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Björklund, A., Stenevi, U., Schmidt, R.H., Dunnett, S.B., Gage, F.H.: Intracerebral grafting of neuronal cell suspensions. II. Survival and growth of nigral cells implanted in different brain sites. Acta Physiol. Scand., Suppl. 522, 9-18, 1983.

Schmidt, R.H., Björklund, A., Stenevi, U., Dunnett, S.B., Gage, F.H.: Intracerebral grafting of neuronal cell suspensions. III. Activaty of intrastriatal nigral suspension implants as assessed by measurements of dopamine synthesis and metabolism. Acta Physiol.Scand., Suppl. 522, 19-28, 1983

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Intracerebral Grafting of Neuronal Cell Suspensions VII. Recovery of Choline Acetyltransferase Activity and Acetylcholine Synthesis in the Denervated Hippocampus Reinnervated by Septal Suspension Implants

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The time-course and magnitude of fibre outgrowth from septal suspension grafts injected into the previously denervated hippocampal formation was monitored by measurements of choline acetyltransferase (ChAT), and the activity of the grafted neurons was assessed by measurements of [^{14}C]acetylcholine (ACh) synthesis from [^{14}C]glucose in vitro. Graft-derived ChAT activity was barely detectable 10 days after grafting, but increased sharply between 10 days and 1 month in the areas of the hippocampus located close to the septal implants. By 6 months ChAT activity was restored to near normal levels in all segments of the previously denervated hippocampus. The overall hippocampal [^{14}C]ACh synthesis was also restored to normal levels in the grafted animals, and estimates of the ACh turnover rate suggested that the transmitter machinery of the newly established "septo-hippocampal" connections operated at a rate similar to that of the intrinsic septohippocampal pathway. The intrahippocampal septal suspension grafts, similar to the intrastriatal nigral grafts, thus seem to be capable of maintaining function at a relatively "physiological" level despite their abnormal positions.

INTRODUCTION

Like dopaminergic neurones, some functional aspects of central cholinergic neurones can be monitored biochemically. In the parallel histochemical study reported in Chapter VI, the cholinergic neurones in the septal suspension grafts were visualized with the acetylcholinesterase (AChE) staining method. Although in the hippocampal formation AChE staining provides a useful and selective demonstration of the septo-hippocampal cholinergic projection system, the AChE enzyme is generally not a specific marker for cholinergic neurones. The acetylcholine (ACh) synthetic enzyme, choline acetyltransferase (ChAT), which is an enzyme specifically confined to cholinergic neurones, is a better index of cholinergic neurotransmission in the CNS. In the present study, therefore, we have monitored the hippocampal ChAT activity in animals with septal suspension implants reinnervating the previously denervated hippocampus in order to obtain some quantitative assessment of the time-course and magnitude of fibre outgrowth from the implanted cholinergic neurones. In order to obtain further information on the activity of the implanted neurones at the transmitter level, we have in addition made use of a method, recently introduced by Sims et al (10), for the measurement of [^{14}C]ACh synthesis from [^{14}C]glucose in vitro, under resting and activated conditions.

METHODS

A total of 65 young female Sprague-Dawley rats (180-200 g at the time of surgery) were used as recipients in the present study.

The grafts consisted of cell suspensions prepared from the septal-diagonal band area dissected from 14-16-day old rat embryos (CRL 12-16 mm), according to the procedure described in Chapter I. The final suspension volume corresponded to 10 µl per dissected piece. Each animal received two 5 µl aliquots of the suspension, i.e., in total the number of cells recovered from the septal-diagonal band area of one embryo. The injections were made at a speed of 1 µl/min into the dorsal hippocampal formation at the following coordinates: (i) A: +4.5 mm (rostral to inter-aural line), L: 3.5 mm, V: 3.0 mm below dura; (ii) A: +3.0 mm, L: 3.7 mm, V: 3.7 mm below dura. Incisor bar was set at the level of the inter-aural line.

ChAT experiment

Fifty rats were subjected to a unilateral complete aspirative lesion of the hippocampal fimbria, as described previously (2, 11). The lesion was made through the overlying cortex and corpus callosum and completely transected the fimbria, the dorsal fornix, the ventral hippocampal commisure and the supracallosal stria (Fig. 1). In the same surgical

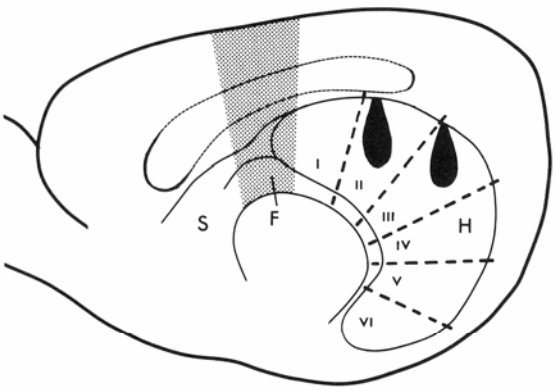

Fig. 1 Extent of the fimbria-fornix lesion and position of the two septal suspension implants as illustrated schematically on a medial view of the septum (S), fimbria (F) and the hippocampal formation (H). The hatched area indicates the lesion and dashed lines indicate the approximate extent of the slices I-VI used for the biochemical assays in Figs. 2 and 3.

session 25 of the lesioned rats received 2 x 5 µl of septal cell suspension injected into the ipsilateral hippocampal formation, as indicated schematically in Fig. 1.

The rats were killed for biochemical analysis at 7 days (5 rats), 10 days (10 rats), 3 weeks (4 rats), 1 month (10 rats), 3 months (10 rats) and 6 months (11 rats). The ipsi- and contralateral hippocampi were dissected free, flattened out on a chilled glass plate and cut into 6 equal transverse slices (I-VI), as indicated in Fig. 1. In this dissection the two implants were included in slices no. II and III, respectively, counted from the septal end.

ChAT was assayed according to the micromethod of Fonnum (3, 4). Protein content was measured according to Lowry et al (6) and the values were expressed as nmoles of ACh formed per hour per mg of protein (in Fig. 2) or as nmoles of ACh formed per hour per hippocampus (slices I-VI pooled) (Fig. 3).

[^{14}C]*ACh synthesis in vitro*

Fifteen rats were used in this experiment. 5 rats received bilateral fimbrial lesions plus bilateral septal suspension grafts, as above; 5 rats received bilateral fimbrial lesions alone; and remaining 5 rats served as normal controls. All rats were killed for biochemical analysis 6 months after operation.

The entire hippocampus from each side (or hippocampus including the septal implants) was taken for measurements of [^{14}C]ACh synthesis according to the method of Sims et al (10), with the exception that the $^{14}CO_2$ production was not measured in parallel. The hippocampus was minced into fine fragments with a pair of scissors. Two aliquots of the minced sample were taken for duplicate ChAT assay, as above, and two further aliquots were taken for duplicate protein determinations. The remaining tissue was split into four samples: two were incubated at 37°C in [U-^{14}C]glucose (>230 Ci/mol; The Radiochemical Centre, Amersham, U.K.) at a low K^+ concentration (5 mM), and two were incubated in [^{14}C]glucose at a high K^+ concentration (31 mM). Cortical tissue incubated at 0°C served as blanks.

RESULTS

ChAT activity

Fig. 2 summarizes the time-course of recovery of ChAT activity in the grafted animals (filled circles and solid lines) as compared with the lesioned but non-grafted controls (open circles and dashed lines). The ChAT levels rose most rapidly in slices II and III, which were the slices that contained the implant tissue. Here, some of the animals showed high ChAT values (above 50% of normal) already by 3 weeks after transplantation, and by 1 month the increase was highly significant ($p < 0.001$), reaching an average of 50-70% of normal in slices II and III. Between 1 and 6 months the increase progressed further in these slices to reach a mean of 100-120% by 6 months. The highest individual values recorded at 3 and 6 months were up to 70% above normal.

In the hippocampal slices outside the immediate vicinity of the grafts (slice I rostrally and slices IV-VI ventrally; see Fig. 1), the reappearance of ChAT tended to be slower and more protracted than in the slices including the grafts. A significant increase, to an average of 40% of normal, was nevertheless noted at 1 month in slices IV and V (no values were obtained from slice I in the 1 month group due to the larger size of the fimbrial lesion in those animals). The ChAT levels continued to increase between 1 and 3 months and between 3 and 6 months. In slices I and IV, which represent the parts of the hippocampus located approx. 1-2.5 mm from the nearest graft, the mean ChAT level attained by 6 months was 90-110% of normal. In slices V and VI, representing distances of approx. 2.5-5.5 mm from the nearest graft, the values were on the average 60-70% of normal by 6 months. In slice VI, ChAT levels significantly above control were only reached between 3 and 6 months.

The time-dependent changes are further illustrated in Figs. 3 and 4. In Fig. 3 the graft-derived ChAT levels are plotted for the different slices at 4 different postoperative time-points. Each point gives the difference in ChAT activity for each slice between the means of the grafted

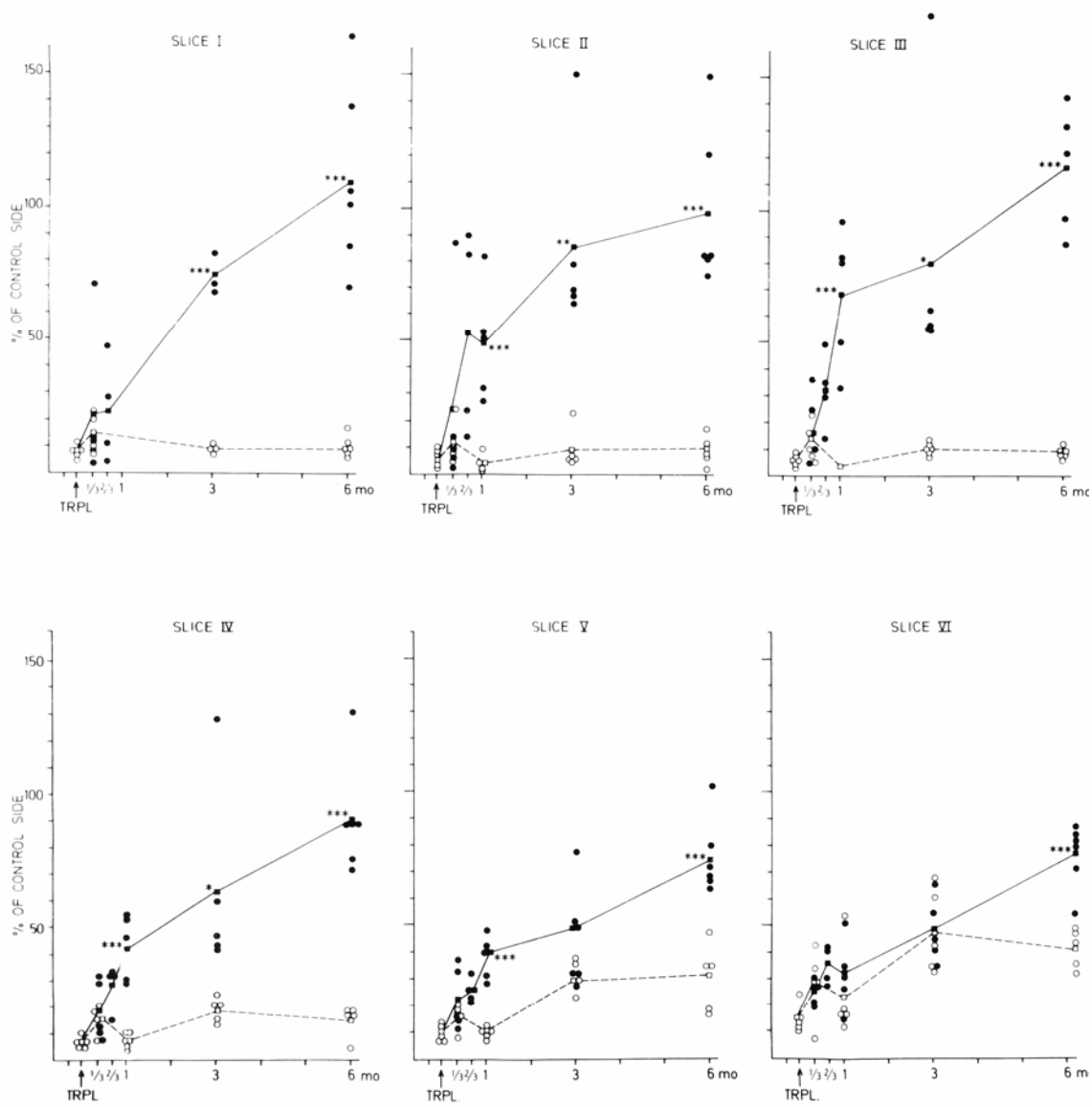

Fig. 2 Recovery of ChAT activity (expressed as nmoles of ACh formed per mg protein per hour) in slices I-VI in animals with septal suspension grafts (solid lines and filled circles) and in animals with fimbria-fornix lesions alone (dashed lines and open circles). Each individual is represented by a filled or open circle, and the mean values by squares. Differences between animals with and without grafts: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Student's t-test.

and the lesioned control values in Fig. 2. This figure shows that graft-derived ChAT was barely detectable by 10 days, that it appeared particularly in the region of the grafts by 3 weeks, and that it expanded progressively over the entire hippocampal formation over the subsequent months. Fig. 4 gives the *total* ChAT content for the entire grafted hippocampus (filled circles) and for the entire denervated hippocampus in the lesioned controls (open circles). This curve levels off at about 70-95% of normal, but since the hippocampus undergoes about 25% shrinkage after the fimbrial lesion (1) the ChAT concentration levels attained will nevertheless be close to normal, as seen in Fig. 2.

For comparison, Fig. 4 gives also the total ChAT values calculated from our previous study (2) on solid septal grafts reinnervating the hippocampus. This curve (crosses and dashed line) gives the total ChAT activity measured in the solid grafts plus the reinnervated ipsilateral hippocampus. Although the amount of tissue

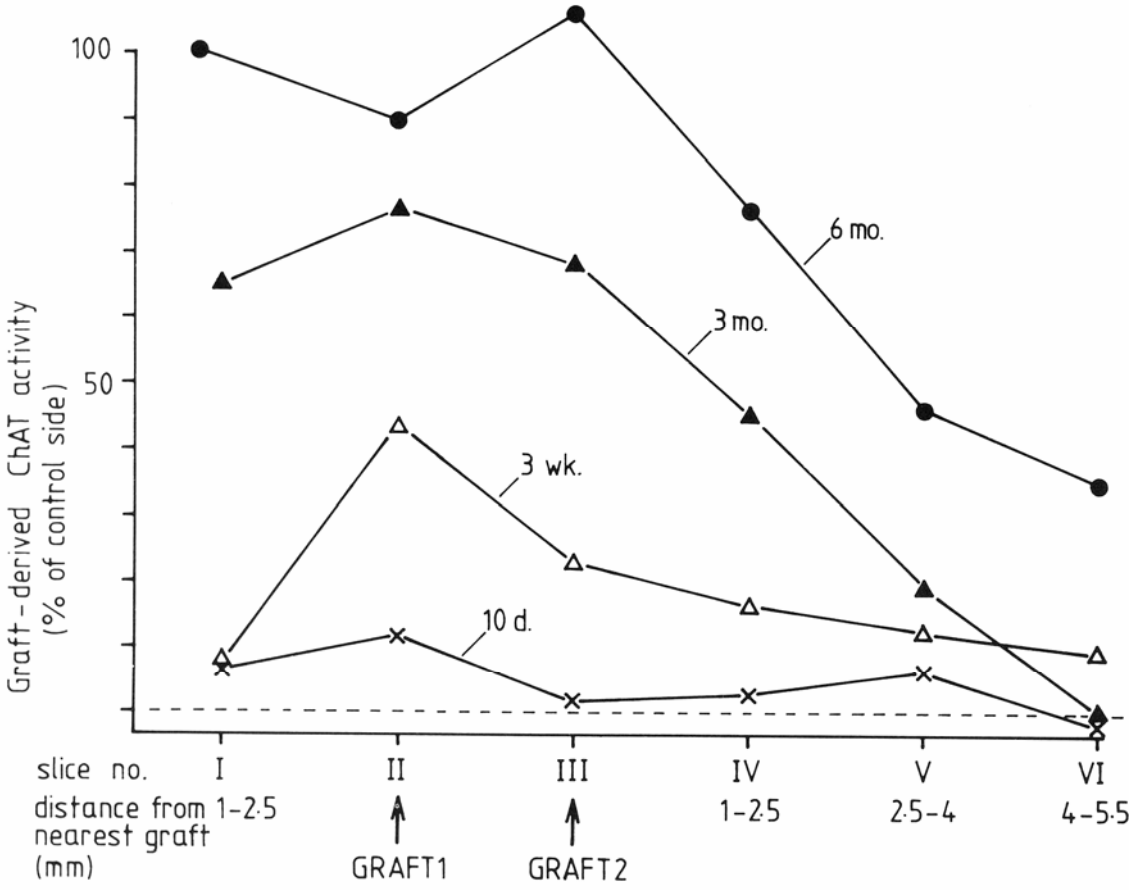

Fig. 3 Graft-derived ChAT activity in slices I-VI at different postoperative time-points, calculated as the differences between the means in the grafted and lesioned controls groups in Fig. 2.

grafted is comparable in each case (i.e. the tissue obtained from one embryo), the suspension grafts appear to be about twice as effective as the solid grafts in terms of the total ChAT level expressed.

[^{14}C]*Acetylcholine synthesis*

The hippocampal ACh synthesis rate, measured from [^{14}C]glucose in vitro, was in the lesioned rats reduced by about 60% under resting condition (low K^+) and by about 65% under activated conditions (high K^+), and it was restored to normal levels in the grafted animals (Fig. 5A). These differences were highly significant ($p < 0.001$). In the normal hippocampus K^+-induced neuronal activation resulted in an 81% increase in the [^{14}C]ACh synthesis (open column in Fig. 5B). The hippocampus reinnervated by the septal grafts responded in a similar manner (hatched column in Fig. 5B), whereas the denervated hippocampus in the lesioned control animals was impaired in this respect (filled column in Fig. 5B). In fact, the K^+-induced increase in [^{14}C]ACh synthesis in the lesioned controls was non-significant.

Like the ACh synthesis, ChAT (measured in aliquots from the same specimens) was reduced in the lesioned controls and recovered to normal level in the grafted hippocampus (Fig. 5C). Interestingly, however, the reduction in ChAT in the denervated animals (about 90%) was more pronounced than the reduction in [^{14}C]ACh synthesis (60-65%). As a result the [^{14}C]ACh : ChAT ratio (Fig. 5D) was increased 7-10 fold over that recorded in the normal, intact hippocampus ($p < 0.05$), suggesting that the ACh turnover was markedly increased in the residual cholinergic innervation spared by the fimbrial lesion (i.e. the innervation which reaches the hippocampal formation along the so-called

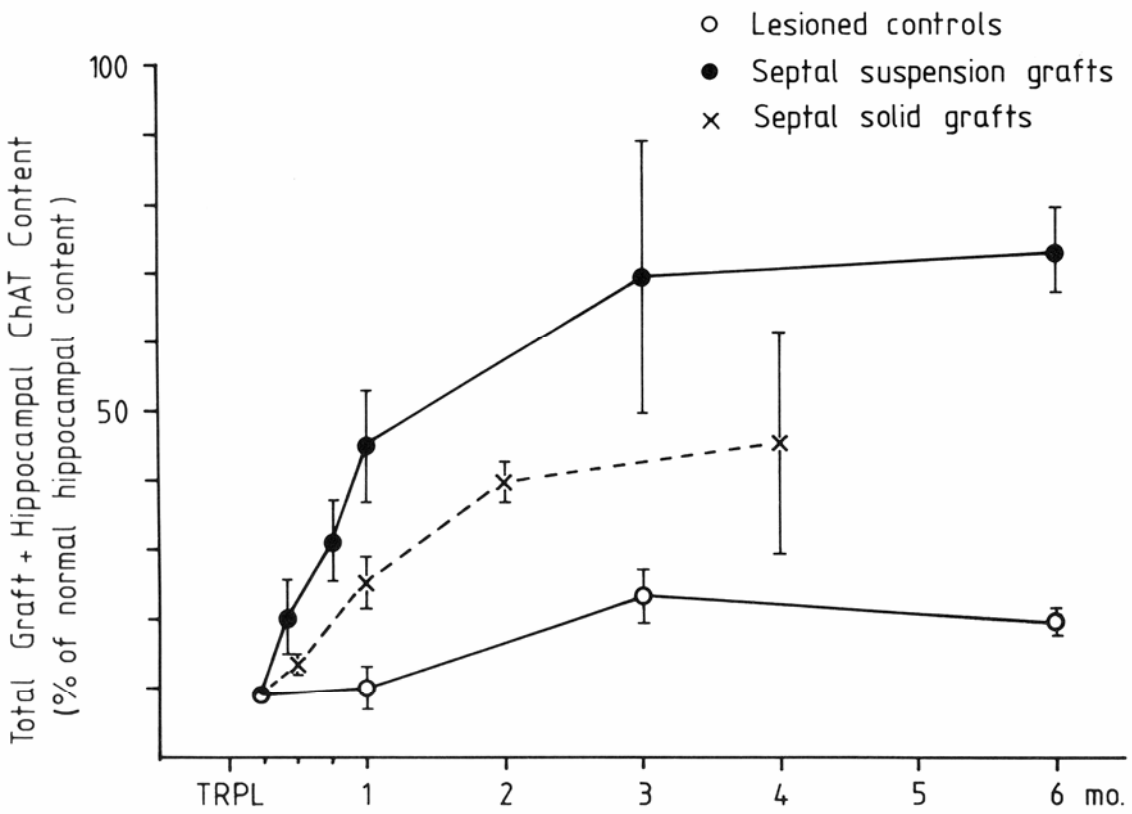

Fig. 4 Total ChAT content expressed as nmoles of ACh formed per hour per hippocampus (slices I-VI pooled) in the grafted animals (filled circles) and in the lesioned controls (open circles) at different postoperative time-points. The dashed line and crosses give the total ChAT content in animals with solid septal grafts reinnervating the hippocampus (graft plus hippocampus combined), calculated from ref. 2.

ventral route; see ref. 5). In the lesioned animals with septal suspension grafts the [^{14}C]ACh : ChAT ratios were significantly reduced, and the values recorded in the grafted hippocampi at both low and high K^+ concentration (0.41 and 0.91, respectively) were close to the normal ratios (0.42 and 0.75, respectively), indicating that the ACh turnover rate had been normalized in the hippocampi reinnervated by the septal grafts.

DISCUSSION

The results show that intrahippocampal implants of septal cell suspensions are capable of restoring ChAT activity to near normal levels in the previously denervated hippocampal formation. In the area of the implant the most rapid phase of ChAT increase occurred between 10 days and 1 month after grafting. In the areas of the hippocampus located further away from the grafts the recovery was more protracted and proceeded also beyond 3 months. The levels attained by 6 months corresponded to between 80 and 120% of normal in all segments of the hippocampus, suggesting that the septal implants had provided a relatively complete reinnervation of the surrounding denervated target.

The calculation of the graft-derived ChAT activity, obtained by subtracting the values of the lesioned controls from those of the grafted specimens (Fig. 3), provides a good picture of the dynamics of development and growth of the implanted cholinergic neurones. These "profiles" demonstrate a clear-cut gradient in the recovery of ChAT activity in the directions away from the implants, and this gradient still persisted in the direction of the temporal pole of the hippocampus by 6 months. This is consistent with the parallel histochemical observations (Chapter VI) that the outgrowth of AChE-positive fibres from the implants appears first in the vicinity of the grafts, and progresses later along the hippocampus in the septal and temporal directions. The ChAT data indicate that the outgrowing fibres had reached slice IV

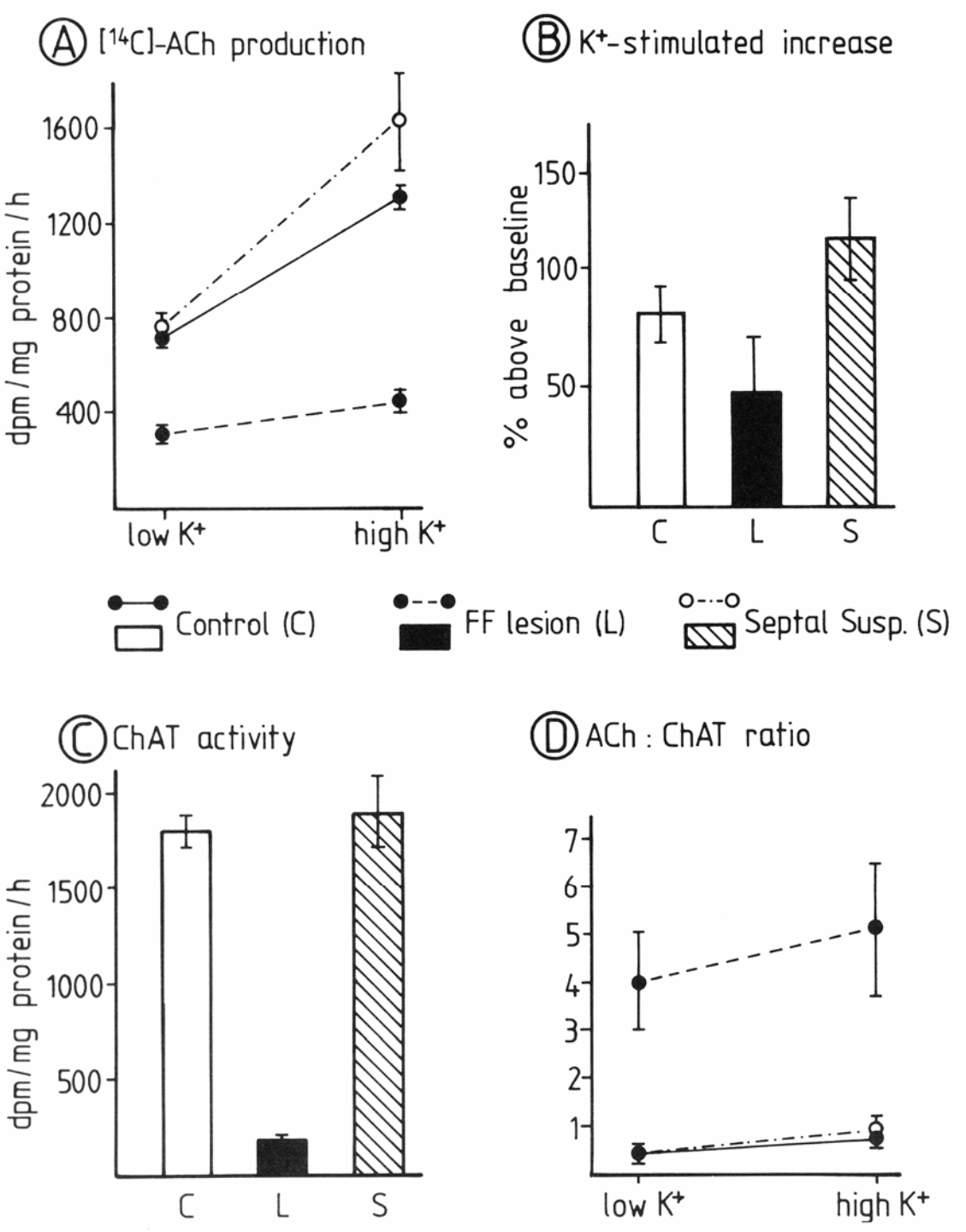

Fig. 5A and B [14C]ACh synthesis from [14C]glucose in vitro in whole hippocampus control rats (C), fimbria-fornix lesioned rats (L), and lesioned rats with septal suspension grafts (S). *C*: ChAT activity measured on aliquots of the same samples as in A and B. *D*: Ratio between [14C]ACh production to ChAT activity at resting (low K^+) and activated (high K^+) conditions. Means ± S.E.M. of 10 determinations in each group.

(i.e. about 1-2.5 mm from the nearest graft) by 3 weeks, slice V (2.5-4 mm away) by 1-3 months and slice VI (4-5.5 mm away) by 6 months. This gradient is notably similar to that previously observed in hippocampi reinnervated by solid septal grafts implanted into the fimbrial lesion cavity (see Fig. 3 in ref. 2).

The total suspension volume injected into each animal in the present study was equivalent to the amount of cells recovered from one dissected septal-diagonal band area tissue piece. In our previous study using the solid graft technique, one such piece was capable of restoring only about 1/3 of the total hippocampal ChAT activity (see Table I in ref. 1). The present suspension grafts were clearly more efficient in this respect. It is conceivable that this difference could be due to topographic factors, such as shorter growth distances and more intimate contacts between graft and host in the suspension grafted specimens. Fig. 4 shows however that the total expression of ChAT in the graft and hippocampus combined was about twice as high in the suspension grafted animals as in our previous animals with solid septal grafts. It seems more likely, therefore, that the difference in efficiency between the solid and suspended septal grafts is due to a difference in survival and/or total fibre outgrowth of the implanted cholinergic neurones.

Since ChAT probably is not a rate limiting step in ACh synthesis under normal conditions (7, 8) the activity measures of this enzyme do not give a direct measure of actual turnover rates of the transmitter. The [^{14}C]ACh synthesis from [^{14}C]glucose provides a better index of the functional state of the ACh transmitter machinery, and Sims et al (10) have shown that the synthesis rates measured *in vitro* with this technique are similar to *in vivo* estimates of ACh turnover rates. The present results can therefore be taken as evidence that hippocampal cholinergic neurotransmission (which was severely impaired in the lesioned control animals) was restored by the septal suspension implants. This was seen both in the overall [^{14}C]ACh synthesis rate, as well as in the [^{14}C]ACh synthesis : ChAT ratio. If one assumes that the ChAT level primarily reflects the magnitude of the cholinergic terminal network, then the [^{14}C]ACh : ChAT ratio would provide an estimate of the average synthesis rate in the individual terminals. This seems consistent with the observation that the [^{14}C]ACh : ChAT ratio was greatly increased in the denervated hippocampi, and that the ratio was normalized in the hippocampi reinnervated by the septal grafts. This pattern parallels that seen with dopamine-rich nigral grafts reinnervating the dopaminergically denervated striatum (Chapter III; ref. 9). It is interesting to note, however, that using the same implantation technique and similar suspension volumes, the overall recovery of ACh synthesis seen in the hippocampus with the septal suspension grafts was about 3-fold greater than the recovery in DA synthesis seen in striatum with nigral grafts (i.e. 105% of normal for ACh synthesis and 28% of normal for DOPA accumulation; cf. Table 4 in Chapter III).

In conclusion, the present results indicate that the intrahippocampal septal suspension grafts can reinstate cholinergic neurotransmission in the previously denervated hippocampus to levels close to those seen in the normal hippocampus. The implanted septal cholinergic neurones appear to reinnervate the entire hippocampal formation with a time-course spanning several months. The in vitro ACh synthesis measurements indicate that the ACh transmitter machinery in the newly established "septo-hippocampal" connections operates at a rate similar to that of the intrinsic septo-hippocampal cholinergic pathway. Thus, similar to the observations on the dopamine-rich nigral grafts in Chapter III, it appears that the implanted septal neurones can maintain function at a relatively "physiological" level despite their abnormal position. Whether this reflects an intrinsic property of the neurones themselves, or some regulatory feedback mechanisms operating between the graft and the host, remains an interesting topic for further investigation.

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