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Abstract

Basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) were administered into the rat brain following unilateral fimbria-fornix transection. Both bFGF and NGF stimulated the sprouting of acetylcholinesterase (AChE) positive fibers in the hippocampus on the lesioned side. Furthermore, a small number of AChE-positive fibers were regenerated even when only the vehicle was administered. Rats treated with NGF as well as control group had only thin fibers, whereas those treated with bFGF had not only thin fibers but also thick fibers. These results indicate that intrinsic NGF is released and acts on damaged neurons directly, while bFGF acts them on directly and/or indirectly after brain injury.

KEYWORDS: bFGF, NGF, regeneration, acetylcholinesterase positive fibers, sprouting

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Basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) were administered into the rat brain following unilateral fimbria-fornix transection. Both bFGF and NGF stimulated the sprouting of acetylcholinesterase (AChE) positive fibers in the hippocampus on the lesioned side. Furthermore, a small number of AChE-positive fibers were regenerated even when only the vehicle was administered. Rats treated with NGF as well as control group had only thin fibers, whereas those treated with bFGF had not only thin fibers but also thick fibers. These results indicate that intrinsic NGF is released and acts on damaged neurons directly, while bFGF acts them on directly and/or indirectly after brain injury.

Key words : **bFGF, NGF, regeneration, acetylcholinesterase positive fibers, sprouting**

Neurotrophic factors (NTFs) may play important roles in the adult nervous system by sustaining normal cell function (1-5). Brain damage stimulates the synthesis of NTFs which take part in damage repair mechanisms (6-10). Furthermore, administration of neurotrophic factors prevents neuronal death after lesion (11-14). Nerve growth factor (NGF) is the most completely characterized of NTFs, especially its action on cholinergic neurons (12, 13, 15, 16). Basic fibroblast growth factor (bFGF) as well as NGF have, during recent years, also been established as pluripotent growth factors for many kinds of cells including neurons. For example, bFGF promotes survival of embryonic neurons *in vitro* (2, 5) and prevents neuronal death following fimbrial transection (11, 14). Moreover, in cell cultures, bFGF as well as NGF protects central neurons against hypoglycemic damage (17). Finklestein *et al.* (6) reported that bFGF immunoreactivity increased at the site of focal brain wounds. These results suggest that bFGF may protect neurons against brain injury and stimulate neural regeneration the same as NGF. However, the precise action of bFGF on neurons after brain injury is still a matter of debate. Neurotrophic effects of bFGF may be mediated by the enhancement of NGF synthesis and secretion by astrocytes (18). On the other hand, Cheng *et al.* (17) reported that bFGF may act directly on neurons after hypoglycemic damage *in vitro*. In this study, we compared the action of bFGF and its mechanism with NGF on the regeneration of cholinergic fibers in the hippocampus following fimbria-fornix transection.

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Materials and Methods

A total of 25 young adult female Sprague-Dawley rats weighing between 220-250 g at the beginning of the experiment were used. The animals were anesthetized with ether and pentobarbital (20 mg/kg), and placed in a stereotaxic apparatus. They received unilateral transections of the right fimbria-fornix (F-F) by aspiration through the medial parietal cortex and corpus callosum, 1-2 mm posterior to bregma (19). In all cases the commissure of the fornix was intact. This procedure transects cholinergic afferents reaching the hippocampus via the two dorsal routes through the cingulate bundle and F-F. After F-F transection, sterile pieces of gel foam (4 × 4 × 6 mm, 1 mg; Upjohn Inc., Kalamazoo, MI)

were soaked in 25 μl of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Inc., Tokyo) with or without the addition of the following additives: a) 25 μg of NGF from mouse submaxillary gland (Chemicon Inc., Temecula, CA); and b) 12.5 μg of bFGF from bovine brain (R & D Systems Inc., Minneapolis, MN). A piece of gel foam was placed into a cavity made by aspiration in the right parietal cortex (coordinates of the center of the cavity: AP 4.3; L 3.0 mm)(19), according to NIH guidelines. Four weeks after FF transection, these rats (n = 5, per group) were deeply anesthetized with barbiturate (70 mg/kg) and then killed by intracardial perfusion with 150 ml of phosphate buffered saline (PBS), followed by 200 ml of ice cold 4 % paraformaldehyde in PBS (pH 7.4). The brains were removed and post-fixed at 4 °C in the same fixative for 4 h, and then immersed in 30 % sucrose at 4 °C until they sank.

Caudal hippocampal areas were serially sectioned in the coronal planes at 40 μm thickness. These sections were collected for acetylcholinesterase (AChE) staining based on the thiocholine method (20), using ethoproprazine as the inhibitor of nonspecific esterases and with 0.25 % silver nitrate to enhance the sulfide reaction product. Presumptive cholinergic axons were identified by using thiocholine methods of AChE staining (21), and the distribution of the cholinergic axons after F-F lesion have been discussed by using AChE histochemical method the same as Henderson reported (22). Counterstaining was lightly done with Cresly Violet. The diameter of AChE-positive fibers in the hippocampus was computed using a digitizer tablet, which serves for the direct input into the computer of X and Y coordinates of a point (image analysis system-IBAS, Karl Zeiss Inc., Oberkochen, Germany), connected to the light microscope (magnification × 1000). Two coronal planes separated by about 100 μm from each other were measured (n = 5, per group). The fiber diameter of at least 40 randomly selected AChE-positive fibers per section was determined. The quantity of AChE-positive fibers in the hippocampus was also expressed by color imaging and shown as mm^2 in 1.68 mm square, using IBAS. Statistical analysis of the data was performed by Student's *t*-test.

Results

In F-F transected rats, AChE-positive fibers in the hippocampus side of the incision completely disappeared (Fig. 1B, Fig. 2B) 4 weeks after unilateral fimbria-fornix transection, but rats that received exogenous NGF or bFGF treatment clearly regenerated the AChE-positive fibers in the hippocampus (Fig. 1E-H, Fig. 2D, E). Only a small number of AChE-positive fibers were regenerated even in the case of the cavity with a gel foam (Fig. 1C, D, Fig. 2C). The quantity of fibers in NGF (1.041 ± 0.133 mm^2) or bFGF (1.026 ± 0.102 mm^2) treated rats was not the same as normal rats (1.704 ± 0.148 mm^2, $p < 0.001$), but larger than DMEM rats (0.370 ± 0.083mm^2, $p < 0.001$) (Table 1). In the 6FGF treated rats (Fig. 1G, H), some AChE-positive fibers showed a thicker and knottier appearance than the other groups. As indicated in Table 1, the diameter of AChE-positive fibers in bFGF treated rats (0.497 ± 0.242 μm) was significantly thicker than normal control (0.425 ± 0.133 μm, $p < 0.001$).

Table 1 The diameter and area of AChE-positive fibers in the hippocampus

	Diameter (μm)	Area (mm^2)
Normal	0.425 ± 0.133	1.704 ± 0.148
DMEM	0.403 ± 0.117	0.370 ± 0.083*
NGF	0.401 ± 0.133	1.041 ± 0.087*†
bFGF	0.497 ± 0.242*†	1.026 ± 0.102*†

Values are given as the mean ± SEM of 5 animals per treatment group. *$p < 0.001$ *vs.* Normal group; †$p < 0.001$ *vs.* Dulbecco's modified Eagle's medium (DMEM) group by Student's *t*-test. AChE : acetylcholinesterase; NGF : nerve growth faotor; bFGF : basic fibroblast growth factor.

Discussion

The results of this study suggent that bFGF as well as NGF stimulates regeneration of cholinergic neurons in the denervated hippocampus. In addition, the appearance of these regenerating fibers, especially in the bFGF treated rats, was different from normal rats; some fibers in the bFGF treated rats were thicker and knottier than normal rats. This means that the regenerating AChE-

Fig. 1 Acetylcholinesterase (AChE)-positive stained fibers in the hippocampus 4 weeks after unilateral fimbria-fornix (F-F) lesion and implantation of gel foam with following additives. A: intact control rat; B: F-F transection only rat; C, D: Dulbecco's modified Eagle's medium (DMEM) only; E, F: nerve growth factor (NGF) ; G, H: basic fibroblast growth faetor (bFGF) ; D, F, H: high power micrographs of dentate gyrus. AChE-positive fibers disappeared completely in the F-F transection only rats (B), but exogeneous NGF and bFGF treatment clearly regenerated AChE-positive fibers (E, F, G, H). Note a small number of fibers regenerated even by administration of vehicle into the gel foam cavity (C, D). Furthermore, administered bFGF, not only thin fibers but also thick fibers with varicosities (arrowheads) were regenerated (G, H). The arrow in C, E and G indicates the cavity in which the gel foam was implanted. Scale bars = 250 μm.

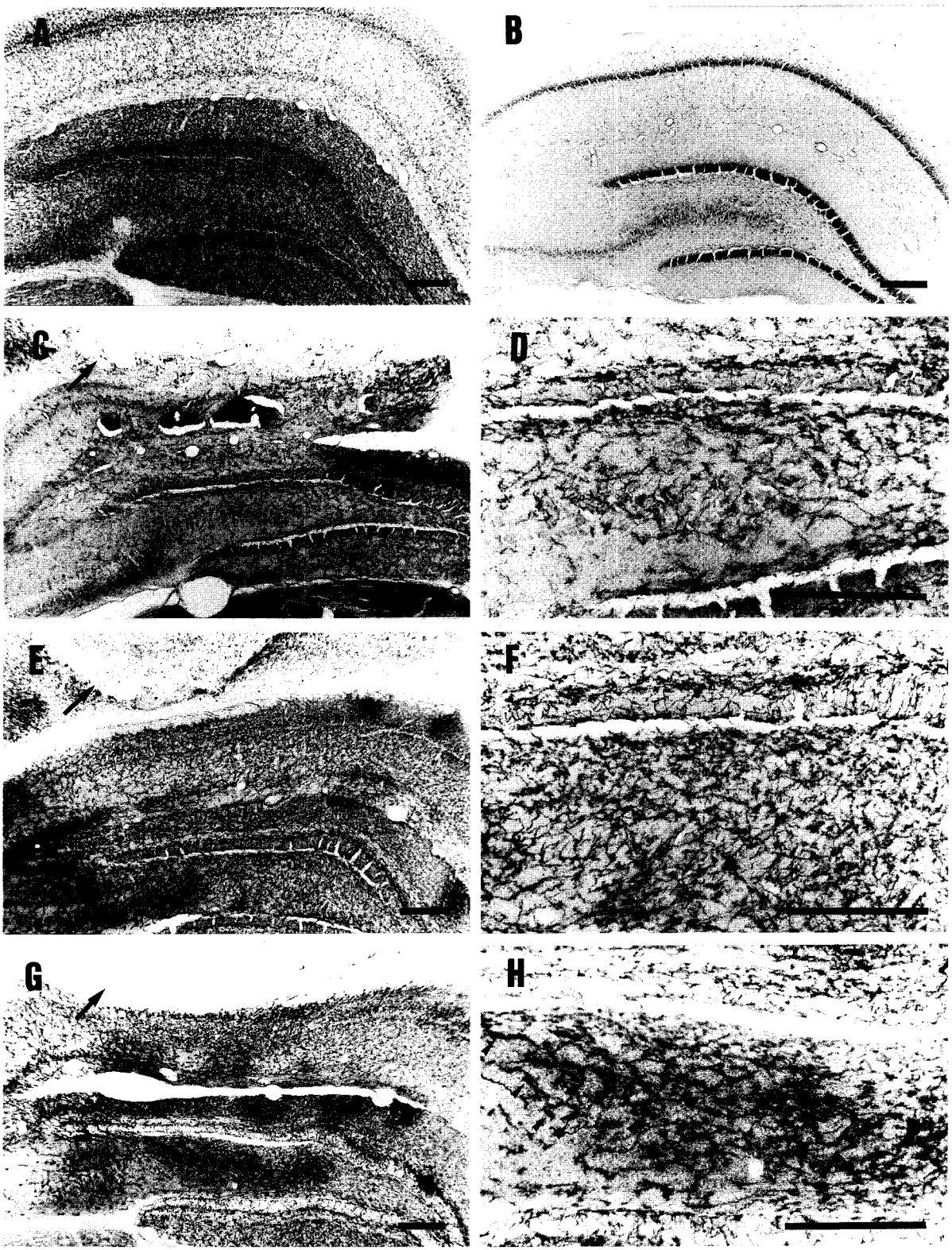

Fig. 1

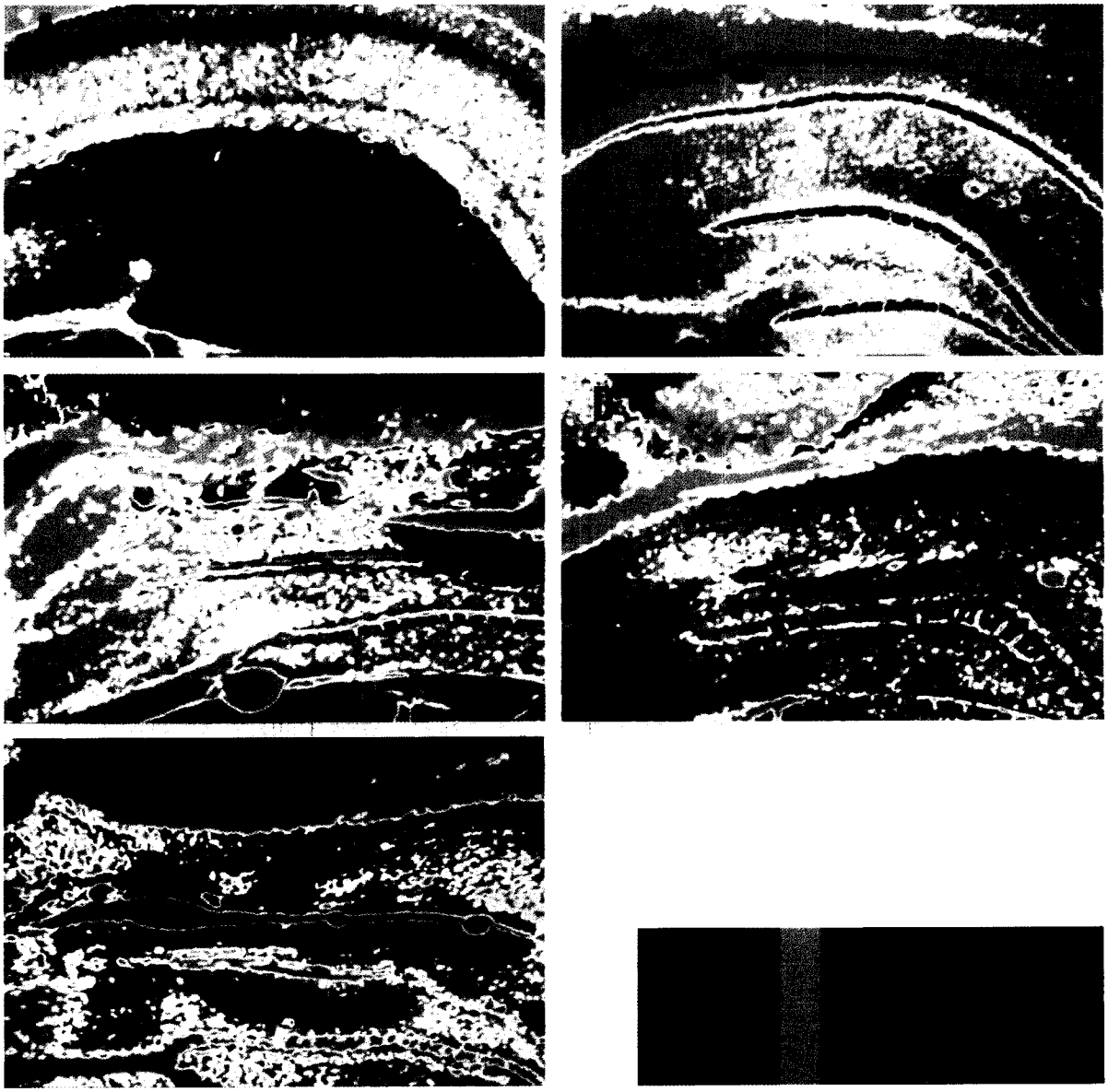

Fig. 2 Color imaging of a quantity of AChE-positive fibers in the hippocampus. A: Normal; B: F-F transection only; C: DMEM; D: NGF; E: bFGF rats. A considerable amount of fibers regenerate in the NGF (D) or bFGF (E) rats. Abbreviations: See Fig. 1.

positive fibers were different from the persisting fibers, resulting in sprouting and reinnervation of cholinergic neurons. There was no regeneration of AChE-positive fibers after F-F transection in the untreated rats, whereas a few regenerating fibers were associated with the gel foam cavity which received vehicle only. After administrating a gel foam with anti NGF antibody into the cavity following F-F transection, regeneration of AChE-positive fibers did not occur in the hippocampus (data not shown). Furthermore, when a smaller dose of NGF or bFGF was administered, fewer fibers were seen (data not shown). These results suggest that some intrinsic NTFs (mainly NGF) may be released after brain injury (6–10) and stimulate the regeneration of cholinergic fibers, although

it may be a matter of dosage that determines whether these NTFs trigger a sprouting response or not.

However, the source of these cholinergic fibers is not clear. There are three separate routes by which cholinergic afferents reach the hippocampal formation: a supracallosal pathway, a subcallosal pathway (along the fimobriafornix), and a ventral pathway (along the ansa lenticularis and the ventral amygdaloid pathway)(23, 24). In the present study, the two dorsal pathways were transected, but the ventral pathway was left intact. Gage *et al.* (23) reported that the recovery in cholinergic fibers after F-F transection is due to proliferation and expension of the spared terminal networks which enter the hippocampal formation along the ventral route. There is also the possibilty that sprouting of cholinergic fibers generates from the contralateral hippocampus through the commissures as a result of unilateral F-F transection and/or cingulate-entorhinal-dentate pathway.

In the study by Gage *et al.* (23), the sprouting response in the hippocampus was first detectable between 1 and 3 months after F-F transection and continued for at least 6 months. The time course of the present post-lesion reinnervation phenomenon matches well the slow and protracted functional recovery that can occur after experimental brain lesions in rats (24). In our data, intense sprouting response occurred only one month after transection when bFGF and NGF were administered, and this time course of reinnervation was more rapid than shown by the data of Gage *et al.* (23). From this point of view, exogenous administration of NTFs may promote recovery of amnesia and motor function in patients with brain injuries.

According to Yoshida *et al.* (18), FGFs (both acidic and basic) may work in autocrine fashion, ensuring their own release by astrocytes, and stimulating the synthesis and release of NGF in astrocytes. Accordingly bFGF may act on damaged neurons via NGF. In our data, bFGF regenerated thin fibers and thick fibers, but both NGF and DMEM treated rats had only thin fibers. Moreover, NGF receptors are located in basal forebrain cholinergic neurons and the hippocampus of rats (25, 26), while FGF receptors are widely expressed within the rat brain, including the hippocampus and pontine cholinergic neurons (27). These results suggest that one of the intrinsic NTFs which directly act on cholinergic neurons after brain injury may be NGF, and bFGF may also affect cholinergic neurons in addition to its effect through NGF.

In conclusion, NGF and bFGF appear to stimulate neural regeneration after brain injury. This result supports previous papers demonstrating neurotrophic effect of NGF and bFGF (3, 4). In addition, our data indicate that bFGF directly and/or indirectly acts on cholinergic neurons *in vivo*.

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