Sleep and memory

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CHAPTER 2

Sleep deprivation impairs spatial working memory and reduces hippocampal AMPA receptor phosphorylation

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ABSTRACT

Sleep is important for brain function and cognitive performance. Sleep deprivation (SD) may affect subsequent learning capacity and ability to form new memories, particularly in the case of hippocampus-dependent tasks. In the present study we examined whether SD for 6 or 12 hours during the normal resting phase prior to learning affects hippocampus-dependent working memory in mice. In addition, we determined effects of SD on hippocampal glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and their regulatory pathways, which are crucially involved in working memory. After 12 hours of SD, but not yet after 6 hours, spatial working memory in a novel arm recognition task was significantly impaired. This deficit was not likely due to stress since corticosterone (CORT) levels after SD were not significantly different between groups. In parallel with the change in cognitive function, we found that 12 hours of SD significantly reduced hippocampal AMPA receptor phosphorylation at the GluR1-S845 site, which is important for incorporation of the receptors into the membrane. SD did not affect protein levels of cyclic-AMP dependent protein kinase (PKA) or phosphatase calcineurin (CaN), which regulate GluR1 phosphorylation. However, SD did reduce the expression of the scaffolding molecule A-kinase anchoring protein 150 (AKAP150), which binds and partly controls the actions of PKA and CaN. In conclusion, a relatively short SD period during the normal resting phase may affect spatial working memory in mice by reducing hippocampal AMPA receptor function through a change in AKAP150 levels. Together, these findings provide further insight into the possible mechanism of SD-induced hippocampal dysfunction and memory impairment.

INTRODUCTION

Sleep is thought to be a process that facilitates neuronal and synaptic plasticity, which in turn is crucial for brain function and cognitive performance (Benington and Frank, 2003; Tononi and Cirelli, 2006; Meerlo et al., 2009). Numerous studies suggest the importance of post-training sleep for the consolidation and strengthening of memories (Graves et al., 2001; Maquet, 2001; Walker and Stickgold, 2004). However, sleep prior to learning may affect memory processes as well, by determining the capacity of neuronal networks to process new information and the ability to encode new memories. Several studies in rodents have shown that 1-5 days of sleep deprivation (SD) or rapid-eye-movement (REM) SD preceding a learning task affects subsequent behavioral performance (Stern, 1971; Dametto et al., 2002; McDermott et al., 2003; Ruskin et al., 2004; Silva et al., 2004; Alvarenga et al., 2008; Ruskin and LaHoste, 2008; Tiba et al., 2008). Recent studies in humans show that even a single night of SD or mild sleep disruption impairs acquisition and memory encoding (Yoo et al., 2007; Van der Werf et al., 2009). With the exception of the latter studies in humans and few studies in rodents (Guan et al., 2004), most experiments on sleep loss and subsequent learning applied relatively long, multiple day SD. There is a shortage of data on SD durations that are more relevant to what happens in everyday life, particularly in animal models that can be applied for detailed studies on molecular and neuronal mechanisms underlying the changes in learning.

SD prior to a learning task may affect acquisition in several ways, not only through a general decrease in alertness or motivation, but also through more specific effects on brain regions and neuronal substrates involved in memory processes. The finding that some tasks and brain regions are more easily affected by SD indeed suggests that disruption of more specific mechanisms play a role as well. SD is known to particularly affect the formation of memories that require the hippocampus. For example, in rats, multiple days of REM SD prior to acquisition impair hippocampus-dependent contextual fear conditioning but not amygdala-mediated tone-cued fear conditioning (McDermott et al., 2003; Ruskin et al., 2004). Also, in humans, a single night of sleep disruption attenuates hippocampal activation and subsequent memory in a hippocampus-dependent declarative task, while it has no effect on a hippocampus-independent memory task (Van der Werf et al., 2009). Importantly, whereas many studies show that SD prior to a learning task affects hippocampus-dependent memory tested later on, it remains uncertain in most cases whether this is due to impaired acquisition and memory encoding or due to impaired memory consolidation.

One potential mechanism through which SD might directly affect acquisition in hippocampus-dependent tasks is by decreasing the function and efficacy of the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. These receptors consist of different subunits (GluR1-GluR4) assembled in various combinations (Hollmann and Heinemann, 1994). Several studies have shown that GluR1-containing AMPA receptors are critically involved in working memory. Mice lacking the GluR1 subunit show impaired spatial working memory (Reisel et al., 2002; Bannerman et al., 2003; Sanderson et al., 2007), and genetic rescue of the GluR1 deficiency leads to restoration of working memory (Schmitt et al., 2005). A recent study shows that AMPA receptor function is reduced by multiple days of REM SD (Ravassard et al., 2009), but data on effects of SD

are inconsistent (McDermott et al., 2006; Vyazovskiy et al. 2008).

In the present study, we examined the effects of relatively brief periods of 6 or 12 hours of SD during the normal resting phase on hippocampus-dependent spatial working memory in mice. In addition, we examined the effects of these SD periods on AMPA receptor GluR1 protein levels and phosphorylation state in the hippocampus as a potential mechanism underlying changes in working memory. We further determined effects of short SD on the intracellular elements that regulate AMPA receptor phosphorylation at the GluR1 site, i.e., the expression of the cAMP-dependent protein kinase A (PKA) and the calcium-dependent protein phosphatase calcineurin (CaN; Banke et al., 2000), as well as the expression of the scaffolding molecule A-kinase anchoring protein 150 (AKAP150), which binds and partly controls the actions of PKA and CaN (Coghlan et al., 1995; Bauman et al., 2004).

METHODS

Animals and housing conditions

Ten-week-old male C57Bl/6J mice (Harlan, Horst, The Netherlands) were individually housed in standard macrolon cages with ad libitum water and food. Animals were maintained on a 12-hour light/12-hour dark cycle (lights on at 7.00 a.m.) and a room temperature of 21 ± 1°C. A layer of sawdust served as bedding. The procedures described in the present study were approved by the Dutch Animal Experiment Committee of the University of Groningen in compliance with Dutch law and regulations.

Experiment set-up

In a first experiment we assessed the effect of brief SD on spatial working memory using a novel arm recognition task (Etkin et al., 2006). In the second experiment, we examined the effect of SD on hippocampal AMPA receptor GluR1 protein levels and phosphorylation, as well as effects on PKA, CaN, and AKAP150 protein levels. In experiment 2, we also assessed the number of stimuli required to keep the animals awake, and at the end of the SD period blood was collected to assess plasma levels of corticosterone (CORT). In both experiments, three groups of mice were used: a control group; a group subjected to 6 hours of SD; and a group subjected to 12 hours of SD. For both SD groups, the SD was performed during the light phase and ended shortly before lights-off. Experiment 1: n = 8, 10, and 10, respectively; Experiment 2: n = 8 in each group. In the first experiment, one outlier in the 12-h SD group was excluded from the analysis because it displayed much lower activity than all other animals. In the second experiment, one animal in the 6-h SD group was not included because of failed brain processing.

Sleep deprivation

During the SD period, mice were continuously observed. SD was accomplished by mild stimulation, which consisted of a standardized procedure of 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). This procedure is effective in keeping rodents awake for several hours, as established by electroencephalic recordings (Meerlo and Turek, 2001; Meerlo et al., 2001), without being a major stressor (Meerlo and Turek, 2001; Van der Borght et al., 2006).

Novel arm recognition task

In the first experiment, the effect of 6 and 12 hours of SD on hippocampus-dependent spatial working memory was assessed by using a novel arm recognition task (Etkin et al., 2006). The task was performed at the end of the 6 or 12 hour SD period, during the last 2 hours of the light phase. The actual duration of SD in the 12-h SD group ranged from 10 to 12 hours, and in the 6-h SD group from 5 to 6 hours. To perform the task for all animals as close to the end of the light phase as possible, the experiment was performed with two identical mazes in parallel and animals were divided over two consecutive days. Animals from different experimental groups were mixed for test day, test order and test set-up. Tubular, transparent Plexiglas Y-mazes were used, consisting of three interconnected arms forming the Y. All three arms were 5 cm in diameter, 27.5 cm long, and at a 120^{o} angle from each other. The experimental room contained various distal spatial cues and light intensity was similar to that in the home cage room (about 50 lux). Mice were extensively habituated during the 5 days preceding the experiment. Twice a day at random times during the light phase they were transported to the test room, picked up and handled for 1 minute. The actual task consisted of an exploration and test session, separated by a brief interval of 2 minutes during which the mice stayed in their home cage. Each session started by placing the mice in the centre of the maze. During the exploration session, which lasted 10 minutes, the animals were allowed to freely explore two arms of the maze while access to the third arm was blocked. During the 5-minute test session, all three arms were accessible. The maze was cleaned with damped paper towel and dried with dry paper towel between all trials and subjects. This task is based on the innate explorative behavior of rodents. If the animals have good spatial working memory, they will spend more time in the novel arm relative to the previously explored arms. Behavior during the sessions was recorded on videotapes, which were analyzed afterwards for the number of entries and time spent in each arm. The experimenter was blind for treatment during the analyses of the tapes. An arm entry was defined as four paws in an arm. Exploration ratios were calculated, being the time spent in the novel arm relative to the average time spent in the previously explored arms, according to the following formula: (time in novel arm)/(time in familiar arms/2)*100%. A value of 100% indicates no arm preference.

Processing of brain material and Western blotting

In the second experiment, we examined whether 6 or 12 hours of SD affects hippocampal AMPA receptor GluR1 protein levels and phosphorylation. Mice were sacrificed at the end of the light phase immediately after the SD period. A group of undisturbed home cage control mice was sacrificed in parallel. They were deeply anesthetized with a mixture of CO_2 and O_2, followed by quick removal and dissection of the brain. Hippocampi were dissected, snap-frozen in liquid nitrogen, and stored at -80ºC until further processing for Western blot analysis. Subcellular fractionation for Western blotting was performed as previously described (Smith et al., 2006). Hippocampal homogenates were prepared

using a pellet pestle (Sigma-Aldrich, St. Louis, MO, USA) in ice-cold homogenization buffer (in mM: Tris base, 10, pH 7.6; sucrose, 320; NaCl, 150; EDTA, 5; EGTA, 5; benzamidine, 1; 4-(2-aminoethyl) benzenesulfonyl fluoride, 1; NaF, 50) with an inhibitor cocktail (complete Mini EDTA free, Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 1000 g for 10 minutes to remove nuclei and large debris. The supernatant was centrifuged at 10 000 g for 15 minutes resulting in a new, final supernatant (cytosol fraction) and a synaptosomal fraction (membrane fraction). The pellet was resuspended in homogenization buffer. Protein concentrations of the cytosol fraction and membrane fraction were determined using the method of Bradford (Bradford, 1976). Samples were diluted using homogenization buffer. Sodium dodecyl sulphate (SDS) buffer (50% glycerin, 321.5 mM Tris/HCl pH 6.8, 10% SDS, 25% β-mercaptoethanol, 0.1% bromophenol blue) was added, followed by 5 minutes heat denaturation at 95°C. Subsequently, the samples were aliquoted and stored at -80°C until further processing.

Equal concentrations of protein were resolved in 10% SDS-polyacrylamide gels, blotted electrophoretically to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer [0.1% Tween-20, 0.2% I-block, Tropix, Bedford, MA, USA, in phosphate buffered saline (PBS), pH 7.4] at 5 °C. Separate membranes were incubated with primary antibodies for proteins of interest overnight in buffer (containing 0.05% Tween-20, 0.1% I-block, Tropix, MA, USA, in PBS). The following antibodies were used: GluR1, phospho GluR1-Serine 845, PKA-RIIα,β subunit (Upstate, Charlottesville, VA, USA), CaN catalytic subunit (Sigma-Aldrich, St. Louis, MO, USA), and AKAP150 C-terminal (Santa Cruz, Santa Cruz, CA, USA). In each case, actin was used as a loading control (MP Biomedicals, Solon, OH, USA). After rinsing with blocking buffer, membranes were incubated with the proper alkaline-phosphatase-conjugated secondary antibodies (Tropix, Bedford, MA, USA; Santa Cruz, Santa Cruz, CA, USA) in PBS (containing 0.05% Tween-20, 0.1% I-block; Tropix, Bedford, MA, USA) for 30 minutes at room temperature. Following rinsing with blocking buffer, membranes were rinsed in assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, pH 10.0) for 2 x 5 minutes at room temperature. For chemoluminescent labeling, membranes were incubated with Nitroblock II (1 : 40; Tropix, Bedford, MA, USA) in assay buffer, rinsed with assay buffer, and finally incubated with CDP star substrate (1 : 1000; Tropix, Bedford, MA, USA) in assay buffer for 5 minutes at room temperature. The immunoreactive bands were captured on autoradiography film (Kodak X scientific image film, Rochester, NY, USA). Densitometric scans of the immunoreactive bands were digitized, and grey levels and surface area of each individual band were measured using a Quantimet 500 image analysis system (Leica, Cambridge, UK). Integrated optical densities (IOD) were calculated by multiplication of the values for grey level and surface area.

Blood sampling and hormone plasma levels

To examine the effect of 6 and 12 hours of SD on plasma CORT levels, trunk blood was collected at the end of the SD period in the second experiment. Blood was collected in pre-cooled plastic centrifuge tubes containing 0.01% ethylenediaminetetraacetic acid (EDTA) as anticoagulant and antioxidant. Blood was centrifuged at 4ºC for 15 minutes at 2600 g in a precooled centrifuge. Plasma was stored at -80ºC until further processing. CORT levels were determined by radio-immuno-assay

(MP Biomedicals, Orangeburg, NY, USA).

Statistical analysis

Effects of SD on all relevant parameters were analyzed using one-way analyses of variance (ANOVAs). When appropriate, *post hoc* comparisons were made with a Tukey test. Additionally, for within-group analysis of arm preference in the novel arm recognition task, one-sample t-tests were applied. A Pearson correlation was used to determine the relationship between AMPA receptor GluR1 phosphorylation and protein levels of membrane-associated AKAP150. All data in text and figures are expressed as mean ± S.E.M. $p < 0.05$ was considered as significant.

RESULTS

Mild stimulation and plasma corticosterone levels

Mice were kept awake by means of mild stimulation. Initially, the animals required little stimulation and spent a big part of their time exploring their cage. During the last hours of SD, much of their behavior consisted of rearranging their nest after disturbance, and increasing attempts to curl up and sleep. The number of interventions needed to keep mice awake increased during ongoing SD, indicating an increased drive for sleep (Fig. 1). CORT levels appeared to be slightly elevated in the 6-h SD group (16.3 ± 1.1 μg/dl, n = 7) and 12-h SD group (16.3 ± 2.1 μg/dl, n = 8) compared with the control group (11.9 ± 2.0 μg/dl, n = 8). However, while ANOVA shows an overall treatment effect ($F_{2,20} = 4.072$, $p = 0.033$), *post hoc* Tukey tests did not reveal significant differences between any of the three groups ($p > 0.05$). Overall, the behavior and CORT levels do not indicate that the mice were particularly stressed or anxious.

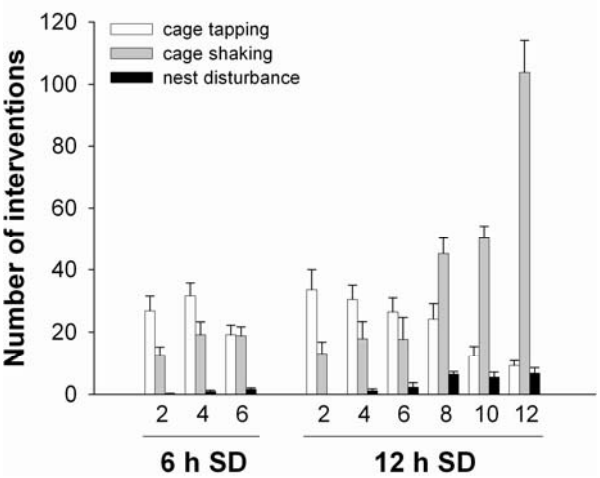

Figure 1. Effects of sleep deprivation (SD) on sleep drive. Two groups of mice were deprived of sleep for 6 hours (6 h SD, n = 7) or 12 hours (12 h SD, n = 8) by tapping on the cage, carefully shaking the cage or disturbing the sleeping nests. The number of interventions that was needed to keep the animals awake increased in the course of the SD period indicating an increased drive for sleep.

Short sleep deprivation impairs spatial working memory

To test whether 6 or 12 hours of SD affects subsequent spatial working memory, mice were subjected to a novel arm recognition task immediately after the SD period, at the end of the light phase. SD had no effect on the total number of arm entries, neither during the exploration phase with two arms of the maze accessible ($F_{2,24}$ = 0.282, p > 0.2; Fig 2A) nor during the test phase with three arms available ($F_{2,24}$ = 0.125, p > 0.2, data not shown). This indicates that overall activity during the task was not affected by prior SD. Also, none of the groups displayed an arm preference during the initial exploration session of the task. Both control and SD mice spent similar times exploring the two available arms (one sample t-tests, p > 0.2 for all groups). In contrast, control animals showed a clear preference for the novel arm during the test phase. These animals spent significantly more time exploring the novel arm relative to the previously visited arms (128.2 ± 7.9%; one-sample t-test, p = 0.009), while both the 6-h SD and 12-h SD groups showed no difference in time spent in the new arm compared with the average time spent in the previous visited arms (106.0 ± 5.0% and 97.7 ± 7.8%, respectively, p > 0.2 for both). In line with this, ANOVA revealed a significant main treatment effect for the exploration ratio during the test session ($F_{2,24}$ = 4.974, p = 0.016; Fig. 2B). *Post hoc* comparisons indicated that the exploration ratio in the 12-h SD group was significantly reduced compared with controls (Tukey test, p = 0.014; Fig. 2B). In the 6h-SD group the difference with controls nearly reached statistical significance (Tukey test, p = 0.088).

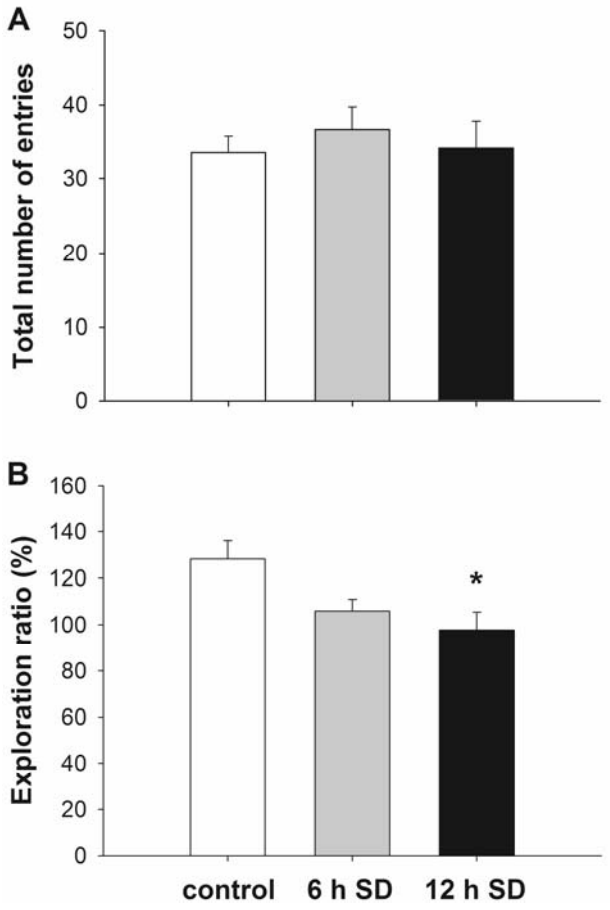

Figure 2. *Effects of sleep deprivation (SD) prior to acquisition on spatial working memory in a novel arm recognition task. (A) SD did not lead to differences in general activity levels indicated by the total amount of arm entries during the exploration session. (B) Twelve hours of SD (n = 9) lead to a significant reduction in novel arm exploration ratio compared with controls (n = 8), while the effect of 6 hours of SD (n = 10) did not reach statistical significance. Exploration ratio was calculated as the percentage of time spent in the novel arm relative to the average time spent in the familiar arms. A 100% level indicates no arm preference. Data are expressed as mean ± S.E.M. * p < 0.05.*

Sleep deprivation reduces hippocampal AMPA GluR1 phosphorylation

In the second experiment, we examined effects of SD on the GluR1 subunit of the AMPA receptor, which plays a pivotal role in hippocampus-dependent working memory (Reisel et al., 2002; Bannerman et al., 2003; Sanderson et al., 2007). We particularly assessed whether 6 and 12 hours of SD affect phosphorylation levels of the serine 845 (S845) site on the GluR1 subunit as this site is thought to be important for the incorporation of GluR1-containing AMPA receptors into the membrane (Banke et al., 2000; Esteban et al., 2003). Fig. 3A shows representative bands for GluR1 and GluR1-S845p. The IODs of the immunoreactive bands for GluR1 and GluR1-S845p are shown in Fig. 3B and 3C, respectively. Six or 12 hours of SD did not change the overall levels of the hippocampal GluR1 protein (ANOVA, $F_{2,20} = 0.157$, $p > 0.2$; Fig. 3B), but it did affect the phosphorylation state of the GluR1-S845 site (ANOVA, $F_{2,20} = 3.961$, $p = 0.036$; Fig. 3C). After 6 hours of SD, S845 phosphorylation levels were reduced but not significantly different from controls yet (67.8 ± 7.1%, $p = 0.163$). Twelve hours of SD resulted in a further and significant reduction of S845 phosphorylation levels (55.0 ± 12.0%, $p = 0.032$).

No changes in hippocampal PKA or CaN protein levels

The phosphorylation state of the GluR1 subunit is largely determined by the opposing actions of PKA and CaN (Banke et al., 2000). PKA-mediated phosphorylation of GluR1-S845 is thought to promote membrane incorporation (Banke et al., 2000; Esteban et al., 2003), while CaN-mediated dephosphorylation initiates endocytosis of GluR1-containing AMPA receptors (Lee et al., 1998; Carroll et al., 1999; Lee et al., 2000; Lin et al., 2000; Smith et al., 2006). Therefore, we investigated whether the reduction in hippocampal S845 phosphorylation levels after SD were paralleled by changes in protein levels of PKA and CaN. Protein levels of the regulatory subunit II of PKA (PKA RII) in both membrane fraction (6 h SD: 113.1 ± 22.6%; 12 h SD: 112.5 ± 24.0% of control levels; ANOVA: $F_{2,20} = 0.142$, $p > 0.2$) and cytosol fraction (6 h SD: 87.6 ± 12.5%; 12 h SD: 77.8 ± 12.1% of control levels; ANOVA: $F_{2,20} = 0.702$, $p > 0.2$) were not significantly affected by SD. Similarly, CaN protein levels were not changed by SD, neither in the membrane fraction (6 h SD: 100.2 ± 9.3%; 12 h SD: 120.3 ± 12.5% of control levels; ANOVA: $F_{2,20} = 1.347$, $p > 0.2$) nor in the cytosol fraction (6 h SD: 102.4 ± 15.1%; 12 h SD: 121.8 ± 15.6% of control levels; ANOVA: $F_{2,20} = 0.614$, $p > 0.2$).

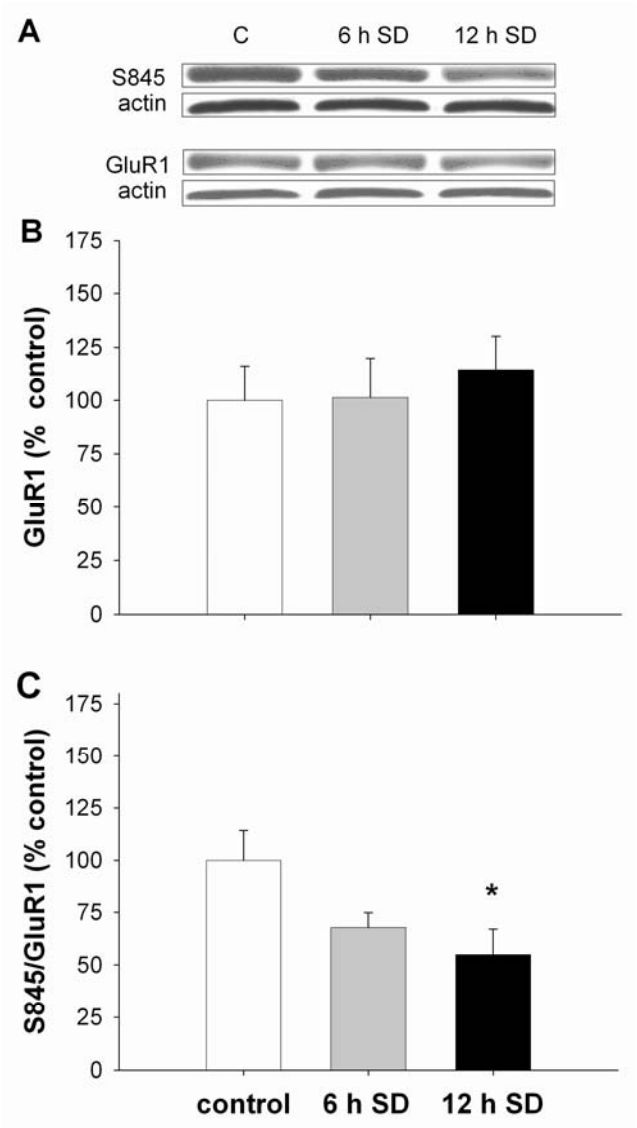

Figure 3. *Effects of sleep deprivation (SD) on hippocampal AMPA receptor GluR1 subunit protein level and phosphorylation state in the membrane fraction. (A) Representative immunoreactive bands for GluR1 and S845 phosphorylation in the membrane fraction. (B) Both 6 hours of SD (n = 7) and 12 hours of SD (n = 8) do not change GluR1 protein levels compared with controls (n = 8). (C) However, 12 hours of SD does significantly decrease phosphorylation at the GluR1-S845 site. Data are expressed as mean ± S.E.M. * p < 0.05.*

Short sleep deprivation reduces levels of AKAP150

The actions of PKA and CaN are partly controlled by binding to the scaffolding molecule AKAP150, which targets both proteins to the membrane (Carr et al., 1992; Klauck et al 1996; Colledge et al., 2000; Gomez et al., 2002). Because AKAP150 is strongly expressed in the mouse hippocampus (Ostroveanu et al., 2007) and plays an important role in AMPA receptor phosphorylation (Tunquist et al., 2008), we investigated whether the SD-induced reduction in GluR1-S845 phosphorylation was accompanied by a reduction in AKAP150 protein levels in the membrane fraction. Representative bands for membrane-AKAP150 and actin are shown in Fig. 4A. ANOVA revealed a main treatment effect on hippocampal AKAP150 protein levels ($F_{2,20}$ = 4.817, p = 0.020). *Post hoc* comparisons indicated that 6 hours of SD resulted in a non-significant decrease of AKAP150 protein levels in the membrane fraction (83.8 ± 10.8%, p > 0.2). Twelve hours of SD further reduced membrane-bound AKAP150 to a level that was significantly different from controls (50.3 ± 9.6%, p = 0.017; Fig. 4B). Furthermore, changes in protein levels of membrane-associated AKAP150 were positively correlated

with the changes in S845 phosphorylation (R^2 = 0.36, p = 0.003). Protein levels of AKAP150 in the cytosol fraction were not different between the groups (ANOVA $F_{2,20}$ = 0.938, p > 0.2; Fig. 4C).

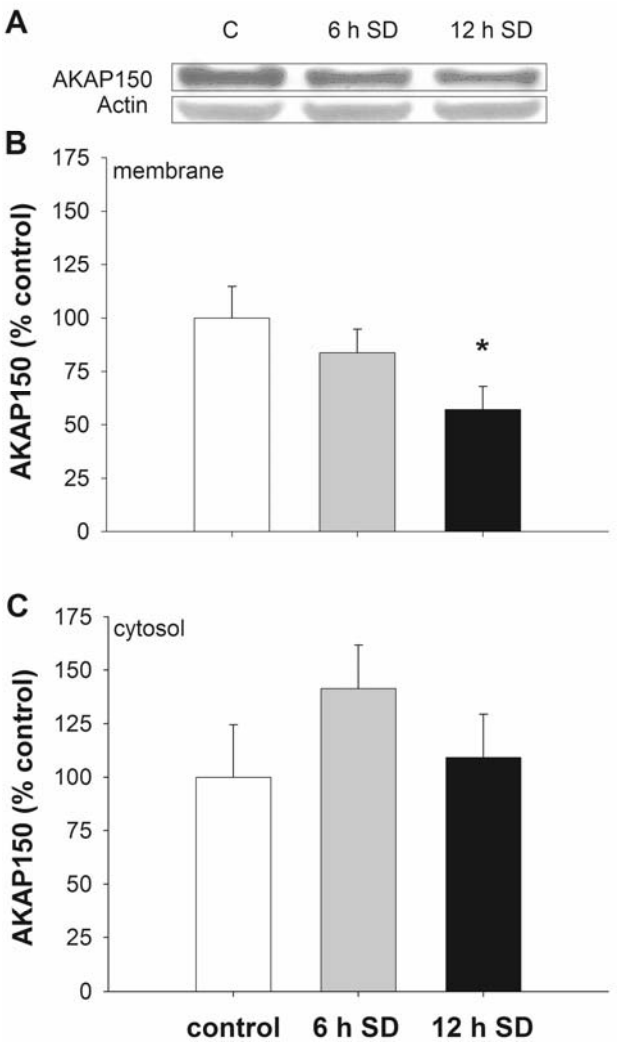

Figure 4. *Effects of sleep deprivation (SD) on hippocampal A-kinase anchoring protein 150 (AKAP150) protein levels in the membrane and cytosol fractions. (A) Representative immunoreactive bands for AKAP150 and actin. (B) Twelve hours of SD significantly decreases hippocampal AKAP150 protein levels in the membrane fraction. (C) However, it does not significantly affect hippocampal AKAP 150 protein levels in the cytosol fraction. Data are expressed as mean ± S.E.M. * p < 0.05.*

DISCUSSION

In the present study, we examined whether 6 or 12 hours of SD in mice affects hippocampal function at the behavioral and cellular level. SD caused an impairment of spatial working memory in a novel arm recognition task, which reached statistical significance after 12 hours. In line with this change in cognitive function, we found that 12 hours of SD leads to a significant reduction in hippocampal AMPA receptor phosphorylation at the GluR1-S845 site and a reduction in levels of the scaffolding protein AKAP150. These data suggest that SD may affect spatial working memory by reducing hippocampal AMPA receptor function through a decrease in AKAP150 protein levels.

SD may have affected performance in the novel arm recognition task in several ways, including a reduction in activity and motivation because of fatigue. However, even after 12 hours of

SD, the mice appeared sufficiently aroused when they were placed in the novel maze environment. Indeed, the total number of arm entries during the exploration and test session was not different between SD mice and controls, indicating that the impaired behavioral performance in SD mice was not likely due to reduced motivation or explorative activity.

It is often argued that memory deficits found after SD are due to stress and stress hormones, particularly in studies with animals, which necessarily involve some sort of enforced wakefulness. Although one may never be able to fully separate effects of sleep loss and stress (Meerlo et al., 2008), it does not seem likely that stress induced by our SD method caused the changes in working memory and hippocampal AMPA receptor phosphorylation. On average, plasma levels of the stress hormone CORT were slightly elevated in SD mice, yet neither of the two SD groups was significantly different from the control group. Also, other studies using longer and more stressful methods of SD prior to learning showed that experimentally blocking the glucocorticoid stress response did not prevent SD-induced memory impairments (Ruskin et al., 2006; Tiba et al., 2008). Moreover, several studies show that low levels of CORT may have positive rather than negative effects on hippocampal AMPA receptor function (Clark and Cotman, 1992; Karst and Joëls, 2005).

Our data suggest a cumulative effect of SD on both spatial working memory and AMPA receptors. After 6 hours of SD, performance in the novel arm recognition task and AMPA GluR1 phosphorylation were both reduced but this change had not yet reached statistical significance. Twelve hours of SD lead to a further and significant reduction in both behavioral performance and AMPA receptor GluR1 phosphorylation. Thus, somewhere between 6 and 12 hours the effects of SD reached a critical point where it noticeably impaired spatial working memory and its molecular substrates.

Most studies in laboratory animals that examined effects of SD prior to a learning task used relatively long, multiple-day periods of SD or REM SD (Stern, 1971; Dametto et al., 2002; McDermott et al., 2003; Ruskin et al., 2004; Silva et al., 2004; Alvarenga et al., 2008; Ruskin and LaHoste, 2008; Tiba et al., 2008; Ravassard et al., 2009). Here we show in mice that a relatively short period of total SD is sufficient to affect memory processes. These findings are in line with recent studies in humans showing that a single night of SD or even mild sleep disruption reduces hippocampal activity and subsequent memory encoding (Yoo et al. 2007; Van der Werf et al., 2009).

Given the parallel decrease in spatial working memory and hippocampal AMPA receptor phosphorylation in our study, we propose that the two may be related. Particularly, the deficit in novel arm recognition may in part have been caused by the reduction in AMPA receptor phosphorylation at the GluR1 subunit. Several studies show that the AMPA GluR1 subunit is critically involved in spatial working memory (Reisel et al., 2002; Bannerman et al., 2003; Sanderson et al., 2007). The GluR1 subunit has different phosphorylation sites, including the S845 site, which is thought to be important for the incorporation of receptors into the membrane (Banke et al., 2000; Esteban et al., 2003). To the opposite, dephosphorylation of S845, such as occurs with long-term depression (LTD), is associated with endocytosis of GluR1-containing AMPA receptors from the membrane (Carroll et al., 1999; Beattie et al., 2000; Lee et al., 2000). Therefore, the finding of a reduction in GluR1-S845 phosphorylation after SD suggests a reduced incorporation of GluR1-containing AMPA receptors in

the membrane. Our findings are in agreement with a recent study in rats showing that multiple days of REM SD cause a reduction in AMPA receptor function, including reduced phosphorylation at the S845 site (Ravassard et al., 2009). In contrast, one other recent study in mice reported an increase in GluR1 expression and phosphorylation after 6 hours of SD (Vyazovskiy et al., 2008). One difference is that the latter study performed Western Blot analysis specifically on post-synaptic density fractions whereas the other two used membrane fractions and whole homogenates that did not differentiate between neurons and glia. Therefore, because hippocampal glia cells may express GluR1 (Fan et al., 1999), the reduction in hippocampal GluR1 may have occurred in neurons as well as glia cells. It cannot be excluded that these procedural differences have affected the outcome of the studies; however, whether it explains the opposite results needs to be determined. It is worth noting that recent studies indicate that glia cells may be involved and important for sleep regulation and cognitive function (Halassa et al., 2009).

Although SD reduced AMPA GluR1 phosphorylation in the hippocampus, we did not find changes in the PKA and CaN protein levels that might have explained this reduction. Instead, the decrease in GluR1 phosphorylation may have been related to changes in the intracellular distribution and activity of PKA and CaN rather than a change in the overall level. The latter might be due to alterations in levels of the scaffolding molecule AKAP150, which binds, targets and controls PKA and CaN activity (Coghlan et al., 1995; Colledge et al., 2000; Tavalin et al., 2002; Bauman et al., 2004). Indeed, it has been shown that LTD leads to loss of AKAP150 in the synapse (Gomez et al., 2002; Smith et al., 2006), accompanied by S845 dephosphorylation and endocytosis of AMPA receptors (Lee et al., 1998; Beattie et al., 2000; Lee et al., 2000; Smith et al., 2006). In addition, experimental disruption of PKA anchoring to AKAP150 leads to removal of AMPA receptors from the cell surface in a way similar to what is seen during LTD (Snyder et al., 2005). Therefore, an important outcome of the present study is the finding that SD reduces hippocampal AKAP150 levels in the membrane fraction, a reduction that was correlated to the decrease in AMPA GluR1 phosphorylation. Thus, despite the fact that PKA and CaN protein levels were not significantly changed by SD, the reduction of membrane-bound AKAP150 levels may be sufficient to explain the reduction of S845 phosphorylation.

In conclusion, our study shows that a relatively short period of SD is sufficient to impair spatial working memory in mice. Such a change might be related to a decrease in glutamate AMPA receptor function, one of the established molecular substrates of working memory. Indeed, our data show that SD reduces hippocampal AMPA receptor phoshorylation levels at the GluR1-S845 site, which is important for incorporation of the receptor into the membrane. Our data further suggest that this reduction may be the result of a decrease in membrane-bound AKAP150 protein, which regulates GluR1 phosphorylation by controlling the opposing actions of PKA and CaN. Together, these findings provide further insight into the possible mechanism of SD-induced hippocampal dysfunction and memory impairment.

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