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Circadian variation in mouse hippocampal cell proliferation

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Abstract

Hippocampal cell proliferation and concomitant motor activity were examined in adult male mice (C57BL/6J) across a 12:12 h light–dark cycle. 5-Bromo-2′-deoxyuridine (BrdU) (200 mg/kg, i.p.) was administered at six equally spaced time points across 24 h. A significant change in cell proliferation was found in the hilus (light phase > dark phase), but not in the granule cell layer (GCL)/subgranular zone (SGZ). Since it is generally believed that proliferating cells in the hilus and GCL/SGZ give rise primarily to glia and neurons, respectively, these data suggest a possible circadian influence on gliogenesis, rather than neurogenesis.

Keywords: BrdU; Dentate gyrus; Neurogenesis; Gliogenesis; Hilus; Circadian rhythm

Recently, several studies, including our own, have reported that total sleep deprivation of 24 h or more is associated with a strong suppressant effect upon hippocampal cell proliferation and neurogenesis [7,8,14,16]. No information is available regarding the conditions or factors responsible for this effect. Changes in any of a number of biochemical substances, such as glucocorticoids, growth hormone, or adenosine, would be candidates for mediating these hippocampal changes. In addition, the influential two factor theory of sleep regulation [4] proposes that the need for sleep is regulated by the interaction of the circadian cycle (*C* factor) and the amount of sleep loss (*S* homeostatic factor). Therefore, to evaluate the influence of the circadian factor, we examined endogenous variation in hippocampal cell proliferation across the 24 h light–dark (L–D) cycle in adult mice. To ensure entrainment to the L–D cycle, the behavioral activity of all animals was quantified.

Several previous studies have examined this issue, either directly or secondarily. None of these studies, however, has employed a combination of a sufficient number of time points with measures of entrainment. Van der Borght et al. [17] examined 8 time points across the L–D cycle in rats and found no significant variation in proliferation, but no evidence for L–D entrainment was reported. In addition, they utilized Ki-67, an endogenous marker of cell proliferation, which labels all cells in the cell cycle, except those in G_0. This was done to avoid the potential stress of injecting the commonly used exogenous proliferation marker 5-bromo-2′-deoxyuridine (BrdU). However, labeling cells across most of the 16–20 h cell cycle with Ki-67 would potentially mask any short-term variation. Thus, the time resolution of Ki-67 labeling is not well-suited for these types of studies. (Furthermore, our own unpublished work indicates that single i.p. drug injections have no significant effect upon hippocampal cell proliferation.) Ambrogini et al. [1] examined 4 time points across the L–D cycle in rats and found no significant variation in hippocampal cell proliferation, but, once again, no evidence of entrainment was presented. This study also employed a 24 h survival time post-BrdU, allowing for the possibility that factors during this period might influence survival of BrdU-labeled cells. Finally, an incidental observation in a study by Holmes et al. [10] appears to show no significant variation in hippocampal cell proliferation in home cage control mice across 3 time points of the L–D cycle.

In the present studies, adult male C57BL/6J mice (20–25 g) from Jackson Laboratory (Bar Harbor, ME) were individually housed in a dedicated vivarium at Rider University, with *ad libitum* access to commercial rodent chow and water. Animals were acclimated to controlled lighting and temperature conditions (12–12 h standard or reversed light/dark cycle; lights on 0700 h or 2100 h, respectively) for 3 weeks prior to experimentation to ensure that all animals were fully entrained to the appropriate

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L–D cycles. Mice on each L–D cycle were randomized to create groups of $n = 6$. All animal use was conducted in accordance with University guidelines and with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*.

Cell proliferation was assessed at 6 time points across the circadian cycle (ZT 0, ZT 4, ZT 8, ZT 12, ZT 16, ZT 20), where zeitgeber time (ZT) 0 corresponds to the beginning of the light portion of the L–D cycle. At each time point, animals were injected with the thymidine analog BrdU (200 mg/kg; i.p., Sigma–Aldrich, St. Louis, MO) and sacrificed 2 h post-BrdU to assess the number of proliferating cells in the dentate gyrus (DG). Animals were deeply anesthetized with an overdose of sodium pentobarbital (300 mg/kg, i.p.) and were perfused transcardially with 4.0% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4).

Brains were removed, postfixed in the same paraformaldehyde solution for 24 h at 4 °C, and then transferred to 30% sucrose (in 0.1 M PBS) until equilibrated. Frozen serial coronal sections (40 μm) were cut through the entire rostrocaudal extent of the hippocampus, and one series of tissue (every sixth section) was then processed using a slide-mounted immunoperoxidase technique.

Sections were mounted onto adhesive microscope slides, dried overnight at 37 °C, boiled in citric acid (0.01 M, pH 6.0) for 7 min, allowed to cool for 20 min, digested with trypsin (0.1% in 0.1 M Tris buffer, pH 7.5, containing 0.1% $CaCl_2$) for 8.5 min, denatured with 2.4 M HCl (in PBS) for 30 min, and then incubated with a mouse monoclonal antibody raised against BrdU (1:200 in PBS containing 0.5% Tween-20; NCL-BrdU; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) for 24 h at 4 °C. The next day, sections were incubated with a biotinylated horse anti-mouse IgG (1:200 in PBS; Vector Laboratories, Burlingame, CA, USA) and with avidin–biotin complex (1:100 in PBS; Vectastain® Elite ABC kit, Vector Laboratories) for 60 min, and then reacted with 3,3′-diaminobenzidine and urea hydrogen peroxide (Sigma Fast™ tablet sets, Sigma–Aldrich) for 10 min to visualize labeled cells. Sections were then counterstained with cresyl violet, dehydrated, and cover-slipped under DPX (Fluka BioChemika, Steinheim, Switzerland).

All slides were analyzed blindly using an Olympus BX-60 light microscope. BrdU+ cells were counted bilaterally in the DG at 400× magnification. The cell counts for each animal were summed across all sections and then multiplied by 6 (section periodicity) to obtain an estimate of the total number of BrdU+ cells in the hippocampus. Cell counts were also obtained for the granule cell layer (GCL)/subgranular zone (SGZ) and hilus. Cells located three or more cell body widths from the edge of the GCL were considered to be in the hilus. Cells located at the border of the GCL and hilus were considered to be in the SGZ and were summed with those in the GCL. In addition, the DG was divided into anterior and posterior portions, using the criteria of Guzmán-Marín et al. [8] for rats. Briefly, the boundary separating the anterior and posterior portions of the DG corresponded to the region where the CA2 and CA3 pyramidal cell layers coalesce into a continuous cell layer in the coronal plane (approximately −2.92 mm from Bregma, according to the atlas of Paxinos and Franklin [12]). Typically, there were eight anterior and four posterior sections for each animal.

To monitor general motor activity, animals were singly housed in cages equipped with infrared motion detectors (Slimline 12 V detectors, SmartHomePro.com), which are sensitive to both stationary and ambulatory activity, and score equally for both. Thus, low activity scores reflect low absolute movement. Behavioral data was recorded and analyzed using Clocklab data collection software (Actimetrics Inc., Evanston, IL). Changes in motor activity for each time period are expressed as a percentage of the overall behavior for each animal on that day.

For the BrdU and locomotor data, a one-way analysis of variance (ANOVA) was used to analyze all the time points independent of the L–D cycle, whereas a two-way ANOVA was used to take into account the L–D cycle, followed by *post hoc* Newman-Keuls multiple comparison test. For comparisons of means between two groups, a paired or unpaired *t*-test was used. All data are expressed as mean ± S.E.M. A probability value ≤ 0.05 was considered statistically significant.

Motor activity, expressed as an hourly percentage of total counts, significantly varied across the 24-h day ($p < 0.0001$, $F_{(23, 840)} = 97.71$) and in relation to the L–D cycle ($p < 0.0001$, $F_{(1, 840)} = 1653$), as shown in Fig. 1 (top). The percent of total daily activity across all animals was significantly higher in the dark phase as compared to the light phase ($p < 0.0001$, $t = 65.11$, $n = 35$). Overall, 87.4% ± 0.8% of activity occurred in the dark versus 12.6% ± 0.8% in the light. Nearly half (47.7%) of the total activity in the light period occurred during the first hour after light onset (ZT 0–ZT 1), which in part represents continued activity from the dark phase. Thus, throughout the remainder of the light phase, animals were almost completely inactive.

Using a one-way ANOVA to examine circadian change, no significant differences were found in the number of BrdU-labeled cells in the DG as a whole ($p = 0.38$, $F_{(5, 29)} = 1.097$), the hilus ($p = 0.06$, $F_{(5, 29)} = 2.387$), or the GCL/SGZ ($p = 0.45$, $F_{(5, 29)} = 0.971$) (see Fig. 1, bottom, and Table 1). Using a two-way ANOVA, which takes into account the L–D cycle, a highly significant variation in cell proliferation was found in the hilus (light > dark; $p = 0.005$, $F_{(1, 29)} = 9.148$), but not in the GCL/SGZ ($p = 0.37$, $F_{(1, 29)} = 0.815$) or entire DG ($p = 0.85$, $F_{(1, 29)} = 0.038$).

Comparison of the 2 time points which fell 8 h into the light phase (ZT 8) or the dark phase (ZT 20), so that the entire S-phase was exclusively in the light or dark, showed a significantly greater number of BrdU-labeled cells in the hilus at the light phase time point ($p = 0.026$, $t = 2.651$, d.f. = 9). Furthermore, comparison of the 2 time points at which phase transitions occurred (ZT 0 and ZT 12) showed a significant difference in the hilus (light transition > dark transition; $p = 0.015$, $t = 2.919$, d.f. = 10). Similar time point comparisons for the GCL/SGZ or entire DG revealed no significant changes in cell proliferation ($p = 0.25$–0.70, $t = 0.400$–1.209, d.f. = 9–10).

Finally, we examined cell proliferation in the anterior (dorsal) and posterior (ventral) DG, since in a previous rat study using sleep deprivation we noted differential changes in cell proliferation as a function of hippocampal region [16], perhaps related to different inputs and functional roles [11]. However,