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Hippocampal activation during the recall of remote spatial memories in radial maze tasks

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ABSTRACT

Temporally graded retrograde amnesia is observed in human patients with medial temporal lobe lesions as well as in animal models of medial temporal lobe lesions. A time-limited role for these structures in memory recall has also been suggested by the observation that the rodent hippocampus and entorhinal cortex are activated during the retrieval of recent but not of remote memories. One notable exception is the recall of remote memories for platform locations in the water maze, which requires an intact hippocampus and results in hippocampal activation irrespective of the age of the memory. These findings raise the question whether the hippocampus is always involved in the recall of spatial memories or, alternatively, whether it might be required for procedural computations in the water maze task, such as for calculating a path to a hidden platform. We performed spatial memory testing in radial maze tasks to distinguish between these possibilities. Radial maze tasks require a choice between spatial locations on a center platform and thus have a lesser requirement for navigation than the water maze. However, we used a behavioral design in the radial maze that retained other aspects of the standard water maze task, such as the use of multiple start locations and retention testing in a single trial. Using the immediate early gene c-fos as a marker for neuronal activation, we found that all hippocampal subregions were more activated during the recall of remote compared to recent spatial memories. In areas CA3 and CA1, activation during remote memory testing was higher than in rats that were merely reexposed to the testing environment after the same time interval. Conversely, Fos levels in the dentate gyrus were increased after retention testing to the extent that was also observed in the corresponding exposure control group. This pattern of hippocampal activation was also obtained in a second version of the task that only used a single start arm instead of multiple start arms. The CA3 and CA1 activation during remote memory recall is consistent with the interpretation that an older memory might require increased pattern completion and/or relearning after longer time intervals. Irrespective of whether the hippocampus is required for remote memory recall, the hippocampus might engage in computations that either support recall of remote memories or that update remote memories.

1. Introduction

In human patients, damage to the medial temporal lobe or to the hippocampus alone causes retrograde amnesia – a loss of memory for information gained prior to the brain damage. In many cases, the pattern of retrograde amnesia is temporally graded, such that memories acquired close to the onset of the brain damage are lost while memories that were acquired a long time before the brain damage are not affected (Shrager, Bayley, Bontempi, Hopkins, & Squire, 2007; Squire, 1982). These findings suggest that the hippocampus has only a temporary role in memory storage and that long-term memories can be stored and retrieved by neocortical brain areas without a continued hippocampal contribution (Frankland & Bontempi, 2005; McClelland, McNaughton, & O'Reilly, 1995; Nadel & Hardt, 2011; Nadel & Moscovitch, 1997; Squire, Stark, & Clark, 2004; Winocur, Moscovitch, Rosenbaum, & Sekeres, 2010). Temporally graded retrograde amnesia is generally supported by data obtained from experimental animals with medial temporal lobe and hippocampal lesions (Kim, Clark, & Thompson, 1995; Kim & Fanselow, 1992; Lesburgueres et al., 2011; Maren, Aharonov, & Fanselow, 1997; Zola-Morgan & Squire, 1990). However, there are also studies that suggest a longer, if not continuous

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involvement of the hippocampal formation in some types of memory, in particular if the retention testing requires pathfinding or the planning of routes (Broadbent, Squire, & Clark, 2006; Clark, Broadbent, & Squire, 2005a, 2005b; Kee, Teixeira, Wang, & Frankland, 2007; Martin, de Hoz, & Morris, 2005).

In addition to lesion studies, non-invasive functional brain imaging studies in behaving animals have importantly contributed to identifying how brain circuits reorganize during the transition from recent to remote memories (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Maviel, Durkin, Menzaghi, & Bontempi, 2004). These studies used the visualization of immediate early genes (IEGs) to map neuronal circuits for remote memory formation. IEG expression is rapidly upregulated with neuronal activity that is associated with synaptic plasticity (Cole, Saffen, Baraban, & Worley, 1989), and these markers have generally shown that the hippocampus remains active during task acquisition as well as during retrieval testing within a period of several days. In contrast, if retrieval is tested after several weeks, the hippocampus and its adjacent cortical areas (e.g., subiculum, entorhinal cortex) are more weakly activated and prefrontal areas are more strongly activated (Maviel et al., 2004). While these findings were obtained using the radial maze, studies that used the Morris water maze found high levels of hippocampal activation during remote memory testing (Kee et al., 2007; Lopez et al., 2012).

Although testing in the water maze as well as in the radial maze requires memory for a specific spatial location, the experimental procedures necessarily differ in many other aspects, which might engage the hippocampus to different extents. The higher hippocampal activation on the water maze task, for example, could emerge from the higher demand for navigation (i.e., pathfinding or the planning of routes) in the water maze task compared to radial maze tasks. However, the testing procedures in previous studies that found lower levels of hippocampal engagement during remote memory testing also differed in other important aspects. For example, in a recent study by Lopez et al. (2012) memory testing in the water maze was performed during a single probe trial, while testing in the radial maze included multiple trials (Bontempi et al., 1999). Furthermore, testing in the water maze is usually performed using multiple start locations (Kee et al., 2007; Lopez et al., 2012), while testing in a radial maze task used a single start location (Maviel et al., 2004). With a single start location, the task might, after prolonged training, be solved with a striatum-dependent turning strategy rather than a hippocampus-dependent spatial strategy (Packard & McGaugh, 1996; Teather, Packard, Smith, Ellis-Behnke, & Bazan, 2005). This raises the possibility that a diminished hippocampal contribution at the end of the acquisition period might result in a lesser hippocampal engagement during remote memory testing. In addition, previous studies differed also in the construction of the retention test from the water maze. For example, Maviel et al. (2004) included a forced-choice presentation phase, which preceded the test phase by 20 min. Rather than remembering the location of the goal over several weeks, the task could thus also be solved by remembering the rule to return to the most recently presented arm over long intervals and by remembering the spatial location only over the short time interval between the sample and the choice phase.

To explicitly test whether the long-term retention of a goal location or whether procedural aspects of radial maze tasks result in diminished hippocampal activation, we performed a spatial memory task in the radial maze with multiple start locations and in which retention testing was limited to a single trial. This task has been shown to be hippocampus dependent throughout training (Cassel et al., 1998) and can, because of the single retention trial, only be solved by long-term memory for the goal location. The radial maze task thus shares these features with the standard version of the water maze but, in contrast to the water maze, has low demand for spatial navigation because it merely requires that a choice is made on a central platform. To further reduce the navigational demand, we also used a radial maze task that required rats to learn a fixed trajectory. By visualizing the immediate–early gene product Fos as a marker for brain activation during a single retention trial, we could therefore distinguish whether the recall of spatial memories rather than high navigational demand might result in sustained hippocampal activation.

2. Material and methods

2.1. Animals and housing conditions

Male Long Evans rats (n = 46) weighing 250–290 g at the beginning of the experiment were obtained from Taconic (Petersburg, USA) and group housed (4–5 rats per cage) in polycarbonate cages (60 × 38 × 20 cm). The environmental temperature was 22 ± 2 °C and the relative humidity 55 ± 10%. The animals were submitted to a reversed light–dark cycle with artificial fluorescent lighting of 80 Lux from 8:00 p.m. to 8:00 a.m. and were tested during the dark phase of the cycle. Rats were food deprived to 85–90% of their free-feeding body weight and maintained at this level throughout the period of behavioral training and 1 week prior to the training and testing phases of the experiment. Water was available ad libitum. All experiments were carried out in accordance with the Norwegian Animal Welfare Act and the European Convention for the Protection of Vertebrate Animals used for Experimentation and Other Scientific Purposes.

2.2. Apparatus and testing environment

A 6-arm radial maze was placed in a 2.4 m × 3.0 m room. The maze consisted of a hexagonal central platform with a diameter of 30 cm from which 6 arms (85 cm long, 10 cm wide) radiated at equal angles. The maze was covered with black contact paper and was elevated 50 cm above the floor. A black food-well was placed at the end of each arm. A camera was mounted at the ceiling above the central platform and connected to a monitor and DVD recorder to track and record the rats' performance on the maze. During the intertrial intervals the animals were placed in opaque boxes (30 × 40 cm) in an area that was approximately 2 m from the maze and separated from the training area by 1.8 m high opaque dividers.

2.3. Behavioral tasks

Each rat was trained on one of two different reference memory tasks in the 6-arm radial maze (Fig. 1A). In each of the two tasks, rats were given five training trials per day with 20-min intertrial intervals. Sixteen rats were trained in a version of the task with a fixed goal location and five possible start positions that varied pseudorandomly from trial to trial (multiple start-arm task). Each start arm was used once during the five trials of each day. The use of multiple start locations in the radial maze renders the task hippocampus dependent (Cassel et al., 1998) and is a feature that is also used in the standard version of the water maze. Fourteen rats were trained on a version of the task with a fixed goal location and a single start arm (single start-arm task). Because both the start and the goal arm remained the same throughout training, the rats took the same trajectory during each correct trial. This task is similar to a task that has previously been used for imaging hippocampal activity patterns (Maviel et al., 2004), and it can be solved by using either a hippocampus-dependent place strategy or a striatal-dependent response strategy. However, an important difference between our version and the version that was used by

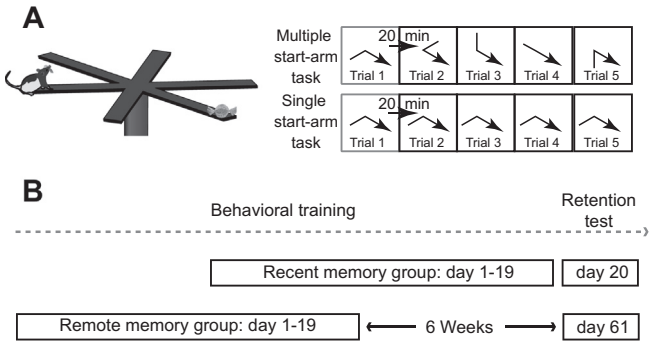

Fig. 1. Behavioral design. (A) Illustration of the 6-arm radial maze, and examples of daily training sessions in the task with multiple start locations (multiple start-arm task, top) and in the task with a single start location (single start-arm task, bottom). Rats were trained to find a reward at a constant location at the end of one of the arms. The multiple start-arm version of the task used each of the remaining five arms as start locations, varying in pseudorandom order. The single start-arm version of the task used the same arm as the start location for each trial of an individual rat. Different start locations were used for different rats. The intertrial intervals between the five trials within a training day were 20 min. (B) Experimental timeline. Rats were trained for 19 consecutive days in either the multiple start-arm task or the single start-arm task. Half of the rats in each task version were tested for recent memory retention 1 day after the last training session (day 20), and the other half was tested for remote memory retention 6 weeks after the last training session (day 61). A single trial rather than a full set of five trials was used for retention testing.

Maviel et al. (2004) is that their trials included a forced-choice presentation phase which preceded the test phase by 20 min, while our version never used forced-choice trials. Each rat was randomly assigned to either the multiple start-arm or the single start-arm version of the task.

The experimental design included four different control groups. A caged control group consisted of rats ($n = 3$) that never left their home cages until perfusion. A novelty control group consisted of rats ($n = 3$) that were naïve to all behavioral procedures until they were allowed to explore the 6-arm radial maze for 10 min during a time window that began 75 min prior to the perfusion. Two additional control groups experienced the same schedule of exposure to the testing room as the memory retention groups, but were not subjected to behavioral testing on the radial maze. The animals in the exposure groups were food-deprived and brought to the behavioral testing room in the same way as the animals in the memory groups. They were kept in the same room and in the same type of holding box as the experimental animals during each training day and during either the recent testing day (recent exposure group, $n = 5$) or the remote testing day (remote exposure control group, $n = 5$).

2.4. Experimental procedures – habituation and training

The rats were handled for 3 consecutive days before the start of the behavioral procedures. They were then habituated to the testing environment and apparatus. During habituation, three pieces of chocolate cereal were placed on the goal arm, one into the food well at the end of the arm, one on the middle of the arm, and one at the beginning of the arm close to the center platform. At the beginning of each trial, the rat was placed at the outer end of a start arm with its path to the center platform obstructed by a block. After 30 s, the block was removed and the rat was allowed to search for the food rewards. The rat was returned to its holding box after a maximum of 9.5 min or, if it found and consumed all three chocolate loops, immediately after eating the third chocolate loop. One daily habituation session was given per animal.

After 3 days of habituation, rats were trained for 19 consecutive days (Fig. 1B) to find food at the end of the goal arm that was introduced during habituation. The food well at the end of the goal arm contained one chocolate loop as a reward. The food wells at the end of arms other than the goal and the start arm contained small chocolate cereal crumbs to render olfactory cues uninformative about the goal location. None of the rats were observed to consume the small food crumbs, and it was therefore not necessary to insert a physical barrier to prevent rats from eating the crumbs. At the beginning of each trial, the rat was placed at the outer end of the arm that was the start arm for the trial. The path to the center platform was obstructed by a block for 30 s. Once the block was removed, the rat was allowed to run to the center platform from where it could choose to enter any of the arms. An arm entry was recorded as soon as the rat moved into the arm by its entire body length, but rats would typically continue to the end of the arm. If an incorrect arm was chosen, the rat was allowed to return to the center platform and to continue to choose arms. The trial was terminated immediately after the rat found and consumed the food reward, or after a maximum of 4.5 min after removing the block. Trials only needed to be terminated at the maximum time on few occasions during the initial training days, and the few trials in which the reward was not found and consumed were excluded from the analysis. The number of reference memory errors was obtained by counting how many unrewarded arms the rat had entered during a trial. Entries back into the start arm were also considered errors, and repeated entries into an incorrect arm were counted as a single reference memory error. The maximal number of errors in the six-arm maze thus corresponded to the number of arms other than the goal arm (i.e., 5). Chance level was determined by considering that, without memory for the goal, there is an equal likelihood (1/6th) for each number of reference memory errors (0, 1, 2, 3, 4, or 5). The expected number of errors would thus be the mean of these six possibilities (i.e., 2.5). The number of reference memory errors in the first trial of a training day indicate whether the rat remembered the goal location for at least 1 day. The number of reference memory errors in the subsequent training trials of the day (trial 2–5) indicate whether the rats remembered the goal location for at least 20 min. For trial 2–5, the total number of errors during the four trials was divided by the number of trials such that the mean number of errors per trial is reported.

2.5. Experimental procedures – retention test

Animals were tested for spatial memory retention either 1 day or 6 weeks after the end of the 19th training day. To temporally match histological processing steps for rats tested for recent and remote memory, the behavioral training schedule was arranged in a way that allowed recent and remote memory testing to take place on the same day (Fig. 1B). During retention testing, the initial behavioral procedures were identical to those that were used during each training day except that only the first trial was completed. After the single retention trial, each rat was placed into the same holding box in which it had also been placed during previous training. Each rat remained in the holding box for 75 min, before it was transferred into a different room and perfused. The testing procedure did thus not differ from a standard training day until 20 min after the rat was placed into the holding box (i.e., the intertrial interval during training was 20 min). The time window that is most effective for the induction of Fos protein (Guzowski et al., 2005; Morgan, Cohen, Hempstead, & Curran, 1987; Zangenehpour & Chaudhuri, 2002) therefore included the period in the holding box before

the first trial, the retention testing, and the 20-min period in the holding box after the first trial.

2.6. Histology

The rats were transcardially perfused 75 min after retention testing. They were first anesthetized in a chamber that was filled with isoflurane (3%) and were subsequently deeply anesthetized by an intraperitoneal injection of an overdose of pentobarbital (2 ml of 150 mg/ml; Vortech Pharmaceutical LTD, Michigan, USA). The perfusion was conducted with 150 ml of phosphate buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde (PFA) in PBS. The brains were postfixed in 4% PFA in PBS for 24 h at 4 °C. This solution was then replaced by 30% sucrose in PBS and the brains were stored at 4 °C until they sunk to the bottom of the tube. The brains were first cut along their midline and the left hemisphere was sectioned sagittally at 40 μm with a freezing sliding microtome. Free floating sections were collected and were stored in PBS containing 0.02% sodium azide at 4 °C until further processing. For staining, sections at regular intervals (240 μm) were selected to fulfill the criteria for systematic random sampling such that stereological methods for unbiased counting could be applied.

2.7. Immunohistochemical staining

The activity in different hippocampal regions was visualized using immunohistochemistry to stain the translational product of the activity-dependent immediate early gene c-fos with a Fos-protein specific primary rabbit polyclonal antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, USA). The primary antibody was used at a dilution of 1:2500 in PBS containing 0.1% Triton X-100 and the incubation time was 24 h at room temperature. No blocking solution was used. A biotinylated goat anti-rabbit IgG antibody (1:1000; VectaStain, Vector Laboratories, Burlingame, USA) was used as the secondary antibody (incubation time: 24 h at room temperature). The secondary antibody was coupled to a horse radish peroxidase using the avidin–biotin peroxidase method (VectaStain ABC Kit, Vector Laboratories, Burlingame, USA) and was visualized with diaminobenzidine (DAB; Sigma–Aldrich Chemie GmBH, Steinheim, Germany) at a concentration of 0.25 g/ml in 0.1 mol/l Tris-buffered saline (TBS) for 12 min. To enhance the intensity of the DAB precipitation, $NiCl_2$ (2.3 mmol/l) was added to the staining solution. To ensure consistency in staining, the brains were divided into two batches of 22–24 brains, and the brains in each batch were stained in parallel.

2.8. Quantification

In order to estimate the number of Fos-positive cells in different subregions of the entire hippocampus, we used design-based stereological counting methods (Stereo Investigator, mbf Bioscience; MicroBrightField, CA, USA). The CA1 and the CA3 region of the pyramidal cell layer of the hippocampus and the granule cell layer of the ventral (vDG) and dorsal blade (dDG) of the dentate gyrus were traced using darkfield imaging. For vDG, dDG, CA3, and CA1, a systematic sampling grid with adjacent counting frames was randomly placed over each image, and the number of distinctly stained nuclei within the cell layer of each hippocampal subregion was counted. The sampling fraction along the mediolateral axis was 1/12 for the dentate gyrus and CA3 (every 480 μm for 40 μm thick sections) and 1/6 for CA1 (every 240 μm for 40 μm thick sections).

3. Results

3.1. Spatial reference memory persisted for up to 6 weeks

Rats ($n = 16$) were trained for five trials per day over 19 days to find a food reward at the end of a goal arm in a 6-arm radial maze. The goal arm remained constant throughout the training period and each trial began by placing the rat in the end of one of the remaining five arms (multiple start-arm task; Fig. 1A) such that the rats had to run to the central platform and then select the goal arm. The intertrial intervals between the trials of a day were 20 min. In the second, third, fourth, and fifth trial of each day, rats therefore needed to remember the goal location over at least 20 min. During the first trial of each day, rats had to remember the goal location over an interval of at least 1 day because they were last rewarded on the previous training day. During the initial phase of training rats occasionally were not able to find the food reward within the maximum allowed time on the maze. However, most rats ($n = 13$) found and consumed the reward on the first trial of the first training day. The remaining three rats found the reward within the time limit on the first trial of the day on day 2 or on day 3. As expected for the first completed training trial, the rats' performance did not differ from chance (Fig. 2A; $df = 15$, $t = -0.97$, N.S.). On the last training day (day 19) the mean number of errors during the first trial of the day was substantially lower than chance ($df = 15$, $t = -5.51$, $p < 0.001$) and lower than in the beginning of the training period ($df = 15$, $t = -2.85$, $p < 0.05$). As expected when rats showed improved memory retention over 1-day intervals, their memory performance over 20-min intervals also improved (trial 2–5 tested retention over intertrial interval of 20 min; $df = 15$, $t = -7.01$, $p < 0.001$).

Following the last training session, rats were tested for memory retention in a single trial (see Fig. 1B). Half of the rats were tested 1 day after the last training day (i.e., recent memory group) and half of the rats were tested 6 weeks after the last training day (i.e., remote memory group). The mean number of reference memory errors during the first trial of the last training day was not different between the recent compared to the remote memory group ($df = 7$, $t = -0.20$, N.S.). Furthermore, the rats' performance during either recent or remote memory testing did not decline compared to the first trial of the last training day (Fig. 2B; recent, $df = 7$, $t = -0.93$, N.S.; remote, $df = 7$, $t = 0.42$, N.S.). Importantly, the mean number of errors during retention testing did not differ between the recent and remote memory groups ($df = 14$, $t = 1.17$, N.S.) and was significantly below chance in both groups (recent, $df = 7$, $t = -8.08$, $p < 0.001$; remote, $df = 7$, $t = -3.24$, $p < 0.05$).

3.2. Hippocampal activation during the recall of remote spatial memories

The activation of the hippocampus during retention testing, either 1 day or 6 weeks after the last training day, was measured by visualizing the translational product of the immediate early gene c-fos. Expression of the Fos protein is essential for normal LTP and spatial learning (Fleischmann et al., 2003) suggesting that it is associated with synaptic plasticity. In behaving animals, its expression is low at resting level and is rapidly induced by behavioral states such as novelty detection (Albasser, Poirier, & Aggleton, 2010; Jenkins, Amin, Pearce, Brown, & Aggleton, 2004; Zangenehpour & Chaudhuri, 2002) and spatial learning (He, Yamada, & Nabeshima, 2002). Fos induction is therefore commonly used as a marker for recent neural activation in the intact brain (Guzowski et al., 2005; Morgan & Curran, 1991; Morgan et al., 1987; Sagar, Sharp, & Curran, 1988). Using stereological methods, we counted the total number of Fos-positive cells in hippocampal regions

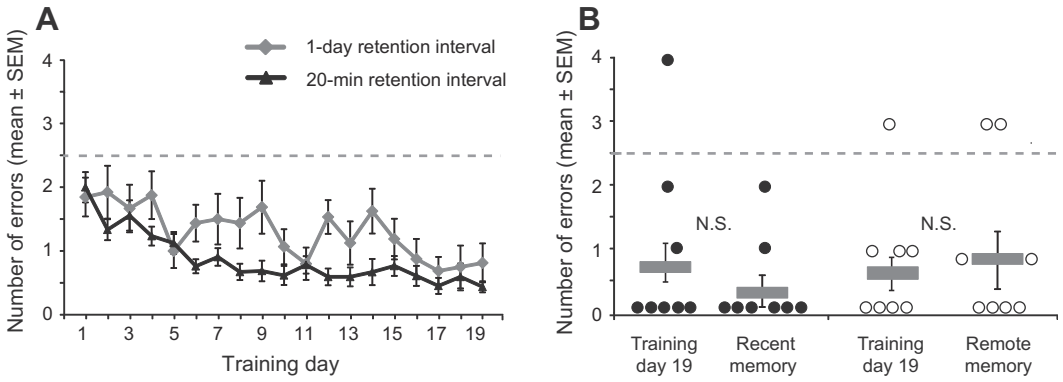

Fig. 2. Acquisition and memory retention in a radial arm task with multiple start locations. (A) Training. The number of reference memory errors per trial (mean ± SEM) for the first trial (1-day retention) and trials 2–5 (20-min retention) are shown. For the 1-day and for the 20-min retention interval, the number of reference memory errors decreased between the beginning of the training period and the last training day (see text for statistics). (B) Retention testing. The number of reference memory errors (mean ± SEM) during recent memory testing (recent memory) did not differ from the number of errors during remote memory testing (remote memory). Each animal was given a single probe trial. Performance on the first trial of the last training day (training day 19) is shown for comparison. The number of errors for individual rats in the recent and remote memory groups is shown as filled and open circles, respectively. The mean number of reference memory errors during either recent or remote retention testing was not different from the last training day. The performance during recent and remote retention testing was different from chance (see text for statistics). The chance level is shown as a dashed line.

CA3 and CA1 as well as in the dorsal and ventral blade of the dentate gyrus (vDG and dDG, respectively). The ventral blade was distinguished from the dorsal blade because substantial differences in the number of cells that express immediate early genes have previously been reported between these dentate subregions (Chawla et al., 2005, 2013).

The number of Fos-positive cells in dDG, CA1 and CA3 was higher in the remote compared to the recent memory group (all p values < 0.01; see Fig. 3 for additional statistics and Fig. 4 for photographs). No differences between the recent and remote memory groups were found for vDG ($df = 14$, $t = 1.81$, N.S.). In the recent memory group, the number of Fos-positive cells did not exceed the number of cells in the caged control group in any of the brain regions. In the remote memory group, the number of Fos-positive cells in dDG, CA3, and CA1 was higher compared to the cage control group and at the same level as in the novelty control group (Fig. 3). Moreover, the comparison between caged control and the novelty group also showed the expected pronounced difference in the number of Fos-positive cells in all hippocampal subregions (all p values < 0.05).

3.3. Activation in CA3 and CA1, but not in DG was specific to remote memory recall

Elevated Fos levels during remote memory testing could arise due to memory-specific activation, or could result from the mere reexposure to the experimental environment after a long time interval. To distinguish between these possibilities, we examined hippocampal activity in control groups which were given the same schedule of exposure to the testing environment as the experimental groups, but were not subjected to memory testing on the radial maze (recent and remote exposure control groups; Fig. 5A). We found that, in the dDG, the number of Fos-positive cells in the remote exposure control group was significantly higher than in the recent exposure control group ($df = 8$, $t = 3.58$; $p < 0.01$). In contrast, no significant differences were observed for CA3 and CA1 (CA3, $df = 8$, $t = 0.18$, N.S.; CA1, $df = 6$, $t = 0.55$, N.S.). These results demonstrate that, after a 6-week-long retention interval, the dDG is activated by mere re-exposure to the experimental environment, while CA3 and CA1 remain at caged-control levels without retention testing.

To determine the degree of activation that was induced by memory performance compared to reexposure, we calculated the difference between the recent and remote memory groups and the corresponding exposure control groups. In the DG, a small increase above exposure control levels was observed for the recent as well as the remote memory group, but the magnitude of the increase above exposure control levels did not differ between the two memory groups (dDG, $df = 14$, $t = 1.86$, N.S.; vDG, $df = 14$, $t = 1.05$, N.S.). In areas CA3 and CA1, there was more increase above exposure group levels in the remote memory group than there was in the recent memory group (CA3, $df = 14$, $t = 3.04$, $p < 0.001$.; CA1, $df = 13$, $t = 3.89$, $p > 0.01$; Fig. 5B). We also calculated the increase above exposure control levels by dividing the number of Fos-positive cells in the recent and remote memory groups by the mean values that were obtained for the recent and remote exposure control groups, respectively (Fig. 5C). For this ratio, we found the same pattern of results as we had obtained by calculating the difference. The increase above exposure control levels did not differ between the recent and remote memory group in the DG (dDG, $df = 14$, $t = 0.85$, N.S.; vDG, $df = 14$, $t = 0.81$, N.S.). In contrast, in the CA3 and CA1 area, the ratio was significantly higher for the remote compared to the recent memory group (CA3, $df = 14$, $t = 2.97$, $p < 0.05$; CA1, $df = 13$, $t = 3.73$, $p < 0.005$).

3.4. Hippocampal activation patterns in a radial maze task with a single start location resembled the patterns that were observed in the task with multiple start locations

We found hippocampal activation during remote memory testing in a radial maze task that uses multiple start arms. Because a previous study found hippocampal deactivation in a task that tested spatial memory retention with a single start location (Maviel et al., 2004), we asked whether a version of the radial maze task that uses a single start arm (see Fig. 1A) would, compared to the version with multiple start arms, result in decreased hippocampal activation during remote memory testing. As observed for rats that were trained with multiple start locations, the rats that were trained with a single start location showed learning for 1-day retention intervals (first completed training trial compared to first trial of last training day; $df = 13$, $t = -2.58$, $p < 0.05$) as well as for 20-min retention intervals (Fig. 6A, trial 2–5; $df = 13$, $t = -3.82$, $p < 0.05$). On the first completed training trial the number of reference memory errors was not different from chance ($df = 14$, $t = -1.87$, N.S.), but performance was well below chance during the first trial of day 19 ($df = 14$, $t = -9.99$, $p < 0.001$). On the last

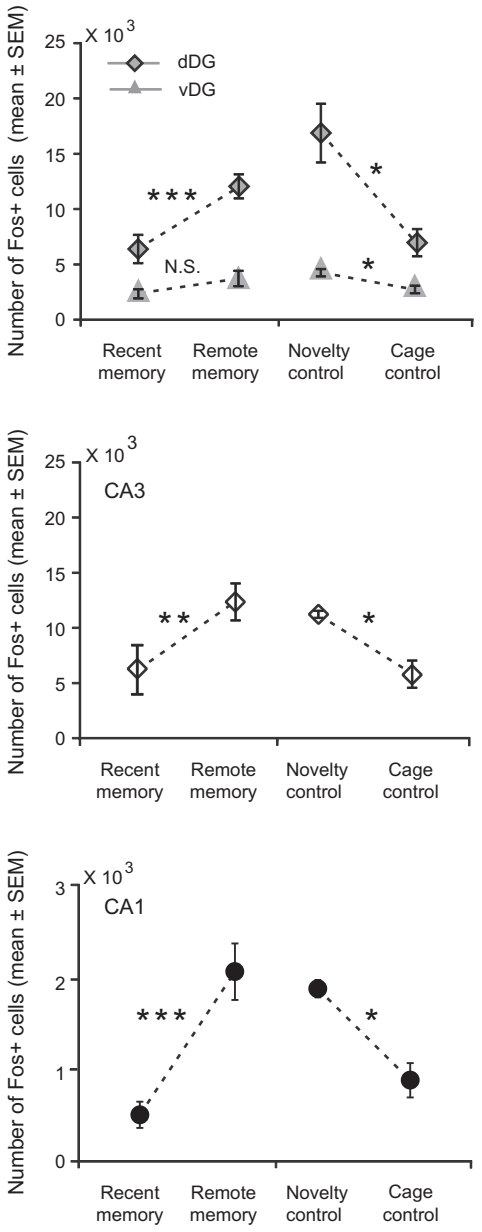

Fig. 3. Hippocampal activation during remote memory testing. Hippocampal subregions are shown in separate panels. The number of Fos-positive cells in the dorsal blade of the dentate gyrus (dDG), CA3 and CA1 was higher in the remote memory group compared to recent memory group (dDG, $df = 14$, $t = 4.26$, $p < 0.001$; CA3, $df = 14$, $t = 3.16$, $p < 0.01$; CA1, $df = 13$, $t = 4.47$, $p < 0.001$). The number of Fos-positive cells in the recent memory group was not different to the caged control group (dDG, $df = 9$, $t = 0.08$, N.S.; vDG, $df = 9$, $t = 0.04$, N.S., CA3, $df = 9$, $t = -0.63$, N.S.; CA1, $df = 8$, $t = 1.49$, N.S.), while it was higher in the remote memory group compared to the caged control group (dDG, $df = 9$, $t = 2.84$, $p < 0.05$; CA3, $df = 9$, $t = 2.48$, $p < 0.05$, CA1, $df = 9$, $t = 2.29$, $p < 0.05$). Moreover, the number of Fos-positive cells was higher in the novelty control group compared to the recent memory group (vDG, $df = 9$, $t = 2.57$, $p < 0.05$; dDG, $df = 9$, $t = 5.11$, $p < 0.001$; CA3, $df = 9$, $t = 2.43$, $p < 0.05$; CA1, $df = 9$, $t = 5.92$, $p < 0.001$), but was not different between the novelty control group and the remote memory group (dDG, $df = 9$, $t = 1.88$, N.S.; vDG, $df = 9$, $t = 0.04$, N.S., CA3, $df = 9$, $t = -0.71$, N.S.; CA1, $df = 9$, $t = 0.38$, N.S.). As expected, activation was higher in the novelty compared to the caged control group (dDG, $df = 4$, $t = 3.26$; $p < 0.05$; vDG =, $df = 4$, $t = 3.37$; $p < 0.05$; CA3, $df = 4$, $t = 2.96$; $p < 0.05$; CA1, $df = 4$, $t = 4.74$; $p < 0.01$). Significance levels ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) are shown for comparisons between recent and remote groups and for comparisons between novelty and cage control groups.

training day, the number of errors of rats assigned to the recent memory group was not significantly different from those assigned to the remote memory group ($df = 7$, $t = -0.20$, N.S.; Fig. 6B). Furthermore, memory performance during the recent as well as during the remote memory retention test had not declined compared to the first trial of the last training day (recent, $df = 7$, $t = 0.93$, N.S., remote, $df = 7$, $t = 0.42$, N.S.; Fig. 6B). Importantly, the mean number of errors during retention testing did not differ between recent and remote memory testing ($df = 6$, $t = 1.86$, N.S.) and was different from chance for both retention intervals (recent, $df = 6$, $t = -4.2$, $p < 0.01$; remote, $df = 6$, $t = -3.6$, $p < 0.05$). Quantification of hippocampal activation revealed that the number of Fos-positive cells in all hippocampal subregions and over both retention intervals (recent and remote) was corresponding between rats trained with the single start location and rats trained with multiple start locations on the radial maze task (Fig. 6C).

4. Discussion

In single-trial retention testing for spatial memory in radial maze tasks, we showed higher hippocampal activation during the recall of remote memories compared to the recall of recent memories. This pattern of results was observed in tasks that used multiple start locations as well as in tasks that required learning of a fixed trajectory. Although we found that all subregions of the hippocampus were activated during remote memory testing, only activation in hippocampal areas CA3 and CA1 was specific to memory recall, while the dentate gyrus was activated by mere reexposure to the environment after a 6-week long interval. Our results, obtained by visualizing Fos protein as a marker of neural activation, are consistent with the activation patterns that have been reported for the water maze (Kee et al., 2007; Lopez et al., 2012), but differ from the deactivation of the hippocampal system that has been observed in studies using the radial maze (Bontempi et al., 1999; Maviel et al., 2004). An important difference between our study and previous studies in the radial maze is that we used a single-trial testing procedure that exclusively relied on the long-term retention of a spatial memory, while previous results were obtained in procedures in which the long-term memory could either have been for the spatial location or for the rule of the task (Maviel et al., 2004). Our results therefore suggest that the patterns of hippocampal activation during remote memory recall in different spatial memory tasks cannot be entirely explained by the higher navigational demand in the water maze task, but that the degree of hippocampal activation in the radial maze also depends on whether retention testing was performed in a single-trial.

Lesion and inactivation studies have consistently shown that the hippocampus is required to solve the water maze task, independent of the length of the time period between hippocampal damage and the retention test (Broadbent et al., 2006; Clark et al., 2005b; Martin et al., 2005; Sutherland et al., 2001). In addition, it is known from studies using immediate early genes that the intact hippocampus is activated during recall of remote spatial memories (Kee et al., 2007; Lopez et al., 2012). The findings on the water maze for remote memory are inconsistent with studies using non-spatial memory tasks which usually report that the hippocampus is engaged in and required for the recall of recent but not remote memories (Kim & Fanselow, 1992; Kim et al., 1995; Lesburgueres et al., 2011; Maren et al., 1997; Zola-Morgan & Squire, 1990). These data raise the possibility that non-spatial memories become independent of the hippocampus over time, while the recall of spatial memories always engages the hippocampus. It should be taken into consideration, however, that the water maze task not only requires learning of a specific escape location, but also requires the animal to reorient itself within the recording apparatus in the beginning of each trial and to calculate the route to the learned escape location (Whishaw, Cassel, & Jarrad, 1995; see Knierim & Hamilton, 2011 for review). At least one of these

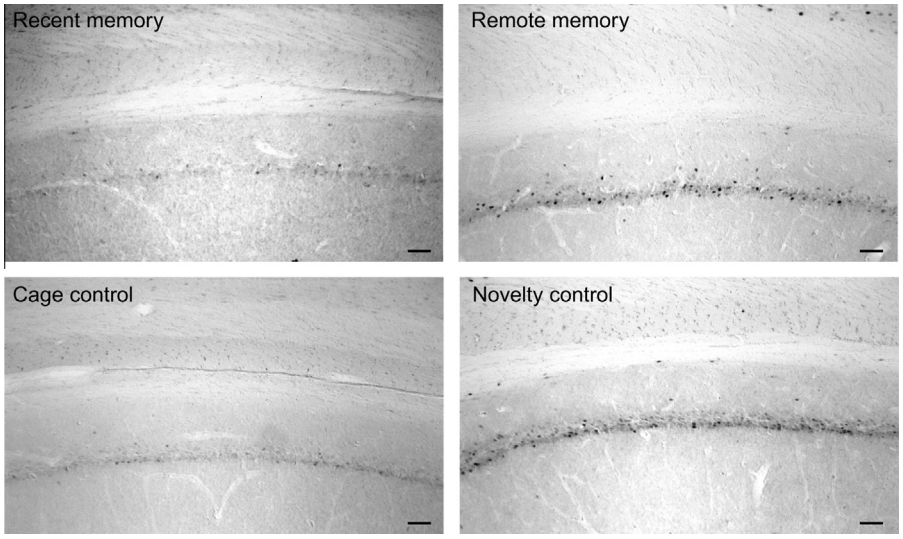

Fig. 4. Increased number of Fos-positive cells in the hippocampal CA1 region in the remote memory group and in the novelty control group. Fos immunoreactivity in the hippocampal CA1 area of representative animals tested for recent and for remote memory retention (upper panels) as well as of representative animals in the caged control and novelty control group (lower panels). Bright field images with Fos-positive cells are shown. Scale bars are 100 μm.

procedural components, the navigation to a learned location, is thought to require the intact hippocampus (Whishaw et al., 1995). Previous results can therefore be interpreted as an indication that a continued requirement for navigation rather than the recall of the remote spatial memory might account for the hippocampal activation and for the need of hippocampal processing during remote memory testing.

The notion that the navigational demand, rather than recall of remote spatial memory, results in hippocampal activation during remote memory testing is supported by studies investigating spatial memory in tasks with a lesser need to navigate. When testing memory for a specific reward location in radial maze tasks, animals are merely required to make a choice between several arms rather than to perform ongoing calculations of the path to the goal location. For the radial maze it was found that the hippocampus was only activated during the recall of recent but not of remote spatial memories (Bontempi et al., 1999; Maviel et al., 2004). These previous findings thus make the important suggestion that non-spatial as well as spatial memories could become independent of the hippocampus over time. Furthermore, they suggest that differences between studies that find engagement or disengagement of the hippocampus during remote memory testing are a result of ongoing computational demands rather than memory demands.

Here, we find increased hippocampal activation during the recall of remote spatial memories using a spatial memory task on the radial maze. Our results are thus consistent with water maze studies, but differ from previous observations obtained on the radial maze. An additional key difference, other than the navigational demand, between the water maze task and previously used radial maze tasks is in the construction of the retention test. In previous studies using the radial maze, retention testing was performed over a series of at least two trials. For example, a forced-choice reminder trial preceded the test trial in the Maviel et al. (2004) study. Accurate performance on the retention tests could thus have been supported by working memory for the location that was visited 20 min ago along with remote memory for the rules of the task. Similarly, Bontempi et al. (1999) used multiple trials for retention testing. This raises the possibility that recall of spatial memories over shorter time intervals or that repeated testing in the radial maze resulted in a decline of hippocampal activation. Previously used radial maze tasks therefore do not only differ from the water maze in their requirement for spatial navigation, but also with respect to the possible use of a strategy that enabled the return to a recently rewarded goal location. Our behavioral procedures addressed this difference and found that the use of a single retention trial in the radial maze resulted in hippocampal engagement rather than disengagement. Taken together, the imaging results in the radial maze are therefore consistent with the interpretation that the design of the retention test can determine the level of engagement of the hippocampus.

An additional difference between studies that have shown that the hippocampus remains active during remote memory testing – as opposed to those that demonstrated a decrease in hippocampal activation – is the use of behavioral tasks that engage different memory systems. While the water maze task uses multiple start locations and is hippocampus dependent during learning (Broadbent, Squire, & Clark, 2004; Moser & Moser, 1998), the task used by Maviel et al. (2004) required mice to run to a single goal location while being released from a single start location. A task with a stereotyped path from a start to a goal location has been shown to rely on a hippocampal-dependent place strategy during the initial phase of training, which can be replaced by a striatal-dependent turning strategy during the later phase (Packard & McGaugh, 1996). In the present study, we used two different versions of the radial maze task, one that used multiple start arms and is dependent on the integrity of the hippocampus (Cassel et al., 1998) and one that used a single start location and can be solved using either a place or a turning strategy. We found that the activity patterns in different hippocampal subregions did not differ between the two tasks. We thus do not find support for the notion that differential hippocampal engagement during learning results in differential activation during remote memory testing.

While all examined areas of the hippocampus showed an elevated number of Fos-positive cells during remote memory testing, careful examination of a number of control conditions revealed that individual hippocampal areas are differentially involved in the processing of different aspects of the memory. The dDG, CA3, and CA1 areas of the hippocampus were highly activated during first exposure to the experimental apparatus, indicating that all of those areas are involved in the initial encoding of the environment. Reexposure to the testing room after the same interval that was used for remote retention testing resulted in the activation of dentate granule cells,

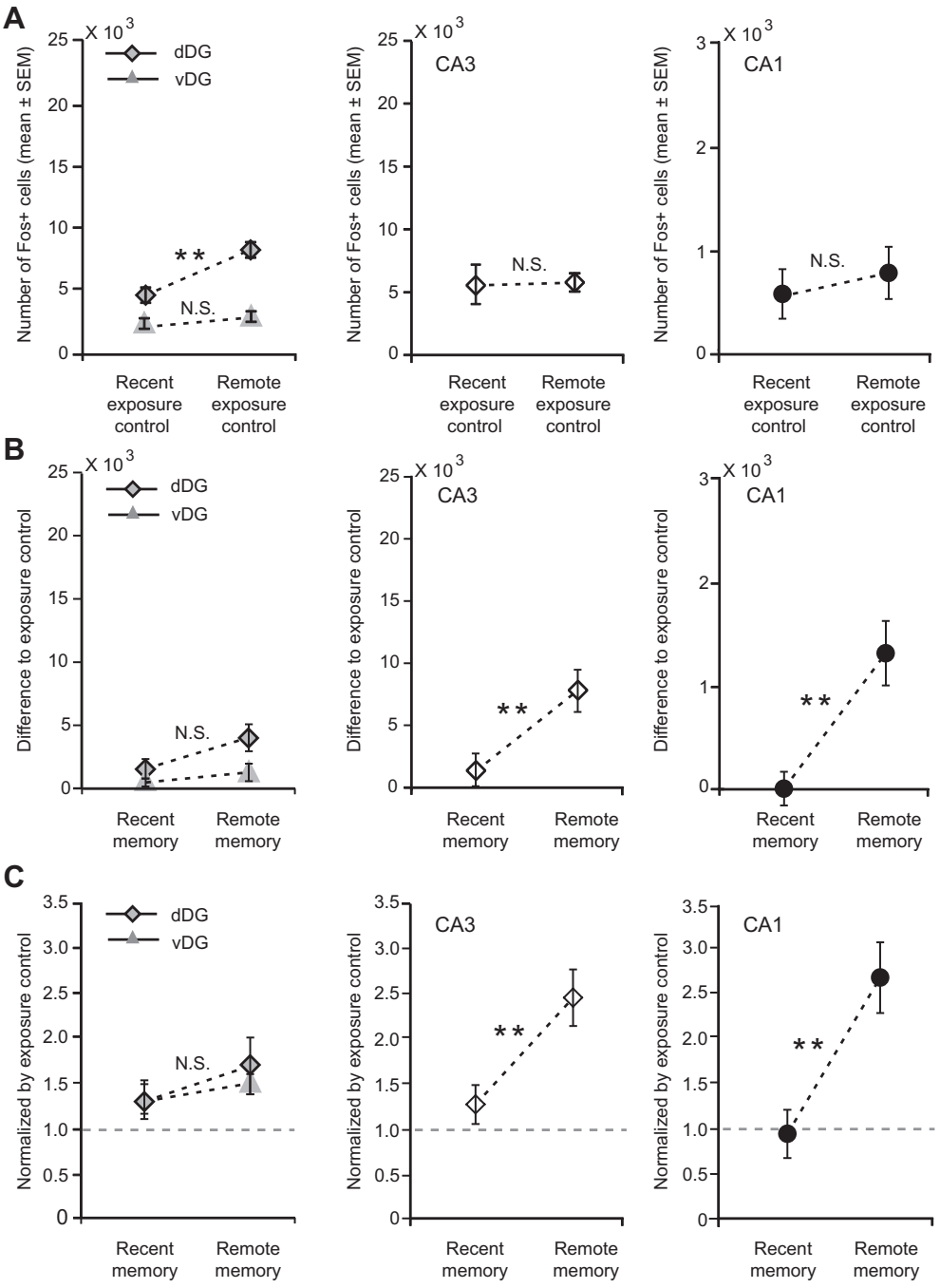

Fig. 5. Fos-positive cells are selectively activated by remote memory testing in CA3 and CA1 and are activated by mere reexposure to the testing environment in DG. Reexposure testing was performed at time points that corresponded to either recent or remote memory testing. (A) Exposure control groups. In the dorsal dentate gyrus (dDG), the number of Fos-positive cells was higher in the remote exposure control group compared to the recent exposure control group (** $p < 0.01$). No significant differences were found in the ventral dentate gyrus (vDG), CA1, and CA3. (B) Difference between memory groups and corresponding exposure control groups. An increase in the number of Fos-positive cells above exposure control levels was found in CA3 and CA1, but not in DG (see text for detailed statistics). (C) Ratio between the number of Fos-positive cells in each memory group divided by the average in the corresponding exposure control group. In CA3 and CA1, the ratio of Fos-positive cells was significantly higher in the remote compared to the recent memory group. No significant differences were found for DG. The gray dashed line at a ratio of 1.0 indicates a corresponding activation for exposure and memory testing.

but not of CA3 or CA1 cells. Single-unit recording studies in comparable conditions show that the average CA1 and CA3 population activity is similar during repeated exposure to an environment, irrespective of the relative familiarity. The differences in Fos activation that we find between conditions can thus not be interpreted as differences in the neuronal firing patterns but rather reflect that the induction of the Fos protein is related to synaptic plasticity (Cole et al., 1989; Guzowski et al., 2005).

The finding that activity levels in the dentate gyrus are similar between memory groups and exposure control groups suggests that the dentate gyrus is involved in the recall and/or relearning of general aspects of the environment (Kee et al., 2007; Tashiro, Makino, & Gage, 2007). Remarkably, activation in CA3 and CA1 did not exceed caged-control levels during mere reexposure to the testing environment over either 1-day or week-long intervals. This raises the possibility that, during mere reexposure to general

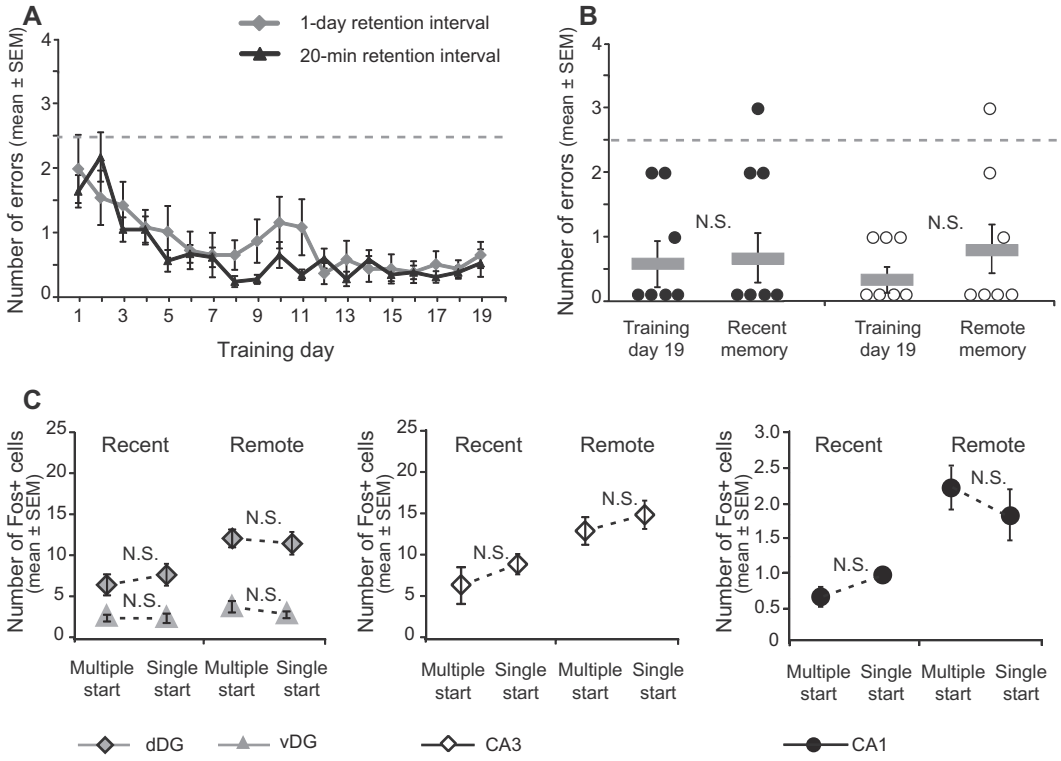

Fig. 6. Hippocampal activation is higher during remote compared to recent memory testing in a spatial memory task with a single start location. (A) Rats were trained to find a reward at a constant location at the end of one of the arms. For each rat, a single start-arm was used as the start location for all training trials and for the retention trial. Rats were trained for five trials per day, and the intertrial interval was 20 min. (A) Training. The number of errors per trial is shown for the first trial of each training day (1-day retention) and for trials 2–5 (20-min retention). Performance was substantially below chance at the end of training (see text for statistics). (B) Retention testing. The number of reference memory errors (mean ± SEM) during retention testing was not different from the number of errors on the first trial of the last training day. The number of errors for individual rats in the recent and remote memory groups is shown as filled and open circles, respectively. (C) Total number of Fos-positive cells during recent and remote memory testing in rats trained on the single start version of the task. The values from the multiple start-arm task are repeated from Fig. 3 for comparison. The number of Fos-positive cells was not different between the multiple start-arm and the single start-arm version of the task for any of the hippocampal subregions at either retention interval (recent, all t values < 1.43, N.S.; remote, all t values < 0.83, N.S.).

aspects of the environment, CA3 and CA1 do not respond if they do not subserve specific mnemonic functions. In fact, these areas did, in the recent memory group, not even respond to running on the maze or recent memory recall. In contrast, during remote memory testing, pyramidal cells in the CA3 and CA1 area reached activity levels that corresponded to those in the novelty control group. When considering that the behavioral performance during remote memory testing did not differ from performance on the last training day, these data are consistent with the interpretation that the CA3 network may be particularly important to accurately recall a faded memory after a week-long retention interval through the process of pattern completion. Activity patterns that are completed in CA3 may then be projected to the CA1 area, which is the main hippocampal output region and projects back to neocortical structures (Witter, Wouterlood, Naber, & Van Haeften, 2000). Importantly, activation of the CA3 area but not of DG has also been observed by He et al. (2002), who examined hippocampal activation during the acquisition of a spatial memory task on the radial maze that was similar to the task with multiple start locations in our study. Interestingly, He et al. (2002) observed an increased number of Fos-positive cells during the initial, but not during the later phase of training. These results are consistent with our observation of low Fos expression during recent memory testing and suggest that activation of the immediate early gene c-fos in the CA3 area is limited to the initial learning phase and to the initial recall of memories after a long retention interval. These observations are also supported by a study in the water maze that showed elevated hippocampal Fos levels during remote but not during recent memory testing (Lopez et al., 2012). The activation of the CA3 area during the initial encoding of spatial memories raises the possibility that activation observed during remote memory testing in our study might not only be related to the recall of the spatial memory, but also to mechanisms that update memories after long retention intervals (Nader & Einarsson, 2010).

Taken together, our results indicate that the activation of hippocampal CA3 and CA1 areas is specific to the single-trial processing of remote spatial memories, while the DG is involved in the recall and/or relearning of more general aspects of the environment. Moreover, the CA3 and CA1 activation during remote memory recall is consistent with the interpretation that a fading memory might require increased pattern completion and/or relearning after longer time intervals. Hippocampal activation therefore does not solely emerge from the high navigational demand of the water maze task, but is rather generated during the initial recall event. Irrespective of whether the hippocampus is required for memory performance in a particular task or at a particular time interval, our result thus supports the notion that the hippocampus is engaged in computations that support the retrieval and/or update spatial memories that are stored over more distributed memory systems.

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