Hippocampal lesions halve immediate–early gene protein counts in retrosplenial cortex: distal dysfunctions in a spatial memory system

Mathieu M. Albasser,1 Guillaume L. Poirier,1 E. Clea Warburton2 and John P. Aggleton1
1School of Psychology, Cardiff University, Tower Building, Park Place, Cardiff, CF10 3AT, UK
2MRC Centre for Synaptic Plasticity, Department of Anatomy, University of Bristol, Bristol BS8 1TD, UK

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Abstract
The present study examined whether hippocampal lesions disrupt retrosplenial cortex function. The immediate–early genes c-fos and zif268 provided markers of cellular activity, and their levels were compared in different cytoarchitectonic subregions (dysgranular, granular a and granular b) and different layers (superficial or deep) within retrosplenial cortex. Experiments 1–3 examined the impact of hippocampal lesions on retrosplenial cortex function, with the variations in protocol (e.g. lesion method, rat strain, behaviour prior to gene activity measurement) testing the generality of the findings. Experiment 1 showed that radio-frequency hippocampus lesions result in very striking losses of Fos and Zif268 activity in both superficial and deep laminae of all retrosplenial subregions. This pattern of results was repeated for Fos in experiments 2 and 3. Despite the loss of Fos and Zif268, there was no evidence of retrosplenial cortex atrophy as measured by Nissl counts (experiments 1–3) or NeuN-positive cell counts (experiment 3). Likewise, there was little evidence of any overt changes in cellular size, shape or appearance. The specificity of these hippocampal lesion effects was confirmed in experiment 4 as entorhinal cortex lesions did not change retrosplenial Fos levels. These results provide strong support for the notion that the retrosplenial cortex is unusually sensitive to deafferentation from some of its inputs, so that hippocampal damage might produce permanent ‘covert pathology’ in the retrosplenial cortex. Such dysfunctions could contribute to the pattern of cognitive changes associated with hippocampal lesions and also help to explain the functional interdependency of these two structures.

Introduction
Anatomical, behavioural and neuropsychological studies increasingly reveal the importance of hippocampal interactions with the retrosplenial cortex (areas 29 and 30). The direct projections from the subiculum to the retrosplenial cortex (Van Groen & Wyss, 1990, 1992, 2003; Insauti et al., 1997; Kobayashi & Amaral, 2003) provide a nonfornical route by which the hippocampus can influence many sites, including prefrontal cortex. Lesion studies have not only confirmed that retrosplenial cortex lesions disrupt many spatial tasks that are also dependent on the hippocampus (Cooper & Mizumori, 1999; Vann & Aggleton, 2002, 2004), but have also shown their interdependence for aspects of spatial memory (Sutherland et al., 1988; Mizumori et al., 2000). Further support comes from studies of patients with focal lesions and from fMRI studies, which link the hippocampus and retrosplenial cortex for spatial navigation and episodic memory (Maguire, 2001; Iaria et al., 2007). The present study addressed one possible explanation for this interdependence, namely that hippocampal lesions produce permanent dysfunctions in retrosplenial cortex activity.

There is growing evidence that the retrosplenial cortex is vulnerable to the loss of its distal connections. Related studies, based on the dense projections from the anterior thalamic nuclei to retrosplenial cortex, showed that large thalamic lesions reduce levels of cytochrome oxidase and markers for acetyl choline but do not cause retrosplenial cell loss (Van Groen et al., 1993). Subsequent studies of selective anterior thalamic lesions found a dramatic, and permanent, fall in the expression of the immediate–early genes (IEGs) c-fos and zif268 in retrosplenial cortex (Jenkins et al., 2004). This loss was especially marked in the superficial layers (up to 90% loss), and subsequent slice studies revealed a loss of plasticity in the local circuits within the same superficial layers, indicative of covert pathology (Garden et al., 2006). Positron emission tomography studies have also shown that the retrosplenial hypoactivity in various neurological conditions (Korsakoff’s disease, mild cognitive impairment and Alzheimer’s disease) is characterized by both a loss of memory and prominent pathology in sites connected with the retrosplenial cortex (Braak & Braak, 1991a; Fazio et al., 1992; Minoshima et al., 1997; Nestor et al., 2003a,b; Reed et al., 2003). In Alzheimer’s disease this hypoactivity is evident at very early stages in the disease, when overt pathology is present in afferent sites (e.g. the hippocampus and entorhinal cortex) but not in the retrosplenial cortex itself (Braak & Braak, 1991a,b).

The present study examined whether hippocampal formation lesions, like anterior thalamic lesions, markedly disrupt retrosplenial IEG activity. Levels of Fos and Zif268 proteins were compared in different cytoarchitectonic subregions within retrosplenial cortex. These IEGs were selected as they have high expression levels in the retrosplenial cortex, both have repeatedly been implicated in plastic processes (Herdegen & Leah, 1998; Tischmeyer & Grimm, 1999), and
there are no previous reports on the impact of hippocampal lesions upon IEG expression in the retrosplenial cortex. In experiments 1, 3 and 4 the rats had previously been used for other behavioural studies. The resultant variations in procedure (e.g. lesion method, rat strain) helped to test the generality of the findings.

Materials and methods

General methods

In experiments 1, 3 and 4, tissue was obtained from cohorts of rats involved in separate behavioural experiments that were designed to study the impact of hippocampal or entorhinal cortex lesions (Table 1). These behavioural tests were completed a minimum of 14 days prior to the present IEG study. These cohorts were chosen as they involved different surgical methods (radio-frequency lesioning, injection of a cytotoxin, different anaesthetic), the impact of the lesions had been behaviourally confirmed, and overall animal usage could be minimized. The rats in experiment 2 had unilateral hippocampal lesions and so did not receive prior behavioural tests, apart from those integral to the study. These variations in procedure (Table 1) helped to test the reliability of any findings. At the same time, the methods for visualizing and analysing IEG expression were consistent across all experiments. In addition to standard Nissl staining (experiments 1–4), tissue from the rats in experiments 3 and 4 was stained for neuronal nuclei protein (NeuN; Jongen-Relo & Feldon, 2002) in order to help detect whether there was any neuronal loss in retrosplenial cortex following either hippocampal or entorhinal cortex lesions.

In all four experiments rats were exposed to either a novel environment or novel visual stimuli 90 min prior to perfusion (Table 1). These environmental manipulations ensured that IEG expression in the retrosplenial cortex in control hemispheres was sufficiently high to detect lesion-induced changes.

For all surgeries, animals were first anaesthetized (see details below) and then placed in a stereotaxic head holder (Kopf Instruments, Tujunga, CA, USA). A longitudinal incision was made in the scalp, which was retracted to expose the skull. Craniotomies were made directly above the target regions, and the dura cut to expose the cortex. After every surgery, the skin was sutured, antibiotic powder applied (Acramide; Dales Pharmaceuticals, UK), and the animal placed in a temperature-controlled recovery box. Animals also received 5 mL of glucose saline subcutaneously (s.c.) and the analgesic agent Meloxi-

cam, 1.0 mg/kg (s.c.). Paracetamol was added to the water for three days postsurgery.

The rats were kept in pairs, housed under diurnal conditions (14 h light, 10 h dark) and were thoroughly habituated to handling before the study began. All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Experiment 1: bilateral radio-frequency hippocampal lesions

Animals

The subjects were 12 male Dark Agouti (DA) rats (Harlan, UK), a fully pigmented strain. The rats were 12–14 weeks old and weighed 208–257 g at the time of surgery. Animals had previously been trained for six sessions (36 trials) on reinforced T-maze alternation (Table 1). This task, which confirmed the effectiveness of the hippocampal lesions, was completed 3–4 weeks before the immunohistochemistry study.

Surgery

The 12 DA rats were divided into two groups: bilateral lesions of the hippocampus (RF Hpc, n = 6) and surgical controls (RF Hpc Sham; n = 6). All rats were deeply anaesthetized with isoflurane gas. The incisor bar of the stereotaxic frame was set at +5.0 mm. Diazepam was administered systemically (2.5 mg/kg, i.p.). Lesions were made with radio-frequency signals using an RFG4-A Lesion Maker (Radionics, Burlington). The electrode (0.3 mm tip length, 0.25 mm diameter) was lowered vertically, and at each site the temperature at the tip of the probe was also raised to 75 °C for 60 s.

Each hippocampal lesion was formed by making 17 lesions per hemisphere (in 15 separate tracts). The stereotaxic coordinates of the lesions correspond to those used by Sanderson et al. (2006). The control surgical procedure was identical to that for hippocampal lesions except the electrode tip was lowered only 1.7 mm from the top of the cortex using the same AP and laterality coordinates (15 times per hemisphere). The temperature at the tip of the electrode was not raised. The IEG expression study was conducted 8 months postsurgery.

Behavioural procedure to induce IEG expression: spontaneous exploration of radial-arm maze

Each rat was allowed to explore spontaneously an unfamiliar eight-arm radial maze in an unfamiliar room. The maze consisted of an octagonal central platform (34 cm diameter) and eight equally spaced radial arms (87 cm long, 10 cm wide) with food wells at the end. The floor of the maze was made of wood, while the side walls were clear Perspex (24 cm high). All animals were tested in the same distinctive rectangular room (295 × 295 × 260 cm).

The rat was first placed in the central platform and then freely allowed to visit every arm for 30 min. The eight arms were baited and continually rebaited with sucrose pellets (45 mg; Noyes Purified Rodent Diet, Lancaster, NH, USA). Each animal was placed in a holding box in a quiet, dark room for 30 min before and 90 min after the single radial-arm maze session. Animals were habituated to this dark room for 2 h every day for the 7 days prior to radial-arm maze exploration.

<table>
<thead>
<tr>
<th>Table 1. Summary of procedural differences for experiments 1–4</th>
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<td>Experiment number and lesion site</td>
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<td>1. Bilateral hippocampus</td>
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<td>2. Unilateral hippocampus</td>
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<td>3. Bilateral hippocampus</td>
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<td>4. Bilateral entorhinal cortex</td>
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CTA, conditioned taste aversion. *Ninety minutes after completing the radial arm-maze exploration, rats were deeply anaesthetized and perfused intracardially to prepare brains for immunohistochemistry.
Experiment 2: unilateral NMDA hippocampal lesions

Rats were trained on the ‘paired-viewing’ task using the behavioural procedure originally described by Zhu et al. (1996). The data in the present study come from an IEG expression experiment solely designed to measure the impact of hippocampal lesions on cortical function. For this procedure, the rats were shown visual images on computer monitors 90 min before perfusion. The visual stimuli were selected as they cause Fos activation in either the perirhinal cortex (‘novel stimuli’) or the hippocampus (‘novel arrangements’) in normal rats (Wan et al., 1999).

Animals

The study used 11 adult male DA rats (Bantin and Kingman, Hull, UK) weighing between 180 and 200 g at the time of surgery.

Surgery

All rats received unilateral hippocampal lesions by injecting N-methyl-D-aspartate (NMDA). All animals were deeply anaesthetized by intraperitoneal injection (60 mg/kg) of pentobarbitone sodium (Sagatal; Rhone Merieux). Unilateral lesions of the hippocampus (NMDA Hpc) were made by injecting 0.15 μL of 0.06 m NMDA (Sigma Chemical Company Ltd, Poole) dissolved in phosphate buffer (pH 7.2) into 12 sites in one hippocampus and 0.1 μL in one further site (see Jarrard, 1989). Each injection was made gradually over a 4-min period through a 1-μL Hamilton syringe, and the needle was left in situ for a further 3 min before being withdrawn. Anteroposterior (AP) and lateromedial (LM) stereotaxic coordinates were calculated relative to bregma, and dorsoventral (DV) coordinates were calculated relative to the top of the cortex (Paxinos & Watson, 1997). The incisor bar was set so that the top of the skull was horizontal. The co-ordinates (AP, LM and DV) of the 13 sites correspond to those reported by Jenkins et al. (2006). The left or right hemisphere was targeted in equal numbers of animals.

Behavioural procedure to induce IEG expression: paired-viewing procedure (‘novel stimuli’ and ‘novel arrangements’)

The rats were trained on one of two conditions on the paired-viewing procedure (Wan et al., 1999). One condition (‘novel stimuli’) was chosen as it increases Fos in the perirhinal cortex while the other condition (‘novel arrangements’) induces Fos changes in the hippocampus (Wan et al., 1999). Each rat was trained in an open-topped viewing chamber (30 × 30 × 35 cm), the front of which was transparent (Perspex) with a central observing hole 3 cm in diameter, 6 cm above the floor. When the rat’s head was positioned in the observing hole, an infrared beam was interrupted so signalling the computer (Viglen P5-100) to start a trial. Pictures could be shown on two computer monitors placed 30 cm in front of the observing hole. A black partition ensured that the rat’s left eye could not see the right monitor screen and his right eye could not see the left screen.

Training began after a 2-week recovery period. During training the rats were allowed ad libitum access to water for 2 h each day. Each rat was first trained for 3 days to go to the observing hole for blackcurrant juice reward. For the next 6 days the rats were shown different visual images on the two computer monitors set behind the observer hole. When the rat held its head in the observer hole for a variable interval of 3–4 s, pictures (each 15 × 12 cm) were displayed for 4.5 s on the two monitors. After the pictures had been displayed for 4 s a drop of blackcurrant juice was delivered via a metal tube that the rat could just reach and lick. As the tube was reached through the observer hole the rats remained orientated towards the visual stimuli. Each day consisted of two morning training sessions and one afternoon session. The second morning session followed the first without a delay and in each morning session 30 pictures (the familiar set) were presented to both eyes. Both eyes saw the same 30 pictures, but the order of presentation differed for the two monitors so that a different picture was shown by each eye on every trial. In the afternoon training session one eye was exposed to the familiar pictures while the other eye was exposed to a set of 30 novel pictures. A different set of 30 novel pictures was shown each afternoon to familiarize the animal with seeing novel and familiar stimuli simultaneously. The different sets of novel and the familiar set of pictures were so presented that by the end of the experiment each eye had seen the same number of novel and familiar stimuli. The final set of novel stimuli was shown with the familiar set on the afternoon of day 6, one eye seeing each set. Each animal was placed in a holding box in a quiet and dark room for 30 min before and 90 min after daily sessions.

For three rats each monitor only displayed one picture at a time. ‘Novel stimuli’ consisted of completely new pictures presented on just one monitor (Wan et al., 1999). ‘Novel arrangements’ (n = 8) were created by presenting familiar groups of the same three pictures but with rearranged spatial relationships. Once again, only one eye was shown the novel arrangements. Which eye saw the novel stimuli was counterbalanced across animals and side of lesion.

Experiment 3: bilateral ibotenic acid hippocampal lesions

Animals

The experiment comprised 15 male Lister Hooded rats (Harlan, UK) weighing 280–400 g at the time of surgery. These rats had previously been trained in a water maze to discriminate local geometric features (Table 1). The training, which was completed 2 weeks before the IEG study, confirmed the effectiveness of hippocampal lesions.

Surgery

Each rat was placed in an induction box in which anaesthesia was induced with a mixture of oxygen and isoflurane, prior to being placed in a stereotaxic apparatus. Nine rats received ibotenic acid hippocampal lesions (IBO Hpc; n = 9). The infusions of ibotenic acid (Biosearch Technologies, San Rafael, CA, USA), which was dissolved in sterile phosphate-buffered saline (PBS; pH 7.4) to produce a 63 mM solution, were made via a 2-μL Hamilton syringe. Volumes of between 0.05 and 0.10 μL ibotenic acid were infused in 28 sites per hemisphere (for stereotaxic coordinates of the injection sites, see Coutureau et al., 1999). Infusions were made at a rate of 0.03 μL/min, and the needle was left in situ for 2 min after completing each infusion. Rats in the sham-operated control group (IBO Hpc Sham; n = 6) underwent a similar surgical procedure to those in lesion groups in which the skin was incised, the cortex exposed and the dura perforated with a 25-gauge Microlance3 needle (Becton Dickinson, Drogheda, Ireland), but neither cortical damage nor injection were made. Post-operative procedures matched other studies, and the IEG analysis followed from 2 to 2.5 months postsurgery.

Behavioural procedure to induce IEG expression

The rats were exposed to a novel environment by placing them individually in a new cage with clean sawdust placed on a rack in a novel room. Rats were first left for 15 min in this novel cage, after which they were returned to their home cages for 90 min prior to perfusion.
Experiment 4: bilateral ibotenic acid entorhinal cortex lesions

Animals
The animals were 19 male Lister Hooded rats (Harlan, UK) weighing 280–400 g at the time of surgery. These rats had previously undergone a conditioned taste aversion task (Mundy et al., 2005) that had been completed 2 weeks before training for IEG expression (Table 1).

Surgery
Rats received bilateral lesions of the entorhinal cortex (IBO Ent; n = 11) or sham surgeries (IBO Ent Sham, n = 8). The basic procedures were essentially the same as for experiment 3. Volumes of between 0.05 and 0.10 μL were infused in 14 sites. The details of these infusions and their coordinates have been described elsewhere (Coutureau et al., 1999). The immunohistochemistry was carried out 3 months postsurgery.

Behavioural procedure to induce IEG expression
The behavioural procedures were exactly the same as in experiment 3.

IEG immunohistochemistry: Fos and Zif 268.
Ninety minutes after completing the radial arm-maze exploration, the paired-viewing final session, or being placed in a novel cage, rats were deeply anaesthetized with sodium pentobarbital (140 mg/kg; Euthatal; Rhone Merieux, UK) and perfused intracardially with 0.1 m PBS followed by 4% paraformaldehyde in 0.1 m PBS (PFA). The brains were removed and postfixed in PFA for 4 h and then transferred to 25% sucrose overnight at room temperature with rotation. Sections were cut at 40 μm on a freezing microtome in the coronal plane for experiments 1 and 2 and in the horizontal plane for experiments 3 and 4. Series of sections were collected in 0.1 m PBS containing 0.2% Triton X-100 (PBST). A peroxidase block was then carried out in which the sections were transferred to 0.3% hydrogen peroxide in PBST for 10 min to inhibit endogenous peroxidase and then washed several times with PBST. Sections were incubated in PBST containing Fos rabbit polyclonal antibody (1 : 5000; Ab-5, Oncogene Science, several times with PBST. Sections were incubated in PBST containing PBST for 10 min to inhibit endogenous peroxidase and then washed. The reaction was then visualized using diaminobenzidine (DAB in PBST). A peroxidase block was then carried out in which the sections were transferred to 0.3% hydrogen peroxide in PBST for 10 min to inhibit endogenous peroxidase and then washed several times with PBST. Sections were incubated in PBST containing Fos rabbit polyclonal antibody (1 : 5000; Ab-5, Oncogene Science, UK); Zif268 (also known as Egr-1/Krox-24/NGFI-A) for experiment 1 (antibody 1 : 3000; C-19, Santa Cruz Biotechnology, USA), for 48 h at 4 °C with periodic rotation. Sections were washed and then incubated for Fos and Zif268 in biotinylated goat antirabbit secondary antibody (diluted 1 : 200 in PBST; Vectastain, Vector Laboratories, Burlingame, USA) and 1.5% normal goat serum. Sections were then washed and processed with avidin-biotinylated horseradish peroxidase complex in PBST (Elite Kit, Vector Laboratories) for 1 h at room temperature, again with constant rotation. Sections were washed again in PBST and then in 0.05 m Tris buffer. The reaction was then visualized using diaminobenzidine (DAB Substrate Kit; Vector Laboratories) and then stopped by washing in cold PBS. Sections were mounted on gelatine-coated slides, dehydrated through a graded series of alcohols and coverslipped.

IEG cell counts: experiments 1–4
Estimates were made of IEG-positive cells and also of the total numbers of cells present in the retrosplenial cortex by using an automated cell counting procedure. Wherever possible, counting procedures were without knowledge of the group assignments. Images were viewed on a Leica DMRB microscope, photographed using an Olympus DP70 camera and transferred to a computer. Counts of the stained nuclei were carried out using the program analySIS® D (Soft- Imaging Systems). Regions of interest were assessed using counts of nuclei labelled above threshold. The counts were made in a frame area of 0.84 × 0.63 mm, which enabled all laminae to be included in one image.

Retrosplenial cortex (Wyss & Van Groen, 1992) can be subdivided (Fig. 1) into granular b cortex (Rgb), granular a (Rga) and the dysgranular cortex (Rdg). Separate counts were made in all three subregions for experiments 1 and 2 (coronal sections). Counts were made only in Rgb for experiments 3 and 4 (horizontal sections), i.e. not in Rdg or Rga. The reason for this difference concerns the shape and location of Rdg and Rga. Area Rdg spans the dorsal convexity of the cortex; as a consequence, horizontal slices give very unequal amounts of the various cell laminae and can often completely miss the deeper laminae. Area Rga lies more caudal within area 29, and the border between caudal Rgb and Rga is extremely difficult to detect in horizontal sections. Accordingly, only Rgb was examined in experiments 3 and 4. Here, separate counts were made for rostral Rgb (rostral to splenium) and caudal Rgb (caudal to splenium). The distinctive border between caudal Rgb and Rdg enabled the latter counts.

Separate counts of IEG-positive cells were made in the superficial (layer II and upper III) and deep (lower layer III to VI) layers of Rgd, Rgb and Rga. This border is signalled by an abrupt change in packing density. For each brain area analysed, counts of positive cells were taken from three different sections in each hemisphere for experiments 1, 3 and 4 (i.e. six sections in total) and from four different sections into the same hemisphere for experiment 2 (unilateral lesion). These area counts were then averaged to produce a mean for a given animal. The Nissl and NeuN counts were made by the same protocol as that used for IEG-positive cells. These counting procedures were not stereological and so while providing information about relative numbers of cells they do not provide accurate counts of absolute cell numbers.

Nissl and NeuN staining
A one-in-four series of sections was mounted directly onto slides and stained using cresyl violet, a Nissl stain, for experiments 1–4. In addition, for experiments 3 and 4 (ibotenic hippocampal and entorhinal lesions, respectively), tissue was stained for a specific neuronal marker, NeuN (Jongen-Relo & Feldon, 2002). NeuN was identified using immunohistochemistry and procedures very similar as those for Fos and Zif268. The NeuN protein was identified using a horse antimonue-ter-adsored secondary antibody and 1.5% normal horse serum.

Neuronal cell counts: experiments 1–4
In addition to the relative counts of Nissl-stained cells, several parameters were also analysed using the same software (analy-SIS® D, Soft-Imaging Systems, Olympus, UK). In order to reduce counts of glial cells and so improve the validity of the neuronal counts, cells were only counted if they had a feret mean between 5 and 20 μm (the feret mean refers to the minimum diameter of a cell). Using this constraint, six parameters were investigated within each region of interest: (1) the mean area of all cells; (2) the mean intensity of the signal (the ‘grey value’) for all cells; (3) the mean perimeter of all cells; (4) the mean diameter of all cells; (5) the mean of the sphericity values (a measure of how similar the cells are to a sphere) for all cells; and (6) the mean convexity value of the cells in an area (a measure of the extent that the cells are infolded or indented).
**Parietal cortical damage: experiment 1**

As a control measure, the amount of damage in the overlying cortex was assessed quantitatively in order to determine whether it was correlated with Fos level changes. Most of this damage was to the parietal cortex, but it often extended caudally to include rostral parts of area 18b. Cortical damage was quantified using the analySIS®D programme. The total size of the cortical lesion ($\text{cm}^2$) was measured from drawings of the extent of parietal and area 18b cell loss on captured images of the Nissl-stained sections. Coronal sections were captured and measured every 40 $\mu$m, starting from the first visible damage; the number of images thus depended of the extent of the cortical lesion.

**Statistics**

Cell counts were compared using an ANOVA with one between-subject factor (group, two levels: control vs. lesion) and one within-subject factor (region: experiments 1–2, six levels: Rdg superficial, Rdg deep, Rgb superficial, Rgb deep, Rga superficial and Rga deep; experiments 3–4, four levels: rostral superficial and deep Rgb, caudal superficial and deep Rgb). Because the SDs were proportional to the mean, the raw cell counts were subject to logarithmic transformations (Howell et al., 2002). Simple effects were examined when a significant ($P < 0.05$) interaction was found.

In a final series of analyses designed to compare results across the four experiments the counts for each individual animal for each area were normalized against the mean score of the respective control group for that area. The scores were normalized by dividing the mean number of activated neurons in a lesioned animal by the mean of the appropriate control group, and calculating the proportion score as a percentage. Thus, a score of 100 would correspond to a score that matched that of the appropriate control group, and a score lower than 100 reflects a lower number of cells. Group comparisons used either $t$-tests or one-way ANOVA, and Newmann–Keuls post hoc tests were used as appropriate.

**Results**

**Experiment 1: radio-frequency bilateral hippocampal lesions**

*Lesion analysis*

All hippocampal lesions (Fig. 2A) resulted in substantial damage to the structure. Cell loss was most complete in the dorsal hippocampus,
involving areas CA1, CA2, CA3, dentate gyrus and, for most cases, the dorsal subiculum. There was consistent partial damage to the fimbria–fornix which, in some cases, was substantial. All rats had damage to the ventral hippocampus, but typically the lesions spared part of the most posterior regions of the ventral hippocampus which were, nevertheless, markedly shrunken. In approximately half of RF Hpc cases the damage in the overlying cortex extended beyond the tract marks to involve limited parts of the parietal cortex and rostral 18b (Fig. 2A).

IEG counts

Fos. Counts of Fos-positive cells in Rga, Rgb and Rdg were markedly reduced following the bilateral radio-frequency hippocampal lesions (Figs 3 and 4). There was a significant decrease in Fos counts (group, $F_{1,10} = 54.8$, $P < 0.001$; group × region interaction, $F_{5,50} = 11.0$, $P < 0.001$). The significant interaction reflected the fact that the degree of Fos protein loss differed between regions (Fig. 3), with the greatest decrease occurring in the superficial layers of Rgb. Subsequent inspection showed that the difference between the two groups was significant in both the superficial layers (II and upper III) and in the deep layers (lower III to VI), as confirmed by the simple effects (Rdg: superficial, $F_{1,60} = 29.7$, $P < 0.001$; deep, $F_{1,60} = 5.2$, $P < 0.05$; Rgb: superficial, $F_{1,60} = 93.1$, $P < 0.001$; deep, $F_{1,60} = 15.1$, $P < 0.001$; Rga: superficial, $F_{1,60} = 32.9$, $P < 0.001$; deep, $F_{1,60} = 7.1$, $P < 0.01$). In addition to the very evident loss of Fos-positive cells in layer II, there also appeared to be a band of cells in layer V that showed abnormally low levels of Fos (Fig. 4).

Fig. 2. Diagrammatic reconstructions of the lesions in experiments 1–4 showing the cases with the largest (grey) and smallest (black) lesions. The numbers refer to the distance (mm) from Bregma according to the atlas of Paxinos & Watson (1997). (A) Experiment 1, radio-frequency hippocampal lesions (RF Hpc) on coronal sections; (B) experiment 2, unilateral NMDA hippocampal lesions (NMDA Hpc) on coronal sections; (C) experiment 3, ibotenate hippocampal lesions (IBO Hpc) on horizontal sections; (D) experiment 4, entorhinal cortex lesions (IBO Ent) on horizontal sections.
As with Fos, a significant reduction in Zif268 cells was observed in the lesion group (group, \( F_{1,10} = 19.0, P < 0.001 \); group \( \cdot \) region, \( F_{5,50} = 5.2, P < 0.001 \); Fig. 5). Similarly to Fos counts, the significant interaction reflected a greater decrease in the superficial layers of Rgb (Fig. 5). Subsequent analyses revealed that the decrease in Zif268-positive nuclei was found across all subregions of the retrosplenial cortex (simple effects; Rdg: superficial, \( F_{1,60} = 6.9, P < 0.05 \); deep, \( F_{1,60} = 11.3, P < 0.001 \); Rgb: superficial, \( F_{1,60} = 38.1, P < 0.001 \); deep, \( F_{1,60} = 17.1, P < 0.001 \); Rga: superficial, \( F_{1,60} = 8.6, P < 0.01 \); deep, \( F_{1,60} = 6.0, P < 0.05 \)). The pattern of Zif268 changes was very similar to that observed for Fos, with the most striking losses in layers II, upper III and V.

**Experiment 2: NMDA unilateral hippocampal lesions**

**Lesion analysis**

All hippocampal lesions caused near total cell loss in the dorsal hippocampus, with only minor sparing of the ventral horn of the dentate gyrus in one case (Fig. 2B). The amount of sparing of the ventral hippocampus varied between animals. In eight of the 11 cases there was almost total cell loss, while in three cases there was sparing of the most posterior regions of ventral hippocampus, in particular CA3 and the medial blade of the dentate gyrus. Cell loss also occurred in the cortex overlying the dorsal hippocampus. This cortical damage was restricted to the parietal cortex overlying CA1. In all animals the ventral subiculum was partially spared, while the dorsal subiculum was more consistently damaged.

**IEG counts: Fos**

Unilateral NMDA hippocampal lesions markedly reduced Fos levels across the retrosplenial cortex (Fig. 6). Raw counts were significantly lower in the lesioned hemisphere (group, \( F_{1,20} = 34.1, P < 0.001 \); group \( \cdot \) region interaction, \( F_{5,100} = 7.0, P < 0.001 \)), while the interaction showed that this loss was more marked in the superficial layer.

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**Fig. 4.** Experiment 1. Photomicrographs comparing Nissl and Fos Levels in Rgb in rats with either (C and D) a bilateral radio-frequency hippocampal lesion (RF Hpc) or (A and B) sham surgery (Control). The brightfield photomicrographs of coronal sections show the similar appearance of Nissl-stained sections (cresyl) in (A and C) the retrosplenial cortex, which contrast with (D vs. B) the striking loss of Fos-positive cells following hippocampal lesions. The asterisk denotes the zone of Fos-free cells in layer V. Scale bar, 100 μm.
of Rgb. Subsequent analyses showed that the difference between the two hemispheres was significant for all the layers of the three subregions (simple effects; Rdg: superficial, $F_{1,120} = 8.5, P < 0.01$; deep, $F_{1,120} = 4.1, P < 0.05$; Rgb: superficial, $F_{1,120} = 59.5, P < 0.001$; deep, $F_{1,120} = 6.6, P < 0.05$; Rga: superficial, $F_{1,120} = 26.5, P < 0.001$; deep, $F_{1,120} = 6.9, P < 0.01$).

**Experiment 3: ibotenic acid bilateral hippocampal lesions**

**Lesion analyses**
All rats with hippocampal lesions sustained bilateral damage to both the dentate gyrus and the CA subfields at all dorsal and ventral levels (Fig. 2C). The range of cell loss amounted to 65–100% of the intact hippocampus (dentate gyrus, CA1–3). The majority of rats also sustained some cell loss in the subiculum (including the pre- and parasubiculum). This damage was most evident in ventral regions of the subiculum. There was no detectable cell loss in the postsubiculum or in the medial or lateral entorhinal cortex in any of the lesioned rats.

**IEG counts: Fos**

Raw counts were taken only from the granular b retrosplenial cortex. Rgb was subdivided into the rostral and caudal area (pre- and postsplenium), as well as superficial and deep layers. In the lesioned animals (IBO Hpc) there was a Fos decrease across the whole of Rgb (group, $F_{1,13} = 9.6, P < 0.01$; group x area interaction, $F_{3,39} = 0.3$; Fig. 7). This lesion effect was significant in both layers and in both rostral and caudal areas of Rgb (simple effects; rostral Rgb: superficial, $F_{1,52} = 8.5, P < 0.01$; deep, $F_{1,52} = 6.3, P < 0.05$; caudal Rgb: superficial, $F_{1,52} = 7.0, P < 0.05$; deep, $F_{1,52} = 9.6, P < 0.01$). Once again, there was a striking loss of Fos-positive cells not only in layers II and upper III but also in layer V (Fig. 8).

**Experiment 4: ibotenic acid bilateral entorhinal cortex lesions**

**Lesion analyses**
All lesioned rats sustained substantial cell loss within the medial entorhinal cortex, and cell loss that was more variable in the lateral entorhinal cortex (Fig. 2D). There was, as intended, substantial sparing of cells in the subicular complex. Typically, the lesion extended from −4.6 to −7.6 mm ventral to the surface of the brain (Paxinos & Watson, 1997).

**IEG counts: Fos**
Raw counts were taken only from the granular b retrosplenial cortex. A nonsignificant reduction in Fos levels was found in rostral and caudal Rgb (group, $F_{1,17} = 1.7, P = 0.2$; group x area interaction, $F < 1$; Fig. 9).
Neuronal cell counts: experiments 1–4

Nissl-stained cell counts. There were no significant differences in the total Nissl cell counts taken across all layers of the retrosplenial cortex for any of the experiments (Fig. 3, RF Hpc: group, $F_{1,10} = 3.5$, $P = 0.09$; group × region interaction, $F < 1$; NMDA Hpc: group, $F < 1$; group × region interaction, $F < 1$; IBO Hpc: group, $F < 1$; group × area interaction, $F_{3,36} = 1.2$, $P = 0.3$; IBO Ent: group, $F < 1$; group × area interaction, $F < 1$).

Nissl-stained cell parameters. In addition to counting cell numbers, other cell parameters were analysed and compared to help determine whether the lesions caused morphological abnormalities in the retrosplenial cortex. The cell parameters were: cell area, intensity (‘grey value’) of stain, perimeter, diameter, sphericity and convexity. In experiments 2–4, there were no significant changes in any of the six studied parameters ($P > 0.05$) for the areas under investigation in each experiment. In experiment 1 no group differences were found.

**Fig. 8.** Photomicrographs comparing NeuN and Fos Levels in Rgb in rats with either (C and D) a bilateral ibotenate hippocampal lesion (IBO Hpc), (E and F) a bilateral ibotenate entorhinal lesion (IBO Ent), or (A and B) a sham surgery (Control). The brightfield photomicrographs of horizontal sections show (A, C and E) the similar levels of neurons in the retrosplenial cortex, which contrast with (D vs. B and F) the selective, striking loss of Fos-positive cells following hippocampal lesions. The asterisk denotes the zone of Fos-free cells in layer V. Scale bar, 100 μm.
The main findings of this study are very clear. Lesions of the rat hippocampus resulted in consistent, striking decreases in the counts of Fos-positive cells in the retrosplenia cortex. Typically this reduction was at least one-half. A very similar result was found for Zif268 in the one experiment in which this IEG was studied. These IEG decreases were found across all three subareas of area 29 (Rga, Rgb, Rdg). Furthermore, these IEG reductions were found in both the superficial and deep layers. Although the superficial layers (II and upper III) often displayed the most dramatic decreases in IEG-positive cells, there was an additional band of cells in layer V of the granular retrosplenia cortex that often appeared to be largely devoid of Fos-positive cells (Figs 4 and 8). In contrast, entorhinal cortex lesions led to little or no change in retrosplenia IEG levels. These novel findings show that hippocampal lesions leave the retrosplenia cortex abnormal, raising the possibility that retrosplenia dysfunction contributes to the impact of hippocampal damage. The entorhinal lesion results show that this hippocampal lesion effect is selective and not simply due to any deafferentation of the subiculum.

Comparisons of IEG findings across experiments 1–4

For each of the three subregions within retrosplenia cortex (Rga, Rgb, Rdg) it was found that hippocampal lesions reduced overall Fos-positive cell counts (superficial and deep laminae combined) by between 49% (Rgb, experiment 3) and 70% (Rgb, experiments 1 and 2). Thus, for experiments 1–3 the Fos counts were typically less than half of those of the control cases. In order to compare statistically the magnitude of these IEG changes across the different experimental techniques (different lesion method, different rat strain, different behavioural task) the data were first normalized against their respective controls. (For experiment 3 the data were taken from just rostral Rgb to aid comparability with the other experiments). These comparisons (Fig. 10) further confirmed how hippocampal lesions, but not entorhinal cortex lesions, consistently reduced retrosplenia cortex activity as measured by Fos expression. Zif268 was only visualized in experiment 1.

Comparisons across the four experiments indicated that the magnitude of the Fos loss in Rdg or Rga did not differ significantly when the lesions were made either with radio-frequency signals or with NMDA (Rdg: superficial, \( P = 0.067 \), deep, \( P = 0.4 \); Rga; superficial, \( P = 0.67 \), deep, \( P = 0.3 \)). There was, however, a significant effect of lesion method for Rgb (\( F_{3,33} = 38.5, P < 0.001 \)). Of the three lesion methods, the IBO Hpc rats had the least reduction in Fos activity in the superficial layers (IBO Hpc vs. RF Hpc, Newman–Keuls, \( P < 0.01 \); IBO Hpc vs. NMDA Hpc, \( P < 0.01 \)). There were, however, no differences in the deep layers (IBO Hpc vs. RF Hpc, \( P = 0.99 \); IBO Hpc vs. NMDA Hpc, \( P = 0.91 \)). The RF Hpc and NMDA Hpc rats showed similar Fos decreases (\( P = 0.99 \)) in superficial and deep layers. Finally, post hoc analyses revealed a significant difference between the entorhinal cortex lesions and all three other hippocampal surgeries (\( P < 0.001 \)).

Discussion

The main findings of this study are very clear. Lesions of the rat hippocampus resulted in consistent, striking decreases in the counts of Fos-positive cells in the retrosplenia cortex. Typically this reduction was at least one-half. A very similar result was found for Zif268 in the one experiment in which this IEG was studied. These IEG decreases were found across all three subareas of area 29 (Rga, Rgb, Rdg). Furthermore, these IEG reductions were found in both the superficial and deep layers. Although the superficial layers (II and upper III) often displayed the most dramatic decreases in IEG-positive cells, there was an additional band of cells in layer V of the granular retrosplenia cortex that often appeared to be largely devoid of Fos-positive cells (Figs 4 and 8). In contrast, entorhinal cortex lesions led to little or no change in retrosplenia IEG levels. These novel findings show that hippocampal lesions leave the retrosplenia cortex abnormal, raising the possibility that retrosplenia dysfunction contributes to the impact of hippocampal damage. The entorhinal lesion results show that this hippocampal lesion effect is selective and not simply due to any deafferentation of the subiculum.

The present study deliberately examined a variety of methods to lesion the hippocampus, and also varied the behavioural methods used to raise IEG levels above their resting baseline levels in the retrosplenia cortex. In spite of the many procedural changes the findings were remarkably consistent (Fig. 10) and all led to significant reductions in Fos-positive cells across the retrosplenia cortex. These IEG depletions, which were found across both superficial and deep layers, were of similar magnitude across experiments in spite of the variations in procedure. The only minor exception was the evidence of a relatively smaller Fos decrease in Rgb in experiment 3 (Fig. 10). While this subtle difference might reflect the change in rat strain, it is more probably due to the differential involvement of the dorsal and ventral subiculum in experiments 1–3. In experiment 3 there was the
most sparing of the dorsal subiculum, which projects to Rgb (Van Groen & Wyss, 2003), so potentially limiting the IEG disruption.

Assessments of the numbers of retrosplenial cells and the physical parameters of these same cells showed that the region appeared grossly normal in spite of the reductions in both IEGs. The hippocampal lesions did not alter significantly the numbers of Nissl-stained cells in any of the subareas within the retrosplenial cortex. Likewise, there were no changes in the numbers of NeuN-positive cells in experiment 3, both results showing the lack of cortical atrophy following the various hippocampal lesions. More detailed measurements of the physical properties of the Nissl-stained material in Rga, Rgb and Rdg again found no evidence of robust changes in volume, staining intensity or shape. No changes were found in experiments 2–3. The only exception was for Nissl staining in experiment 1 (radio-frequency lesions) where a decrease in cell area was found just in Rdg and an increase in staining intensity was noted in Rdg and Rgb. These selective changes may be associated with the use of radio-frequency lesioning in experiment 1 and the inevitable greater damage to white matter. This damage also often included parts of the overlying parietal cortex and area 18b, and it may be relevant that area 18b projects directly to Rdg and Rgb (Van Groen & Wyss, 1992, 2003) but not to Rga (Van Groen & Wyss, 1990). It is therefore plausible that the loss of white matter would have resulted in slightly greater cell packing.

Studies into the impact of retrosplenial cortex dysregulation in rats have shown how lesions in this area consistently impair tests of spatial memory (Sutherland et al., 1988; Mizumori et al., 2000; Alexinsky, 2001; Cooper et al., 2001; Whishaw et al., 2001; Vann & Aggleton, 2002, 2004). These same spatial tests are also sensitive to hippocampal lesions. Furthermore, there are dense, reciprocal connections between the two structures (Wyss & Van Groen, 1992), with hippocampal efferents from CA1, the subiculum and postsubiculum projecting directly to Rga, Rgb and Rdg. This arrangement suggests that the two areas work conjointly to support spatial memory (Mizumori et al., 2000). This notion was tested directly by Sutherland & Hoesing (1993) who examined the effects of crossed, unilateral lesions in the hippocampus and retrosplenial cortex. The subsequent deficits in the Morris water maze (Sutherland & Hoesing, 1993) showed that the two areas work conjointly to support spatial memory (Mizumori et al., 2000). This notion was tested directly by Sutherland & Hoesing (1993) who examined the effects of crossed, unilateral lesions in the hippocampus and retrosplenial cortex. The subsequent deficits in the Morris water maze (Sutherland & Hoesing, 1993) showed that the two areas work conjointly to support spatial memory (Mizumori et al., 2000).
but are relatively unresponsive due to the lack of one set of excitatory afferents. In order to demonstrate the former (covert pathology) it would be necessary to show that the responsiveness of some retrosplenal neurons to all other (intact) inputs is permanently abnormal. There are, in fact, several reasons to believe this ‘covert pathology’ account. First, the IEG depletions were very marked (especially in Rga) and were found across both the superficial and deep laminae in retrosplenal cortex. This global effect on area 29 can be contrasted to the innervations from the hippocampal region, which appear more restricted as they terminate in laminae I-III (Wyss & Van Groen, 1992). Second, the surgeries would have left intact the other numerous afferents to area 29, e.g. from the anterior thalamic nuclei, the lateral dorsal thalamic nucleus, the anterior cingulate cortex, contralateral area 29 and areas 17 and 18b (Van Groen et al., 1993), yet these inputs were not sufficient to compensate for the lost hippocampal afferents. Third, there is growing evidence that the retrosplenal cortex shows covert pathology following anterior thalamic nuclei lesions. Not only do all three subareas (Rga, Rgb and Rdg) show very marked decreases in Fos and Zif counts (Jenkins et al., 2004) but electrophysiological studies using retrosplenal cortex slices taken from rats with unilateral anterior thalamic lesions reveal a loss of synaptic plasticity in superficial Rgb on the same side as the thalamic lesion (Garden et al., 2006). This loss of plasticity is revealing as it cannot be a mere deafferentation effect as the stimulated microcircuits are still present in the slice (Garden et al., 2006). Given that the IEG losses in the superficial lamina appear similar after hippocampal lesions to those observed after anterior thalamic lesions (Jenkins et al., 2004) it becomes increasingly probable that the present lesions might also be sufficient to disrupt plasticity. Four, the retrosplenal cortex is abnormally sensitive to systemic NMDA receptor modulation (Olney et al., 1993, 1998), highlighting its potential sensitivity to the loss of these glutamatergic inputs. Finally, the growing evidence that the activity of c-fos and zif268 is integral to many neuronal functions, including neuronal plasticity (Herdegen & Leah, 1998; Hughes et al., 1999; Tischmeyer & Grimm, 1999), makes it increasingly unlikely that tissue showing chronically low IEG levels could contribute normally to tests of learning and memory.

The lack of any marked changes in retrosplenal Fos levels after entorhinal cortex lesions provides evidence for the specificity of the response to hippocampal damage. This region provides an ideal control as the lesion methodology was identical to that used in experiment 3 and the lesion placement was immediately adjacent to the hippocampus. Not only does the entorhinal cortex have some direct projections to the retrosplenal cortex (Van Groen & Wyss, 1992; Insauti et al., 1997) but the granular retrosplenal cortex is also innervated by the subiculum (Van Groen & Wyss, 1990, 1992) which, in turn, is innervated by the entorhinal cortex. The surprising lack of a clear change in retrosplenal cortex accords with the report that aspiration lateral entorhinal cortex lesions need not decrease, and can indeed increase, c-fos in the hippocampus, parietal cortex and piriform cortex (Bernabeu et al., 2006). The present null result can also be added to that seen after postrhinal cortex lesions (Jenkins et al., 2004), and so highlights the specificity of the hippocampal lesion effects on retrosplenal cortex.

The patterns of Fos and Zif268 changes following hippocampal lesions can be compared with those seen after anterior thalamic lesions. Like the hippocampus, the anterior thalamic nuclei have dense, reciprocal connections with area 29. While lesions in both the hippocampus and anterior thalamic nuclei produce a dramatic fall in retrosplenal Fos and Zif268 levels (Jenkins et al., 2004), there is at least one clear difference. The IEG changes following anterior thalamic lesions are extremely evident in the superficial laminae but often not noted in the deeper laminae (Jenkins et al., 2004). This selectivity was not seen in the present study (Fig. 10) as in addition to the striking loss of superficial label there was also a clear loss of deeper IEG label, sometimes seen as a cell-free band in layer V (Figs 4 and 8). The difference presumably reflects the fact that hippocampal inputs to the retrosplenal cortex are focused in laminae I-III (especially II), while the anterior thalamic inputs terminate in I, III and IV with much lighter terminations in lamina II (Van Groen & Wyss, 1990, 1992, 2003). Nevertheless, it is striking that lesions in both the anterior thalamic nuclei and the hippocampus have such remarkable effects on IEG levels in the retrosplenal cortex, and this common effect adds weight to other evidence from rats showing that these three regions (the hippocampus, anterior thalamic nuclei and retrosplenal cortex) function together to support aspects of memory (Sutherland & Hoesing, 1993; Warburton et al., 2001).

The importance of retrosplenal dysfunction to various neurological conditions is only gradually emerging. Of especial potential relevance to the present study are the findings of retrosplenal hypoactivity in Alzheimer’s disease, mild cognitive impairment, Korsakoff’s disease and other forms of diencephalic amnesia (Fazio et al., 1992; Aupe et al., 2001; Nestor et al., 2003b; Reed et al., 2003). The retrosplenal cortex is one of the first brain sites to show metabolic hypoactivity in Alzheimer’s disease (Minoshima et al., 1997; Nestor et al., 2003a). While the same region does not show overt pathology in the early stages of the disease, three of the very earliest sites to show overt pathology are the entorhinal cortex, the hippocampus and the anterior thalamic nuclei (Braak & Braak, 1991a,b). It has therefore been suggested that retrosplenal hypoactivity is a response to deafferentation from such sites (Fazio et al., 1992; Meguro et al., 1999; Hirao et al., 2006). It is striking, therefore, that when two of these three sites (hippocampus and anterior thalamus) are damaged a very marked IEG hypoactivity is found in retrosplenal cortex. These findings support the notion that the retrosplenal hypoactivity seen in Alzheimer’s disease is a secondary response to distal damage in these limbic sites.

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Abbreviations

DA, Dark Agouti (rats); IBO Ent Sham, sham surgery; IBO Ent, (group with) bilateral lesions of the entorhinal cortex; IBO Hpc Sham, sham-operated control group; IBO Hpc, (group with) ibotenic acid hippocampal lesions; IEG, immediate–early gene; NeuN, neuronal nuclei protein; NMDA Hpc, (group with) unilateral lesions of the hippocampus; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PBST, 0.1% Triton X-100; PFA, 4% paraformaldehyde in 0.1 M PBS; Rdg, dysgranular cortex; RF Hpc Sham, surgical controls; RF Hpc, (group with) bilateral radio-frequency lesions of the hippocampus; Rga, granular a cortex; Rgb, granular b cortex.

References
