

		MS	VDB	Striatum	Nucleus basalis	Retention
Aged impaired, NGF-treated	L	129.8 ± 9.5	136.6 ± 7.9	122.0 ± 10.7	155.6 ± 6.2	6.6 ± 1.0**
	R	136.8 ± 8.8	143.4 ± 5.6	156.2 ± 11.7*	184.8 ± 10.0*	
Aged impaired, vehicle-treated	L	119.9 ± 5.3	146.4 ± 6.3	140.1 ± 6.5	165.4 ± 3.9	24.4 ± 3.5
	R	132.0 ± 7.4	133.9 ± 10.0	143.4 ± 4.8	162.1 ± 3.8	
Young	L	170.2 ± 7.4	226.6 ± 11.2	236.8 ± 6.6	268.8 ± 23.4	
	R	172.7 ± 2.6	229.5 ± 16.7	233.3 ± 3.8	270.1 ± 18.9	

Values are means ± SEM of AChE positive cell body size given as cross-sectional area in μm^2. The mean escape latency (± SEM) in the memory retention test on day 1 of the second test week is also given for the two subgroups of impaired rats.
MS, medial septum; VDB, vertical limb of diagonal band; L, non-infused side; R, infused side
* $P < 0.01$ compared to the non-infused side; Student's related t test
** $P < 0.01$ compared to the vehicle injected controls; Student's unrelated t test

Additionally, based on our initial observations of the trophic effect of NGF on cholinergic neurons in the retrograde degeneration model described above, we infused NGF into the lateral ventricles of cognitively impaired aged rats and found that all aged rats with intact NGF pumps showed an improvement in retention of a complex spatial learning task relative to matched, noninfused, cognitively impaired rats (FISCHER et al. 1987). In the same study, we observed a significant increase in the size of the cholinergic neurons in the basal forebrain region on the side of the brain into which the NGF was intraventricularly infused (Table 1).

8 NGF Functions as a Tropic Factor

In addition to its function of maintaining the normal integrity of central cholinergic basal forebrain neurons and of peripheral sympathetic and neural crest-derived sensory neurons (*neurotrophic*), NGF has been postulated to have a role in the axonal growth of these neurons after damage to the nervous system (*neurotropic*). Two forms of axonal growth are commonly observed in the mature hippocampus: *regeneration*, or the regrowth of axons previously damaged, and *collateral sprouting*, or the new growth of remaining (intact) axons (GAGE and BJORKLUND 1986b). Collateral sprouting occurs within the hippocampus from two distinct populations of neurons following fimbria/fornix transection. Superior cervical ganglion (SCG)-derived sympathetic axons, which normally surround the hippocampal vasculature, undergo a robust sprouting response into the dentate gyrus and CA3 pyramidal cell region of the hippocampal parenchyma (LOY and MOORE 1977; STENEVI and BJORKLUND 1978). In addition, the magnocellular midline cholinergic neurons on the dorsal hippocampal formation respond by sprouting into the dorsal subiculum and CA1 pyramidal

cell layers (BLAKER et al. 1988). To test the postulate that NGF has tropic effects on cholinergic axons in the hippocampus and that the lesion-induced increase in NGF in the hippocampal formation may serve as a chemoattractant of NGF-responsive axons towards the source of its production (CRUTCHER 1987), we examined the fimbria/fornix lesioned hippocampal formation to determine whether the sprouting neurons stain positively for NGFr. We found that the two populations of neurons which undergo collateral sprouting, namely, the midline magnocellular cholinergic neurons of the dorsal hippocampus and the sympathetic neurons of the SCG, stain strongly for NGFr with a monoclonal antibody (TANIUCHI et al. 1986, 1988). In contrast, the small intrinsic cholinergic neurons of the hippocampus exhibited neither sprouting response nor staining for NGFr (BATCHELOR et al. 1989). In view of these results we suggest that the differing sprouting responses demonstrated by these three neuronal populations may be due to their responsiveness to NGF, as indicated by the presence or absence of NGFr.

9 NGF Induces Regeneration in the CNS

Attempts to restore this severed fimbria fornix pathway have been made by grafting fetal tissue or other substances that may act as bridges (TUSZYNSKI et al. 1990; GAGE et al. 1988b; KROMER et al. 1981) between the disconnected septohippocampal pathway. Increases in ChAT activity and AChE fiber innervation in the host hippocampus have been consistently reported in these studies, but the extent of reinnervation is small.

A possible explanation for the limited restoration of the cholinergic circuitry may be that the majority of the cholinergic neurons in the medial septum and diagonal band of Broca degenerate, become dysfunctional, and die within a month following the transection (DAITZ and POWELL 1954; GAGE et al. 1986; ARMSTRONG et al. 1987). This observation, and the evidence of a link between NGF and cholinergic neurons (HEUMANN et al. 1985; HONEGGER and LENOIR 1982; SHELTON and REICHARDT 1986), have prompted several groups to test and subsequently demonstrate the dependence of adult denervated cholinergic neurons on exogenous NGF for survival in the absence of endogenous NGF previously transported from the hippocampus (GAGE et al. 1988a; HEFTI 1986; KROMER 1987; WILLIAMS et al. 1986).

These results led to the prediction that the exogenous delivery of NGF could not only promote the survival of septal neurons, but also would then promote cholinergic axon extension across a bridge of hippocampal fetal tissue placed in the fimbria/fornix cavity. Thus, in a recent study we combined the exogenous infusion of NGF into the lateral ventricle adjacent to the denervated septum with the simultaneous grafting of fetal hippocampal tissue to the fimbria/fornix cavity as a set of procedures that may more fully and functionally restore the severed

septohippocampal circuitry (TUSZYNSKI et al. 1990). Embedded in the design of this study were two, additional, related questions: (1) Does the transient 2 week NGF infusion period, which has been shown to result in significant cholinergic cell rescue, have an enduring effect on the medial septal cells 6–8 months following termination of the NGF infusion? (2) Will fetal hippocampal grafts alone, in the absence of exogenous NGF infusion, support the survival of the axotomized cholinergic neurons of the medial septum?

A combination of intracerebral grafting and intraventricular infusion of NGF was used to attempt reconstruction of the cholinergic component of the septohippocampal pathway following fimbria/fornix lesions. Four groups were used: lesion only (FF); lesion and fetal hippocampal graft (FF-HPC); lesion and NGF (FF-NGF); and lesion, graft, and NGF (FF-HPC-NGF). ChAT immunoreactivity (ChAT-IR), AChE fiber staining, and behavior-dependent theta electrical activity were used to assess the extent of pathway reconstruction. The NGF infusion only lasted the first 2 weeks following the FF lesion, while measurement of theta activity and histological analysis were conducted 6–8 months after lesioning. Only the FF-HPC-NGF group had long-term rescue of ChAT-IR cells as compared to the FF and FF-HPC group. In addition, the FF-HPC-NGF group had more extensive reinnervation of the hippocampus than any other group. Further, the FF-HPC-NGF had the most complete evidence of behavior-dependent theta activity restoration. These results demonstrate clearly that a combination of short-term intraventricular NGF infusion and fetal hippocampal grafts can result in a more complete reconstruction of the damaged septohippocampal circuit.

10 A Role for Glia in Mediating the Tropic Effects of NGF

Damage to the fimbria-fornix and separately to the perforant path leads to distinct and dramatic time dependent increases in glial fibrillary acidic protein immunoreactivity (GFAP-IR) in specific areas of the hippocampal formation (GAGE et al. 1988c). Specifically, FF lesions resulted in an increase in GFAP-IR in the pyramidal and stratum oriens layers of the CA3 region as well as in the inner molecular layer of the dentate gyrus. In addition, in the septum ipsilateral to the lesion, there was a rapid and robust increase in GFAP-IR in the dorsal lateral quadrant of the septum, but not in the medial septal region. Only after 30 days did GFAP-IR reach the medial septum. Following perforant path lesions, there was a selective increase in GFAP-IR in the outer molecular layer of the dentate gyrus. Most of these changes were transient and disappeared by 30 days post-lesion. We speculated that the increase in GFAP-IR, reflecting activation of astrocytic cells in these target areas, is a necessary requirement for sprouting responses (GAGE et al. 1988c).

Considerable in vitro and in vivo evidence supports the presumption that astrocytes make and secrete NTF which can subsequently support the survival and/or axonal outgrowth of a variety of central and peripheral neurons (BANKER 1980; HATTEN and LIEM 1981; LIESI et al. 1984; LINDSAY 1979; TARRIS et al. 1986). At present, when a new putative factor is tested in vitro for its neurotrophic activity, rigorous controls must be used to establish that the neuronal population is not contaminated with glia and that the presumed NTF is not acting through the glial cell population in vitro. To date, the evidence supports the notion that only reactive and/or proliferating astrocytes secrete trophic and tropic substances; thus, it is essential to understand the signals for this activation in vivo. Microglial proliferation often precedes astrocytic proliferation (GALL et al. 1979; VIJAYAN and COTMAN 1983). Recently, GIULIAN et al. (1986) and HETIER et al. (1988) showed that activated microglia produce interleukin-1 (IL-1) in vitro and that IL-1 stimulates astrocyte proliferation in vitro and in vivo (GIULIAN et al. 1988). Very recently it has been shown that IL-1 regulates the synthesis of NGF in non-neuronal cells of the damaged rat sciatic nerve (LINDHOLM et al. 1987) and that IL-1 is most likely secreted from activated macrophages in the vicinity of the damaged nerve (HEUMANN et al. 1987b).

Work in the CNS has demonstrated time dependent changes in microglial and astrocytic populations in the outer molecular layer of the dentate gyrus denervated by perforant path transection (FAGAN et al. 1988). Reactive microglia stained with the monoclonal antibody OX-42 appear in this area within 24 h after transection. The appearance of cells immunostained for IL-1 in this area parallels that of microglia, suggesting that the reactive microglia themselves produce IL-1 in vivo in response to deafferentation. The astrocyte response, as indexed by GFAP-IR, is not observed until a later time point coincident with the disappearance of IL-1 staining. These observations provide evidence that reactive microglia produce IL-1 in vivo in response to injury and that IL-1 may be the signal responsible for the documented astrocyte response in the outer molecular layer of the dentate gyrus observed after damage to the entorhinal input to the hippocampus.

11 Working Hypothesis of NGF Effects

The results of data summarized in the previous sections suggest: (1) a role for IL-1 in the proliferation of astrocytes, (2) that activated microglia and macrophages can secrete IL-1, and (3) that IL-1 can activate NGF synthesis in non-neuronal cells. We have made the following suggestion for the outline of events that lead to the NGF-sensitive sprouting responses in the hippocampus and septum following fimbria/fornix and perforant pathway lesions.

Perforant pathway damage induces terminal degeneration of the axons of cells which were transected in the entorhinal cortex. This terminal degeneration in the outer molecular layer of the dentate gyrus activates microglia to phagocytize in the restricted zone of terminal degeneration. These activated

microglia release IL-1 into the surrounding environment, which induces the proliferation of astrocytes. The activated astrocytes, in turn, secrete NGF which results in the attraction of cholinergic fibers that express NGFr on their membrane surfaces.

Similarly, following fimbria/fornix lesions, cholinergic terminals are disconnected from their cell bodies in the septum and, once again, there is terminal degeneration, this time in the areas of heaviest cholinergic innervation in the CA3 region and dentate gyrus. This degeneration leads to a microglial proliferation, IL-1 secretion, and an astrocytic mitogenic reaction in CA3 and inner molecular layer of the dentate gyrus. The activated astrocytes secrete NGF and promote the ingrowth of NGFr-bearing sympathetic fibers of the SCG.

Concurrently in the septum, degeneration of terminals from the hippocampus to the dorsolateral quadrant results in microglial reactivity and subsequent IL-1 secretion, proliferation of glia, and increased NGF concentration. This, in turn, induces the growth of cholinergic fibers from the medial septum, which are undergoing retrograde degeneration, into the dorsolateral quadrant. This NGF source in the dorsolateral quadrant is not sufficient to support all the medial cholinergic cells, but some of the medial septal cells are always spared even with a complete bilateral fimbria/fornix lesion. Meanwhile, the absence of reactive astrocytes in the medial septum soon after the fimbria/fornix lesion is the paramount reason for the death of these cells, because, as in development, these cells survive only in the presence of adequate glia-derived NGF.

This working hypothesis generates several specific testable predictions, which when examined should reveal more about the mechanisms underlying survival, growth, and function of normal and damaged cholinergic neurons in the CNS.

12 Conclusions

Based on this review, the following points summarize our knowledge of NGF function in the CNS:

1. NGF is synthesized in target areas of the projecting cholinergic neurons of the forebrain.
2. NGFr is synthesized in basal forebrain cholinergic projecting neurons and anterogradely transported to its terminals in target areas which produce NGF.
3. Survival of the projecting basal forebrain neurons is dependent on the retrograde transport of NGF to basal forebrain neurons.
4. Disconnection of the projecting neurons from the targets results in retrograde degeneration which can be reversed by exogenous administration of NGF.
5. NGF exogenous infusion can also promote axonal regrowth, provided a substrate for axonal growth is available.

6. NGF appears to have a positive effect on its own receptors, such that increased levels of NGF result in increased expression of NGFr and increased responsiveness to NGF.
7. In the aged rat with cognitive impairments in behavior which are correlated with cholinergic cell size and number, chronic NGF infusion can result in improved behavioral performance and partial restoration of cholinergic cell size.
8. While NGF appears to be synthesized in neurons of the intact target areas, damage-induced activated astroglia may also have the ability to synthesize and secrete NGF.
9. IL-1 may regulate NGF expression and activate astrocytes: this regulation of NGF may contribute to sprouting responses of cholinergic neurons in the CNS.

While a complete understanding of NGF regulation and function is not available, both a trophic and tropic role for NGF exists in the CNS. The specificity of NGF for cholinergic neurons, while not perhaps exact, may be unique in its specificity. To date, the other candidate neurotrophic molecules have a broader range of function. Thus while NGF may be the first and best characterized NTF, it may not be the prototype for other trophic factors in the CNS.

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Altered levels of amyloid protein precursor transcripts in the basal forebrain of behaviorally impaired aged rats

(Alzheimer disease/*in situ* hybridization/neuritic plaques/growth factors)

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ABSTRACT **The β/A4 protein is a constituent of plaque and vascular amyloid deposits in Alzheimer disease. Previous studies have shown increased levels of amyloid protein precursor (APP) mRNA in basal forebrain neurons in the disease. Morphological and neurochemical changes occur within the forebrain in Alzheimer disease and are also correlated with behavioral impairments in aged rats. Recent studies suggest that decreased nerve growth factor responsiveness of basal forebrain neurons is a feature of normal aging and of Alzheimer disease. We have used *in situ* hybridization to show that the abundance of specific forms of APP mRNA, which contain an inserted Kunitz-type serine protease inhibitor motif (APP-751, APP-770, and APP-related 563), are increased relative to the noninserted form (APP-695) of APP mRNA in the basal forebrain of aged rats. This increase appears to be specific to animals who exhibit spatial memory deficits but not aged rats without behavioral impairments.**

The amyloid β/A4 protein is a major constituent of neuritic (senile) plaque and cerebrovascular amyloid deposits in Alzheimer disease (AD) (1, 2). Although a direct causal link between aberrant β/A4 protein accumulation and AD has not yet been proven, one possible mechanism of plaque formation may be increased amyloid expression in the disease. Increased expression of mRNA that encodes the amyloid protein precursor (APP) has been observed in the basal forebrain and hippocampal formation in AD (3, 4), suggesting a mechanism by which pathological deposits of the β/A4 protein could accumulate in the disease. A "brain-specific" form of APP, which encodes a 695-residue molecule (APP-695), was originally cloned by several different laboratories (5–8). Three alternate forms of APP mRNA, including APP-751, APP-770, and APP-related 563, have been shown to contain an "in-frame" insertion that encodes 57 amino acids homologous to the Kunitz domain of serine protease inhibitors (KPI) (9–12). Other serine protease inhibitors, such as the glia-derived neurite-promoting factor (13), have been shown to promote neurite extension and to inhibit neuronal migration. A recent study shows that a secreted form of APP-751 is identical with protease nexin II (14), a protein that has been shown to bind with growth-factor-associated proteases, such as the γ subunit of nerve growth factor (NGF). Secreted forms of APP have been shown to be involved in growth regulation (15), whereas the membrane-bound carboxyl-terminal portion of the APP molecule appears to be neurotoxic to differentiated neuronal cells in culture (16). Recent studies have shown that NGF increases APP mRNA expression in the developing and adult rodent basal forebrain (17, 18). Thus, it is possible that APP may be involved in the regulation of neuronal growth and atrophy in the aging central nervous system (CNS).

An experimental animal model of aberrant APP expression would be valuable for elucidation of the potential role of this molecule in the age-related neurodegenerative process of AD. Some aged rodents show learning and memory deficits correlated with degenerative changes in the basal forebrain that may resemble certain pathological and behavioral features of AD in humans (19, 20), and thus the behaviorally impaired aged rat has become a model system in which to study spatial learning deficits associated with an age-related decline in cholinergic integrity in the basal forebrain (21). Cholinergic neuronal atrophy and/or cell loss in rodents have been observed not only with basal forebrain nuclei such as the nucleus basalis, diagonal band, and medial septum, which send long projections to the cortex and hippocampal formation, but also within cholinergic interneurons of the striatum (22–26). NGF, which is important for the survival and maintenance of cholinergic neurons in the periphery, can reverse cholinergic neuronal atrophy in the basal forebrain of behaviorally impaired aged rats, emphasizing the importance of NGF in the normal maintenance of cholinergic neuronal integrity in the adult brain (27).

We examined APP gene expression in the aged rat brain. Elevated levels of total APP mRNA within presumptive cholinergic neurons of the nucleus basalis have been reported in AD (3), raising the possibility that APP expression may be correlated with neuronal atrophy in the basal forebrain. In this study, we have attempted to determine whether similar phenomena may also occur in the aged rodent brain by asking the following: (*i*) Is expression of any specific form of APP mRNA differentially increased in aged rat brain? (*ii*) Are changes in APP gene expression observed in all aged rats, or are they related to cognitive impairments in aged animals? *In situ* hybridization has been used to discriminate between the noninserted (APP-695) and KPI-inserted (APP-751/770/563) forms of APP mRNA in young adult, nonimpaired aged, and behaviorally impaired aged rats. Our data suggest that KPI-containing APP mRNAs, which contain an inserted serine protease inhibitor sequence, are selectively elevated relative to APP-695 mRNA within forebrain neurons in a subpopulation of aged rats that exhibit spatial memory deficits.

MATERIALS AND METHODS

A total of 8 young adult female (age: 7 months) and 11 aged female (age: 25 months) Sprague–Dawley rats were used in this study. Four aged and 4 young control animals were sent from San Diego to Rochester for *in situ* hybridization (batch

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Abbreviations: APP, amyloid protein precursor; NGF, nerve growth factor, AD, Alzheimer disease; CNS, central nervous system; KPI, Kunitz-type serine protease inhibitor.

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1). Following these initial studies, an additional group of 4 young and 7 aged animals, which had been tested and behaviorally characterized, were processed for *in situ* hybridization (batch 2). *In situ* hybridization and data analysis were performed without prior knowledge of the experimenter as to the behavioral status of the animals.

Behavioral Studies. Spatial learning and memory were tested in the circular water maze (28). Rats were tested on the water maze for 5 consecutive days using a visible platform, followed by 5 consecutive days using an invisible platform, with 2 intervening days between the 5-day blocks. Each rat was given one block of four trials every test day. On the fifth day of invisible platform testing, the platform was removed from the water maze for a fifth trial ("spatial probe trial"). The water maze consisted of a tank 152 cm in diameter that was painted black and filled with room temperature water to a depth of 34 cm. The invisible platform was a 5 × 5 cm black Plexiglas square supported by a clear Plexiglas base placed 3 cm below the surface of the water. The visible platform consisted of an elevated platform with a 6-cm-high base and 20-cm-high wooden rods placed at the corners on top of the hidden platform. The water maze was located in the corner of the room containing numerous extramaze cues (e.g., posters, the computer, tables). The escape platform was kept in a constant position in the water maze for all trials. Start locations were semirandomized such that the first trial each day began from the same location as the last trial from the previous day, and the first trial began from one of the locations farthest from the escape platform. If a rat was unable to locate the platform in 90 sec, it was removed from the water and placed on the platform for 20 sec. If a rat was able to locate the platform, it was allowed to remain on the platform for 20 sec before beginning the next trial. The swim path, path distance, and escape latency were monitored using a video camera, and videotrack images were digitized and analyzed by a computer programed for water maze data analysis (San Diego Instruments, San Diego, CA).

***In Situ* Hybridization and Densitometry.** *In situ* hybridization was performed on paraformaldehyde-fixed coronal tissue sections using oligonucleotide probes end-labeled with [^{32}P]ATP or tailed with dCTP[^{35}S] using methods described previously (4). For initial *in situ* hybridization analysis, a 39-mer specific for the KPI motif present in human APP-751/770/563 mRNA (5'-CTTCCCTTCAGTCACATCAAAGTACCAGCGGGAGATCAT-3') was used for *in situ* hybridization (29). To increase the specificity of the hybridization experiments, ^{35}S-labeled 40-mer oligonucleotide probes based on recently published rat sequences (30, 31) corresponding to the KPI motif shared by APP-751/770/563 mRNA sequences, as well as a "splice-junction" probe corresponding to rat APP-695 mRNA (Fig. 1), were hybridized to adjacent tissue sections that had been collected from all of the animals used in this study. Aged and young rat brain tissue sections were hybridized on the same slides. The slides were hybridized overnight at 37°C, and the posthybridization rinses were taken to a final stringency of 0.5× SSC (0.075 M NaCl/7.5 mM sodium citrate) at 56°C for the APP-695 probe and 1× SSC (0.15 M NaCl/15 mM sodium citrate) at 42°C for the KPI-APP probe. The hybridization and rinsing conditions allow the discrimination of the 40-mer probe for APP-695 mRNA from its component 20-mers. The slides were exposed to x-ray film (DuPont Cronex-4) and subsequently processed for emulsion autoradiography using Kodak NTB-2 emulsion. Manual grain counting was used to determine relative APP mRNA levels following hybridization with transcript-specific probes within the nucleus basalis as described (32). "Grain clusters" were taken to represent isotopic emission from single cells (4). "Within-section" ratios were then used to normalize to cell groups that did not show variation correlated with aging or behavioral impairment, such as the lateral nucleus of the amygdala. These normalized values were then used to construct ratios of the relative levels of KPI-containing APP transcripts to APP-695 for comparisons between impaired and nonimpaired aged rats.

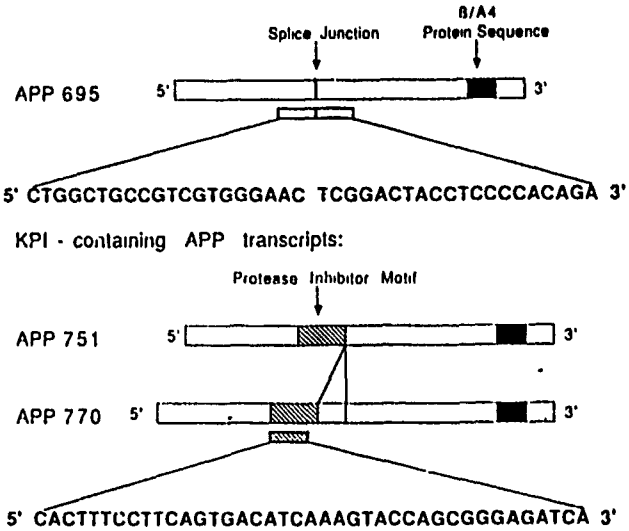

FIG. 1. Schematic depiction of the oligonucleotide probes used for *in situ* hybridization and their corresponding APP-695 and KPI-containing (APP-751/770) mRNAs. Forty-base oligonucleotide probes specific for APP-695 and KPI-containing forms of APP message were synthesized based on published rat sequences (30, 31). In addition to APP-751 and APP-770 mRNAs, another APP-related mRNA (APP-563) contains the same KPI motif (12) but is not depicted. The black region at the 3' end of the APP mRNAs represents the β/A4 protein motif, whereas the shaded region represents the protease inhibitor motif that is inserted into the splice junction shown in APP-695 mRNA.

RESULTS

Three of four aged rats in the initial group of experimental animals (batch 1) showed dramatic increases in KPI forms of APP mRNA in basal forebrain regions relative to young adult rats that were processed for *in situ* hybridization at the same time. Because of the variable results in the aged rats of batch 1, a second group (batch 2) of experimental animals was examined, which consisted of three aged rats with clear cognitive deficits (impaired), four aged rats with no detectable cognitive deficit (nonimpaired), and four matched young adult rats. Fig. 2 shows x-ray film images of *in situ* hybridization using a ^{32}P-labeled oligonucleotide for human KPI-containing APP mRNA to coronal tissue sections (*Left*) and behavioral profiles (*Right*) of an aged rat with spatial memory deficits (*A*), a nonimpaired aged rat (*B*), and a young adult rat (*C*). At this level of the decussation of the anterior commissure, large increases in this inserted form of APP mRNA can be seen within the diagonal band/ventral pallidal complex and within the striatum of behaviorally impaired rats compared with either a nonimpaired aged rat or young adult rat (Fig. 2). Retrospective analysis of results obtained with batch 1 animals showed a pattern consistent with a correlation of increased KPI-containing APP message levels with behavioral impairment.

To examine cellular changes in APP gene expression that occur in forebrain neurons during aging, *in situ* hybridization with ^{35}S-tailed oligonucleotides, based on the published rat sequences (30, 31), were used for discrimination of KPI-containing and APP-695 mRNAs on tissue sections processed for emulsion autoradiography. Relative increases in KPI-containing mRNA levels were observed in the medial septum, ventral pallidum/diagonal band complex, and nucleus basalis magnocellularis in behaviorally impaired aged rats

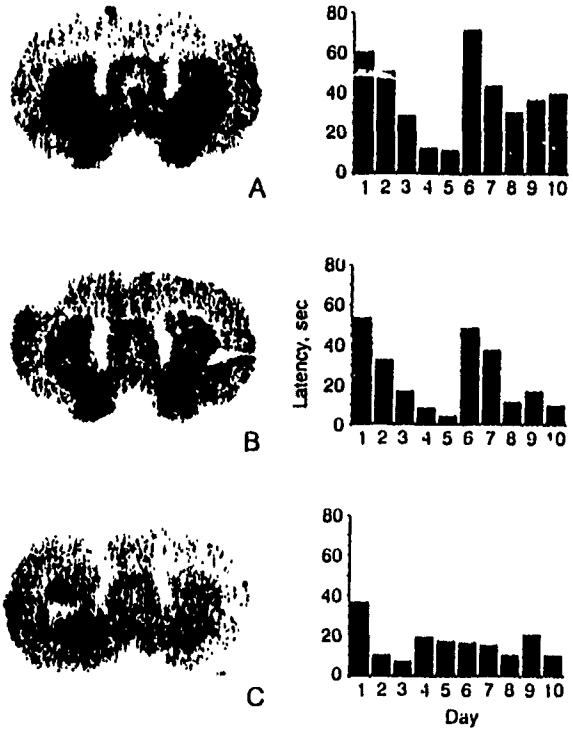

FIG. 2. Elevated expression of KPI-containing (APP-751/770/563) mRNA transcripts in the telencephalon of an aged rat with spatial memory deficits. (*Left*) X-ray film images of *in situ* hybridization of KPI-containing APP mRNA transcripts on coronal tissue sections of rat forebrain at the level of the decussation of the anterior commissure. Tissue sections in *A* and *C* were hybridized on the same slide, and the section in *B* was hybridized in the same run. Tissue sections in *B* and *C* have tears, and all are notched for identification purposes. Note the elevated abundance of KPI-containing APP mRNA in the ventral pallidum/diagonal band and striatum of a behaviorally impaired aged rat (*A*) versus a nonimpaired aged rat (*B*) and a young adult rat (*C*). (*Right*) Behavioral data from the same animals obtained in water maze trials. The rat in *A* shows no apparent deficit in locating an observable platform in days 1–5, as indicated by short latencies to find the visible platform during the training period. In contrast, this animal shows a severe spatial learning deficit when trained to find the hidden platform located in days 6–10, as indicated by long latencies to find the hidden platform maintained over the trials. The nonimpaired rat (*B*) shows similar decreases in swim latencies during the visible platform trials as in the hidden platform trials. The young rat (*A*) shows short swim path latencies over all of the trials. A human KPI probe (see text) was used in this experiment.

versus nonimpaired rats. Increased levels of KPI-containing forms of APP mRNA in the nucleus basalis magnocellularis and adjacent basal ganglia are shown in Fig. 3. The cellular specificity of KPI-containing APP mRNA changes was confirmed by examination of other cell types, such as those in the cerebral cortex and lateral nucleus of the amygdala, which showed similar levels of these forms of APP mRNA in the same tissue sections in aged impaired rats as were observed in aged nonimpaired rats (Fig. 3). Examples of increases in KPI-containing APP mRNA levels combined with decreases in APP-695 message levels are shown for the medial septum of a behaviorally impaired aged rat versus a nonimpaired aged rat in Fig. 4. More variable increases in KPI-containing APP mRNA expression were observed in the striatum in half of the behaviorally impaired aged rats (data not shown).

To provide a relative estimate of the increase in the ratio of KPI-containing APP transcripts to APP-695 mRNA abundance associated with behavioral impairment in the basal forebrain of the aged rat, we measured the intensity of ^{35}S-labeled probe hybridization from tissue sections processed for emulsion autoradiography. Grain counts were made from cells of the nucleus basalis in three behaviorally impaired aged rats versus three nonimpaired aged animals (n = 100 cells per animal). In impaired aged rats, the ratio of grain counts on KPI-containing to APP-695 mRNA levels was significantly higher than that in nonimpaired aged rats (Fig. 5). In contrast, cells of the lateral nucleus of the amygdala measured on the same tissue sections had similar ratios of APP mRNA hybridization in impaired and nonimpaired aged animals (Fig. 5). Thus, it appears that relative increases in the ratio of KPI-containing to APP-695 mRNA hybridization are anatomically restricted to certain cell populations in behaviorally impaired aged rats.

DISCUSSION

The discovery of human APP mRNA transcripts containing inserted sequences encoding serine protease inhibitor domains (9–11) has prompted new efforts to understand how differential APP expression may contribute to amyloid pathology in AD. The originally described APP cDNA sequence predicts a protein of 695 amino acids (APP-695) (6), whereas two forms of β/A4 protein-containing APP mRNA have been described that also encode a shared protease inhibitor domain (APP-751 and APP-770) (9–11), and this same motif has recently been described in a form of APP mRNA that does not encode the β/A4 protein (APP-related 563) (12). A simple hypothesis suggests that overexpression of one form of APP mRNA may be preferentially associated with neuritic plaque formation and cerebrovascular amyloidoses. However, recent studies show that APP-695 and APP-751/770 mRNAs are expressed widely in neurons of the human brain, and their anatomical distribution does not simply correlate with neuronal vulnerability to amyloid pathology (29). Expression of the noninserted APP-695 mRNA is largely restricted to the brain (10), and its abundance varies between different cortical regions of human CNS (11, 29). Northern blotting studies show that APP-751/770 mRNA is expressed in a variety of peripheral tissues (9–11) and is expressed at relatively equal levels in different human brain regions (11), except for the hippocampal formation, where elevated levels of KPI-containing APP mRNA have been described (29). In the rodent brain, KPI-containing forms of APP mRNA have been suggested to represent a more minor component of total APP mRNA than in the human CNS (33).

Previously demonstrated increases in total APP mRNA expression within certain neuronal subpopulations in AD may result from differential APP gene expression. For example, *in situ* hybridization has been used to suggest that elevated expression of total APP mRNA within nucleus basalis neurons in AD is due to a selective increase in APP-695 (noninserted) mRNA levels (34). In contrast, Johnson *et al.* (35, 36) used Northern blotting to show decreased levels of APP-695 message in the cortex and hippocampal formation in AD, which they suggest is not a simple result of the preferential loss of APP-695-containing neurons in the disease (37). In addition, increased levels of KPI-containing (APP-770) mRNA have been observed in the brains of AD patients versus age-matched controls (38). Our results show that levels of KPI forms of APP mRNA are increased relative to APP-695 mRNA in the telencephalon of aged rats whose spatial memory deficits and cholinergic neuronal atrophy are analogous to some features of AD pathology in the human. A possible explanation for these findings is that aged rodents, who apparently never develop mature amyloid pathology (39), overexpress a form of APP that is not processed to yield the pathological fragment of β/A4 protein. However, recent immunocytochemical studies using anti-β/A4 protein antibodies have revealed a more extensive distribution of "diffuse," immature amyloid deposits in AD brain (40), suggesting the possibility that a wide

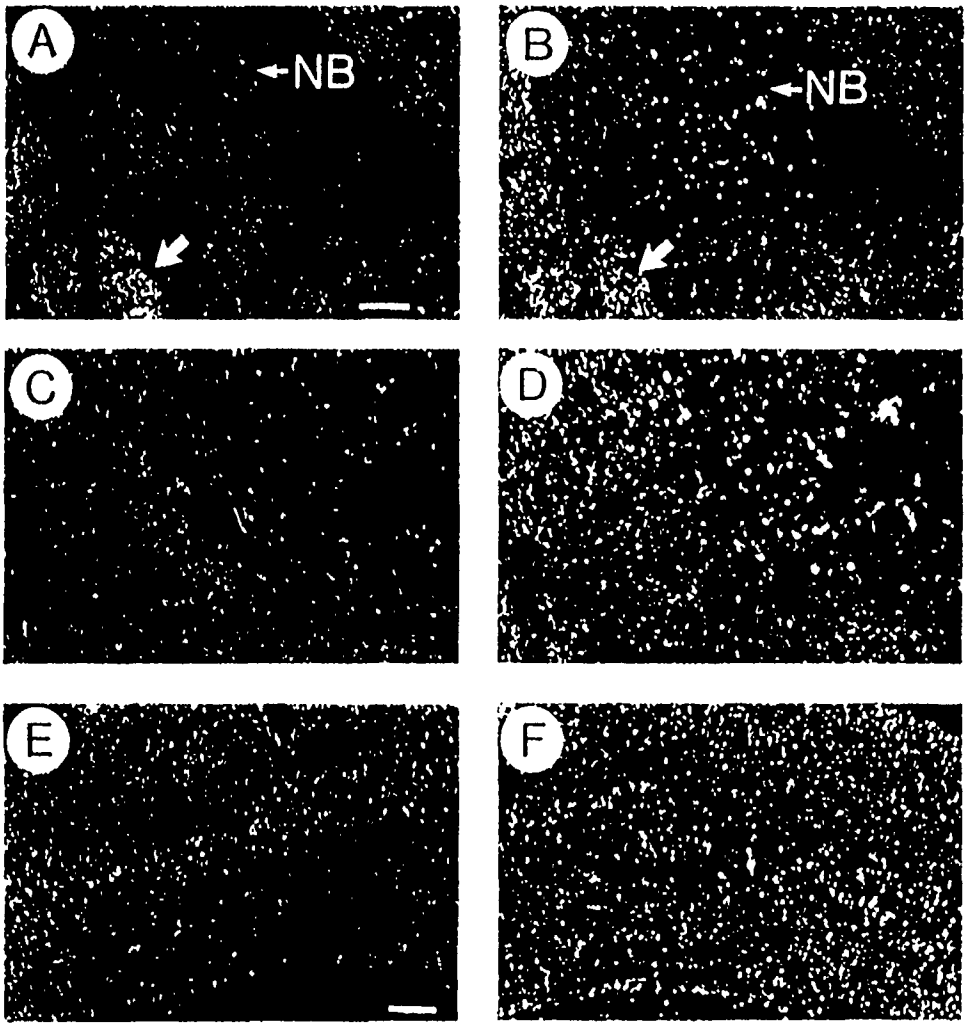

FIG. 3. KPI-containing mRNA levels are increased in the nucleus basalis magnocellularis (NB) in aged rats with spatial memory impairments. Dark-field photomicrographs show *in situ* hybridization using a rat ^{35}S-labeled oligonucleotide probe. (*A* and *C*) Non-impaired aged rat. (*B* and *D*) Behaviorally impaired aged rat. (*C* and *D*) High power views of *A* and *B*. Large arrows indicate the lateral nucleus of the amygdala, which appears to contain similar levels of APP mRNA in both animals. The cerebral cortex also has similar levels of KPI forms of APP mRNA in nonimpaired (*E*) and impaired (*F*) animals. (*A* and *B* bar = 500 μm; *C*–*F* bar = 200 μm.)

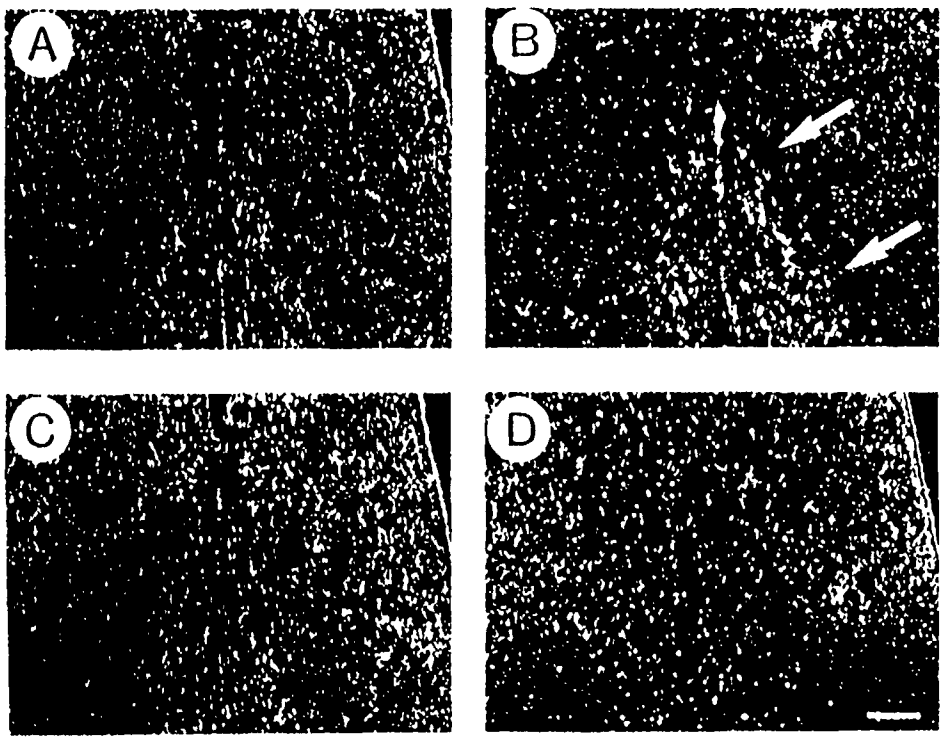

FIG. 4. Differential expression of APP mRNAs in the medial septum in behaviorally impaired aged rats. KPI-containing APP mRNA levels appear to be increased in the medial septum of an impaired aged rat (*B*, arrows) versus a nonimpaired aged rat (*A*), whereas a more global decrease in APP-695 mRNA hybridization is apparent in this region in these same animals (*C*, nonimpaired aged rat; *D*, impaired aged rat). (Bar = 200 μm.)

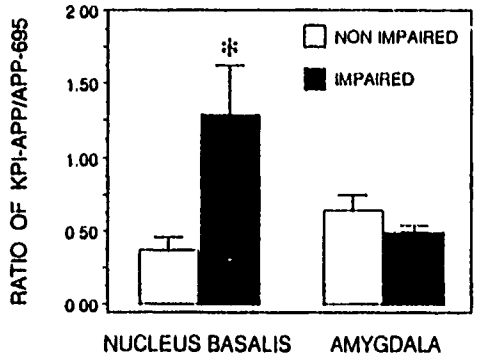

FIG. 5. Quantification of grain density measurements from *in situ* hybridization data shows that the ratio of KPI-containing to APP-695 mRNA hybridization is increased in the nucleus basalis of aged rats with behavioral impairments ($P < 0.05$; nonimpaired; 0.37 ± 0.09; impaired: 1.29 ± 0.33; n = three animals). Similar ratios of APP transcripts are found in the lateral nucleus of the amygdala in impaired versus nonimpaired aged rats ($P > 0.25$; nonimpaired: 0.64 ± 0.11; impaired: 0.50 ± 0.04; n = three animals).

spectrum of aged mammals may accumulate amyloid deposits reminiscent of the human but not previously visualized using traditional staining methods. Thus, although it appears that an increase in the neuronal ratio of KPI-containing to noninserted APP mRNAs is emerging as a common theme in AD and in aged rats, it is also possible that other cell types such as microglia or astrocytes may be a source of the β/A4 protein. In this context, β/A4 protein immunoreactivity has recently been found within reactive astrocytes in lesioned rat brain (41) and within skin cells in AD patients (42), suggesting the possibility that shifts in APP gene expression or processing may be a common feature of many cell types following damage and/or in association with neurodegenerative disease.

Previous studies have demonstrated the presence of NGF-responsive cholinergic neurons within some of the same regions of the basal telencephalon that showed increased expression of KPI-containing APP mRNAs in this study. Magnocellular cholinergic neurons, which comprise the Ch1–Ch4 cell groups and innervate the cerebral cortex and the hippocampal formation, are affected by AD pathology (19, 20), and decreased NGF responsiveness of these basal forebrain neuronal population appears to be a primary feature of the disease (43). Neuronal atrophy has been described in homologous regions of rat forebrain during aging and is correlated with behavioral deficits in learning and memory tasks reminiscent of those seen in AD patients (21). Our results suggest the possibility that alterations in the ratio of APP mRNAs may be associated with neuronal atrophy in a subpopulation of aged rats that exhibit behavioral impairments. Studies aimed at a systematic evaluation of the time course of APP expression in relationship to development of the cognitive disorder should provide further insight into the mechanism of behavioral deterioration in the aging nervous system.

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Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes

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Nerve growth factor (NGF) is produced and secreted by astrocytes and fibroblasts, but not by microglia, in a primary non-neuronal cell culture derived from newborn rat brains under a standard culture condition. NGF secretion by astrocytes was highest just after passage and then gradually decreased. There is no significant difference in NGF secretion by astrocytes from five sites of origin tested: cerebral cortex, striatum, hippocampus, septum, and cerebellum. Acidic and basic fibroblast growth factors (aFGF and bFGF) significantly increased NGF secretion by astrocytes. The effect of aFGF was greater than that of bFGF, and the effect of both FGFs was not additive at the maximum concentration. The peak of NGF secretion stimulated by aFGF occurred 3–12 h after the addition of aFGF. On the other hand, the dramatic increase in cell numbers was observed 12–48 h after stimulation, and the morphological change became significant 24 h after aFGF stimulation. NGF synthesized by astrocytes is rapidly secreted into the culture medium and aFGF enhances NGF secretion from the transcription level, because cycloheximide and actinomycin-D completely inhibited NGF secretion by astrocytes in the presence or absence of aFGF. Epidermal growth factor (EGF), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) also increased NGF secretion by astrocytes to a certain extent. NGF secretion by astrocytes in the presence of a maximum dose of aFGF was enhanced by the addition of IL-1β or TNF-α, but not EGF. However, platelet-derived growth factor, interleukin-3, and interleukin-6 had no significant effects. FGFs also enhanced NGF secretion by fibroblasts derived from meninges, but not by microglia.

INTRODUCTION

Astrocytes are the supporting cells for neurons in the nervous system and have unique neurotrophic effects. These neurotrophic effects have been investigated mainly by in vitro experiments. Astrocytes enhance neuronal survival and neurite elongation, and suppress the proliferation of neuroblasts[4,35,41,42,60]. Neurotrophic effects of astrocytes are considered to be mainly mediated by neurotrophic factors (NTFs)[41]. Nerve growth factor (NGF), which was originally found as a neurite-promoting factor for peripheral sensory and sympathetic neurons[6,33], has recently been revealed to be produced and be functioning as a NTF in the central nervous system (CNS)[31]. The most well-characterized NGF-sensitive neurons in the CNS are cholinergic neurons in the basal forebrain[20,21,27]. Hippocampal neurons, which are the target cells of cholinergic neurons in medial septum, have also been shown to produce NGF[2,48,57]. Although there is no direct evidence for NGF production by astrocytes in vivo, our previous data have shown that astrocytes play important roles in the sprouting of NGF-sensitive septal cholinergic neurons after axotomy[14]. Moreover, NGF production by astrocytes in vitro has been demonstrated previously[13,28,59]. In the injured brain, astrocytes might be stimulated to produce NTFs, including NGF, which can affect the damaged neurons. The purpose of this study is to discover the mechanism regulating the production of NGF by astrocytes in vitro. We examined the effects on NGF secretion by astrocytes of several growth factors and lymphokines, including acidic and basic fibroblast growth factors (aFGF and bFGF), which are abundant in the brain and are the most potent proliferation factors for astrocytes[37].

MATERIALS AND METHODS

Growth factors and lymphokines

Acidic and basic FGFs from bovine brain were purchased from R&D Systems, Inc. Human recombinant platelet-derived growth factor (PDGF-BB) and human recombinant interleukin-1β (IL-1β) were from Genzyme. Murine recombinant IL-3 was from Gibco. Human IL-6 was from Endogen. Mouse submaxillary epidermal growth factor (EGF) and human recombinant tumor necrosis factor-α (TNF-α) were obtained from Sigma.

Cell culture

Several brain regions were dissected out from newborn rats (Fischer 311), and the meninges, blood vessels, and choroid plexus were carefully removed under a dissection microscope. The brain

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fragments made by passing through a stainless-steel mesh (pore size 200 μm) were suspended in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2.5 mg/l Fungizone, and 40 mg/l gentamicin, and were seeded in poly-L-lysine-coated culture flasks (25 cm^2, Corning). Astrocyte-enriched culture obtained after two passages with the enzymatic treatment was used for the present study.

Meninges removed from newborn rat brain were seeded in poly-L-lysine-coated culture flasks (25 cm^2). Fibroblast cultures were obtained after several passages with enzymatic dissociation from this primary culture in untreated culture flasks.

Microglia were collected from over-confluent astroglial cultures according to Giulian and Baker[15].

All of the cells used in the present study were grown in 10% FCS-DMEM at 37 °C in 5% CO_2–95% room air.

The cells were seeded on 24-well plates (Corning) for the measurement of NGF secretion, and were incubated with 0.3 ml/well of medium for NGF measurement. The cell numbers were counted using a Coulter counter.

NGF protein measurement

NGF protein levels were measured by two-site enzyme-immunoassay. (1) Polystyrene microtiter plates (PRO-BIND assay plate, Falcon) were incubated with 100 μl/well of 50 mM sodium carbonate/sodium bicarbonate buffer, pH 9.6, containing 0.1 μg of mouse monoclonal anti-mouse βNGF (Boehringer-Mannheim) for 2 h at 37 °C. (2) Each well was incubated for 16 h at 4 °C with 50 μl of the samples or with a standard solution (5–160 pg of mouse βNGF/ml of sample buffer: Tris HCl 50 mM, NaCl 200 mM, $CaCl_2$ 10 mM, bovine serum albumin (BSA) 1% (w/v), Triton X-100 0.1% (w/v); pH 7.0). An additional 3 blank wells were incubated with sample buffer but without NGF. (3) β-Galactosidase-conjugated monoclonal anti-mouse βNGF (Boehringer-Mannheim) was diluted to 0.4 U/ml with sample buffer. Each well was incubated with this solution for 4 h at 37 °C. Each step described above was followed by 3 washes with sample buffer without BSA. (4) β-Galactosidase activity was determined by incubation with 100 μl of the freshly prepared substrate solution (0.3 mg/ml of *o*-nitrophenyl galactopyranoside in the substrate buffer: HEPES 100 mM, NaCl 150 mM, $MgCl_2$ 2 mM, BSA, 1% (w/v); pH 7.0) for 3 h in the dark at 37 °C. The optical density of the generated colored product was measured at 405 nm by using ELISA-reader (Titertek Multiskan Plus, Flow Labs, Inc.). The detection limit of this assay is 5–10 pg/ml.

Immunocytochemistry and cell labelling method

Cells were grown on poly-L-lysine coated (for astrocytes and fibroblasts) or untreated (for microglia) sterilized round coverslips (diameter 13 mm) placed in 24-well culture plates for histological evaluation. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature. For glial fibrillary acidic protein (GFAP) immunocytochemistry, cells were treated with methanol at −4 °C for 5 min. Subsequently cells were treated sequentially with the following reagents: (1) 0.3% H_2O_2 in PBS for 30 min; (2) 0.1 M glycine for 15 min; (3) 5% BSA for 30 min; (4) rabbit anti-cow GFAP (1:2,000, Dakopatts), rabbit anti-human fibronectin (1:20,000, Cappel), or mouse monoclonal antibody MAC OX-42 (1:100, Serotec) in 1% BSA–PBS for 16 h at 4 °C; (5) biotinylated anti-mouse or anti-rabbit IgG (Vector; 1:200) in 1% BSA–PBS for 60 min; (5) horseradish peroxidase-conjugated biotin–avidin complex (Vector; 1:100) in 1% BSA–PBS for 60 min; (6) 0.05% solution of diaminobenzidine tetrahydrochloride, 0.01% H_2O_2, and 0.04% nickel chloride in 0.1 M Tris-buffered saline. Immunolabelled cells were dehydrated in graded alcohol and mounted on slideglass with Permount (Fisher).

Microglia grown on untreated coverslips were incubated with culture medium containing 10 μg/ml of acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetrathyl-indocarbocyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies, Inc.) for 3 h at 37 °C in 5% CO_2–95% room air. Cells were washed with culture medium 3 times, fixed with 3.7% formaldehyde in PBS for 5 min, and mounted on slideglass with Fluoromount-G (Southern Biotechnology Associates, Inc.).

RESULTS

Cell types and NGF secretion

The cultures obtained after two passages with an

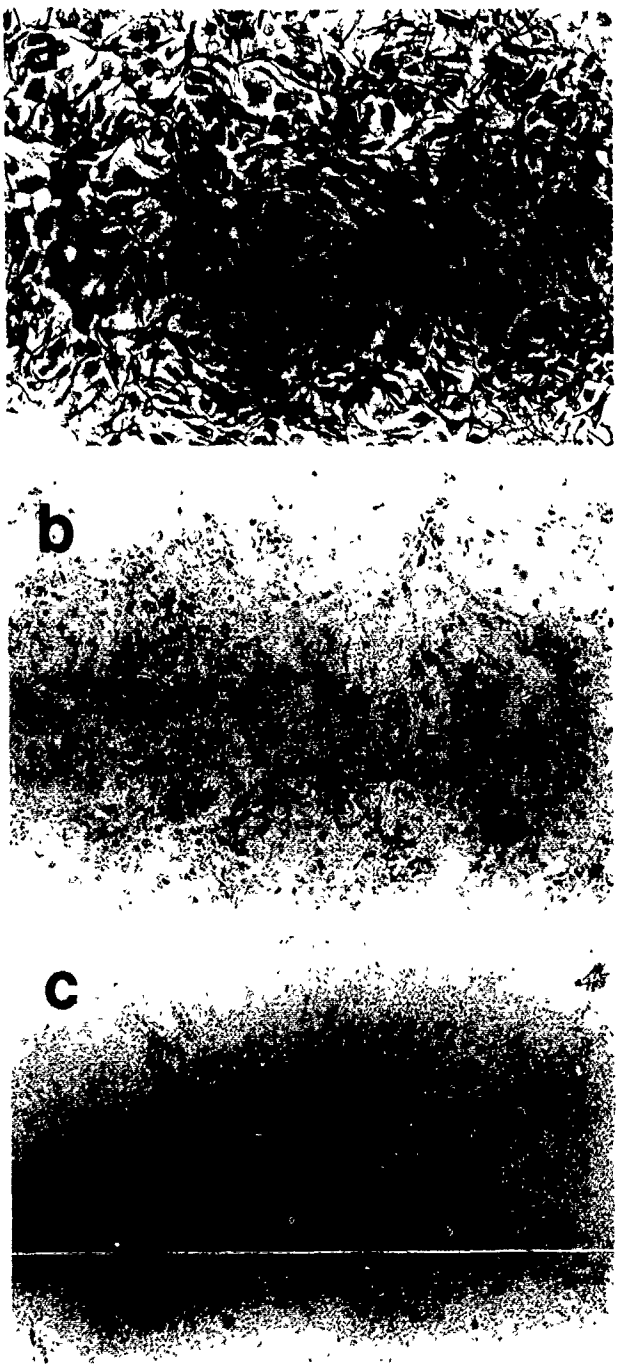

Fig. 1. Immunocytochemistry of astrocyte-enriched cultures derived from hippocampus for GFAP (a), MAC OX-42 (b), and fibronectin (c). Arrowheads and arrows indicate typical OX-42-positive microglia and fibronectin-positive fibroblasts, respectively. ×112.

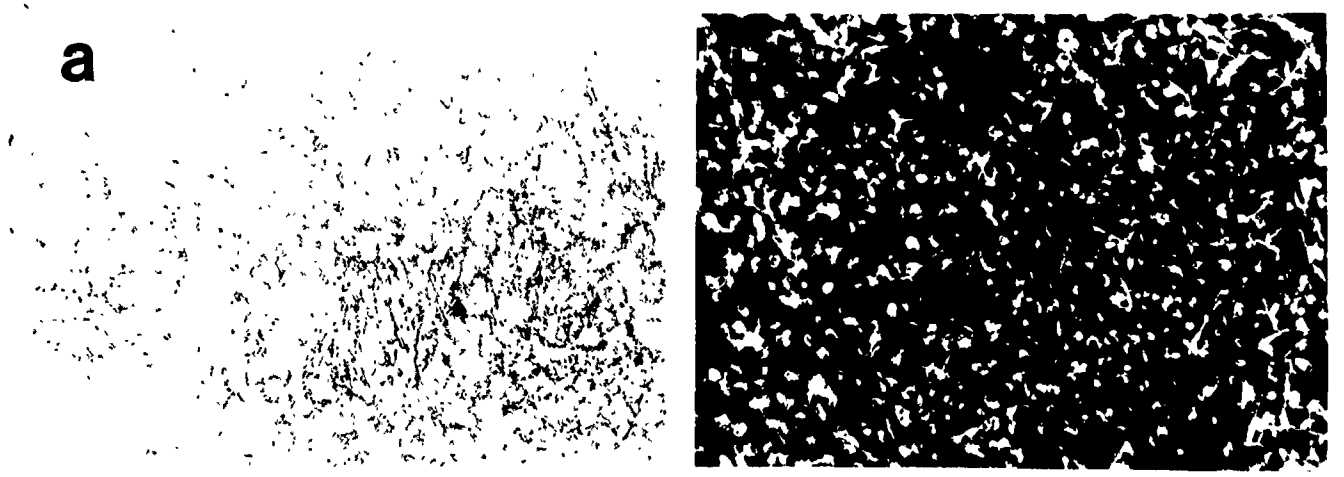

Fig. 2. Fibroblasts from meninges stained for fibronectin (a), and purified microglia labeled with DiI-Ac-LDL (b). ×120.

enzymatic treatment from newborn rat brains are mainly composed of GFAP-positive astrocytes (Fig. 1a), but other types of cells are also present. The major population of contaminated cells are microglia (Fig. 1b). We tried to keep the contamination of microglia to less than 5% by an earlier passage from a primary culture and by removing the detached cells at the washing process with Dulbecco's PBS during the passage procedure. Fibroblasts may also be present in our cultures (Fig. 1c), but the percentage of fibroblasts is much less than 1%. Although our astrocyte-enriched cultures are pure enough to be characterized as astrocytes, we obtained the pure fibroblast culture from meninges (Fig. 2a) and microglia-enriched culture from over-confluent astroglial cultures (Fig. 2b).

We first examined NGF secretion by astrocytes from newborn rat hippocampus under a standard culture condition. NGF secretion by astrocytes is highest just after passage and then gradually decreases (Fig. 3). NGF secretion by astrocytes at the overconfluent stage is sometimes lower than the detection limit (data not shown).

NGF secretion by astrocytes derived from cerebral cortex, striatum, hippocampus, septum, and cerebellum was tested just after passage. NGF contents in the conditioned media (CM) are well correlated with the final cell number at the lower cell density, and reach a plateau at the higher cell density. All of the astrocytes derived from the 5 different sites of origin can secrete NGF in the same manner (Fig. 4). NGF production by fibroblasts and microglia was also examined under the same conditions. The NGF level of microglia CM at each cell density tested (0.2–1.2 × 10^5 cells/well) was lower than the detection limit; however, fibroblasts secreted significant levels of NGF (Fig. 4). Although NGF secretion per cell by fibroblasts is higher than that by astrocytes, the number of fibroblasts contaminating to our astrocyte-enriched culture is not high enough to

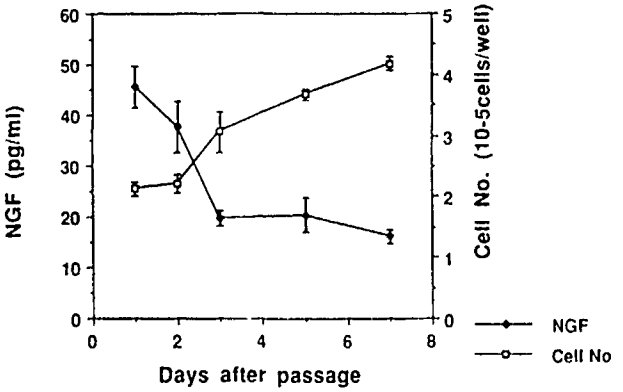

Fig. 3. Astrocytes derived from rat hippocampus were seeded at a density of 1.6 × 10^5 cells/well. NGF levels in the CM after 24 h of incubation and the final cell numbers were determined at various time points after passage. The values represent the means ± S.D. of 3 determinations.

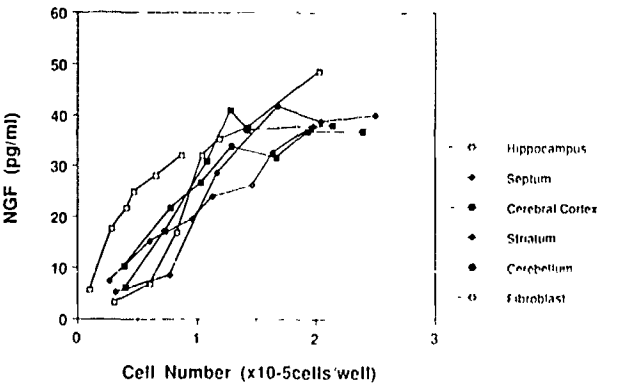

Fig. 4. Astrocytes from 5 different sites of origin and fibroblasts obtained from meninges were seeded at various cell densities. NGF contents in the CM were examined 24 h after seeding to compare the ability of astrocytes from various sites of origin and fibroblasts to secrete NGF.

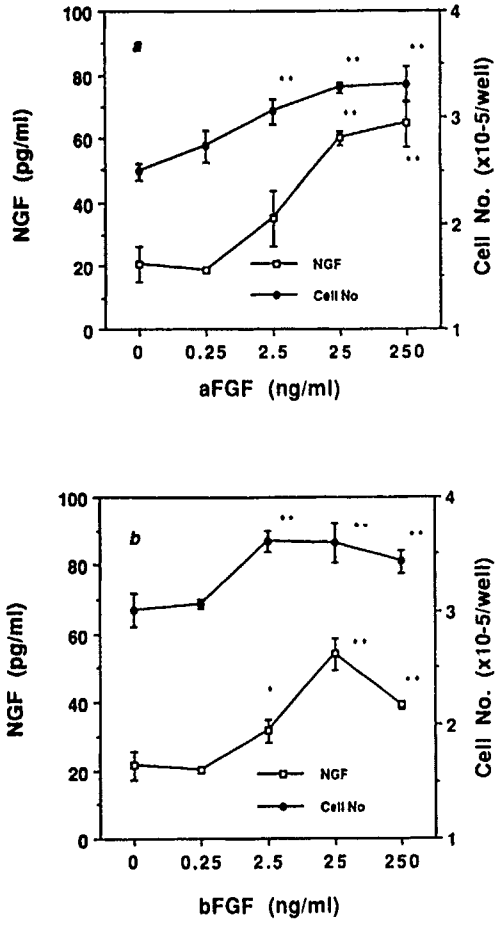

Fig. 5. The effects of aFGF (a) and bFGF (b) on astrocytes derived from cerebral cortex 3 days after passage were examined. Different doses of aFGF and bFGF were added to the culture media. NGF levels in the CM and cell numbers after 24 h of incubation were determined. The values represent the means ± S.D. of 3 determinations (**$P < 0.01$, *$P < 0.05$; Student's t-test).

secrete a detectable level of NGF.

Based on these results, we decided to use the astrocytes from cerebral cortex three days after passage for our experiments.

Effects of FGFs on NGF secretion by astrocytes

Fig. 5 shows the effects of aFGF and bFGF on NGF secretion by astrocytes. Both types of FGFs dramatically enhanced NGF secretion by astrocytes in a dose-dependent manner, and the effect of bFGF slightly decreased at the highest concentration. The effects of both types of FGFs on NGF secretion were not additive at the concentraton which elicits the maximum effect, although they were additive at a lower concentration (Fig. 6). These results suggest that both FGFs stimulate the same transduction pathway to NGF synthesis and secretion.

Subsequently the time course of NGF secretion by astrocytes stimulated by aFGF was examined, because

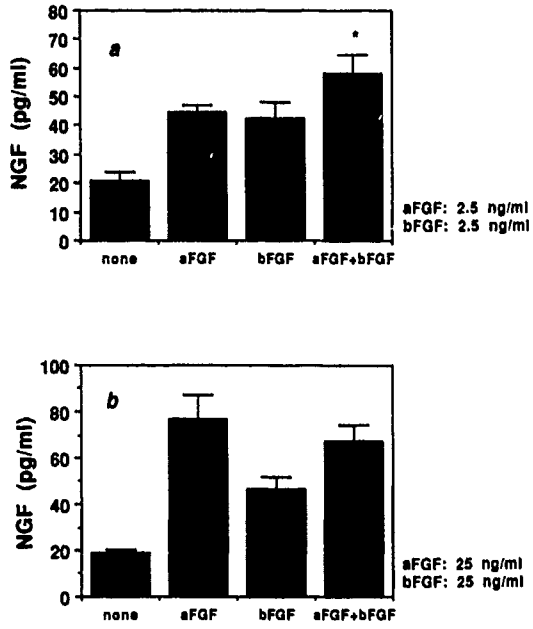

Fig. 6. The combined effect of aFGF and bFGF on NGF secretion by astrocytes three days after passage was examined. NGF levels in CMs 24 h after incubation were measured. The values represent the means ± S.D. of 3 determinations (*$P < 0.1$; compared to aFGF, bFGF, and none. Student's t-test).

the previous data had shown that the effects of both FGFs were similar and that aFGF had a stronger effect on NGF secretion than bFGF. The enhancement of NGF secretion by the addition of 25 ng/ml of aFGF was detected within 3 h, and the peak of NGF secretion occurred 6–9 h after stimulation (Fig. 7a). NGF secretion 24–48 h after aFGF stimulation was almost the same as that seen in control cells (Fig. 7a). On the other hand, a remarkable increase in cell number was observed 12–48 h after the aFGF stimulation (Fig. 7b), and the morphological changes became significant 24 h after the addition of aFGF (Fig. 8). As part of this experiment, the effects of cycloheximide (CH) and actinomycin-D (AC-D), which are a protein synthesis inhibitor and an RNA

TABLE I

The effects of cycloheximide (CH) and actinomycin-D (AC-D) on NGF secretion by astrocytes

NGF concentrations in CM determined after 9 h of incubation. Cell numbers were counted at the end of the experiment. Values represent means ± S.D. of 3 determinations.

Treatment	*NGF conc. (pg/ml)*	*Cell no. (×10⁵ cells/well)*
None	15 ± 2	3.46 ± 0.09
aFGF (25 ng/ml)	63 ± 3	3.38 ± 0.18
aFGF + CH (10 μg/ml)	<5	3.30 ± 0.17
aFGF + AC-D (10 μg/ml)	<5	3.37 ± 0.07

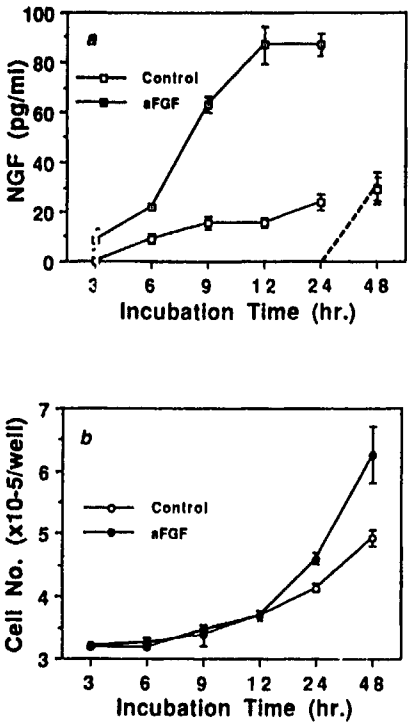

Fig. 7. NGF contents (a) and final cell numbers (b) were measured after various incubation times in the presence of aFGF (25 ng/ml). At the last time point both FGF-stimulated and control cells were incubated with the medium without FGF for the last 24 h. Values represent means ± S.D. of 3 determinations (**$P < 0.01$, *$P < 0.05$; Student's t-test).

synthesis inhibitor, respectively, were tested. The results shown in Table I indicate that NGF protein synthesized by astrocytes is rapidly secreted, and aFGF apparently stimulates NGF production and secretion from the transcription level.

TABLE II

The effects of various growth factors and lymphokines on NGF production by astrocytes

NGF concentrations in CM determined after 24 h incubation. Cell numbers were counted at the end of the experiment. NGF secretion standardized by the cell number determined at the end of the experiment. Effects of lymphokines were examined by a separate experiment. Values represent means ± S.D. of 3 determinations.

Treatments	*Conc. of NGF in CM (pg/ml)*	*Cell no. ($\times 10^5$/well)*	*NGF secretion (pg/10^5 cells/24 h)*
None	30 ± 6	2.19 ± 0.12	4.2 ± 0.5
aFGF (25 ng/ml)	113 ± 14**	2.65 ± 0.08**	12.9 ± 2.0**
bFGF (25 ng/ml)	90 ± 11**	2.48 ± 0.03*	10.8 ± 1.4**
PDGF (10 ng/ml)	24 ± 2	2.27 ± 0.01	3.1 ± 0.3
EGF (20 ng/ml)	46 ± 3**	2.33 ± 0.20	5.9 ± 0.3*
None	18 ± 3	1.97 ± 0.06	2.7 ± 0.5
IL-1β (30 U/ml)	42 ± 8*	2.06 ± 0.09	6.1 ± 1.0*
IL-3 (100 U/ml)	14 ± 1	1.82 ± 0.05	2.3 ± 0.3
IL-6 (30 U/ml)	15 ± 2	1.79 ± 0.05	2.6 ± 0.2
TNF-α (100 ng/ml)	43 ± 9*	2.08 ± 0.03	6.2 ± 1.2*

**$P < 0.01$, *$P < 0.05$ (Student's t-test).

NGF secretion by astrocytes induced by growth factors and lymphokines

The effects of other growth factors and lymphokines are summarized in Table II. PDGF, IL-3, and IL-6 do not have a significant effect on NGF secretion by astrocytes. However, EGF, IL-1β, and TNF-α also increased NGF secretion. Among the growth factors and lymphokines tested in the present study, FGFs were found to be the most potent stimulators of NGF production by astrocytes.

Subsequently the effects of EGF, IL-1β, and TNF-α were examined in the presence of aFGF. IL-1β and TBF-α increased NGF secretion under this condition,

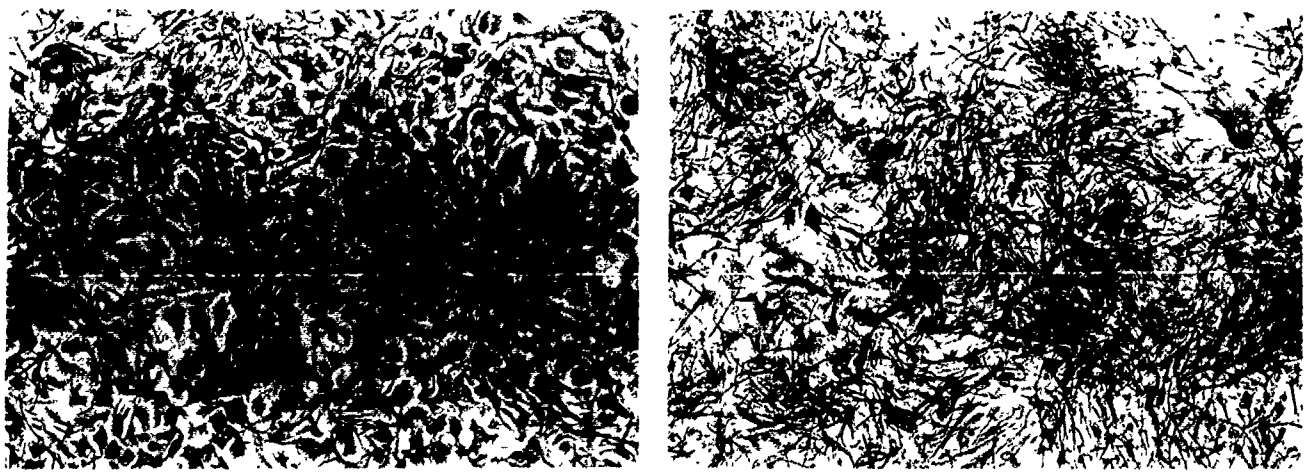

Fig. 8. GFAP immunocytochemical staining of untreated astrocytes (a) and aFGF-treated astrocytes (b; 24 h after the addition of aFGF (25 ng/ml)). ×120.

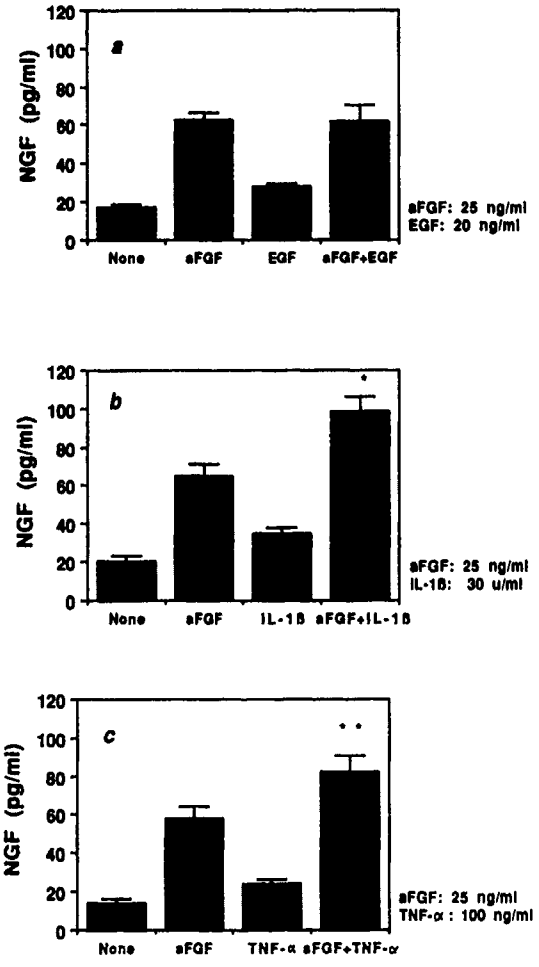

Fig. 9. The effects of EGF (a), IL-1β (b), and TNF-α (c) on NGF secretion by astrocytes were tested in the presence of aFGF under the same culture conditions described in the legend of Fig. 6. Values represent means ± S.D. of 3 determinations. (*$P < 0.01$; compared to aFGF, IL-1β, and none. **$P < 0.05$; compared to aFGF, TNF-α, and none. Student's t-test).

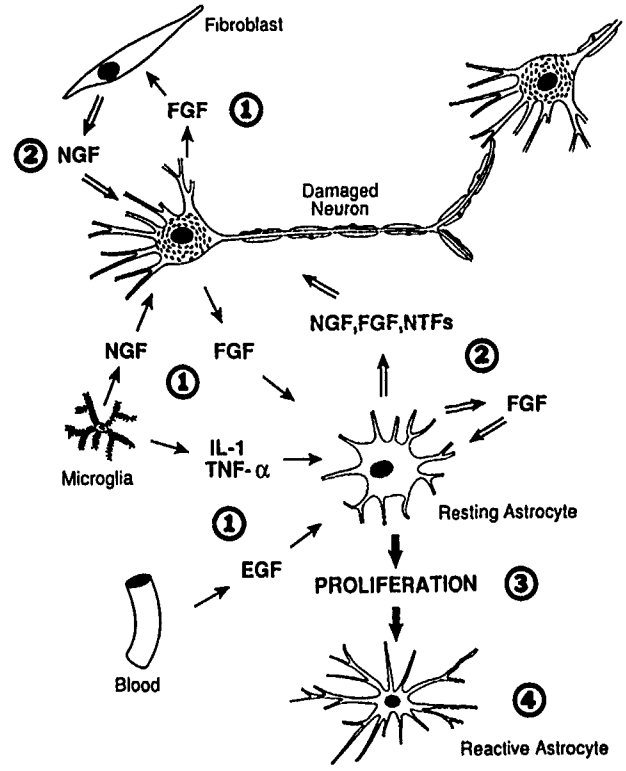

Fig. 10. The proposed mechanism of FGF activation of astrocytes in the damaged brain. In the normal brain, FGFs are mainly localized in the neurons. (1) In the case of brain damage, FGFs are released into the extracellular space from neurons, and act on astrocytes as a paracrine type growth factor. The disrupted blood–brain barrier may allow the influx of EGF in blood into the damaged brain. Microglia activated by the injury secrete IL-1, TNF-α, and NGF. (2) FGFs, EGF, TNF-α and IL-1 stimulate the secretion of NGF, and possibly other unknown NTFs, by astrocytes to save the damaged neurons. FGFs also stimulate NGF production by fibroblasts. (3) Subsequently, the number of astrocytes increases remarkably. (4) Astrocytes become so-called 'reactive' astrocytes at the end of this activation process. These reactive astrocytes no longer actively secrete NGF.

and the results showed that aFGF and IL-β or TNF-α synergically regulate NGF secretion by astrocytes (Fig. 9b,c). However, EGF had no significant effect in the presence of aFGF (Fig. 9a).

TABLE III

The effects of FGFs on NGF secretion by fibroblasts from meninges

NGF concentrations in CM determined after 24 h incubation from 3 days after passage. Cell numbers were counted at the end of the experiment. NGF secretion standardized by the cell number determined at the end of the experiment. Values represent means ± S.D. of 3 determinations.

Treatments	*Conc. of NGF in CM (pg/ml)*	*Cell no. (×10⁵/well)*	*NGF secretion (pg/10⁵ cells/ 24 h)*
None	18 ± 1	0.42 ± 0.03	12.8 ± 1.2
aFGF (25 ng/ml)	104 ± 12**	0.61 ± 0.03*	50.9 ± 4.0**
bFGF (25 ng/ml)	43 ± 3**	0.45 ± 0.03	28.7 ± 0.5**

**$P < 0.01$, *$P < 0.05$ (Student's t-test).

TABLE IV

The effects of FGFs and LPS on NGF secretion by microglia

NGF concentrations in CM determined after 24 h incubation from 2 h after passage. Cell numbers were counted at the end of the experiment. NGF secretion standardized by the cell number determined at the end of the experiment. Values represent means ± S.D. of 3 determinations.

Treatments	*Conc. of NGF in CM (pg/ml)*	*Cell no. (×10⁵/well)*	*NGF secretion (pg/10⁵ cells/ 24 h)*
None	<10	1.17 ± 0.01	–
LPS (10 μg/ml)	23 ± 8	1.28 ± 0.07	5.4 ± 2.1
aFGF (25 ng/ml)	<10	1.26 ± 0.05	–
bFGF (25 ng/ml)	<10	1.24 ± 0.08	–

Effects of FGFs on NGF secretion by fibroblasts and microglia

Because FGFs were revealed to be potent regulators of NNF production by astrocytes, the effects of FGFs on NGF secretion by fibroblasts and microglia were tested. Both of the FGFs also increased NGF secretion by fibroblasts (Table III); however, the FGFs had no significant effects on NGF secretion by microglia. Lipopoly-saccharide (LPS; Sigma) enhanced NGF production by microglia (Table IV).

DISCUSSION

FGFs, which were originally purified from the brain and the pituitary gland as potent mitogens for fibroblasts[18,51], are pluripotent growth factors for many kinds of cells, including astrocytes[52]. The typical effects of FGFs on astrocytes are: (1) stimulation of proliferation, (2) remarkable morphological changes (astrocytes become more fibrous-shaped in the presence of FGFs), and (3) induction of astrocyte-specific proteins, such as GFAP and glutamine synthetase[37,38,46]. We have demonstrated here the role of FGFs as potent regulators of NGF synthesis and secretion by astrocytes and fibroblasts.

The most well-characterized NGF-sensitive cells in the CNS are the cholinergic neurons in the basal forebrain, and the NGF-producing cells are their target neurons. In the normal brain, NGF molecules synthesized in the target neurons are retrogradely transported to the NGF-sensitive neurons and act as a maturation and maintenance factor[20,21,30]. It is well known that astrocytes have an exclusive neurotrophic effect in vitro, and this effect is considered to be mediated by NTFs, including NGF. NGF produced by astrocytes may not be important during the normal development of the CNS neurons, because the proliferation and maturation of astrocytes occur later than the maturation of neurons[9]. However, in the pathological condition, NGF produced by astrocytes may play an important role in repair, sprouting, and aberrant regeneration. We assume that astrocytes are stimulated to produce and secrete NTFs, including NGF, in the damaged brain and support the survival of damaged neurons. The present results strongly support our hypothesis. In the case of brain damage, FGFs, which are mainly localized in the neurons in the normal brain[29,47], might be released into the extracellular space and stimulate proliferation of and NGF secretion by astrocytes. FGFs are also considered to be produced by astrocytes and to act as an autocrine growth factor[11,22]; thus, exogenous FGF might stimulate FGF production by astrocytes.

The increase in NGF production and secretion by astrocytes is an early and transient response to FGF stimulation, relative to the morphological changes in the cells. The morphological appearance of astrocytes induced by FGFs is similar to that of reactive astrocytes in vivo. Our present results suggest that the so-called 'reactive astrocytes' do not actively secrete NGF. FGFs may also enhance the production by astrocytes of other kinds of NTFs, in addition to NGF, as evidenced by the fact that brain injury increases non-NGF type neurotrophic activity at the lesion site[43].

Among the growth factors and lymphokines we tested, EGF, IL-1, and TNF-α enhanced NGF production by astrocytes. A significant level of EGF exists in the blood and the cerebrospinal fluid (CSF)[7,26]. The disrupted blood–brain barrier and blood–CSF barrier in the case of brain injury may allow the influx of EGF into the brain. On the other hand, IL-1 and TNF-α are products of the activated microglia (macrophage)[8,24], and IL-1 has been shown to be a proliferation factor for astrocytes[16,17]. EGF and IL-1 have also been shown to exist in the brain[5,10], and IL-1-like activity was shown to be increased by brain injury[44]; thus EGF and IL-1 may act on astrocytes in vivo. Although the effects of EGF, IL-1 and TNF-α are less than those of FGFs, these 3 biologically active proteins can significantly increase NGF secretion by astrocytes. Furthermore, IL-1 and TNF-α act as regulators of NGF production in concert with FGF. IL-1, TNF-α and EGF may also play an important role in NGF production by astrocytes in the case of brain damage. Fibroblasts and microglia can also produce NGF. Activated microglia with LPS were found to produce NGF[36], and we have reconfirmed that finding in the present study. On the other hand FGFs do not have significant effects on NGF secretion by microglia, but stimulate NGF production by fibroblasts derived from meninges. Fibroblasts are a major component of a scar which is the vestige of injury, and thus NGF produced by fibroblasts and by activated microglia may act as a neurotrophic factor in the case of brain injury. This hypothesis is presented schematically in Fig. 10.

Although there is no direct evidence for NGF production by astrocytes in vivo, NGF production by cultured astrocytes has recently been shown[13,28]. More recently, Spranger et al.[50] reported the effects of transforming growth factor-α, bFGF, EGF, and IL-1 on NGF synthesis by astrocytes cultured with the medium containing a very low amount of FCS. The accumulated evidence clearly demonstrates that astrocytes can produce NGF (or NGF-like molecules) in vitro. However, NGF production by astrocytes is extremely variable. Many molecules can regulate NGF production by astrocytes, and among these, FGF is the most potent regulator of NGF production by astrocytes, and the only factor which can display the mitogenic function by astrocytes in the

presence of FCS. This suggests that FGFs stimulate the function of 'healthy' astrocytes.

FGFs are heparin-binding proteins, and heparin is known to modulate the activity of aFGF[53]. Heparin enhances the activity of aFGF in a serum-free condition, but reduces it in the presence of serum[53]. All of the present experiments were done in the presence of fetal calf serum. Heparin reduced the effect of aFGF on NGF secretion by astrocytes in our culture condition (data not shown), which agrees with the findings by Uhlrich et al.[53]. The modulation of the effect of aFGF on NGF secretion by heparin, and the effects of other growth factors and lymphokines in a serum-free condition are under investigation.

In the peripheral nervous system, Schwann cells and fibroblasts produce NGF after injury[25]. NGF production by fibroblasts from the sciatic nerve is mainly regulated by IL-1, which is a product of the activated macrophages, and not by FGFs[34]. PDGF, which can increase NGF production by the fibroblasts from the sciatic nerve[34], has no effect on NGF production by astrocytes. More interestingly, FGFs which are shown to have no effect on NGF production by fibroblasts from sciatic nerve[34], were found to significantly enhance NGF production by fibroblasts derived from meninges. Our results demonstrate that the mechanism regulating NGF production by astrocytes and fibroblasts in the CNS is different from that of fibroblasts from the sciatic nerve.

FGFs and EGF are considered to have neurotrophic effects on certain types of neurons in the CNS[3,12,19,39,40,54–56]. For example, bFGF can reduce axotomized cholinergic cell death to a certain extent[1,45]. Most of these cholinergic neurons are NGF-sensitive, and the exogenous application of NGF can ameliorate the cell death after axotomy[23,32,49,58]. The present findings strongly suggest that at least some of the neurotrophic effects of FGFs and EGF could be mediated by the enhancement of the neurotrophic activities of astrocytes.

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