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GRAFTS OF FETAL SEPTAL CELLS AFTER CHOLINERGIC IMMUNOTOXIC DENERVATION OF THE HIPPOCAMPUS: A FUNCTIONAL DISSOCIATION BETWEEN DORSAL AND VENTRAL IMPLANTATION SITES

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Abstract—Three-month-old Long–Evans rats were subjected to intraseptal infusions of 0.8 μg of 192 IgG–saporin followed, 2 weeks later, by intrahippocampal suspension grafts containing fetal cells from the medial septum and the diagonal band of Broca. The suspensions were implanted in the dorsal or the ventral hippocampus. Sham-operated and lesion-only rats were used as controls. Between 18 and 32 weeks after grafting, all rats were tested in a water maze (using protocols placing emphasis on reference memory or on working memory) and an eight-arm radial maze. The lesion produced extensive cholinergic denervation of the hippocampus, as evidenced by reduced acetylcholinesterase-positivity and acetylcholine content. Depending upon their implantation site, the grafts restored an acetylcholinesterase-positive reinnervation pattern in either the dorsal or the ventral hippocampus. Nevertheless, the grafts failed to normalize the concentration of acetylcholine in either region. The cholinergic lesion impaired working memory performance in both the water maze and the radial maze. To a limited degree, reference memory was also altered. Grafts placed in the ventral hippocampus had no significant behavioral effect, whereas those placed in the dorsal hippocampus normalized working memory performance in the water maze.

Our data show that infusion of 192 IgG–saporin into the septal region deprived the hippocampus of its cholinergic innervation and altered spatial working memory more consistently than spatial reference memory. Although the cholinergic nature of the graft-induced reinnervation remains to be established more clearly, these results further support the idea of a functional dissociation between the dorsal and the ventral hippocampus, the former being preferentially involved in spatial memory.

Key words: acetylcholine, 192 IgG–saporin, lesion, radial maze, spatial memory, water maze.

Lesions of the cingular bundle/fimbria–fornix pathways have often been used in rodents as a hippocampal denervation model in order to investigate structural and functional effects of intrahippocampal grafts rich in cholinergic or in other neurochemical types of developing neurons. Most studies undertaken so far have shown that such grafts not only survive, develop and provide the hippocampus with a new functional innervation, but also reduce the extent of lesion-induced cognitive deficits (e.g. Cassel et al., 1997; Dunnett, 1990; Sinden et al., 1995; Tarricone et al., 1996). Nevertheless, there is evidence demonstrating that well integrated grafts inducing cognitive benefits often fail to bring the altered functions back to a level found in intact control animals (e.g. Cassel et al., 1992, 1997; Dunnett, 1990; Dunnett and Björklund, 1994; Tarricone et al., 1996). The reasons of such limitations are multiple. For example, they might comprise the ectopic placement of the graft (e.g. Duconseille et al., 2001), the delay elapsed from grafting to functional evaluations (e.g. Cassel et al., 1991), the tests used to assess the graft-induced effects (e.g. Cassel et al., 1991, 1992), the hippocampal subfield into which the graft is placed (e.g. Hofferer et al., 1996), and even the multitransmitter deficits associated with the cingular bundle/fimbria–fornix lesion models as opposed to mainly cholinergic effects of the grafts (e.g. Balse et al., 1999; Jeltsch et al., 1994; Leanza et al., 1998).

Unlike in previous research involving standard lesion techniques (electrolysis, radio-frequency, aspiration), the immunotoxin 192 IgG–saporin permits selective damage to the cholinergic neurons of the basal forebrain (e.g. Wrenn and Wiley, 1998). Leanza et al. (1998) have shown that spatial navigation deficits induced by i.c.v. injections of 192 IgG–saporin could be completely reversed by grafts of fetal cholinergic neurons transplanted to both the hippocampus and the frontoparietal cortex, although the grafts produced weaker and less consistent effects in a delayed matching-to-position task. In this study, the lesions were not focused on the septo-hippocampal cholinergic neurons, as i.c.v. injec-

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Abbreviations: A, anterior; ACh, acetylcholine; AChE, acetylcholinesterase; ANOVA, analysis of variance; HPLC, high-performance liquid chromatography; L, lateral; PBS, phosphate-buffered saline; SAPO, 192 IgG–saporin lesion; SAPO+DHG, SAPO lesion+graft in the dorsal hippocampus; SAPO+VHG, SAPO lesion+graft in the ventral hippocampus; SHAM, sham-operated; V, ventral.

tions of the immunotoxin also damage the cholinergic neurons of the nucleus basalis magnocellularis (e.g. Leanza et al., 1998; Wrenn and Wiley, 1998). Therefore, in the present experiment, we have focused on the septo-hippocampal cholinergic system. The lesion approach consisted of injecting 192 IgG–saporin directly into the septal region. Also, as data from the literature suggest that there may be a functional difference between the dorsal and the ventral hippocampus (e.g. Hock and Bunsey, 1998; Hugues, 1965; Moser and Moser, 1998), the effects of fetal septal cell suspension grafts were evaluated in rats with grafts being placed in the dorsal or in the ventral hippocampus. Behavioral testing assessed the effects of the lesions and of both implantation sites of the grafts in a Morris water maze and a radial maze. The effects of the lesions and the grafts were also determined by histochemical and neurochemical verifications.

EXPERIMENTAL PROCEDURES

Subjects and design

All procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (council directive #87848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animales; permission #6212 to J.-C.C. and 6714-bis to H.J.; M.G., C.L., F.B. and R.G. under the responsibility of J.-C.C. and H.J.) and international (NIH publication, No. 85-23, revised 1985) laws and policies. All efforts were made to minimize both suffering and the number of animals used.

The study was performed on 55 male Long–Evans rats (R. Janvier, Le Genest St-Isle, France). At approximately 90 days of age (body weight approximately 350 g), the rats were allocated to one of four experimental groups. In three groups, the rats received bilateral infusions of 192 IgG–saporin (Advanced Targeting Systems, San Diego, CA, USA) into the septal region. Rats from the fourth group were sham-operated. All rats were housed in transparent Makrolon cages (42×26×15 cm) under a 12:12 h dark–light cycle (lights on at 7.00 h), with *ad libitum* access to food (except during radial maze) and water throughout the experiment. The colony and testing rooms were under controlled temperature (21°C).

Lesion and grafting surgeries

All surgical procedures were conducted under aseptic conditions, using pentobarbital anesthesia (75 mg/kg, i.p.). Injections of 192 IgG–saporin (in phosphate-buffered saline, PBS) into the septal region were performed stereotaxically through a 1-µl Hamilton syringe at the following coordinates (in mm from bregma, Paxinos and Watson, 1998): anterior (A) +0.6, lateral (L) ±0.2; ventral (V1) −7.2 and V2 −6.5, with the incisor bar set at the level of the interaural line. After the most ventral injection on one side of the brain (V1: −7.2 mm), the needle was left *in situ* for 4 min and then retracted 0.7 mm before the second injection was performed. After another delay of 6 min, the needle was completely retracted and injections on the contralateral side were made. An amount of 0.2 µg 192 IgG–saporin (1 µg/µl PBS) was injected at each site. Rats subjected to sham operations were injected with an equal volume of PBS.

Cells to be grafted were prepared from brains of Long–Evans fetuses aged 15 days (embryonic day 15, average CRL = 15 mm). Approximately 2 weeks after lesion surgery, two subgroups of lesioned rats received bilateral intrahippocampal grafts of a cell suspension prepared from the region including the septum and the diagonal band of Broca, a region rich in cholinergic neurons. Tissue fragments were collected into 0.6% glucose saline, incubated for 30 min at 37°C in the same solution containing 0.1% trypsin (Sigma, Grade II), washed three times with 5 ml of glucose saline and brought to a final volume of approximately 10 µl per septal tissue piece. The tissue pieces were dissociated using a fire-polished Pasteur pipette until a milky suspension was obtained. Injections (2 µl/site, 1 µl/min) of the resulting suspension were performed stereotaxically through a Hamilton syringe. In a first subgroup of rats (SAPO+DHG, $n = 14$), the suspension was injected into the dorsal hippocampus at A −3.6 mm, L ±1.8 mm, V −3.6 mm and A −4.5 mm, L ±2.6 mm, V −3.4 mm (from bregma, Paxinos and Watson, 1998). In the second subgroup of rats (SAPO+VHG, $n = 14$), it was injected in the ventral hippocampus at A −4.8 mm, L ±4.9 mm, V −6.6 mm and A −5.8 mm, L ±4.7 mm, V −6.6 mm (from bregma). The syringe was left *in situ* for 2 min after each injection. The number of cells injected was counted in a hemocytometer (Thoma chamber) and non-viable cells were identified with 0.05% Trypan Blue. The suspensions contained 50 000 cells/µl and at least 91% cells were viable. In our hands, the number of viable cells generally remains relatively stable for about 4 h after preparation of the suspension (J.-C.C. and C. Kelche, unpublished observations). Thus, cell suspensions were used within a maximum of 3 h after preparation. On each day, an equivalent number of rats were injected with the same solution in the dorsal or in the ventral hippocampus. Sham-operated rats (SHAM, $n = 16$) and lesion-only (SAPO, $n = 13$) rats were injected with 2 µl of 0.6% glucose saline at each of the four sites, half in the dorsal hippocampus, the other half in the ventral hippocampus.

Behavioral tests

Behavioral studies begun during the 18th week after grafting surgery and continued for approximately 3 months. They assessed spatial memory in both a water maze (using protocols placing emphasis on either reference or working memory) and a radial maze.

Morris water maze. This test, run from the 18th to the 20th week after grafting, was performed with two procedures, one placing emphasis on reference memory, the other on working memory. The Morris water maze consisted of a circular pool (diameter 160 cm, height 60 cm) filled with water to half the height. The water (22°C) was made opaque with powdered milk. The pool was located in an experimental room with many extra-maze cues (e.g. chair, computer, desk, cages, lights, pictures on the wall, fan, etc.) and was virtually divided into four equal quadrants with four starting points identified as north, east, south and west. A circular platform, 11 cm in diameter, was placed in the pool, 1 cm underneath the water surface. For each trial, the rat was placed in the pool, facing the wall at a randomly designed starting point from where it was released and given a maximum of 60 s to reach the submerged platform. When the rat had climbed onto the platform, it was allowed to remain there for 10 s before being removed and placed on the next starting point. If the rat failed to find the platform within 60 s, it was placed on it for 10 s by the experimenter. Using a video-tracking system (Noldus, The Netherlands), the latency to reach the platform and the distance swum by the rat were recorded for each trial.

Reference memory procedure. During 5 consecutive days, the platform was placed in the northwest quadrant. Each day, the rats were given four trials for which they were released from each starting point in a randomized order. When the third trial of the last day was completed, the platform was removed and all rats were given a probe trial for 60 s. The testing procedure used before the probe trial was considered to provide a measure of learning reflecting spatial reference memory, while the probe trial was considered to measure the strength of spatial learning.

Working memory procedure. During 10 other consecutive days, the platform was placed in a new location on each day. On each day, the rats were released from two different starting points, equidistant from the platform. This testing procedure is assumed to measure primarily working memory. On day 11, all

rats were subjected to the same testing procedure, but the platform was visible. The present protocol is at some variance with a protocol used in previous experiments (e.g. Balse et al., 1999) in which four consecutive trials were run on each day and a single starting point was used. In case of a four-trial protocol using a single start point, the performances recorded on the last two trials may be related to memory for procedural aspects of the task, a type of automatic processing in which repetition of a memorized pattern may be determinant for good performance, and in which striatal mechanisms rather than hippocampal ones may be relevant (e.g. Jog et al., 1999; White, 1997). Therefore, we have decided to use a testing procedure based on only two successive trials on each testing day.

Radial-maze test. Radial-maze training and testing were run using two identical gray polyvinylchloride radial-maze placed in an experimental room with several different visual cues placed around the mazes. The octagonal central platform was 40 cm in diameter. Arms radiating from the platform were 56 cm long and 10 cm wide, with a food well located 3 cm from the end of each arm. A 3-cm-high border was fixed to the arms and 30×20-cm walls were fixed to each arm entrance. Each maze was elevated 68 cm above floor level. In each maze, 16 infrared photocells (two per arm, one at 12 cm from the entrance and the other 10 cm from the end, with the infrared beam 4 cm above floor level) enabled the entries and movements of the rats to be followed. The sequence of photocell beam interruptions was monitored with a microcomputer and errors were defined as re-entries into already visited arms within a given trial.

The body weight of all rats was reduced progressively (over 10 days, starting during the 22nd week after grafting surgery) and subsequently maintained at about 80% of the free-feeding value. Water was available *ad libitum*. All rats were habituated to eat food pellets (45 mg, Noyes, distributed by Sandow Scientific, UK) in the maze on 5 consecutive days according to a training schedule described in detail by Jeltsch et al. (1994). Following training, all rats were tested for a series of 24 trials between the 24th and 27th week after grafting surgery.

Neurochemichal determinations

Thirty-two weeks after grafting, eight rats from each group were killed by microwave irradiation (2.0 s; 6.3 kW; Sairem, Villeurbanne, France) in order to rapidly inactivate brain enzymes such as acetylcholinesterase (AChE) (Stavinoha et al., 1973). After decapitation, the brain was extracted and dissected on a cold plate in order to collect both hippocampi that were separated into a dorsal (septal pole) and a ventral (temporal pole) portion. The left and right structures from each rat were pooled, weighed and kept at −80°C until neurochemical determination. Concentrations of acetylcholine (ACh) were measured using high-performance liquid chromatography (HPLC) with electrochemical detection. The tissue samples were prepared for HPLC by homogenization in 1 N formic acid/acetone (15/85, v/v), and the formic extracts were used for ACh determinations. ACh concentrations were measured after a purification of the formic acid extracts. This consisted in tetraphenylboron exchange of the amines in 3-heptanone, followed by 0.1 N HCl extraction (Beley et al., 1987). HPLC analysis was performed on a C18 Spherisorb ODS2 reverse phase column (3-μm pore size, 7 mm in diameter, 10 cm long). The mobile phase consisted of 0.05 M KH_2PO_4, pH 7, containing 600 mg/l of tetramethylammonium chloride and 25 mg/l sodium octane sulfate. The flow rate was 0.8 ml/min. ACh was converted into betaine and hydrogen peroxide in a post-column reactor with covalently bound AChE (EC 3.1.1.7.) and choline oxidase (EC 1.3.17.). The resulting hydrogen peroxide was detected electrochemically using a 5040 ESA cell working electrode at 0.3 V.

Concentrations of ACh were determined with data analysis software (Baseline 810, Waters) and were expressed as ng/mg microwaved tissue.

Histochemistry and evaluation of AChE density

The rats not killed for neurochemical determinations were injected with an overdose of pentobarbital (100 mg/kg, i.p.; Sanofi, France) and transcardially perfused with 60 ml of saline followed by 60 ml of 0.1 M phosphate-buffered paraformaldehyde (4°C, pH 8). After extraction, the brains were post-fixed for about 4 h and transferred into a 0.1 M phosphate-buffered 20% sucrose solution in which they remained for 36–40 h. The brains were then quickly frozen and cut into 30-μm-thick coronal sections using a cryostat (−23°C). From the anterior septum to the posterior region of the hippocampus, each fifth section was collected onto gelatin-coated slides. The sections were dried at room temperature for 36 h and stained for AChE according to a method similar to that described by Koelle (1954). Ethopropazine (0.3 mM; Sigma, St. Louis, MO, USA) was used to block non-specific cholinesterases and acetylthiocholine iodide (4 mM; Sigma) was used as the substrate.

The quantification of AChE-positive reaction products was assessed by an adaptation of a method described by Turchi and Sarter (2000). The various locations in which the measurements were made are shown in Fig. 1. In brief, an AChE staining index was obtained by measuring the exposure time (in seconds) indicated by a Vanox Olympus Microscope (model AHBT) when the apparatus was switched into the photograph mode (ISO 100, reciprocity: 0, exposure adjustment: 1, magnification: 10×5) and the objective was focused on different areas of the dorsal or the ventral hippocampus (see Fig. 1). Seven

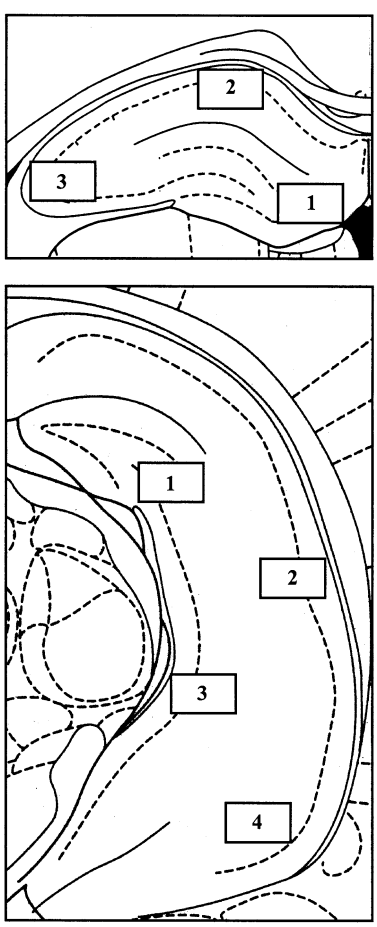

Fig. 1. Different areas (rectangles 1, 2, 3 and 4) in which the density of the AChE-positive reaction products were measured. All counts were taken bilaterally. The section drawings are from Paxinos and Watson, 1998.

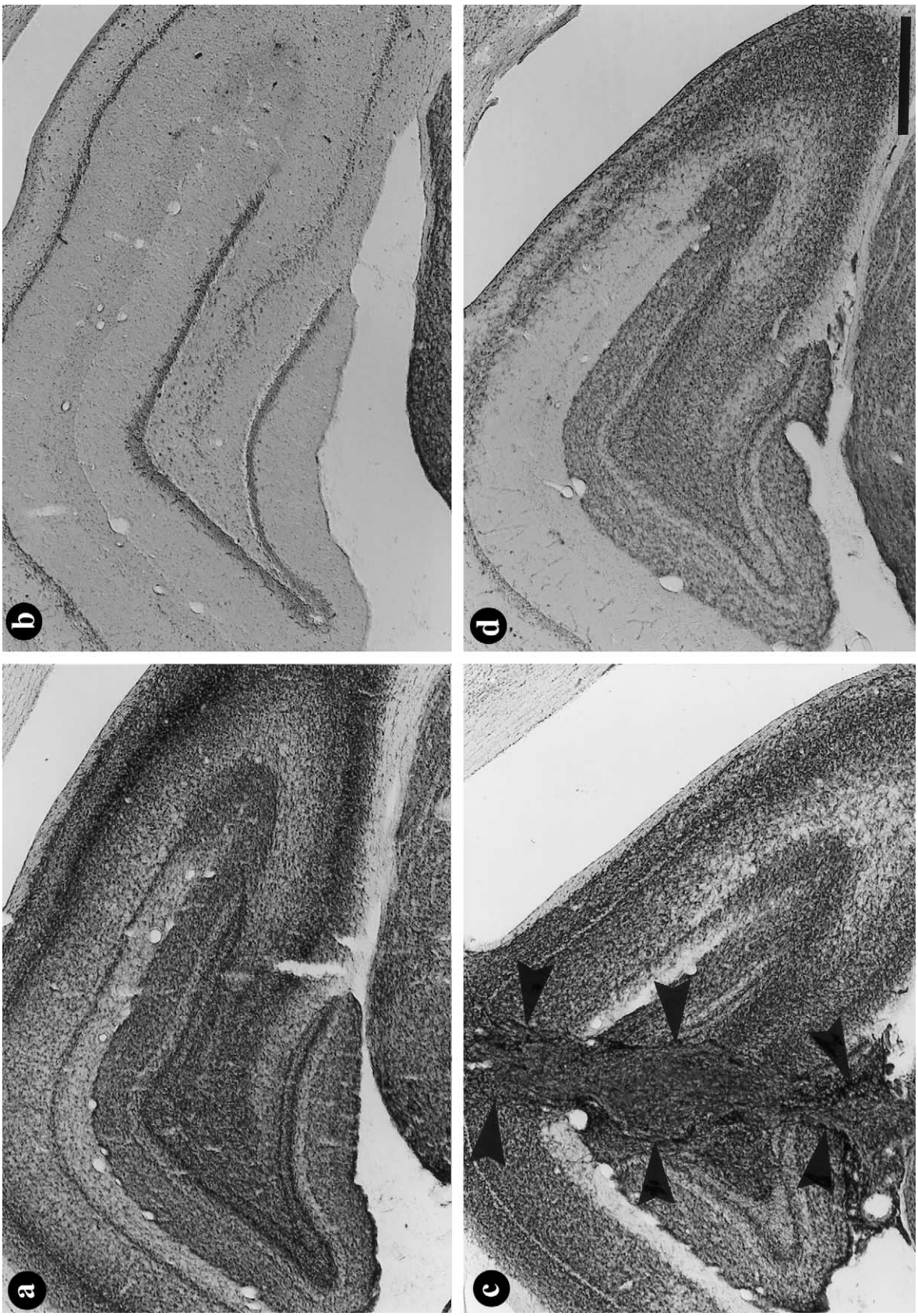

Fig. 2. Coronal sections through the dorsal pole of the hippocampus stained for AChE of SHAM (a), SAPO (b), SAPO+ DHG (c) and SAPO+VHG (d) rats. Group abbreviations are SHAM for sham-operated rats, SAPO for lesion-only rats, SAPO+DHG for lesioned rats with grafts in the dorsal hippocampus and SAPO+VHG for lesioned rats with grafts in the ventral hippocampus. The limits of the grafts are indicated by the arrowheads. There is no graft shown in (d) because the graft is located more ventrally. Note the weaker staining in (d) as compared to (c). Scale bar = 500 μm.

Table 1. AChE-positivity index (mean ± S.E.M.; arbitrary values) and percent difference from controls in the dorsal hippocampus (anteriority from bregma −3.3 mm) and the ventral hippocampus (anteriority from bregma −5.6 mm; Paxinos and Watson, 1998)

Group	Hippocampal region			
	Dorsal	% of SHAM	Ventral	% of SHAM
SHAM	0.55 ± 0.04	–	0.65 ± 0.07	–
SAPO	0.06 ± 0.01*	−89	0.10 ± 0.02*	−85
SAPO+DHG	0.46 ± 0.09#	−17	0.14 ± 0.03*	−78
SAPO+VHG	0.19 ± 0.04*,†	−66	0.50 ± 0.06#,†	−23

The values measured in each hippocampal subregion from each brain side (see Fig. 1) were all averaged in order to obtain one value per rat. Statistics: *significantly different from SHAM, $P < 0.05$; #significantly different from SAPO; †significantly different from SAPO+DHG, $P < 0.05$.

measures for each hemisphere were taken from the sections of each brain corresponding to a given anteriority. Three measures were taken at −3.3 mm and four at −5.6 mm posterior to bregma. A minimal and constant exposure time of 0.47 s was found in regions where there were no AChE-positive reaction products at all (i.e. in a totally denervated hippocampal subregion in the rats given 192 IgG–saporin). This value was considered a 'background' (section without AChE-positive staining, gelatin, slide and cover-glass) and subtracted from all measures before analysis. All measurements were made by the same experimenter in a single uninterrupted session.

Statistical analysis

All data were analyzed by an analysis of variance (ANOVA) followed, where appropriate, by 2×2 comparisons based on the Newman–Keuls multiple range test (Winer, 1971). Factors considered were the group (SHAM, SAPO, SAPO+DHG, SAPO+VHG) and, depending on the variable, the implantation region (dorsal, ventral), the testing day (1, 2, 3, 4, 5 in the water-maze reference memory test), the trial (1, 2 in the water-maze working memory test), and the trial block (six four-trial blocks in the radial-maze test). For analysis of the AChE density, data from the left and the right side were averaged, as no significant side effect could be evidenced. For each rat, only the mean of the three values in the dorsal hippocampus and of the four values in the ventral hippocampus was considered for analysis. For all analyses, the null hypothesis was rejected at $P < 0.05$.

RESULTS

Histochemistry

Representative examples of sections stained for AChE are shown in Fig. 2 (dorsal hippocampus) and Fig. 3 (ventral hippocampus). The lesions induced an almost complete decrease of AChE-positive reaction products throughout the entire hippocampus (see Figs. 2b and 3b). Rats subjected to intraseptal injections of 192 IgG–saporin exhibited depletions of AChE reaction products which were quite equivalent in the dorsal and the ventral hippocampus. It is also noteworthy that the variability across subjects was extremely small in the lesion-only group (SAPO), an observation that accounts for good reproducibility of the cholinergic lesions. Finally, a reduced AChE-positivity was also found in cortical regions, in particular in areas 1 and 2 of the frontal cortex, in the hindlimb and the forelimb area and in mainly area 1 of the parietal cortex. In the region of the nucleus basalis magnocellularis and the substantia innominata, the AChE-positivity was also reduced, but not abolished.

In the rats which received grafts in the dorsal hippocampus (Fig. 2), well delineated cell aggregates could be found in the dentate gyrus and the cornu Ammonis at about −3.5 mm posterior to bregma. They extended generally from about −2.0 mm to about −4.0 mm below the level of bregma. In some rats, the cluster extended through the corpus callosum to cortical areas overlying the injection sites. In the vicinity of the graft, there was an organotypic AChE-positive reinnervation pattern that covered the septal pole of hippocampus (Fig. 2c), but failed to reach more ventral regions (Fig. 3c). When the grafts were located or had extended into the cortex, it seemed that the cortical AChE-positivity was also increased in comparison with the equivalent cortical regions of the lesion-only rat with the largest cortical denervation, but as the cortical denervation was not found in all rats with lesions, it is not possible to draw any conclusion from these observations (graft-derived or weaker denervation of the cortex).

In rats that had received grafts in the ventral hippocampus (Fig. 3), well delineated cell aggregates could also be found, but they had developed in more posterior and more ventral regions of the hippocampus. The major part of the cluster was found at about −5.0 mm posterior to bregma and extended from about −5.0 mm to about −8.0 mm below the level of bregma. Again in the vicinity of the graft, there was a dense organotypic AChE-positive reinnervation pattern which covered the temporal pole of hippocampus (Fig. 3d), but the reinnervation was much more limited at the dorsal level (Fig. 3d, see also Fig. 2d).

These observations were confirmed by the quantitative analysis of the density of the AChE-positive reaction

Table 2. Concentrations of ACh (mean ± S.E.M. in ng/mg irradiated tissue) in the dorsal and the ventral hippocampus of rats from the four experimental groups used for neurochemical determinations

Experimental group	Dorsal hippocampus	Ventral hippocampus
SHAM	1.22 ± 0.08	1.36 ± 0.13
SAPO	0.43 ± 0.07*	0.62 ± 0.13*
SAPO+DHG	0.64 ± 0.09*	0.54 ± 0.09*
SAPO+VHG	0.57 ± 0.07*	0.71 ± 0.12*

Statistics: *significant lesion effect, $P < 0.05$.

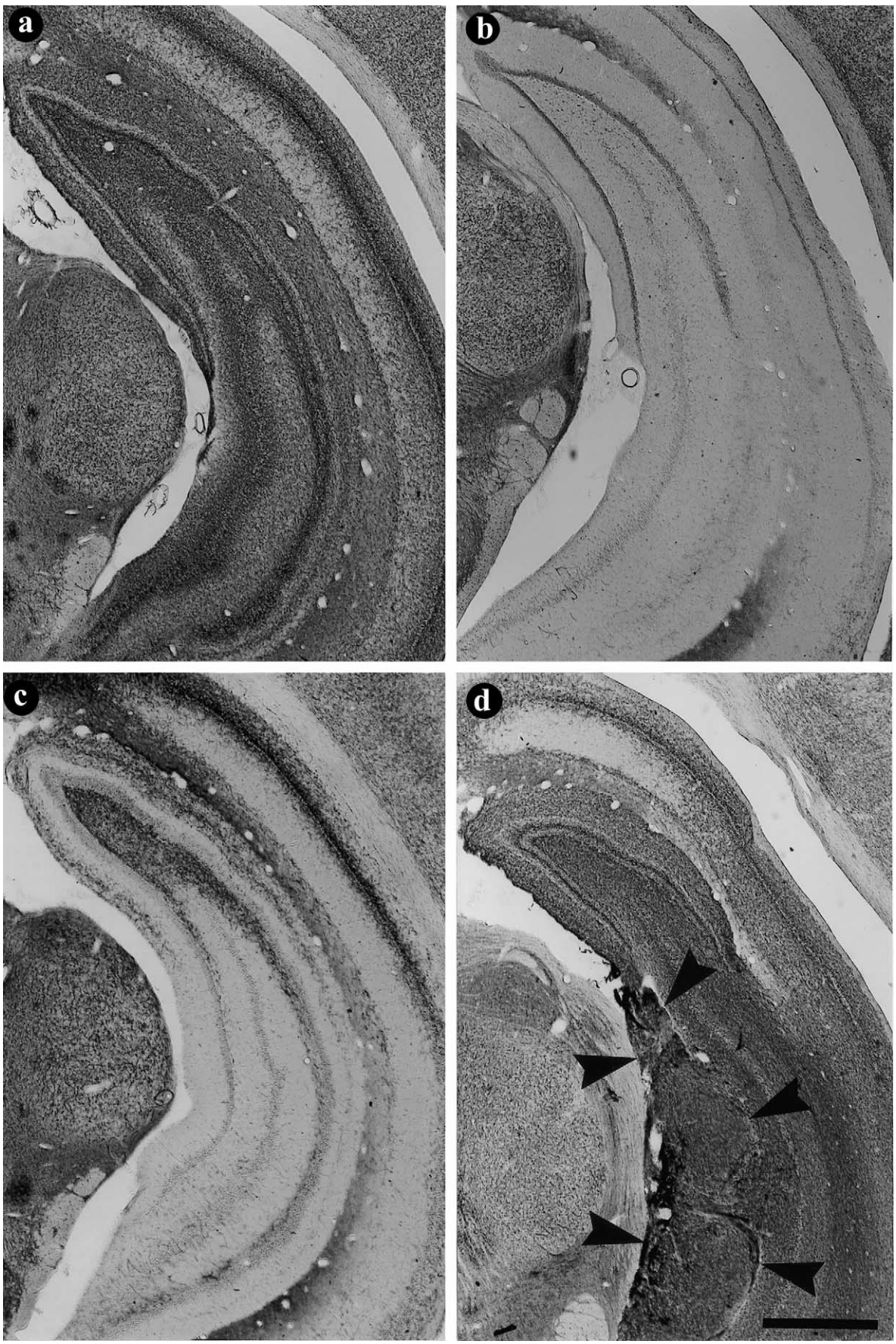

Fig. 3.

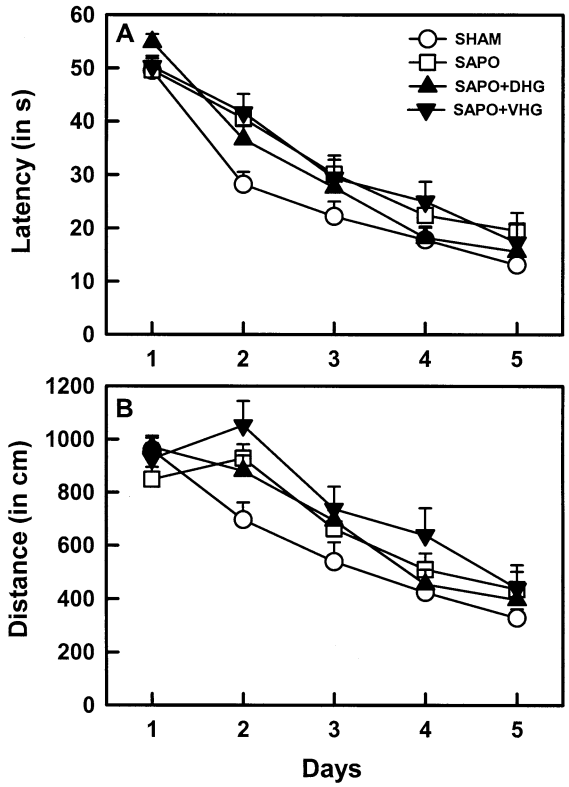

Fig. 4. Mean plus S.E.M. latencies (A) and distances (B) to reach the platform in the water-maze test performed according to a protocol placing emphasis on reference memory. Group abbreviations are SHAM for sham-operated rats, SAPO for lesion-only rats, SAPO+DHG for lesioned rats with grafts in the dorsal hippocampus and SAPO+VHG for lesioned rats with grafts in the ventral hippocampus.

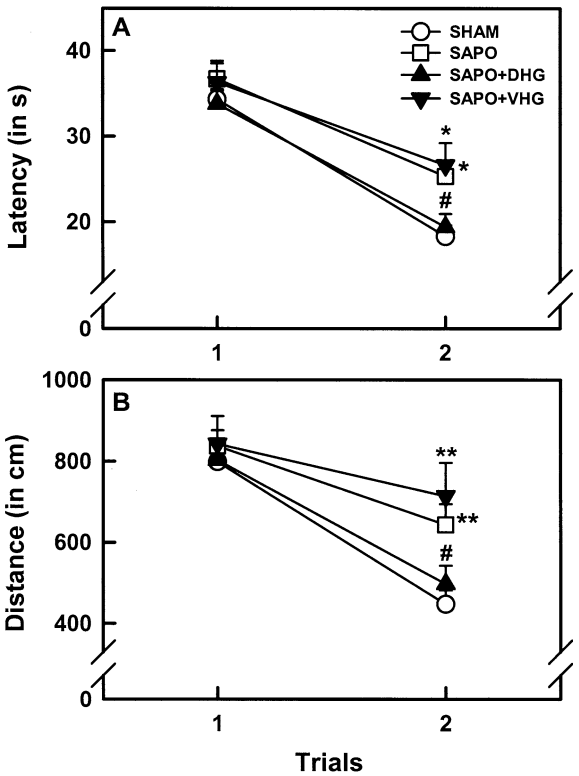

Fig. 5. Mean plus S.E.M. latencies (A) and distances (B) to reach the platform in the water-maze test performed according to a protocol placing emphasis on working memory. Group abbreviations as in Fig. 4. Statistics: significantly different from SHAM, $^{*}P<0.05$; $^{**}P<0.01$; significantly different from SAPO, $^{\#}P<0.05$.

products in the dorsal and the ventral hippocampus (see Table 1). In the dorsal hippocampus, the ANOVA showed a significant group effect ($F[3,20]=18.34$, $P<0.001$). This effect was due to an AChE-positivity which was significantly lower in SAPO and SAPO+VHG rats, as compared to either SHAM or SAPO+DHG rats ($P<0.001$, in all cases). The difference between SAPO+DHG and SHAM rats was not significant. In the ventral hippocampus, the ANOVA also showed a significant group effect ($F[3,20]=25.36$, $P<0.001$). This effect was due to an AChE-positivity which was significantly lower in SAPO and SAPO+DHG rats, as compared to either SHAM or SAPO+VHG rats ($P<0.001$, in all cases). The difference between SAPO+VHG and SHAM rats only tended to be significant ($P=0.09$).

Neurochemistry

Data are shown in Table 2. ANOVA of ACh concentrations showed a significant group effect ($F[3,28]=21.19$, $P<0.001$) which was due to a significant reduction (about −55%) in SAPO, SAPO+DHG and SAPO+VHG rats as compared to SHAM rats ($P<0.001$, in all cases). There was no significant difference amongst the three lesion groups, whether grafted or not. Also, there was no significant effect of the implantation region ($F[1,28]=2.41$), and no significant group×implantation region interaction ($F[3,28]=1.11$).

Behavioral tests

Water-maze test, reference memory. Data are shown in Fig. 4. ANOVA of the latencies showed a significant group effect ($F[3,51]=5.03$, $P<0.01$), a significant day effect ($F[4,204]=119.24$, $P<0.001$), but no significant interaction between both factors ($F[12,204]=1.19$). The group effect was due to overall latencies which were significantly longer in SAPO, SAPO+DHG and SAPO+VHG rats as compared to SHAM rats ($P<0.05$, in all cases). The day effect was due to overall latencies which decreased significantly from day to day ($P<0.05$, in all cases).

Fig. 3. Coronal sections through the ventral pole of the hippocampus stained for AChE of SHAM (a), SAPO (b), SAPO+DHG (c) and SAPO+VHG (d) rats. Group abbreviations as in Fig. 2. The limits of the grafts are indicated by the arrowheads. There is no graft shown in (c) because the graft is located more dorsally. Note the weaker staining in (c) as compared to (d). Scale bar = 500 μm.

Table 3. Time and distance (mean ± S.E.M.) in the different quadrants (Q1–Q4) during the probe trial of the water-maze reference memory test

Group	Quadrant			
	Q1	Q2	Q3 (PF)	Q4
Time (s)				
SHAM	11.7 ± 0.5	12.8 ± 0.9	24.5 ± 1.2	11.06 ± 1.0
SAPO	11.0 ± 0.9	16.5 ± 0.9	22.2 ± 1.3	15.25 ± 1.3
SAPO+DHG	9.9 ± 1.1	13.0 ± 1.2	25.6 ± 1.8	11.53 ± 1.6
SAPO+VHG	9.9 ± 0.8	10.9 ± 1.5	23.0 ± 1.8	16.15 ± 1.6
Distance (cm)				
SHAM	277.5 ± 13.7	322.5 ± 20.1	552.2 ± 23.6	277.8 ± 25.1
SAPO	244.7 ± 22.9	279.3 ± 20.9	493.4 ± 25.2	343.8 ± 24.7
SAPO+DHG	231.7 ± 29.4	303.0 ± 21.0	587.3 ± 39.2	294.1 ± 42.7
SAPO+VHG	234.8 ± 18.9	262.1 ± 36.1	552.3 ± 39.4	387.9 ± 33.6

The platform (PF) was located in Q3 during the acquisition trials.

ANOVA of the distances yielded a different picture: there was a significant group effect ($F[3,51] = 4.22$, $P < 0.01$), a significant day effect ($F[4,204] = 56.74$, $P < 0.001$), but no significant interaction between both factors ($F[12,204] = 1.36$). The group effect was due to overall distances which were significantly longer in SAPO+VHG rats as compared to SHAM rats ($P < 0.05$). Thus, there was no significant effect of the lesion. The day effect was due to overall latencies which decreased significantly from day to day ($P < 0.05$, in all cases).

Finally, in the probe trial (Table 3), there was no significant group effect, whether in terms of time spent in the probe quadrant or of distance swum ($F[3,51] = 1.01$ and 1.44, respectively). In other words, the analysis showed that the lesion did not alter reference memory performance consistently and that the strength of learning the platform location was not different among the four groups. In all groups did the rats exhibit a preferential search pattern in the appropriate quadrant of the pool (Q3).

Water-maze test, working memory. Data are shown in Fig. 5. ANOVA of the latencies showed a significant group effect ($F[3,51] = 4.78$, $P < 0.01$). The trial effect was also significant ($F[1,51] = 111.36$, $P < 0.001$), but the interaction between the two factors was not ($F[3,51] = 1.35$). The group effect was due to overall latencies which were longer in SAPO and SAPO+VHG rats ($P < 0.05$) as compared to SHAM rats. The trial effect was due to a decrease of the overall latencies in all second trials as compared to all first trials ($P < 0.001$, in all cases). Although there was no significant interaction between both factors, a visual inspection of the figure shows that the difference due to the group effect is mainly due to differences between the average performances of the second trials. When the four groups were compared according to the performances of the second trial, the group effect was still significant, and two by two comparisons showed that SAPO and SAPO+VHG rats were significantly impaired as compared to either SHAM or SAPO+DHG rats. The difference between SHAM and SAPO+DHG rats was not significant, and so was also the case for the difference between SAPO and SAPO+VHG rats.

ANOVA of the distances yielded a comparable picture. There was a significant group effect ($F[3,51] = 4.33$, $P < 0.01$), and a significant trial effect ($F[1,51] = 77.89$, $P < 0.001$), but the interaction between the two factors was not significant ($F[3,51] = 2.14$). The group effect was due to overall distances that were significantly longer in SAPO and SAPO+VHG rats as compared to SHAM rats ($P < 0.01$). The overall performances of SAPO+DHG rats did not differ significantly from those found in SHAM rats, but were significantly better than those of SAPO rats ($P < 0.05$). The trial effect was due to a decrease of the overall distances in all second trials as compared to all first trials ($P < 0.001$, in all cases). Also the between-group comparison of the performances found on the second trial yielded a picture similar to that found for latencies.

Water-maze test, visible platform. There was no significant group effect, whether on latencies or distances to reach the platform ($F[3,51] < 2.0$, data not illustrated).

Radial-maze test. Data are shown in Fig. 6. ANOVA of the number of errors showed significant group ($F[3,51] = 13.20$, $P < 0.001$) and trial block ($F[5,255] =$

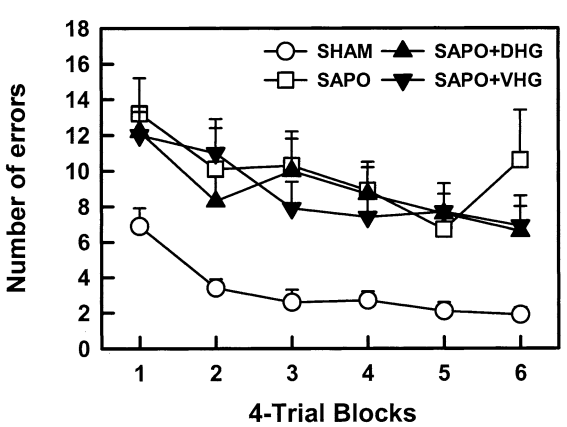

Fig. 6. Mean plus S.E.M. number of errors in the radial-maze task. Group abbreviations as in Fig. 4.

8.63, $P < 0.001$) effects, but no interaction between both factors ($F[15,255] = 0.7$). The group effect was due to an overall number of errors which was significantly higher in SAPO, SAPO+DHG and SAPO+VHG rats as compared to their sham-operated counterparts ($P < 0.001$, in all cases). There was no significant difference between the three lesion groups, whether grafted or not. The trial block effect was due to performances which improved significantly over trials, essentially over the trials comprised in the two first blocks. Analysis of the number of arms visited before the first error was committed yielded a statistical picture identical to that found for errors (data not illustrated).

DISCUSSION

Our results showed that intraseptal injections of 192 IgG–saporin induced a cholinergic denervation of the hippocampus, even though some damage was also found in cortical regions. The septal grafts provided the hippocampus with a new AChE-positive organotypic reinnervation pattern which was preferentially located in the hippocampal region into which the cell suspension had been implanted. In some rats which received the grafts in the dorsal hippocampus, the AChE-positivity was also increased in the cortex overlying the transplantation site (but a similar pattern was also found in some lesion-only rats). The lesions resulted in a more than 50% depletion of the concentration of ACh in the dorsal and the ventral hippocampus. But curiously the grafts failed to induce any significant effect on this depletion. Concerning cognitive functions, intraseptal injections of 192 IgG–saporin induced a consistent impairment of working memory in both the water maze and the radial maze. In the reference memory task, the latencies were increased by the lesion, not the distances. Only the grafts placed in the dorsal hippocampus induced a significant behavioral effect: they normalized working memory performances in the water-maze task. This observation accounts for a functional dissociation between the dorsal and ventral implantation sites of the cell suspension in some aspects of spatial working memory.

Effects of 192 IgG–saporin lesions

Consistent with previous reports, 192 IgG–saporin injected directly into the septal area induced preferential damage to the AChE-positive innervation of the hippocampus (e.g. Torres et al., 1994; Walsh et al., 1996; Wrenn and Wiley, 1998). The cholinergic depletion was confirmed by concentration of ACh, which was also reduced. The immunotoxin partially altered the AChE-positive innervation pattern of the cortex. This alteration might be due to intraparenchymal diffusion of the toxin to the nucleus basalis and the substantia innominata and/or, along the cannula track, to the cortex. Although limited, this non-specific damage was probably the consequence of an injection of a slightly excessive amount of 192 IgG–saporin and/or of some variability in the placement of the needle from one rat to another. The first interpretation is in line with previous observations showing that the neuroanatomical selectivity of the lesion may decrease when the amount of 192 IgG–saporin used increases (e.g. Pizzo et al., 1999, but see Sarter et al., 2000; Walsh et al., 1995; Wrenn and Wiley, 1998). Also, our histochemical and neurochemical data do not allow us to claim that the lesions induced by 192 IgG–saporin were specifically cholinergic, because we did not evaluate possible effects on other neurotransmitter systems.

According to previous reports (e.g. Dornan et al., 1997) and to our own experience with the immunotoxin, it may be assumed that the spatial working-memory deficits found in the present study were essentially the consequence of the cholinergic denervation of the hippocampus. It is interesting that injections of 192 IgG–saporin into the nucleus basalis magnocellularis in two strains of rats failed to alter spatial memory in the radial maze and the water maze (Galani et al., 2002; O. Lehmann, A. Grottick, C.L., J.-C.C. and G. Higgins, unpublished observations).

The reported cognitive effects of selective lesions of the basal forebrain cholinergic nuclei have been inconsistent. Reports show that intraparenchymal or i.c.v. injections produce no alterations of spatial memory processes (e.g. Baxter and Gallagher, 1996; McMahan et al., 1997), whereas others suggest that the induction of deficits may depend upon the amount of 192 IgG–saporin injected (e.g. Walsh et al., 1996). In the present study, intraseptal injections have been performed on four sites. The overall picture of our behavioral data suggests a spatial memory deficit in injected rats. However, these deficits seem to affect spatial working-memory more consistently than spatial reference-memory processes. This observation confirms a previous report on cognitive effects of i.c.v. injections of the immunotoxin (Lehmann et al., 2000). In the reference memory task, the lesion increased the latency to reach the platform, but did not significantly alter the distance, a variable considered less sensitive towards non-cognitive biases than the latencies (Lindner, 1997). Thus, it is suggested that spatial working memory is more sensitive to cholinergic disruption than reference memory, at least in a water-maze task. This possibility is in line with earlier, but also very recent work showing that working memory is more sensitive than reference memory to treatment with an antimuscarinic drug such as scopolamine (Beatty and Bierley, 1985; Lydon and Nakajima, 1992; Varvel et al., 2001; Wirsching et al., 1984).

Effects of the grafts

Many studies have been published on the effects of intrahippocampal grafts rich in cholinergic neurons after more or less extensive lesions denervating the hippocampus, but all these lesions lacked neurochemical selectivity (e.g. Cassel et al., 1997; Dunnett, 1990; Tarricone et al., 1996). Given the theoretical problems that accompany the absence of cholinergic selectivity (see Introduction and Leanza et al., 1998), it seems surprising that only a few experiments were designed to investigate

the effects of grafts rich in cholinergic neurons in a model of selective cholinergic lesions based on intraparenchymal or i.c.v. injections of 192 IgG–saporin. To our knowledge, beside the study by Leanza et al. (1998), only four other experiments using septal grafts were based on 192 IgG–saporin lesions. Two investigated the morphological or behavioral effects of grafts of porcine cells (Deacon et al., 1999; LeBlanc et al., 1999), and another one looked at seizure development after intrahippocampal grafts of septal cell suspensions prepared from the brain of rat fetuses (Ferencz et al., 1998). The fourth study (Leanza et al., 1996) was on rats subjected to lesions immediately after birth and, later on, to homotopic grafts (i.e. septal cells grafted into the septal region). Giving more details about these studies is beyond the scope of this discussion. Nevertheless, the major finding in these studies is that the grafts provided the hippocampus with a new cholinergic innervation, and that this reinnervation was able to produce functional effects. Based on our histochemical data, we may draw a similar conclusion as to the graft-induced reinnervation of the hippocampus. However, this reinnervation was not supported by an increased concentration of ACh in the hippocampal regions where the grafts were implanted. Such a discrepancy between histochemical and neurochemical effects of the grafts points towards several interpretations. First, it might be that ACh concentration, seldom used in studies investigating the effects of intrahippocampal grafts, is not the most appropriate marker for graft-induced cholinergic effects. However, such an explanation is probably wrong as we found clear-cut effects on this type of marker in a previous experiment with fetal septal cells transplanted into the hippocampus deprived of the fimbria–fornix pathways by aspiration (Balse et al., 1999). Second, it could be that the grafts eventually failed to induce the expected cholinergic effects and that the increased AChE-positivity found was associated with non-cholinergic processes. Such a finding would be possible because AChE is not found exclusively in cholinergic neurons (e.g. Eckenstein and Sofroniew, 1983; Satoh et al., 1983). However, that specifically the cholinergic neurons have failed to survive the transplantation in the present experiment remains somewhat enigmatic, although it cannot be excluded that residual 192 IgG–saporin has damaged this particular subpopulation of cells right after transplantation. Further progress along this line requires investigation of the half-life of the immunoglobulin–saporin complex after intracerebral injections. Indeed, the immunotoxin may be active for the first several days after injection (Advanced Targeting Systems, San Diego, CA, USA; D. Higgins, personal communication). A third possibility would be that cholinergic neurons have survived, but the cholinergic turnover was extremely high in the grafted cells, explaining why the activity of AChE was elevated, but also why the concentration of ACh was not significantly increased in comparison with the lesion-only rats. An opposite possibility might be considered as well, namely that of a decrease of the cholinergic tone in the hippocampus bearing the grafts. Due to the cholinergic selectivity of the immunotoxin, all hippocampal neurotransmitter systems other than the cholinergic one should be preserved by the lesion technique. Therefore, it cannot be excluded that part of these systems, which were usually damaged in other studies using non specific lesion models mentioned in the introduction (e.g. Cassel et al., 1997), have interacted functionally with the grafted cholinergic neurons to reduce the cholinergic tone in the hippocampus. To give just one example, in the hippocampus, serotonergic fibers may have contributed to a tonic activation of the inhibitory 5-HT_{1B} heteroreceptors present on the terminals of cholinergic neurons (e.g. Cassel et al., 1995). Should the effects of the grafts not be cholinergic, a final possibility to account for their behavioral consequences would be to consider a mechanism based on neurotrophic influences not targeted on cholinergic neurons, axons or terminals (e.g. Cassel et al., 1992). Further experiments based on functional markers more usual in the literature on cholinergic grafts (e.g. choline acetyltransferase activity or immunostaining, high affinity uptake of choline) should be a first step to progress on this issue.

Whether or not the effects of the grafts were cholinergic, our behavioral data clearly demonstrate that the grafts transplanted in the dorsal hippocampus normalized working memory performances in the Morris water maze, but had no effect in the radial maze. This test- and memory-specificity of the graft-induced effect might be questioned in terms of insufficient or inappropriate integration of the grafts. Such an explanation was provided by Leanza et al. (1998) to account for inconsistent effects of septal grafts on short-term memory capabilities, despite complete graft-induced recovery of water-maze performances in rats subjected to i.c.v. injections of 192 IgG–saporin. The demand on working memory is greater in the radial maze (eight arms) than in the water maze (one position of the platform for a short period of time). It is therefore possible that the graft-induced restoration may have been sufficient to produce behavioral effects in a simpler working memory task (find the platform in the water maze, a requirement that is constant within both trials given on each working memory testing day), but not in a more complex task (remember the arms visited in the radial maze, and thus information that changes while the rat is choosing arms on a given trial).

Our behavioral results also demonstrate that the effects of the grafts did not only depend upon the task in which they were assessed, but also upon the hippocampal region in which the cell suspensions had been transplanted. While working memory performance in the water maze was normalized by the grafts placed dorsally, it was not improved by those placed ventrally. This is an interesting observation which may be related to reports suggesting that the dorsal hippocampus has a preferential involvement in spatial memory (in comparison with the ventral hippocampus). Specifically, Jung et al. (1994) compared the spatial firing characteristics of CA1 neurons in the dorsal and the ventral hippocampus and found that, in the dorsal hippocampus, a greater number of neurons had place fields. In addition, the spatial selectivity of these neurons was better than that

found in the ventral hippocampus. In rats, Moser et al. (1993, 1995) have shown that lesions of the dorsal hippocampus impaired spatial memory while comparable lesions of the ventral hippocampus did not. Using a similar lesion technique, Bannerman et al. (1999) have confirmed the hypothesis of a functional dissociation along the septo-temporal axis of the hippocampus in rats. Vann et al. (2000) used a c-*fos* imaging approach in rats subjected to different spatial memory tasks. They found that the activity of c-*fos* during a task requiring learning of novel spatial landmarks increased in all hippocampal regions, but this increase was more pronounced in the dorsal as compared to the ventral portion of this structure. Even more recently, Ferbinteanu and McDonald (2001) have used a conditioned place–preference task, and thus a task for which normal performance requires an intact capability of acquiring spatial information, and found that while rats with lesions of the ventral hippocampus performed even better than controls, those with lesions of the dorsal hippocampus were impaired. The idea of a functional dissociation in the hippocampus along the septo-temporal axis seems also to apply to monkeys regarding the posterior (equivalent to dorsal) vs. the anterior areas of the hippocampus (Moser and Moser, 1998, for a review). Based on a completely different approach, we have provided additional support for such a view.

Conclusion

The present data show that selective lesions of the cholinergic neurons of the medial septum and the diagonal band of Broca, preferential targets of 192 IgG–saporin infused into the septal region, deprive the hippocampus of cholinergic innervation and impair spatial working memory more than spatial reference memory. The cholinergic nature of the graft-induced AChE-positive reinnervation is less clear-cut. Depending on the injection sites of the cell suspension, this reinnervation was mainly confined either to the dorsal or to the ventral hippocampus. Taken together, our results point towards a clear functional dissociation between the involvement of the cholinergic innervation of the dorsal and the ventral hippocampus in a water-maze working memory task: grafts placed dorsally not only ameliorated but even normalized working memory performances in this test, whereas those placed ventrally had no effect.

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