Neurobiological and functional consequences of chronic partial sleep deprivation
Roman, Viktor

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Sleep restriction by forced activity reduces hippocampal cell proliferation

Viktor Román, Karin Van der Borght, Susan A. Leemburg,
Eddy A. Van der Zee, Peter Meerlo

Department of Molecular Neurobiology, University of Groningen,
P.O. Box 14, 9750 AA Haren, The Netherlands

Brain Research (2005) 1065:53-59
Chapter 8

ABSTRACT

Mounting evidence suggests that sleep loss negatively affects learning and memory processes through disruption of hippocampal function. In the present study, we examined whether sleep loss alters the generation, differentiation, and survival of new cells in the dentate gyrus. Rats were sleep restricted by keeping them awake in slowly rotating drums for 1 day or repeatedly for 20 h/day over a period of 8 days. In addition to home cage controls, we included forced activity controls which, compared to sleep restricted rats, walked at double speed for half the time. These animals thus walked the same distance but had sufficient time to sleep. The results show that a single day of sleep deprivation significantly reduced hippocampal cell proliferation in the hilus of the dentate gyrus as measured by immunostaining for the proliferation marker Ki-67. Repeated partial sleep deprivation reduced cell proliferation in both the hilus and the subgranular zone. However, the latter was also found after chronic forced activity, and may not have been specific for sleep loss. To study neuronal survival, rats received a single intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU) 5 days before the experiment. The number of surviving, BrdU-positive cells was not affected by sleep restriction. Also, the differentiation of BrdU-positive new cells into NeuN-positive neuronal and GFAP-positive glial phenotypes was not significantly altered by sleep loss. In conclusion, since new cells in the hilus mostly differentiate into glia, our findings indicate that sleep loss may reduce hippocampal gliogenesis.
INTRODUCTION

Several lines of evidence suggest that sleep loss may have negative effects on learning and memory processes in humans as well as animals (Graves et al., 2001; Maquet, 2001; Walker and Stickgold, 2004). One brain area that seems particularly sensitive to disruptive effects of sleep loss is the hippocampus, a structure that plays an important role in the formation, storage, and recall of memories. Sleep deprivation alters molecular and electrophysiological properties of hippocampal neurons (McDermott et al., 2003; Marks and Wayner, 2005), and it impairs the performance in hippocampus-dependent learning tasks (Graves et al., 2003; McDermott et al., 2003).

It has been suggested that hippocampal function may in part depend on the generation, differentiation, and integration of new cells in the dentate gyrus (Gould and Tanapat, 1999; Van Praag et al., 1999; Shors et al., 2001). The production and survival of new hippocampal cells is affected by a variety of harmful factors including chronic stress and glucocorticoids (McEwen, 1999; Fuchs et al., 2001; Gould et al., 2001). A recent study also suggests that prolonged total sleep deprivation for 72 h suppresses cell proliferation in the hippocampus, an effect that could not be attributed to stress (Guzman-Marin et al., 2003). The question remains however, what happens to hippocampal cell proliferation under conditions of chronic sleep restriction or repeated partial sleep deprivation, as it more commonly occurs in modern society (Ferrara and De Gennaro, 2001). Also, it is yet unknown how sleep loss affects the survival and phenotypical differentiation of newly produced cells in the dentate gyrus.

In the present study, rats were subjected to a single session of sleep loss (one day of sleep deprivation) or multiple sessions of sleep loss (eight days of partial sleep deprivation). We applied immunohistochemical stainings against Ki-67 (marker of cell proliferation) and BrdU injected 5 days before the experiment (marker of survival of newly produced cells) (Scholzen and Gerdes, 2000; Cameron and McKay, 2001; Kee et al., 2002). In order to test whether the fate of newly generated hippocampal cells is altered by sleep loss, we performed triple immunohistochemical stainings for BrdU together with the neuronal marker NeuN and the glia cell marker GFAP.

MATERIALS AND METHODS

Animals and housing
In the present study, we used 42 adult male Wistar rats bred at the local animal facility of the University of Groningen, Haren, The Netherlands. The rats were ± 350 g at the beginning of the experiments. Animals were housed under a 12 h light/12 h dark cycle, with lights on at 09.00 h. The average temperature of the room was 21 ± 1°C. In both experiments, rats were provided with bedding material, and received food and water ad libitum. Experiments were approved by the Animal Experimentation Committee of the University of Groningen.

Experiment I: one day of sleep deprivation
In this experiment, 18 rats were used. Six rats were sleep deprived for 24 h starting at 09.00 h in slowly rotating drums (40 cm in diameter) driven by an engine at constant speed (0.4 m/min). Since the sleep deprivation procedure includes mild forced locomotion, we used forced activity controls in order to see whether the effects of sleep deprivation are due to forced activity or sleep loss per se (Roman et al., 2005). Six rats were subjected to a schedule of forced activity by placing them in rotating drums similar to the ones used for sleep deprivation, however, these wheels rotated at double speed (0.8 m/min) for half the time (12 h).
Therefore, the rats in the forced activity group walked the same distance as the sleep deprived rats, but had sufficient time to sleep. The 12 h of forced locomotion was divided in 2-h blocks evenly distributed during the 24 h corresponding to the 24-h sleep deprivation protocol, and ended at the same time as sleep deprivation. Six rats served as home cage controls.

Experiment II: eight days of partial sleep deprivation
In this experiment, 24 rats were used. Eight rats were subjected to a protocol of repeated partial sleep deprivation for 8 days allowing them to sleep in their home cage for 4 h per day from 09.00-13.00 h, at the beginning of the light phase (Roman et al., 2005). The remainder of the time, animals were kept awake by forced locomotion in slowly rotating drums (see experiment I). Eight animals were placed in similar drums and served as forced activity controls (Roman et al., 2005). Wheels for activity controls rotated at double speed for half the time (10 h per day). The 10 h of forced activity was divided in five blocks of 2 h separated by 2.5 h of rest. In other words, blocks of forced activity were stretched out over a period of 20 h, corresponding to the 20 h of sleep deprivation in the sleep restricted group. These rats thus walked the same distance as sleep restricted rats, but had sufficient time to sleep (14 h per day). Eight rats served as home cage controls.

BrdU injections
In order to study the survival of newly produced cells in the hippocampus under the two conditions of restricted sleep (single session versus repeated partial sleep deprivation), we labelled proliferating cells with the thymidine analogue BrdU, which incorporates into the DNA during the synthetic phase of the cell cycle. Rats received a single intraperitoneal injection with 300 mg/kg BrdU (20 mg/ml in saline, pH 7.0; Sigma, St. Louis, MO, USA) five days prior to the experiments to generate a pool of BrdU-labelled cells. This concentration of BrdU was chosen because it labels a maximal number of dividing cells without being toxic, while lower doses may label only a fraction of new born cells (Cameron and McKay, 2001). The rats were injected 5 days before the start of the experiment so that the chronically sleep restricted animals would be perfused two weeks after labelling of the cells, when normally up to 40% of new cells in the subgranular zone of the dentate gyrus have reached a mature state and express adult neuronal markers such as NeuN (McDonald and Wojtowicz, 2005; Rao et al., 2005). With this protocol we aimed to establish whether restriction of sleep inhibits or alters the speed of differentiation and fate of new cells.

Perfusions and sectioning
On the last day of the experiments, animals were sacrificed during the light phase, immediately after the end of the sleep deprivation. Under deep pentobarbital anaesthesia, animals were transcardially perfused with physiological saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed from the skull and rinsed overnight in 0.01 M phosphate buffered saline (PBS, pH 7.4). After dehydration in 30% buffered sucrose solution, brains were frozen with liquid nitrogen and stored at -80°C. With a cryostatic microtome, 12 series of 30-μm-sections were cut from the whole hippocampus (between bregma -2.12 and -6.04) (Paxinos and Watson, 1986). Brain sections were stored in 0.01 M PBS containing 0.1% sodium azide until further processing.

Immunohistochemistry
Immunohistochemical staining for the cell proliferation marker Ki-67 was performed using a standard protocol (Van der Borght et al., 2005a). In brief, sections were pretreated with 0.3% H2O2 for 30 min. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Fransisco, CA, USA). Subsequently, sections were incubated for 48 h at 4°C with mouse-anti-Ki-67 primary antibody (1:200, Novocasta, Newcastle upon Tyne, UK). After a second blocking step, the biotinylated secondary antibody (goat-anti-mouse 1:400, Jackson, West Grove, PA, USA) was added for 2 h. This was followed by incubation with Avidin-Biotin-Complex (1:400, ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). Labelled cells were visualized with 0.2 mg/ml diaminobenzidine (DAB) and 0.003% H2O2.

In order to study the survival of newly produced hippocampal cells, brain sections were stained against BrdU injected 5 days before experiments (Van der Borght et al., 2005a). The procedure started with a DNA denaturation step. Sections were incubated for 2 h at 65°C in 2 Malcolm saline citrate (2 x SSC) containing 50% formamide. After successive rinses with 2 x SSC, 2 M HCl (37°C for 30 min) and 0.1 M borate buffer (pH 8.5), sections were exposed to the rat-anti-BrdU primary antibody (1:800, Oxford Biotechnology, Oxfordshire, UK) overnight at 4°C. As secondary antibody, biotinylated donkey-anti-rat antibody was used (1:400, Jackson). Then, sections were incubated in streptavidin-horseradish-peroxidase (1:200, Zymed) for 2 h at room temperature, rinsed, and reacted with DAB and H2O2.

In the rats subjected to chronic partial sleep deprivation or forced activity, we applied triple immunolabelling for BrdU, the glia marker GFAP, and the neuronal marker NeuN to establish the fate and differentiation of newly labelled cells (Van der Borght et al., 2005b). The triple labelling was preceded by a DNA denaturing procedure as described above. Then, sections were incubated for 72 h in a solution containing the following primary antibodies: rat-anti-BrdU (1:200, Oxford Biotechnologies), rabbit-anti-GFAP (1:400, DAKO, Denmark), and mouse-anti-NeuN (1:400, Chemicon, Temecula, USA). Thereafter, the following secondary antibodies were applied: biotinylated donkey-anti-rat, Cy5-conjugated donkey-anti-rabbit, and Rhodamine Red-conjugated donkey-anti-mouse (F(ab') fragments (1:200, all from Jackson). The BrdU staining was visualized by incubation in Fluorescein (DTAF)-conjugated streptavidin (1:200, Jackson). After the stainings, the sections were mounted onto glass slides for microscopic analysis.
Analysis

For the analysis of the Ki-67 and BrdU immunohistochemistry, we focussed on the subgranular zone and the hilus of the dentate gyrus. The subgranular zone has long been recognized as one of the main sites of adult mammalian neurogenesis (Cameron and McKay, 2001). The hilus was chosen because it is an area within the dentate gyrus containing proliferating cells that possibly develop into glia (Niquet et al., 1994), which are important for proper functioning of the hippocampus (Schousboe et al., 2004). Other hippocampal structures such as the CA1-CA3 subfields contained very few scattered Ki-67-positive or BrdU-labelled cells per section and were not included in quantification. The Ki-67 and BrdU-labelled cells were counted in the subgranular zone and the hilus at 40-times magnification in 12 sections per animal. Care was taken, that labelled cells were counted throughout the entire thickness of the section along the whole anteroposterior extent of the hippocampus. The approximate estimation of labelled cells in the subgranular zone and the hilus was calculated from the total number of counted cells per rat multiplied by 12 (Van der Borght et al., 2005a).

Triple-labelled sections were examined in a multi-track analysis by using a LSM 510 META confocal laser and scanning microscope (Zeiss, Jena, Germany). Digital photographs of the subgranular zone of the dentate gyrus were taken at 30 consecutive focal planes with a thickness of 1 μm each. Then, these photographs were examined with the program LSM Image Browser (Zeiss), and the number of BrdU+/NeuN+/GFAP− (i.e. new neurons), BrdU+/NeuN−/GFAP+ (i.e. new glia), and BrdU+/NeuN−/GFAP− (i.e. new cells of unknown phenotype) cells was counted in three sections for each animal. Approximately 20 labelled cells per animal (from 3 photographs of the dorsal hippocampus in each animal) were phenotypically classified. For statistical analysis, one-way ANOVA and post hoc Tukey tests were used. The level of significance was set at P<0.05.

RESULTS

In order to determine the effects of sleep deprivation on cell proliferation in the hippocampus, brain sections were immunostained for Ki-67 (Fig. 1A). After one day of sleep deprivation or forced activity, the number of Ki-67-positive cells in the subgranular zone was not significantly affected (Fig. 2A). However, ANOVA revealed a significant treatment effect for the number of proliferating cells in the hilus (F(2,13)=9.901, p=0.002). This was caused by a significant decrease of Ki-67-positive cells in the sleep deprived animals compared to both home cage controls and the forced activity controls (Fig. 2B). After 8 days, the number of Ki-67-positive cells was significantly decreased in both the subgranular zone (F(2,20)=10.942, p=0.002) and the hilus (F(2,20)=4.192, p=0.039) (Fig. 2C and 2D). In the subgranular zone, this decrease occurred in sleep restricted and forced activity rats as well as in animals subjected to the chronic forced activity schedule (Fig. 2C). In the hilus, this decrease in cell proliferation was only significant in the sleep deprived animals (Fig. 2D).

To examine the survival of newly generated hippocampal cells, brain sections were immunostained for BrdU that was injected and incorporated 5 days prior to the experiment (Fig. 1B). Counts of BrdU-positive cells showed that a single day or repeated partial sleep deprivation did not significantly affect survival of newly generated cells in the subgranular zone and in the hilus (Fig. 3).

In order to follow the differentiation of newly produced cells in the subgranular zone, triple fluorescent immunostaining for BrdU, the neuronal marker NeuN, and the glial marker GFAP was applied. The analysis of triple-labelled sections showed that most of the newly formed cells had an astrocytic phenotype (approx. 60%). The proportion of subgranular zone cells differentiating into neurons was slightly higher in sleep restricted and forced activity rats compared to home cage controls but the difference was not significant (F(2,10)=1.368, p=0.308) (Table 1).
Figure 1. Representative photomicrographs of the Ki-67 and BrdU immunohistochemical stainings in the dentate gyrus. Cells were counted in the hilus and the subgranular zone (SGZ) of the granular cell layer. The arrows point at Ki-67 or BrdU-immunopositive cells. Scale bar: 50 μm.

Table 1. Phenotypical distribution of newly produced cells in the subgranular zone after eight days of repeated partial sleep deprivation. Brain sections were stained for BrdU, NeuN, and GFAP using triple fluorescent immunohistochemistry. Digital photomicrographs of the sections were examined in a multi-track analysis to reveal co-localization of markers. The distribution of the different phenotypes was not significantly altered by the experimental treatment. Data are represented as average percentages ± SEM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NeuN+/GFAP⁺</th>
<th>NeuN+/GFAP⁻</th>
<th>NeuN'/GFAP⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Home cage</td>
<td>13.5 ± 6.3</td>
<td>68.5 ± 7.9</td>
<td>17.9 ± 8.1</td>
</tr>
<tr>
<td>Forced activity</td>
<td>22.8 ± 5.6</td>
<td>58.9 ± 4.9</td>
<td>18.2 ± 4.8</td>
</tr>
<tr>
<td>Sleep restriction</td>
<td>26.7 ± 4.2</td>
<td>61.4 ± 6.5</td>
<td>13.3 ± 3.2</td>
</tr>
</tbody>
</table>

DISCUSSION

In the present study, acute sleep deprivation for a single day significantly reduced cell proliferation in the hilus of the dentate gyrus as measured by Ki-67 immunostaining. After chronic partial sleep deprivation for eight days, cell proliferation was reduced in both the hilus and the subgranular zone. Sleep loss did not significantly affect the survival of new cells that were labelled with BrdU shortly before the sleep restriction protocol, and it also did not appear to affect the differentiation of newly produced cells.

Sleep deprivation in this study was achieved by placing the animals in slowly rotating drums. To establish whether the effects of sleep restriction could be due to forced locomotion, we included additional control groups that, compared to the sleep restricted animals, were forced to walk at double speed for half the time. These animals thus covered the same distance but had sufficient time to sleep. In this group of animals, there was no change in cell proliferation in the
hilus, but eight days of forced activity reduced the number of new cells in the subgranular zone to a similar extent as sleep restriction. Thus, while the reduction in hilar cell proliferation appears to be specific for sleep loss, we cannot exclude that the suppression of cell proliferation in the subgranular zone was partly due to the forced locomotor activity associated with the sleep deprivation.

**Figure 2.** Ki-67-positive cell numbers after one day of sleep deprivation and eight days of repeated partial sleep deprivation in the subgranular zone and the hilus of the dentate gyrus. [A, B] A single session of sleep deprivation did not significantly affect cell proliferation in the subgranular zone, but significantly decreased the number of new cells in the hilus. [C] Repeated partial sleep deprivation and forced activity significantly reduced cell proliferation in the subgranular zone. [D] Repeated partial sleep deprivation significantly reduced cell proliferation in the hilus. *P<0.05; post hoc Tukey test after one-way ANOVA.

Reduced hippocampal cell proliferation after sleep deprivation has been shown in one other study (Guzman-Marin *et al.*, 2003). This paper reported a reduction of cell proliferation in rats after 72 h of total sleep deprivation but, it was not specified whether the decrease in cell divisions occurred in the subgranular zone or in the hilar region of the dentate gyrus. In the present study, one day of sleep deprivation did not significantly decrease cell proliferation in the subgranular zone as it did in the hilus. However, the two areas together still showed a significant decrease in proliferation (data not shown). The two studies may thus be similar in that sleep deprivation primarily reduces cell proliferation in the hilus of the dentate gyrus. On the other hand, it may be that in the study of Guzman-Marin *et al.* (2003) cell proliferation also was reduced in the subgranular zone, perhaps because rats were subjected to a more strenuous protocol of prolonged total sleep deprivation for 72h. In other words, it may be that acute and partial sleep deprivation
initially reduce hilar cell proliferation but prolonged total sleep deprivation may eventually decrease proliferation in the subgranular zone as well.

The two locations, that is the subgranular zone and the hilus, represent different populations of dividing cells. Whereas many cells produced in the subgranular zone migrate a short distance in the granular cell layer and differentiate into granular neurons (Seri et al., 2001; McDonald and Wojtowicz, 2005), newly produced cells in the hilus give rise mainly to new astrocytes (Niquet et al., 1994). Thus, the finding of the present study that sleep loss reduces cell proliferation in the hilus suggests a reduction in gliogenesis (Steiner et al., 2004). Consequently, sleep loss-induced reductions in the production of new astroglia may ultimately limitate glial functions, such as the release of growth factors (Takuma et al., 2004) or glutamate buffering (Schousboe et al., 2004), both important processes for proper functioning of the dentate gyrus and the hippocampus.

The fact that in our study there are baseline differences in hilar cell proliferation between the single session and the repeated partial sleep deprivation experiment (Figs 2B and 2D) needs some consideration. The two experiments were done in two separate sets of animals. Therefore, batch or even minor age differences might explain the deviations in cell proliferation between the two experiments. However, since we used appropriate control groups in both experiments, the differences are not likely to bias the interpretation of the data.

Figure 3. BrdU-positive cell numbers after one day of sleep deprivation [A, B] and after eight days of repeated partial sleep deprivation [C, D] in the subgranular zone and the hilus of the dentate gyrus. Single or repeated sessions of sleep deprivation and forced activity did not significantly affect cell survival as measured by BrdU-positive cell numbers.

90
Although cell proliferation was reduced by sleep deprivation, the survival or phenotypical differentiation of new cells in the dentate gyrus was not significantly altered. If anything, there was a tendency for a reduction in the survival of newly generated cells that had been labelled shortly before the experiment in the animals subjected to the chronic forced activity protocol (P=0.083). This finding appears to be opposite to the positive effects of exercise on neurogenesis reported in mice (Van Praag et al., 1999) and rats (Trejo et al., 2001; Persson et al., 2004). These earlier studies showing increased neurogenesis used voluntary running in contrast to our protocol which is based on forced locomotion. Thus, this apparent contradiction between our present findings and the exercise literature might be explained by the nature of physical activity applied. Perhaps forced exercise has opposite effects because it is stressful (Roman et al., 2005). Indeed, stress has been shown to have adverse effects on cell proliferation and neurogenesis (Gould et al., 1999; McEwen, 1999; Fuchs et al., 2001).

In conclusion, this study shows that both acute and repeated partial sleep deprivation have detrimental effects on hippocampal cell proliferation, particularly in the hilus. However, restricted sleep does not appear to have a major effect on the survival and phenotypical differentiation of newly generated hippocampal cells.