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Sleep deprivation induces hippocampal AMPA receptor dephosphorylation: a possible role of A-kinase anchoring protein (AKAP)-150

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Chapter 6

Abstract

It is well established that sleep loss has adverse effects on cognitive function and memory formation and in particular, hippocampus-dependent memory. However, the neurobiological correlates of a sleep deprivation are not well understood. Here, we examined the effect of 6h or 12h sleep deprivation (SD) on hippocampal AMPA receptor functioning in adult mice. The results show that: 1) sleep loss decreases AMPA receptor phosphorylation at the glutamate receptor 1 (GluR1) serine 845 site (S845); 2) SD did not affect membrane associated protein levels of the cyclic-AMP dependent protein kinase (PKA) or phosphatase calcineurin (CaN); 3) sleep loss decreased protein levels of the A-kinase anchoring protein 150 (AKAP150). The reduction in S845 phosphorylation induced by loss of sleep is not directly related to changes in PKA or CaN protein levels but, instead, may be related to changes in binding of these molecules by AKAP150 and targeting to their substrates. Because PKA activity is targeted to the GluR1 subunit by AKAP150, the SD-mediated decrease in AKAP150 protein levels could therefore diminish PKA dependent S845 phosphorylation. At the end of SD, plasma levels of the stress hormone corticosterone were not significantly enhanced suggesting that the reduction in GluR1 phosphorylation is due to sleep loss rather than a non-specific consequence of stress. Together these findings provide further insight into the possible mechanism of hippocampal dysfunction as a consequence of sleep loss.
Introduction

A growing body of evidence shows an important role for sleep in the regulation of neuronal excitability and neuronal plasticity (for review, see Benington and Frank, 2003). Such sleep-mediated neuronal plasticity is thought to be important for proper brain function, in particular for learning and memory formation (for review, see Maquet, 2001; Stickgold and Walker, 2005). Support for sleep-mediated neuronal plasticity facilitating learning and memory comes from studies showing that sleep deprivation (SD) deteriorates subsequent learning (Stern, 1971; McDermott et al., 2003; Ruskin et al., 2004; Yoo et al., 2007). Furthermore, various studies revealed that SD directly after training also affects memory consolidation (Karni et al., 1994; Smith and Rose, 1996; Smith et al., 1998; Palchykova et al., 2006). SD effects are particularly pronounced when such tasks require the hippocampus (Graves et al. 2003; Ruskin et al. 2004). Yet, the neurobiological correlates of the dysfunctional sleep-deprived hippocampus are not well established.

Hippocampal long term potentiation (LTP) and long term depression (LTD) are widely accepted cellular models for neuronal synaptic plasticity (for review, Malenka and Bear, 2004). It is well known that bidirectional synaptic plasticity is crucially dependent on glutamate NMDA and AMPA receptors. Recent studies suggest that total or partial SD lead to impaired LTP via changes in NMDA receptor functioning (Campbell et al., 2002; Davis et al., 2003; McDermott et al., 2003). In contrast, few studies have examined the effects of sleep and sleep loss on AMPA receptor function.

AMPA receptors consist of different subunits (GluR1 to GluR4) assembled in various combinations (Hollmann and Heinemann, 1994). The efficacy of AMPA mediated neurotransmission depends on channel activity and synaptic location, which are regulated by receptor phosphorylation (Malinow and Malenka, 2002; Bredt and Nicoll, 2003). The phosphorylation state of the GluR1 subunit is the result of a balanced activity of the cAMP-dependent protein kinase (PKA) and the calcium-dependent protein phosphatase calcineurin (CaN) (Banke et al., 2000). PKA mediated phosphorylation of AMPA GluR1 Serine 845 (S845) is thought to promote synaptic incorporation and open channel time (Banke et al., 2000; Esteban et al., 2003). In contrast, CaN mediated dephosphorylation has opposite effects and reduces AMPA receptor efficacy (Banke et al., 2000). LTD induces dephosphorylation of S845 through pathways involving CaN and protein phosphatase 1 initiating endocytosis of GluR1 containing AMPA receptors (Lee et al., 1998; Lee et al., 2000; Carroll et al., 1999; Lin et al., 2000; Smith et al., 2006). The actions of PKA and CaN in turn are partly controlled by binding to the scaffolding molecule A-kinase anchoring protein 150 (AKAP150) which targets both proteins to the postsynaptic density (Carr et al., 1992; Coghlan et al., 1995; Klauck et al., 1996; Colledge et al., 2000).

Although several studies have shown that sleep loss has a major impact on hippocampus function, effects that are partly mediated by changes in NMDA receptor function, little is known regarding the effects of sleep loss on hippocampal AMPA receptor function. Therefore, in the present study, we investigated the effects of SD on AMPA receptor GluR1 phosphorylation state in the hippocampus of adult mice.
Materials and methods

Animals and housing conditions
Twenty-three male C57Bl/6J mice (Harlan, Horst, The Netherlands), 10 weeks old were individually housed in standard macrolon cages with ad libitum water and food. Animals were maintained on a 12h light / 12h 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 internal regulations.

Sleep deprivation
SD was accomplished by mild 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. We used this approach because it effectively keeps animals awake without inducing significant stress as measured by plasma levels of glucocorticoids (Van der Borght et al., 2006). The experiment included three groups of animals; a control group (n=8), one group subjected to 6 hours of SD (6 h SD, n=7) and one subjected to twelve hours of SD (12 h SD, n=8). For both of the latter groups, SD ended at the end of the light phase. Control mice were left undisturbed.

Processing of brain material
At the end of the light phase, mice were deeply anesthesized with a mixture of CO2 and O2, followed by quick removal and dissection of the brain. Hippocampi were dissected, snapfrozen in liquid nitrogen, and stored at -80°C until further processing for Western blot analysis.

Western blotting
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 (10mM Tris base pH 7.6, 320mM sucrose, 150mM NaCl, 5mM EDTA, 5mM EGTA, 1mM benzamidine, 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 50 mM NaF) with an inhibitor cocktail (complete Mini EDTA free, Roche Diagnostics, Mannheim, Germany).

Homogenates were centrifuged at 1000 x g for 10 minutes to remove nuclei and large debris. The supernatant was centrifugated at 10,000 x 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 buffer (50% glycerin, 321.5 mM Tris/HCl pH 6.8, 10 % SDS, 25 % ß-mercaptoethanol, 0.1% bromophenol blue) was added followed by 5 min 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, in phosphate buffered saline (PBS, pH 7.4)) at 5°C. Membranes were incubated with combinations of primary antibodies overnight in buffer (containing 0.05% Tween-20, 0.1% I-block, Tropix, MA in PBS). Pilot experiments with single primary antibodies and combinations of primary antibodies did not reveal any signs of crossreactivity. 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
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(containing 0.05 % Tween-20, 0.1 % I-block (Tropix, Bedford, MA, USA) for 30 min at room temperature. Following rinsing with blocking buffer, membranes were rinsed in assay buffer (0.1 M diethanolamine, 1 mM MgCl2, pH 10.0) for 2x5 min 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, grey levels and surface levels 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. To directly correct for variation in protein levels, blots were probed with antibody against actin (MP Biomedicals, Solon, OH, USA) together with one of the following antibodies: phospho GluR1-Serine 845, GluR1, PKA-RIIα,βα subunit (Upstate, Charlottesville, VA, USA), CaN catalytic subunit (Sigma-Aldrich, St. Louis, MO, USA), and AKAP150 C-terminal (Santa Cruz, Santa Cruz, CA, USA).

Statistical analysis
Protein levels of PKA, CaN, AKAP150, GluR1 as well as phosphorylation levels of S845 were analyzed with ANOVA followed by post-hoc Tukey tests. A Pearson correlation was used to analyze the relationship between AMPA receptor GluR1-S845 phosphorylation and protein levels of membrane associated AKAP150. Data are expressed as mean ± S.E.M. in all figures.

Results

Mild stimulation and plasma corticosterone levels
Mice were kept awake by means of mild stimulation. The number of interventions needed to keep mice awake gradually increased during ongoing SD indicating an increased drive for sleep (Fig. 1). Plasma levels of the stress hormone corticosterone were measured at the end of SD. On average, corticosterone levels were slightly elevated after both 6 and 12 hours of SD, but there were no significant differences between the groups (control: 11.9 ± 2.0 µg/dl, 6 h SD: 16.3 ± 1.1 µg/dl, 12 h SD: 16.3 ± 2.1 µg/dl). Therefore, despite an sing number of stimuli needed to keep the mice awake, the animals did not appear to be severely stressed.

Hippocampal PKA and calcineurin protein levels
The phosphorylation state of the S845 site of the AMPA GluR1 subunit is largely determined by the opposing actions of PKA and CaN. 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 (6h SD: 113.1 ± 22.6 %; 12h SD: 112.5 ± 24.0 % of control levels; ANOVA: F<1) and cytosol fraction were not affected by SD (6h SD: 87.6 ± 12.5 %; 12h SD: 77.8 ± 12.1 % of control levels; ANOVA: F<1). Similarly, CaN protein levels were not changed by SD in either membrane fraction (6h SD: 100.2 ± 9.3 %; 12h SD: 120.3 ± 12.5 % of control levels; ANOVA: F<1), or cytosol fraction (6h SD: 102.4 ± 15.1 %; 12h SD: 121.8 ± 15.6 % of control levels; ANOVA: F<1).
Hippocampal AMPA receptor GluR1-S845 phosphorylation
Figure 2A shows representative bands for GluR1-S845p and GluR1. The IODs of the immunoreactive bands for GluR1-S845p and GluR1 are shown in figure 2B and 2C. Six or 12 hours of SD did not change the overall levels of the hippocampal GluR1 protein (Fig. 2B), but it did affect the phosphorylation state of the GluR1-S845 site (Fig. 2C). After 6 hours of SD, S845 phosphorylation levels were slightly (but not yet significantly) reduced compared to controls (67.8 ± 7.1 %, \( P > 0.1 \)). Twelve hours of SD resulted in a further and significant reduction of S845 phosphorylation levels (55.0 ± 12.0 %, \( P < 0.05 \)).

Hippocampal AKAP150 protein levels
Since AKAP150 is strongly expressed in the mouse hippocampus (Ostroveanu et al., 2007) and plays an important role in AMPA receptor phosphorylation by binding and targeting PKA and CaN, we investigated if the SD induced reduction in GluR1 S845 phosphorylation was accompanied by a reduction in AKAP150 protein levels. Representative bands for membrane-AKAP150 and actin are shown in figure 3A. Six hours of SD resulted in a mild but non-significant decrease of AKAP150 protein levels in the membrane fraction (83.8 ± 10.7959 %, \( P > 0.1 \)). Twelve hours of SD resulted in a further reduction in membrane-bound AKAP150 levels (50.3 ± 9.6 %, \( P < 0.05 \), Fig. 3B). Protein levels of AKAP150 in the cytosol fraction were not different between the groups (ANOVA \( F < 1 \), Fig. 3C).
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Discussion

The present study shows that SD affects the AMPA receptor by a reduction in phosphorylation of the GluR1 subunit at the S845 site. The GluR1 S845 phosphorylation state is the result of a balance between PKA induced phosphorylation and CaN-mediated dephosphorylation. Although the levels of PKA and CaN protein were not significantly affected by SD, their activity and combined effect on the GluR1 S845 phosphorylation state may have been altered by changes in the protein levels of the anchoring protein AKAP150. Binding by AKAP150 normally promotes PKA activity targeted to GluR1-containing AMPA receptor resulting in enhanced phosphorylation of S845. Since SD caused a significant reduction in AKAP150 protein at the membrane, this may have diminished targeted activity of PKA and thereby the phosphorylation of the AMPA GluR1 subunit.

The GluR1 subunit has different phosphorylation sites, including the S845 site that is thought to be important for the incorporation of GluR1 containing AMPA receptors into the membrane (Banke et al., 2000; Esteban et al., 2003). To the opposite, dephosphorylation of S845 by CaN such as occurs with LTD is associated with endocytosis of GluR1 containing AMPA receptors from the membrane (Lee et al., 1998; Caroll et al., 1999; Beattie 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, as shown for LTD.

Figure 2. Effects of sleep deprivation on hippocampal AMPA receptor GluR1 subunit protein levels and phosphorylation state. Representative immunoreactive bands for S845 phosphorylation and GluR1 (A). SD does not affect GluR1 protein levels (B), but it does decrease phosphorylation at the GluR1 S845 site (C). * P<0.05
The phosphorylation state of receptors is regulated by opposing actions of protein kinases and protein phosphatases, in case of the AMPA GluR1 S845 site, PKA and CaN. Although S845 phosphorylation in the hippocampus was reduced after SD, we did not find any significant differences in the PKA and CaN protein levels that might have explained this reduction. Therefore, it seems that the decrease in phosphorylation was not directly related to robust changes in the amount of PKA and CaN protein but, rather, to changes in their intracellular distribution and activity. We hypothesized that the latter might be due to alterations in protein levels of AKAP150 which binds, targets and controls PKA and CaN activity. The available literature suggest that PKA activity targeted to the AMPA receptor is promoted by binding to AKAP150 (Colledge et al., 2000; Tavalin et al., 2002). Indeed, it has been shown that NMDA receptor dependent LTD leads to the loss of AKAP150 in the synapse of “young” cultured neurons (Gomez et al., 2002; Smith et al., 2006) accompanied by S845 dephosphorylation and endocytosis of AMPA receptors (Lee et al., 1998; Lee et al., 2000; Beattie 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). In agreement with this, SD resulted in a significant reduction in AKAP150 levels in the membrane fraction. Furthermore, the changes in protein levels of membrane associated AKAP150 were positively correlated with the changes in S845 phosphorylation ($R^2=0.36$, $P=0.005$). This may suggest that the decreased AKAP150 levels may have impaired PKA activity targeted to the AMPA receptor, leading to a decreased phosphorylation level of the S845 site. Thus, despite the fact that PKA and CaN protein levels themselves were not significantly changes by SD, the reduction of membrane bound AKAP150 levels may be sufficient to explain the reduction of S845 phosphorylation levels.
Although the protein levels of AKAP150 at the membrane were decreased after SD, levels in the cytosol were not affected. This may suggest that AKAP150 removed from the membrane is degraded rather than accumulating in the cytosol. Alternatively, it may be that removal from the membrane and translocation to the cytosol is paralleled by a reduction in de novo synthesis.

Exposure to stressful events is known to elevate corticosterone levels and modulate fast excitatory amino acid mediated synaptic plasticity (for review, see Alfarez et al., 2006). Kopp and co-workers (2006) recently suggested that effects of SD on glutamate receptor function reported in some studies may have been confounded by stress and elevated levels of glucocorticoids independent of SD. Although the number of interventions needed to keep the animals awake increased towards the end of SD, plasma levels of the stress hormone corticosterone were not significantly elevated. The absolute corticosterone levels measured after six and twelve hours of SD were close to normal circadian peak levels and well below the levels found after stress (e.g. Meerlo and Turek, 2001). Furthermore, although it is commonly assumed that glucocorticoids have adverse effects, several studies suggest that they may have a positive rather than a negative effect on hippocampal AMPA receptor function (Clark and Cotman, 1992; Karst and Joels, 2005). The reduction in AMPA GluR1 phosphorylation reported here is therefore most likely due to insufficient sleep rather than stress or glucocorticoids.

Most studies investigating the role of sleep in the regulation of synaptic plasticity and glutamate receptor function so far focused on the NMDA type glutamate receptor. The effects of sleep and sleep loss on AMPA receptor function have received scarce attention. Here, we show that SD reduces AMPA receptor phosphorylation at the GluR1 S845 site and in parallel decreases AKAP150 protein levels at the membrane as has been described previously for LTD. These findings provide new insight in the mechanisms by which sleep loss affects the hippocampus and they may further explain how insufficient sleep can lead to hippocampal dysfunctioning and deteriorated memory formation and consolidation.

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Author contributions

R.H., E.A.V.d.Z. and P.M. generated the hypotheses and designed the experiments. R.H., R.H., A.N., J.N.K. and P.M. carried out the experiments. R.H. wrote the manuscript with input from E.A.V.d.Z. and P.M.

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