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Spatial memory formation differentially affects c-Fos expression in retrosplenial areas during place avoidance training in rats

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The retrosplenial cortex is involved in spatial memory function, but the contribution of its individual areas is not well known. To elucidate the involvement of retrosplenial cortical areas 29c and 30 in spatial memory, we analyzed the expression of c-Fos in these areas in the experimental group of rats that were trained in a spatial place avoidance task, i.e. to avoid shocks presented in an unmarked sector of a stable arena under light conditions. Control rats were trained in the same context as the experimental rats either without (Control-noUS) or with shocks (Control-US) that were delivered in a random, noncontingent manner for three days. On the first day of place avoidance learning, the experimental group exhibited c-Fos induction in area 29c, similar to both control groups. In area 30, similarly high levels of c-Fos expression were observed in the experimental and Control-US groups. On the third day of training, when the experimental group efficiently avoided c-Fos expression in areas 29c and 30 was lower compared with the first day of training. In area 29c c-Fos level was also lower in the experimental group in comparison to the Control-US group. In area 30, c-Fos expression in the experimental group was lower than in both control groups. In conclusion, areas 29c and 30 appear to be activated during spatial memory acquisition on the first day of training, whereas area 30 seems suppressed during long-term memory functioning on the third day of training when rats effectively avoid.

Key words: granular retrosplenial areas, dysgranular retrosplenial area, c-Fos expression, spatial learning, spatial memory, place avoidance task

INTRODUCTION

The retrosplenial cortex (RSC) contains head-direction cells (Chen et al. 1994a, 1994b) and belongs to the limbic network that mediates spatial memory processes in rodents (for review see Vann et al. 2009). The mapping of immediate-early genes (IEGs) in mice has shown that the RSC is engaged in the encoding and storage of spatial memory that is acquired in the water maze task (Czajkowski et al. 2014). The RSC is morphologically heterogeneous, but two main regions (granular and dysgranular) have been distinguished (Vogt 1985). These retrosplenial areas are closely connected with the hippocampal formation, the parahippocampal region, and anterior thalamic nuclei. Each of these areas is characterized by distinct connectivity patterns (Jones and Witter 2007, van Groen and Wyss 1992, 2003, Sugar et al. 2011, Shibata 1993). The morphological and connectional heterogeneity of RSC subdivisions provides a basis for their functional diversity. Understanding the functions of retrosplenial areas appears to be particularly important because the RSC, which belongs to the hippocampal-diencephalic network, is implicated in episodic memory processes, disturbances of which may lead to amnesic dysfunction in humans (Aggleton 2010). To date, few behavioral

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studies have investigated the involvement of individual retrosplenial areas in spatial memory function. Selective lesions of the granular cortex caused deficits in spatial working memory in rats that were trained in a water maze task (van Groen et al. 2004) and radial arm maze (Pothuizen et al. 2010). Such lesions also caused deficits in long-term spatial memory in the water maze task (van Groen et al. 2004). Lesions of the dysgranular cortex impaired allocentric spatial working memory in rats in the radial arm maze task (Vann and Aggleton 2005). No studies of the involvement of the dysgranular cortex in spatial long-term memory have been performed. Additional evidence of the functional specialization of retrosplenial subdivisions comes from IEG imaging studies, showing that distinct patterns of activation of granular and dysgranular cortices depend on the specific conditions of allothetic/idiothetic spatial working memory tasks (Pothuizen et al. 2009). Jenkins and others (2004), Garden and colleagues (2009), and Amin and others (2010) reported that granular and dysgranular areas play diverse roles in memory dysfunction following lesions of anterior thalamic nuclei. Notably, such lesions and commonly applied IEGs mapping studies utilize tests that are based on appetitive reinforcement to form approach responses (e.g., finding a platform or finding food in an arm maze). Another approach, however, is to use aversively motivated conditioning combined with *c-fos* mapping based on nonspatial learning and memory (for review see Kaczmarek 2002).

The present study used c-Fos mapping to investigate the engagement of retrosplenial areas in spatial memory formation in rats that were trained in a (Room and Arena)+ variant of the place avoidance task. The measure of c-Fos expression was the density of c-Fos-immunopositive nuclei in granular area 29c and dysgranular area 30 after one and three days of avoidance training.

MATERIALS AND METHODS

Animals

The experiments were performed with 48 male Long-Evans rats, 3 months of age and weighing 300–325 g. The rats were obtained from the breeding colony of the Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw. The animals were housed four per cage under standard conditions (lights on from 7:00 a.m. to 7:00 p.m., 22°C, 23% humidity) and received food and water *ad libitum*. All of the procedures were performed in accordance with the regulations of the Polish Communities Council for the care and use of laboratory animals and the European Community Directive for the ethical use of experimental animals.

Apparatus and procedure

The place avoidance apparatus consisted of an 80-cm-diameter arena that was constructed of aluminum, flanked by a 5 cm peripheral metal rim. The arena was elevated 80 cm above the floor and placed in a room that was lit by dim light and had many stable visual cues. Small portions of food (commercial seeds) were delivered randomly to the arena surface every 20 s from a food dispenser that was mounted above the arena. Infrared light-emitting diodes (LEDs) were positioned between the rat's shoulders using a latex harness. A cable that carried the shock current was attached to a shock electrode on the rat's back. The V-shaped shock electrode was prepared from a 25-gauge hypodermic needle. Whenever the rat entered the 60° shock sector, a computer-triggered, constant current (50 Hz, 0.5 s) was delivered across the low-impedance (100 Ω) shock electrode and high-impedance (100 KΩ) contact of the rat's paws to the grounded arena surface. The shock amplitude (0.2–0.5 mA) was adjusted for individual rats so their responses to the shock were moderate and did not induce freezing or attempts to escape from the arena. The shock was repeated after 1.5 s in cases in which the rat stayed inside the shock sector. The infrared LED allowed the position of the rat to be tracked every 20 ms using an infrared-sensitive television camera that was attached to a computer system. To maintain constant experimental conditions, the arena was located in a separate room, and the experiment was monitored in an adjacent room where the place avoidance system was located. Commercial software was used for off-line data analyzes (Bio-Signal Group). Shocks on shock sector were applied automatically by program. Application of shocks in a random, noncontingent manner throughout the arena were administered manually by the experimenter because program does not have the option of automatically delivering and recording random shocks.

The place avoidance procedure was described previously (Fenton et al. 1998). Briefly, for three weeks before the experiment, the rats were food-deprived to 85% of their free-feeding weight to prompt searching for food that was randomly scattered in the arena. This ensured that freely moving rats that explored the arena and searched for food during the experiment would enter the shock sector (Bures et al. 1997). In a variant of the place avoidance task that was used in the present study, the position of the arena was constant in the lit room. Thus, the unmarked shock sector was defined by its position relative to the room and arena frame cues (i.e., distal visual stimuli that were always in mutually constant positions and proximal stimuli from arena, like urination and defecation). Both sets of stimuli were relevant for mapping the position of the shock sector in the (Room and Arena)+ variant, distal landmarks across all experimental days, whereas proximal stimuli, changing from day to day, across one daily session.

Place avoidance and control training

All of the rats received identical treatments in their home cages and during two weeks of habituation. During this period food-deprived animals received seeds in their home cages to familiarize them with this type of food and were thoroughly habituated to handling by the experimenters to avoid the effects of novelty during training. During the five last days of habituation rats were pretrained in the experimental setup in 10-min sessions per day. Following pretraining the Exp group was trained to avoid the shock sector in the stable arena under light conditions in the (Room and Arena)⁺ task. Rats in the two control groups were exposed to the same experimental context as the Exp rats. One control group (Control-noUS) did not receive shocks after entering the shock sector, and the other control group (Control-US) received the same number of shocks as the Exp group, but the shocks were administered throughout the entire arena in a random, noncontingent manner. Thus, only the Exp group had the possibility of learning place avoidance. Rats in the Control-noUS group gained experience only in motor activity and exploration of the arena while searching for food, whereas exploration in the Control-US group was disturbed by the shocks. The Exp, Control-US, and Control--noUS groups were subjected to one or three session with one 20-min session per day (Table I).

After the completion of behavioral training, all of the rats were maintained under familiar and constant conditions for 90 min, after which they were perfused for further c-Fos analysis.

Table I. Number of rats in behavioral training and c-Fos analysis.

Group	Number of rats in group	
	Day 1	Day 3
Exp	Exp	Exp
Behavioral analysis	n=8	n=8
c-Fos count	n=7	n=4
Control-noUS	Control-noUS	Control-noUS
Behavioral analysis	n=8	n=8
c-Fos count	n=8	n=4
Control-US	Control-US	Control-US
Behavioral analysis	n=12	n=12
c-Fos count	n=5	n=6

Measures

During training, the computer program recorded the number of entrances (ENTR) to the shock sector and the time to the first entrance to the shock sector (T1). These parameters represented avoidance learning only in the Exp group. In the Control-noUS group, the computer program calculated the number of ENTR and T1 on the shock sector, however the shocks were disconnected. The experimental procedure that was used for the Control-US group (i.e., application of shocks on entire arena in a random, noncontingent manner) excluded the calculation of the aforementioned parameters by computer system.

To compare performance in the Exp and Control-noUS groups, the path trajectories in the arena were recorded, and the percentage of time spent in the following quadrants was calculated: quadrant where 60° place with shocks was present ("TARGET"), quadrant that was opposite to the TARGET quadrant ("OPP"), quadrant that was clockwise from the TARGET quadrant ("CW"), and quadrant that was counterclockwise from the target quadrant ("CCW"). For the Exp and Control-noUS groups, the time in the TARGET quadrant was calculated. For the Control-US group, only the path trajectories were recorded. In the Exp, Control-US, and Control-noUS groups, the total path length (TPL) in meter was used as a measure of locomotor activity.

Data analysis

All of the results were expressed as mean ±standard error of the mean (SEM). The data had a skewed (non-normal) distribution for the measured parameters. Therefore, we transformed the values with a common logarithm. A constant of 1 was added to T1 and the time in the TARGET quadrant prior to this transformation to ensure that the resulting values were not less than zero. Comparisons between consecutive four 5-min blocks of twenty min session on day 1 or between three days of training for Exp and Control-noUS group was evaluated separately using a one-way ANOVA followed by Tukey's Honestly Significant Difference *post hoc* test. A two way ANOVA (group×day: 3×3), with repeated measures on the last factor, was used for comparisons the total path length for Exp, Control-noUS and Control-US rats. The c-Fos expression data were analyzed using three-way ANOVA (group×day×area: 3×3×2), with repeated measures on the last factors. The Huynh-Feldt (H-F)-adjusted *post hoc* test followed the ANOVAs when appropriate. Values of *P*<0.05 were considered statistically significant. The statistical calculations were performed using Statistica 6 software (StatSoft, Nencki Institute).

Retrosplenial cortex divisions

To demarcate retrosplenial areas in the rats, we followed the division that was described by Vogt and Peters (1981). Rodent and primate retrosplenial cortices

are similar in their essential architecture. Thus, for the nomenclature, we followed Vogt and Paxinos (2014), based on the anatomical account of Brodmann (1909). The areas were identified based on the relative size and shape of neuronal perikarya, cell-packing density, and Nissl staining intensity (Fig. 1).

Area 29c: At the rostral level, this area adjoins area 30 (Figs 1A, 1B) and is posteriorly situated between areas 30 and 29b (Figs 1C, 1D). Its superficial layers are more differentiated and encompass smaller neurons compared with area 29b. Layer V is strongly differentiated and contains two sublayers with different neuron-packing densities, with neurons in the
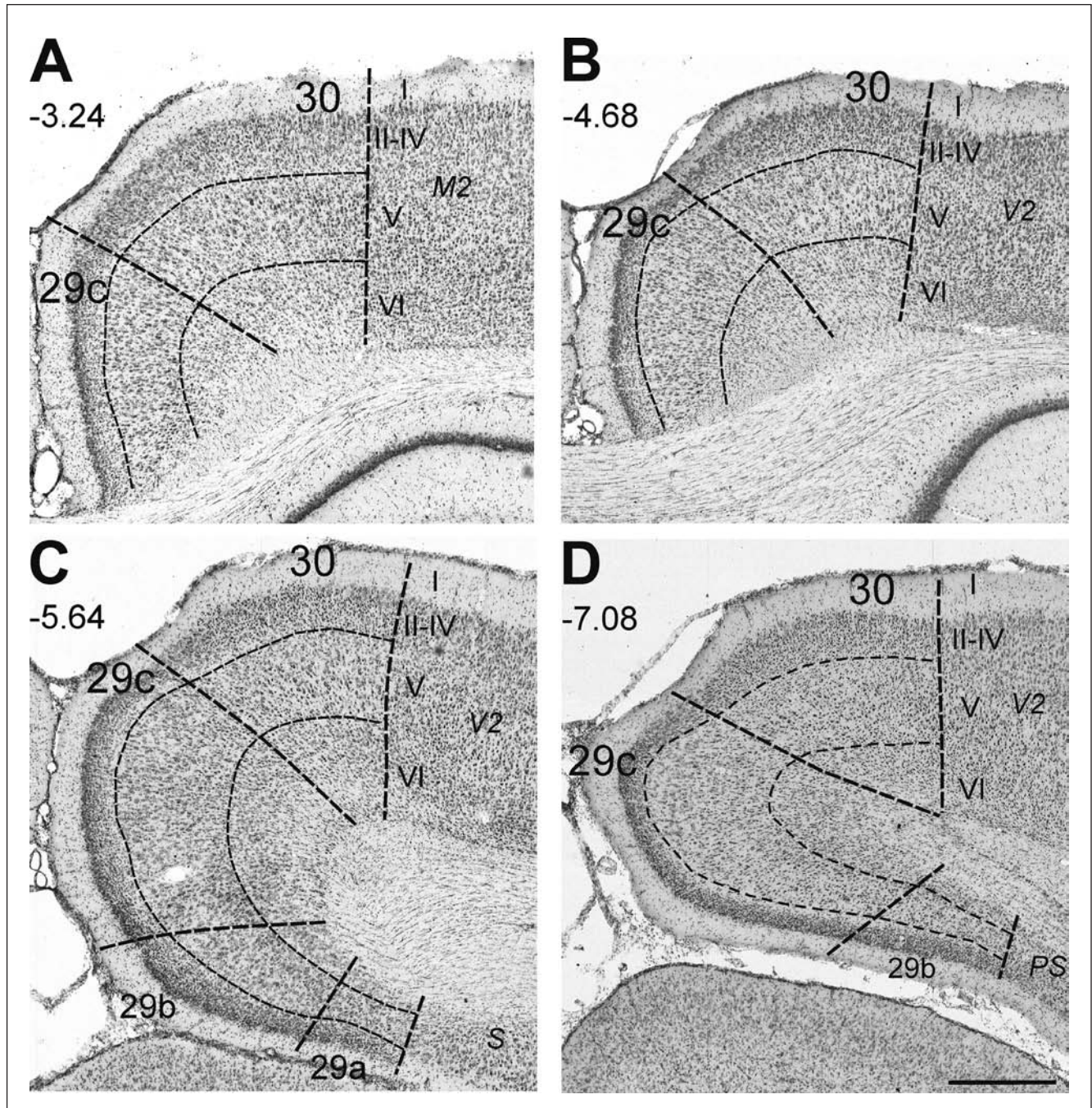

Fig. 1. Division and cytoarchitecture of the retrosplenial cortex in the rat. The figure shows photomicrographs of the cytoarchitecture of retrosplenial areas, revealed by Nissl staining of 40 μm coronal sections at four anterior-posterior levels: −3.24, −4.68, −5.64, and −7.08 mm from bregma (A–D, respectively). At these levels, c-Fos expression was measured. The borders between retrosplenial areas are shown as thick dotted lines. Cortical laminae are indicated by Roman numerals, and their borders are shown as thin dotted lines. Detailed descriptions of the cytoarchitectonic features of each area are provided in the Results section. Scale bar =0.5 mm.

deeper sublayer that are more densely distributed. Layer VI in area 29c is wider than in area 29b.

Area 30: This area distinctly differs from adjacent, medio--ventrally localized area 29c. Layers II and III in area 30 are much wider than in area 29c but less differentiated and with fewer neurons. Layer IV is very thin and encompasses a few neurons. Layer V is conspicuous and involves large, darkly stained neurons. Layer VI in area 30 is generally wider than in area 29c and consists of two sublayers (Figs 1A–1D).

c-Fos immunohistochemistry

Ninety minutes after completing the last training the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, Vetbutal) and transcardially perfused with 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The brains were removed, postfixed in PFA overnight, transferred to 30% sucrose in 0.01 PBS, and kept in a refrigerator for 3–5 days. Sections were cut at 40 μm in the coronal plane in a freezing microtome. Six series of sections were collected in PBS. One series was processed for c-Fos immunohistochemistry, with all stages under agitation. The activity of aldehyde groups (i.e., sources of background staining) was diminished by incubating the sections in 1% borohydride in PBS. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide/70% methanol in distilled water for 20 min. The sections were incubated in 0.3% PBST/5% normal goat serum (NGS) that contained c-Fos rabbit polyclonal antibody (1:100,000, Oncogene) for 48 h at 4°C. Incubation was stopped with PBST/5% NGS, and the sections were incubated in biotinylated goat anti-rabbit antibody in PBST/2% NGS (1:200; Vectastain, Vector Laboratories, Burlingame, CA, USA). The sections were then washed and processed with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite Kit, Vector Laboratories) in PBST for 1 h at room temperature. The reaction was visualized using diaminobenzidine (DAB; Sigma, St. Louis, MO, USA), intensified with nickel salt. The sections were mounted on gelatin-coated slides, dehydrated in a graded series of alcohol, and coverslipped. The adjacent series of sections was Nissl--stained using Cresyl violet for the histological identification of retrosplenial areas. For the immunocytochemistry procedures, 2–4 adequate sections from experimental and control rats were processed simultaneously in sieves-at--bottom four-well Plexiglas organizers to ensure access of the sections from both groups to the same sets of solutions.

c-Fos-positive nuclei counts

In the Exp, Control-US, and Control-noUS groups, c-Fos expression was analyzed on the first and third day of training. Digital images of the retrosplenial areas were captured using a Sony charge-coupled device camera that was attached to an Optiphot-2 light microscope (Nikon) under a 10× objective (10×10 total magnification). Images were acquired in 8-bit gray scale, and no adjustment of contrast or intensity or gamma-correction was applied to the images that were used for automatic counting. Image analysis was performed with ImagePro Plus 6.3 imaging software (Media Cybernetics). c-Fos expression reflected the density of c-Fos-immunopositive nuclei that were labeled above a set threshold. For all of the sections, a brightness threshold of 25% of the background level was applied. Nuclei that were darker than the threshold value were considered c-Fos-positive. The threshold was set individually for each section as the mean of gray levels of the frame area (rectangular sample; 44 μm width×21 μm height) from four random localizations within the background. The thresholds were determined individually for areas 29c and 30. To calculate the density of c-Fos nuclei in each area for each section, the number of c-Fos-immunopositive nuclei in a given retrosplenial field was counted and divided by the area (mm^2) that was occupied by this field. For each brain, counts were taken from four anterior-posterior levels (–3.24, –4.68, –5.64, and –7.08 mm from bregma). For each level, 2–4 consecutive coronal sections across the right hemisphere were analyzed and then averaged.

RESULTS

Place avoidance performance during (Room and Arena)$^+$ training

In the Exp group acquisition of place avoidance began on the first day of the (Room and Arena)$^+$ training. The number of entrances into the place where shocks were applied decreased in the Exp group over four 5-min blocks in a 20-min session (the one-way ANOVA, $F_{3,18}$=4.73, P<0.01; Fig. 2A, ENTR). During the first block number of entrances was higher than in the next three blocks (*post hoc* test; P<0.01). Rats from the Control-noUS group, which never received shocks, visited the particular place in the consecutive 5-min blocks by chance, the one-way ANOVA for blocks was not significant ($F_{3,21}$=0.98; P=0.42).

The total path length (TPL) on day 1 was calculated in the Exp, Control-noUS and Control-US groups (mean values in meters: 78.20±10.35, 51.94±6.03, and 26.12±3.52, respectively). The one-way ANOVA on day 1 revealed a significant effect of group ($F_{2,18}$=9.78, P<0.001). The Exp and Control-noUS groups travelled a similar distance, whereas the distance travelled in the Control-US group was shorter compared with the Exp group (*post hoc* test; P<0.002).

Long-lasting place avoidance learning was analyzed over three days of training in the (Room and Arena)[+] task in the Exp group. The Exp rats exhibited efficient avoidance, reflected by a low number of entrances into the place with shocks and an increase of latency to the first entrance (Figs 2B, 2C, respectively). The one-way ANOVA analysis, which was performed on the number of entrances or T1 over the three days of avoidance training, revealed significant effect of day (ENTR $F_{2,14}$=8.28, P<0.004; T1 $F_{2,14}$=3.77, P<0.04). The number of entrances decreased on the third day in comparison to the first day of avoidance training (*post hoc* test; P<0.003). Time to the first entrance was shorter on day 1 than on day 2 and day 3, however statistical evaluation was on the border of significance (*post hoc* test; P=0.06).

Similar statistical comparison was done for Control--noUS rats, which freely walked on arena without shocks administration and could entered by chance the shock place.

The one-way ANOVA revealed significant effect of day on the number of entrances but not for T1 over the three days of training (ENTR $F_{2,14}$=4.58; P<0.02; T1 $F_{2,14}$=0.67; P=0.52). The high level of entrances at the beginning of training decreased on the second and third day, however statistical evaluation was on the border of significance (*post hoc* test; P=0.06).

The time spent in the quadrant, where shocks were presented ("TARGET" quadrant), by rats from the EXP group was changing across days. The one-way ANOVA revealed significant effect of day ($F_{2,14}$=8.10, P<0.005). Time spent in the "TARGET" quadrant was shorter on day 2 and day 3 in comparison to day 1 (*post hoc* test; P<0.03) (Fig. 2D). The same comparison for Control-noUS group was not significant.

During avoidance training the percentage of time spent by rats from Exp group in the "TARGET" quadrant decreased, with consequent increases of percentage of time in the counter-clockwise ("CCW") quadrant and in the quadrant opposite to the target ("OPP") (Fig. 3B). Although for the Control-noUS group the percentage of time in the "OPP" quadrant increased, but they also visited the "TARGET" quadrant on each consecutive day (Fig. 3D). The behavior of the rats in the three groups was assessed by recording the path trajectories during each session (Fig. 3). The Exp group walked mainly peripherally and in the "OPP" quadrant (Fig. 3A), whereas the Control-noUS group explored the entire arena (Fig. 3C). The Control-US group exhibited different pattern of exploration compared with the other two groups. On the first day of training, the path trajectory covered the entire arena, although the rats received a similarly high number of shocks as the Exp group (Fig. 3E). On the second and third day, the path trajectory in the Control-US group was limited to the peripheral part of the arena.

The total path length (TPL) in the Exp and Control-noUS groups differed from the Control-US group (Fig. 2E). The two-way ANOVA (group×day; 3×3, with repeated measures on the last factor) revealed a main effect of group ($F_{2,25}$=51.30; P<0.000001), with effect of day ($F_{2,50}$=15.73; P<0.000001), and group×day interaction ($F_{4,50}$=5.2, P<0.001). The Control-US group walked less than rats in the Exp and Control-noUS groups (*post hoc* test; P< 0.0001) and rats walked more on day 1 than on days 2, and 3 (P<0.0001). The Exp rats covered similar distance every day across
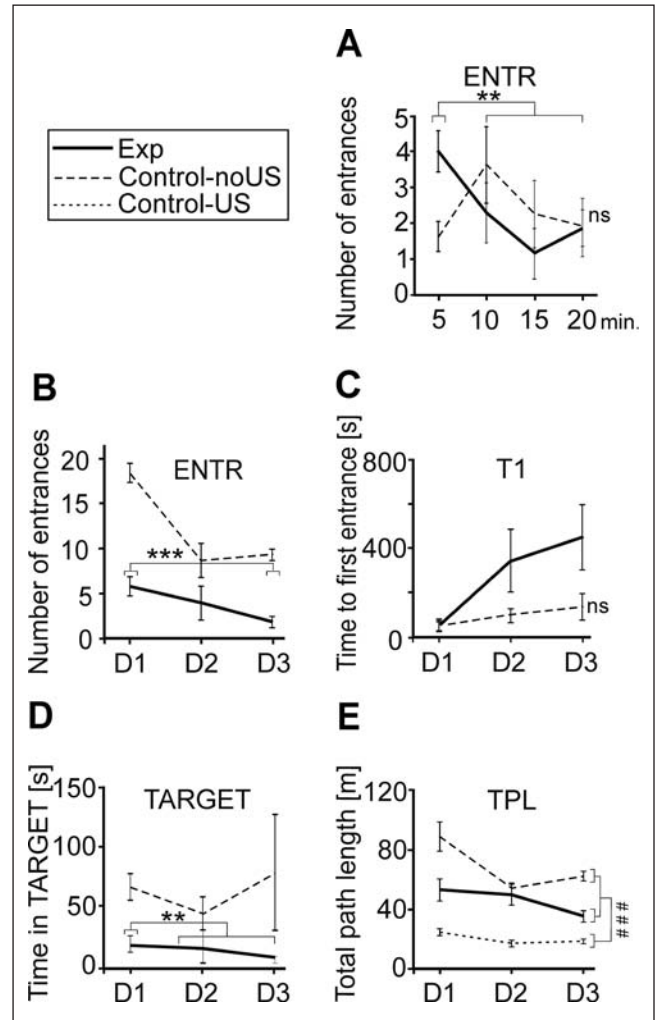

Fig. 2. Formation of spatial memory at different stages of place avoidance training, demonstrated by changes in behavioral parameters. (A) On the first day of training (D1), the number of entrances (ENTR) decreased in rats in the experimental (Exp) group in consecutive 5-min blocks. *Post hoc* test results marked by asterisk was equivalent to ** (P<0.01). (B) On third day of training (D3), the Exp rats exhibited a lower number of entrances (ENTR), (C) a longer latency to the first entrance (T1), and (D) a shorter time spent in the TARGET quadrant of the arena compared with the first day of training (the *post hoc* results for ENTR *** P<0.003; TARGET ** P<0.03; T1 P=0.06 border level of significance). (E) The total path length (TPL), as measure of locomotor activity, was similar in the Exp and Control-noUS groups and it was higher than in the Control-US group (*post hoc* test; ###P<0.001). The data are expressed as mean ±SEM. Numbers of rats in each group is presented in Table I.

three days of training. The interaction group by day showed that the Control-US rats also covered similar distance every day, however its level was lower than in the Exp and Control-noUS rats. Moreover, the Control-noUS rats on day 1 presented longer distance than all rats in all days of training (*post hoc* test; *P*<0.001).
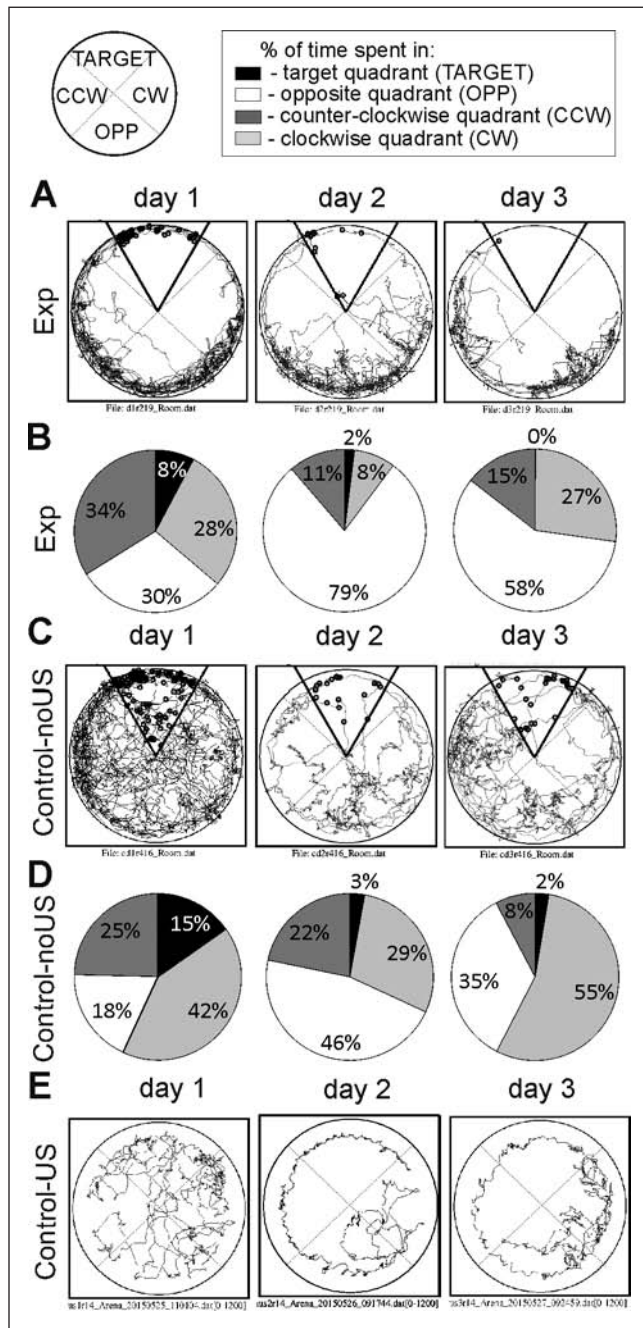

Fig. 3. Illustration of performance of rats from the Exp, Control-noUS and Control-US groups over three days of training. (A, C, E) Representative path trajectories on the arena. (B, D) Percentage of time spent by rats (average) in the quadrant with 60° shock sector ("TARGET," black), in the quadrant opposite to the target ("OPP," white), in the clockwise quadrant ("CW," light grey) and the counter-clockwise ("CCW," dark grey). Borders of quadrants are showed by broken lines.

Pattern of c-Fos expression following (Room and Arena)+ and control training

Patterns of c-Fos expression on the first and third days of training differed in the Exp, Control-US, and Control-noUS groups. The three way ANOVA (group *vs.* day *vs.* area) for c-Fos nuclei density confirmed significant effect of group ($F_{2,10}$=4.73, *P*<0.035), day ($F_{1,10}$=5.79, *P*<0.036) and area ($F_{1,10}$=28.22, *P*<0.0003). The level of c-Fos expression was higher in the Control-US group then in the EXP and the CONTR-noUS groups, on border level of significance (*post hoc* test; *P*<0.056). The level of c-Fos expression was higher on day 1 than on day 3 (*post hoc* test; *P*<0.03). In area 29c, the density of c-Fos nuclei was higher than in area 30 (*post hoc* test; *P*<0.0005).

The group×day interaction was significant ($F_{2,10}$=5.35, *P*<0.026). In the Exp group level of c-Fos on day 3 was lower than on day 1, and lower than c-Fos on day 1 and day 3 in the Control-US group (*post hoc* test; *P*<0.02). In the Control-noUS group level of c-Fos expression does not differ from the EXP and the Control-US groups on day 1 and 3. The group×day×area interaction was significant ($F_{2,10}$=12.08, *P*<0.0021). The *post hoc* test for the above interaction showed that on day 1 in area 29c in the Exp rats the level of c-Fos expression was the same as levels in the Control-noUS and the Control-US rats (Figs 4A, 5, day 1). The *post hoc* test for the same interaction shows, that on day 3 the density of c-Fos nuclei in area 29c was lower on day 3 in the Exp rats
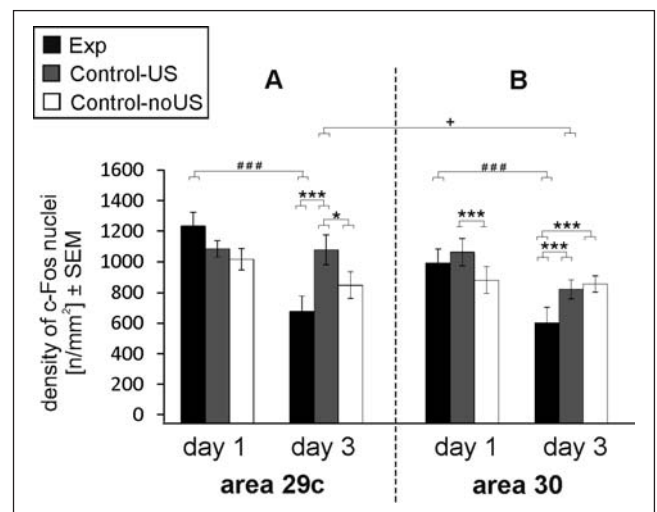

Fig. 4. Expression of c-Fos in (A) retrosplenial area 29c and (B) retrosplenial area 30 over the course of training in the Exp, Control-US, and Control-noUS groups. Notice the similar induction of c-Fos in the Exp and Control-US groups after the first day of training and lower level of c-Fos in the Exp group than in the Control-US group after the third day of training in both areas. *P*<0.05, **P*<0.01, ***P*<0.001, group differences within days; ###*P*<0.001, group differences between days; +*P*<0.05, area differences within days and groups. The data are expressed as mean ±SEM.

compared with the Control-US rats (*P*<0.0003), but no difference was found in the density of c-Fos nuclei compared with the Control-noUS rats (Figs 4A, 5, day 3). Contrary, the Control-US rats exhibited a higher density of c-Fos nuclei compared with the Control-noUS rats (*post hoc* test: *P*<0.03; Figs 4A, 5, day 3). The *post hoc* test further showed that on day 1 the density of c-Fos nuclei in area 30 in the Exp and the Control-US rats was similar (Figs 4B, 5, day 1), whereas the density of c-Fos nuclei was higher in the Control-US rats than in the Control-noUS rats (*post hoc* test: *P*<0.001; Fig. 4B, 5, day 1). On day 3 the level of c-Fos in area 30 was lower in the Exp rats than in the Control-US and the Control-noUS rats (*post hoc* test: *P*<0.007 and *P*<0.005, respectively), which exhibited similar expression levels (Figs 4B, 5, day 3). On day 3 density of c-Fos nuclei in area 30 was lower than in area 29c for Control-US group (*post hoc* test; *P*<0.006).

DISCUSSION

The main finding of the present study was that the level of activation of the granular (29c) and dysgranular (30) retrosplenial areas, measured by c-Fos expression, differed according to the stage of spatial memory that was acquired during place avoidance training in the (Room and Arena)+ variant. Generally, on the first day of avoidance training, c-Fos induction was observed in both retrosplenial areas compared with a lower level of expression on the third day of training. The patterns of c-Fos expression in the Exp group that exhibited avoidance learning were different in areas 29c and 30 compared with the two control groups at each analyzed day.

On the first day of training, a high level of c-Fos expression was found in area 29c in the Exp, Control-US, and Control-noUS groups. In contrast, a high level of c-Fos expression was observed in area 30 in the Exp and Control-US groups, whereas the level of c-Fos expression in the Control-noUS group was lower than in the Control-US group. On the third day of training c-Fos level in area 29c in the Exp group was similar to the Control-noUS group and c-Fos levels in both of these groups were significantly lower than in the Control-US group. In area 30 the level of c-Fos expression was lower in the Exp group than in both control groups.
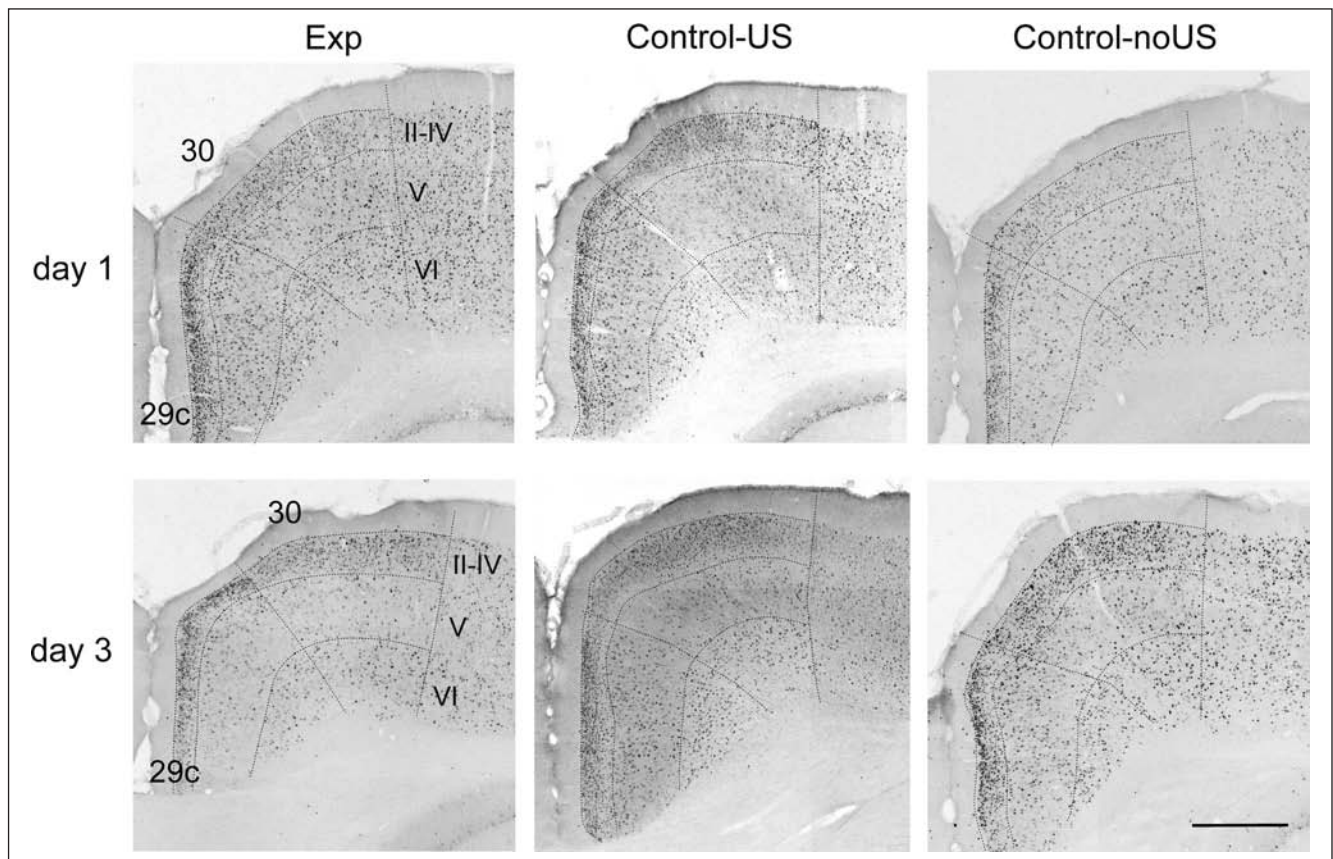

Fig. 5. Photomicrographs showing c-Fos expression in retrosplenial areas 29c and 30 on 40 µm coronal sections. On day 1, similar increases in the levels of c-Fos expression were observed in areas 29c and 30 in the Exp and Control-US groups. On day 3, a high level of c-Fos expression was observed in both areas in the Control-US group, and a lower level was observed in both areas in the Exp group. Scale bar =0.5 mm.

Behavior during spatial memory formation

Spatial learning begins on the first day of place avoidance training, reflected by changes in behavioral measures on the next day of the (Room and Arena)+ task (Wesierska et al. 2009). In the present study, rats in the Exp group effectively learned to avoid the place where shocks were delivered, reflected by a reduction of the number of entrances into the shock place, in subsequent blocks in the first session. Across three days of training the behavioral results indicated improvements in avoidance behavior. This was reflected by a low number of entrances into the shock sector, which approached zero on the third day, and an increase in the time to the first entry. As avoidance training progressed, the rats in the Exp group stayed longer in the quadrant of the arena that was opposite to the TARGET quadrant. This result of avoidance training in the (Room and Arena)+ task is consistent with previous studies (Cimadevilla et al. 2000, Wesierska et al. 2005, Wesierska et al. 2009).

Control training

The expression of *c-fos* during behavioral training, apart from changes that are directly attributable to learning and memory processes, may reflect concomitant factors, such as sensory stimulation, motor activity, stress, and attention (Kaczmarek 2000, Tischmeyer and Grimm 1999). To eliminate the influence of some of these factors (e.g., the effect of the novelty of contextual cues), the rats underwent 5 days of pretraining under experimental set-up conditions in 5 min sessions/day. During subsequent experiment, all of the rats were exposed to the same contextual cues in the room (i.e., a stable arena) in 20-min sessions/day. Under these conditions, the rats in the Exp group received short-lasting shocks in a fixed location in the arena. This shock sector was not marked by detectable demarcations and was defined only by its position relative to contextual cues within the room. Contrary to cues from room, which were in mutual fixed location, cues from arena were stable across session but changing from day to day (defecation and urination). This allowed trained rats to build spatial representations of the place that was to be avoided and consequently avoid it (Fenton et al. 1998). To determine the influence of the experimental conditions on c-Fos expression, we included two control groups. One control group was exposed to contextual cues only (Control-noUS group), and the other control group (Control-US group) was placed in the same context and received the same number of shocks as the Exp group during place avoidance training but in a random, noncontingent manner on the entire arena.

The Control-US group was included in the present study to determine the way in which shocks themselves affect the level of c-Fos expression and whether changes in c-Fos expression in trained rats in the Exp group were specifically associated with spatial learning processes. Unexpectedly, we found that the level of c-Fos was similar in the Control-US and Exp groups on the first day of training. One explanation for this finding is that exploration of the arena was extended from 5 min during pretraining to 20 min during avoidance training. The presence of shocks, shock-associated stress, and the novelty of this situation appeared to potently activate c-Fos in control with shocks group of rats (Tischmeyer and Grimm 1999). The difficulty in designing appropriate controls for aversive conditioning tasks was raised by Grimm and Tischmeyer (1997) and Nikolaev and others (1992). For control rats that are exposed to random, noncontingent shocks, assigning the induction of IEGs to distinct attributes of the learning process is impossible.

In the Control-noUS group we found that the density of c-Fos-positive nuclei exhibited a tendency toward a gradual decline on subsequent days of control training without shocks. A similar tendency in the pattern of c-Fos expression was previously observed in sham-trained animals that were used as a control for mice that were trained for 1, 2, and 5 days in an appetitive operant conditioning task (Bertaina-Anglade et al. 2000). This finding may be explained by diminishing levels of non-associative learning and a lack of an effect of novelty. The level of c-Fos expression in sham-trained mice compared with untrained caged mice was three-times higher in the CA3 region of the dorsal hippocampus and two-times higher in the anterior cingulate cortex. Thus, the context control group (Control-noUS group) and shock control group (Control-US group) that were included in the present study seems appropriate as a reference for avoidance-trained rats that formed spatial memory in the place avoidance task.

Initial place avoidance learning

In the present study, c-Fos expression in area 29c and area 30 on the first day of place avoidance training in the presence of shocks, which were used as contingent reinforcement in the Exp group, was at a similarly high level as in the Control-US group that received random, noncontingent shocks. Similar c-Fos induction in these groups might be explained simply by the influence of shock-associated stress (Tischmeyer and Grimm 1999). The involvement of anterior thalamic nuclei, which are strongly connected with area 29c and engaged in affective cognition (Dupire et al. 2013), may be functionally important at this stage. Grimm and Tischmeyer (1997) reported a lack of differences in *c-fos* mRNA expression in the hippocampus and cerebral cortex between rats that were trained in one session in a visual discrimination task

and reinforced by footshocks and rats that were exposed to footshocks that were presented in a noncontingent manner. This is consistent with the greater induction of *c-fos* mRNA in the cortex after one session in rats that were trained in 40 trials for 45 min in a two-way shuttle-box avoidance learning task compared with sham-trained rats (Nikolaev et al. 1992). Also, the presentation of control footshocks *vs.* contextual cues that were associated with footshocks resulted in similar *c-fos* upregulation in the amygdala after contextual fear conditioning (Milanovic et al. 1998, Rosen et al. 1998). Notably, in our place avoidance procedure, each shock lasted a maximum of 0.5 s, with a shock intensity of 0.2–0.6 mA, whereas in the aforementioned studies, the rats received 1–1.5 mA shocks in 40 trials in a 45-min session.

The induction of c-Fos in the Control-noUS group in the present study may be explained by the fact that exploration of the arena increased from 5 min during the pretraining sessions to 20 min during the training sessions, which may constitute a novel experience for the animals (Kaczmarek 2002).

Effect of long-lasting place avoidance learning

Area 29c

On the third day of training, when place avoidance was highly effective compared with the first day of training, a decrease in c-Fos expression in area 29c was observed in avoidance-trained rats in the Exp group compared with the first day of training. Markedly higher levels of c-Fos expression in the Control-US group compared with the Exp group, suggest that *c-fos* expression is controlled by different processes. Rats from both group received the same number of shocks, but the Control-US group received random, noncontingent shocks, whereas in the Exp group shocks were given on the to-be avoided place and acted as reinforces of the conditioned response (i.e., place avoidance). In the Control-US rats under the same contextual conditions the conditioned response was not acquired. Thus, sporadic shocks may have lost their emotional value during the course of associative learning in the Exp group but still had emotional value in the Control-US group that underwent non-associative learning. The involvement of area 29c in the hippocampal formation network, which is modulated by emotional inputs from the amygdala (Bergado et al. 2011), may be partially responsible for the strong affective influence of random, noncontingent shocks, which elicit stress and arousal, on c-Fos expression. The same level of c-Fos expression in the Exp and Control-noUS groups may indicate that the relatively few shocks that were received by rats that acquired avoidance learning did not affect c-Fos expression.

The involvement of the granular cortex in long-term spatial memory was demonstrated in rats that were subjected to lesions of area Rgb (equivalent to areas 29c and 29b) and were trained in a water maze task (van Groen et al. 2004). Lesions of the entire granular cortex caused deficits in allothetic and idiothetic spatial working memory variants of the radial maze task (Vann and Aggleton 2005, Pothuizen et al. 2010), which is consistent with a previous c-Fos mapping study (Pothuizen et al. 2009). The increase in the number of c-Fos-positive neurons in area 29c may reflect the augmentation of excitatory inputs from anterior thalamic nuclei, the anteroventral and anterodorsal, and subicular complex (Meibach and Siegel 1977, van Groen and Wyss 1992, 2003), and the presubiculum, and homological areas 29c and 30 (van Groen and Wyss 1992, Jones and Witter 2007, Sugar et al. 2011). Anterior nuclei appear to play a significant role in mnemonic processes in the granular cortex by affecting neuronal plasticity, neurotransmission, and metabolic activity (Amin et al. 2010, Garden et al. 2009, Mendez-Lopez et al. 2013). An electrophysiological study by Finch and others (1984) showed that the stimulation of anterior thalamic and subicular afferents evoked excitatory postsynaptic potentials and action potentials in the RSC.

Area 30

On the third day of training we found lower c-Fos expression in area 30 than in area 29c in the Control-US group. It might may have resulted from weaker connections between area 30 and anterior thalamic nuclei compared with stronger connections between area 30 and neocortical areas (Shibata and Naito 2008, Shibata et al. 2004, van Groen and Wyss 1992). Thus, the sensory aspects of the shocks may have prevailed over their affective value. We also found a decrease in the density of c-Fos-positive nuclei in area 30 in avoidance-trained rats in the Exp group, which was lower than in both control groups. The lower level of c-Fos in avoidance-trained rats in the Exp group compared with the Control-noUS group may reflect specific suppression of area 30, in which spatial memory of a specific location that is to be avoided enables efficient avoidance. A decrease in the number of c-Fos-positive nuclei in area 30 in trained rats below sham-trained levels was reported after spatial working memory training in a radial maze task that was conducted in the dark (Pothuizen et al. 2009). Possible explanation for such a decrease may be the withdrawal of a set of excitatory inputs to area 30. After hippocampal lesions, the depletion of c-Fos-positive neurons was particularly strong in granular area Rgb (equivalent to areas 29c and 29b; Albasser et al. 2007). Temporary blockade of the hippocampus, which likely inactivates a portion of hippocampal-retrosplenial inputs, diminished the level of Arc-positive neurons in granular area Rgb in rats (Kubik et al. 2012). Similarly, lesions of anterior

thalamic nuclei resulted in the pronounced depletion of c-Fos-positive nuclei in all granular areas (Jenkins et al. 2004). Another hypothesized reason for the decrease in the density of c-Fos-positive neurons in area 30 could be the enhancement of inhibitory inputs to this area from g-aminobutyric acid-ergic hippocampal-retrosplenial projections that arise from the CA1 and subiculum, and terminate in layer I of the anterior part of area 30 (Miyashita and Rockland 2007, Jinno 2009) or inhibition from local interneurons (Finch et al. 1984). Such a decrease in c-Fos-positive neurons may also reflect the deactivation or inhibition of some functions of area 30 at a specific time point of spatial memory formation. Area 30 is reciprocally connected with visual, motor, association prefrontal, and posterior parietal cortices (Shibata and Naito 2008, Shibata et al. 2004, van Groen and Wyss 1992), suggesting that it is involved in the sensorimotor integration and execution of spatial behavior (Vogt and Miller 1983). On the third day of place avoidance training in the present study, the role of area 30 in performing such a demanding avoidance task may rely on inhibition of the strategies that do not serve to optimize it.

Electrophysiological studies (Gabriel 1980, 1990, 1993, Gabriel et al. 1991) on the retrosplenial cortex have reported an increase in its activity during early training and a decrease in activity during late training in rabbits that were trained in a discriminative avoidance task. Whether our observations are consistent with the aforementioned electrophysiological mapping studies, in which both areas 29c and 30 are activated during the early stages of training and area 30 is suppressed during later stages of training, requires further investigation.

In conclusion, retrosplenial areas 29c and 30 were activated during spatial memory acquisition on the first day of training, whereas area 30 appeared to be suppressed during long-term memory formation on the third day of training when the rats effectively learned to avoid the place that was associated with shock.

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