

Combined Damage to Entorhinal Cortex and Cholinergic Basal Forebrain Neurons, Two Early Neurodegenerative Features Accompanying Alzheimer's Disease: Effects on Locomotor Activity and Memory Functions in Rats

Natalia Traissard^{1,2}, Karine Herbeaux^{1,2}, Brigitte Cosquer¹, Hélène Jeltsch¹, Barbara Ferry¹, Rodrigue Galani¹, Anne Pernon¹, Monique Majchrzak¹ and Jean-Christophe Cassel^{*1}

¹Laboratoire de Neurosciences Comportementales et Cognitives, FRE 2855 CNRS, Université Louis Pasteur, IFR 37 Neurosciences, GDR 2905 CNRS, Strasbourg, France

In Alzheimer's disease (AD), cognitive decline is linked to cholinergic dysfunctions in the basal forebrain (BF), although the earliest neuronal damage is described in the entorhinal cortex (EC). In rats, selective cholinergic BF lesions or fiber-sparing EC lesions may induce memory deficits, but most often of weak magnitude. This study investigated, in adult rats, the effects on activity and memory of both lesions, alone or in combination, using 192 IgG-saporin (OX7-saporin as a control) and L-N-methyl-D-aspartate to destroy BF and EC neurons, respectively. Rats were tested for locomotor activity in their home cage and for working- and/or reference-memory in various tasks (water maze, Hebb-Williams maze, radial maze). Only rats with combined lesions showed diurnal and nocturnal hyperactivity. EC lesions impaired working memory and induced anterograde memory deficits in almost all tasks. Lesions of BF cholinergic neurons induced more limited deficits: reference memory was impaired in the probe trial of the water-maze task and in the radial maze. When both lesions were combined, performance never improved in the water maze and the number of errors in the Hebb-Williams and the radial mazes was always larger than in any other group. These results (i) indicate synergistic implications of BF and EC in memory function, (ii) suggest that combined BF cholinergic and fiber-sparing EC lesions may model aspects of anterograde memory deficits and restlessness as seen in AD, (iii) challenge the cholinergic hypothesis of cognitive dysfunctions in AD, and (iv) contribute to open theoretical views on AD-related memory dysfunctions going beyond the latter hypothesis.

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INTRODUCTION

Alzheimer's disease (AD) is characterized by various neuropathological changes, such as the formation of amyloid plaques, hyperphosphorylation of Tau protein, formation of paired helical filaments, by degeneration of various types of neurons among which the basal forebrain (BF) cholinergic ones have received major interest over the past 30 years (eg Bowen *et al*, 1976; Davies and Maloney,

1976; Farlow, 1998; La Ferla and Oddo, 2005), and by severe cognitive (mnemonic and attentional) deficits (eg Buckner, 2004). Diagnosis of AD, however, relies mainly upon the presence of amyloid plaques and neurofibrillary tangles in the limbic system and neocortical structures of the brain (Hyman and Trojanowski, 1997). Based on these major neuropathological aspects of AD, several transgenic animal models have been developed over the 10 last years in order to elucidate the pathogenic mechanisms leading to AD (German and Eisch, 2004; Gotz *et al*, 2004; Morishima-Kawashima and Ihara, 2002). More than 20 years ago, the cholinergic hypothesis of geriatric memory dysfunctions (Bartus, 2000; Bartus *et al*, 1982; Perry *et al*, 1978) proposed that cholinergic alterations in the BF accounted for cognitive impairments related to aging and AD. Although supported by experimental data in aged animals (eg Fisher *et al*, 1989), this view has been challenged recently by the failure of selective cholinergic lesions in the BF to induce memory deficits as predicted by this hypothesis (see Parent

*Correspondence: Dr J-C Cassel, Laboratoire de Neurosciences Comportementales et Cognitives, FRE 2855 CNRS, Université Louis Pasteur, IFR 37 Neurosciences, GDR CNRS 2905, 12 rue Goethe, F-67000 Strasbourg, France, Tel: + 33390241952, Fax: 33390241958, E-mail: jean-christophe.cassel@psycho-ulp.u-strasbg.fr

²These authors contributed equivalently to the work.

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and Baxter (2004) for a review; Dornan *et al* (1997), Torres *et al* (1994), Wenk *et al* (1994) for early research reports), especially in rats, although such lesions were shown to induce dramatic impairments in attentional performance (Baxter *et al* (1997), Chiba *et al* (1995), McGaughy *et al* (1996) for a review; see Wenk, 1997). Furthermore, patients suffering from either mild cognitive impairment (MCI), an early manifestation of age-related neurological disease (Bennett *et al*, 2005), or mild AD, fail to exhibit reduced cholinergic markers in the cortex (eg Davis *et al* (1999), Dekosky *et al* (2002); see also Frölich, 2002; Terry and Buccafusco, 2003). Finally, the first evidence of AD-related degeneration appears in the entorhinal cortex (EC), but not in the BF (Palmer, 2002; Pennanen *et al*, 2004). Thus, cholinergic depletion in cortical structures is probably not the earliest neurodegenerative event in AD (DeKosky *et al*, 2002; Gilmor *et al*, 1999; Mesulam, 2005) and, as recently emphasized by Mesulam (2005), is not the principal correlate of the clinical manifestations of the disease. Degenerative processes in the EC can even be considered the earliest signs of AD (Gomez-Isla *et al*, 1996; Pennanen *et al*, 2004; Stoub *et al*, 2005), good predictors of conversion from MCI to AD (deToledo-Morrell *et al*, 2004), and one potentially important cause of memory dysfunctions (eg Du *et al*, 2001, 2003; Rodrigue and Raz, 2004). In rodents, however, fiber-sparing (unlike more conventional) EC lesions generally induce minor or no effects on memory performance, particularly in spatial tasks (eg Bannerman *et al*, 2001). This view has been recently challenged in terms of lesion location when Steffenach *et al* (2005) demonstrated that lesions encroaching onto the dorsolateral band of the EC, contrary to many formerly performed lesions that only encroached onto more ventral EC portions, were actually able to alter spatial learning.

As there is evidence not only for cholinergic degeneration, but also for EC damage in the brain of AD patients, it seems reasonable to hypothesize that both alterations might conjointly, perhaps even synergistically participate in memory dysfunctions. In other words, coincident degeneration in both systems might account for the memory deficits associated with AD, more than degeneration in only one of them. This possibility may have important implications for our understanding of memory dysfunctions in AD, but appears impossible to test in humans. It can nevertheless be experimentally approached in animals.

To address this question in the rat, we used BF cholinergic neurons and EC lesions either in combination or separately. BF lesions were made by intracerebroventricular injections of 192 IgG-saporin such as to induce extensive though submaximal lesions of cholinergic neurons in both the basolateral and septohippocampal systems (Schliebs *et al*, 1996; Browne *et al*, 2001). EC lesions were performed using multiple sites L-N-methyl-D-aspartate (NMDA) injections to produce neuronal loss in the whole EC. The behavioral (activity and memory) consequences of these lesions were evaluated in a variety of tasks, between a few days and several months after surgery. As one of the earliest and most prominent cognitive features of AD is alteration of episodic memory—a faculty involved in remembering most recent events (eg Collie and Maruff, 2000)—we used a battery of tests in which rats had either to process information that remained useful only

for a short period of time (working memory) or to fix information remaining valid from day to day, and which is therefore likely to be consolidated (reference memory).

MATERIALS AND METHODS

Animals

Seventy-two adult male Long-Evans rats (300 g at the time of the first surgery; Centre d'Élevage R Janvier, Le Genest-St-Isles, France) were used. They were housed individually in transparent Makrolon cages ($42 \times 26 \times 15 \text{ cm}^3$) under controlled temperature (21°C) and a 12/12 h light/dark cycle (lights on at 0700 h). Food and water were provided *ad libitum*, except when testing required a restricted food diet. After arrival, the animals were allowed to acclimate to the laboratory conditions for a period of 2 weeks before surgery. All experimental procedures were conducted in conformity with the institutional guidelines (council directive 87/848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale; NIH publication, 86–23, revised 1985). Permission references were 67–251 for BF, 67–191 for RG, 6714-bis for HJ, 72–94 for MM, and 67–215 for J-CC; all other co-authors were under the responsibility of the formers.

Surgery

All surgical procedures were conducted under aseptic conditions by those authorized to do so.

Lesions of the EC. For EC lesions, the rats were anaesthetized using a intraperitoneal (i.p.) injection of pentobarbital (68.4 mg/kg; Ceva Santé Animale, Libourne, France) in saline (Laboratoire Aguettant, Lyon, France). Lesions of the EC were performed with multiple injections of small amounts of NMDA (40 mM in phosphate-buffered saline (PBS), pH 7.4). NMDA was injected through a thin glass micropipette ($1 \mu\text{m}$ at the extremity) connected via polyethylene tubing to a $10 \mu\text{l}$ Exmire microsyringe (ITO Corporation, Fuji, Japan) driven by an automated syringe pump (CMA model 100). The cannula was lowered into the brain at six sites according to the following coordinates from Bregma (Paxinos and Watson, 1998): *Site 1* A -5.6 mm , L $\pm 6.5 \text{ mm}$, V -8.2 mm ; *Site 2* A -6.3 mm , L $\pm 6.2 \text{ mm}$, V -8.2 mm ; *Site 3* A -6.3 mm , L $\pm 4.8 \text{ mm}$, V -8.4 mm ; *Site 4* A -7.0 mm , L $\pm 5.1 \text{ mm}$, V -8.0 mm ; *Site 5* A -7.6 mm , L $\pm 5.1 \text{ mm}$, V -7.0 mm ; *Site 6* A -8.3 mm , L $\pm 4.6 \text{ mm}$, V -5.0 mm . Injection volumes were $0.1 \mu\text{l}$ in site 1, $0.2 \mu\text{l}$ in sites 2 and 3, $0.3 \mu\text{l}$ in sites 4 and 5, and $0.4 \mu\text{l}$ in site 6. NMDA was injected at a flow rate of $0.2 \mu\text{l}/\text{min}$ at each site and the micropipette was left in place for 1–4 additional minutes before slow retraction. Surgery in control rats was similar except that no injection was made, but a micropipette was lowered at each site (ie in rats from SHAM, OX7, and SAPO groups; see below).

Lesions of BF cholinergic neurons. Five days after EC lesions, rats were anesthetized with i.p. injections of ketamine $100 \text{ mg}/\text{kg}$, 15 min after having received an i.p.

injection of diazepam 3 mg/kg. Lesions of the BF cholinergic neurons were produced with intracerebroventricular (i.c.v.) injections of the cholinergic immunotoxin 192 IgG-saporin (ATS, San Diego, CA; batch 24#87), using a 1 µg/µl PBS concentration (Heckers *et al*, 1994). Injections were performed stereotactically through a 10-µl Hamilton syringe according to Paxinos and Watson (1998), with the incisor bar of the stereotaxic frame set at the level of the interaural line. Injections into the lateral ventricles (5.0 µl/rat, ie 5.0 µg 192 IgG-saporin/rat) were administered at the following coordinates from Bregma: A -0.4 mm, L \pm 1.4 mm, V -4.3 mm (2.5 µl/side). After each injection, the needle was left in place for 5 min, retracted 2 mm, and a second delay of 5 min was allowed before complete retraction. This procedure was used to minimize aspiration of the toxin in the track during retraction of the needle. As one drawback of i.c.v. administration of 192 IgG-saporin is the damage of Purkinje cells in the cerebellum (eg Heckers *et al*, 1994; Waite *et al*, 1995) and possible accompanying alterations in sensory-motor function (eg Gandhi *et al*, 2000, Waite *et al*, 1999), additional groups with selective lesions of Purkinje cells in the cerebellum were included as controls.

Lesions of cerebellar Purkinje neurons. Rats were injected with 2.3 µg/µl/lateral ventricle OX7-saporin (ATS, San Diego, CA; batch 21-146a) using the same coordinates as for 192 IgG-saporin injections. OX7-saporin produces Purkinje cell damage but does not alter cholinergic neurons in the BF (Angner *et al*, 2000), and is used to control for behavioral effects of cerebellar damage (Waite *et al*, 1999).

Based on these surgical procedures, six experimental groups were constituted: rats given two sham operations (SHAM, $n=8$), EC lesions and a sham operation (EC, $n=15$), OX7-saporin lesions and a sham operation (OX7, $n=10$), 192 IgG-saporin lesions and a sham operation (SAPO, $n=11$), and rats subjected to EC lesions combined to either an OX7-saporin (EC + OX7, $n=14$) or a 192 IgG-saporin (EC + SAPO, $n=14$) injection.

Behavioral Testing

Locomotor activity in the home cage. Locomotor activity of the rats was measured in the home cage 12 days before and 12 days after the second surgery (see Table 1). The cages were placed on shelves (eight cages/shelf) located in a dedicated room. Two infrared light beams, passing through each cage, were targeted on two photocells, 4.5 cm above floor level and 28 cm apart. The number of cage crossings by the rats was recorded by a microcomputer over 21 h (5 h light, 12 h dark, 4 h light). Recording was started at the end of a 3-h habituation period which enabled the rats to habituate to the novelty of the test situation (eg Galani *et al*, 2001).

Morris water maze. This test used two procedures with a hidden platform, one placing emphasis on reference memory (which stores context-independent information such as rules on a long-term basis), and the other one on working memory (which deals with context-dependent information that remains valid for short periods of time). Subsequently, all rats were also tested with a

Table 1 Timeline for the Series of Experiments

Behavioral test or experimental step	Time (days) from second surgery (day 0)
Locomotor activity in the home cage	-12
First surgery (EC lesions)	-5
Second surgery (192 IgG-saporin injections)	0
Home cage locomotor activity	+12
Reference memory in the WM	30-35
Probe trial in the WM (24 h delay)	36
Visible platform test in the WM	37
Working memory in the WM	38-46
Reduction of body weight	47-57
H-W maze (training and testing)	57-77
Sensory-motor coordination in the beam test (training and testing)	94-99
Reduction of body weight	100-110
Radial maze (training and testing)	111-169
Euthanasia and histological verifications	180

Abbreviations: WM, water maze test; H-W, Hebb-Williams test; RM, radial maze test.

visible platform to check for possible motivation or sensory-motor biases.

The Morris water maze consisted of a circular pool (diameter 160 cm, height 60 cm) filled with water up to a height of 30 cm (22°C). Water was made opaque with powdered milk. The pool was located in an experimental room with many extra-maze cues (eg a chair, a computer, a desk, cages, lights, pictures on the wall, fan, etc) and was virtually divided into four equal quadrants with four starting points identified as north (N), east (E), south (S), and west (W). A circular platform, 11 cm in diameter, was placed into the pool, 1 cm beneath the water surface. For each trial, the rat was placed into the pool, facing the wall at a starting point designed randomly. The rat was given a maximum of 60 s to reach the escape platform. When the rat had climbed on it, it was allowed to remain there for 10 s before being removed and placed onto the next starting point. If the rat failed to find the platform within 60 s, it was placed on top of it for 10 s by the experimenter. The latency and the distance to reach the platform, as well as the swimming velocity were recorded for each trial, using a video-tracking system (Noldus, Wageningen, The Netherlands).

Reference memory procedure: During 5 consecutive days, the platform was placed in the northwest quadrant. Each day, the rats were given four trials in which they were released from four out of eight possible starting points (N, NE, E, SE, S, SW, W, NW) in a random order. On the sixth day (delayed probe trial), the platform was removed from the tank and all rats were given a 60-s probe trial. The testing procedure used before the probe trial (ie acquisition) is generally considered to provide a measure of spatial learning, whereas the probe trial is considered to measure the strength and precision of spatial memory.

Visible platform: After the reference memory test, the rats were tested on one single day, using a protocol with a visible platform. The platform, which protruded 1 cm above water level and was colored black to enhance its visibility, was placed in another location. Four successive trials, in which the position of the platform was not changed, were conducted in the same way as during the reference memory task. This testing procedure is considered to be sensitive to sensory-motor bias or lack of motivation to escape from the water.

Working memory procedure: During nine other consecutive days, the platform was placed in a new location each day, and the rats were released from each of two starting points on two consecutive trials separated by 10 s. The platform locations were those described by Jeltsch et al (2004a).

Hebb-Williams maze. The body weight of all rats was reduced progressively (over 10 days) and maintained at about 80% of the free-feeding value throughout testing. Water was available *ad libitum*. All rats were habituated to eat calibrated food pellets during weight reduction (45 mg, Noyes, distributed by Sandow Scientific, UK). The maze consisted of a square box ($75 \times 75 \text{ cm}^2$) made of black painted wood walls (height 12 cm) and covered with a transparent plastic top. The white floor was divided into 36 black-outlined squares, which allowed the experimenter to define error zones and to arrange appropriately the removable black walls (height 12 cm) used to build up the daily different maze patterns. Start and goal boxes ($40 \times 15 \times 12 \text{ cm}^3$) were located in two diagonally opposite corners and equipped with sliding doors. The illumination was provided by a 40-W white light placed centrally, 120 cm above the maze. Following a standardized procedure, the rats were pretrained over 10 days in the maze. During the first two days of pretraining, groups of two rats were placed into the maze (without walls inside) and were allowed to explore it freely for 10 min and to have access to food reinforcement in the goal box. During the remaining 8 days, six trials per day were performed using simple maze patterns. Following pretraining, all rats were tested on the standard series of 12 Hebb-Williams maze-learning problems (Rabinovitch and Rosvold, 1951), with one problem per day and six trials per problem. Initial and repetitive errors were recorded. An initial error was defined as the first entrance with at least the two forepaws into a given error zone of a blind alley on a given trial; repetitive errors were defined as further errors made in the same zone on the same trial.

Sensory-motor coordination in the beam-walking test. Rats were fed *ad libitum* during the delay separating the Hebb-Williams' test from the evaluation of beam-walking performance. Assessment of sensory-motor coordination was performed by placing each rat on a $2 \times 200 \text{ cm}^2$ wooden beam, divided into four 50-cm segments, elevated 80 cm above the floor level, and which was in contact with the home cage on one extremity. A net was spread out 15 cm beneath the beam in order to catch the rat when it slipped and fell from the beam. All rats were trained according to

the following protocol. On the first session (day 1), the rats were placed on the beam, 50 cm away from the goal box (ie their home cage) on five consecutive occasions. On the next session (day 2), the rats were placed 50, 100, 150 and 200 cm away from the goal box, successively, with only one run allowed for each distance. On the third session (day 3), the rats were placed twice 100 cm away and then twice 200 cm away from the goal box. On the fourth session (day 4), the rats were placed 200 cm away for three consecutive runs. Regardless of the session and the group, all rats always went to their home cage after they were released on the beam. Whenever a rat fell from the beam into the net, the experimenter gently placed it back on the beam, the head facing the home cage, on the exact place from where it had slipped. On the next day, all rats were tested for three consecutive trials as in the fourth session, and their performance was rated. For each virtual 50-cm segment of the beam, the experimenter rated the locomotor behavior a score of 1 per segment when the rat traversed the segment with all paws on the upper surface of the beam. Conversely, a score of 0 was given for each segment on which the rat slipped, placed its toes on the side surface of the beam or fell from the beam. The overall score was calculated by adding the scores of the three runs (maximal score = 12, ie four for each trial), and the interval between each run was of 5 s. For both training (days 1–4) or testing (day 5), there was no time limit for a rat to finish a trial or a session. In fact, a trial or a session was completed when the rat had reached the home cage after the last release on the beam. Generally, under the present training conditions and in our hands, 'normal' rats reached an average score of about 8 after 4 days of training. This score may further improve to slightly above 9 in the case of additional training days, but it rarely exceeds 10, even with intense training. The observer was not aware of the rat's treatment.

Radial maze. The body weight of all rats was reduced progressively (over 10 days) and subsequently maintained at about 80% of the free-feeding value for the whole testing period. Training and testing were run using two identical gray polyvinylchloride radial arm mazes placed in an experimental room with several different visual cues around the mazes (eg animal cages, stools, a trash basket, a curtain, etc). The maze, elevated 68 cm above floor level, had eight arms (60 cm long and 10 cm wide) radiating from a central octagonal platform (diameter 40 cm), with a concave food well located 3 cm from the end of each arm. A 3-cm-high border was fixed to the arms and $30 \times 20 \text{ cm}^2$ walls were fixed to each arm entrance. Guillotine doors controlled access to the arms. Sixteen infrared photocells (two per arm, one at 12 cm from the entrance and the other 8 cm from the end, with the infrared beam 4 cm above the floor level) enabled the entries and movements of rats to be followed. Sequences of photocell beam interruptions were monitored with a microcomputer.

Pretraining lasted 5 days. On the first day, only one arm was accessible and its food well contained eight pellets. On the second day, two adjacent arms containing four pellets per food well were accessible. For the following 3 days, three adjacent arms were accessible with two pellets placed into each food well. Following pretraining, all rats were tested

once a day for 32 trials. On a single trial, the rat was placed on the central platform with all guillotine doors open. Four arms were baited according to two different patterns: 1, 3, 4, 6 or 2, 5, 7, 8. The baited arms were always the same for each rat, but changed from one rat to another. The rats remained in the maze until all four reinforcements had been eaten or until 5 min had elapsed, whichever occurred first. The procedure used allowed to determine two types of memory (working and reference memory) failures within the same session. Reference memory supposes information that remains constant over time to be stored and used appropriately (ie the never-baited arms) and difficulties in reference memory are thus reflected by first visits of arms that were never baited. Working memory relies on information that remains pertinent only for a short period of time, and impairments in working memory are indicated by repeated entries into always baited arms that have already been visited within the ongoing trial, and all visits occurring in the never-baited arms after a first visit. The former type of errors is called working memory 'correct' errors, whereas the latter is termed working memory 'incorrect' errors according to Jarrard *et al* (1984).

Histological Verifications

Perfusion and preparation of tissue sections. After completion of all behavioral testing, each rat was given an overdose of sodium pentobarbital (100 mg/kg) and was transcardially perfused with 60 ml of phosphate-buffered 4% paraformaldehyde (pH 7.4; 4°C). The brain was then extracted, postfixed for 4 h in the same fixative (4°C) and transferred into a 0.1 M phosphate-buffered 20% sucrose solution for about 36–40 h (4°C). All brains were frozen using isopentane (−40°C), and subsequently kept at −80°C until sectioning. Coronal sections, 30 or 60 µm, were cut on a freezing microtome (−23°C) and, depending on the region of interest, were collected serially (from the septum to the nucleus basalis magnocellularis (NBM)) or according to a schedule in which four successive sections were discarded before one was collected.

Acetylcholinesterase histochemistry and cresyl violet staining. Sections, 30 µm in thickness, were collected onto gelatine-coated slides. These sections were dried at room temperature and stained either with cresyl violet, according to Klüver and Barrera (1953) in order to identify the extent of EC lesion sites, or for acetylcholinesterase (AChE) histochemistry according to Koelle (1954) to verify the extent of the cholinergic denervation in both the cortical mantle and the hippocampus.

Quantification of AChE-positive staining. The extent of cholinergic denervation was quantified by optical density (OD) measurements. Using a computer-assisted image analysis system (SAMBA Technologies, Meylan, France) coupled to a monochrome CCD digital Sony (Japan) video camera (Model XC 77CE) equipped with a 60 mm Nikkon objective (Nikkor) and a Triplux extension tube, the mean OD was measured on digitalized images after precise delineation of each brain region of interest (ie the somatosensory, piriform, retrosplenial, auditory and perirhinal, visual and entorhinal cortices, the amygdala, and

both the dorsal and ventral hippocampus). For digitalization, sections were placed on a Kaiser Prolite 5000 light box (Kaiser Fototechnik, Buchen, Germany). Magnification from section to computer screen was 2.5. The mean OD considered as a 'background' and subtracted from all measures before analysis was obtained from a value taken for each rat in the corpus callosum, where almost no AChE-positive reaction products could be identified. The experimenter performing the OD assessments was not aware of the rat's treatment. Owing to the obvious reduction of AChE positivity in rats given 192 IgG-saporin, it was nevertheless easy to distinguish at first sight sections from SAPO or EC + SAPO rats from all the other ones.

Anti-cholineacetyltransferase, anti-parvalbumine, and anti-calbindin immunostaining. Anti-cholineacetyltransferase (ChAT) immunostaining was used to visualize the effects of 192 IgG-saporin on cholinergic neurons in the septum, diagonal band of Broca, NBM, and as a control, for the selectivity of the toxin for BF cholinergic neurons, in the striatum. Anti-parvalbumine (Parv) immunostaining was used to control for possible effects of 192 IgG-saporin on GABAergic neurons in the septal region. Finally, anti-calbindin immunostaining was used to visualize the effects of both 192 IgG- or OX7-saporin on Purkinje neurons in the cerebellum.

For ChAT and Parv immunostaining, 60-µm-thick coronal sections through the septum and the basal nucleus were used. For calbindin immunostaining, 30-µm-thick coronal sections through the cerebellum from the same rats were used.

The sections were rinsed three times for 10 min in PBS (0.1 M, pH 7.4) containing 0.02% merthiolate (PBSM) before being soaked for 1 h in 5% normal donkey serum (BioWest, Nuaille, France) in PBSM containing 0.5% Triton X-100. The sections were then transferred without rinsing into the primary antibody solution, a goat polyclonal antibody directed against ChAT (1:500; Chemicon International, AB 144 P, Temecula, CA) or a mouse monoclonal antibody directed against Parv (1:4000; Sigma-Aldrich, P 3088, St Louis, MO) or a rabbit polyclonal antibody directed against calbindin (1:4000; Chemicon International, AB 1778, Temecula, CA). The incubation at room temperature for 18 h with the primary antibody was followed by three PBSM rinses. Omission of the primary antibody for some additional slices served as a negative control. Then, all the sections preincubated with the anti-ChAT primary antibody were soaked for 2 h in a buffer solution containing biotinylated donkey anti-goat antibody (1:500; Vector Laboratories International, AP 180 B, Burlingame, CA); those preincubated with the anti-Parv primary antibody were soaked in a buffer solution containing biotinylated horse anti-mouse antibody (1:500; Vector Laboratories International, BA 2001, Burlingame, CA); and those preincubated with the anti-calbindin primary antibody were soaked in a buffer solution containing biotinylated goat anti-rabbit antibody (1:500; Vector Laboratories International, BA 1000, Burlingame, CA). After three PBSM washes, the sections were incubated for 2 h in a standard avidin-biotin-peroxidase complex (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). The slices were then rinsed twice in PBSM and once in 0.6% Tris-buffer (pH = 7.6) and subsequently exposed to a solution of 0.0125% 3,3'-diaminobenzidine tetrahydrochloride

(Sigma-Aldrich, St Louis, MO) in Tris-buffer containing 0.0075% H₂O₂ until background staining saturation. Finally, after three PBSM rinses, the sections were mounted onto gelatine-coated slides, dried at room temperature, dehydrated and cover-slipped.

All the sections had pronounced cell staining with a little background. In all the sections processed without primary antibody, no staining was observed.

ChAT- and Parv-positive cell counting. To get an estimation of the lesion extent/selectivity induced by SAPO and OX7 alone or combined with EC lesion, it is worth emphasizing that only a limited number of sections were processed for immunostaining (two per region/antibody). Sections were obtained from seven randomly selected rats in groups SHAM, SAPO, EC + SAPO, OX7, and EC + OX7. Sections from EC rats were not immunostained, as we considered that information provided by material from OX7 and EC + OX7 groups should be sufficient to check for an effect of OX7 or/and EC lesions on cholinergic and GABAergic markers in the BF. Anatomical landmarks were used to select, define, and standardize the location of counting frames of a set size in the medial septum (MS), the vertical and horizontal limbs of the diagonal band of Broca (vDBB and hDBB, respectively), the NBM, and the striatum. For cell counting, we did not use an unbiased stereological method. In fact, counting was made on a particular section corresponding to an anteriority of Bregma +0.20 mm for counting of MS, vDBB, hDBB and striatal neurons, and Bregma -1.4 mm for counting of NBM neurons. The number of ChAT- and Parv-positive neurons was determined separately in the MS, vDBB, and hDBB (left and right) as illustrated in Figure 1. The MS was defined dorsally and laterally by the distribution of stained neurons, and ventrally by a virtual line joining the most dorsal level of the anterior commissures. The same line was used to define the dorsal limit of the vDBB. The ventral limits of the vDBB corresponded to a virtual line joining the medial and most dorsal part of each anterior commissure and the midline on the ventral edge of the section. Finally, the regions containing the hDBB (left and right) were delimited as follows: the ventrolateral limits of the vDBB also served as the dorsomedial limits of the hDBB, the ventral limits of the brain were used as ventral limits of this region, and virtual lines prolonging the lateral limits of the lateral ventricles towards the ventral edge of the section were used as lateral borders of this region. Care was taken not to count the neurons in the islands of Calleja. For cell counting in the striatum, a 3 × 3 mm² square area was defined as shown in Figure 1, and all ChAT- or Parv-positive neurons located in this area were counted in the left and right hemispheres. NBM ChAT-positive neurons were counted in the region shown in Figure 1; this region comprised both the NBM and the *substantia innominata*. Parv-immunoreactive neurons in this region were almost always weakly stained, sparse or rare, but were nevertheless counted.

Statistical Analyses

All data were processed using an analysis of variance for a single factor (ANOVA) or repeated measures (rANOVA). For the analysis of morphological data, only a Group factor

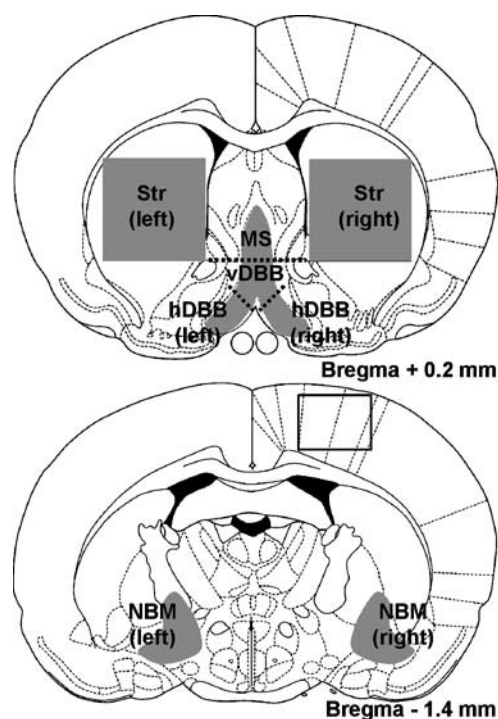


Figure 1 Diagram of coronal sections through the rat brain (at +0.2 mm and -1.4 mm from Bregma; according to Paxinos and Watson, 1998) showing the delimitation of the regions (grayish) in the septum (MS, vDBB, and hDBB), striatum (Str), and NBM in which ChAT-positive and Parv-positive neurons were counted. The rectangle in the cortex at Bregma -1.4 mm indicates the location and limits of the photographs shown in Figure 6 (AChE-stained sections, photographs g-l).

was considered. For the analyses of behavioral measures, factors considered were Group (SHAM, EC, SAPO, EC + SAPO, OX7, OX7 + SAPO for all analyses), Operation (before and after, for home cage activity), Day (1–5 for water maze reference memory), Trial (1–4 for water maze visible platform; 1, 2 for water maze working memory; 1–6 for Hebb-Williams maze), and Trial-block (1–9 for radial maze). When appropriate (ie in case of a significant effect of a factor or an interaction), multiple *post hoc* comparisons were performed with the Newman-Keuls test (Winer, 1971). All these tests were performed using the software Statistica 6.0 (Statsoft, Inc., Tulsa, OK).

RESULTS

EC Lesions

Lesions were drawn from transversal sections stained with cresyl violet. Typical (ie average, smallest, and largest) lesion extents are illustrated in Figure 2. Rats with only unilateral EC lesions, with lesions restricted only to the superficial layers of the EC, or with lesions encroaching bilaterally onto the three layers of the dentate gyrus were excluded from the study. A virtually complete lesion of the lateral and medial EC subdivisions was observed in 44% ($n = 14$) of the rats. In 34% of the rats ($n = 11$), the most rostral part of the lateral EC was preserved unilaterally, whereas in 19% of the rats ($n = 6$) it was preserved on both

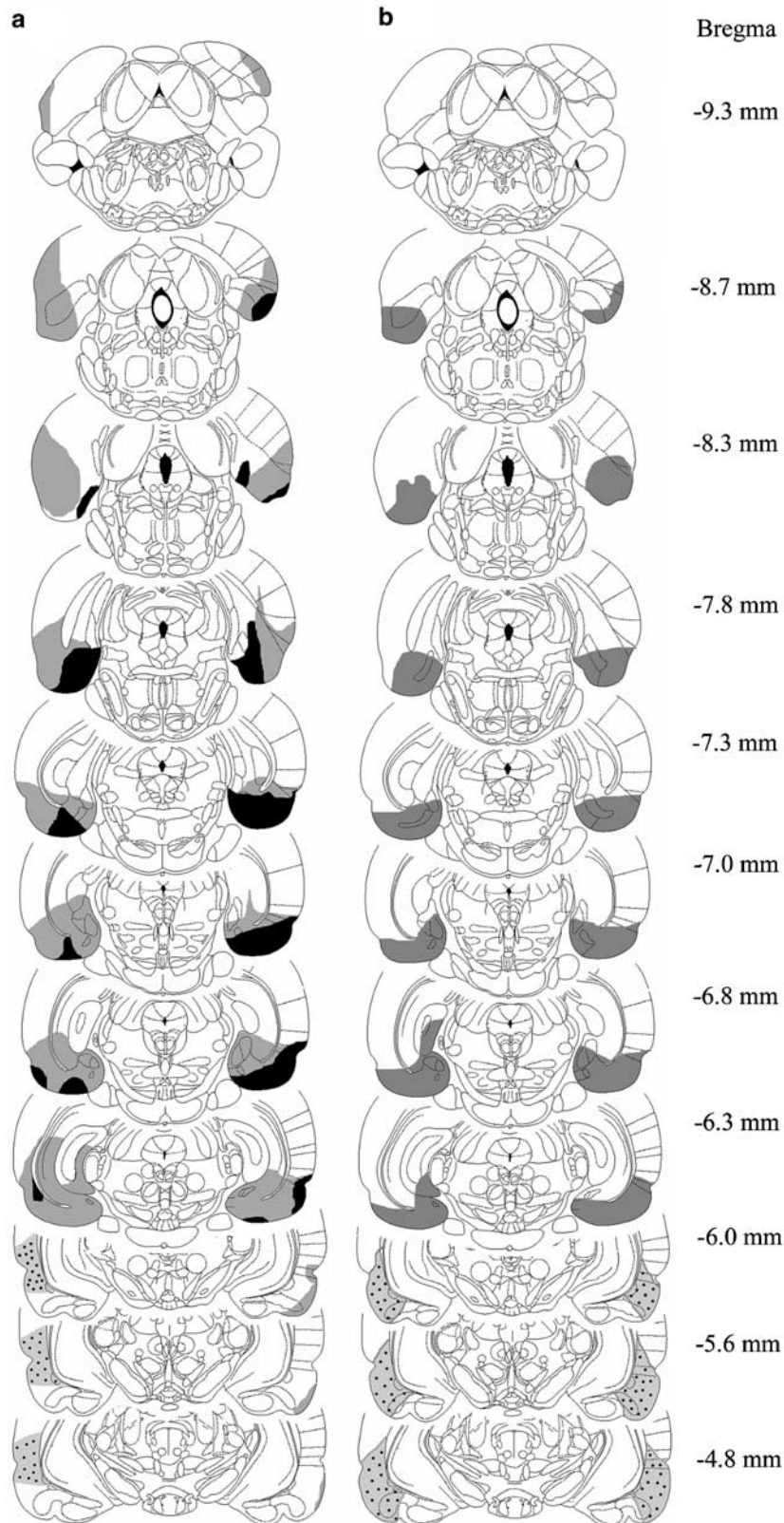


Figure 2 Diagram of coronal sections through the rat brain (between -9.3 and -4.8 mm from Bregma; according to Paxinos and Watson, 1998) showing the extent of the smallest (dark in a) and largest (gray + dark in a) EC lesion, or a typical lesion as was found in most rats (gray in b). The stippled grayish area corresponds to regions in which no particular evidence for cell loss was observed but where tissue shrinkage was clearly identified.

sides. In two rats, a small portion of the most posterior part of EC was preserved in the right hemisphere. In two other rats, this part was preserved bilaterally. Most lesions

involved the posterior part of the perirhinal and ectorhinal cortices (unilaterally in 38% of the rats, $n = 12$; bilaterally in 34% of the rats, $n = 11$), as well as onto the ventral

subiculum (unilaterally in 41% of the rats, $n = 13$; bilaterally in 38% of the rats, $n = 12$). Some lesions also included a very small portion of the outer molecular layer in the ventral hippocampus, predominantly unilaterally, in 12 rats (38% of the rats). In nine rats (28%), the track owing to the introduction of the injection cannula had caused visible although very limited neocortical damage. Most rats, whether lesioned or not, showed some enlargement of the lateral ventricles. This dilation was accompanied by some compression of neighboring tissue (Figure 2). The lesions' extents and variability were comparable in EC, EC + SAPO, and EC + OX7 groups. As Steffenach *et al* (2005) reported that the lesion of the dorsolateral band of the EC accounted for spatial memory deficits detected in a water-maze task, we also paid particular attention to this region of the EC. Indeed, the lesions encroached onto all or almost all of the dorsolateral band of the EC in all rats except two EC, one EC + SAPO and one EC + OX7 rat. In the latter animals, the damage was partial (<50%). These rats were kept for statistical analyses because, overall, but also particularly in the water maze, their scores did not appear to be marginal regarding the group means.

After exclusion of the rats with inappropriate lesions, the group sizes were as follows: SHAM $n = 7$, EC $n = 11$, SAPO $n = 11$, EC + SAPO $n = 9$, OX7 $n = 9$, and EC + OX7 $n = 12$.

192 IgG-Saporin and OX7-Saporin Lesions

ChAT-positive and Parv-positive neurons in the septum, NBM, and striatum. In rats subjected to 192 IgG-saporin injections, there was a clear loss of ChAT-positive neurons in both the septal region and the NBM (Figure 3). Such loss was not observed after OX7-saporin injections (Figure 3d, i, e, j), whether combined or not with EC lesions. Parv-positive neurons were observed in the septal region in all groups from which rats were used for immunostaining (Figure 4). In the striatum, many ChAT- (Figure 3k–o) and Parv-positive neurons (not illustrated) were observed, regardless of the lesion group considered.

Immunoreactive cell counting. For ChAT-positive neurons (Figure 5), the analysis considered the total number of neurons within each structure on a particular section (Septum = MS + vDBB + left and right hDBB; NBM = left + right NBM; Striatum = left + right striatum). The ANOVA showed a significant Group effect in the septum ($F_{(4,30)} = 50.34$, $p < 0.001$) and the NBM ($F_{(4,30)} = 15.50$, $p < 0.001$), but not in the striatum ($F_{(4,30)} < 1.0$). Multiple comparisons showed that the number of ChAT-positive neurons was significantly reduced in SAPO and EC + SAPO rats as compared to SHAM, OX7, or EC + OX7 rats ($p < 0.001$), whether in the septum or the NBM. The difference between SAPO and EC + SAPO rats was not significant.

Neurons immunostained for Parv were observed only occasionally within or in the vicinity of the NBM. They were always weakly stained. As a consequence of this staining and the uncertainty about the exact location of these neurons, counting was performed but not analyzed. In the septum and striatum, conversely, many densely stained Parv-positive neurons could be identified and counted. Examples of Parv-positive cells in the septum are shown in Figure 6. Cell counts (mean \pm SEM) were SHAM 114 ± 15 ,

SAPO 121 ± 41 , 374 ± 20 ; EC + SAPO 139 ± 22 , 372 ± 31 ; OX7 163 ± 40 , 349 ± 24 ; EC + OX7 186 ± 35 , 341 ± 17 , in the septum and striatum, respectively. There was no significant difference among the groups in either the septum ($F_{(4,30)} < 1.0$) or the striatum ($F_{(4,30)} < 1.0$). In the NBM of rats from SHAM, SAPO, EC + SAPO, OX7, and EC + OX7 groups, in average (\pm SEM), there were 2.6 ± 0.4 neurons per NBM region that were lightly immunoreactive for Parv (all subjects regardless of grouping). More precisely, for left and right NBM, cell counts (mean \pm SEM) were SHAM 2.7 ± 1.3 ; SAPO 6.5 ± 1.9 ; EC + SAPO 5.7 ± 1.2 ; OX7 2.5 ± 1.0 ; and EC + OX7 8.6 ± 1.0 .

AChE-staining and OD measurements. The i.c.v. injections of the toxin induced a strong depletion of AChE-positive reaction products in both the cortical mantle and the entire hippocampus, indicating damage to basolocortical and septohippocampal cholinergic neurons (Figure 6). Under the microscope, these changes appeared to be of comparable extent in SAPO and EC + SAPO rats. Compared to SHAM rats, EC, OX7, or EC + OX7 rats showed no detectable modification of AChE reaction products in all these regions, except that EC and EC + OX7 rats exhibited a denser staining in the medial and outer molecular layers of the dentate gyrus (Figure 6d, f). This denser staining was strongly reduced or not observed at all in EC + SAPO rats (Figure 6c). There was no clear-cut alteration of AChE-positivity in the thalamus or the striatum of SAPO or EC + SAPO rats (data not illustrated). 192 IgG-saporin-induced damage of septohippocampal and basolocortical projections was confirmed by both OD analyses of AChE-positivity. The ANOVA of the OD values (Table 2) showed significant Group effects in all regions ($F_{(5,52)} = 6.0$, at least; $p < 0.001$; see Table 2 for further details). These effects were due to values that were significantly lower in SAPO and EC + SAPO rats as compared to any of the four other groups ($p < 0.05$, at least). Whatever the region was considered, the differences between SAPO and EC + SAPO rats were not significant. This was also the case for differences between SHAM, EC, OX7, and EC + OX7 rats, except in the perirhinal and piriform cortices where the values in EC + OX7 rats were significantly lower than in SHAM rats ($p < 0.05$).

Purkinje cells in the cerebellum. Purkinje cells were visualized by anticalbindin immunohistochemistry, which is expressed in these cells in intact rats. Following 192 IgG-saporin or OX7-saporin injections, there was a clear-cut reduction of calbindin immunoreactivity in both the dorsal and ventral folia of the cerebellum, the effect being more pronounced in the dorsal one (Figure 7). This reduction was assessed only at a qualitative level. The inspection of the sections under a light microscope indicated that the cell loss was of comparable extent in SAPO (Figure 7b) and EC + SAPO rats (Figure 7c), and might have been slightly weaker in OX7 and EC + OX7 rats (Figure 7d, e).

Behavioral Data

Locomotor activity in the home cage. Locomotor activity recorded during the light phase was analyzed separately from that recorded during the dark phase. The rANOVA

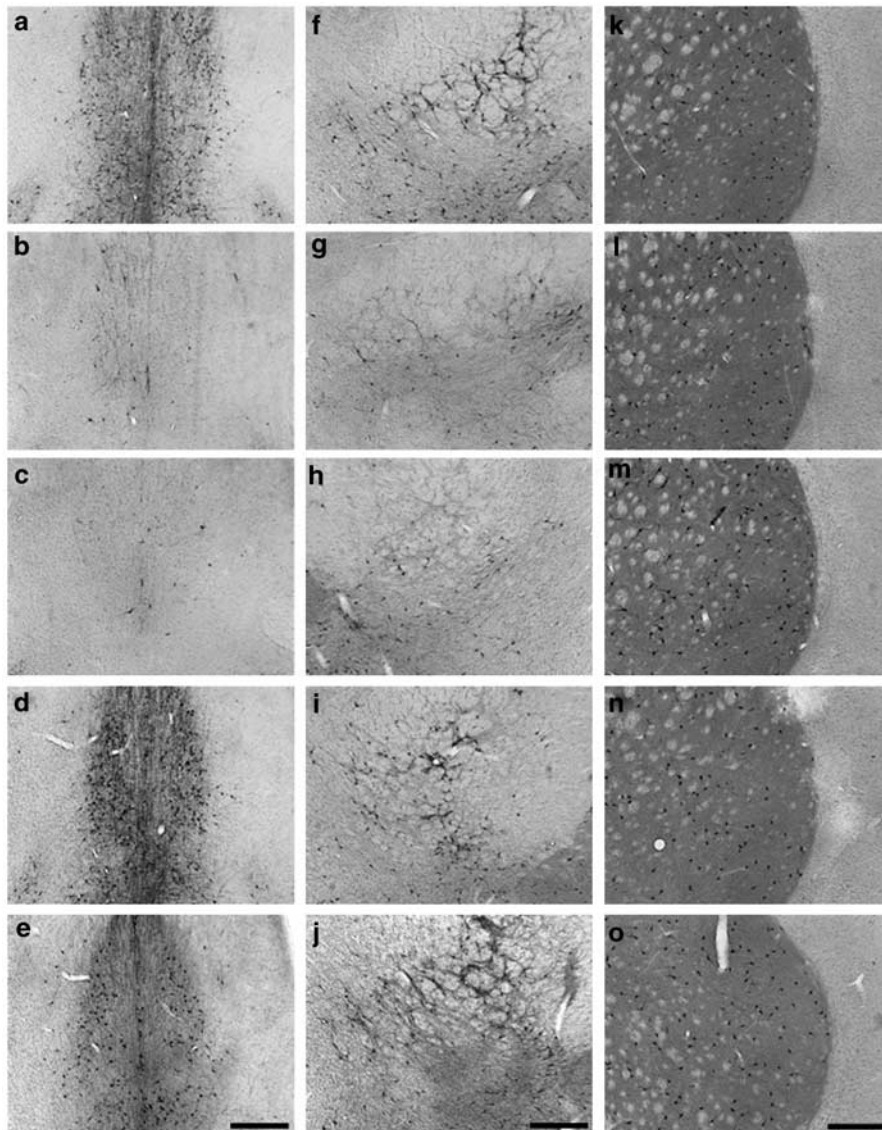


Figure 3 Microphotographs illustrating ChAT-immunoreactivity on coronal sections through the septum (a–e), the nucleus basalis (f–j), and the striatum (k–o) in rats subjected to sham operations (SHAM: a, f, k), injections of 192 IgG-saporin (SAPO: b, g, l), entorhinal cortex lesions and 192 IgG-saporin injections (EC + SAPO: c, h, m), entorhinal cortex lesions and OX7-saporin injections (EC + OX7: d, i, n), or OX7-saporin lesions (e, j, o). Note the reduction of ChAT-positive neurons in the septum and the NBM of rats subjected to 192 IgG-saporin injections, and their preservation in the septum of rats subjected to i.c.v. OX7-saporin injections. Sections from EC rats were not processed for anti-ChAT immunostaining. Staining in OX7 was comparable to that found in SHAM rats. Scale bar = 500 μ m.

indicated significant Group (Day: $F_{(5,53)} = 2.54$, $p < 0.05$; Night: $F_{(5,53)} = 5.03$, $p = 0.01$), Surgery (Day: $F_{(1,53)} = 42.46$, $p < 0.001$; Night: $F_{(1,53)} = 8.48$, $p < 0.001$) and Group \times Surgery interaction effects (Day: $F_{(5,53)} = 6.43$, $p < 0.001$; Night: $F_{(5,53)} = 7.56$, $p < 0.001$). Multiple comparisons showed that, after surgery, locomotor activity was higher in EC + SAPO rats as compared to all other groups, whether during the diurnal ($p < 0.05$) or the nocturnal period ($p < 0.001$). Other differences among groups were not significant, whether before or after surgery (Figure 8).

Sensory-motor coordination in the beam-walking test. The ANOVA showed a significant Group effect ($F_{(5,53)} = 14.83$, $p < 0.001$), which was due to dramatically reduced scores in rats given 192 IgG-saporin or OX7-saporin

injections, as compared to SHAM or EC rats ($p < 0.001$; Figure 9). The differences between SAPO, EC + SAPO, OX7, and EC + OX7 rats, or between SHAM and EC rats were not significant.

Morris water-maze task: reference memory. The rANOVA of the distances to the platform indicated significant Group ($F_{(5,53)} = 7.24$, $p < 0.001$), Day ($F_{(4,212)} = 53.30$, $p < 0.001$) and Group \times Day interaction effects ($F_{(20,212)} = 1.99$, $p < 0.001$). Overall performance was impaired in EC and EC + OX7 rats as compared to SHAM and OX7 rats ($p < 0.05$), and in EC + SAPO rats as compared to all other groups ($p < 0.05$). As shown in Figure 10, performance improved over trials in all but EC + SAPO rats: the only group in which performance on day 4 or 5 was not significantly better

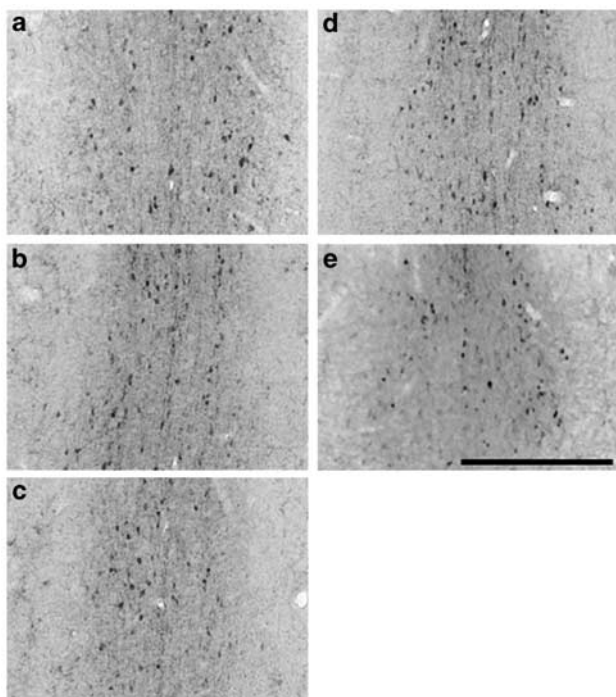


Figure 4 Microphotographs illustrating Parv-immunoreactivity on coronal sections through the septum of rats subjected to sham operations (a), injections of 192 IgG-saporin (b), lesions of the EC and 192 IgG-saporin injections (c), EC lesions and OX7-saporin injections (d), or injections of OX7-saporin (e). Note that neither lesion altered the Parv-positive staining pattern. Scale bar = 500 μ m.

than on day 1 was EC + SAPO ($p = 0.87$). Similar conclusions were drawn from the analysis of latencies to reach the platform (Group: $F_{(5,53)} = 6.64$; $p < 0.001$, Day: $F_{(4,212)} = 51.30$; $p < 0.001$, and Group \times Day $F_{(20,212)} = 2.05$; $p < 0.001$). Despite evidence for acquisition of the platform position in SHAM, EC, SAPO, OX7, and EC + OX7 rats, the probe trial given 24 h after the last testing day revealed that only SHAM and OX7 rats focused their research on the former location of the platform (Table 3); they spent 38.7 and 40.5% of the probe trial time, respectively, in the appropriate quadrant ($p < 0.05$ when compared to random, ie 25%; $t_{(6)} = 2.65$ and $t_{(9)} = 2.94$, respectively). A Group \times Quadrant ANOVA of the probe trial scores showed no significant overall Group effect ($F_{(5,53)} = 1.0$), but there was a significant overall Quadrant effect ($F_{(3,159)} = 7.32$, $p < 0.001$) as well as a significant Group \times Quadrant interaction ($F_{(15,159)} = 2.41$, $p < 0.01$). The Quadrant effect was due to the fact that, regardless of the surgical group, the rats spent significantly more time in the target quadrant than in each of the three other ones ($p < 0.05$ for each comparison). For the significant interaction, two by two comparisons showed that only SHAM and OX7 rats spent significantly more time in the target quadrant than in any of the three other quadrants ($p < 0.01$, in each case).

Morris water-maze task: visible platform. The rANOVA of distances and latencies (Figure 11) over trials showed a significant Trial effect ($F_{(5,53)} = 26.8$ and 3.79 , respectively, $p < 0.05$), owing to significantly higher values on the first as compared to subsequent trials ($p < 0.05$).

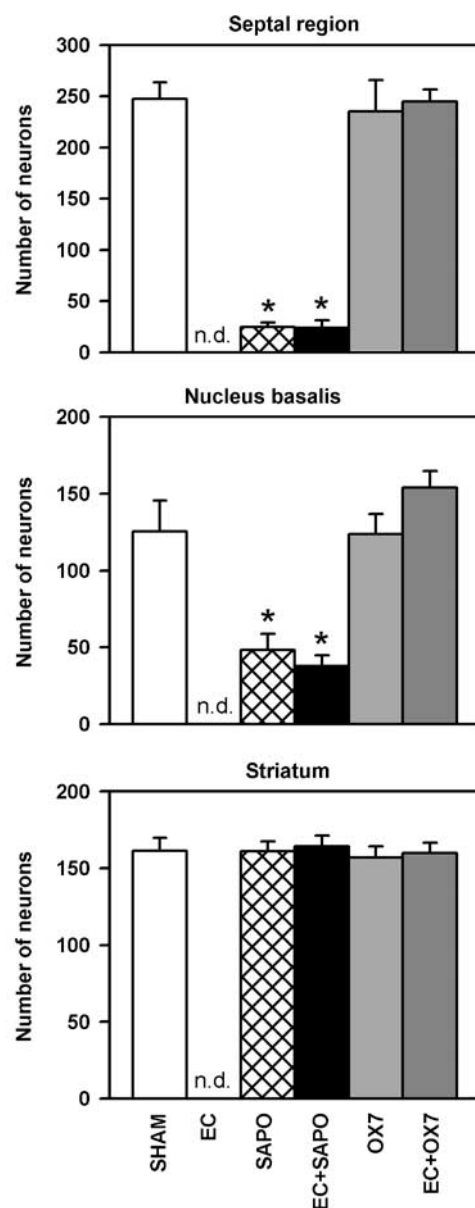


Figure 5 Number of ChAT-positive neurons counted in the septum (the entire structure as appearing on a transversal section located at Bregma +0.2 mm), nucleus basalis and *substantia innominata* (the entire structure as appearing on a transversal section located at Bregma -1.4), and striatum (an area of 9 mm² on a transversal section at Bregma +0.2 mm, as illustrated in Figure 1) of rats subjected to sham operations (SHAM), i.c.v. injections of 192 IgG-saporin (SAPO), or OX7-saporin (OX7), whether combined or not to EC lesions. Note that sections from EC rats were not processed for anti-ChAT immunostaining (ND). *Significantly different from SHAM, OX7, and EC + OX7, $p < 0.001$.

Group and Group \times Trial interaction effects were not significant.

Morris water-maze task: working memory. According to our experience, rats generally have some trouble in the acquisition of the new rule of a test when switching from the reference to the working memory task. Therefore, working memory performance for the first 4-day period was analyzed separately from that of the last 5-day period

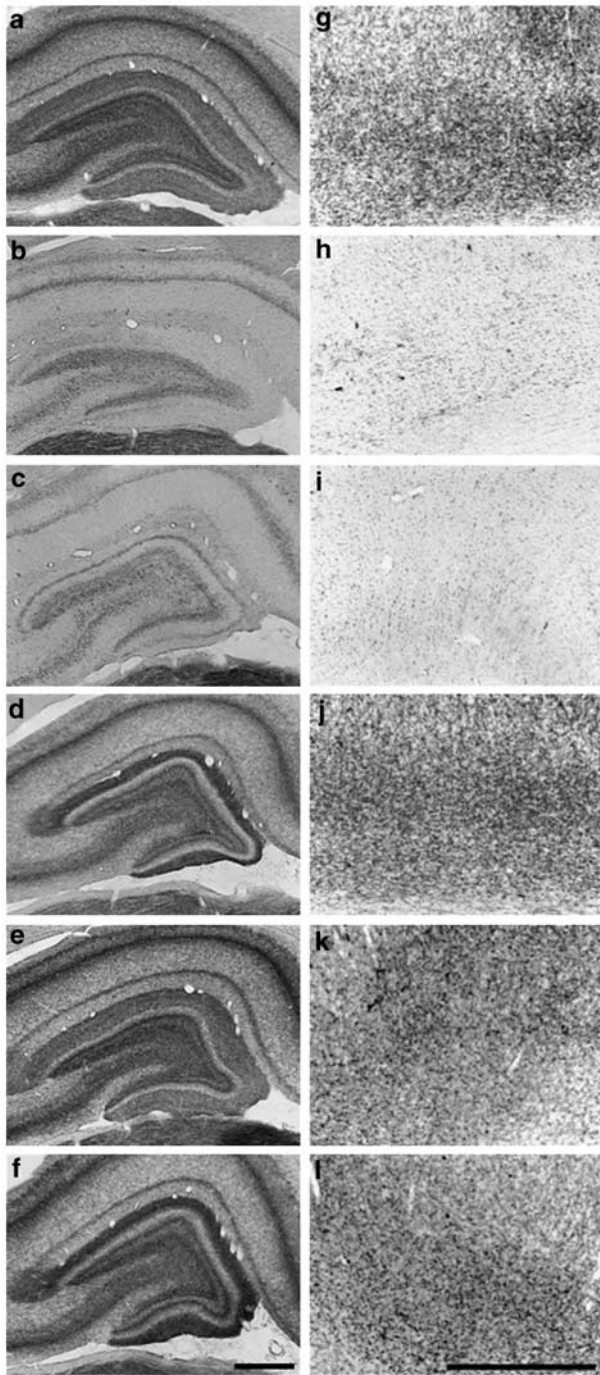


Figure 6 Microphotographs illustrating histochemical staining of AChE activity on coronal sections through the dorsal hippocampus (a–f) or the fronto-parietal cortex (g–l) in rats subjected to sham operations (a, g), injections of 192 IgG-saporin (b, h), entorhinal cortex lesions and 192 IgG-saporin injections (c, i), lesions of the entorhinal cortex and OX7-saporin injections (d, j), injections of OX7-saporin alone (e–k), or lesions of only the entorhinal cortex (f, l). Note the reduction of AChE-positive staining in the hippocampus and cortex of rats subjected to i.c.v. 192 IgG-saporin injections. The denser staining in the molecular layers of the dentate gyrus in (d) and (f) (and traces seen in c) corresponds to the septo-hippocampal cholinergic sprouting elicited by entorhinal lesions. Scale bar = 500 μ m.

(Figure 12). For latencies, the rANOVA only showed a significant Trial effect in the first period ($F_{(5,53)} = 41.51$, $p < 0.001$). For distances, the Trial effect was significant for

either period ($F_{(5,53)} > 21.66$, $p < 0.001$). From Figure 12, it appears that the absence of significant Group and Group \times Trial interaction effects was partly due to a rather high variability in performance. To refine the analysis, performance in each trial was compared within each group and for each period using a paired *t*-test. This analysis showed that none of the groups exhibited significant improvement in the first period. Conversely, performance improvement in the second period was significant, whether in terms of distance or latency in SHAM ($t_{(6)} = 3.88$ and 6.24, respectively; $p < 0.01$), SAPO ($t_{(11)} = 3.46$ and 2.25, respectively; $p < 0.05$), and OX7 rats ($t_{(9)} = 5.43$ and 4.00, respectively; $p < 0.01$), but not in the other three groups.

Hebb-Williams maze. The rANOVA of the number of initial errors (the first time an error is made in a given trial) showed significant Group ($F_{(5,53)} = 6.69$, $p < 0.001$), Trial ($F_{(5,265)} = 146.14$, $p < 0.001$), and Group \times Trial interaction ($F_{(25,265)} = 2.57$, $p < 0.001$) effects (Figure 13). EC + SAPO rats again showed the greatest deficit. Multiple comparisons revealed that the overall number of errors in EC + SAPO rats was significantly larger than in SHAM, SAPO, and OX7 rats ($p < 0.01$). Also, EC and EC + OX7 rats made more errors than SHAM rats ($p < 0.05$). There was a significant improvement over trials in all groups ($p < 0.05$), but this improvement was more marked in SHAM, SAPO, and OX7 rats than in the three other groups. When the number of repetitive errors was considered (all errors subsequent to the first one within the same trial), Group ($F_{(5,53)} = 9.23$, $p < 0.001$) and Trial ($F_{(5,265)} = 65.57$, $p < 0.001$) effects were significant, and the Group \times Trial interaction tended towards significance ($F_{(25,265)} = 1.50$, $p = 0.065$). The overall number of repetitive errors was significantly larger in EC + SAPO rats as compared to any of the five other groups. It was also significantly larger in EC as compared to SHAM, SAPO, and OX7 rats. The rANOVA of the number of total errors (initial and repetitive; not illustrated), showed significant Group ($F_{(5,53)} = 9.58$, $p < 0.001$) and Trial ($F_{(5,265)} = 127.18$, $p < 0.001$) effects, but no significant interaction between both factors ($F_{(25,265)} < 1.0$). EC + SAPO showed the greatest deficit, as their overall number of errors was significantly higher than in each of the five other groups ($p < 0.05$). EC and EC + OX7 rats made more errors than those from the SHAM, SAPO, or OX7 groups ($p < 0.05$). There was no significant difference between EC and EC + OX7 groups, as well as between SHAM, SAPO, and OX7 groups.

Radial maze. The rANOVA of the number of reference memory errors (Figure 14) showed significant Group ($F_{(5,53)} = 10.47$, $p < 0.001$), Trial ($F_{(8,424)} = 16.98$, $p < 0.001$), and Group \times Trial interaction ($F_{(40,424)} = 1.75$, $p < 0.01$) effects. The overall number of errors was significantly smaller in SHAM rats as compared to any of the five other groups ($p < 0.05$). In EC and EC + OX7 rats, the number of errors tended to be significantly larger than in OX7 rats ($p = 0.071$ and 0.078, respectively). The difference between SAPO and OX7 rats was significant ($p < 0.05$). Multiple comparisons revealed that a significant improvement of performance over trials was found only in the SHAM group: performance in trial blocks 6, 7, 8, 9 was significantly better

Table 2 Mean Optical Density (Arbitrary Units) of AChE-Positive Staining in Various Brain Structures

Brain region	SHAM	EC	SAPO	EC+SAPO	OX7	EC+OX7	F (1,52)
Somatosensory cortex	7.2±0.9	7.1±0.5	1.0±0.6*	1.1±0.4*	7.1±0.7	5.9±0.4	29.27 [#]
Piriform cortex	16.1±0.9	14.3±1.1	6.6±1.5*	5.6±1.0*	12.8±0.7	11.3±0.6*	15.59 [#]
Retrosplenial cortex	8.2±0.6	8.8±0.5	0.5±0.2*	0.9±0.3*	8.7±0.7	8.2±0.4	74.51 [#]
Auditory and perirhinal cortices	9.6±0.5	8.7±0.6	3.8±0.9*	2.7±0.6*	8.4±0.4	6.9±0.4*	20.69 [#]
Amygdala	15.0±1.0	14.2±1.0	10.2±0.9*	9.0±1.2*	14.6±1.3	13.9±0.7	6.00 [#]
Dorsal hippocampus	15.7±0.8	16.5±1.2	2.6±0.7*	2.0±0.6*	15.9±0.9	16.1±0.6	100.77 [#]
Ventral hippocampus	16.2±0.9	16.5±1.2	4.3±1.0*	3.8±0.8*	17.8±1.3	18.5±0.7	44.24 [#]
Visual cortex (V1, V2)	4.0±0.4	4.8±0.6	0.3±0.3*	0.1±0.1*	4.5±0.3	4.1±0.4	30.80 [#]
Entorhinal cortex	8.3±1.1	7.5±1.0	2.8±0.8*	3.1±1.1*	8.5±1.0	8.7±0.6	9.47 [#]

Data are presented as means±SEM and illustrate the effects of 192 IgG-saporin (SAPO), OX7-saporin (OX7) and entorhinal cortex (EC) lesions, separately or in some combinations (EC+SAPO, EC+OX7) as compared to sham-operated rats (SHAM).

Statistics: *significantly different from SHAM, $p < 0.05$; [#]significant overall group effect as shown by the ANOVA (F-value in the right column).

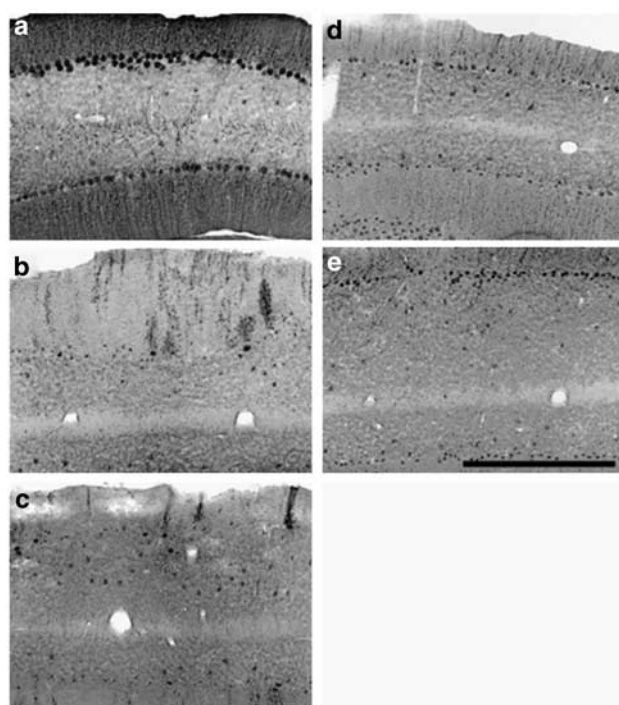


Figure 7 Microphotographs illustrating calbindin immunoreactivity on coronal sections through the cerebellum of rats subjected to sham operations (a), injections of 192 IgG-saporin (b), lesions of the entorhinal cortex and 192 IgG-saporin injections (c), entorhinal cortex lesions and OX7-saporin injections (d), or injections of OX7-saporin alone (e). Note the loss of calbindin-positive Purkinje cells in rats subjected to i.c.v. 192 IgG-saporin or OX7-saporin injections, whether combined or not to EC lesions. Scale bar = 500 μm.

than in trial blocks 1, 2, 3, or 4, and in trial block 5 compared to 1 ($p < 0.05$). The rANOVA of the number of working memory errors also showed significant Group ($F_{(5,53)} = 8.38$, $p < 0.001$), Trial ($F_{(8,424)} = 15.55$, $p < 0.001$), and Group \times Trial interaction ($F_{(40,424)} = 3.10$, $p < 0.001$) effects. Multiple comparisons showed that the overall performance of EC+SAPO rats was significantly impaired as compared to any of the five other groups ($p < 0.01$). EC made ($p = 0.05$) and EC+OX7 rats tended to make

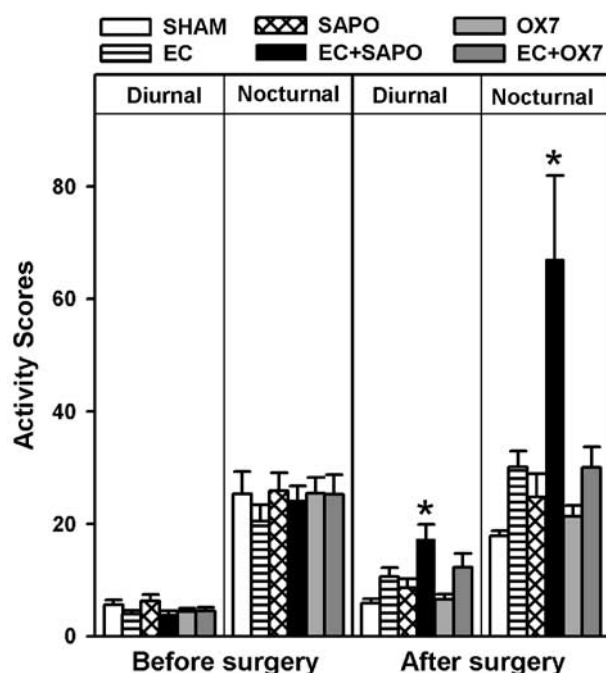


Figure 8 Home cage activity: average activity scores (+ SEM) during the diurnal and nocturnal periods of a 24-h cycle 12 days before (left) and 12 days after (right) the second surgery in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC+SAPO and EC+OX7 rats sustained combined lesions. Note the dramatic increase of nocturnal activity in EC+SAPO rats. *Significantly different from all other groups, $p < 0.05$ (diurnal) and $p < 0.001$ (nocturnal).

($p = 0.077$) more errors than SHAM rats. Because previous reports (eg Jarrard *et al*, 1984) distinguished working memory errors made in the always baited arms ('correct') from those made in the never baited ones ('incorrect', ie re-entries after an initial visit), we also performed an additional analysis on these errors separately from each other. Roughly, half the working memory errors were of the 'correct' type, the other half being of the 'incorrect' one. The outcome of the analyses was the same as when all working memory errors were analyzed together (data not illustrated).

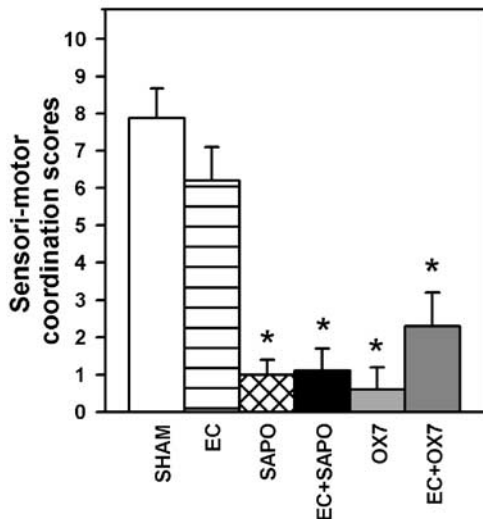


Figure 9 Beam-walking test: average sensory-motor coordination scores (+SEM; maximal score=12) in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC+SAPO and EC+OX7 rats sustained combined lesions. Note the dramatic alteration of sensory-motor coordination following injections of 192 IgG-saporin or OX7-saporin. *Significantly different from SHAM and EC groups, $p < 0.001$.

DISCUSSION

Our data, summarized in Table 4, show that rats subjected to EC lesions alone faced essentially two kinds of memory problems. First, they were less efficient in performing tasks taxing working memory processes with either high (radial maze and water maze) or low (Hebb-Williams maze) spatial constraints. Second, they also showed impaired anterograde memory capabilities in tasks in which the same information was presented repeatedly over a few (water maze) or even many days (radial maze); in the radial maze, however, rats had to deal with more items (four baited and four unbaited arms) than in the water maze (the platform location). Rats with lesions of basolateral and septohippocampal cholinergic neurons showed reference memory deficits, but only in the probe trial of the water-maze task (acquisition was normal but retention was impaired at 24 h) and in the radial maze (acquisition of a complex pattern was disrupted).

When both lesions were combined, some of the reference- and working memory deficits owing to EC lesions were larger than those observed with EC damage alone. With the exception of alterations in radial-maze reference memory performance, none of these deficits was mimicked or amplified by OX7-saporin, a lesion which, under circumstances, was previously shown to alter water-maze performance (Gandhi *et al*, 2000). Only rats given combined lesions showed marked hyperactivity in both the diurnal and nocturnal phases of the cycle. Both 192 IgG-saporin and OX7-saporin lesions induced sensory-motor deficits, which can be attributed to lesions of cerebellar Purkinje cells (Gandhi *et al*, 2000; Waite *et al*, 1999). Given the differences between SAPO, OX7, EC + SAPO, and EC + OX7 rats in cognitive performance, these sensory-motor deficits most probably did not bias the measures of cognitive alterations in our battery of tests.

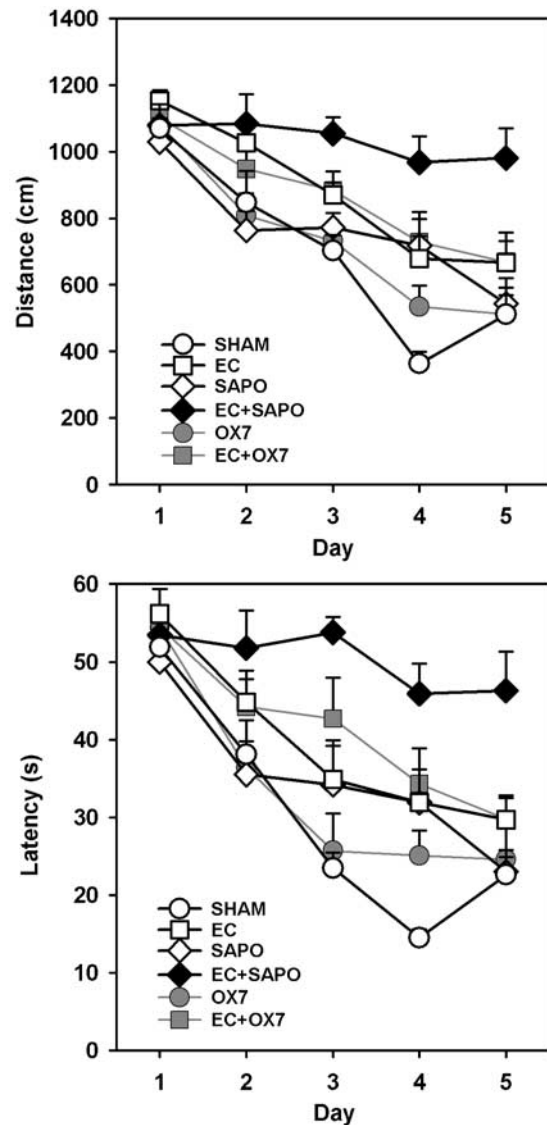


Figure 10 Water-maze reference-memory task: average distances (+SEM; top) and latencies (+SEM; bottom) to reach the platform in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC+SAPO and EC+OX7 rats sustained combined lesions. Rats were given four consecutive trials on each day with a constant platform location on all days. Note the absence of improvement in EC+SAPO rats.

EC Lesions and Spatial Memory

In rats subjected to EC lesions, the pattern of septo-hippocampal sprouting observed in the dentate gyrus (eg Naumann *et al*, 1997; see Phinney *et al*, 2004) confirmed that the lesion damaged the medial and the lateral parts of the EC. As the EC is the major source of hippocampal afferents from the neocortex (Witter and Amaral, 2004; Witter *et al*, 1989), such a lesion is assumed to disrupt hippocampal information-processing, crucial for spatial memory functions (Burgess *et al*, 2002; Shapiro and Eichenbaum, 1999). Our data showing that EC lesions impaired spatial memory are in agreement with findings based upon 'conventional' (aspiration, electrolysis) EC lesions (Aggleton *et al*, 2000), or electrophysiological approaches (eg Fyhn *et al*, 2004), and clearly support

Table 3 Time (s) Spent in Each of the Quadrants during the 60 s Probe Trial of the Water-Maze Task

Groups	Quadrants			
	Q1	Q2	Q3	Q4 (target)
SHAM	12.1 ± 1.8	11.8 ± 1.8	12.9 ± 1.5	23.2 ± 3.1*
EC	11.2 ± 1.7	18.6 ± 2.8	13.7 ± 1.1	16.5 ± 2.3
SAPO	10.0 ± 1.3	15.6 ± 1.8	17.0 ± 1.6	17.5 ± 1.6
EC+SAPO	16.8 ± 2.7	14.2 ± 1.5	16.7 ± 2.2	12.3 ± 2.3
OX7	11.2 ± 1.7	13.2 ± 1.4	11.3 ± 2.1	24.3 ± 3.2*
EC+OX7	13.1 ± 1.7	13.4 ± 0.9	17.1 ± 0.8	16.5 ± 1.5

Data are presented as means ± SEM in rats subjected to sham operations (SHAM), lesions of the entorhinal cortex (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC+SAPO and EC+OX7 rats sustained combined lesions. *Significantly different from random (15 s), $p < 0.05$.

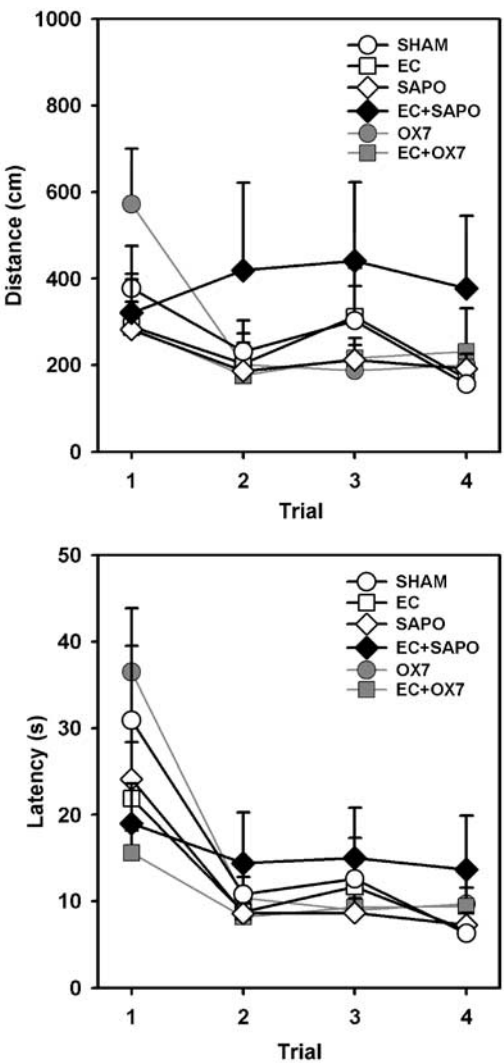


Figure 11 Water-maze task with visible platform: average distances (+ SEM; top) and latencies (+ SEM; bottom) to reach the platform in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC + SAPO and EC + OX7 rats sustained combined lesions. There was no significant difference between groups.

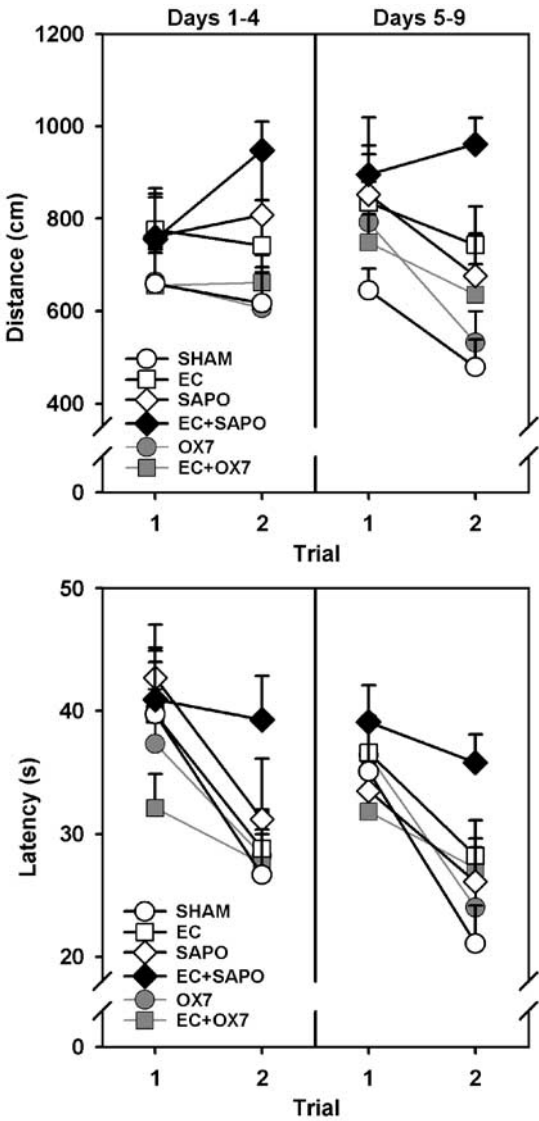


Figure 12 Water-maze working-memory task: average distances (+ SEM; top) and latencies (+ SEM; bottom) to reach the platform in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC + SAPO and EC + OX7 rats sustained combined lesions. Rats were given two consecutive trials on each day with the platform location changed from day to day. A transition phase in which rats had to acquire the new rule (days 1–4; left) was distinguished from the testing phase (days 5–9; right). Note the absence of improvement in EC + SAPO rats.

participation of this region in spatial memory. Until recently, however, it was considered that excitotoxic fiber-sparing lesions of the EC produced minor alterations in spatial learning (Aggleton *et al*, 2000; Pouzet *et al*, 1999), in contrast to the proposed role of the EC in linking the neocortex and the hippocampus during encoding and consolidation of information. This view has been challenged by Steffenach *et al* (2005), who showed that spatial learning was actually disrupted by fiber-sparing EC lesions, but only when the lesion encroached onto the dorsolateral band of the EC. These authors also demonstrated that other lesions encroaching onto the ventromedial band of the EC, the perirhinal or postrhinal cortex did not produce such effects.

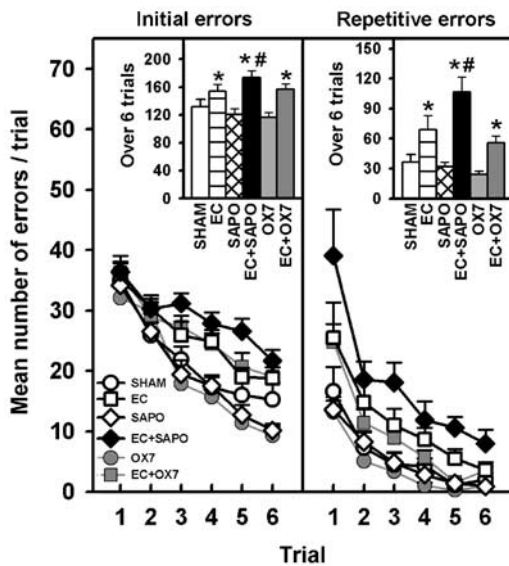


Figure 13 Hebb-Williams maze test: average number of initial (left) and repetitive (right) errors in the Hebb-Williams maze task in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC + SAPO and EC + OX7 rats sustained combined lesions. Note the larger number of repetitive errors in EC + SAPO rats. *Significantly different from SHAM, $p < 0.05$; #significantly different from all other groups, $p < 0.05$.

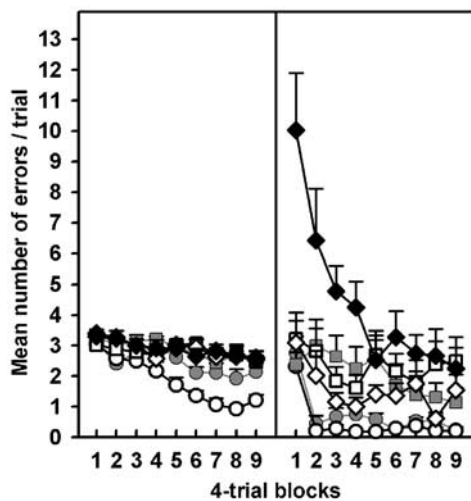


Figure 14 Radial arm-maze test: average number of reference memory (in never baited arms; left) and working memory (in always baited arms; right) errors in the radial-arm maze in rats subjected to sham operations (SHAM), entorhinal cortex lesions (EC), injections of 192 IgG-saporin (SAPO), or OX7-saporin injections (OX7). EC + SAPO and EC + OX7 rats sustained combined lesions. Note the larger number of repetitive errors in EC + SAPO rats. *Significantly different from SHAM, $p < 0.05$; #significantly different from all other groups, $p < 0.05$.

The authors suggested that the absence of any alterations on spatial memory described in previous studies were due to lesions that spared partly or entirely this dorsolateral band of the EC. Our results in EC and EC + OX7 groups, in which rats also exhibited damage in the dorsolateral part of the EC, are in line with the conclusions drawn by Steffenach *et al* (2005). Despite repeated testing, EC and EC + OX7 rats

Table 4 Summary Table of the Behavioral Effects of the Different Experimental Treatments

Variable/group	EC	SAPO	EC+SAPO	OX7	EC+OX7
Diurnal activity	—	—	h	—	—
Nocturnal activity	—	—	h	—	—
Sensory-motor coordination	—	+	+	+	+
Reference memory in WM	+	—	++	—	+
Probe trial in WM (24 h delay)	+	+	+	—	+
Visible platform	—	—	—	—	—
Working memory in WM	+	—	+	—	+
Initial errors in H-W maze	+	—	+	—	+
Repetitive errors in H-W	+	—	++	—	—
Total errors in H-W	+	—	++	—	+
Reference memory in RM	+	+	+	+	+
Working memory in RM	+	—	++	—	(+)

Abbreviations: —, no effect; h, hyperactivity; +, deficit; ++, deficit significantly larger than in rats subjected to entorhinal cortex lesions (EC), such lesions combined to OX7-saporin injection (EC+OX7), or to 192 IgG-saporin injections (SAPO); WM, water maze test; H-W, Hebb-Williams test; RM, radial maze test.

A sign between parentheses indicates a strong tendency towards significance.

were impaired in the acquisition and recall of the platform location in the water maze, and failed to learn the arms that were never baited in the radial maze, two tasks in which information must be encoded and consolidated appropriately. EC rats also showed deficits in the Hebb-Williams maze, as previously found (Galani *et al*, 1997). In this task, information does not require long-term consolidation (as problem configuration is changed from day to day), but requires processing in short-term memory. Altogether, our present findings in EC rats, as those previously reported by Steffenach *et al* (2005), are in line with a recent report showing that in humans, shrinkage of the EC may be a good predictor of immediate and delayed episodic memory decline (Rodrigue and Raz, 2004). It is nevertheless noteworthy that, in the rat, the memory deficits found after EC lesions were less pronounced than after additional BF cholinergic lesions.

192 IgG-Saporin vs OX7-Saporin Control Lesions

Highly selective lesions of the BF cholinergic neurons can be obtained by intraparenchymal (ie directly into the NBM or the septal region) or i.c.v. injections of 192 IgG-saporin (eg Parent and Baxter, 2004; Wrenn and Wiley, 1998). In the present study, i.c.v. injections were preferred to intraparenchymal injections of the immunotoxin in order to obtain a substantial cholinergic lesion in both the septum and the NBM using a single injection in each hemisphere, thereby reducing the surgical stress. Another reason for preferring i.c.v. administrations was to minimize the risk of damaging GABAergic neurons of the septal region. Indeed, intraseptal injections of 192 IgG-saporin may alter GABAergic Parv-positive neurons (eg Johnson *et al*, 2002; Marques Pereira *et al*, 2005), and it cannot be excluded that these neurons can contribute to memory performance (eg Smith and Pang (2005), Wu *et al* (2000); but see Pang *et al*, 2001). In

the study reported by Marques Pereira *et al* (2005), the lower number of Parv-positive neurons in the septum was significantly correlated with performance in a learning task. Although we did not verify the number of Parv-positive neurons in the present study using stereological methods, our estimation, as assessed from counting on one section from seven rats selected randomly in each group, points to a relative preservation of this cell population. Therefore, consideration of possible GABAergic damage does not seem to become a priority in the interpretation of our behavioral data.

As one drawback of i.c.v. administration of 192 IgG-saporin, however, is the damage of Purkinje cells in the cerebellum (eg Heckers *et al*, 1994; Waite *et al*, 1995) and possible accompanying alterations in sensory-motor function (eg Gandhi *et al*, 2000, Waite *et al*, 1999), additional groups with selective lesions of Purkinje cells in the cerebellum were included as controls (OX7 and EC + OX7). I.c.v. injections of 192 IgG-saporin or OX7-saporin resulted in sensory-motor deficits, which are compatible with the role of Purkinje cells in motor coordination and balance (Gandhi *et al*, 2000; Waite *et al*, 1999). A memory deficit selectively related to OX7-saporin injections was also found, but only for the reference memory task in the radial maze. This test probably placed maximal constraints on memory systems, as rats had to operate conjointly with both their working and their reference memory in the same trials. Although Wrenn and Wiley (2001) have shown that working memory tested in a radial maze was not altered by OX7-saporin lesions, Gandhi *et al* (2000) found such lesions to impair reference memory in the water maze, and concluded that Purkinje cells might be involved in spatial processing. Our present findings in the water maze do not corroborate the latter contribution. As an altered performance does not necessarily provide direct information on the nature of the dysfunctions accounting for a deficit, it is also possible that this particular effect of OX7-saporin could be an example whereby a motor deficit might have biased cognitive performance. Thus, it seems that further studies could be required to address this possibility more accurately. It is noteworthy, nevertheless, that in all other memory tests used in the present study, rats with OX7-saporin lesions performed at a level that was not significantly different from that of sham-operated rats, suggesting that the effects of 192 IgG-saporin lesions on Purkinje cells, whether combined or not to EC lesions, most probably had no significant impact on performance in these other tests.

192 IgG-Saporin Lesions: Memory vs Attention

In rats given 192 IgG-saporin lesions, combined or not with EC damage, we observed that the number of ChAT-positive neurons was reduced by about 90% in a particular section through the septal region and by about 65% in a particular section through the NBM. In the striatum, however, the number of ChAT-positive neurons was normal. These modifications do not reflect exact lesion-induced changes in the whole structures (which had required stereological analyses), but rather provide an approximate order of magnitude indicating that the lesions were efficient and resulted in substantial loss of cholinergic neurons.

This cholinergic damage was confirmed by reduced AChE-positivity in the corresponding projection areas of the BF nuclei. Parv-immunoreactive neurons were preserved in the septal area, indicating acceptable cholinergic selectivity of the lesions. Thus, although i.c.v. injections of 192 IgG-saporin induced a substantial loss of cholinergic neurons in the BF nuclei, the population of GABAergic neurons, which may contribute to memory performance (eg Smith and Pang (2005); but see Pang *et al*, 2001; Wu *et al*, 2000), was spared. All these observations are in agreement with previous reports (eg Schliebs *et al*, 1996). We also confirmed the report by Naumann *et al* (1997) who showed, to our knowledge in the only yet published study that relied upon the combination of EC and 192 IgG-saporin lesions, that the destruction of septal cholinergic neurons abolished septohippocampal cholinergic sprouting in the dentate gyrus. In our present study, however, cholinergic sprouting was strongly reduced, not abolished, probably because of the few septal cholinergic neurons that were not affected by the toxin.

Our behavioral data from SAPO rats are in line with several studies showing that 192 IgG-saporin lesions induce rather modest memory deficits, perhaps particularly in case of i.c.v. injections of the toxin (eg Parent and Baxter (2004), Wrenn and Wiley (1998) for reviews). The only memory deficit found in SAPO rats and not mimicked by OX7-saporin was on probe-trial performance in the water maze after a 24-h delay, suggesting that BF cholinergic systems might contribute to some consolidation or recall processes, a possibility that is in line with studies aimed at characterizing hippocampal plasticity, particularly as concerns long-term potentiation after cholinergic denervation (eg Jouvenceau *et al*, 1996; Motooka *et al*, 2001) or stimulation (eg Leung *et al*, 2003). Such a memory deficit was not found after i.c.v. injections of 192 IgG-saporin when the probe trial was given immediately after the last acquisition trial (Lehmann *et al*, 2000).

In the present study, the rats were tested in a battery of tasks commonly used to tax various aspects of memory performance. It may be worth mentioning that in these tasks, however, it can be conceived that memory function is not the sole cognitive process enabling rats to perform efficiently. Attention resources, and perhaps particularly visual attention ones, may influence the efficiency of learning and memory (Sarter *et al*, 2003). Previous studies having shown that damage to corticopetal cholinergic neurons could induce dramatic alterations of attention in a rather selective way (eg Lehmann *et al*, 2003; McGaughy *et al* 1996, 2002), it is possible that, owing to cholinergic damage in the NBM, part of the deficits reported herein might in fact be an indirect effect of altered attentional functions. Unfortunately, none of our tests enabled to discriminate between attention-related deficits and memory-related ones. Although the issue of an attentional bias cannot be rebutted, it may be qualified on the basis of a few previous findings. First, Pappas *et al* (2005) performed neonatal lesions of the BF cholinergic system by i.c.v. injections of 192 IgG-saporin. Although the lesions were submaximal in both the septal region and the NBM (as was also the case in the present study), they impaired acquisition of a water-maze task, but had no effect in a five choice serial reaction time task taxing visual attention

(Pappas *et al*, 2005). In another study, it was shown that after close to complete immunotoxic lesions of NBM cholinergic neurons, however, rats exhibited dramatic impairments in the latter task (Lehmann *et al*, 2003). Second, rats given selective cholinergic lesions in the NBM were not found to exhibit clear deficits in water-maze (Galani *et al*, 2002) and radial-maze tasks (Galani *et al*, 2002; Lehmann *et al*, 2003). Third, Chudasama *et al* (2004) showed that following injections of 192 IgG-saporin into the NBM, trained rats performed normally under conditions of low- and even high-attentional demands in a combined attention-memory task, but were impaired when tested for memory of the stimulus location. Previously, Risbrough *et al* (2002) reported similar conclusions on attention after submaximal cholinergic lesions in the NBM. All these studies point to the fact that, after cholinergic lesions in the BF, memory deficits can occur independently from alterations of attention, and that partial damage to NBM cholinergic neurons is perhaps not sufficient to disrupt attention.

The Combination of EC and 192 IgG-Saporin Lesions

192 IgG-saporin lesions increased the magnitude of the EC lesion-induced deficits on water-maze acquisition performance, repetitive and total errors in the Hebb-Williams maze and working memory in the radial maze. Some of the deficits resulting from combined EC and cholinergic lesions were found in tasks in which the cholinergic lesions alone had no significant effect (reference memory in the water maze, working memory in the radial maze, performance in the Hebb-Williams maze; see Table 4), suggesting that the increased amplitude of the deficits induced by combined lesions cannot be reduced to a simple addition of the specific effects of each lesion. Interestingly, Bannon *et al* (1996) found that whereas intraseptal injections of 192 IgG-saporin induced similar depletions of ChAT activity in the hippocampus of mature and aged rats, aged rats with lesions exhibited poor performance. It is not to exclude that this higher sensitivity to cholinergic lesions in aged rats might also be related to an interaction between the cholinergic lesion and existing damage in other structures. In that concern, although Merrill *et al* (2001) found no evidence for age-related damage in the EC of rats, Smith *et al* (2000) have reported that individual differences in cognitive capabilities of aged rats could be linked to a decline in the 'fidelity' of entorhino-hippocampal connections, suggesting that aging could affect processes in the EC.

Our present behavioral observations suggest that damage restricted to BF cholinergic neurons and neurons in the EC obliterated or dramatically altered the capability to encode or consolidate spatial information (reference memory in the water maze), but also the possibility for the rat to deal with information remaining valid for a short period of time, whether spatial (water maze, radial maze) or not (Hebb-Williams maze). Because 192 IgG-saporin was administered i.c.v., cholinergic denervation was verified in various target structures of the BF (Table 2). Owing to this widespread denervation profile, it was not possible to know whether the cholinergic contribution to the deficit found in EC + SAPO rats was due to the denervation of a particular cholinergic target rather than to global reduction of cholinergic

functions. For instance, basolocortical damage may have weakened attentional capabilities, whereas septohippocampal damage may have weakened memory functions (Lehmann *et al*, 2003; see also above). There is also some evidence that the EC may contribute to some attentional function (eg Coutureau *et al*, 1999; Oswald *et al*, 2001). On the other hand, the cholinergic denervation of the retrosplenial cortex could have contributed more directly to memory dysfunction in interaction with EC lesions, not only because both regions project to the hippocampus and are cholinergic targets, but also because the retrosplenial cortex seem directly involved in spatial cognition (Aggleton *et al*, 2000; Parron and Save, 2000). Experiments with more focal cholinergic damage should help to progress on this issue.

Our behavioral data may have implications not only for understanding the role of cholinergic alterations in age-related memory dysfunctions, and perhaps more specifically in AD, but also for setting up symptomatic drug therapy strategies that would go beyond the cholinergic hypothesis of memory dysfunctions. Indeed, it is possible that cholinergic degeneration, rather than accounting directly for cognitive malfunction, might amplify the impact of earlier alterations occurring in memory-related cortical areas such as the EC. If so, disruption of the cooperation between several structures/routes might be more efficient in disrupting spatial information, and perhaps even more generally memory processing. Interestingly, such a view would still be compatible with data showing a good correlation between markers of cholinergic and cognitive functions, as this amplification could grow proportionally to the cholinergic degeneration and might to some degree be sensitive to cholinomimetic treatments. A possible explanation to this might be that spatial information conveyed to the hippocampus follows multiple, perhaps to some respect redundant routes, and that selective damage to only one relay structure or transmitter system is not sufficient to produce major or at least easily detectable deficits (Aggleton *et al*, 2000). This view on the redundancy of information processing is compatible, for instance, with the observation that firing of hippocampal place cells, which are thought to play a crucial role in spatial coding, is not triggered by a unique sensorial modality (eg Quirk *et al* 1990). Thus, disruption of the cooperation between several structures/routes might be more efficient in perturbing spatial information, and perhaps even more generally in disrupting memory processing.

Interestingly, such a view would still be compatible with data showing a good correlation between markers of degradation of cholinergic and cognitive functions, as this amplification could grow proportionally to the cholinergic degeneration. Also, it would not contradict data showing some (even limited) efficacy of cholinomimetic-based therapies (eg Francotte *et al*, 2004; Jones, 2003; Palmer, 2002), as such therapies might act by reducing the amplification effect rather than the initial cause (eg degeneration of the retrohippocampal region). In that concern, it is noteworthy that our data indicate that the retrohippocampal area, and perhaps particularly the (dorsolateral band of the) EC, might interact synergistically with BF cholinergic mechanisms, and that this interaction is relevant to memory function. In fact, we demonstrated that

selective lesions to cholinergic neurons of the BF increased the magnitude of some of the (not necessarily) spatial memory deficits elicited by EC lesions without having an effect *per se* on them. This effect of the cholinergic lesions, however, does not generalize to all aspects of memory tested herein, and might be more or less pronounced depending on the complexity of the task and the kind of memory process required to deal with a particular task.

Finally, combined lesions also resulted in an increased level of activity, which neither of the single lesions could explain. Interestingly, at the behavioral level, AD is not only a matter of cognitive dysfunction. The disease is also associated to noncognitive manifestations such as restlessness, wandering, aggressiveness, agitation, etc (eg Klein *et al*, 1999; Meguro *et al*, 2004; Profenno and Tariot, 2004). It is therefore tempting to make a parallel between the hyperactivity found in EC + SAPO rats and restlessness or increased ambulation in demented or AD patients. As to the neurobiological bases of this hyperactivity, which is usually interpreted as reflecting dopaminergic hyperactivity in the nucleus accumbens, it is difficult to propose a precise mechanism. In previous studies, however, no or weak hyperactivity was found after i.c.v. injections of 192 IgG-saporin (Lehmann *et al*, 2000), or after excitotoxic lesions of the EC (Coutureau *et al*, 2000; Mittleman *et al*, 1998; Yee *et al*, 1995). Interestingly, in some reports, each of these lesions was shown to exacerbate the locomotor responsiveness to amphetamine, a psychostimulant drug increasing the catecholaminergic tone (Coutureau *et al* (2000), Mattsson *et al* (2002), but see Jeltsch *et al*, 2004b; Yee *et al*, 1995). This increased sensitivity to amphetamine might indicate that each lesion alone is able to induce modifications in the regulation of the dopaminergic tone, but in a way that might be insufficient to trigger hyperactivity under normal conditions. In the case of the lesion combinations, it is possible that an addition or even a potentiation of the facilitatory effects of each lesion may have driven the dopaminergic tone over a threshold from where locomotor manifestations of dopaminergic hyperactivity are observed. Although this hypothesis may appear attractive, it is worth mentioning that in AD, reports mostly show dopaminergic hypofunction in the striatum, and that noncognitive disturbances such as wandering are sensitive to risperidone, a dopamine (and also serotonin) antagonist (Meguro *et al*, 2004). It is also possible that the cholinergic loss in the retrosplenial cortex (see Table 2), which is a structure contributing to processing of the geometric properties of the environment (Parron and Save, 2004), has participated in both disorientation and impaired topographical memory in a way that could be related to home-cage 'wandering'. A last possibility to account for hyperactivity in the double-lesion group may be weakened septo-hippocampal cholinergic sprouting in EC + SAPO rats. Shortly after EC lesions, one generally observes hyperactivity which is compensated for over approximately 1 week (Lasher and Steward, 1981; Steward *et al*, 1977). This return to normal activity levels could be due to the EC lesion-induced septo-hippocampal cholinergic sprouting. As this sprouting was severely reduced by the lesion of cholinergic neurons in the septum, the hyperactivity might be due to a too limited sprouting response. In any case, further studies should enable to

clarify the neuropsychopharmacology of the hyperactivity found in EC + SAPO rats.

Conclusion

We conclude that combined damage to BF and EC neurons might model some aspects of dementia, and perhaps even aspects of restlessness or wandering described in AD more accurately than single-lesion approaches aimed at the BF cholinergic system. Although they do not mimic the progressive development of the degeneration process, lesion approaches in rodents and primates represent one among several possibilities to progress on the understanding of the link between neurodegenerative processes and cognitive malfunctions in AD. Other approaches relying upon transgenic models that mimic amyloid deposits and/or neurofibrillary tangles (German and Eisch, 2004; Gotz *et al*, 2004; Morishima-Kawashima and Ihara, 2002) are certainly more appropriate than those based on lesions when the aim is to elucidate the (causes and timing of) physiopathological events leading to AD. Based on our present data, it is nevertheless tempting to speculate that cholinergic degeneration might basically not be a major causal but rather an aggravating factor of cognitive disorders related to AD. At an early stage of the disease, these disorders could be more accounted for by conjoint degeneration and dysfunctions of both the retrohippocampal region and the BF nuclei. This view does not contradict a recent statement by Mesulam (2005): 'the cholinergic lesion is neither as central as its enthusiasts had once claimed nor as peripheral as some of its detractors would seem to imply'. Our present data illustrate that a better understanding of how cholinergic lesions are able to potentiate the impact on memory of other types of (cognitively relevant) damage in the brain of AD, perhaps particularly of the degeneration of the EC, could be worth further investigation efforts.

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