Sleep Restriction Suppresses Neurogenesis Induced by Hippocampus-Dependent Learning

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Sleep deprivation impairs hippocampal-dependent learning, which, in turn, is associated with increased survival of newborn cells in the hippocampus. We tested whether the deleterious effects of sleep restriction on hippocampus-dependent memory were associated with reduced cell survival in the hippocampus. We show that sleep restriction impaired hippocampus-dependent learning and abolished learning-induced neurogenesis. Animals were trained in a water maze on either a spatial learning (hippocampus-dependent) task or a nonspatial task. This increase was abolished by sleep restriction that caused overall reduced cell survival in all animals. Sleep restriction also selectively impaired spatial learning while performance in the nonspatial task was, surprisingly, improved. Further analysis showed that in both training groups fully rested animals applied a spatial strategy irrespective of task requirements; this strategy interfered with performance in the nonspatial task. Conversely, in sleep-restricted animals, this preferred spatial strategy was eliminated, favoring the use of nonspatial information, and hence improving performance in the nonspatial task. These findings suggest that sleep loss altered behavioral strategies to those that do not depend on the hippocampus, concomitantly reversing the neurogenic effects of hippocampus-dependent learning.

INTRODUCTION

Several studies indicate that sleep facilitates memory consolidation (Born and Wagner 2004; Gais et al. 2000; Karni et al. 1994; Mednick et al. 2003; Plihal and Born 1999; Smith 1996). While sleep deprivation studies in animals have shown diverse molecular effects (Cirelli 2002; Graves et al. 2001), it remains unclear how these molecular changes elucidate processes mediating the effects of sleep on memory consolidation.

Recently it has been shown that prolonged (72 h) sleep deprivation suppresses cell proliferation in the granule cell layer of the hippocampus (Guzman-Marin et al. 2003). Hippocampus-dependent learning and memory correlate with neurogenesis in the granule cell layer. For example, prolonged stress inhibits neurogenesis and behavioral measures of hippocampal function (Gould and Tanapat 1999; Gould et al. 1992), whereas physical activity, such as wheel-running, augments neurogenesis as well as improves cognitive function (van Praag et al. 1999a,b). More compelling were the observations that behavioral tasks that specifically engage the hippocampus were associated with increased survival of newborn cells in the granule cell layer (Gould et al. 1999; Leuner et al. 2004) and that suppression of neurogenesis specifically impaired the formation of hippocampal-dependent memories (Shors 2001; Snyder et al. 2005). These studies suggest that neurogenesis is modulated by hippocampal activity and may even be a necessary component for the formation of some types of hippocampus-dependent memories.

We set out to test whether sleep deprivation would impact learning-induced neurogenesis in concert with its behavioral outcome. To simulate conditions similar to human sleep restriction, animals were deprived of sleep only for part of their rest phase, between training sessions. The effects of sleep restriction on memory were evaluated by following performance on daily training sessions. Rats were trained in a water maze, eight trials per day for 4 days in one of two training paradigms: a hippocampus-independent cue task, wherein a visible platform was moved to a different quadrant of the maze every four trials; and a hippocampus-dependent spatial task, using a submerged (hidden) platform, which remained in the same location on all trials. Because rats are nocturnal animals, training occurred during the dark phase, with the end of each training session coinciding with light onset and beginning of the rest phase. One-half of the animals were kept awake for 6 h and allowed to sleep the remainder of the day until the next training session. This protocol allowed the animals to recover from sleep loss before the next training session, thus reducing the impact of fatigue on performance.

As expected, cell survival and the proportion of cells with neural fate were higher in rested animals that were trained in the hippocampus-dependent task; sleep-restricted animals were impaired in spatial learning. In addition, sleep restriction reduced learning-induced neurogenesis by eliminating the task-dependent increase in cell survival and preference for neuronal fate.

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**METHODS**

Forty-two rats were maintained on a 12:12-h light/dark cycle, with ambient temperature of 22°C and food and water ad libitum. Of these, 24 underwent surgery for polysomnography, and 12 were used for histology. All husbandry and experimental procedures were reviewed and approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC).

### Polysomnography

Ten days before the beginning of the behavioral experiment, rats were anesthetized with rodent ketamine (Ketaset, Fort Dodge 22–44 mg/kg; X-Ject, Vetus 2.5 mg/kg; Aceproject, Vetus 0.75 mg/kg) administered intraperitoneally. Four EEG electrodes (no. 000 stainless steel screws) were fastened bilaterally in frontal and parietal bones. Three EMG electrodes (stainless steel wire) were inserted into the neck muscles. Electrodes were attached to a socket and fastened to the skull with dental acrylic. Rats were acclimated to the recording apparatus for 24 h, and subsequently, baseline was determined 1 day before training started. For polysomnography data acquisition, animals were lightly anesthetized with halothane (Halocarbon Laboratories) to connect the rats to the head cables; data acquisition started ~30 min thereafter. To avoid halothane affecting learning and performance, polysomnography data were collected during the light phase only. These animals were not used for histology, because the effect of halothane on neurogenesis is unknown.

Polysomnography data were acquired using a Grass 7 polygraph, with one different EEG channel and one differential EMG channel. EEG, from either left or right fronto-parietal derivation, was filtered at 0.3 and 35 Hz (1/2 max, 6 dB/octave), digitized at 100 Hz, and stored in 10-s epochs. EMG was filtered at 3 and 75 Hz, full-wave rectified, and integrated for each epoch. For statistical analysis, EEG was Fourier transformed, and vigilance states were scored using an automated scoring system (Benington et al. 1994). Artifact-free recording time was scored as slow-wave sleep (SWS), paradoxical sleep (PS), or wake, based on the spectral content of the EEG. Percent of recording time spent in SWS and in PS, and the power in the delta band (1–4 Hz), were averaged into 6-h bins (Zeitgeber Time, ZT 0–6; ZT 7–12). The effects of sleep restriction on sleep patterns were assessed by analyzing SWS and PS percent at ZT 7–12 using repeated-measures ANOVA, with days as the repeated-measures factor and sleep and learning condition as independent variables. Dunnett’s test for treatment versus control, with baseline assigned as control, was used to determine the significance of change in sleep amounts from baseline. Changes in the delta band were assessed by averaging hourly bin values across the days and determining percent change from baseline. The impact of the repeated sleep deprivation on delta power was determined by a repeated-measures ANOVA, with time bins (ZT 7–12) as the repeated-measures variable and sleep and learning condition as independent variables. Finally, time spent in either SWS or PS, during the first 6 h of the light phase (ZT 0–6) on each day, was correlated with the latency to find the platform in the training session preceding sleep.

### Behavioral assessment and sleep restriction

Rats were trained in a dimly lit room, in a water maze (175 cm diameter) filled with ~28°C water, with prominent posters and objects on the wall surrounding the maze. Each animal was trained in one of the following paradigms. 1) A spatial task using a submerged platform (10 cm diam). The pool and platform were black, which when submerged was invisible from the surface. 2) A cue task, where the platform was raised an inch above the surface of the water, covered with vertical white strips, and with a black and white block with lemon odor placed on top. There were eight trials per day (2 blocks of 4) for 4 days, with an intertrial interval of 60 s, and an interblock interval of ~40 min. The entry point to the maze, on each trial, was pseudo-randomly varied so that no more than two adjacent entry points occurred within a block. Rats remained on the platform for 10 s at the end of each trial. Latency to reach the platform was timed, and the swim path was videotaped and quantified.

Training occurred during the last 3 h of the dark phase, ensuring that the animals returned to their cages before lights came on. Sleep restriction was achieved by depriving one-half the animals of sleep for 6 h, starting immediately at lights-on (ZT 0), of each training day. Animals were deprived of sleep by removing the cage lids, allowing the rats out of their cages, and introducing novel objects (e.g., tissue paper, plastic tubes, fresh bedding). During this time, the animals could play and explore their environment. The animals were visually monitored throughout this period, and interventions only occurred if animals tried to go to sleep (i.e., built a nest or curled up). These interventions consisted of introducing more novelty. This method of sleep deprivation was effective in keeping the animals active and required minimal handling. At the end of sleep deprivation, animals were returned to their cages without further interference until the next training day.

For statistical analysis, mean time to reach the platform (latency) was averaged across the whole training session. Repeated-measures ANOVA tests were performed with training day as the repeated-measures variable and sleep and training conditions as the independent variables. Swim path length, in centimeters, was calculated for each animal and analyzed, similarly, with repeated-measures ANOVA. In addition, regression analysis was performed on latency values from days 2–4 from each animal. The r coefficients from each animal were used in a further two-way ANOVA with independent variables of sleep and learning condition and single group t-test with predicted means of zero. These provided information on whether the slope was affected by learning or sleep conditions and whether the animal’s performance improved over days, respectively.

To determine the effects of sleep restriction on the search strategy used by the rats to locate the platform, video files from each trial were evaluated for the animal’s tendency to use spatial landmarks. Landmarks were defined as a point in the maze to which the rats tended to return to within a trial. An experimenter, blinded to the experimental condition of the animals, analyzed each trial for each animal. Trials in which rats repeatedly returned to a certain location (landmark) received a score of 1 (see Fig. 3D); trials in which rats did not show a preferred location in the maze were scored as 0 (Fig. 3C). Scores were summed across trials for each animal, and t-tests were performed on the summed scores comparing sleep-restricted and non–sleep-restricted animals for spatial and cue animals separately.

### BrdU administration and histology

Seven days before training, animals were injected with BrdU (5-bromo-2′-deoxyuridine; B-5002, Sigma-Aldrich). BrdU incorporates into the DNA during the S-phase of the cell cycle only and is a reliable marker for mitotic cells in studies of adult neurogenesis (Cameron and Mckay 2001; Gratzner 1982). To maximize labeling of dividing cells in the hippocampus, two injections of 100 mg/kg each (i.e., 200 mg/kg total) were administered during the light phase, 8 h apart. After the last day of training, rats were anesthetized with pentobarbital sodium (50 mg/kg) and perfused with 4% paraformaldehyde (prills, 95%; Aldrich Chemical Co.), and their brains were removed. After equilibrating the brains in 30% sucrose, they were sectioned to 40-μm sections in the coronal plane with a freezing microtome and stored at ~20°C in a cryoprotectant solution.

To assess the number of proliferating cell in the hippocampus, sections were processed for BrdU incorporation. Sections were incubated in H₂O₂, permeabilized with 3 N HCl at 37°C, blocked in 3% normal donkey serum (17-000-121, Jackson ImmunoResearch Laboratories), and incubated in primary rat monoclonal antibody against BrdU (2 μl/ml; MAS 250c ascites, Accurate Chemical and Scientific Laboratories).
Corporation) and secondary anti-rat biotin-conjugated antibody [2 μg/ml; Biotin-SP-AffiniPure Donkey Anti- Rat IgG (H+L); 712-065-150, Jackson Immunoresearch]. Sections were reacted using Vectastain ABC Elite Kit (PK-6100, Vector Laboratories) and nickel-enhanced diaminobenzidine (DAB; SK-4100, Vector Laboratories). BrdU incorporation was quantified using the Optical Fractionator method with Microward Stereoinvestigator software, on a z-series of sections 240 μm apart. The hilus, dentate gyrus (DG), and the granule cell layer (gcl) were defined as the region of interest (ROI; Fig. 4A). All cells were counted within this ROI, using an overlay grid of 50 μx50 μm, and cell density was estimated as a function of the ROI volume for each animal.

Neuronal fate was assessed using combined fluorescent labeling of BrdU and doublecortin (DCX), a selective marker for immature neurons (Couillard-Despres et al. 2005; Francis et al. 1999). Sections were incubated in primary goat anti-rat anti-DCX (2 μg/ml; sc-8066, Santa Cruz Biotechnology) and secondary goat-anti-FITC (2 μg/ml; fluorescein AffiniPure; 705-095-147, Jackson Immunoresearch Laboratories). After fixation in 4% paraformaldehyde, sections were permeabilized and incubated with rat monoclonal antibody against BrdU and with a secondary anti-mouse CY-3 [2 μg/ml, Cy3 AffiniPure donkey anti-rat IgG (H+L); 712-165-150, Jackson Immunoresearch Laboratories). Double-labeling was assessed by visualizing the sections on a confocal microscope (BIORAD MRC-1034). Total BrdU+ and colabeled DCX+Brdu-labeled cells were counted. A minimum of 150 BrdU-labeled cells, in a minimum of four sections, were counted per animal.

For both confocal and stereology assessments, the experimenter was blind to the conditions of the experiment. Statistical analyses of BrdU cell counts and DCX-BrdU double labeling were done using the 

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\chi^2 \text{ median test}, \quad \text{and the HsuMSB test was used to determine the source of the effect for each comparison.}
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Radioimmunoassay of corticosterone

To determine whether the method of sleep restriction by enrichment, used in this study, caused an increase in corticosterone (CORT) levels, a separate group of 10 animals was sleep deprived for 6 h and light phase (ZT 7–12). Repeated-measures ANOVA, with days as the repeated-measure variable and learning and sleep conditions as the independent variables, yielded an increase in SWS (F(1,15) = 18.12, P = 0.007; Fig. 1A and B), with no difference in the amount of PS (F(1,15) = 0.89, P = 0.430). Dunnett’s test on the amount of PS from baseline (ZT 7–12) expressed as percent recording time.

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\text{FIG. 1. Mean sleep time averaged across the last 6 h of the light phase (Zeitgeber time, ZT 7–12) expressed as percent recording time.} \quad A: \text{sleep-restricted (SR, solid lines), but not non–sleep-restricted (nSR, dashed lines) spatial animals showed an increase in slow-wave sleep (SWS) from baseline (Dunnett’s LSD, } P < 0.05); \text{ sleep restriction had no effect on the amount of paradoxical sleep (PS) during this time.} \quad B: \text{both SR and nSR cued animals the proportion of SWS relative to baseline (Dunnett’s LSD, } P < 0.05), \text{ with no change in PS.} \quad C: \text{percent change from baseline of delta power (DP) during SWS, in hourly bins, from the end of sleep restriction.} \quad \text{SR spatial (solid dark bars and line) and SR cue (solid light bars and line) expressed increased DP (} P = 0.050 \text{ and } P = 0.017, \text{ respectively).} \quad \star \text{ and } \diamond \text{ significant differences between SR and nSR, and between each day from baseline, respectively.}
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of SWS on each day, with baseline as control, revealed that sleep-restricted spatial animals had an increase in SWS (Dunnett’s LSD = 2.87, *P < 0.05) but that non–sleep-restricted spatial animals did not (Dunnett’s LSD = 2.54, *P > 0.05). However, both sleep-restricted and non–sleep-restricted cue animals showed an increase in SWS amounts (Dunnett’s LSD = 2.54, *P < 0.05 and Dunnett’s LSD = 2.88, respectively, *P < 0.05; Table 1), although in the non–sleep-restricted animals SWS was significantly higher in days 2 and 3 but not day 1. This effect may be caused by a slightly lower amount of SWS in non–sleep-restricted cue animals at baseline. In consequence, Tukey’s post hoc comparisons of sleep-restricted versus non–sleep-restricted animals yielded a significant difference in SWS amounts between sleep-restricted and non–sleep-restricted spatial animals (*q = 2.31, *P < 0.05), an effect that was absent in cue animals.

Spectral power of the delta band within SWS was calculated for each hour from the end of sleep deprivation, normalized to baseline value at the same time-point, and averaged across days. A repeated-measures ANOVA, with hourly values of the delta power as the repeated measures variable, yielded an increase in power in sleep-restricted animals, mainly in the first 2 h of sleep (spatial: $F(1,15) = 5.67, P = 0.050$; cue: $F(1,15) = 10.59, P = 0.017$; Fig. 1C). Paired post hoc comparisons to determine the differences between sleep-restricted and non–sleep-restricted groups, as well as difference from baseline, were applied. These analyses revealed that both cue and spatial sleep-restricted animals had increased delta power in SWS (*q = 2.98, *P < 0.05) compared with baseline, and sleep-restricted groups differed from non–sleep-restricted groups during the first 2 h of sleep after sleep deprivation (*q = 2.31, *P < 0.05).

To assess whether sleep quality during the first 6 h of the light phase (ZT 0–6) was affected by performance in the water maze, the latency to reach the platform was correlated with the amounts of SWS and PS during the following 6 h of sleep in the non–sleep-restricted training groups on each day (i.e., each animal contributed 3 data points). Figure 2 shows that SWS was correlated with latency to reach the platform in non–sleep-restricted cue animals ($R^2 = 0.21, t = -2.40, P = 0.025$; Fig. 2B) but not in non–sleep-restricted spatial animals ($R^2 = 0.01, t = 0.50, P = 0.961$; Fig. 2A). This difference was associated with a higher proportion of SWS in the cue group compared with the spatial group (*t(7) = 3.643, *P = 0.008; Fig. 2B, inset). Conversely, the proportion of recording time spent in PS was significantly correlated with performance of the non–sleep-restricted spatial ($R^2 = 0.19, t = -2.58, P = 0.016$; Fig. 2C), but not non–sleep-restricted cue ($R^2 = 0.008, t = 0.43, P = 0.67$; Fig. 2D). This difference was associated with a higher proportion of PS in the spatial group compared with cue (*t(7) = -2.362, *P = 0.050; Fig. 2D, inset).

**Effects of sleep restriction on learning and performance in the Morris water maze**

Performance was assessed by determining the latency for each animal to find the platform on each trial, wherein the shorter the latency the better the performance. The difference in task difficulty between the two learning tasks was apparent from the first training session, whereby cue animals located the platform faster than spatial animals ($F(1,36) = 8.32, *P = 0.007$; Fig. 3A). Because of the apparent difference in task difficulty, the effects of sleep restriction on performance were assessed for each group separately.

Repeated-measures ANOVA on mean daily latency values yielded opposite effects on spatial and cue learning groups (Fig. 3B). Sleep-restricted spatial animals had higher latencies than the non–sleep-restricted spatial animals ($F(1,38) = 4.01, *P = 0.050$). However, sleep restriction enhanced performance of the sleep-restricted cue animals, resulting in shorter latencies in this group ($F(1,36) = 4.5, *P = 0.045$). A similar analysis performed on path length revealed that path length did not differ between the sleep-restricted and non–sleep-restricted animals (spatial: $F(1,38) = 0.08, P = 0.408$; cue: $F(1,36) = 1.07, P = 0.334$). However, in both groups, path length declined across days (spatial: $F(1,38) = 22.27, P < 0.001$; cue: $F(1,36) = 4.44, P = 0.019$; Table 2). This suggests that animals that were better at locating the platform swam a bit slower.

An ANOVA on the r values derived from regression analyses performed on the latency values of each animal showed that spatial animals had steeper learning curves than the cue animals ($F(1,45) = 10.84, *P = 0.002$). There was a significant interaction between task type and sleep group ($F(1,45) = 5.16, *P = 0.028$), wherein sleep-restricted spatial animals had steeper learning curves than non–sleep-restricted spatial animals, while the opposite was true for the cue animals. However, pairwise Bonferroni comparisons within learning condition did not yield significant differences between sleep-restricted and non–sleep-restricted animals. We also tested if the r values were significantly different from zero to determine whether animals in the different groups actually improved over days, using a single group r-test with a predicted mean of zero. This analysis revealed that non–sleep-restricted cue animals did not have a decline in latencies (*t(1) = -1.05, *P = 0.313); this was probably because of the increase in latencies observed on day 4 (Fig. 3B). The three other groups showed decline in latencies indicative of improved performance (sleep-restricted cue: *t(1) = -3.885, *P = 0.003; non–sleep-restricted spatial: *t(1) = -3.885, *P = 0.003).

### Table 1. Percent recording time spent in SWS during the last 6 h of the last phase

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cue nSR</td>
<td>60.0 ± 7%</td>
<td>65.5 ± 6% *</td>
<td>68.4 ± 7% *</td>
<td>66.8 ± 3% *</td>
</tr>
<tr>
<td>Cue SR</td>
<td>64.6 ± 6%</td>
<td>72.2 ± 6%</td>
<td>73.6 ± 4% †</td>
<td>75.4 ± 3% †</td>
</tr>
<tr>
<td>Spatial nSR</td>
<td>65.5 ± 5%</td>
<td>63.7 ± 2%</td>
<td>62.3 ± 7%</td>
<td>64.5 ± 3%</td>
</tr>
<tr>
<td>Spatial SR</td>
<td>62.4 ± 9%</td>
<td>75.6 ± 6% †</td>
<td>74.1 ± 4% †</td>
<td>77.6 ± 3% †</td>
</tr>
</tbody>
</table>

Values are means and SD of percent slow-wave sleep of total recording time during the latter half of the light phase, after animals were released from sleep deprivation. Repeated-measures ANOVA yielded significantly more SWS in sleep-restricted (SR) animals ($F(1,45) = 18.12, *P = 0.007$). Dunnett’s test, with baseline as control, revealed that SR spatial animals had increased amounts of SWS. In the cue animals, both SR and non–sleep-restricted (nSR) groups had increased SWS amounts compared to baseline. *P < 0.05 for paired comparisons with baseline. †Significant differences between SR and nSR.
We hypothesized that non–sleep-restricted animals may preferentially use spatial information for navigation even when presented with varying platform locations, relying on prior experience to return to old and irrelevant locations of the platform, and hence increasing their search time. Thus for each animal, each trial was scored depending on whether the rat repeatedly returned to certain locations in the maze (Fig. 3D) or searched in an apparent random fashion (Fig. 3C). t-tests showed that, regardless of learning condition (spatial or cue), non–sleep-restricted animals tended to use landmarks more frequently than animals that were sleep-restricted \( t(16) = 1.74, P = 0.05 \); spatial: \( t(16) = 1.75, P = 0.05 \); Fig. 3E).

Effects of sleep restriction on cell survival and cell fate in the hippocampus

Animals were injected with BrdU 13 days before training and sleep restriction. BrdU is only incorporated into the cells during the DNA replication phase (S-phase, \( \sim 3 \) h) of the mitotic cycle (Cameran and Mckay 2001). Assuming asymmetrical division, daughter cells that maintain their mitotic capacity will continue to divide, diluting out the BrdU immunosignal. Thus most cells expressing BrdU immunoreactivity are surviving cells that did not further divide after BrdU administration; cells that coexpress BrdU and DCX are surviving cells that matured into neurons.

Unbiased stereology was used to assess the effects of learning experience and sleep restriction on newborn cell survival within the ROI (Fig. 4A). No main effect of learning condition was found (\( \chi^2(1) = 0.923, P = 0.337 \)). Sleep restriction, however, decreased the total number of BrdU cells (\( \chi^2(1) = 4.33, P = 0.033 \); Fig. 4, B and C). This effect was most pronounced in the spatial group where the non–sleep-restricted animals had the highest BrdU cell count compared with all other groups (Tukey HSD; \( q = 3.202, P = 0.05 \); Fig. 4G).

To assess cell fate, a separate set of tissue sections were evaluated for the proportion of BrdU-labeled cells double-labeled for DCX (Fig. 4, D–F). Kruskal-Wallis test, performed on the percent of DCX cells, corrected for volume (estimated by stereology), yielded significant differences between the four groups (\( \chi^2(3) = 7.82, P = 0.05 \)). Hsu MSB test, used to determine the source of this effect, revealed that non–sleep-restricted spatial animals had the highest proportion of DCX+ cells, whereas all other groups did not differ from each other (critical \( d = 2.42 \), \( P < 0.05 \); Fig. 4H).

FIG. 2. Relationship between performance and sleep patterns during the first 6 h of the light phase in nSR animals. A: spatial—proportion of recording spent in SWS (ordinate) was not correlated with the number of seconds to reach the platform (\( R^2 = 0.01, t = -0.50, P = 0.961 \)). B: SWS was correlated with latency to reach the platform in nSR cue animals (\( R^2 = 0.21, t = -2.40, P = 0.025 \)). Insert: cue animals had a higher proportion of SWS compared with spatial animals (\( P = 0.008 \)). Proportion of recording time spent in PS was significantly correlated with performance of the nSR spatial (C: \( R^2 = 0.19, t = -2.58, P = 0.016 \)) but not in nSR cue (D: \( R^2 = 0.008, t = 0.43, P = 0.67 \)). Insert: higher proportion of PS in the spatial group compared with cue (\( P = 0.050 \). * Statistically significant difference, \( P < 0.05 \).
Effects of sleep restriction on corticosterone levels

A separate group of 10 animals were sleep deprived using the enrichment method for 6 h and compared with controls that were undisturbed during this time (n = 5 in each group). Sleep-deprived animals had higher levels of CORT (t(11) = 3.14, P = 0.007, data not shown) compared with non–sleep-deprived animals.

Discussion

In this study, we assessed the effects of repeated sleep restriction on hippocampus learning-induced neurogenesis. As expected, cell survival and the proportion of cells with neural fate were higher in animals that were trained in the hippocampus-dependent task; sleep-restricted animals were impaired in spatial learning. We also found that sleep restriction, by enrichment, eliminated the neurogenic effects of learning. These findings lend further support to the notion that selective activation of hippocampal networks is an important factor in neurogenesis in this region. Our findings further suggest that the waking experience per se is only one aspect of plasticity and that postlearning sleep has an important contribution to experience-induced neurogenesis.

TABLE 2. Effects of sleep restriction on the distance rats swam to reach platform

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>P Value Days 2–4</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Cue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nSR</td>
<td>176 ± 62.2</td>
<td>105.3 ± 58.3</td>
<td>78.5 ± 29.4</td>
<td>72.3 ± 36.6</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>164.4 ± 62.5</td>
<td>79.9 ± 23.6</td>
<td>78.6 ± 51.6</td>
<td>54.3 ± 24.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spatial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nSR</td>
<td>230 ± 57.8</td>
<td>136.8 ± 37.2</td>
<td>88.1 ± 39</td>
<td>83 ± 34.3</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>196.5 ± 52.6</td>
<td>155.6 ± 74.9</td>
<td>93.1 ± 55.3</td>
<td>90.5 ± 46.2</td>
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</table>

Mean distance from entry point to the platform and SD, in centimeter declined with days in each of the learning groups (P values in far left column). Sleep restriction did not affect the overall distance the animals swam. Day 1 was not included in the statistical analysis as sleep restriction started subsequent to the training on day 1.

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The specificity of the behavioral outcome of sleep restriction observed in this study is similar to findings in rodents that show a specific role for sleep, especially PS, in spatial learning (Guan et al. 2004; Peigneux et al. 2004; Smith and Rose 1996; Youngblood et al. 1997); contextual fear-conditioning (Ruskin et al. 2004); and reference but not working memory in an eight-arm radial maze (Smith et al. 1998). Studies in human subjects have shown that SWS in particular may play a role in the consolidation of declarative/explicit memory (Gais et al. 2000; Huber et al. 2004; Plihal and Born 1997, 1999).

In this study, the cue task tapped into implicit memory resources, because the odor and sight of the platform were paired with “safety,” whereas the spatial task tapped into declarative/explicit memory resources, because rats needed to access spatial maps of the training environment to locate the platform (Morris et al. 1982; Olton and Papas 1979; White and McDonald 2002). We found better performance (shorter latencies) in the cue task was correlated with increased amounts of SWS; in agreement with previous studies (Smith and Rose 1997; Smith and Wong 1991), better performance in the spatial task was correlated with increased amount of PS (Fig. 2).

### TABLE 3. Effects of training paradigm and sleep restriction on linear fit of the learning curve

<table>
<thead>
<tr>
<th>Spatial nSR</th>
<th>Spatial SR</th>
<th>Cue nSR</th>
<th>Cue SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-0.39 \pm 0.12^*$</td>
<td>$-0.27 \pm 0.07^*$</td>
<td>$-0.07 \pm 0.07$</td>
<td>$-0.21 \pm 0.06^*$</td>
</tr>
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Mean $r$ and SD derived from regression analysis performed on latencies values from days 2–4 from each animal. ANOVA yielded significant differences between spatial and cue animals, indicating “spatial” animals had a steeper learning curve ($F_{(1,42)} = 10.84, P = 0.002$). A significant interaction of learning group by sleep condition ($F_{(1,42)} = 5.16, P = 0.028$) indicated steeper curves for the non-sleep restricted (nSR) “Spatial” group compared with SR “Spatial” animals, while the reverse was true for the “Cue” animals. The mean $r$ of each group was analyzed with a single group $t$-test, with 0 as hypothesized mean, to determine whether each group was displaying improved performance across days. All but the non-sleep restricted “Cue” animals displayed improvement. $^*$ denotes mean $r$ values significantly different than zero.

The specificity of the behavioral outcome of sleep restriction observed in this study is similar to findings in rodents that show a specific role for sleep, especially PS, in spatial learning (Guan et al. 2004; Peigneux et al. 2004; Smith and Rose 1996; Youngblood et al. 1997); contextual fear-conditioning (Ruskin et al. 2004); and reference but not working memory in an eight-arm radial maze (Smith et al. 1998). Studies in human subjects have shown that SWS in particular may play a role in the consolidation of declarative/explicit memory (Gais et al. 2000; Huber et al. 2004; Plihal and Born 1997, 1999).

In this study, the cue task tapped into implicit memory resources, because the odor and sight of the platform were paired with “safety,” whereas the spatial task tapped into declarative/explicit memory resources, because rats needed to access spatial maps of the training environment to locate the platform (Morris et al. 1982; Olton and Papas 1979; White and McDonald 2002). We found better performance (shorter latencies) in the cue task was correlated with increased amounts of SWS; in agreement with previous studies (Smith and Rose 1997; Smith and Wong 1991), better performance in the spatial task was correlated with increased amount of PS (Fig. 2).
However, as the landmark analysis suggests, animals in both learning groups applied spatial memory to navigate the maze, although spatial maps were irrelevant information in the cue task and interfered with localization of the platform (Fig. 3E). Thus the effects of the sleep restriction were not equivalent in both tasks: spatial sleep-restricted animals, which could not effectively use spatial maps, tended to use a random search strategy that was less effective because the platform was invisible. In the cue group, however, this strategy afforded benefit over animals who searched for the platform in previous locations. These observations are consistent with recent findings by Bjorness et al. (2005) that showed that selective PS deprivation impaired learning of a spatial navigation task by altering the behavioral strategies employed by the rats to solve the maze.

In addition, sleep-restricted animals compensated for their lost SWS in the latter half of the rest phase, which may have “protected” processes that support explicit memory consolidation. PS, on the other hand, was not compensated for during this time—thus resulting in impaired spatial learning. It should be noted, however, that polysomnographic recordings were not obtained during the dark phase; thus the possibility that PS was recovered during the active phase cannot be ruled out.

It is interesting to speculate that the impairment of hippocampal function played a role in defining the spatial search strategy that worked best in sleep-restricted animals. Degradation in declarative memory dependent processing may have removed a strongly competing cognitive process (spatial maps) and improved the efficiency of a cue-directed search strategy. This interpretation is supported, in part, by the increase in latencies on day 4 observed in the non–sleep-restricted cue animals, suggesting that repeated exposures to the maze environment reinforced the use of spatial maps over a cue oriented search strategy.

Finally, it should be noted that on day 4 of training, sleep-restricted spatial animals caught up with their non–sleep-restricted counterparts. This suggests that sleep restriction did not eliminate the capacity to acquire and use spatial maps but simply delayed the stabilization of the hippocampal-dependent behavior.

The effects of the learning task on neurogenesis are consistent with prior findings. Gould et al. (1999) showed that spatial but not cue animals had increased survival in the granule cell layer. Similarly, we showed that spatial animals had a significantly higher rate of cell survival than cue animals if they were allowed to sleep after training. Thus our findings confirm that experience that engages the hippocampus can increase neurogenesis (Leuner et al. 2004; Shors et al. 2002). We also observed comparatively high levels of cell survival. A possible explanation for this is the fact that we used female rats, in contrast to other studies that used male rats. Sex differences account for different levels of proliferation (higher in males) and survival (higher in females) (Perfilieva et al. 2001). The effects of sleep restriction on cell survival are complementary to Guzman-Marin et al. (2003), who showed that sleep restriction significantly reduced proliferation in the granule cell layer. Our findings extend this observation to a much milder sleep restriction paradigm and suggest that sleep restriction, and subsequent alterations in the quality of sleep, may also affect survival and fate determination of newborn cells, resulting in a net decrease in neurogenesis. Notably, our method of sleep restriction was such that animals remained spontaneously active through social interaction and stimulation with novel objects. Such an experience is more similar to situations of enriched environment rather than standard methods of sleep deprivation (e.g., automated drum, gentle handling). Environmental enrichment has been shown to augment, rather than attenuate, neurogenesis in the hippocampus (e.g., Kempermann et al. 1997, 1998; Nilsson et al. 1999), underscoring the selectivity of the effects of sleep loss on hippocampus-dependent learning.

It is currently unclear what mechanism underlies the effects of sleep loss on cell fate and survival. Acute sleep deprivation induces increases in CORT levels (Hairston et al. 2001; Meerlo et al. 2002), and the method of sleep restriction used in this study had a similar effect as well. Increased CORT level are known to reduce cell proliferation in the hippocampus (Gould et al. 1992). However, Garcia et al. 2004 have recently shown that newborn neurons expressing DCX do not coexpress glucocorticoid receptors, suggesting that the effects we report are not caused by the stressful effects of sleep restriction. Moreover, both exposure to a water maze (Schaaf et al. 1999) and to a novel environment (Miserendino et al. 2003) increase CORT levels, although both have been shown to increase cell proliferation and survival of hippocampal cells (e.g., Gould et al. 1999; Kempermann et al. 1997). It is unlikely that the stress response will provide a simple resolution to this issue.

An alternate hypothesis would be that posttraining sleep promotes processes that underlie plasticity. For example, on the molecular level, we have recently shown that fibroblast growth-factor-2, which promotes cell survival (Walicke and Baird 1988) and neuronal fate (Palmer et al. 1999), is highly expressed during sleep in brains of animals previously sleep-deprived (Hairston et al. 2003). Similarly, it has been suggested that during posttraining sleep, hippocampal networks that were involved in learning are activated (or reactivated) and thus may further enhance plasticity, including increased survival of new neurons. Evidence of such postlearning reactivation has been shown in rats (Lee and Wilson 2002; Louie and Wilson 2001; Ribeiro et al. 2004; Wilson and McNaughton 1994) and recently in humans (Huber et al. 2004; Peigneux et al. 2004).

Interestingly, most rodent and human studies have shown reactivation during SWS. Conversely, selective sleep deprivation studies have shown that the loss of PS impairs hippocampal function (Bjorness et al. 2005; Davis et al. 2003; Smith and Rose 1996) and that PS amounts selectively increase after learning that involves the hippocampus (Smith and Rose 1997; Smith and Wong 1991). Thus which sleep state contributes the most to memory consolidation remains an open question (for review, see Rauchs et al. 2005).

The findings reported in this study reaffirm the importance of sufficient sleep to neural and behavioral plasticity and provide insight into neural processes that are affected by sleep loss. We chose a sleep restriction paradigm over prolonged total elimination of sleep because this is more similar to the type of sleep restriction adult humans normally incur in a standard work week. The improved performance in the sleep-restricted cue animals compared with their rested counterparts was unexpected and suggests that accumulated fatigue may alter behavioral strategies that an organism uses. This may be significant in human learning as well, and implies that it may...
be possible to optimize the way information is presented to rested versus fatigued individuals to take advantage of the specific neural substrates that are unaffected by sleep loss. That said, while the cognitive impairment may be overcome, our findings indicate that mild, chronic sleep restriction may have long-term deleterious effects on neural function.

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