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Reformation of the Severed Septohippocampal Cholinergic Pathway in the Adult Rat by Transplanted Septal Neurons

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Summary. Transplants containing developing cholinergic neurons were obtained from the septum-diagonal band area of rat fetuses and were implanted into a lesion of the septohippocampal cholinergic pathway or into a cavity of the occipital cortex in adult recipient rats. The growth of new cholinergic fibres from the implant into the hippocampal formation was followed with choline acetyltransferase (ChAT) determinations and acetylcholine esterase (AChE) histochemistry. A fimbrial lesion alone, transecting the septohippocampal pathway, caused an almost complete cholinergic denervation of the hippocampal formation that persisted throughout the five month experimental period. A septal transplant implanted into the cavity of the fimbrial lesion restored a new AChE-positive innervation pattern in the hippocampus and the dentate gyrus that closely mimicked the original innervation removed by the lesion. In parallel, there was a progressive recovery in the ChAT levels, starting in the septal end, and progressing in a temporal direction. A new cholinergic fibre supply could be established in the hippocampal formation also along an abnormal route, i.e. from the transplants implanted into a cavity in the occipital cortex (involving also the dorsal part of the entorhinal cortex). Provided the hippocampus previously had been denervated of its normal cholinergic innervation, a partly normal AChE-positive terminal pattern was thus re-established also from this abnormal position. If, on the other hand, the cholinergic afferents were left intact, the ingrowing fibres were restricted mainly to the outer portion of the dentate molecular layer, i.e. the terminal zone of the lesioned entorhinal perforant path fibres. This suggests that the growth of the sprouting AChE-positive fibres into the normal cholinergic terminal fields was blocked by the presence of an intact cholinergic innervation. It is concluded that regrowing cholinergic axons can be guided over large distances within the hippocampal formation, and that their patterning within the terminal fields is very precisely regulated by mechanisms released by deafferentation.

Key words: Neuronal regeneration – Cholinergic neurons – Hippocampus – Neural transplantation.

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Introduction

The possibility of re-establishing degenerated neural connections in the mammalian central nervous system (CNS) is traditionally regarded with pessimism. A number of reasons have been put forth in order to explain why true regeneration would not occur in mature central nervous tissue of mammals. Thus, even if the axotomized central neurons were able to sustain sprouting fibres, a series of obstacles have generally been assumed to operate in preventing the sprouting fibres from growing back to their original terminal sites. Thus, for example, the glial scar has been said to be impenetrable for growing axons, and the absence of adequate guiding mechanisms (or the dominance of inappropriate attractive stimuli) would make the reconnection with the denervated target cells extremely unlikely.

These views have, however, recently been challenged by observations that central monoaminergic neurons do sprout and are partly able to grow back to their original terminal areas after chemically induced selective axotomy (Björklund et al., 1973, 1975; Nobin et al., 1973; Nygren et al., 1974; Nygren and Olson, 1977). Moreover, grafts of such neurons implanted into the brain or the spinal cord have been shown to sprout new axons that are able to grow over long distances in the host CNS (Stenevi et al., 1976; Björklund et al., 1976; Björklund and Stenevi, 1977; Nygren et al., 1977). In the present study evidence is provided in the adult rat that embryonic septal cholinergic neurons, transplanted to the transected septohippocampal cholinergic pathway or into a cavity of the occipital cortex, can grow for considerable distances to regenerate a new cholinergic innervation pattern in the hippocampal formation, closely mimicking the original innervation removed by the lesion.

Materials and Methods

The transplants comprised the septum-diagonal band area, taken from rat embryos with a crown-rump length of 15–20 mm (corresponding to a gestational age of about 16–17 days). The dissected piece was in most cases (44 rats) placed at the bottom of a cavity transecting the hippocampal fimbria in young adult female Sprague-Dawley rats (180–200 g body weight at the time of surgery) as described previously (Stenevi et al., 1976). The cavity, approximately 2×2 mm wide, was made through the parietal cortex, the corpus callosum and the hippocampal fimbria, as shown in Fig. 1A. Care was taken to achieve a complete transection of the fimbria, the dorsal fornix, and the ventral hippocampal commissure. The transplant was placed onto the vessel-rich pia overlying the anterior thalamus. From here the transplant is efficiently revascularized, thus ensuring a good survival of the graft (see Stenevi et al., 1976). In a second series of eight rats the transplant was placed in a cavity in the occipital cortex, onto the pia overlying the superior colliculus (see Stenevi et al., 1976, and Fig. 1B). In five of these, the fimbria were lesioned (as above) at the time of transplantation, in the remaining three rats the fimbria were lesioned 5 days before killing. All these rats were killed 12 weeks after transplantation.

The transplanted animals were killed at 2 (10 rats), 4 (12 rats), 6 (4 rats), 8–10 (12 rats), 12 (8 rats) and 16–20 weeks (6 rats) after transplantation. In the control rats a fimbrial lesion identical to that of the transplanted animals was made but no transplant was introduced. These rats were killed 1 week (6 rats), 3 weeks (4 rats) and 3–5 months (10 rats) after operation.

Acetylcholine Esterase (AChE) Staining. Rats were perfused with 150 ml of an ice cold 4% paraformaldehyde solution (pH 7.4). The brains were postfixed for 2 h in the same solution, and then left overnight in 30% sucrose (Butcher and Bilezikjian, 1975). The transplant plus the hippocampal complex was serially sectioned, in the coronal plane, in a cryostat at 25 µm. Every second or third section was

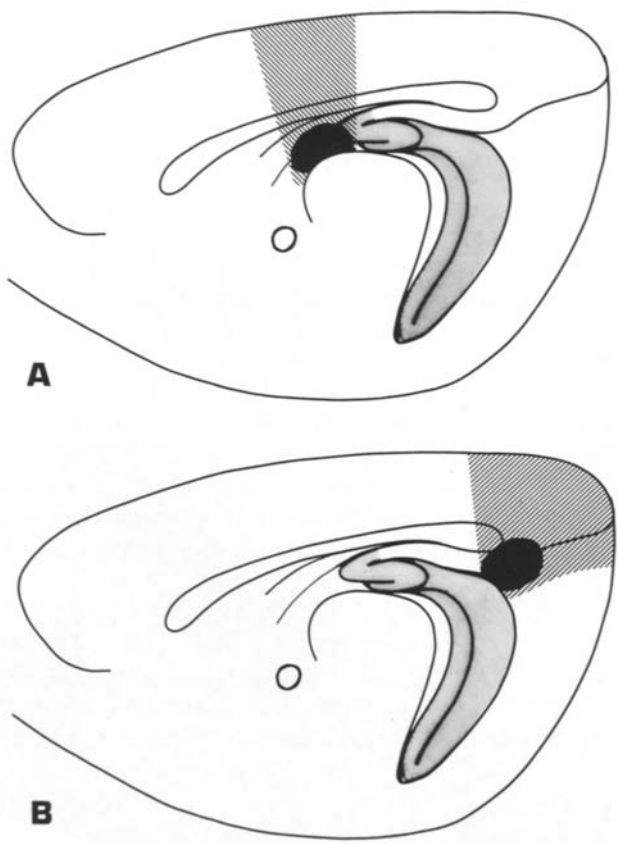


Fig. 1. Extent of lesion (cross hatching) and position of transplant (black) in the hippocampal fimbria (**A**) and the dorsal entorhinal cortex (**B**). The hemisphere is viewed from its medial side after the diencephalon-brain stem has been removed. The stippled area represents the hippocampal formation

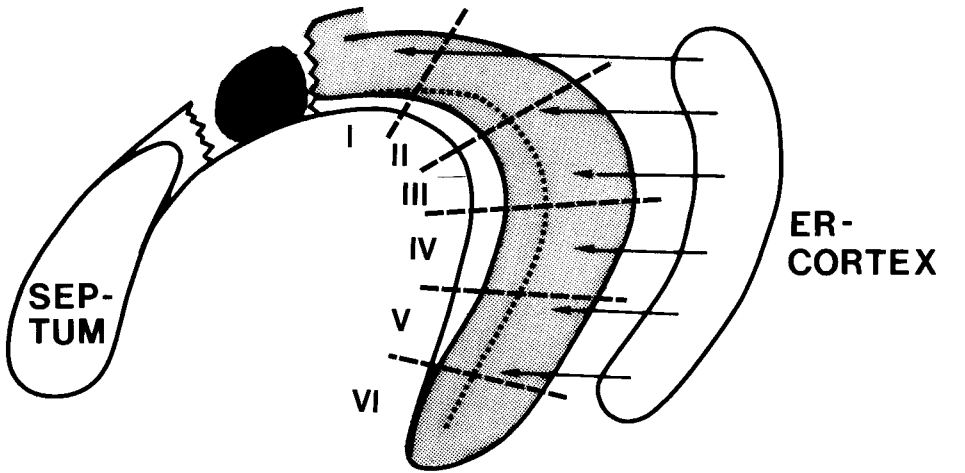
stained for AChE according to the Koelle thiocholine method, using Mipafox (4×10^{-6} M) as inhibitor of non-specific esterases (Holmstedt, 1957). Incubation time was 6–8 h. The sections were very lightly counterstained with haematoxylin and eosin, or with eosin alone

Choline Acetyltransferase (ChAT) was assayed with the micromethod of Fonnum (1969, 1975). The hippocampus of the operated and the control sides was dissected free and divided into six equal portions, as illustrated in Figure 2A. The transplant, dissected free from surrounding tissue under a dissection microscope, was assayed separately. The protein content was measured in an aliquot of the homogenates using the method of Lowry et al. (1951).

Results

The overall survival rate of the transplants (as judged from the AChE stained sections or by visual inspection at the time of dissection) was about 60–70%. At longer survival times, six weeks or more, the transplants that failed to survive (as well as the covering gel-foam pad) had been resorbed; such specimens were discarded at dissection.

Fig. 2.
A Position of the knife-cuts dividing the hippocampal complex in the six slices used for the ChAT determinations. The black field denotes the septal transplant.
B ChAT levels in slices I–VI in animals bearing septal-diagonal band transplants (solid lines and filled circles) and in animals subjected to the fimbrial lesion alone (broken lines and open circles). Each determination is represented by a filled or open circle, the mean values by squares. Values expressed as percent of the non-operated control side. Differences between animals with and without transplants: *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$. Student's *t*-test



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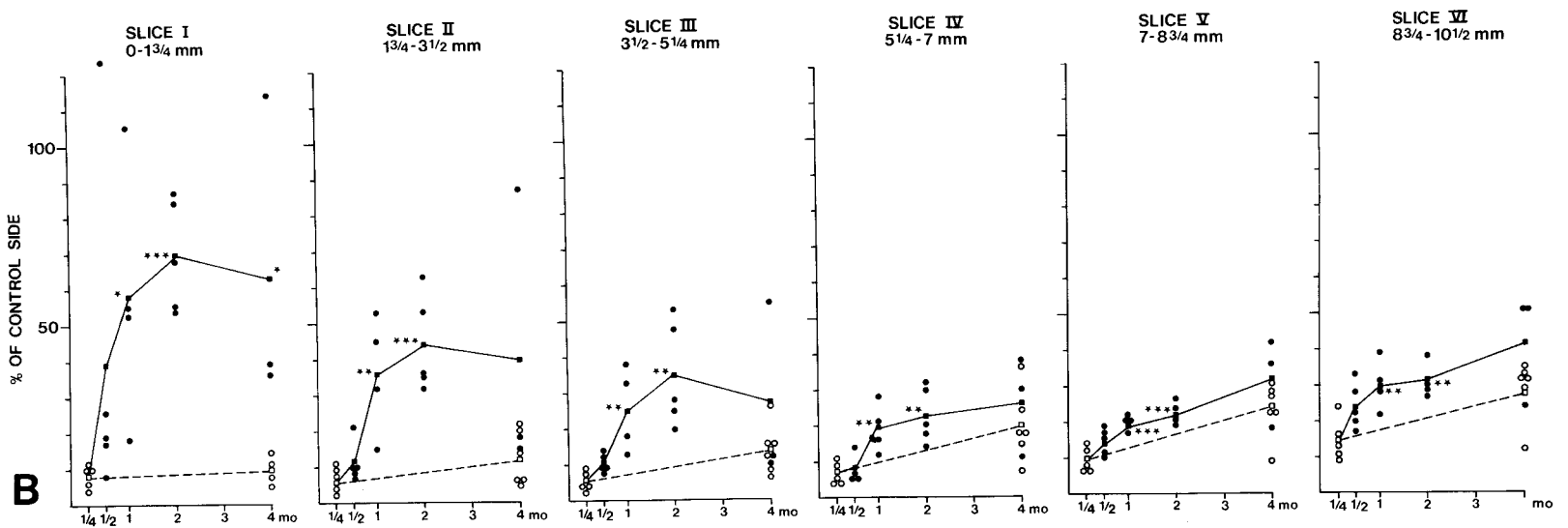

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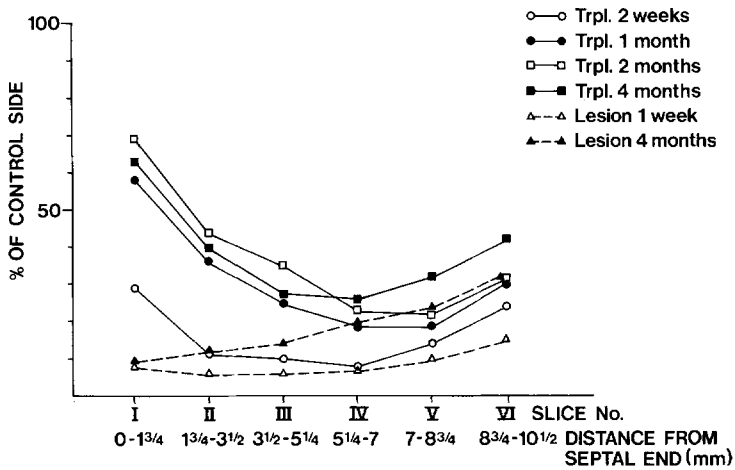

Fig. 3. Septo-temporal gradient in the ChAT levels in animals bearing septal-diagonal band transplants (solid lines) and in animals subjected to the fimbrial lesion alone (broken lines). Symbols give means of 3–6 animals after different postoperative times (see Fig. 2)

1. Transplantations into the Hippocampal Fimbria

As determined from the *ChAT assays,* the fimbrial lesion caused, by one week, a 90–95% reduction in the enzyme level in all portions of the ipsilateral hippocampus, except in the most temporal portion where the reduction was somewhat less, about 85% (Figs. 2 and 3). This indicates that the lesion used caused a complete transection of the cholinergic septohippocampal pathway. Within the experimental period, which was four months, no recovery of ChAT was noted in the septal portion of the hippocampus, whereas tendency to a partial recovery in the temporal part was noted (Figs. 2 and 3).

In the animals bearing a septal implant there was a progressive recovery of ChAT in the hippocampus, starting in the septal end closest to the transplant, and progressing in a temporal direction (Figs. 2 and 3). During the early stages after transplantation the size of the transplants (as assessed visually at dissection) was markedly reduced. Subsequently they recovered their size, and by four months the grafts had generally grown to become larger than at the time of transplantation. These size changes are also reflected in the protein content of the transplanted tissue, as shown in Table 1. By two to four weeks the protein content was thus reduced by about 40–50%, by two months it was about 100% and by four months 160% of the initial content. The ChAT activity of the transplant followed a different pattern (Table 1): In the embryonic septal-diagonal band area taken for transplantation the ChAT level was very low, only about 5% of that measured in the corresponding area of the adult rat. After transplantation the enzyme level rose approximately 20-fold within two months (Table 1), indicating a continued maturation of the tissue and growth of its contained neural elements in its new location in the brain.

In the hippocampus, there was a clear septo-temporal gradient in ChAT at all postoperative times, the highest activities being found closest to the transplant

Table 1. Protein content and ChAT activity of septal-diagonal band transplants and in the septal-diagonal band area of adult rats. Means ± S.E.M. of 4–6 determinations

	Protein content μg	Total ChAT activity cpm × 10^3	Specific ChAT activity cpm/μg protein
Before transplantation	476± 29	57.8± 6.8	116± 10
2 weeks	290± 55**	86.5± 14.9	361±119*
1 month	245± 43***	245.7± 83.4*	1049±223**
2 months	501± 36	1187.8±205.5***	2369±371***
4 months	766±242	1550.4±594.3**	1972±182***
Adult rat	3313±141	7256.3±156.5	2214±128

Differences from pre-transplantation values: * $0.05>p>0.01$; ** $0.01>p>0.001$; *** $p<0.001$. Student's *t*-test

(Fig. 3). In the part of the hippocampus bordering on the transplant (0–1.75 mm from the septal end; slice I in Fig. 2A) ChAT was increased already by two weeks, and by two to four months the ChAT level had recovered to about 60–70% of control. In individual cases a ChAT level close to or even above normal was recorded (Fig. 2B). In the subsequent two portions (slices II and III; about 1.75–5.25 mm from the transplant) the enzyme activity remained low at two weeks, but started to recover at one month after transplantation. By two to four months the ChAT level had, on the average, recovered to 30–45%. In some cases, enzyme activities more than 50% of controls were found in the more caudally located segments. In the temporal half of the hippocampus (more than 5.25 mm from the transplant) the ChAT recovery was clearly less, but still statistically significant.

The *AChE histochemistry* revealed an almost total disappearance of the AChE-positive staining patterns in the animals subjected to the fimbrial lesion alone. As reported previously (Storm-Mathisen, 1974; Mellgren and Srebro, 1973), part of the staining in the stratum lacunosum-moleculare of CA1 remains after complete septal deafferentation. This staining, which has a different microscopical appearance (Fig. 7), remained the same at all post-operative times. A sparse pattern of AChE-positive fibres remained in the most temporal portion of the hippocampus and the dentate gyrus, consistent with the residual ChAT activity found in this portion. By three to four months after lesion this AChE-positive fibre pattern in the temporal end appeared to have increased, whereas all other portions of the hippocampus remained denervated (Fig. 4B).

In the short term transplants, histochemistry revealed many AChE-positive cell bodies. With time, a heavily staining neuropil developed, and by four to eight weeks this neuropil filled the entire transplant, partly obscuring the AChE-positive perikarya (Fig. 8). The microscopical picture indicated that the AChE-positive fibres grew from the transplant into the septal end of the dentate gyrus and hippocampus. Interestingly, few growing fibres were found in the fimbria and the dorsal fornix (i.e., the normal route for the caudally running septohippocampal fibres). Rather, the growing AChE-positive fibres from the transplant appeared to reach the more temporal portions of the hippocampus within the hippocampal grey matter. The first few AChE-positive fibres appeared by two weeks in the part of the dentate gyrus bordering on the transplant. During the subsequent weeks (four to six

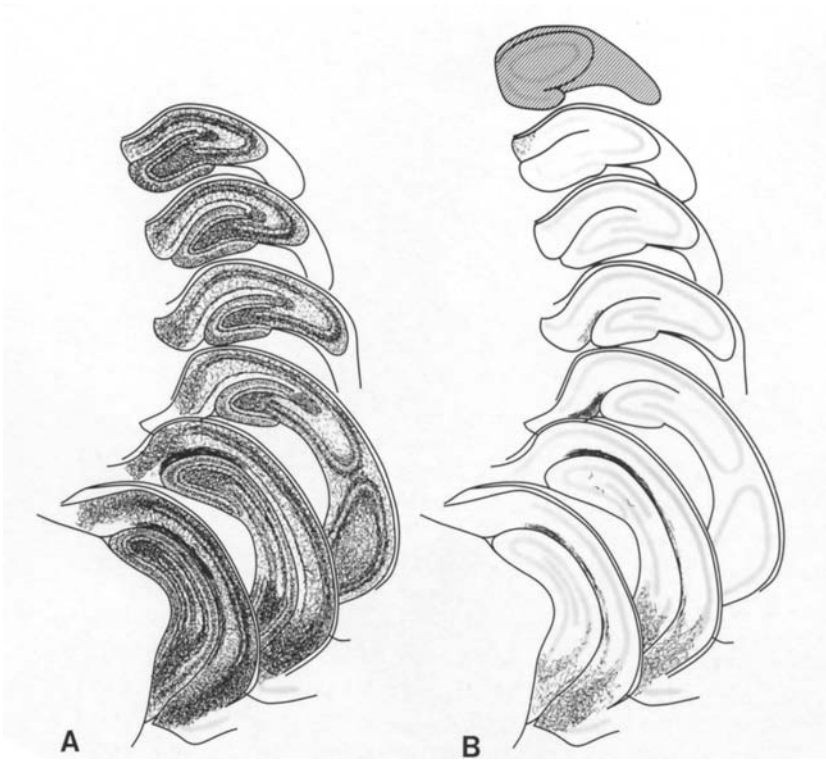

Fig. 4A and B. Distribution of AChE-positive fibres in a normal rat (**A**) and in a rat subjected to the fimbrial lesion 3 months before sacrifice (**B**). The drawings in this figure and in Figure 5 give a semidiagrammatic representation of authentic specimens in 6 frontal planes, spaced at approximately 0.5 mm distance. The lesion in **B** is represented by cross hatching

weeks survival) there was a rapid development of a new AChE-positive fibre pattern in the septal half of the hippocampal complex, both in the dentate gyrus and the hippocampal CA3 and CA1 fields. As in the ChAT measurements, there was a marked septo-temporal gradient in the density of the newly-appearing AChE-positive fibres. In the most successful cases, such as the one shown in Figs. 5A and 6C, the new fibre pattern had an almost normal staining density in the part closest to the transplant. By four months, the regrowing fibres had reached further along the septo-temporal axis, into the temporal half of the hippocampus (Fig. 5B). Here, the fibres coming from the transplant mingled with those remaining after the fimbrial lesion (see Figs. 4B and 5B). In the most extensively reinnervated specimens, the advancing AChE-positive fibres were estimated to have covered approximately $^2/_3$–$^3/_4$ of the hippocampus, corresponding to a distance of about 6–8 mm from the transplant. The density of the AChE-positive fibre pattern remained, however, much below normal in the temporal parts of the hippocampal complex (see Figs. 4A and 5A).

The patterning of the newly-formed AChE-positive fibres was remarkably similar to that of the normal AChE-containing fibres, removed by the transplantation lesion. In the normal rat (Figs. 4A and 6A) the AChE-positive

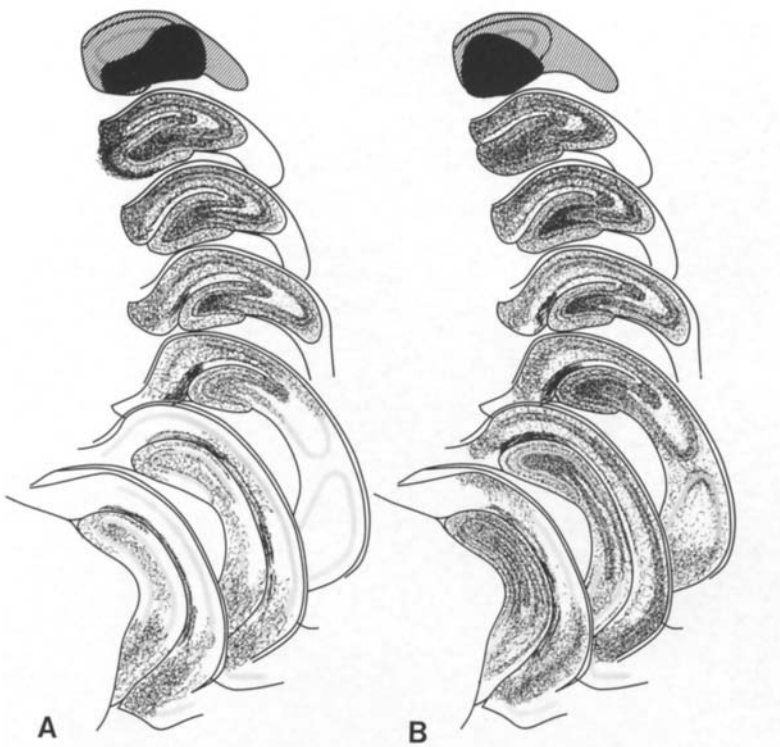

Fig. 5A and B. Extension and distribution of ingrowing AChE-positive fibres in animals bearing septal-diagonal band transplants. **A** 6 weeks survival; **B** 4 months survival. The lesion is represented by cross hatching and the transplant by the black field

fibres, as well as AChE and ChAT activities, exhibit a distinct laminar pattern in the dentate gyrus and hippocampus (Storm-Mathisen and Blackstad, 1964; Storm-Mathisen, 1970; Fonnum, 1970), and this laminar pattern was consistently seen also in the reinnervated transplanted specimens (Figs. 5–7). Thus, in the dentate gyrus the densest pattern occurred, as in the normal animal, in the hilus and in a narrow supragranular zone of the molecular layer (Fig. 7B). In the CA1 and CA3 areas the AChE-positive fibres were aggregated in the supra- and infrapyramidal zones and in the stratum lacunosum-moleculare of CA3, also resembling the normal distribution. Also the thin stripe of AChE-positive fibres normally seen along the stratum lacunosum of CA1 was very precisely reproduced in the transplanted specimens (Fig. 6A and C).

2. Transplantations into the Occipital Cortex

In order to determine whether the AChE-positive fibres from the septal implants could successfully invade the hippocampus also from an anomalous direction, along an abnormal route, a preliminary experiment was made in which eight rats received a septal transplant implanted into the occipital cortex, as shown in Figure 1B.

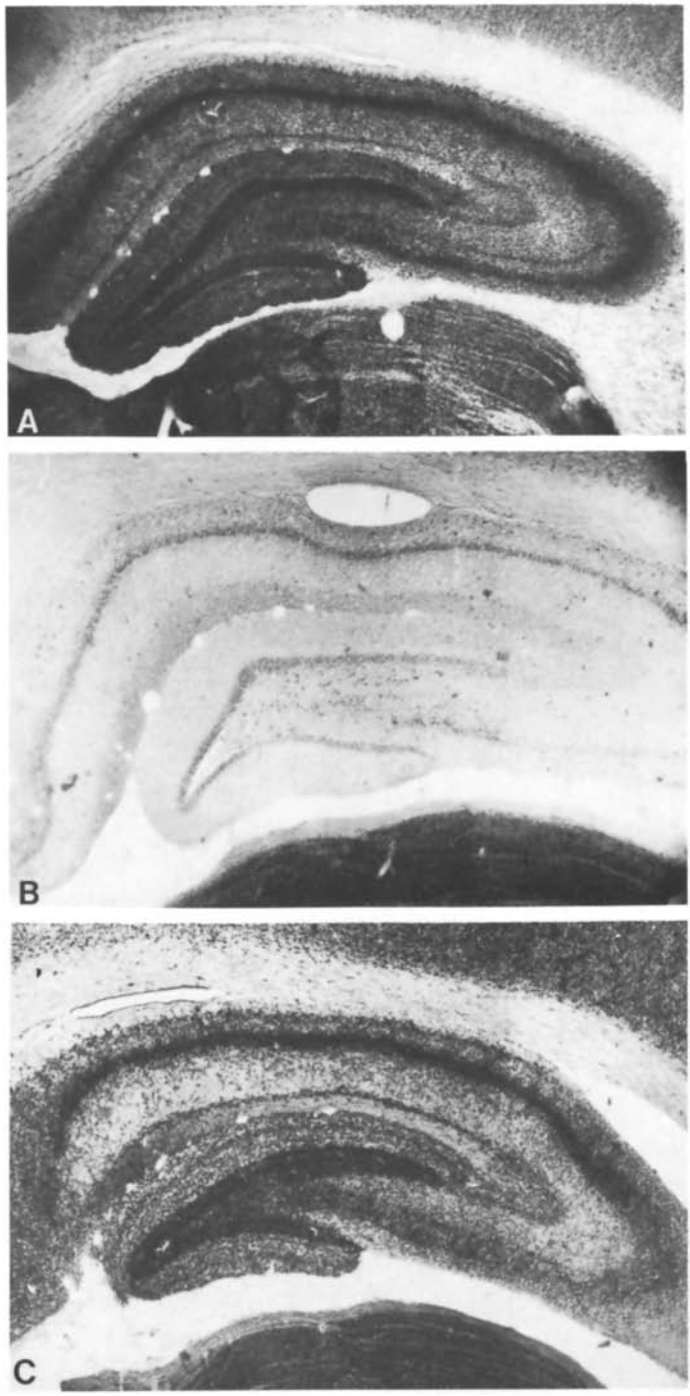

Fig. 6A–C. Photomicrographs of coronal sections through the dorsal hippocampus, stained for AChE. **A** normal rat; **B** fimbrial lesion alone, 5 months survival; **C** animal with septal-diagonal band implant, 6 weeks survival

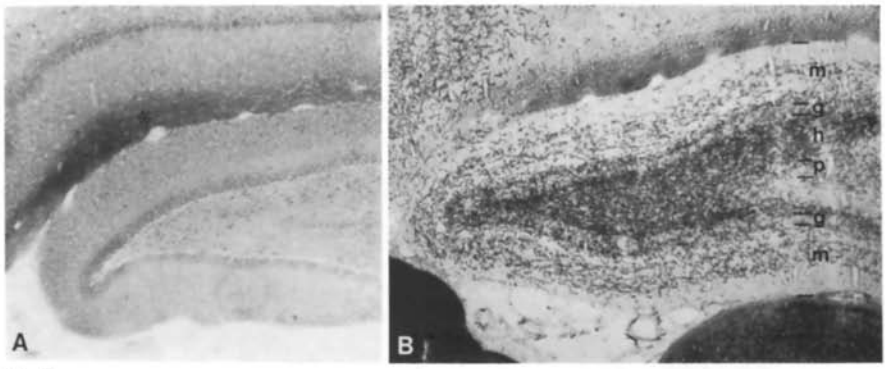

Fig. 7

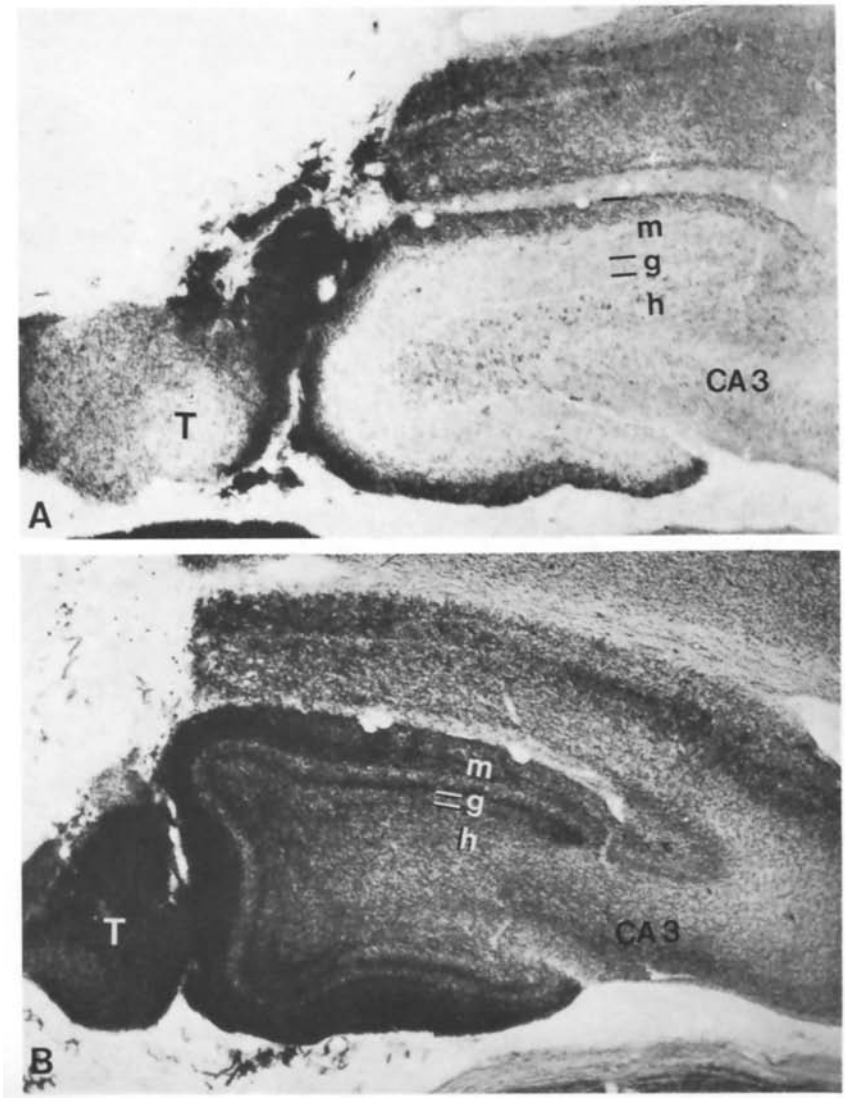

Fig. 8

The transplantation cavity involved the dorsal part of the entorhinal cortex and the angular bundle, thus removing the part of the temporo-ammonic perforant path fibres innervating the dorsal hippocampus (Hjort-Simonsen and Jeune, 1972). In five of the animals an additional lesion of the fimbria was made at the time of transplantation in order to remove the normal cholinergic innervation of the hippocampus. In the remaining three, the fimbrial lesion was made five days before killing. This latter lesion served the purpose of eliminating the normal AChE-positive fibres prior to microscopy. All rats were killed 12 weeks after transplantation.

In both experimental situations the AChE-positive fibres had invaded the dorsal hippocampus in great numbers, but their patterning was markedly different (Fig. 8). In the rats where the fimbria had been left intact up to the last five days the ingrowing fibres were restricted to the outer portion of the dentate molecular layer, where they formed a very densely stained neuropil, and to part of stratum radiatum and oriens of CA1 (Fig. 8A). In the dentate this fibre distribution conformed to the terminal field of the lesioned perforant path fibres (Hjort-Simonsen and Jeune, 1972; Steward, 1976). In rats having the fimbria lesioned at the time of transplantation, the AChE-positive fibres had extended also into the supragranular zone and the hilus of the dentate gyrus, into the infra- and suprapyramidal zones and the molecular layer of CA3 (Fig. 8B), i.e., into the terminal fields of the normal septal cholinergic afferents.

Discussion

The combined histochemical and biochemical observations provide strong evidence for a cholinergic reinnervation of the denervated hippocampal region from the embryonic septal implant. Like the noradrenergic, dopaminergic and indolaminergic neurons previously investigated (Stenevi et al., 1976; Björklund et al., 1976), the developing AChE-positive neurons of the septal-diagonal band area survive well a transplantation to the pia of the choroidal fissure. Following an initial reduction in size, probably due to partial necrosis, the implant grew to become on the average about twice as large as at the time of transplantation. The

Fig. 7A and B. AChE-positive fibres in the dentate gyrus and the adjacent CA1 area at a level corresponding to slice IV in Figure 2A (coronal sections). **A** is from a fimbria-lesioned animal, 3 months survival, and **B** is from an animal with a septal-diagonal band transplant, 2 months survival. Due to the subnormal density of AChE-positive fibres the differential laminar distribution of the fibres is particularly well demonstrated in **B.** *m* Molecular layer, *g* granule cell layer, *h* hilar zone, *p* CA3 pyramidal cell layer. Asterisk in **A** denotes the diffuse AChE staining that remains in the stratum lacunosum-moleculare after complete fimbrial transection (see text)

Fig. 8A and B. Photomicrographs of AChE-positive coronal sections through the dorsal hippocampal formation in animals bearing septal-diagonal band transplants in the occipital cortex (see Fig. 1B), 12 weeks survival.
In **A** the fimbria (and hence the normal cholinergic innervation) was left intact up to 5 days before killing. In **B** the fimbria were lesioned ipsilaterally at the time of transplantation. Note the marked difference in distribution of the AChE-positive fibres. From a portion of the transplant (*T*) fibres are seen to extend into the outer molecular layer (*m*) of the dentate gyrus in **A**, and further into the supra- and subgranular zones (*g*) and the hilus (*h*) in **B**. Also the CA3 which is devoid of fibres in **A** is densely supplied in B

AChE staining and the ChAT determinations indicate that the cholinergic neurons sprout vigorously in their new location, and similar to transplanted dopaminergic and indolaminergic neurons (Stenevi et al, 1976), the newly formed fibres form a dense neuropil throughout the transplant. In fact, the ChAT activity reaches, within two months after transplantation, levels similar to those found in the septal-diagonal band area of the adult rat. From the fimbrial implants the regrowing sprouts extended in abundance (across the narrow gap separating the transplant from the hippocampus) into the cut septal end of the hippocampal formation, and within the hippocampus they expanded in a caudal-temporal direction over a distance of at least 6–8 mm to restore a remarkably accurate laminar pattern in the dentate gyrus and the hippocampal CA1 and CA3 zones.

Regarded as a reinnervation process the cholinergic ingrowth from the fimbrial implant is efficient as well as remarkably specific. In transplants of the embryonic locus coeruleus region about 10% of the noradrenergic neurons have been estimated to survive grafting (Stenevi et al., 1976). Thus, despite the fact that only a fraction of the septal-diagonal band neurons can be expected to survive in the transplant, near normal ChAT levels and AChE staining were restored in the most successful specimens in the septal part of the hippocampus. In fact, it seems quite possible that the number of surviving neurons in the graft could be a limiting factor for the magnitude of regrowth into the denervated hippocampus.

The orderly ingrowth of the AChE-positive fibres was evidently independent of the route by which the fibres reached the hippocampal terminal fields. This is illustrated by the fact that the fibres extending caudally from the transplantation site in the fimbria grew within the hippocampal grey matter rather than along the dorsal fornix and the fimbria where the septohippocampal axons normally run (Lewis and Shute, 1967; Meibach and Siegel, 1977). Moreover, the septal implants were able to restore a partly normal AChE-positive terminal pattern in the hippocampal formation also from the abnormal position in the dorsal entorhinal cortex, provided that the hippocampus previously had been denervated of its normal cholinergic afferents. This indicates that whatever mechanisms are operating to guide the axons to their terminal fields, they do not depend on guidance along the neural tubes normally carrying the septohippocampal cholinergic axons.

A comparison of the mode of regrowth of the AChE-positive fibres from the two transplantation sites point to very precise regulatory mechanisms in the formation of terminal patterns. Thus, in the transplantations to the fimbrial site, it is notable that despite the lesion removing not only the septal inputs but also the commissural ones, the ingrowing AChE-positive fibres were not observed to extend into areas innervated predominantly by the commissural afferents. This holds true above all for the stratum radiatum of CA1 and CA3, which for the greater part receives dense commissural but only sparse septal innervations (see Blackstad, 1956; Raisman et al., 1965; Storm-Mathisen and Blackstad, 1964), but partly (in the temporal regions) also for the zone of the dentate molecular layer just above the supragranular zone of septal AChE-positive terminals (Fig. 7B) (Mosko et al., 1973). Only scattered AChE-positive fibres had grown into these areas, indicating a preference of the sprouting cholinergic fibres for the areas denervated of their homologous cholinergic afferents.

Further, in the (yet preliminary) transplantations to the entorhinal site, the extension of the AChE-positive fibres into the terminal fields of the normal cholinergic afferents was apparently blocked by the presence of an intact cholinergic innervation. This indeed suggests that the growth of the sprouting fibres is somehow related to the filling of vacated terminal space in the denervated target tissue. Although this points to some degree of specificity of the reinnervation process, it is evident that pattern formation from the sprouting cholinergic neurons is not entirely specific for vacated cholinergic terminal sites. Thus, with the transplants placed in the entorhinal perforant path there was an abundant growth also into the outer portion of the dentate molecular layer, which is a principal terminal zone for the non-cholinergic perforant path fibres. In fact, in the dentate having an intact cholinergic innervation, the ingrowing fibres were mainly confined to this entorhinal terminal field. This is in line with the observations of Lynch et al. (1972) and Storm-Mathisen (1974) of an expansion of the AChE-positive septal terminals into the outer molecular layer of the dentate gyrus, triggered by entorhinal deafferentation. Furthermore, in a previous study (Björklund and Stenevi, 1977) we found that adrenergic neurons transplanted into the septohippocampal pathway give rise to a new hippocampal adrenergic innervation with a pattern corresponding not only to that of the normal adrenergic innervation, but partly also to that of the lesioned septal innervation. These various observations yield proof for the notion that axonal regeneration in the hippocampal formation are very precisely regulated by mechanisms released by deafferentation, and that the specificity of these mechanisms partly overlap with respect to the different afferent systems.

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