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Neurotrophic factor-like effect of FPF1070 on septal cholinergic neurons after transections of fimbria-fornix in the rat brain

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Summary. FPF1070 is an aqueous protein-free solution, which consists of 85% free amino acids and 15% small peptides. Our previous study showed a potent neurotrophic factor-like activity in cultured embryonic cells of dorsal root ganglia. The present study investigated whether FPF1070 regenerated the cholinergic cells in the medial septal nucleus after axonal transections by cutting the fimbria-fornix. Fimbrial transections reduced the number of septal cholinergic cells by 30 ± 3.6%, compared with the number on contralateral sides at 4 weeks. Intrapretioneal injections of FPF1070 caused 49.9 ± 6.3% of the cholinergic neurons to survive. Furthermore, the cell sizes of the cholinergic neurons were significantly different: 16.4 ± 4.2 μm, 14.3 ± 3.8 μm in FPF1070 treatment and vehicle treatment, respectively. These results indicated that FPF1070 prevents the degeneration and atrophy of impaired cholinergic neurons by systemic administration.

Key words: FPF1070, Neurotrophic factor, Cholinergic neuron, Peptide

Introduction

The cholinergic neurons of the forebrain play an important role in the functional processes of learning and memory (Bartus et al., 1982). Clinically, reduction of the cholinergic cells and a loss of choline acetyl transferase activity correlate with presenile dementia as a morphological feature (Perry et al., 1977; Pearson et al., 1983; Etienne et al., 1986). On the other hand, several findings indicate that nerve growth factor (NGF) has a trophic influence on the cholinergic neurons of the basal forebrain (Hatanaka et al., 1987) and isolated septal neurons (Hartika and Hefti, 1988). In particular, intraventricular injections of NGF prevent retrograde neuronal cell death and promote behavioural recovery (Will and Hefti, 1985; Hefti, 1986; Williams et al., 1986; Tuszynski et al., 1990). As an experimental model, fimbria fornix transection has been developed. This pathway is easily accesible in the lateral ventricle by a surgical approach. Above all, it is a well-verified portion of the ascending cholinergic projections (Gage et al., 1983). Transection of the hippocampal projections to the medial septum (MS) and vertical limb of the diagonal band of Broca (VDB) rapidly ameliorates the cholinergic neurons in the regions. Nearly 70% of the cholinergic neurons die within 2 weeks post-transection (Gage, 1986). Though exogenous NGF saves cholinergic neurons there is a limitation to administration because of the blood-brain barrier. Peptides derived from pig brain by enzymatic breakdown (FPF1070); 85% amino acid component and 15% peptide component have been used for therapy of cerebral dysfunction (Harrer, 1954; Lee An-Shih, 1970). Among the effects reported with these derivatives, Baraschnev (1970) showed a rapid gain of brain weight and found the peptide derivatives promoted recovery from asphysial insult in rabbits. Independently, Bard (1982) reported a new neurotrophic factor extracted from mammalian brain, which was different from NGF because of an improvement in survival of the neurons of older embryos, which no longer respond to NGF by extending processes (Herrup and Shooter, 1975). Our previous experiments (Iwamoto et al., 1989) have shown that these amino acids and the peptide compound, FPF1070, supports the survival of sensory neurons isolated from dorsal root ganglia of the chicken embryo (E8-E10) and also elicit neurite outgrowth from ganglion explants cultured in collagen gel. This effect was not blocked by NGF antibodies. Therefore, it is interesting to determine whether this compound protects against amelioration of cholinergic neurons after fimbrial transection with a systemic administration.

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Materials and methods

Surgical procedures

Adult male Wistar rats (a total of 49), weighing 200-250 gm at the beginning of the experiment were used. A unilateral fimbria-fornix lesion was made according to Gage's method (Gage et al., 1983) under ketamine anesthesia (50 mg/kg Ketalar, Parke-Davis). Briefly, under the surgical microscope, a small bone window was opened posterior to the bregma, then the fimbria-fornix was removed by a fine suction tip. The most rostral portion of CA3 and the cortex over the lesion were consequently aspirated (Fig. 1). The complete transection reduced cholinergic innervations of the hippocampus by up to 90%. The bone flap was returned to the initial position. Every morning, 24 rats received 5ml/kg of FPF1070 intraperitoneally and the other group of 20 rats received the same dose of saline as a control until the planned time of sacrifice. The first injection was done on the day of surgery. Each animal had free access to water and was fed ad libitum on pellets. FPF1070-treated rats were killed at 1 week (n = 5), 2 weeks (n = 7), 3 weeks (n = 5) and 4 weeks (n = 7) after surgery. Five rats of saline-treated controls were killed at the same planned time as the FPF1070-treated group. Five animals were submitted to a sham-operation (craniotomy without a transection of fimbria-fornix and vehicle-treatment) as a normal control.

Histochemistry

FPF1070-treated and control animals were sacrificed with perfusion fixation of 4% paraformaldehyde in PBS. The animals were injected intramuscularly with an irreversible acetyl choline esterase inhibitor, diisopropyl fluorophosphate (DFP, Sigma) 4 hours before sacrifice at a dose of 2 mg/kg according to Butcher's method (1975). The brains were removed and postfixed for 2 hours in the same fixative, then left overnight in 10% sucorse in PBS at 4° C. Nonfrozen sections were cut at 40 μm with a Microslicer (DT-1000, Dosaka EM LTD, Osaka, Japan) through the MS. The sections were processed for the visualization of actylcholinesterase (AChE) according to Koelle (1954), using 0.1 mM prometazine as an inhibitor of nonspecific esterase. Alternate sections were stained for choline acetyltransferase (CAT) by an immunohistochemical method. Incubation of the sections was carried out by flotation overnight at 4° C. Monoclonal antibody to choline acetyltransferase (Boehringer Manheim Biochemica) was used at a dilution of 4 μg/ml. The ABC method was then applied: sections were incubated in a biotinylated rabbit anti-rat immunoglobulin (Vectastain, 1:200) for 1 hour at room temperature; in streptavidin-peroxidase conjugate solution for 1 hour, and were then visualized in a solution of diaminobenzidine hydrochloride (1 mg/ml) containing 0.1% H_2O_2. Every third section was taken for Cresyl violet staining. The extent of fimbriafornix transection was confirmed in the caudal section.

Cell count

CAT-positive cells were counted in the medial septal nucleus between the anteroposterior levels, Plate-12 and Plate-14 (Paxinos and Watson, 1982). The regions on both sides were photographed microscopically, and the cells with dark staining, which were defined as neurons, were counted with a Digiplan (Kontron Co., Ltd., Munich, West Germany). Cell size was estimated with the same analytic system, measuring the length along the major axis. The results are presented as a percentage of the control side in the same animal (ipsilateral region/ contralateral region x 100). A one-factor analysis of variance was used to determine the statistical difference.

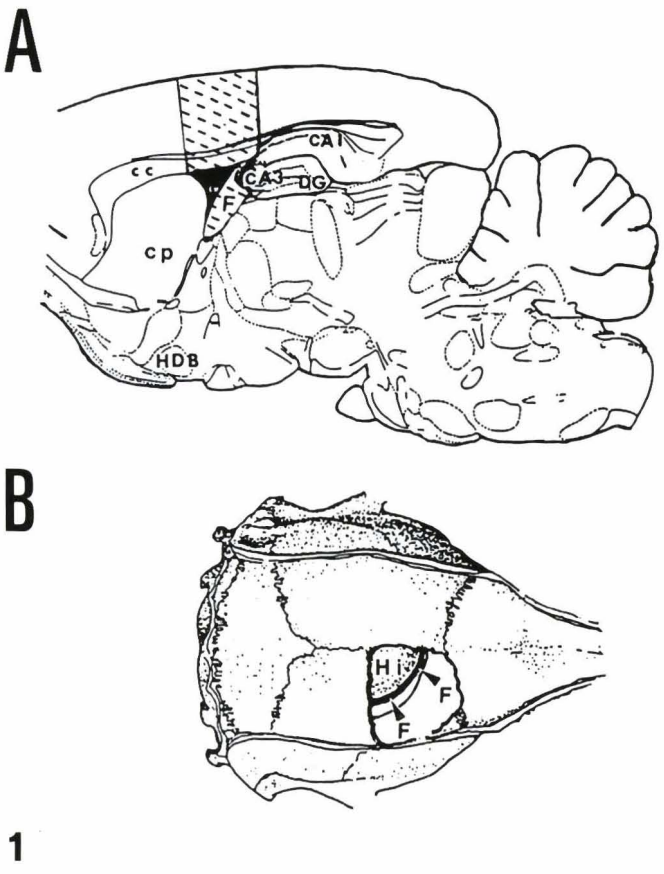

Fig. 1. Surgical approach for transection of the fimbria. A: The cortex and the cingulate gyrus is resected for aspiration of the underlying fimbria-fornix. B: Making a bone window then aspiration of the cortex visualizes the fimbria adjacent to the right hippocampal complex. CA1 and CA2, subfield of hippocampus propria; CC, Corpus callosum; CP, caudate putamen; DG, dentate; F, fimbria; HDB, horizontal limb of the diagonal band of Broca; Hi, hippocampus.

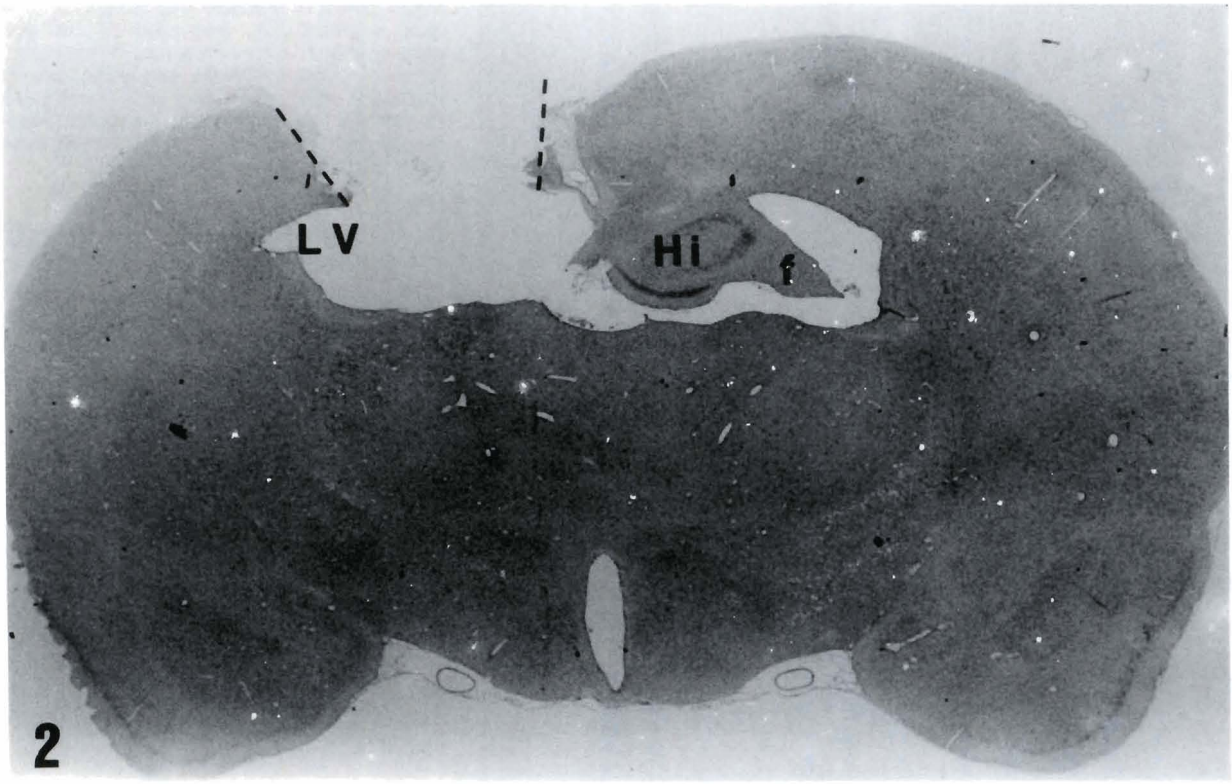

Fig. 2. Coronal section with an aspirated lesion. Note a small part of the dorsal hippocampus is also removed for completing fimbria-fornix transection. Hi, hippocampus; LV, lateral ventricle; f, fimbria.

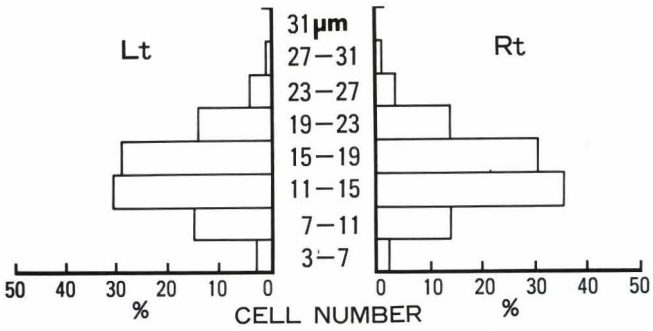

Fig. 3. Frequency histogram of CAT-positive cells in the medial septal area (normal control). Cell size (vertical axis) is indexed by the diameter along the major axis, according to Gage (1986). Cell size and distribution show no difference between both sides.

Results

The lesion was a large cavity with foamy macrophage infiltration. The fimbria-fornix and rostral tip of the hippocampus were removed completely and the damage extended to a small part of the caudate-putamen (Fig. 2). Enlargement of the ipsilateral lateral ventricle was seen in two animals of the FPF1070-treated group for 3 weeks and each one of the animals of the control groups, which were treated for 2 weeks and 3 weeks.

In the normal control rats, which had not been transected, the cell number ratio was 102.36 ± 9.18% between the right and left slides (n = 5). A histogram of CAT-positive cell size showed a peak at the fraction between 11 and 15 μm, and mean diameters were 15.2 ± 4.2 (N = 1359), 15.4 ± 4.3 (N = 1401) μm at the left and right side, respectively (Fig. 3).

Control group

1 week following the transection of fimbria-fornix, the cell loss in the MS and the VDB was evident on the sections stained with CAT (Fig. 4). The cell number was reduced to 62% in the MS of the lesion side. AChE histochemistry in the present study visualized cell bodies in the majority of the cases and fibres in a few cases. Though highly darkly-stained neurons reduced in number, total AChE-positive cell bodies showed no obvious reduction. The number of Nissl-stained cell bodies did not change either, but some of the cells were reduced in size and staining property. The relative decrease in CAT-positive cells became prominent with time. There was a significant loss of neurons in the MS to 44% at 2 weeks following the lesion and then almost to 30% at weeks 3 and 4 (Fig. 5). By 1 week AChE «pile-up» (Gage et al., 1986) was observed as swollen AChE-positive fibres throughout the septal area. However, swollen CAT-

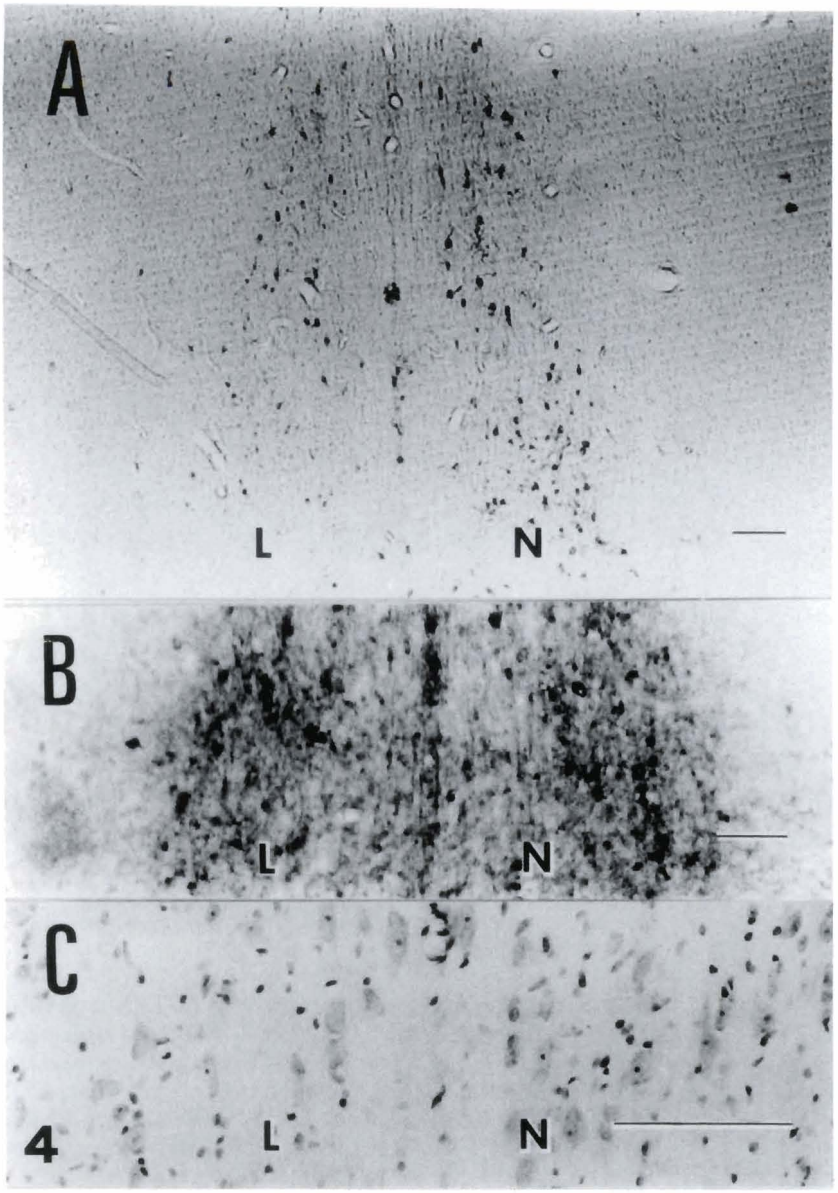

Fig. 4. Effect of fimbria-fornix transection (1 week after operation). A: Reduction of CAT-positive cells is marked in the septal area of the lesion side (L) as contrasted with the non-lesion side (N). B: Reduction of AChE-positive cells is not apparent. C: Nissl's staining shows atrophic neurons, though the cell numbers are not different between both sides. Scales = 100 μm

positive fibres were few. Shrinkage of CAT-positive cells was apparent 1 week after the transection of fimbria-fornix. CAT staining simplified the reliable identification of cholinergic cell bodies, because of its specificity and constant reproducibility. In consequence, relatively small cell bodies less than 12 μm could be countable. The distribution of CAT-positive cell size as indexed by the diameter along the long axis showed a shift of the peak to a decrease in size, compared with the non-lesion side (Fig. 6). The mean cell sizes were 14.0 ± 4.0 μm, 12.5 ± 3.1 μm in the non-lesion and lesion sides respectively 1 week after the lesion. This shrinkage of the remaining neurons did not change during 3 weeks, but it was not apparent at 4 weeks.

The group treated with FPF1070

FPF1070 treatment significantly reduced the loss of CAT-positive neurons in the MS. In control animals fimbrial transection reduced the number of CAT-positive cell bodies to 30% on the contralateral side by 4 weeks. FPF1070 did not prevent the reduction during the first week after transection. However, further reduction was prevented. The numbers of CAT-

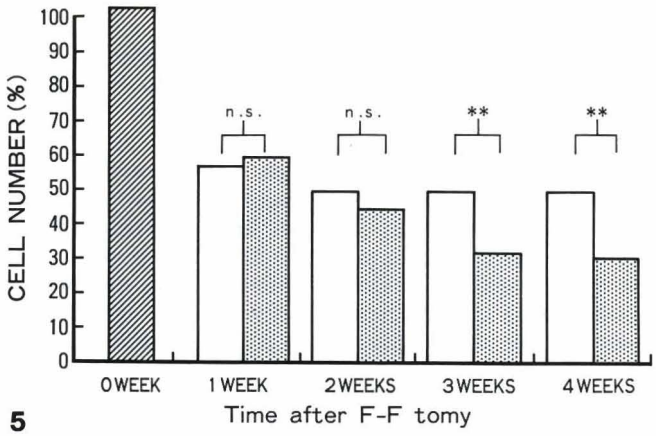

Fig. 5. Bar charts comparing the number of CAT-positive cells expressed as a percentage of the non-lesion side 1 to 4 weeks after transection of fimbria-fornix. Open bars, FPF1070 treatment; dotted bars, saline treatment; n.s., non-significant; **, significant ($P < 0.01$, ANOVA-LSD).

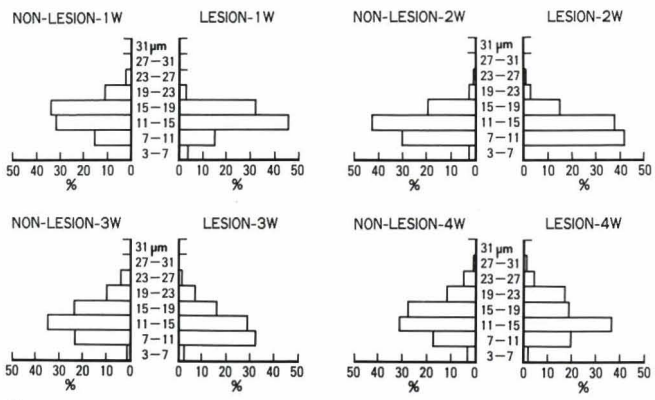

Fig. 6. Frequency histograms of CAT-positive cells after transection of fimbria-fornix in the saline-treated group. Reduction of the cell size reaches maximum at 2-3 weeks. Horizontal axes, cell size (the diameter along the long axis); Vertical axes, cell number (percentages of each side).

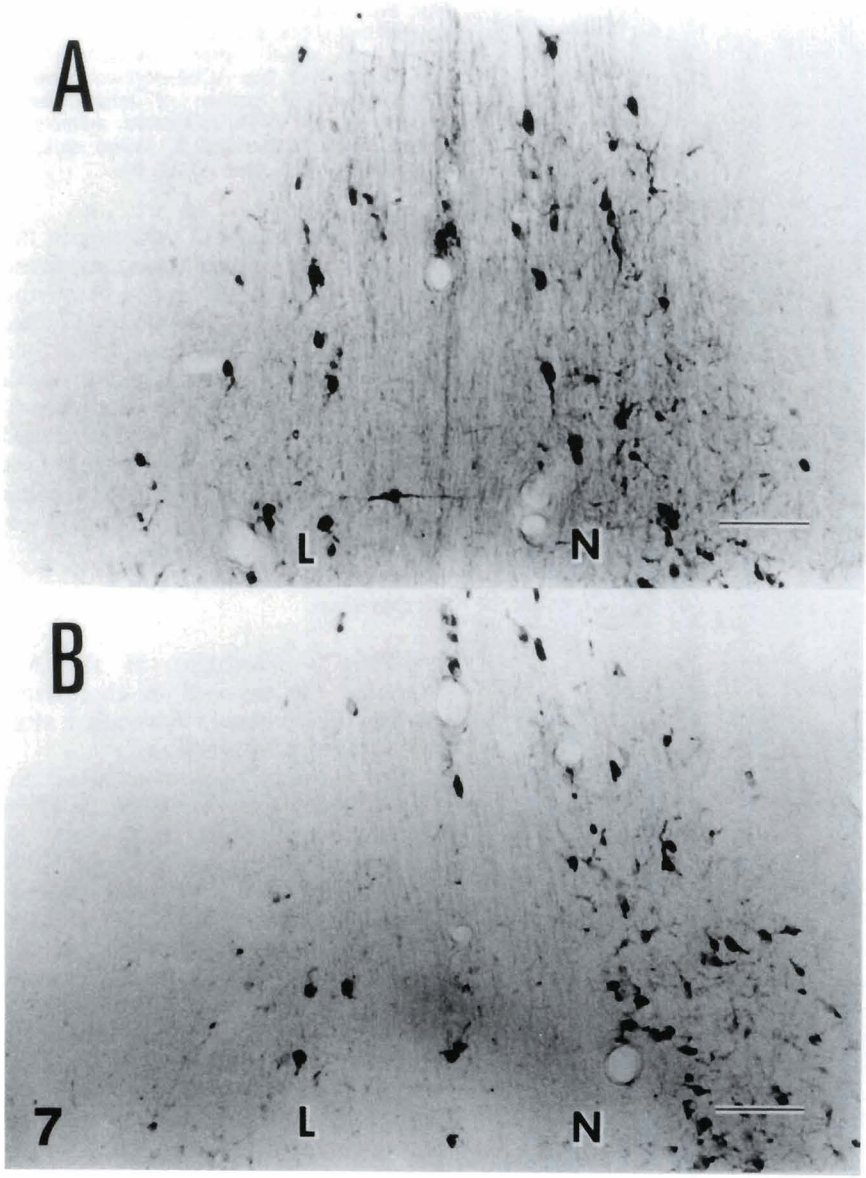

Fig. 7. Effects of FPF1070 on cholinergic neurons in the medial septal area, visualized by CAT immunohistochemistry (3 weeks after transection). Note neurons are preserved in number and size in an FPF1070-treated animals (A) compared with a saline-treated animals (B). L, lesion side; N, non-lesion side. Scales = 100 μm.

positive cells were 49.8%, 49.9% and 49.9% at 2, 3, and 4 weeks respectively (Table 1). Consequently, approximately 20% of CAT-positive cells were saved at 4 weeks by FPF1070. The difference between the two groups was significant ($P > 0.01$). In the Nissl-stained sections, the total number of neurons did not change even 4 weeks after transection, though shrinkage was remarkable. On the contrary, the CAT-positive cell bodies retained their size over the 4 weeks and showed a rather hypertrophic appearance (Fig. 7). Table 2 represents the time courses of cell diameters in the control and FPF1070-treated group. There was no significant difference between the two groups at 1 week on the non-lesion side, though shrinkage was remarkable on the lesion side of the control group. CAT-positive cells in the FPF1070-treated animals preserved their size not only on the lesion side but also on the contralateral side at 4 weeks. The frequency histogram showed a more precise distribution (Fig. 8). After 1 week, the number of small axes (less than 15 μm) increased in the lesion side of both groups. The shrinkage became maximum at the 2nd week in the lesion side of the control animals. On the contrary, in the FPF1070-treated animals, the patterns of distribution after 2 weeks were similar or resembled the pattern of the contralateral side of control group (1 week). The non-lesion side reduced in cell size through 2 weeks and then recovered, though the distribution shifted to a larger size than the control group after 4 weeks.

AChE staining demonstrated swollen fibres in the medial septal area at 1 week, which was the same as in the control group. These large cholinergic axons were never observed after 2 weeks. However, a thick accumulation of AChE became clear in the intermediate portion of lateral septal nucleus on the lesion side at 2 weeks (Fig. 9). There was a significant

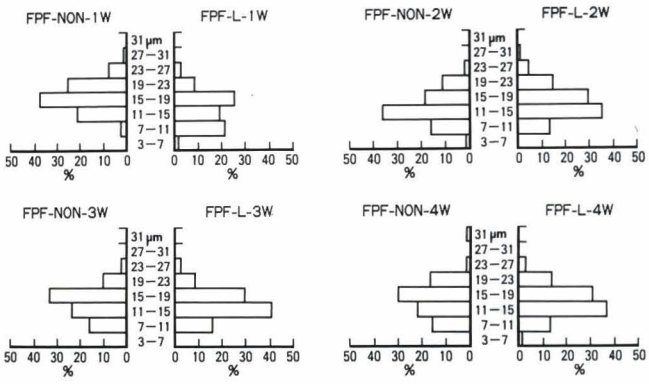

Fig. 8. Frequency histograms of CAT-positive cells after transection of fimbria-fornix in the FPF1070-treated group. Note retaining of cell size through 4 weeks in the lesion side. Horizontal axes, cell size (the diameter along the long axis); Vertical axes, cell number (percentages of each side).

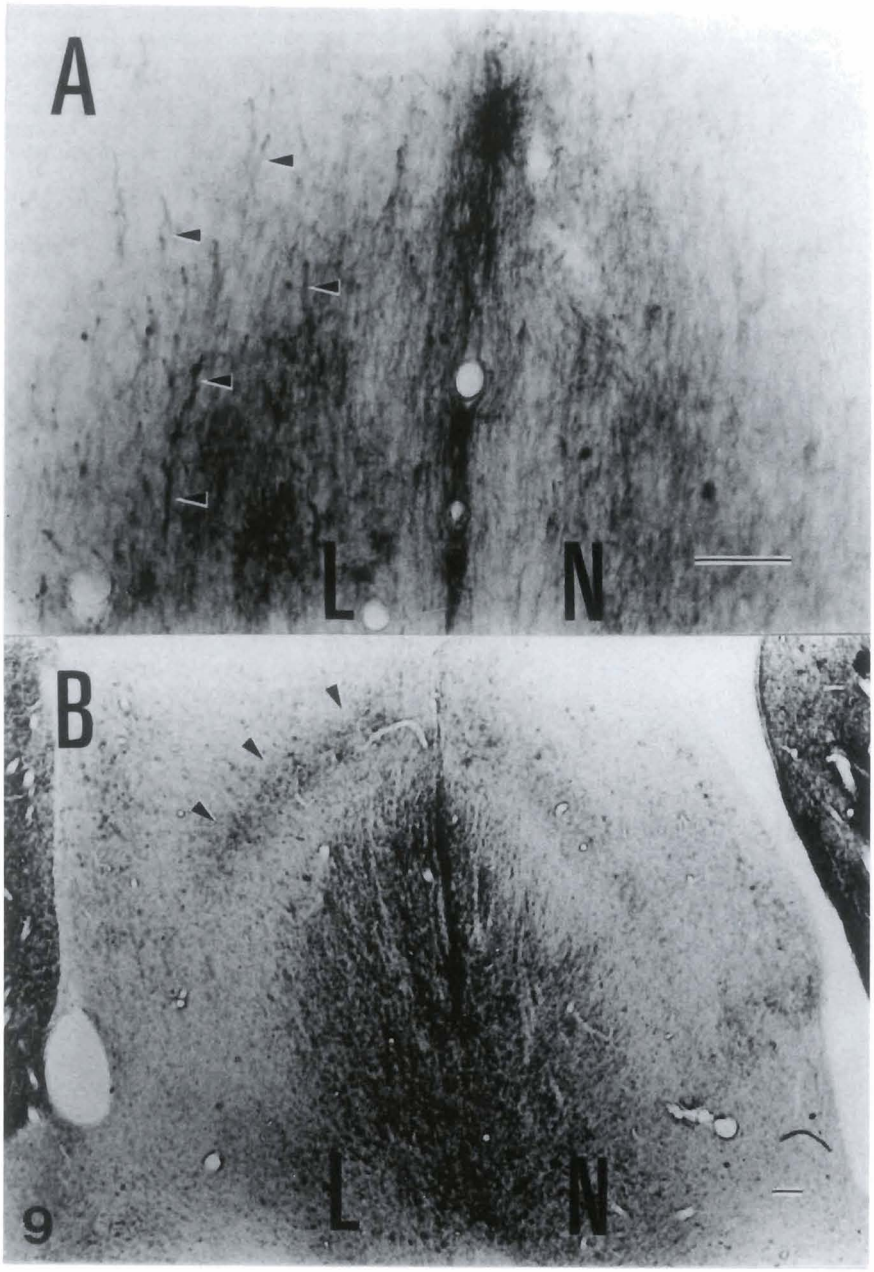

Fig. 9A. Thick AChE-positive fibres in the dorsolateral septal area of an FPF1070-treated animal (1 week after transection). **B.** Accumulation of fine AChE-positive fibres in the intermediate portion of lateral septal nucleus of an FPF1070-treated animals (2 weeks after transection). L, lesion side; N, non-lesion side. Scales = 100 μm.

enhancement of AChE staining in the ipsilateral side of the lesion, compared with the contralateral side. From the viewpoint of size, those staining in the neuropil were different from the swollen fibres in the medial septa area 1 week after the transection. Enhanced staining was not observed in the contralateral side of the lesions of either groups and did not become apparent until 4 weeks on the lesion side of the control group.

Discussion

FPF1070 treatment in the MS promoted the survival of cholinergic neurons which would have died after fimbria fornix transection.

The present study confirmed the results of Gage et al. (1986) or Hefti (1986) that a substantial loss of neurons occurs in the MS and VDB after transection of the septo-hippocampal pathway. About 40% of CAT-positive cells were lost in the medial septal area at 1 week. The loss of CAT-positive cells reached a maximum around 3 weeks following transection of the fimbria-fornix. The number of the CAT-positive cells was reduced to 30% in the contralateral side, which confirms the results of Hefti's study. However, they demonstrated a maximal cell loss within 1 week post-transection. We used an immunohistochemical method of CAT instead of the histochemical presentation of AChE. Regional measurements of AChE and CAT were not always in accordance with the distribution of these two enzymes. Levey et al. (1983) reported the co-localization of AChE and CAT. Although all neurons which contained CAT also had some AChE, many AChE-containing neurons did not have any demonstrable CAT. However, in the neostriatum and basal forebrain, all neurons that stained intensely for AChE also contained CAT. Gage and Hefti defined both cholinergic neurons as dark

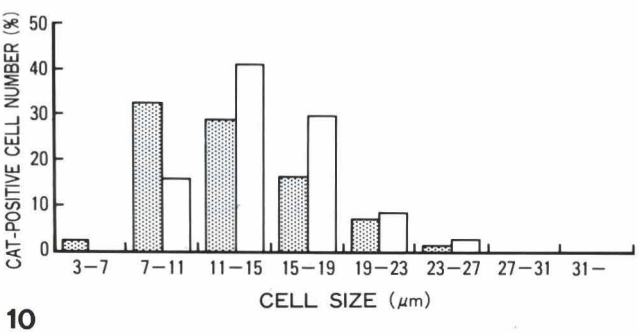

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Fig. 10. Frequency histogram of CAT-positive cells of the lesion side (3 weeks after transection of fimbria-fornix). Open bars, FPF1070-treated animals; dotted bars, saline-treated animals.

Table 1. EFECT OF FPF1070 TREATMENT ON THE NUMBER OF CHOLINERGIC NEURONS IN THE MEDIAL SEPTAL NUCLEI AFTER FIMBRIA-FORNIX TRANSECTIONS

Weeks after transection	Saline (5 mℓ/Kg) mean ± SD%[a]	FPF1070 (5 ml /Kg) mean ± SD%[a]
1 Week	59.8 ± 6.1 (5)	57.3 ± 4.4 (5)[b]
2 Weeks	44.7 ± 5.5 (5)	49.8 ± 6.7 (7)
3 Weeks	32.0 ± 4.2 (5)	49.9 ± 9.8 (5)*
4 Weeks	30.5 ± 3.6 (5)	49.9 ± 6.3 (7)*
Normal control	102.9 ± 9.2 (5)[c]	

a : surviving neurons as % of non-lesion side.
b : number in parentheses indicates number of animals analyzed.
c : number expressed as right/left ratio.
* : P<0.01 compared to saline treatment. (ANOVA-LSD)

Table 2. CELL SIZES OF CAT-POSITIVE CELL BODIES AS INDICATED BY DIAMETER ALONG THE MAJOR AXIS

Weeks after transection	Sides	Saline (5 mℓ/Kg) mean ± SD (μm)	FPF1070 (5 ML/Kg) mean ± SD (μm)
1 Weeks	non-L*	14.8 ± 4.6	15.9 ± 4.1
	lesion	12.5 ± 3.3	15.0 ± 4.1
2 Weeks	non-L	13.8 ± 3.9	15.8 ± 4.3
	lesion	13.0 ± 3.3	16.5 ± 4.3
3 Weeks	non-L	14.5 ± 4.1	16.0 ± 4.1
	lesion	13.6 ± 3.1	15.5 ± 4.0
4 Weeks	non-L	15.5 ± 4.0	16.5 ± 4.7
	lesion	14.3 ± 3.8	16.2 ± 4.2

Normal control : lt. = 15.2 ± 4.2 (N=1359), rt. = 15.4 ± 4.3 (N=1401)
non-L* : non-lesion side, lt.

brown bodies with a minimum diameter of 12 μm along the major axis. The present study revealed a much larger proportion of AChE-positive and CAT-negative cells in the MS and in the VDB. In addition, differentiation between intense and lighter stain is subjective. Moreover, this region displayed a heterogeneous population of intensely stained cells. CAT immunohistochemistry is therefore more sensitive and specific than AChE histochemistry for identifying cholinergic neurons. We included the smaller CAT-positive neurons in our calculations because of sensitive staining. Large CAT-positive neurons may have deteriorated soon after transection. The distribution of the cell size shifted to a smaller diameter during the first 3 weeks then returned to normal after 4 weeks. Therefore, it is conceivable that shrinkage of large CAT-positive cells precedes the death of the smaller ones. This shrinkage is probably equivalent to the death of most neurons, though other types of cholinergic neurons may survive afterwards, and which may be partially axotomized (Gage et al., 1986).

FPF1070 treatment

FPF1070 is an aqueous protein-free solution, prepared from a standardized hydrolysate of brain protein by EBEWE, Austria. It consists of 15% low molecular weight peptides up to a molecular weight of 10,000 and 85% free amino acids. Previous studies in our laboratory have shown that FPF1070 promoted neuronal survival and neurite outgrowth at low concentrations (10 μl/ml - 20 μl/ml) in dorsal root ganglia of embryos 8 to 10 days old. These neurotrophic factor-like effects were distinguished from those of NGF by delayed onset and morphology of neurites. Moreover, antibody against NGF did not inhibit the neurotrophic activity of FPF1070. An amino acid solution of the same components as FPF1070 had no effect on neurites (Iwamoto et al., 1989). In this study, we have demonstrated the neurotrophic factor-like effect on an *in vivo* system, i.e., fimbria fornix transection. FPF1070 promoted the survival of up to 20% of cholinergic neurons in the MS. This reduction of cell loss is apparently small compared with that of NGF treatment, which rescued at least 30% of AChE-positive neurons by 2 weeks after transection of fimbria fornix (Hefti, 1986; Williams et al., 1986). We injected FPF1070 intraperitoneally. On the other hand, NGF was infused into the lateral ventricle continuously or twice weekly. The efficacy of NGF treatment via the systemic route is not mentioned. The operative procedure of fimbria-fornix transection causes local disruption of the blood-brain barrier at least for several weeks. We have no evidence that an active component of FPF1070 passes through the blood-brain barrier, except that enlargement of cells occurred not only in the lesion but also in the contralateral side. However, the FPF1070 influenced brain metabolism; a relative increase the oxygen consumption and an improvement in glucose metabolism (Windisch and Piswanger, 1985). Learning behaviour was also significantly improved, asessed by the passive avoidance procedure in the normal adult rat (Windisch et al., 1990). It is unclear whether these effects of FPF1070 are mediated by cholinergic neurons. A behavioural study in rats with a transected fimbria-fornix may provide an answer.

We have demonstrated preservation and an increase of the cell size of both the lesion side and contralateral side in the rats with FPF1070 treatment (Fig. 10). This was supported by a gradual increase in cerebral protein concentration after normal adult rats had been injected with FPF1070 (Piswanger and Windisch, 1990). In aged rats, which were behaviourally impaired, the size of the AChE-positive cell bodies was significantly reduced in the MS, VDB, striatum and nucleus basalis magnocellularis. Continous infusion of NGF into the lateral ventricle increased the cell size of the aged impaired rats on the sides of the infusion (Fischer et al., 1987).

The characterization of the neurotrophic factor has not been completed for FPF1070. Therefore, the possibility cannot be eliminated that it might be a fragment of the NGF family, especially of brain-

derived neurotrophic factor (BDNF). BDNF supports the survival of embryonic sensory neurons *in vitro* (Johnson et al., 1986). In addition, BDNF mRNA is distributed in the hippocampus at the highest level, especially in the CA3 pyramidal cells and dentate granule cells, where NGF mRNA is expressed at the highest level (Korsching et al., 1985; Ernfors et al., 1990). On the contrary, a fragment of NGF, known to have activity, proved to be inactive (Romani et al., 1987). Fragments of EGF and bFGF appear to retain activity (Komiria et al., 1989; Klagsburn et al., 1987), though active fragments do not pass the blood-brain barrier. It is of value to note that a hydrolyzed protein has neurotrophic property and may also go through the blood-brain barrier or act like a neurotrophic factor via several means. Some molecules may induce mRNA of NGF (Wion et al., 1987) or increase the number of receptors for NGF (Haskell et al., 1987). FPF1070 failed to save the cholinergic neurons, which lost CAT immunoreactivity in the first week after transection. So it is conceivable that FPF1070 promotes neurotrophic factor during the first postoperative week. We did not investigate whether treatment for 4 weeks resulted in permanent survival of the cholinergic neurons. Single injections of FPF1070 produced prolonged, increased activity of AChE in the caudate-putamens for several days (Iwamoto et al., 1989). However, intraventricular injections of NGF for 4 weeks failed to rescue cholinergic neurons from degeneration after withdrawing NGF (Montero and Hefti, 1988). In cases of chronic injection, systemic administration of drugs has a clinical advantage. Impaired cholinergic neurons become independent from the exogenous neurotrophic source, when their transected axons reestablish contact with the target cells, which provide the specific trophic factor. FPF1070 treatment prevented the degeneration of cholinergic neurons. Age-related functional impairments, including Alzheimer's disease, is associated with a deterioration of cholinergic neurons (Bartus et al., 1982; Coyle et al., 1983). Therefore, a therapeutic injection of FPF1070 might promote the survival of affected cholinergic neurons and then prevent behavioural deterioration and memory loss, cardinal symptoms of Alzheimer's disease.

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