Hippocampal cell proliferation across the day: Increase by running wheel activity, but no effect of sleep and wakefulness

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Behavioural Brain Research (2005), in press
Abstract

The present study investigated whether proliferation of hippocampal progenitors is subject to circadian modulation. Mice were perfused using 3-h intervals throughout the light-dark cycle and brains were stained for Ki-67. Since Ki-67 is not expressed during the G0 phase of the cell cycle, we expected a decline in Ki-67 expression at the moment cells synchronously exit the cell cycle. However, despite the fact that various hippocampal factors fluctuate across the day, the number of dividing cells remained constant. In a second experiment, we studied whether disturbance of normal sleep affected the stable rate in cell proliferation. Our data show that twelve hours of sleep deprivation during the light phase did not influence proliferating cell number. A third experiment investigated whether physical activity, a condition known to enhance hippocampal cell proliferation, caused an elevation of the steady baseline number of proliferating progenitors, or a peak directly following the active phase of the animals. Mice were housed with a running wheel for 9 days. On the last day, animals were sacrificed either directly before or directly after the active phase. Exercise significantly promoted cell proliferation and this effect appeared to be strongest directly after the active period and to disappear during the resting phase. Our data suggest that hippocampal cell proliferation is not synchronized under basal conditions and is unchanged by sleep deprivation. However, running affected cell proliferation differentially at two times of day. These data demonstrate that the steady rate in cell proliferation is not indispensable, but can be changed by behavioral activity.
Introduction

The dentate gyrus of the adult hippocampus contains undifferentiated, rapidly proliferating progenitor cells. Approximately 70-80% of the newly formed cells differentiate into granule neurons, which ultimately fully integrate into the hippocampal network (Cameron et al., 1993; Kaplan and Hinds, 1977; Van Praag et al., 2002). The function of the neurons that are formed during adulthood is unclear, although data suggest that they play a role in the regulation of mood (Kempermann, 2002; Malberg et al., 2000; Santarelli et al., 2003) or in hippocampus-dependent (Gould et al., 1999a; Shors et al., 2001; Snyder et al., 2005) learning and memory. In order to investigate the potential role of newly formed hippocampal granule neurons in normal brain function, the identification of factors that influence neurogenesis may be of crucial importance.

In the present study, we investigated basal levels of hippocampal cell proliferation across the day. For various tissue types, such as oral mucosa, the intestinal mucosa, the kidney or the bone marrow, a partly synchronized cell cycle has been observed (Bjarnason et al., 1999; Burns et al., 1972; Scheving et al., 1978). This means that around the same time of day, a large portion of the dividing cells enters the same phase of the cell cycle. Studies in invertebrates suggest that the generation of new neurons may also fluctuate across the day. Neurogenesis in the olfactory pathway of a certain crustacean (the American lobster, Homarus americanus) shows diurnal variations with a peak in cell production around dusk (Goergen et al., 2002). This is the time of day when these crustaceans display the highest levels of activity. Also for rodents it has been suggested that there is an activity-mediated circadian rhythm in hippocampal cell proliferation (Holmes et al., 2004).

We hypothesized that hippocampal cell proliferation varies across the day-night cycle as a consequence of daily rhythms in physiological parameters and neuroendocrine factors (e.g., neurotrophic factors or glucocorticoid hormones) or by spontaneous day-night differences in behavior (e.g., the daily rhythm in sleep and wakefulness and variations in activity level across the day). In addition, external factors, such as the light-dark cycle, and the consequent daily rhythm in light exposure also may affect hippocampal function and the number of proliferating progenitors.

In order to test this hypothesis, mice were sacrificed throughout the day using 3-h intervals. Brains were stained for the proliferation marker Ki-67. Since Ki-67 is expressed during all phases of the cell cycle, except G0 (Scholzen and Gerdes, 2000), a decline in Ki-67 expression at a certain time of day would be expected at the time that a large number of cells concurrently finish the cell cycle (Bjarnason et al., 1999). We avoided the use of BrdU, since this substance has to be administered via injections, which would arouse the animals and disturb their sleep and activity pattern. Here we show that, despite the fact that many hippocampal processes fluctuate during the day, there is no evidence for synchronized hippocampal cell proliferation under a normal sleep-wake cycle.

Next, we investigated whether the stable rate in proliferating cell number is affected by disturbance of normal sleep or by increased physical activity. To determine the role of sleep in the regulation of hippocampal cell proliferation, we sleep deprived mice by
gentle handling during their normal resting phase and determined the number of proliferating cells in the dentate gyrus. The impact of increased physical activity on the constant generation of new hippocampal cells across the day was studied by housing animals with a running wheel for 9 days. Wheel running has repeatedly been shown to enhance the number of proliferating cells in the hippocampus (Holmes et al., 2004; Van Praag et al., 1999b). Here, we explored whether running wheel activity causes an increase in cell proliferation that is constant across the day or whether increased activity results in an acute peak in proliferating cell number, which declines during the resting phase. In order to answer this question, mice were either sacrificed directly following the active phase or at the end of the resting phase, two time points at which the rate of hippocampal cell proliferation is identical under basal conditions. The results suggest that the stable rate of cell proliferation is not affected by sleep deprivation, but may be enhanced during the active phase, by high levels of physical activity.

Materials and Methods

All experiments were performed with male C57Bl/6 mice (Harlan, Horst, The Netherlands). Animals were individually housed under a 12h light/12h dark cycle and were provided with food and drinking water ad libitum. The procedures concerning animal care and treatment were in accordance with the regulations of the ethical committee for the use of experimental animals of the University of Groningen.

Experiment 1: Spontaneous daily fluctuations in hippocampal cell proliferation
To investigate spontaneous fluctuations in hippocampal cell proliferation throughout the light-dark cycle, 64 12-week-old mice were sacrificed using 3-h intervals (n=5-8 per time point), starting 2.5 h after the beginning of the light period (Zeitgeber Time (ZT) 2.5, with light onset designated as ZT 0). Animals were rapidly anesthetized with CO2. Subsequently, mice were transcardially perfused with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer.

Experiment 2: The effect of sleep deprivation on hippocampal cell proliferation
The effects of sleep on cell proliferation in the dentate gyrus were studied by depriving 6-8-week-old mice (n=7) of sleep for 10-12 h starting at the onset of the light phase, the period during which the mice normally sleep most of the time. Sleep deprivation was accomplished by the 'gentle handling' method, which involved tapping on the cage, gently shaking the cage or, when this was not sufficient to keep the animals awake, disturbing the sleeping nests. Control animals (n=8) were left undisturbed. During the sleep deprivation period, the experimenter recorded number and nature of the interventions that were necessary to keep the animals awake. After 8-10 h of sleep deprivation, mice were injected i.p. with 300 mg/kg BrdU (Sigma, St. Louis, MO, USA) dissolved in saline (20 mg/ml, pH 7.0). Two hours after the BrdU injection, animals were perfused. Prior to perfusion, a
blood sample (50 μl) was taken from the heart. Blood samples were collected in prechilled Eppendorf tubes containing EDTA as anti-coagulant. After centrifugation at 2600 rpm for 15 min, the plasma was collected, and stored at -80°C for later analysis of corticosterone by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA, USA).

Experiment 3: The effect of exercise on hippocampal cell proliferation
To assess whether day-night variations in activity level can induce synchronization of hippocampal cell proliferation, 6-8-week-old mice (n=15) were housed with a running wheel for 9 days to increase physical activity. Control animals (n=16) were housed in a standard cage during this period. On the last day of the exercise period, 8 runners and 8 control animals were sacrificed just before the active phase (ZT 10-12). The remainder of the animals was perfused shortly after the circadian activity phase (ZT 0-2).

Brain processing and immunocytochemistry
Following the perfusions, brains were removed from the skull and kept in 0.01 M PBS overnight. Subsequently, the brain material was cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 48 h and 30 μm sections were cut with a cryostat. Sections were kept in 0.01 M PBS containing 0.1% sodium-azide until further processing.

For both BrdU and Ki-67 immunocytochemistry, every sixth section of the dorsal hippocampus was used. The material for the BrdU immunostaining underwent steps for DNA denaturation (van der Borght et al., 2005a). Sections were placed in a 50% formamide/2XSSC solution at 65°C for 20 min, followed by a rinsing step with 2XSSC. Next, sections were incubated with 0.2 M HCl at 37°C for 30 min and neutralized in a 0.1 M borate buffer solution. Sections for the BrdU as well as for the Ki-67 staining were incubated with 0.3% H2O2 for 30 min. This was followed by 3% normal serum (Jackson Immunolabs, West Grove, PA, USA) and 0.1% Triton-X100 in 0.01 M PBS. The primary antibody (rat-anti-BrdU, 1:800, Oxford Biotechnology, Oxfordshire, UK or rabbit-anti-Ki-67, 1:400, Novocastra, Newcastle upon Tyne, UK) was applied for 48 h at 4°C. After a second blocking step with 3% normal serum and 0.1% Triton-X100, the biotinylated secondary antibody (donkey-anti-rat or goat-anti-rabbit, both 1:400, Jackson Immunolabs, 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) for 2 h. The staining was visualized by adding 0.2 mg/ml diaminobenzidine (DAB) and 0.003% H2O2.

Quantification
BrdU or Ki-67 immunopositive cells were counted in every sixth section of the dorsal hippocampus using a 40x objective. Only cells that were located in the subgranular zone, or one cell diameter deviating from this region, were included. Cells throughout the entire thickness of the section were counted. The number of counted cells was multiplied by 6 to get an estimation of the total cell number in the dorsal dentate gyrus (Holmes et al., 2004; van der Borght et al., 2005b).
Statistics

Circadian fluctuations in Ki-67 positive cell number were analyzed using a one-way ANOVA. Effects of exercise on time-of-day variations in cell proliferation were tested with a two-way ANOVA. The sleep deprivation data were analyzed with an independent samples t-test. All data are presented as mean ± S.E.M.

Figure 1: Hippocampal cell proliferation across the day. A) Groups of mice were sacrificed at 8 time points during the day and brains were stained for Ki-67. The white bar represents the light phase, the dark bar the dark phase. There were no significant differences in proliferating cell number in the subgranular zone between the different time points. B) Photomicrograph showing Ki-67 positive cells in the subgranular zone of the dentate gyrus. Scale bar = 50 μm. C) Magnification of the selected area in the inner blade. Scale bar = 10 μm. GCL: Granule cell layer, H: hilus.
Results

No daily rhythm in hippocampal cell proliferation

In order to determine whether hippocampal cell proliferation shows fluctuations across the day, groups of mice were sacrificed throughout the light-dark cycle, using 3-h intervals. The number of proliferating cells was determined by counting Ki-67 immunoreactive nuclei in
the dentate gyrus. At all time points investigated, the subgranular zone contained between 1500 and 1800 Ki-67 positive cells. There were no significant differences between the different time points that were investigated (Fig. 1A).

No effects of sleep deprivation on hippocampal cell proliferation
The influence of sleep on hippocampal progenitor proliferation was investigated by sleep depriving animals for 10-12 h during the light phase. Although the mice were disturbed more frequently as the sleep deprivation period progressed, plasma levels of the stress hormone corticosterone at the end of the experiment were not elevated above control levels, suggesting that the animals were not seriously stressed (control: 11.0 ± 3.5 μg/dl, sleep deprived: 16.4 ± 4.4 μg/dl, P>0.05). Yet, the number of interventions that was necessary to keep the animals awake gradually increased during the sleep deprivation period, thereby indicating an increasing drive for sleep (Fig. 2A). Despite the accumulated sleep debt, neither the number of Ki-67 positive cells (Fig. 2B) nor the number of cells that had incorporated BrdU (Fig. 2C-E) was changed by the sleep deprivation procedure.

Exercise stimulates cell proliferation, with the largest effect directly after the active period
Animals were housed with a running wheel for 9 days and on the last day they were sacrificed at either ZT 0-2 or at ZT 10-12. Animals from both groups ran on average the same distance over the 9-day exercise period (73.4 ± 7.2 km and 69.6 ± 6.3 km, respectively). Brains were stained for Ki-67 to study the effect of an increased activity level on hippocampal cell proliferation and to determine whether this effect is different directly after the active period compared to directly before the start of the dark phase. The data show that exercise caused a significant increase in cell proliferation in the subgranular zone (Fig. 3, two-way ANOVA, F(1,31)=5.63, P<0.05). There was no statistically significant effect of time of sacrifice or an interaction between exercise and the time of sacrifice. However, when tested separately with an independent-samples t-test, the effect of exercise on cell proliferation was only significant at the ZT 0-2 time point (P<0.05) and not at ZT 10-12.
Discussion

The present study shows that, under standard housing conditions, hippocampal cell proliferation in the mouse does not show a circadian rhythm. In addition, the stable number of proliferating cells in the dentate gyrus is not affected by sleep deprivation during the resting phase of the animal. However, providing the mice with a running wheel significantly promoted hippocampal cell proliferation. This effect was strongest directly following the period of highest activity and seemed to disappear in the course of the resting phase.

Circadian regulation of cell proliferation has been reported for many tissue types (Bjarnason et al., 1999; Burns et al., 1972; Scheving et al., 1978; Smaaland, 1996). A clear example of synchronous circadian cell cycling can be found in the liver. Upon ablation of a large part of the liver, resting hepatocytes enter the cell cycle in order to repair the damaged tissue. However, the time point at which the hepatocytes undergo transition from G2 to mitosis is strictly limited to a specific time of day, independent of the time of day at which the lesion was made (Matsuo et al., 2003). In contrast to these cell types, our data indicate that under standard laboratory conditions proliferation in the hippocampus is not subjected to circadian modulation. This is in line with previous studies, in which BrdU was injected at ZT 6, ZT 12 and ZT 18 (Holmes et al., 2004) or at ZT 1, ZT 7, ZT 13 and ZT 19 (Ambrogini et al., 2002) and no differences in BrdU-positive cell number were observed. Importantly, the lack of a circadian rhythm in cell proliferation after BrdU injections in these studies may have been caused by the fact that the animals were aroused and disturbed by the injections, which could have masked an endogenous rhythm. Therefore, we counted the number of Ki-67 positive cells in the dentate gyrus, which is also a reliable marker for dividing cells (Kee et al., 2002; Scholzen and Gerdes, 2000) and which does not require disturbance of the animals. Since Ki-67 is present throughout all phases of the cell cycle, fluctuations in Ki-67 expression indicate the simultaneous exit of cohorts of cells from the cell cycle. For other tissues, Ki-67 has been shown to be a suitable marker for observing circadian rhythms in cell proliferation (Bjarnason et al., 1999). However, in the hippocampus no differences were observed between the 8 time points investigated, suggesting that under basal conditions hippocampal cell proliferation is constant across the 24-h light-dark cycle.

We further hypothesized that circadian fluctuations at the behavioral level (i.e. the sleep-wake rhythm and an uneven distribution of activity across the day) might influence the number of dividing cells at a certain time of day. To test whether disturbance of the normal sleep-wake pattern of the animals affects the steady daily rate in hippocampal cell proliferation, we sleep-deprived mice by gentle handling during their normal resting phase. The number of interventions that were required to keep the mice awake gradually increased in the course of the sleep deprivation period, illustrating an increasing sleep drive. With a single injection of BrdU, we labeled cells during the last two hours of the sleep deprivation period, but no effect of sleep loss on cell proliferation was observed. The brains were also analyzed for the number of Ki-67 expressing cells, because this method may provide

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information on the cumulative effects of 10-12 h of sleep deprivation. However, despite the rigorous stimulation and the increasing sleep debt, also this staining did not show alterations in the number of dividing cells.

Our data do not confirm the findings reported by Guzmán-Marín and colleagues (2003) showing that sleep deprivation in rats significantly reduced cell proliferation. However, their study shows essential differences compared to the protocol we used. Besides the fact that we performed our experiment with mice instead of rats, the duration of the sleep deprivation period (12 h versus 96 h) and the method that was used to sleep deprive the animals (gentle handling versus the treadmill method) also differed between the two studies. Importantly, in the present experiment, we only counted proliferating cells in the subgranular zone, the brain region that is known to be the place were the neuronal progenitors are located (Alvarez-Buylla and Lim, 2004; Seki, 2002b). The cell counts reported by Guzmán-Marín and colleagues did not distinguish between the the subgranular layer and the hilus, which is known to be a more gliogenic region (Liu et al., 1998b; Picard-Riera et al., 2004; Rietze et al., 2000). Perhaps, the changes in cell proliferation that were reported in rats merely reflect changes in the hilar region and not in the subgranular zone. Indeed, a study in our lab showed that acute sleep deprivation in rats suppressed cell proliferation primarily in the hilus (Roman et al., 2005). From our study we can conclude that the amount of time that mice normally spend sleeping during the light phase is not of crucial importance for the regulation of cytogenesis in the granule cell layer.

In a third experiment, we investigated the impact of increased physical activity on hippocampal cell division. We confirmed previous reports in the literature, which did not take time-of-day into consideration, by showing that 9 days of running wheel activity increased hippocampal cell proliferation. We further investigated whether under conditions of increased voluntary exercise, cell proliferation was constantly enhanced across the day, or whether increased activity during the dark phase would alter the stable rate in cell proliferation and cause a peak immediately following a night of running. We therefore sacrificed groups of mice at two times of day, directly following the active phase and at the end of the resting phase. Although there is no statistically significant interaction between exercise and the time of day at which the animals were sacrificed, our data suggest that the exercise-induced increase in cell proliferation is most profound directly after the active phase and disappears in the course of the resting phase.

This finding implies that exercise may acutely stimulate resting progenitors to enter the cell cycle, resulting in an accumulation of proliferating cells during the active period. The increased cell proliferation appears to be somewhat lower at the end of the resting phase. Possibly, as the estimated cell cycle time of hippocampal progenitors in mice is 12-14 h (Hayes and Nowakowski, 2002), many of the cells that entered the cell cycle during the active phase completed the cell cycle before the end of the resting phase. Our data suggest that running wheel activity may not simply cause a continuously elevated number of dividing cells. It appears more likely that running induces certain factors that acutely promote cell proliferation. IGF-1 and vEGF may be potential candidates responsible for this effect since they have been shown to increase cell proliferation under baseline condi-
tions (Anderson et al., 2002; Schanzer et al., 2004) and to be essential for the exercise-induced increase in neurogenesis (Fabel et al., 2003; Trejo et al., 2001). However, additional research is required to determine the temporal dynamics of the exercise-induced upregulation of these two growth factors and of the effects of IGF-1 and vEGF on promoting cell proliferation.

A final remark is that the number of Ki-67 positive cells considerably differed between the 3 independent experiments. This could be caused by the fact that experiments were performed with animals from different batches or by the fact that the mice of experiment 1 were a few weeks older than the other groups (Heine et al., 2004a; Kempermann et al., 2002). However, since we compared the experimental groups with the appropriate control groups, the difference in proliferating cell number between the experiments does not interfere with the interpretation of the data.

In summary, our data show that under baseline laboratory conditions, hippocampal cell proliferation takes place at a constant rate across the day, which is not affected by 12 h of sleep deprivation during the resting phase. Despite the presence of hippocampal daily rhythms in various physiological parameters and in sleep-wake behavior, hippocampal cell proliferation remains stable. However, housing animals with a running wheel, resulting in high levels of physical activity, appears to induce a partly synchronous cell cycle. These data demonstrate that the steady rate in cell proliferation may not be indispensable for optimal hippocampal functioning, but can be changed by behavioral activity.

Acknowledgements

We thank Anouk Funke, Lisa van den Hengel, Ingrid Nijholt and Jan Bruggink for their assistance with the sleep deprivation, the perfusions and the corticosterone assay. This work was supported by the Netherlands Organization for Scientific Research (NWO-Vernieuwingsimpuls; E.A.V.d.Z. (grant 016.021.017) and P.M. (grant 864.04.002)).