Sleep deprivation prevents learning-induced MAPK activation in the mouse hippocampus and impairs behavioral flexibility in a Y-maze task

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Abstract

Sleep loss impairs memory formation, particularly in case of hippocampus-dependent memories. However, it is unclear whether sleep deprivation (SD) also affects the adaptation of previously acquired memories to match with changes in a familiar environment. Here, we studied effects of SD on learning and reversal learning in a Y-maze task. Mice were trained to locate a food reward in one of two accessible arms of a Y-shaped maze. When the animals had mastered the task, the food reward was relocated, after which they had to learn that the previously non-baited arm was now the baited one (reversal training). In addition, we examined whether SD affected hippocampal activation induced by training in the Y maze. The results show that: 1) performance during training was not affected by SD; 2) sleep loss during reversal training did not attenuate performance; 3) SD during training reduced performance during subsequent reversal training; 4) training in the Y maze induced hippocampal expression of the immediate early genes zif268 and c-fos regardless of SD; 5) SD prevented training-induced MAPK activation seen in the hippocampus of control mice. In conclusion, these data show that sleep loss during the learning phase of a task affects plasticity-related signaling in the hippocampus. This may not necessarily affect performance at the behavioral level directly, but it can hamper subsequent adaptation of this acquired memory when the situation changes. Sleep loss may thus impair the flexibility of individuals under changing conditions.
Introduction

Over the recent years, it has become apparent that sleep has a role in learning and memory processes (for review, Maquet, 2001; Stickgold, 2005; Stickgold and Walker, 2005). Various studies show that loss of sleep prior to training deteriorates subsequent learning (Stern, 1971; McDermott et al., 2003; Ruskin et al., 2004; Yoo et al., 2007) and that sleep deprivation (SD) after training disturbs the formation and consolidation of long-term memories (Karni et al., 1994; Smith and Rose, 1996; Smith et al., 1998; Palchykova et al., 2006). Although learning and memory formation are complex processes that involve various brain regions, data suggest that sleep loss may particularly hamper the formation of hippocampus-dependent memories (Graves et al., 2003; McDermott et al., 2003; Ruskin et al., 2004).

With regards to the role of sleep in learning and memory processes, there is one aspect of hippocampal function that has received scarce attention: the hippocampus is not only important for the formation of new memories, but is also essential for the adaptation of previously acquired memories. The hippocampus has a key role in this process by detecting mismatches between previously stored information and actual incoming new sensory information (Lisman and Otma, 2001; Vinogradova, 2001). Via that route, the hippocampus plays an important role in behavioral flexibility and adaptation to changing conditions.

The aim of the present study was to examine if this aspect of hippocampus function is affected by SD as well. Mice were allowed to freely explore a two-arm Y maze in which one of two accessible arms was baited with a food reward. After mice learned to locate the baited arm (training), the food reward was relocated to the previously non-baited arm. With ongoing training, mice had to adapt and learn that the food reward was now located in the previously non-baited arm (reversal training). We examined the effect of SD after the training and reversal training sessions to assess the role of sleep in formation of new memories and the role in subsequent adaptation of these memories when conditions change and a previously learned strategy is no longer valid.

To analyze whether Y-maze training with and without SD affect hippocampal activation, we examined protein expression of the immediate early genes c-fos and zif268, both frequently used markers of neural activity (for review, Lanahan and Worley, 1998; Tischmeyer and Grimm, 1999; Davis et al., 2003). We further studied the effects of Y-maze learning and SD on hippocampal activation of the mitogen activated protein kinase (MAPK) pathway, a signaling cascade that is involved in the formation of several forms of long-term memory including spatial memory (Atkins et al., 1998; Blum et al., 1999; Selcher et al., 1999; Schafe et al., 2000). Importantly, a recent study showed that SD prior to learning not only reduced behavioral performance, but also impaired MAPK activation in the hippocampus (Guan et al., 2004). This suggests that interference with MAPK signaling might be one of the mechanisms by which sleep loss impairs memory formation.
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Materials and Methods

Animals and housing condition
This study was performed with adult, male C57BL/6J mice, 12 to 15 weeks old (Harlan, Horst, Netherlands). The animals were individually housed in standard macrolon cages and maintained on a 12 hour light/ 12h dark cycle (lights on at 7.00 a.m.) with food and water ad libitum. A layer of sawdust served as bedding. Subjects were food deprived to 90 % of their individual body weight, starting four days before the beginning of the experiment. Animals were weighed and fed after the last daily learning session. 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.

Experimental set up
Mice were trained in a Y maze to locate a food-reward in one of two accessible arms. After training, mice were subjected to a reversal training in which the food reward was relocated to the previously unrewarded arm. Three experiments were performed. In the first experiment, one group of mice was subjected to the standard training and reversal training protocol (control n=9) and a second group was subjected to 5h of SD after each daily session of training and reversal training (SD, n=10). The second experiment consisted of a control group (n=6), a group of mice subjected to 5h SD after every training session but not after the reversal training sessions (SDT, n=6), and a group of mice that received 5h of SD only after each reversal training session (SDRT, n=6). The third experiment served to collect brain material after the last training session and consisted of 3 groups: home cage controls (HCC, n=10), normal training without SD (T, n=10), and training with SD (TSD, n=10).

Y-maze learning and reversal learning
To study effects of sleep loss on memory formation and memory adaptation, mice were subjected to a Y-maze task (Havekes et al., 2006, 2007; Van der Borght et al., 2007). Behavioral testing was conducted in a tubular, transparent plexiglass Y maze consisting of a start arm and two test arms forming the Y. All three arms were 5 cm in diameter, 27.5 cm long, and at a 120° angle from each other. The start box was connected to the start arm of the Y-maze. Food crumbs placed below perforations at the end of the two test arms prevented animals to discriminate between baited and non-baited arms by olfactory cues. A guillotine door halfway each arm could be operated manually from the experimenters position. Small grey plastic blocks (1 cm high) were placed 4 cm from the end of the arms to prevent visual inspection for food presence from a distance. The experimental room contained visual cues, which served as distal spatial cues.

On the first day, mice received two habituation trials to familiarize the animals with the experimental set-up. During the first habituation trial, mice were placed in the start box and were given the opportunity to freely enter the maze. Subjects could explore one of the two test arms, which was baited with small crumbs of food 0.05-0.1 g. The other arm was closed. When the reward was consumed the mouse 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 received a daily training session consisting of 6 trials. During the entire training phase, either the right or left arm was baited. This was constant for a given individual, but randomized between subjects and treatments. When during a trial a subject visited one of the two accessible arms, the non-visited arm was closed. After the subject retreated to the start box, the start arm connected to the start box was blocked preventing re-entrance of the maze. After cleaning all arms with damped paper cloth, and re-baiting the same arm, the subject was again allowed to explore either the right or left test arm. A visit to the baited arm was recorded as a correct trial. In experiment 1 and 2, all mice received a training followed by a reversal training. In experiment 3,
Sleep deprivation and Y-maze learning

Mice were sacrificed for brain collection 90 minutes after the last training session. Animals were not subjected to SD after the last training session before brain collection to prevent a direct impact of SD.

Sleep deprivation
Mice were subjected to SD for 5 hours immediately following the daily sessions of training and/or reversal training. 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. This approach effectively keeps animals awake without inducing significant stress as measured by plasma levels of glucocorticoids (Meerlo and Turek, 2001; Van der Borght et al., 2006). Our choice to sleep deprive the mice for 5h following the Y-maze session was based on other studies in rodents suggesting that sleep plays a critical role in formation of memories during this time-window (e.g. Graves et al. 2003, Palchykova et al. 2006).

Brain processing and immunocytochemistry
Ninety minutes after the last training, animals from experiment 3 were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, and post fixated for three hours. Afterwards brains were kept in 0.01 M PBS overnight. Brains were subsequently cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 48h. Next, 30 µm coronal sections, spanning the dorsal hippocampus (Bregma -1.46 to -2.80), were cut on a cryostat microtome. Brains were stained for c-fos, zif268 and phospho-P44/42 MAPK. Sections were treated with 0.3% H2O2 (30 minutes), blocked with 3% normal serum and the cell membrane was permeabilized with 0.1% Triton-X100. Rabbit-anti-c-fos (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit-anti-zif268 (1:750, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit-anti-phospho-P44/P42 MAPK (1:300, Cell Signaling Technology, Beverly, MA, USA) were applied for 72h at 4°C. Sections were incubated with a biotinylated secondary antibody (goat-anti-rabbit, 1:400, Jackson Immunolabs, West Grove, PA, USA) for 2h at room temperature followed by overnight at 4°C. The next day, sections were incubated with Avidin-Biotin-Complex (1:400, ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) for 2h. Staining was processed with 20 mg/100 ml DAB and 0.03% H2O2 as reaction initiator.

Quantification of the immunostainings
Numbers of immuno-positive cells in the dentate gyrus (DG) of the dorsal hippocampus were determined in 3 sections per animal, between -1.82 and -2.06 mm from Bregma. Cells were counted throughout the entire thickness of the sections, using a 40x magnification. For every animal, the average number of immuno-positive cells per DG was calculated. The number of immuno-positive cells was corrected for length of each DG separately, using Quantimed 500 image analysis software (Leica, Cambridge, UK).

Optical densities for P44/42 MAPK and Zif268 immunoreactivity were measured in the CA1 stratum radiatum and stratum pyramidale in 3 sections of the dorsal hippocampus (-1.82 and -2.06 mm from Bregma). The OD is expressed in arbitrary units corresponding to grey levels using a Quantimet 550 image analysis system (Leica, Cambridge, UK). The value of background labeling was measured in the corpus callosum and extracted of the OD of the area of interest, thus reducing the variability in background staining among sections. The experimenter was blind to the group assessment of individual animals during all cell counts and OD measurements.

Statistics
The learning curves of the Y maze were analyzed using a repeated measures ANOVA with a between-subject factor ‘treatment’ (control or SD) and a within-subject factor ‘time’ (daily Y-maze sessions).
Potential differences in phospho-P44/42 MAPK, c-fos, and zif268 immunoreactivity after training with and without SD were statistically analyzed with one-way ANOVA with factor ‘treatment’ (home cage control, training or training with SD). Whenever ANOVA revealed a significant treatment effects, pair wise comparisons were made with a post hoc Tukey test. Data are expressed as averages ± S.E.M. \( P < 0.05 \) was considered as significant.

**Results**

**SD impairs reversal learning in the Y maze**

In experiment 1, we investigated whether SD affected learning and reversal learning in the Y maze. At the beginning of training, both groups performed at chance level, indicating that they had no preference for either of the two arms (control: 40.7 ± 8.4 % and SD: 50.0 ± 7.5 % correct trials, Fig. 1A). With ongoing training, both groups progressively learned to locate the baited arm (effect of session: \( F_{5,85} = 17.356, P < 0.001 \)). After six days of training, the control group and SD group reached scores of 85.3 ± 6.8 % and 95.0 ± 5.0 % respectively. SD did not affect the rate of acquisition during training.

After training, subjects received a reversal training with the food reward relocated to the previously unrewarded arm. During the first reversal training session, performance of the control group dropped to 22.2 ± 8.3 % and that of the SD 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. In other words, they all visited the arm that had been baited during the training phase. During the following trials of the first session, mice of the control group gradually started to visit the correct arm (e.g. the arm that was now baited), while mice of the SD group continued to visit the incorrect arm (data not shown). With further training, both groups gradually learned to locate the baited arm (effect of session: \( F_{6,102} = 24.630, P < 0.001 \), Fig. 1A). However, performance of the SD group was significantly reduced compared to that of the control group (treatment effect: \( F_{1,17} = 14.868, P = 0.001 \)). While the control group reached an average performance of 88.9 ± 4.8 % after 7 sessions of reversal training, the SD group had reached a score of only 45.0 ± 10.4 % (Fig. 1A). These findings indicate that SD does not affect Y-maze learning but does attenuate reversal learning in the Y-maze reference task.

**SD during training affects behavioral performance during reversal training**

In experiment 2, we explored whether the reduced performance during reversal training shown in experiment 1 was a consequence of SD during training or SD during reversal training. Three groups of mice were subjected to training and reversal training in the Y maze: a control group (control), a group that was sleep deprived after each training session (SDT), and a groups that was sleep deprived after each reversal training session (SDRT). Similar to experiment 1, SD did not affect performance during training (data not shown). In contrast, a strong effect of treatment was found during reversal training (\( F_{1,17} = 14.868, P = 0.001 \)). While the control group reached an average performance of 88.9 ± 4.8 % after 7 sessions of reversal training, the SD group had reached a score of only 45.0 ± 10.4 % (Fig. 1B). These findings indicate that SD does not affect Y-maze learning but does attenuate reversal learning in the Y-maze reference task.
mice that had been exposed to SD after each training sessions performed significantly worse than the other two groups (\( P < 0.005 \) versus control group and \( P < 0.05 \) versus SDRT group). After 7 daily sessions of reversal training, the control group and SDRT group reached a score of 94.4 ± 5.5 % and 97.2 ± 2.8 % respectively, whereas the SDT group had reached a score of only 38.9 ± 13.4 %. These findings show that SD during training affects the behavioral performance during subsequent reversal training.

Training enhances expression of the immediate early genes c-fos and zif268 in the dorsal hippocampus regardless of SD
Since the second experiment showed that only SD during the training phase affected performance during the reversal training, we anticipated that possible differences in hippocampal function might be noticeable at the end of the training phase. We therefore collected brain material at the end of the training phase from 3 groups of mice: home cage control (HCC), a group of mice receiving the normal training sessions (T), and a group receiving the training sessions combined with 5h SD (TSD). After 7 sessions of training, the two trained groups had a strong preference for the baited arm with performance scores of 88.9 ± 8.2 % in the T group and 97.2 ± 2.8 % in the TSD group (data not shown). As shown in experiment 1 and 2, SD did not affect performance during training in the Y maze.

Representative pictures of c-fos protein expression in the dorsal hippocampus are shown in figure 2A and B. Ninety minutes after the last daily training session, both groups of trained mice (T and TSD) had increased numbers of c-fos positive cells in the dorsal DG compared to the HCC animals (T versus HCC: \( P < 0.005 \); TSD versus HCC: \( P < 0.05 \); Fig. 3C). This increase in the number of c-fos-positive cells was not affected by SD.
The distribution of zif268 labeling in the hippocampus is shown in figure 3A and B. In line with the training-induced increase in c-fos expression, the number of zif268 positive cells was enhanced in the DG after training and this training-induced increase was not affected by SD (P < 0.005 for T versus HCC and TSD versus HCC, Fig. 3C). Similarly, zif268 expression was increased in the CA1 area of the hippocampus after training irrespective of SD (compared with control group P < 0.005 in both cases).

**Figure 2.** Effects of Y-maze training with or without SD on c-fos expression in the dorsal hippocampus. A representative picture of c-fos immunoreactivity in the dorsal hippocampus (A). Enlargement of the DG region (see insert in A); arrows indicate c-fos positive cells (B). Y-maze training enhanced the number of c-fos positive cells in the DG irrespective of SD (C). HCC (home cage controls), T (training), TSD (training with SD). * P < 0.05, *** P < 0.005.

**SD prevents the training-induced increase in hippocampal MAPK expression**

Finally, we investigated if SD affects MAPK activation in the hippocampus induced by training. Representative pictures of P44/42 MAPK phosphorylation in the dorsal hippocampus are shown in Figure 4A and B. Ninety minutes after the last training session, the number of phospho-P44/42 MAPK cells in the granular cell layer of the DG was significantly enhanced in the trained mice (T) compared to HCC animals (P < 0.05, Fig. 4C). However, this increase in number of MAPK-positive cells was not found in animals that had been subjected to SD after each daily training session (T vs TSD P < 0.005, HCC vs TSD P > 0.25). In contrast to the DG, no changes in P44/42 MAPK immunoreactivity were found in the CA1 area of the hippocampus (Fig. 4D).
Figure 3. Effects of Y-maze training with and without SD on zif268 protein expression in the dorsal hippocampus. A representative picture of zif268 immunoreactivity in the dorsal hippocampus (A). Enlargement of the DG region (see insert in A) (B). SD did not affect the training-induced increase in zif268-positive cells in the DG (C). SD did not affect training-induced increase in zif268 immunoreactivity in area CA1. HCC (home cage controls), T (training), TSD (training with SD). *** P < 0.005.
Figure 4. Effects of Y-maze training with or without SD on MAPK activation in the dorsal hippocampus. A representative picture of phospho-P44/42 MAPK immunoreactivity in the dorsal hippocampus (A). Enlargement of the DG region (see insert in A) (B). Y-maze training enhanced the number of phospho-P44/42 MAPK positive cells in the DG. This training-induced enhancement was impaired by SD (C). Training with or without SD did not affect phospho-P44/42 MAPK immunoreactivity in the stratum radiatum of area CA1 (D). HCC (home cage controls), T (training), TSD (training with SD). * P < 0.05, *** P < 0.005.
Discussion

In this study, we investigated whether SD affected learning and reversal learning in a Y maze. We showed that: 1) SD during training did not affect acquisition performance directly, but it did impair subsequent reversal training; 2) Training in the Y maze was associated with increased hippocampal activity as reflected by enhanced protein expression of the immediate early genes c-fos and zif268, but this was not affected by SD; 3) SD impaired the normal training-induced hippocampal activation of MAPK. Together, these data indicate that SD impairs the adaptation of previously formed memories to match with the current situation, possibly in part by impairing MAPK signaling in the hippocampus.

Various studies focusing on the role of sleep in memory consolidation showed that hippocampus-dependent learning is impaired by loss of sleep (Graves et al., 2003; Ruskin et al., 2004). In our study, SD after the training sessions reduced the activation of plasticity-related MAPK signaling, but it did not affect the rate of acquisition during training. The latter is in line with a recent PET study on hippocampus-dependent place-finding in humans (Orban et al., 2006). Subjects performed a navigation task in a virtual town and the results showed that SD inhibited post-learning restructuring of brain activity although the behavioral performance was not reduced (Orban et al., 2006). Thus, sleep loss may affect learning and memory processes at the neuronal level but such changes are not always immediately noticeable at the behavioral level. One possible explanation for this is that a hippocampal deficiency is compensated by other brain areas. For example, in our study, animals subjected to SD during Y-maze training may have partly shifted from a hippocampus-based strategy to a strategy that involves the striatum. Indeed, our training-procedure with a food reward located in a fixed arm not only allowed mice to use a spatial approach, but also allowed mice to use a response-based approach (learning to always take the same direction, independent of spatial cues). The latter approach is known to be more dependent on the striatum and less on the hippocampus (Kesner et al., 1993; Packard and McGaugh, 1996). This explanation is supported by studies in rats that had to learn which of two accessible arms was baited in a cross maze paradigm. Animals undergoing pharmacological blockade of the hippocampal system were still able to master the task. However, rather than using a spatial strategy requiring a functional hippocampus, rats used a striatum-dependent learning strategy instead to locate the baited arm (Packard and McGaugh, 1996).

In our study, also performance during reversal training in the Y-maze task was not affected by SD that occurred after the reversal training sessions. Sleep loss per se did not interfere with learning the novel location of the food reward. As discussed for training, mice may have switched from spatial learning to response learning during the reversal training, without affecting the behavioral performance.

One important finding of our study is that SD during training, although it had no immediate effect on performance during the training itself, did impair subsequent reversal learning. In other words, sleep loss had a delayed effect that only became apparent later on, when the condition had changed and the previously learned behavior required adaptation. Thus, SD during training had affected information processing and memory formation in
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such a way that adaptation to a change in the condition became more difficult. This delayed effect of SD may in part be related to the shift in hippocampal/striatal balance in SD mice suggested above. In comparison with the hippocampus, the striatum generates more stereotypical and less flexible responses that are more difficult to adapt to changing conditions (Hartley et al., 2003). If SD during Y-maze training facilitated the use of a striatum-dependent approach rather than a strategy dependent on the hippocampus, adapting this response upon relocation of the food reward during the reversal training might indeed be more difficult and slower than adapting the more hippocampus-dependent approach in control animals. Future experiments are required to establish if such a shift from a hippocampal to striatum-dependent strategy indeed occurs in mice under conditions of SD.

Activation of the MAPK-signaling pathway by means of P44/42 MAPK phosphorylation was enhanced in the hippocampus after Y-maze training. This is in agreement with various other studies showing that memory formation induces hippocampal MAPK activation (Atkins et al., 1998; Cammarota et al., 2000; Alonso et al., 2002). Markedly, the normal training-induced activation of the MAPK pathway in the hippocampus was prevented by SD. In agreement with our data, Guan and colleagues (2004) reported that SD prior to water maze training affected the MAPK pathway in the hippocampus. In line with the latter study, our results suggest that impairment of the MAPK signaling pathway may be one route via which SD affects hippocampus function and memory formation.

c-Fos and zif268 expression in the dorsal hippocampus were significantly elevated after Y-maze training, and this elevation was not altered by SD. The fact that these markers of neuronal activation were not changed in sleep deprived animals shows that SD does not does not lead to an overall reduction or general impairment of hippocampal activity, but rather specifically affects some hippocampal signaling pathways.

In conclusion, our study shows that sleep loss affects plasticity-related signaling pathways in the hippocampus (attenuated learning–induced MAPK expression), but such changes do not always immediately show up at the behavioral level (apparently normal acquisition of a Y-maze task). However, sleep loss during the acquisition phase of a task may have delayed effects that only show up later, for example, when the learned behavior requires adaptation (SD during the initial learning phase of a Y-maze task impaired subsequent reversal learning). Thus, while many studies have shown that sleep loss can impair the formation of new hippocampus-dependent memories, our data indicate that sleep loss may also hamper the adaptation of previously acquired memories. This implies that sleep loss may reduce the flexibility of individuals under changing conditions.

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Author contributions

R.H., R.H., E.A.V.d.Z. and P.M. generated the hypotheses and designed the experiments. R.H., R.H., A.N., K.H., PW. and PM. did the behavioral testing, sleep privation and collection of the brain tissue. K.H. and P.W. did the immunohistochemistry and analyses. R.H. wrote the manuscript with input from E.A.V.d.Z and PM.

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