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# Effects of grafts containing cholinergic and/or serotonergic neurons on cholinergic, serotonergic and noradrenergic markers in the denervated rat hippocampus

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Long–Evans female rats sustained aspirative lesions of the septohippocampal pathways and, 2 weeks later, received intrahippocampal suspension grafts prepared from the regions including either the medial septum and the diagonal band of Broca (group S), or the mesencephalic raphe (group R), or from both these regions together (group S + R). Sham-operated (group SHAM) and lesion-only (group LES) rats were used as controls. Six months after grafting, high affinity synaptosomal uptake of choline (HACU) and serotonin (HASU), choline acetyltransferase (ChAT) activity and, using HPLC, the content of serotonin ([5-HT]), 5-hydroxyindolacetic acid ([5-HIAA]) and noradrenaline ([NA]) were determined in three rostro-caudal segments of the hippocampus (designated hereafter as the dorsal, the 'middle' and the ventral segments). In all three segments of the dorsal hippocampus, septohippocampal lesions decreased HACU, ChAT activity, HASU and [5-HT]; [5-HIAA] was decreased only in the middle and ventral hippocampal segments. The lesions also resulted in an above normal increase of [NA]. Septal grafts increased HACU and ChAT in the three hippocampal regions, had no effect on serotonergic markers and attenuated the lesion-induced increase of [NA] in only the dorsal and middle hippocampal segments. Raphe grafts increased HASU, [5-HT] and [5-HIAA] in the dorsal and middle hippocampal segments, had no effects on cholinergic markers and did not affect the lesion-induced increase of [NA]. Co-grafts increased HACU, ChAT activity, HASU, [5-HT] and [5-HIAA], and attenuated the lesion-induced increase in [NA]. These data demonstrate that grafts of fetal neurons placed into the denervated hippocampus may induce a neurochemical recovery which depends upon the anatomical origin of the grafted cells. They also show that co-grafting allows to combine the neurochemical properties of two fetal brain regions grafted separately. Furthermore, our findings suggest that graft-derived cholinergic reinnervation of the hippocampus prevents the lesion-induced increase of noradrenaline concentration which is likely to result from sympathetic sprouting. Thus, our data confirm the results of a previous experiment carried out at a post-grafting delay of 10–11 months, and show that the graft-induced effects reported previously are already massively present by 6 months after surgery.

## INTRODUCTION

One of the major deficits observed in patients who have died from Alzheimer's disease (AD) is a dramatic degeneration of the central cholinergic neurons located in the basal forebrain (e.g. refs. 5,13,35). The extent of this degeneration is correlated with the severity of the cognitive alterations resulting from AD (e.g. refs. 5,13,41,42). To test some potential treatments of these cognitive deficits, several experimental models of AD have been used in animals. Overall, they are aimed at investigating the efficacy of either pharmacological treatments (drugs enhancing the cholinergic neurotransmission, cholinergic agonists or neurotrophic factors) or intracerebral grafts on cognitive dysfunctions induced by brain lesions (e.g. refs. 13,21). One of these models consists of performing grafts rich in cholinergic neurons into the hippocampus deprived of its cholinergic inputs. Over the past decade, many studies have shown that such grafts were able to attenuate or even to fully compensate for some of the lesion-induced electrophysiological (e.g. refs. 30,45–47), neurochemi-

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cal (e.g. refs., 6,28,29), histochemical (e.g. refs. 7,10,37,38), and behavioral (e.g. refs. 10,22,23,30,37,38) disturbances.

However, the neuropathology of AD is also characterized by degenerative processes affecting neuronal populations other than the cholinergic one[26,35,39]. Among these, one can include the population of serotonergic neurons which, in some respects, were also shown to be involved in cognitive functions (e.g. refs. 20,27,52,53).

Interestingly, the extensive damage to the septohippocampal pathways does not only induce a cholinergic denervation of the hippocampus. The hippocampus is also deprived of its serotonergic innervation arising from the mesencephalic raphe and its noradrenergic innervation arising from the locus coeruleus (e.g. refs. 25,50). There are a few studies suggesting that the cholinergic and serotonergic hippocampal afferents may be interactively involved in the modulation of cognitive functions (e.g. refs. 20,44). Therefore, it becomes particularly interesting to use intrahippocampal grafts rich in both cholinergic and serotonergic neurons as a tool to promote concomitant recovery from both the lesion-induced cholinergic and serotonergic neurochemical deficits; such grafts are usually called co-grafts and, to our knowledge, have been used for the first time by Nilsson et al.[37] for intracerebral transplantation.

In a recent experiment that was aimed at trying to attenuate more than only the cholinergic deficit in the hippocampus denervated by extensive septohippocampal damage, we performed intrahippocampal grafts of cells prepared from different regions of the fetal brain[11,12]. On the one hand, grafts rich in cholinergic neurons were prepared from the ventral forebrain which includes the medial septum and the diagonal band of Broca. On the other hand, grafts rich in serotonergic neurons were prepared from the mesencephalic raphe region. These grafts were performed as single grafts (i.e. prepared only from one of both regions) or as co-grafts (i.e. combining the neurons from both regions). In this experiment, we have demonstrated that grafts of cells from the septal region compensated for or attenuated the cholinergic deficits due to the lesion, grafts of cells from the mesencephalic region compensated for or attenuated the serotonergic deficits, whereas co-grafts induced recovery from both types of neurochemical deficits. In addition, we have shown that graft-derived cholinergic, but not serotonergic, reinnervation of the hippocampus was able to prevent the long lasting lesion-induced increase in hippocampal noradrenaline concentration ([NA], which is considered to reflect sympathetic ingrowth into the hippocampus. However, in the previous experiment, assessment of the lesion- and graft-induced neurochemical effects was carried out only in the most dorsal portion of the hippocampus and after a rather long post-grafting delay (10–11 months).

The experiment reported herein was designed to address several issues. First, we wanted to determine, *in the whole hippocampus* (dorsal, 'middle' and ventral segments), the neurochemical effects of lesions and grafts which were virtually identical to those used in our previous experiment. Second, since (i) we have been the first and, so far, the only ones to report an inhibitory effect of graft-derived cholinergic hippocampal reinnervation on the lesion-induced increase of hippocampal [NA] and (ii) since this report was based on only one experiment, we also wanted to confirm this effect. Third, we were interested in whether the recently reported effects of the lesions and the various single- and co-grafts could be detected at a shorter post-grafting delay (6 months) than the delay used in our previous experiment[11,12].

The present study was carried out on rats which were subjected to an aspirative lesion of the septohippocampal pathways. The grafts were placed into the dorsal hippocampus and consisted of a cell preparation from the ventral forebrain region (rich in cholinergic neurons), the mesencephalic raphe (rich in serotonergic neurons) or from both of these regions (co-grafts). Six months after grafting, the neurochemical effects of the lesions and the grafts were assessed in the dorsal, middle and ventral segments of the hippocampus using (i) high affinity uptake of [$^3$H]choline and [$^3$H]serotonin ([$^3$H]5-HT) by hippocampal synaptosomes, (ii) choline acetyltransferase (ChAT) activity, (iii) HPLC determination of 5-HT, 5-hydroxyindolacetic acid (5-HIAA) and noradrenaline (NA) concentrations.

## MATERIALS AND METHODS

### *Subjects*

Rats used in the present experiment were Long–Evans female rats ($n = 29$) obtained from R. Janvier (France). They were housed in transparent Makrolon cages (59×38×20 cm) in groups of five at most, except for surgeries for which they were isolated in smaller cages (42×26×15 cm). Food and water were available ad libitum. The colony room was maintained on a 12 h light/12 h dark cycle (lights on at 07.00 h) under controlled temperature (23°C).

### *Surgeries*

All surgeries were performed under aseptic conditions, using Nembutal anaesthesia (40 mg/kg, i.p.) after an atropine sulfate injection (4 mg/kg, i.p., about 10 min prior to the injection of pentobarbital). For extraction of the fetuses, pregnant females were anaesthetized according to the same procedure, except that (i) no atropine pretreatment was given and (ii) the dose of Nembutal was increased to about 70 mg/kg.

*Lesion surgery*. At 31 (±1) days of age, 25 rats received bilateral aspirative lesions (5.5 mm anterior to Lambda[40]) of the fimbria, the dorsal fornix and parts of the overlying structures. Besides damage to hippocampal efferents and other alterations (including retrograde modifications in the structures projecting fibers on the hippocampus or receiving fibers from the hippocampus), such lesions produce extensive hippocampal denervations since they disrupt cholinergic and GABAergic fibers originating in the septal area, serotonergic fibers originating in the mesencephalic raphe, and noradrenergic fibers originating in the locus coeruleus (e.g. refs. 9,25,50). Hereafter, the expression "septohippocampal lesion" should be understood to refer to all these alterations. To perform the lesions, rats were placed in a stereotaxic apparatus with the incisor bar placed 3.0 mm below the interaural line. Tissue aspiration was made using a curved Pasteur pipette ending in a 7-mm long straight microcap (Drummond) with an external diameter of 0.7 mm. The control group (SHAM, $n = 5$) consisted of rats which were subjected to all surgical steps except that they received no lesion.

*Transplant surgery*. Cells to be grafted were prepared from fetal tissue excised from the brains of fetuses obtained from pregnant females from the same strain. Two weeks after lesion surgery, the first group of rats with lesions (S, $n = 7$) received bilateral intrahippocampal grafts of a cell suspension prepared from the septal–diagonal band region which is rich in cholinergic neurons (CRL: 16 mm, E: 16 days). A second group of lesioned rats (R, $n = 6$) received grafts of a cell suspension prepared from the mesencephalic raphe region which is rich in serotonergic neurons (CRL: 13 mm, E: 14 days). A third group of rats with lesions (S+R, $n = 6$) received co-grafts which consisted of a cell suspension with a total amount of tissue approximately equal to that of each other group with grafts: thus, these co-grafts contained about half the number of cells of septal grafts and half that of raphe grafts (CRL: 13–15 mm, E: 14–15 days). The fourth group (L, $n = 5$) consisted of lesioned rats which did not receive cell suspension grafts. The grafts were performed as described elsewhere[11]. The present study did not include histological verifications of the placement of grafted cells. However, from previous studies using injection coordinates virtually identical to those used in the present study (e.g. refs. 10,11), we know that the grafts are generally located either entirely within the hippocampal parenchyma, or developed partly within both the hippocampal parenchyma and the third ventricle. When one considers the septotemporal axis, in almost all cases the location of the grafts is limited to the dorsal third (septal pole) of the hippocampus; the grafts are rarely located within or along the middle segment of the hippocampus and, when such a case is observed, the fibers only invade this segment over a few hundreds of micrometers.

Briefly, after extraction of the fetal brains, the ventral forebrain region containing the septal area or the mesencephalic raphe region were dissected out, collected in 0.6% glucose–saline, incubated for 20 min in 0.1% trypsin (Sigma, Grade II), washed 3 times with 5 ml of fresh glucose–saline and brought to a final volume of about 10 µl per septal tissue piece and 20 µl per raphe tissue piece. These blocks were then mechanically dissociated and 2 µl of the resulting suspension were injected stereotaxically in each site of the dorsal hippocampus (1 µl/min) using a Hamilton syringe. The coordinates of the four sites were as follows: from Lambda, A = 4.0 mm, L = ±1.6 mm, V = 3.1 mm; A = 2.4 mm, L = ±3.2 mm, V = 3.3 mm. The incisor bar was placed 3.0 mm below the interaural line. Cell suspensions were used within a maximum of 3 h after preparation. The number of cells injected ranged between 60,000 and 70,000 cells/µl, with 88–94% viability. For further details, see Cassel et al.[11].

*Pharmacological and biochemical assessments*

Six months after grafting, all rats were decapitated, the brain quickly removed and both hippocampi dissected free and cut into three segments of approximately equal sizes, thereby isolating a dorsal (septal pole), a middle (intermediate) and a ventral (temporal pole) segment. Respecting their septo–temporal level of origin, the left and the right sub-regions were collected together into 3 ml of 0.32 M sucrose (in 2.5 mM HEPES, pH 7.4) where they were homogenized in a Potter/Elvehjem glass–teflon homogenizer (7 strokes at 500 rpm). 100 µl of this crude homogenate was used for measurements of ChAT activity and protein content, as described below. Further 100 µl aliquots of the crude homogenate were mixed with 100 µl 0.2 N $HClO_4$ (containing 250 mg $Na_2SO_3$ and 200 mg $Na_2EDTA$ per l), and stored at −20°C until HPLC determinations. The remaining homogenate was centrifuged for 10 min at 1,000× *g*; the supernatant was carefully removed by pipette and centrifuged again (10 min at 17,000× *g*).

*Determination of synaptosomal uptake*. The pellet of this second centrifugation was carefully resuspended in 700 µl of KHB containing 60 µM pargyline. 50 µl of this supension was incubated for 5 min at 30°C in a total volume of 250 µl KHB in the presence of 50 nM of either [$^3$H]choline or [$^3$H]5-HT (samples in triplicates). In a series of preliminary experiments, we found that the synaptosomal uptake was linear between 2 and 8 min for [$^3$H]choline and between 2 and 5 min for [$^3$H]5-HT. In order to correct for non-specific binding or uptake, parallel samples (duplicates) were run in the presence of 1 µM (each) of hemicholinium-3 ([$^3$H]choline uptake) or fluvoxamine ([$^3$H]5-HT uptake). The incubation was stopped by the addition of 4 ml of ice-cold KHB+pargyline, followed by rapid filtration through cellulose nitrate filters (0.65 µm pore size; Sartorius) and further washing of the filters (3 times with 4 ml KHB+pargyline). The filters were dissolved in 2 ml of ethyleneglycol monoethylether and radioactivity was assessed by liquid scintillation counting (LSC) after addition of 10 ml of toluene scintillator.

*Determination of ChAT activity*. ChAT activity was determined as described by Fonnum[24] with, however, several modifications. In brief, 100 µl of the crude homogenate of hippocampal slices (see above) were diluted with 100 µl of a medium containing 269 mM NaCl, 90 mM $NaH_2PO_4$, 0.45% Triton-X100, 0.9 mM EGTA, 0.9 mM $Na_2EDTA$ and 179 µM physostigmine. 10 µl of this mixture (all samples in quadruplicates) was added to 5 µl of choline bromide (32 mM). The incubation was started by the addition of 5 µl of [$^{14}$C]acetyl-coenzyme-A (50 nCi/assay; 0.242 mM final concentration). After 15 min at 37°C, the tip of the incubation tube was cut with a razor blade and put into a mixture of 5 ml sodium phosphate buffer (10 mM, pH 7.4) with 2 ml of sodium tetraphenylborate in acetonitrile (5 mg/ml). From this mixture the newly formed [$^{14}$C]acetylcholine was extracted by careful shaking with 10 ml of toluene scintillator. Following separation of the aqueous phase from the organic phase the samples were directly counted by LSC. In order to correct for non-specific effects, in each case four samples were run at 0°C.

*High performance liquid chromatography (HPLC)*. [5-HT], [5-HIAA] and [NA] was evaluated by HPLC with electrochemical detection (Model 300 c pump, Gynkotek). Each sample (see above) was centrifuged and the supernatant (approx. 90 µl) filtered through 0.22 µm filters (Millipore). 20 µl of the filtered supernatant was injected onto a C18 reverse-phase column (ODSII, 5 µm). The separation of the different compounds was obtained using a citrate–acetate buffer (pH 4.1, sodium acetate 39.8 mM, citric acid monohydrate 17.3 mM, $Na_2EDTA$ 86.7 µM) containing 80 ml/l methanol, 6.5 ml/l ethanol and 518 mg/l sodium octane sulfonate. Electrochemical detection (Gynkotek) was done at 0.6 V using a calomel reference electrode. Sensitivity of the detection was 2.5 pg for NA and 5-HIAA, and 7.4 pg for 5-HT.

*Protein content*. Protein content was assessed in both the crude homogenate (used for determination of ChAT activity, [5-HT], [5-HIAA] and [NA]) and in the suspension of synaptosomes, according to the method described by Lowry et al.[31].

*Statistical analyses*

All data were analyzed using an analysis of variance (ANOVA) followed, where appropriate, by multiple comparisons based on the Newman–Keuls test[57]. Data from each sub-region of the hippocampus were analyzed separately. Computation of the correlations between some variables was performed according to the parametric method described by Tallarida and Murray[51]. Due to a technical problem, the HASU could not be assessed successfully in one rat with septal grafts.

RESULTS

*High affinity choline uptake (HACU, Fig. 1)*

An ANOVA showed a significant group effect in all three hippocampal regions ($F_{4,24} > 17.0$, $P < 0.001$). In the lesion-only group (LES), the average HACU was significantly decreased (dorsal −74%; middle −79%; ventral −75%) as compared to that observed in sham-operated rats ($P < 0.01$, in all cases). For all three regions, the HACU found in raphe-grafted rats was significantly lower than in sham-operated rats ($P < 0.01$) and did not differ from the values of lesion-only rats. Whatever region was considered, the HACU observed in septal- or co-grafted rats was significantly higher than that found in both lesion-only and raphe-grafted rats ($P < 0.01$, in all cases). In the dorsal and the middle segments of the hippocampus, rats with septal grafts and rats with co-grafts showed a HACU which did not differ significantly from that of sham-operated rats.

*ChAT activity (Fig. 2)*

An ANOVA showed a significant group effect in the three regions of the hippocampus ($F_{4,24} > 11.3$, $P < 0.001$, whatever region was considered). In the lesion-only group, the average ChAT activity was significantly decreased (dorsal −67%; middle −73%; ventral −72%) as compared to that observed in sham-operated rats ($P < 0.01$, in all cases). The ChAT activity found in raphe-grafted rats was significantly lower than in sham-operated rats ($P < 0.05$, at least) and did not differ from that of lesion-only rats. Whatever region was considered, the ChAT activity observed in septal or co-grafted rats differed significantly from that found in either lesion-only or raphe-grafted rats ($P < 0.05$, at least). In both the dorsal and the middle segments of the hippocampus, the rats with septal grafts and the rats with co-grafts showed a ChAT activity which did not differ significantly from that found in sham-operated rats.

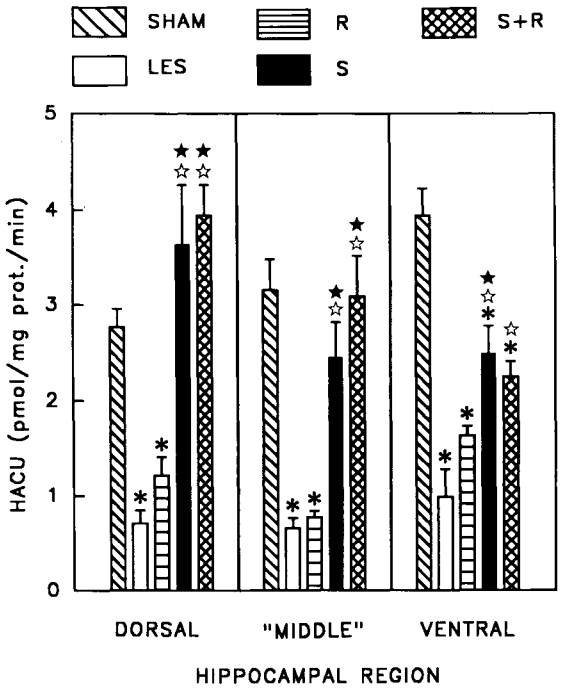


Fig. 1. Average (+S.E.M.) high affinity choline uptake values (HACU) by synaptosomes prepared from the dorsal, middle and ventral hippocampal segments of control (SHAM), lesion-only (LES), raphe-grafted (R), septal-grafted (S) and co-grafted (S+R) rats. Newman–Keuls test: *significantly different from values of sham-operated rats, $P < 0.05$; ☆ significantly different from values of lesion-only rats, $P < 0.05$; ★ significantly different from values of raphe-grafted rats, $P < 0.05$.

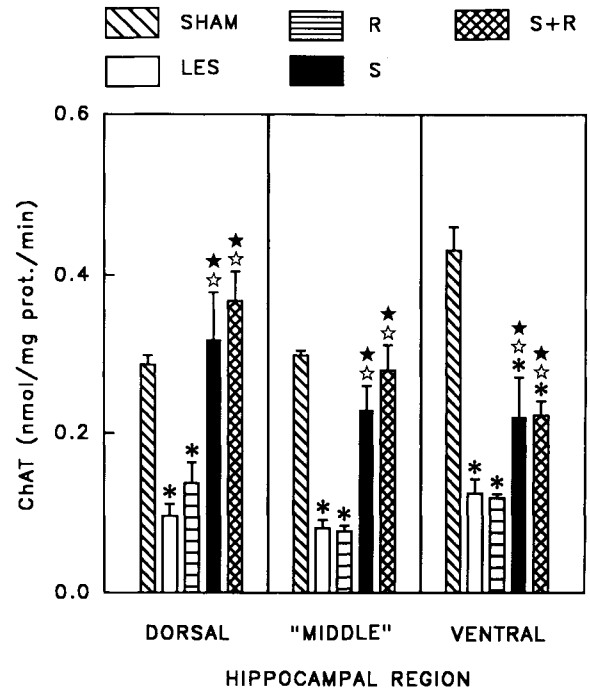


Fig. 2. Average levels (+S.E.M.) of ChAT activity found in homogenates prepared from the dorsal, middle and ventral hippocampal segments in the five experimental groups. Symbols and abbreviations are as in Fig. 1.

*High affinity serotonin uptake (HASU, Fig. 3)*

An ANOVA showed a significant group effect in all hippocampal regions ($F_{4,23} > 6.8$, $P < 0.01$). In the lesion-only group, the average HASU was significantly decreased (dorsal −76%; middle −75%; ventral −63%) as compared to that found in sham-operated rats ($P < 0.01$, in all cases). In the dorsal and the middle segments of the hippocampus, the average HASU found in both raphe-grafted and co-grafted rats was significantly higher than in lesion-only rats ($P < 0.01$). In the dorsal hippocampus, it also significantly exceeded that found in sham-operated rats. This was

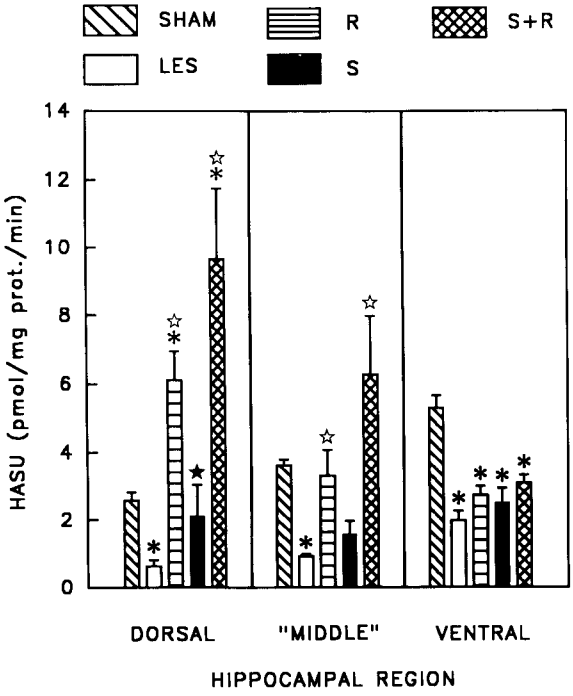


Fig. 3. Average (+S.E.M.) high affinity serotonin uptake values (HASU) by synaptosomes prepared from the dorsal, middle and ventral hippocampal segments in the five experimental groups. Symbols and abbreviations are as in Fig. 1.

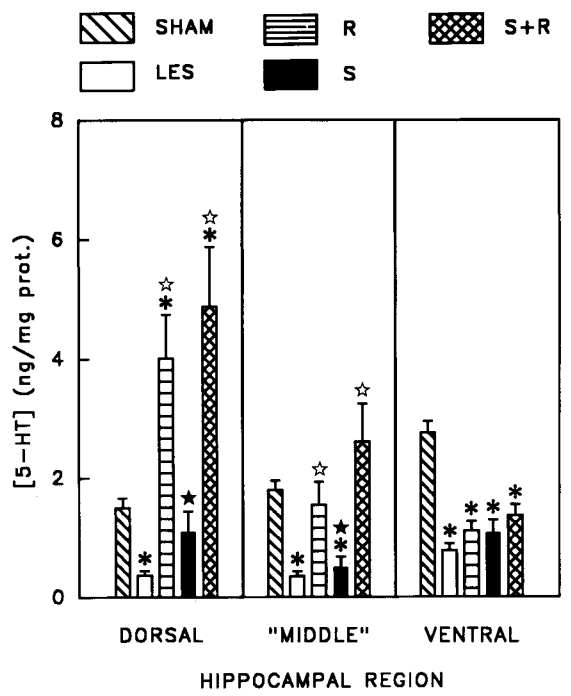


Fig. 4. Average (+S.E.M.) concentration of serotonin ([5-HT]) determined in homogenates prepared from the dorsal, middle and ventral hippocampal segments in the five experimental groups. Symbols and abbreviations are as in Fig. 1.

not the case in the ventral hippocampus, where no significant difference was observed among the values of the four lesion groups, whether grafted or not. In any region, the HASU observed in rats with septal grafts did not differ significantly from that of lesion-only rats.

*Serotonin (5-HT) concentration (Fig. 4)*

The ANOVA revealed a significant group effect in the dorsal ($F_{4,24} = 9.68$, $P < 0.01$), the middle ($F_{4,24} = 6.59$, $P < 0.01$) and the ventral ($F_{4,24} = 14.75$, $P < 0.001$) hippocampal segments. The lesions had decreased [5-HT] by 76% in the dorsal hippocampus, 81% in the middle and 72% in the ventral parts ($P < 0.01$, in all cases). Both the raphe grafts and the co-grafts resulted in increased [5-HT] in the dorsal and middle segments of the hippocampus ($P < 0.05$, at least), but had no significant effect in the ventral hippocampus (comparisons to lesion-only rats). Also, in the dorsal hippocampus, [5-HT] of raphe- and co-grafted rats significantly exceeded that found in sham-operated rats. Whatever hippocampal region was considered, no significant differences were noted between septal-grafted and lesion-only rats.

*5-hydroxyindolacetic acid (5-HIAA) concentration (Fig. 5)*

The ANOVA showed a significant group effect in the three regions of the hippocampus (dorsal $F_{4,24} = 6.6$; middle $F_{4,24} = 5.44$; ventral $F_{4,24} = 8.19$; $P < 0.01$). In the dorsal hippocampus, this effect was due to significantly increased [5-HIAA] in both raphe- and

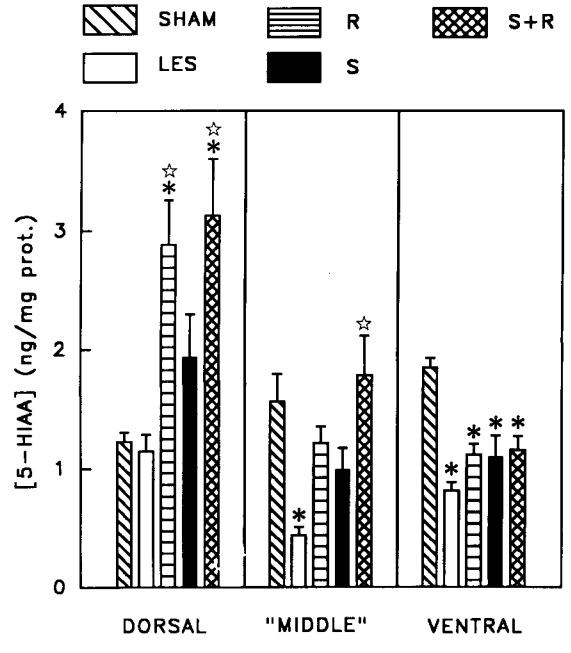


Fig. 5. Average (+S.E.M.) concentration of 5-hydroxyindolacetic acid ([5-HIAA]) determined in homogenates prepared from the dorsal, middle and ventral hippocampal segments in the five experimental groups. Symbols and abbreviations are as in Fig. 1.

co-grafted rats, as compared to the values found in sham-operated and lesion-only rats ($P < 0.01$). In the middle hippocampal segment, the only significant differences to be observed were between lesion-only rats and both co-grafted and sham-operated rats ($P < 0.05$). In the ventral hippocampus, [5-HIAA] of sham-operated rats was significantly different from that found in the four other groups ($P < 0.01$), but no significant difference was found among the latter.

*[5-HIAA] / [5-HT] ratios (Fig. 6)*

The overall group effect was significant in both the dorsal and the middle segments of the hippocampus ($F_{4,24} = 7.64$, $P < 0.01$; $F_{4,24} = 2.94$, $P < 0.05$, respectively), but not in the ventral one. In the dorsal hippocampus, this effect was due to significantly increased ratios in lesion-only and septal-grafted rats as compared to the three other groups ($P < 0.01$). In the middle hippocampal segment, the overall group effect was due to a significant difference between septal-grafted rats and either sham-operated, raphe-grafted or co-grafted rats ($P < 0.05$). Although higher in lesion-only rats, the [5-HIAA]/[5-HT] ratios found in the ventral hippocampus did not differ significantly from those found in the other groups.

*Noradrenaline (NA) concentration (Fig. 7)*

The ANOVA showed a significant group effect in all three hippocampal regions (dorsal $F_{4,24} > 3.08$, $P <$

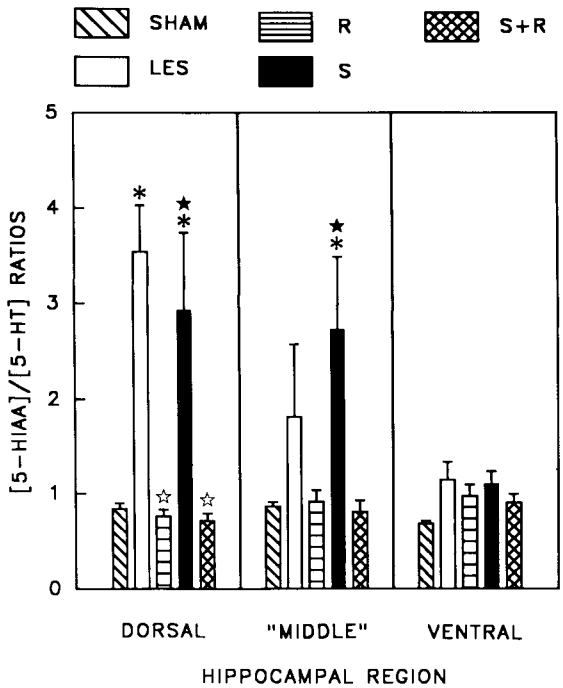


Fig. 6. Average (+S.E.M.) [5-HIAA]/[5-HT] ratios calculated from the data presented in Figs. 4 and 5. Symbols and abbreviations are as in Fig. 1.

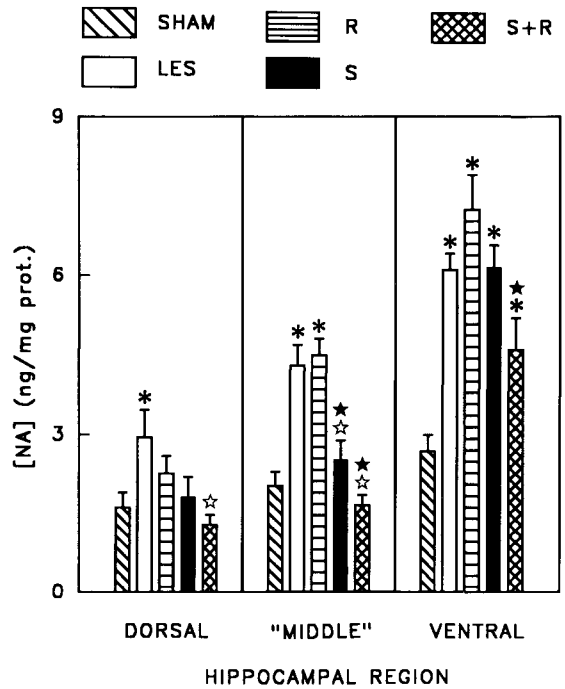


Fig. 7. Average (+S.E.M.) concentration of noradrenaline ([NA]) determined in homogenates prepared from the dorsal, middle and ventral hippocampal segments in the five experimental groups. Symbols and abbreviations are as in Fig. 1.

0.05; middle $F_{4,24} = 17.44$, $P < 0.001$; ventral $F_{4,24} = 10.43$, $P < 0.001$). In the dorsal hippocampus of the lesion-only group, the average [NA] was higher than in the four other groups, but the difference reached significance only when this [NA] was compared to that found in sham-operated or co-grafted rats ($P < 0.05$). In the middle hippocampal segment, [NA] was significantly higher in both lesion-only and raphe-grafted rats than in the three other groups ($P < 0.01$); among the average values of the latter, no significant difference was observed. Finally, in the ventral hippocampus, there was no significant difference between the 4 groups with lesions, whether grafted or not, but all these groups showed an average [NA] which was significantly higher than that found in sham-operated rats ($P <$ 0.01).

*Correlations between neurochemical parameters*

To run these analyses, data from sham-operated rats were not taken into account, since inclusion of these data could lead to erroneous interpretations. The different meaningful and significant correlations (with 22 d.f.) found are the following: apart from the highly significant *positive* correlation between HACU and ChAT activity on the one hand and, on the other hand, between HASU and 5-HT concentration, we found a *negative* correlation between ChAT activity and NA concentration which was significant in all three hip-

pocampal regions (dorsal $r = -0.434$, $P < 0.05$; middle $r = -0.772$, $P < 0.001$; ventral $r = -0.668$, $P < 0.001$). The correlation between HACU and [NA] was also negative and significant whatever region was considered (dorsal $r = -0.649$, $P < 0.001$; middle $r = -0.79$, $P < 0.001$; ventral $r = -0.439$, $P < 0.05$).

## DISCUSSION

About six months after surgeries, we found that the aspirative lesion of the septohippocampal pathways induced a severe cholinergic and serotonergic hippocampal denervation. The cholinergic denervation was evidenced by reduced HACU and ChAT activity, while the serotonergic one was manifested by decreased HASU, [5-HT] and [5-HIAA], as well as by an increased [5-HIAA]/[5-HT] ratio in the dorsal and middle segments of the hippocampus. The latter modification indicated changes in 5-HT turnover, namely the occurrence of an up-regulating adaptative response by which the undamaged serotonergic fibers tend to compensate for the reduced serotonergic innervation of the hippocampus (e.g. refs. 3,19). We also observed that the septohippocampal denervation had increased hippocampal [NA] largely above the normal values. This effect was particularly pronounced in the middle and ventral hippocampal segments; it is currently interpreted as due to sympathetic sprouting (e.g. refs. 14–16,32–34). Previous studies have shown that after septohippocampal lesions, the hippocampus can be reinnervated by, primarily, spared cholinergic and aminergic fibers which undergo lesion-induced homotypic sprouting (e.g. ref. 25). This phenomenon can be particularly pronounced after a partial denervation of the hippocampus (e.g. ref. 25). However, after an extensive aspirative lesion of the dorsal hippocampal afferents, the sprouting of intact fibers remains rather weak and several months may be required to induce significant histological and neurochemical effects (e.g. ref. 25). Therefore, we assume that the major part of the differences reported here between lesion-only and grafted rats (see below) actually resulted from lesion-induced effects (such as, for instance, reactional sprouting).

The single grafts rich in serotonergic neurons (i.e. transplants containing cells from only the mesencephalic raphe) resulted in serotonergic reinnervation of the hippocampus, with a dorsal-to-ventral decreasing gradient: in the dorsal hippocampus, the HASU, [5-HT] and [5-HIAA] exceeded the values found in sham-operated rats, in the middle segment of the hippocampus, these parameters were normalized (comparable to values found in sham-operated rats), whereas in the ventral hippocampus no significant effect of the raphe grafts could be found (see Table I). Also, the raphe grafts had no effect on the lesion-induced increase of hippocampal [NA], whatever region was considered. The single grafts rich in cholinergic neurons (i.e. transplants containing cells from only the ventral forebrain) induced a cholinergic reinnervation of the hippocampus with a dorso–ventral decreasing gradient (see Table I). Actually, these grafts normalized HACU and ChAT activity in both the dorsal and middle hippocampal segments. Although both of these parameters were also significantly increased in the ventral hippocampus, only a partial compensation of the lesion-induced cholinergic deficit was observed in this region. In contrast to single raphe grafts, the single septal grafts also attenuated the lesion-induced increase of [NA] in the dorsal and the middle, but not in the ventral segments of the hippocampus. Finally, in the regions where effects of the single grafts were observed, the co-grafts combined the compensatory properties of each single graft: they reduced both the serotonergic and cholinergic deficits and also attenuated the lesion-induced increase in [NA]. All of these results confirm those of the previous experiment[11,12]; they show that the graft-induced effects which we previously detected between 10 and 11 months after

TABLE I

*Effects of both the lesions and grafts (group abbreviations as in the figures) found in the three hippocampal segments (d,m,v) on five primary neurochemical markers*

The data are expressed as a percentage of the corresponding values (100%) found in sham-operated rats. HACU, high affinity choline uptake; ChAT, choline acetyltransferase activity; HASU, high affinity serotonin uptake; [5-HT], serotonin concentration; [NA], noradrenaline concentration; d, dorsal hippocampus; m, middle hippocampal segment; v, ventral hippocampus; LES, lesion only; R, raphe graft; S, septal graft; R + S, raphe and septal co-graft.

| *Neurochemical marker* | | *Surgical treatments (%)* | | | |
|---|---|---|---|---|---|
| | | *LES* | *R* | *S* | *S + R* |
| HACU | d | 26 | 44 | 131 * | 143 * |
| | m | 21 | 24 | 78 * | 98 * |
| | v | 25 | 41 | 63 | 57 |
| ChAT | d | 33 | 47 | 111 * | 128 * |
| | m | 27 | 26 | 77 * | 93 * |
| | v | 28 | 27 | 51 | 51 |
| HASU | d | 24 | 237 ** | 82 | 375 ** |
| | m | 25 | 92 * | 43 | 173 * |
| | v | 37 | 52 | 47 | 58 |
| [5-HT] | d | 24 | 267 ** | 73 | 325 ** |
| | m | 19 | 86 * | 28 | 145 * |
| | v | 28 | 40 | 39 | 49 |
| [NA] | d | 183 | 140 | 112 * | 80 * |
| | m | 213 | 223 | 125 * | 82 * |
| | v | 228 | 271 | 230 | 172 |

* Indicates a graft-induced normalization (no significant difference between absolute values compared to values found in sham-operated rats); ** indicates a significant graft-induced overcompensation (compared to sham-operated rats).

grafting surgery can already be detected, at least in the dorsal and the middle hippocampal segments, after a post-grafting survival time of only 6 months.

*Graft-induced serotonergic effects*

In the literature, there are now several reports describing various types of neurochemical effects of intrahippocampal grafts containing serotonergic neurons (e.g. refs. 3,43,48,49,54,59,60). One common finding in this series of reports is that such grafts do not only compensate for the lesion-induced neurochemical deficit, but almost always overcompensate for it. Depending upon the experimental paradigm, the variables which indicate the occurrence of graft-derived serotonergic reinnervation of the denervated hippocampus (e.g. HASU or [5-HT]) actually reach levels which vary between 140% (e.g. ref. 3) and about 350% (e.g. refs. 11,12,59) of the levels found in sham-operated control rats. This neurochemical overcompensation is likely to reflect a graft-derived serotonergic hyperinnervation of the host structure as has been verified histologically (e.g. ref. 54). Daszuta et al.[19] reported that the amplitude of these graft-induced effects depended upon the duration of the post-grafting survival time: while there was no overcompensation by 3 weeks after grafting (28% of control values), it was detectable by seven weeks (147%) and continued to increase later on up to 216% (determined 5 months after grafting). Whether in raphe-grafted or in co-grafted rats, our present results are in agreement with those reported by Daszuta et al.[19]. After a 6-month post-grafting survival time, we have found that both types of grafts containing mesencephalic raphe tissue increased the serotonergic markers in the dorsal hippocampus to about 240–375% (see Table I) of the levels found in the corresponding region of virtually intact rats. In our previous experiment carried out at a post-grafting survival delay of 10–11 months[11,12], the graft-derived effects were even larger than those presently reported. Accordingly, it can be assumed that the hippocampal reinnervation provided by serotonergic cells takes time and does not completely fade out over a period of at least 10 months. If the fastest reinnervation rate occurs within the first few months after grafting (e.g. refs. 19,59), this process appears to be continuous and might follow an asymptotical evolution in its latest stages. Although this point has been discussed more extensively in our previous report[11], it can briefly be reiterated that two non-exclusive hypotheses might account for the hippocampal serotonergic hyperinnervation induced by grafts containing mesencephalic neurons. First, it might result from the effect of a trophic factor which is known to be synthetized and released in the hippocampus subsequent to denervation and to specifically stimulate the growth of serotonergic neurons (e.g. refs. 4,59). Second, since in normal animals serotonergic raphe neurons project to many regions of the brain (e.g. refs. 9,36), it can be speculated that when fetal mesencephalic raphe tissue is grafted in a circumscribed structure such as the hippocampus, all the fibers which would have innervated various other brain structures than only the hippocampus remain confined solely to the latter[18].

It should be pointed out that the amplitude of the graft-derived reinnervation is not only dependent upon a chronological factor. Our results also suggest that it depends on the distance between the graft and the target region which has to be reinnervated, even in a given circumscribed structure such as the hippocampus. Actually, the markers for serotonergic innervation reached a maximal level in the dorsal hippocampus, i.e. in the region where the grafts had been implanted (see Table I). In the middle segment of the hippocampus, which was the next nearest region to the grafted tissue, the graft-induced serotonergic effects were still significant, although less pronounced. Finally, in the most distal region, namely the ventral hippocampus, no significant graft-derived effect could be seen. This observation is in line with the conclusion of previous experiments using mesencephalic raphe grafts implanted into the dorsal portion of the extensively denervated hippocampus (e.g. refs. 49,54). The decreasing gradient of the graft-induced hippocampal reinnervation might be related to some limitation in the ability of the graft-derived fibers to grow over large distances through the host parenchyma, as has already been demonstrated with techniques of bridging grafts (e.g. refs. 1,55,56).

*Graft-induced cholinergic effects*

This aspect of our present study closely confirms previous work and, therefore, does not appear to require a detailed discussion. Briefly, intrahippocampal grafts prepared from the ventral forebrain region were shown to compensate for neurochemical (e.g. refs. 2,6,7), neuropharmacological (e.g. refs. 28,29), electrophysiological (e.g. refs. 30,45–47) and, most probably as a consequence of the former compensations, behavioral deficits induced by septohippocampal damage (e.g. refs. 10,17,21–23,37,38,46). One point to underline here is that addition of mesencephalic raphe tissue to the ventral forebrain preparation does not alter the ability of the latter to restore the depleted ChAT activity and the reduced HACU in the hippocampus. It should be noted that our results on serotonergic markers in the presence of co-grafts indicate that the reciprocal assertion can also be accepted. Thus, co-grafting

different neurochemical categories of neurons appears to be an interesting and suitable technique to compensate for more than one of the neurochemical deficits as a result of extensive denervation of a host CNS structure.

As was the case with the graft-induced effects on serotonergic markers, the graft-derived cholinergic effects also showed a decreasing intensity depending upon the distance between the graft and the target region (see Table I): they were maximal in the dorsal segment of the hippocampus, less pronounced in the middle segment and rather low, although significant, in the ventral segment. That cholinergic, but not serotonergic, neurochemical effects were detected up to the ventral region of the hippocampus indicates that grafted cholinergic neurons from the basal forebrain might be able to extent fibers over larger distances than mesencephalic raphe grafts. Indeed, this difference cannot be due to variations in the graft placements along the septotemporal axis, since in the co-grafted group, namely in the group in which both categories of cells had been injected conjointly into the same sites, this difference was still observed.

*Graft-induced noradrenergic effects*

In our previous experiment, carried out after a 10–11 months post-surgical survival delay[12], we found that, in the dorsal half of the hippocampus, septohippocampal lesions had increased [NA] to about 300% of that found in sham-operated rats. Our present results in lesion-only rats, show that the increased [NA] is also observable 6 months after surgeries, although with a lesser amplitude, in all three hippocampal regions. Apparently, this effect follows a growing gradient along the septotemporal axis (see Table I) since it is more pronounced in the ventral than in the dorsal hippocampus. Our data also confirm that graft-derived cholinergic reinnervation of the hippocampus, whether from single ventral forebrain grafts or from co-grafts, partially prevents this lesion-induced effects. As shown in previous work, the [NA] increase, as a result of hippocampal denervation, mainly reflects growth of sympathetic fibers into the hippocampus (e.g. refs. 14–16,32–34). These fibers, which normally innervate blood vessels[14], originate in the superior cervical ganglia and sprout into the hippocampus (but also in other brain structures) in response to a cholinergic denervation (see arguments by Crutcher[14]). The fact that in our previous[12] and present experiments we observed that graft-derived cholinergic ingrowth produced considerable attenuation of this increase may be considered as an additional argument in favor of the hypothesis that cholinergic denervation is a specific and critical phenomenon for sympathetic sprouting to occur. This statement is further supported and reinforced by the following arguments. First, in the presence of single mesencephalic tissue grafts, the hippocampal [NA] was comparable to that found in lesion-only rats. Second, whatever hippocampal region was considered, there was always a significant negative correlation between HACU and [NA], as well as between ChAT activity and [NA]. This negative correlation might reflect a competition between the central (graft-derived) cholinergic and peripheral noradrenergic fibers, both of which are trying to reinnervate the same NGF-releasing targets (e.g. ref. 58). Third, in the regions where the grafts had induced normalization of the cholinergic parameters (dorsal and middle hippocampus), there was a reduction of the lesion-induced increase of [NA], whereas in the ventral hippocampus, in which the cholinergic effects of the grafts remained weak, there was none.

Although these series of data clearly show that cholinergic reinnervation of the hippocampus prevents the lesion-induced increase in hippocampal [NA], it is not possible to definitively conclude that sympathetic sprouting has been hindered. Indeed, an alternative possibility, that we had already raised in our recent report[12], would be that the acetylcholine released by grafted neurons affected the metabolism of noradrenergic sprouted fibers rather than the sprouting phenomenon itself. One possible substrate for such metabolic effects of the grafts could be the muscarinic heteroceptors that sympathetic fibers possess on their terminals[8]. The activation of these receptors is able to inhibit or at least to attenuate the evoked release of noradrenaline[8]. Accordingly, one can speculate that a long lasting graft-derived tonic activation might induce a metabolic breakdown in the sympathetic fibers which have grown into the hippocampus. We would, however, like to underline here, that in a series of preliminary superfusion experiments, we did not find that oxotremorine-induced muscarinic activation inhibited the electrically evoked release of noradrenaline by hippocampal slices prepared from rats which had sustained extensive septohippocampal damage 12 months earlier. Thus, although the question remains open, the first interpretation (sympathetic sprouting) seems more likely than the second one (metabolic breakdown). Future experiments using additional methodological approaches (e.g. ablation of the superior cervical ganglia, tyrosine hydroxylase immunohistochemistry, etc.) should help to clarify this point.

In conclusion, it can be stated that fetal mesencephalic raphe or septal–diagonal band cell suspensions implanted into the extensively denervated hip-

pocampus may provide a neurotransmitter-specific recovery which depends upon the anatomical origin of the grafted cells. Moreover, when a mixture of both these preparations is implanted, the resulting neurochemical effects correspond closely to a combination of the properties of each type of single graft. If all of these effects appear to be lasting in the dorsal half of the hippocampus until at least 11 months of post-grafting survival[11,12], they can already be detected as massive, although less pronounced, effects after a survival time of 6 months. This is true in at least the dorsal and the middle hippocampal segments. Finally, since the present experiment confirms that graft-derived cholinergic reinnervation of the hippocampus can prevent the increase of hippocampal noradrenaline concentration subsequent to septohippocampal lesions, we can accept this finding as being not simply coincidental.

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