Coping with sleep deprivation: shifts in regional brain activity and learning strategy

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ABSTRACT

Dissociable cognitive strategies are used for place navigation. Spatial strategies rely on the hippocampus, an area important for flexible integration of novel information. Response strategies are more rigid and involve the dorsal striatum. These memory systems can compensate for each other in case of temporal or permanent damage. Sleep deprivation (SD) has adverse effects on hippocampal function. However, whether the striatal memory system can compensate for SD-induced hippocampal impairments is unknown. With a symmetrical maze paradigm for mice, we examined the effect of SD on learning the location of a food reward (training) and on learning that a previously nonrewarded arm was now rewarded (reversal training). Five hours of SD after each daily training session did not affect performance during training. However, in contrast with controls, sleep-deprived mice avoided a hippocampus-dependent spatial strategy and preferentially used a striatum-dependent response strategy. In line with this, the training-induced increase in phosphorylation of the transcription factor cAMP response-element binding protein (CREB) shifted from hippocampus to dorsal striatum. Importantly, although sleep-deprived mice performed well during training, performance during reversal training was attenuated, most likely due to rigidity of the striatal system they used. Together, these findings suggest that the brain, when possible, compensates for negative effects of SD on the hippocampal memory system by promoting the use of a striatal memory system. However, effects of SD can still appear later on because the alternative learning mechanisms and brain regions involved may result in reduced flexibility under conditions requiring adaptation of previously formed memories.
INTRODUCTION

Numerous studies have shown that sleep may facilitate learning and memory processes (Graves et al., 2001; Maquet, 2001; Stickgold, 2005; Marshall and Born, 2007). Sleep deprivation (SD) after training disturbs memory consolidation (Karni et al., 1994; Smith et al., 1998; Palchykova et al., 2006), an effect that is particularly prominent in case of memories that rely on the integrity of the hippocampus (Smith and Rose, 1996; Graves et al., 2003; McDermott et al., 2003; Ruskin et al., 2004; Hairston et al., 2005; Ruskin and LaHoste, 2008).

It is recognized that distinct brain systems may compensate for one another if the function of one of the systems is temporally or permanently lost (Bohbot et al., 2004; Voermans et al., 2004; Hairston et al., 2005). For instance, learning to find the way through one’s environment may involve different learning strategies, particularly, a spatial strategy (also known as allocentric strategy, i.e., acquiring a cognitive map based on spatial cues) or a response strategy (known as an egocentric strategy, i.e., making the same turn response regardless of spatial position). The spatial strategy relies on the hippocampus, whereas a response strategy depends on the dorsal striatum (Packard and McGaugh, 1996). Studies in rodents using maze paradigms to investigate the role of specific brain systems in place navigation have shown that temporal hippocampal inactivation results in a shift toward a striatum-dependent response strategy (Packard and McGaugh, 1996; Chang and Gold, 2003). Although SD particularly affects the hippocampus, no studies have been conducted to test whether SD induces a shift in the strategy used for place navigation.

In addition to the potential effects of SD on strategies used for the formation of new memories, one other aspect of the relationship between SD and memory that has received scarce attention is the flexibility of existing memories, i.e., the adaptation of previously acquired memories to match with changes in a familiar situation. The adaptation of memories and learned behaviors is an important aspect of successfully coping with changes that frequently occur in our surroundings, e.g., in the case of moving to a new home, school, or job.

One commonly used approach to study the process of memory formation and adaptation is the Y-maze or T-maze reference paradigm for rodents (Oliveira et al., 1997; Deacon et al., 2002; Havekes et al., 2006). During the initial training, a food reward is located in 1 of 2 accessible arms of the maze. During the subsequent reversal training, the food reward is relocated to the arm that was previously not baited. In the present study, mice were briefly sleep deprived after each daily training and/or reversal training session to assess the effect of SD on the formation of new memories and adaptation of previously acquired memories. In addition, we examined the effect of SD on the cognitive strategy used by mice to locate the food reward. Since distinct cognitive strategies are paralleled by brain-region-specific increases in the phosphorylation of the transcription factor cAMP response-element binding protein (pCREB) (Colombo et al., 2003), we also examined whether SD during training affected pCREB levels in the hippocampus and striatum. Finally, since SD-induced changes in cognitive processes might be mediated by stress and/or anxiety (Packard and Wingard, 2004; Schwabe et al., 2008) we assessed plasma concentrations of the stress hormone corticosterone (CORT) and anxiety in an elevated plus-maze test.
METHODS

Animals and housing conditions
In all experiments, 3 to 3.5-month-old male C57BL/6J mice were used (Harlan, Horst, the Netherlands). Animals were individually housed in standard macrolon cages and maintained on a 12-hour light/12-hour dark cycle (lights on at 09:00 a.m.). A layer of sawdust served as bedding. Water was provided ad libitum throughout the experiment, but, in the maze experiments, the mice were food deprived to 90% of their individual body weight, starting 4 days before the beginning of training. These animals were weighed and fed daily after finishing the 5 hours of SD after training or reversal training. The procedures described in the present study were approved by the Animal Experiment Committee of the University of Groningen in compliance with Dutch law and regulations.

Training and reversal training in the Y maze
To test whether SD affects memory formation and flexibility, mice were subjected to a Y-maze task. The task was conducted in a tubular, transparent, Plexiglas Y maze consisting of a start arm and 2 test arms forming the Y (Havekes et al., 2006; Havekes et al., 2007). All 3 arms were 5 cm in diameter, 27.5 cm long, and at a 120° angle from each other. A start box was connected to the start arm. One of the test arms was baited with a food reward consisting of a small crumb of the regular food (0.05-0.1 g). Food crumbs were also placed below perforations at the end of the other test arm to ensure that both arms would smell alike and thereby prevent animals from discriminating between baited and nonbaited arms by olfactory cues. A small rim (1 cm high) 4 cm before the end of the arms prevented visual inspection for food presence from a distance. A guillotine door located halfway down each arm could be operated manually from the experimenter’s position and was used to allow animals only 1 choice in each training trial. Once the animals chose 1 arm, the other arm was closed. The experiment room contained various distal spatial cues.

On the first day, mice received 2 habituation trials to familiarize the animals with the experiment setup. During the first habituation trial, mice were placed in the start box from which they could explore 1 of the 2 test arms, which was baited with a small crumb of food. The other arm was closed. After the mouse consumed the reward, it was allowed to retreat to the start box. The second habituation trial was given immediately thereafter, but now with the other test arm opened and baited. After the habituation day, mice were subjected to a training protocol consisting of 1 training session per day, including 6 trials in each session. During the entire training phase, either the right or left arm was baited. This was constant for a given individual but was randomly assigned between subjects and treatments. When, during a trial, a subject visited 1 of the 2 accessible arms, the nonvisited arm was closed. A visit was defined as the animal placing all 4 paws in 1 test arm. After the subject retreated to the start box, the start arm connected to the start box was blocked, preventing reentrance of the maze. After we cleaned all test arms and rebaited the same test arm, the animal was again allowed to explore either the right or left accessible arm. A visit to the baited arm was recorded as a correct trial. Animals were trained until the groups reached an average performance of 85% to 90% correct trials per day. The animals were then subjected to reversal training, in which the food reward was relocated...
to the previously unbaited arm. The reversal training again consisted of 1 session per day with 6 trials each. All Y-maze sessions were performed at the beginning of the light phase.

Training in the T maze
To test whether SD causes a shift in the learning mechanism and cognitive strategy used to locate a food reward, mice were subjected to a T-maze paradigm. Testing was conducted in a tubular, transparent, Plexiglas cross maze consisting of 2 start arms (e.g., north and south arm) and 2 test arms (e.g., east and west arm). All 4 arms were 5 cm in diameter and 27.5 cm long. Arms diverged at a 90° angle from each other. During the entire training procedure, only 1 start arm was used (the other start arm was blocked). Food crumbs were placed below perforations at the end of both test arms to prevent animals from discriminating between baited and nonbaited arms by using olfactory cues. Small grey rims (1 cm high) 4 cm before the end of the arms prevented visual inspection for food presence from a distance. A guillotine door positioned halfway down each arm could be operated manually from the experimenter’s position. The experiment room contained various distal spatial cues.

The habituation and training procedure used in the cross maze was similar to the one described for the Y maze. Mice were subjected to daily training sessions, each consisting of 6 trials. The animals were trained until the groups reached an average performance of 85% to 90% correct trials. They were then subjected to a probe trial to assess the learning strategy used during training. During the probe trial, the animals started from the opposite testing arm; the original start arm was now closed. The probe trial had 2 possible outcomes: (1) mice using a spatial strategy would visit the arm that was baited during training, i.e., the same spatial location and (2) mice using a response strategy would make the same turn as they had done during training and would visit the other arm.

Assessment of regional pCREB expression
To assess whether changes in learning strategy after SD were associated with changes in regional brain activity, we performed immunohistochemistry for the transcription factor pCREB, a critical element in memory formation (Silva et al., 1998). One hundred minutes after the T-maze probe trial was completed, mice that were trained in the T maze (T) and mice that had received training followed by 5 hours of SD after each daily training session (SDT) were sacrificed for brain collection. A group of home cage control (HCC) mice was sacrificed in parallel. These HCC mice had been food restricted in a manner similar to that used for the T and SDT mice but were otherwise left undisturbed in their home cage. Importantly, SDT mice were not subjected to SD between probe trial and sacrifice. Therefore, acute effects of SD on pCREB expression on the last day were excluded and differences in pCREB expression between T and SDT animals would have to be a result of SD during the preceding training days. While it seemed unlikely that SD by itself would have a persistent effect on basal pCREB expression independent of training, we performed an additional control experiment to exclude this possibility. This control experiment included a group of HCC mice and a group of mice subjected to 5 hours of SD per day without training but that were otherwise treated in the exact same way as the T and SDT mice in the main experiment. Again, SD was not performed on the day of sacrifice and brain collection. Under deep pentobarbital anesthesia, mice were transcardially perfused
with saline (0.9% NaCl), followed by 4% paraformaldehyde. Brains were collected, postfixed for 24 hours in 4% paraformaldehyde, rinsed for 1 day in 0.01 M phosphate buffered saline (PBS, pH 7.4), and transferred to a 30% sucrose in PBS cryoprotection, where they remained overnight at 4°C. Brains were stored at -80°C until further processing.

Twenty-five micrometer coronal sections of the striatum and hippocampus were collected and stored in PBS containing 0.1% sodium azide. The brain sections were rinsed 3 times in PBS, for 5 minutes each time, and were then placed in 0.3% H$_2$O$_2$ in PBS for 25 minutes. After rinsing the sections in PBS 4 times, for 5 minutes each time, we preincubated them at room temperature for 25 minutes in 5% normal goat serum (NGS; Jackson immuno research laboratories, West Grove, PA) and 0.1% Triton X-100 in PBS to block nonspecific binding of immunoreagents. Subsequently, sections were incubated for 2 hours at room temperature followed by overnight incubation at 4°C with rabbit polyclonal anti-pCREB antibody (1:2000, Upstate, Temecula, CA), in 0.3% Triton X-100 and 1% NGS in PBS. After being rinsed with PBS 4 times, for 10 minutes each time, the sections were incubated at room temperature for 3 hours with the biotinylated goat-anti-rabbit-IgG (1:500, Jackson immuno research laboratories) in 1% NGS, 0.3% Triton X-100 in PBS. After they were rinsed with PBS 3 times, for 5 minutes each time, sections were incubated for 1.5 hours at room temperature with the avidin-biotin-complex (1:500, ABC Elite kit, Vector Laboratories, Burlingame, CA), 0.3% Triton X-100 in PBS. After this step, the sections were rinsed overnight in PBS at 4°C. The next day, after rinsing the sections with PBS 4 times, for 10 minutes each time, we visualized the labeled cells with diaminobenzidine (DAB, 0.7mg/mL in milli q water; Sigma-Aldrich, Steinheim, Germany) with 0.1% H$_2$O$_2$ as a reaction initiator. The reaction was stopped by rinsing with PBS.

For each subject, 3 sections were selected at approximately bregma 1.18 to 0.50 mm for the dorsal striatum and 3 sections at bregma -1.70 to -2.18 mm for the dorsal hippocampus (Franklin and Paxinos, 1997). Phosphorylated CREB immunoreactivity in the striatum was quantified as previously described (Colombo et al., 2003). With a sampling template of 450 $\mu$m$^2$, immunoreactive cells in each section were counted in the dorsolateral and dorsomedial striatum at a 50x magnification using a computerized image analysis system (Quantimet 550, Leica, Cambridge, UK). A threshold was set that marked all cells to be included in the counting. In the hippocampus, the cell layers were densely packed with pCREB immunopositive cells, which made it difficult to distinguish and count individual cells. Instead, optical densities (OD) were measured for the granular cell layer of the dentate gyrus (DG) and for the pyramidal cell layer of the Cornu Ammonis (CA) 3 and 1 of the dorsal hippocampus using a 50x magnification. The OD is expressed in arbitrary units corresponding to grey levels using the Quantimet image analysis system (Leica). To correct for variability in background staining among sections, the background labeling was measured in the stratum radiatum and extracted from the OD of the area of interest. The experimenter was blind to the treatment of individual animals during all cell counting and OD measurements. Data on pCREB immunoreactivity are expressed as percentage of the mean value of the HCC group.

**Sleep deprivation**

Mice were subjected to SD for 5 hours immediately following each daily session of training and/or
Sleep deprivation and learning strategy

reversal training. This time window was chosen because previous studies have shown that it is a critical phase for memory consolidation that is sensitive to SD (Graves et al., 2003; Palchykova et al., 2006). SD was accomplished by mild sensory stimulation, which involved tapping on the cage, gently shaking the cage, or, when this was not sufficient to keep animals awake, disturbing the sleeping nest (Van der Borght et al., 2006; Hagewoud et al., 2010). Previous studies have shown that this procedure is effective in keeping rodents awake for several hours, as established by electroencephalic recordings (Meerlo et al., 2001), without being a major stressor (Meerlo and Turek, 2001; Van der Borght et al., 2006; Hagewoud et al., 2010).

Elevated plus maze
To assess whether a single brief episode of SD and/or repeated SD as applied in the maze experiments might have an effect on memory processes by inducing anxiety, new batches of mice were subjected to an elevated plus maze, a commonly used and well-validated anxiety test in rodents (Pellow et al., 1985). Different batches of mice were subjected to either a single session of 5 hours of SD or to 4 daily sessions of 5 hours of SD, similar to what was applied in the T-maze experiment. Each batch included its own non-sleep-deprived control group. The plus maze consisted of 2 open arms (without walls) and 2 closed arms (16-cm high walls). All arms were 29 cm long and 5 cm wide, and the whole plus was elevated 75 cm above the floor. The test was performed in a separate experiment room under bright-light conditions (100 lux). At the start of the test, each animal was placed on the center of the plus facing 1 of the open arms, after which the experimenter left the room. The test lasted 8 minutes, and behavior of the animals was recorded on videotape for later analysis. Between animals, the maze was thoroughly cleaned with 70% ethanol. The recordings were analyzed for number of entries into closed or open arms and time spent in closed and open arms. An entry was defined as 4 paws in an arm. Data on the time spent in the open arms were expressed as a percentage of the total time in the maze. Data on the number of open-arm entries were expressed as a percentage of the total number of arm entries.

Plasma corticosterone levels
To examine whether a single session of 5 hours of SD and/or repeated 5-hour of SD would affect plasma levels of the stress hormone CORT, an additional experiment was performed with 2 new batches of mice. Both batches consisted of a control group and a group subjected to SD (5 hours of SD once or 5 hours of SD per day for 4 days, respectively). SD was performed using the same method as in the previous experiments. At the end of the SD period, trunk blood was collected in precooled plastic centrifuge tubes containing 0.01% ethylenediaminetetraacetic acid (EDTA) as anticoagulant and antioxidant. Blood was centrifuged at 4°C for 15 minutes at 2600 g, and plasma was stored at −80°C until further processing. CORT levels were determined by radioimmunoassay (MP Biomedicals, Orangeburg, NY).

Statistical analysis
Behavioral performance in the Y or T maze was analyzed using a repeated-measures analysis of
variance (ANOVA) with a between-subject factor ‘treatment’ (control or sleep deprivation) and a within-subject factor ‘time’ (daily Y-maze or T-maze sessions). When appropriate, post hoc comparisons were made with a Tukey test. To determine whether groups showed a significant tendency to use a spatial or response strategy during the T-maze probe trials, binomial tests and a Pearson $\chi^2$ test were computed. pCREB immunohistochemistry data for the different brain regions were analyzed with a one-way ANOVA with a between-subject factor ‘treatment’ (HCC, T, SDT) followed by a post hoc Tukey test. Independent-samples t-test was used to analyze pCREB immunoreactivity in the control experiment, performance in the elevated plus maze, and plasma CORT levels (control vs. sleep deprivation). All data in text and figures are expressed as mean ± S.E.M. p < 0.05 was considered as significant.

RESULTS

Sleep deprivation during training and reversal training in the Y maze

In the first experiment, 1 group of mice was subjected to the Y-maze training and reversal-training protocol without any interference (n = 9). A second group of mice was subjected to 5 hours of SD immediately after each daily training session and reversal-training session (n = 10). At the beginning of training, both groups performed at chance level, indicating that they had no preference for either of the 2 accessible arms (control: 40.7% ± 8.4%, and sleep deprivation: 50.0% ± 7.5%, correct trials, Fig. 1A). The animals in both groups readily consumed the food rewards during correct trials and gradually learned to locate the baited arm (effect of session: $F_{5,85} = 9.30$ p < 0.001). After 6 days of training, the control group and the SD group reached scores of 85.3% ± 6.8% and 95.0% ± 5.0% respectively (Fig. 1A). Overall, there was no effect of SD on the performance during training (effect of treatment and interaction effect: $F_{1,17} = 0.60$, $F_{5,85} = 0.51$, p > 0.1 in both cases, Fig. 1B).

On day 7, the food reward was relocated to the previously nonrewarded arm, and reversal training started. During the first reversal-training session, performance of the control group dropped to 22.2% ± 8.3% and that of the sleep-deprived group to 1.7% ± 1.7% (Fig. 1A). Detailed analysis of this first reversal-training session revealed that all mice of both groups visited the incorrect arm during the first trial of the first session. In other words, they all visited the arm that had been baited during the training phase. During consecutive trials of the first session, mice of the control group eventually started to visit the correct arm (i.e., the newly baited arm), whereas mice of the sleep-deprived group continued to visit the incorrect arm. With further training on the days thereafter, both groups gradually learned to locate the newly baited arm (effect of session: $F_{6,102} = 24.63$, p < 0.001, Fig. 1A). However, performance of the sleep-deprived group remained below that of the control group (treatment effect: $F_{1,17} = 14.87$, p < 0.001). The control group reached an average performance of 88.9% ± 4.8% after 7 daily sessions of reversal training, while the sleep-deprived group reached a score of only 45.0% ± 10.4% (Fig. 1A).
This first experiment indicated that SD selectively impaired the performance during reversal training. Yet, the mice were sleep deprived after each training and reversal training session. Therefore, it was uncertain whether the attenuated performance during the reversal training was caused by SD during training or SD during reversal training. For that reason, in a second experiment, 3 new groups of mice were subjected to training and reversal training in the Y maze: a control group (control, n = 6), a group that was sleep deprived selectively after each daily training session (SD training, n = 6), and a group that was deprived of sleep only after each daily reversal training session (SD reversal training, n=6). In accordance with the results from the first experiment, brief SD after each training session did not affect performance during training (data not shown), whereas a strong effect of treatment was found during reversal training ($F_{2,15} = 9.97$, $p < 0.005$, Fig. 1B). Mice that were sleep deprived during the reversal training phase performed in a manner similar to that of the non-sleep-deprived controls.
indicating that SD does not directly affect performance during reversal training. Remarkably, the mice that were exposed to brief SD after each daily training session performed significantly worse in comparison with the other 2 groups during reversal training (SD training vs. control, p < 0.001, and SD training vs. SD reversal training, p < 0.01). In other words, although SD after each training session did not affect performance during the training itself, it had a delayed effect and reduced performance during the subsequent reversal training. After 7 daily sessions of reversal training, the control group and SD reversal training group reached a score of 94.4% ± 5.5% and 97.2% ± 2.8% respectively, whereas the SD training group reached a score of only 38.9% ± 13.4% (Fig. 1B). These results show that SD during the phase of memory formation hampers the adaptation of this memory later on.

**Sleep deprivation and learning strategy in a T maze**

To test whether SD during Y-maze training caused a shift in the learning mechanism and cognitive strategy used to locate the food reward, we did a new experiment using a 4-arm symmetrical maze. By blocking the arm opposite of the start arm, the maze was essentially used as a T maze with 1 start arm and 2 choice arms (see Fig. 2A). Two groups of mice received 1 training session per day consisting of 6 trials: a control group (control, n = 11) and a group that was deprived of sleep for 5 hours after each training session (SD training, n=10). The results show that both groups of mice started at chance level (control: 47.0% ± 7.0% and SD training group: 41.7% ± 9.0%). Animals in both groups readily consumed the food reward during correct trials and gradually learned to locate the baited arm (effect of session: F3,57 = 31.64, p < 0.001, see Fig. 2A). After 4 days of training, the control group and SD group reached performance scores of 95.0% ± 2.6% and 94.0% ± 2.5%, respectively. As in the Y-maze experiments, SD did not affect behavioral performance during T-maze training (effect of treatment and interaction effect: F1,19 = 0.48, F3,57 = 0.33, p > 0.1 in both cases).

On day 5, all mice were subjected to a probe trial starting from the arm opposite to the one that was used during the training (i.e., the arm that was blocked during training, see Fig. 2B). The start arm that was used during training was now blocked. In the control group, a majority of mice (7 out of 11) visited the arm that was baited during training, indicating a spatial strategy. Overall, this spatial preference did not reach statistical significance, since 4 animals made the same turn, indicating a response strategy (binomial test, p > 0.1, Fig. 2B). In contrast with the majority of control mice, the animals in the SD group displayed a strong preference for a response strategy: 9 out of 10 mice that were deprived of sleep after each daily training session made the same turn as the one they had learned during the training even though the probe trial was given from the opposite start arm (binomial test, p < 0.05, Fig. 2B). Statistical comparison of both groups indicated that SD significantly reduced the use of a spatial strategy and facilitated the use of a response strategy (Pearson χ² test: p < 0.01, Fig. 2B). These results suggest that SD inhibits the use of a spatial strategy in a reference-learning paradigm, resulting in a shift toward the use of a response strategy.
Sleep deprivation, learning strategy and regional CREB phosphorylation

A study by Colombo and colleagues (Colombo et al., 2003) indicates that formation of memory for a spatial strategy is related to prolonged phosphorylation and activation of the transcription factor CREB in the hippocampus, whereas formation of memory for a response strategy is related to increased and prolonged phosphorylation of CREB in the dorsal striatum. To test whether the SD–induced shift from a spatial to a response strategy was accompanied by a shift in CREB phosphorylation levels from the dorsal hippocampus to dorsal striatum, we measured pCREB expression in these brain areas of the mice that received training with and without SD in the T maze (see Fig. 2). Fig. 3 shows representative pictures of pCREB expression in the dorsal hippocampus. ANOVA revealed a
significant main treatment effect for the OD of pCREB immunoreactivity in the DG, as well as the CA3 and CA1 regions (F_{2,23} = 5.05, p < 0.02; F_{2,23} = 8.11, p < 0.01; F_{2,23} = 6.55, p < 0.01, respectively). Subsequent post hoc comparisons indicated that training in the T maze induced a significant increase in pCREB expression in the DG and the CA3 and CA1 regions, compared with the levels in HCC animals. However, SD immediately after each training session prevented this increase (for details, see Fig. 3).

Figure 3. Short sleep deprivation (SD) after each daily T-maze training session prevents a training-induced increase in pCREB immunoreactivity in the dorsal hippocampus. Representative high-resolution pictures of pCREB immunoreactivity in the hippocampus are shown on the left. Optical density of pCREB immunoreactivity was measured for the granular cell layer of the dentate gyrus (DG) and the pyramidal cell layer of the CA3 and CA1 areas. Home cage control mice (HCC), mice that were trained in the T maze (T), and mice that had received training followed by 5 hours of SD after each daily training session (SDT) were sacrificed 100 minutes after the probe trial. Graphs on the right show that normal training was associated with a significant increase in pCREB immunoreactivity in all 3 hippocampal regions, which was not seen in the mice that had been sleep deprived after each training session. Significant differences: * p < 0.05, ** p < 0.01; post hoc Tukey test following one-way ANOVA.
Representative pictures of pCREB expression in the dorsal striatum are shown in Fig. 4. ANOVA revealed a significant main effect of treatment for the number of pCREB immunopositive cells in both the dorsomedial and dorsolateral striatum ($F_{2,23} = 3.81, p < 0.04$ and $F_{2,23} = 3.65, p < 0.05$, respectively). Subsequent post hoc comparisons showed that the number of pCREB positive cells was significantly elevated in both the dorsolateral and dorsomedial striatum in animals that were deprived of sleep after each daily T-maze training session, compared with HCC animals. This increase was not seen in normally trained mice that were not subjected to SD (for details see Fig. 4).

Thus, training accompanied by SD reduced pCREB expression in the hippocampus but facilitated CREB phosphorylation in the dorsomedial and dorsolateral striatum.

Since not all trained control animals displayed a spatial strategy, we compared pCREB expression in the subgroup with a spatial strategy and the subgroup with a response strategy. As expected, animals that used a spatial strategy, on average, had higher pCREB expression in most hippocampal subregions, and animals that used a response-based strategy had higher pCREB levels in the striatum (data not shown). However, these differences did not reach statistical significance because of the small number of animals that used a response strategy and the overall narrow range of pCREB expression.

Importantly, SDT animals were not sleep deprived on the last day between the probe trial and sacrifice. Therefore, the differences in pCREB expression between T and SDT animals were a result of SD during the preceding training days. Although it seemed unlikely that repeated sessions of brief SD by itself, independent of training, would have a long-lasting effect on basal levels of pCREB expression that persisted until brain collection a day after the last SD, we performed a control experiment to exclude this possibility. This control experiment consisted of an HCC group and a group subjected to 5 hours of SD per day for 4 days, followed by brain collection on the fifth day ($n = 10$ and 9, respectively). Repeated brief SD without training did not lead to a persistent change in pCREB expression, compared with HCC animals, in any of the hippocampal brain regions studied: DG ($96.0\% \pm 2.8\%$ vs. $100.0\% \pm 4.4\%$, $p > 0.1$), CA3 ($96.3\% \pm 3.7\%$ vs. $100.0\% \pm 5.3\%$, $p > 0.1$), and CA1 ($102.2\% \pm 3.5\%$ vs. $100.0\% \pm 4.1\%$, $p > 0.1$). Also, SD did not change pCREB expression, compared with pCREB expression in HCC animals, in either the dorsomedial striatum ($100.7\% \pm 2.8\%$ vs. $100.0\% \pm 4.6\%$, $p > 0.1$) or dorsolateral striatum ($99.3\% \pm 3.5\%$ vs. $100.0\% \pm 5.8\%$, $p > 0.1$). Thus, these data indicate that the shift in pCREB expression from hippocampus to striatum in the previous experiment was not an effect of SD on basal pCREB expression but, rather, an effect of SD on the learning process and the subsequent performance in the probe trial.

**Sleep deprivation, anxiety and stress**

It has been reported that anxiety affects cognitive processes and can, in fact, change learning strategy in favor of a response-based strategy (Packard and Wingard, 2004). Therefore, to examine the possibility that SD in our previous experiments caused a shift toward a response-based learning strategy by inducing anxiety, we subjected mice to an elevated plus maze test. In a first batch of mice, we compared animals after a single session of 5 hours of SD with non-sleep-deprived control animals ($n = 10$ in each group). Contrary to the hypothesis that SD might induce anxiety, the sleep-deprived
mice spent significantly more time on the open arms than did the control mice (22.0% ± 2.3% vs. 12.3% ± 3.1%, respectively; $t_{18} = 2.54$, $p < 0.05$) and also entered the open arms more frequently than did control animals (41.5% ± 3.5% vs. 27.6% ± 5.0%, respectively; $t_{18} = 2.28$, $p < 0.05$). The total number of open- and closed-arm entries, however, did not differ between sleep-deprived and control mice (27.5 ± 1.5 and 23.7 ± 2.0, $p > 0.1$), indicating that SD did not affect spontaneous motor activity (Pellow et al., 1985; Walf and Frye, 2007). A second batch of mice was subjected to repeated 5 hours of SD per day for 4 days, similar to what was done in the T-maze experiment ($n = 9$ for both SD and control). The results show a trend toward an increased time in the open arms in sleep-deprived mice compared with control mice (19.1% ± 2.2% vs. 13.8% ± 1.8%, respectively; $t_{16} = -1.83$, $p = 0.09$). The frequency of open-arm entries did not differ between the SD and control groups (34.0% ± 4.1% vs. 27.2% ± 1.9%, respectively; $t_{16} = -1.480$, $p > 0.1$), nor did the total number of open- and closed-arm entries (33.9 ± 3.0 and 28.3 ± 2.5, $p > 0.1$), again indicating that the overall activity was not different.

**Figure 4.** Short sleep deprivation (SD) after each daily T-maze training session induced an increase in pCREB immunoreactivity in the dorsal striatum. This increase was not seen after training alone. Representative pictures of pCREB immunostaining in the dorsal striatum are shown on the left. pCREB immunoreactive cells were counted within the dorsomedial and dorsolateral striatum (MED and LAT, respectively). Home cage control mice (HCC), mice that were trained in the T maze (T), and mice that had received T-maze training followed by 5 hours of SD after each daily training session (SDT) were sacrificed 100 minutes after the probe trial. Although normal training (T) had no effect on pCREB expression in the dorsal striatum, mice that had been subjected to sleep deprivation (SDT) showed a significant increase in pCREB immunoreactivity in both the dorsomedial and dorsolateral striatum. Significant differences: * $p < 0.05$, post hoc analysis following one-way ANOVA.
Also, it is often suggested that effects of SD on learning and memory processes may be related to stress induced by the SD procedure rather than to sleep loss per se. Yet, plasma levels of the stress hormone CORT in sleep-deprived mice were low and not different from levels of control animals, neither after a single session of 5 hours of SD (sleep deprivation: 3.5 ± 0.2 μg/dL, control: 2.6 ± 0.4 μg/dL; n = 9 in each group, t\textsubscript{16} = -1.61, p > 0.1) nor after 4 daily sessions of 5 hours of SD (sleep deprivation: 5.1 ± 1.0 μg/dL, control: 4.3 ± 0.6 μg/dL; n = 10 in each group, t\textsubscript{18} = -0.68, p > 0.5).

All together, these data do not support the hypothesis that changes in learning strategy in our previous experiments were a consequence of SD–induced stress or anxiety.

**DISCUSSION**

In the experiments described in this study, we examined whether brief SD applied after each daily training session affected learning and reversal learning in a Y- or T-maze reference task. SD did not affect performance directly when mice learned the location of a food reward. However, SD applied during the training phase attenuated performance during the subsequent reversal training. Furthermore, the learning strategy that was used for place navigation in a maze was altered by SD. Mice deprived of sleep avoided using a hippocampus-dependent spatial strategy and switched to a response strategy that relies on the dorsal striatum to locate the food reward during training. In line with this switch in learning strategy, we found a shift in CREB phosphorylation in the brain areas thought to underlie these different strategies, i.e., the dorsal hippocampus and dorsal striatum (Colombo et al., 2003). Mice that had been subjected to SD after each daily training session did not show the normal training-induced increase in pCREB levels in the hippocampus, as was seen in control mice, but, instead, showed an increase in pCREB levels in the dorsal striatum. Previous studies in both humans and rodents have emphasized that striatal and hippocampal systems can work cooperatively and may compensate to some extent for the other system under conditions in which either system is temporarily or permanently impaired (Packard and McGaugh, 1996; Oliveira et al., 1997; Chang and Gold, 2003; Bohbot et al., 2004). Our data suggest that SD may represent such a condition, leading to impaired hippocampal function, which is compensated for by shifting to an alternative learning mechanism that involves the dorsal striatum.

Importantly, this shift in learning strategy explains our findings that SD did not affect performance during training in the Y maze: either strategy could be used to correctly locate the food reward. Obviously, there may be conditions and tasks for which no alternative strategies are available. Indeed, in contrast with the present experiments, other studies have reported that SD leads to immediate deficits in performance. For example, contextual fear conditioning is a learning task in which the role of the hippocampus apparently cannot easily be replaced by other brain regions, and several studies have reported SD-induced memory deficits in this task (Graves et al., 2003; McDermott et al., 2003; Ruskin and LaHoste, 2008). Thus, the availability of alternative learning strategies and mechanisms that can compensate for a hippocampal deficit may determine whether or not SD has noticeable effects on cognitive performance. This may explain some of the discrepancies
and discussions in the literature on whether or not deprivation of sleep really affects cognitive performance. Also, in humans, recent imaging studies have shown that SD after learning may be associated with changes in regional brain activity without immediate memory deficits (Orban et al., 2006; Rauchs et al., 2008).

Intriguingly, while a shift from a hippocampus-dependent to striatum-dependent learning strategy in mice in the SD group explains why we did not find a performance deficit during the Y-maze training, it might also account for the fact that we did find an attenuated performance during reversal training. Although either of the 2 available strategies was sufficient to locate the food reward during training, there is a particular difference between the systems underlying these 2 strategies in terms of flexibility. In comparison with the hippocampus, the dorsal striatum generates more stereotypical and less flexible responses that are more difficult to adapt to changing conditions (O’keefe and Nadel, 1978; Hartley et al., 2003). In agreement with this, rats using a response strategy in a T-maze learning paradigm have more difficulties with learning the novel location of the food reward during reversal training (Oliveira et al., 1997). Likewise, mice in the SD group that mastered the Y-maze task by preferentially using a response-based strategy during training had more difficulties during the reversal training. In other words, although the shift in learning strategy initially prevented a performance deficit during training, it may have caused a performance deficit during the subsequent reversal training.

Although the shift to a response-based learning strategy and use of a striatal memory system is the most direct explanation for the reduction in memory flexibility in mice in the SD group, it will be of interest to determine whether SD also affects functional activity of the prefrontal and orbitofrontal cortex, 2 regions that are critically involved in reversal learning (Ragazzino, 2007). It is not excluded that these regions play an additional role in the behavioral deficit seen during reversal training as a consequence of SD during training.

The mechanisms through which SD affects hippocampal function and memory formation are largely unknown. In fact, it is still a matter of debate whether sleep plays an active role in memory consolidation or merely a passive role by preventing waking interference (Ellenbogen et al., 2006). Waking interference and disruption of ongoing memory consolidation might result not only from mental activity related to sensory input and processing of new information, but also from neuronal and neuroendocrine activity related to physical activity or stress. Particularly in animal studies on sleep and memory, which rely on forced SD, it is often argued that interference might occur as a consequence of forced activity, stress, and stress hormones. Similar to our finding with SD, it has indeed been shown that stress and anxiety can modulate learning processes and favor the use of a response-based strategy (Packard and Wingard, 2004; Schwabe et al., 2008). However, in these studies, rodents were exposed to stress paradigms leading to strong increases in the plasma levels of glucocorticoid stress hormones, whereas, in our study, plasma levels of CORT after a single session of SD as well as after repeated SD were low and not different from the levels in control animals. The latter is in agreement with previous studies using the same SD method of mild stimulation, showing no significant elevations in stress-hormone levels (Van der Borght et al., 2006; Hagewoud et al., 2010). Also, in earlier studies, we showed that even more severe sleep restriction does not lead to persistent
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increases in glucocorticoid levels (Roman et al., 2005). In fact, undisturbed and spontaneously behaving rodents show mild increases in plasma CORT levels associated with, for example, eating and grooming that are above the levels we find in mice in the SD groups (Shiraishi et al., 1984). Importantly, it is sometimes overlooked that, particularly in animal studies, the learning task itself involves a degree of arousal and activation of stress systems that is much higher than what is found with SD by mild stimulation (e.g., Palchykova et al., 2006). Also, behaviorally, the mice in our study did not appear to be particularly stressed or anxious. After they were sleep deprived for several hours, most of their activity consisted of simply increasing attempts to curl up and sleep. Moreover, when we exposed sleep-deprived mice to an elevated plus maze, a commonly used and well-validated anxiety test, if anything, they appeared to be less anxious. All together, while we cannot exclude that some sort of interference by waking activities and sensory input plays a role in the effects of SD on learning and memory, it is unlikely that, in the present study, such interference was caused by stress, stress hormones, or anxiety.

On the molecular level, our data suggest that the behavioral effects of SD may result from reduced phosphorylation of the transcription factor CREB in the dorsal hippocampus. Numerous studies have implicated CREB in the formation of long-term memory (Silva et al., 1998), and spatial memory is impaired by suppression of CREB protein through the administration of antisense oligodeoxynucleotides (Guzowski and McGaugh, 1997) or genetic knock-out (Bourtchuladze et al., 1994). One of the many pathways that targets CREB is the extracellular signal-regulated kinase (ERK) pathway, which is known to be involved in synaptic plasticity and memory formation (Impey et al., 1999). Studies in rats have shown that SD attenuates ERK phosphorylation in the hippocampus (Guan et al., 2004; Ravassard et al., 2009). Since the ERK pathway mediates CREB phosphorylation (Bozon et al., 2003), a loss of the training-induced increase in CREB phosphorylation due to SD might be the result of impaired ERK function. A second candidate that may explain the effects of SD on CREB phosphorylation is the cAMP-dependent protein kinase (PKA) pathway. PKA is required for the formation of long-term memories (Abel et al., 1997), and previous studies by our laboratory have shown that hippocampal PKA expression is elevated during Y-maze training (Havekes et al., 2007). Furthermore, inhibitors of PKA and protein synthesis are capable of disturbing memory formation during the same time window after training as SD (Abel et al., 1997; Bourtchouladze et al., 1998; Graves et al., 2003), and a recent study indeed shows that SD selectively impairs cAMP and PKA dependent forms of synaptic plasticity in the mouse hippocampus (Vecsey et al., 2009). Future experiments will have to indicate whether training in the Y-maze indeed activates either or both pathways and whether this activation is attenuated by deprivation of sleep. If either pathway is impaired by SD, then posttraining intrahippocampal application of inhibitors of these pathways should induce a similar shift in learning strategy for place navigation as seen after SD.

In conclusion, our study indicates that the brain can temporarily compensate for the effects of sleep loss on cognitive performance by switching to alternative learning mechanisms. An important implication of these findings is that effects of SD in the brain may not always be directly evident on the level of behavioral performance. However, a second important implication of our data is that effects of SD may still appear later, long after the actual sleep loss itself, because the alternative learning
mechanisms and brain regions involved can result in reduced flexibility under changing conditions that require adaptation of the previously formed memory.

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