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Direction-dependent effects of chronic "jet-lag" on hippocampal neurogenesis

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ABSTRACT

Disruptions in circadian rhythms, as seen in human shift workers, are often associated with many health consequences including impairments in cognitive functions. However, the mechanisms underlying these affects are not well understood. The objective of the present study is to explore the effects of circadian disruption on hippocampal neurogenesis, which has been implicated in learning and memory and could serve as a potential pathway mediating the cognitive consequences associated with rhythm disruption. Circadian rhythm disruptions were introduced using a weekly 6 h phase shifting paradigm, in which male Wistar rats were subjected to either 6 h phase advances (i.e. traveling eastbound from New York to Paris) or 6 h phase delays (i.e. traveling westbound from Paris to New York) in their light/dark schedule every week. The effects of chronic phase shifts on hippocampal neurogenesis were assessed using doublecortin (DCX), a microtubule binding protein expressed in immature neurons. The results revealed that chronic disruption in circadian rhythms inhibits hippocampal neurogenesis, and the degree of reduction in neurogenesis depends upon the direction and duration of the shifts. In two cohorts of animals that experienced phase shifts for either 4 or 8 weeks, a greater decrease in neurogenesis was observed when the phase was advanced versus delayed in both groups. The direction-dependent effect mirrors the findings on clock gene expression in the SCN, suggesting a causal link between the reduction in hippocampal neurogenesis and a disrupted SCN circadian clock.

1. Introduction

Circadian rhythms are endogenous rhythms in bodily processes ranging from gene expression to behaviors that have important implications in both health and disease [20]. In mammals, the control center for circadian rhythms exists in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus [18]. Circadian rhythms are generally synchronized to the environmental light/dark (LD) cycle, which is the most salient factor for setting the phase of the brain clock located in the SCN, and is believed to be the driving force for the initial emergence of the circadian system over evolution [31]. Although there are incremental changes in the LD schedule over the seasons in temperate regions, the circadian system has evolved under relatively stable and predictable LD conditions [15].

However, in modern society, the LD conditions experienced by humans could change markedly in a daily to weekly basis as seen in jet-lag, shift work, or simply associated with personal life styles [1,35]. Alteration in the ambient LD conditions can lead to disruptions in circadian rhythms, which have been shown to be associated with impairments in learning and memory in both human subjects [34] and in animal models [11]. Learning and memory are linked to hippocampal neurogenesis with a reciprocal relation, such that learning enhances neurogenesis and blocking neurogenesis disturbs learning (reviewed in [2]). These studies point to a reduction in hippocampal neurogenesis as a potential downstream pathway mediating the effects of circadian disruption on learning and memory. Consistent with this hypothesis, a recent study has revealed that disrupting the circadian rhythms in female hamsters by shifting their LD schedules results in decreased hippocampal neurogenesis and long-term cognitive deficits [12].

The objective of the present study is to further elucidate the effects of circadian rhythm disruption on hippocampal neurogenesis. We utilized a weekly 6 h shifting paradigm, in which the animals were subjected to either 6 h phase advances (i.e. traveling eastbound from New York to Paris) or 6 h phase delays (i.e. traveling westbound from Paris to New York) every week [6,37]. Hippocampal neurogenesis was assessed using immunochemical detection of doublecortin (DCX), a microtubule binding protein associated with migration and differentiation of neuroblasts, which serves as a marker for immature neurons [9,13]. The expression of DCX within the dentate gyrus (DG) has been shown to be directly related to neurogenesis [5,13]. The number of DCX-expressing cells was

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quantified in the DG, and the results revealed a unique direction-dependent effect of phase shifts on hippocampal neurogenesis.

2. Methods

2.1. Animals and housing

Young male rats (Wistar, 28 days old) were purchased from Charles River Laboratory and were housed in a 12:12 h light:dark (LD) cycle with food (standard rodent chow) and water available ad *libitum*. During the light hours, a fluorescent white light provided approximately 300 lux at cage level. Dim red light (<1 lux) was kept on during the dark hours for husbandry. All animals were kept under the same LD schedule for three weeks before they were subjected to the experimental paradigms (50 days old). All procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University.

2.2. Experimental groups

In experiment 1, the animals were randomly assigned to three groups ($n = 5$/group). Group one was treated with weekly 6 h phase advances (i.e. New York to Paris); group two was treated with weekly 6 h phase delays (i.e. Paris to New York); group 3 remained in the same 12:12 LD cycle. In experiment 2, to further compare the effects between phase delays and advances, two additional groups ($n = 5$/group) were used and were treated with weekly 6 h phase delays or advances for 8 weeks. The animals were sacrificed at the end of the 4th or 8th weekly shift (on day 7 after the last shift) at Zeitgeber time (ZT) 14 (lights off is defined as ZT12). The animals in experiment 1 were sacrificed at 78 days old; and those in experiment 2 were sacrificed at 106 days old.

2.3. Immunocytohistochemistry (ICC)

Rats were overdosed with sodium pentobarbital (200 mg/kg). They were then perfused intracardially with 100 ml saline followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer. Following extraction, brains were post-fixed then cryoprotected in 20% sucrose overnight. Coronal sections (40 μm) were obtained using a cryostat (Leica, IL, USA). Free-floating sections encompassing the area between the caudal end of the SCN and the rostral end of the supramammillary nucleus were incubated in the primary antibody against DCX (goat-anti-DCX, 1:5000, Santa Cruz Biotechnologies, CA) and processed following a standard peroxidase method using 3,3′-diaminobenzadine (DAB) as the chromogen [37]. The specificity of the DCX antibody has been evaluated in previous studies [10,16]. After the reaction, sections were mounted on microscope slides, dehydrated with alcohol rinses, cleared with xylene, and coverslipped with Permount (Fisher Scientific, NJ, USA).

2.4. Data analysis

Sections containing the hippocampus that encompassed its rostro-caudal extent from plane 187 (Bregma −2.68 mm) to 216 (Bregma −3.95 mm) of a rat brain atlas were analyzed [28]. Images of the DG were captured using a CCD camera (CX9000, MBF bioscience, Williston, Vermont, USA) attached to a light microscope (Zeiss, Gottingen, Germany). Photographs were taken bilaterally and centered at the DG. From each animal, six to ten images were used for quantification by cell counting. The DCX-containing neurons were counted in the entire DG manually from captured images (as shown in Figs. 1 and 2) by two observers who were blind to the experimental condition of each animal. The counts obtained by the two observers were highly correlated ($r^2 = 0.7$, $p < 0.01$) and the average counts across the sections and the observers were used to represent the value for each animal. A one-way ANOVA, followed by post hoc Tukey tests, were performed to assess the effect of shifts after 4 weeks. An unpaired t-test was performed to compare the difference between phase advances and delays after 8 weeks. In all cases, the statistical significance was defined as $p < 0.05$.

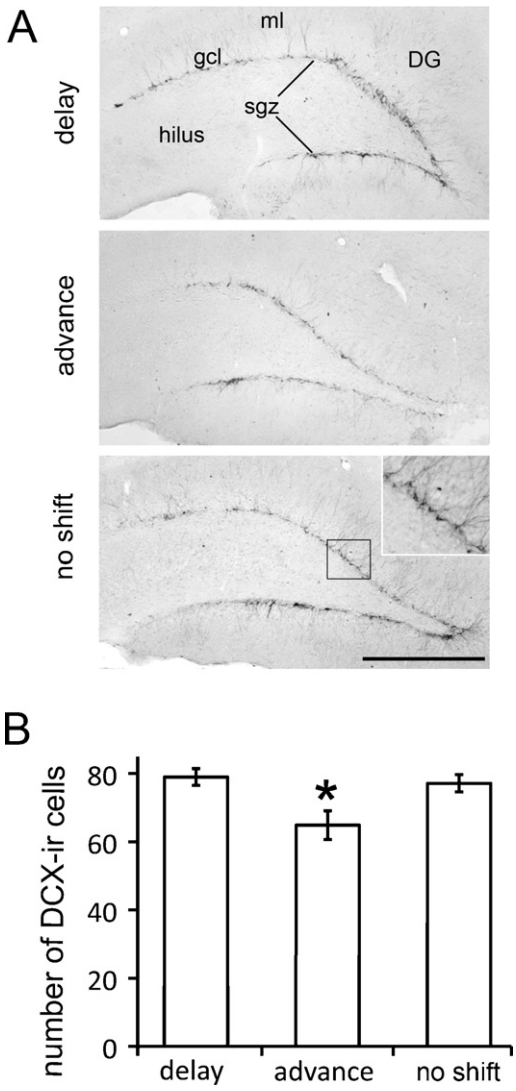

Fig. 1. DCX in the dentate gyrus (DG) of animals that experienced 4 weekly phase advances or delays or those that remained in the same LD cycle (non-shift). Representative photomicrographs show DCX staining in the DG of animals from each condition (A). Inset shows higher magnification of DCX-containing cells. The scale bar is 500 μm. The histograms show the number of DCX-immunoreactive (ir) cells in the DG (B). The results are shown as mean ± sem, $n = 5$. * indicates $p < 0.05$. sgz, subgranular zone; gcl, granular cell layer; ml, molecular layer.

3. Results

DCX-stained cells were observed in the DG of hippocampus (Figs. 1 and 2). The cell bodies were found mostly in the subgranular zone, with processes projecting to the molecular layer of the DG. The numbers of the DCX-stained cells were compared among animals that experienced 4 weekly advances or delays or without any shifts. The one-way ANOVA revealed a significant effect of phase shifts on the number of DCX neurons (Fig. 1B, $F_{2,14} = 6.31$, $p = 0.013$). Post hoc comparisons revealed that the number of DCX neurons in the 4th advance group was significantly lower than that from both the 4th delay group ($p = 0.023$) and the non-shift group ($p = 0.041$). To further test the different effects of phase advances and delays, the next experiment compared two groups of animals

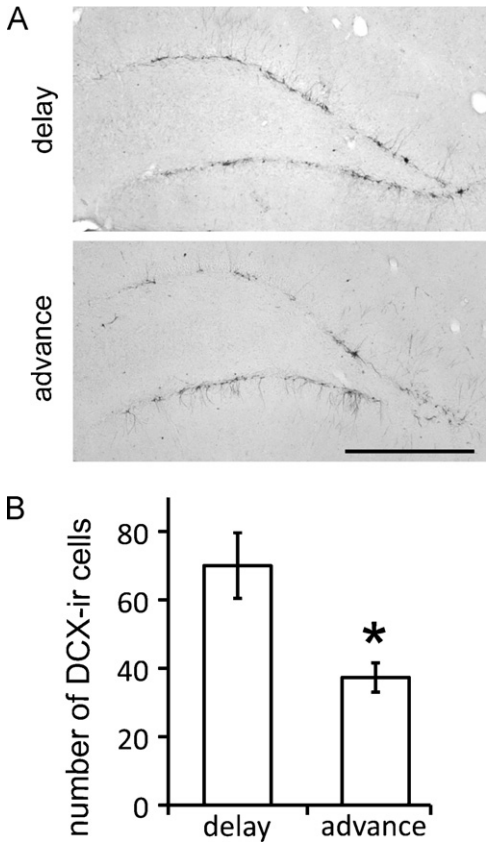

Fig. 2. DCX in the dentate gyrus (DG) of animals that experienced 8 weekly phase advances or delays. Representative photomicrographs show DCX staining in the DG of animals from each condition (A). The scale bar is 500 μm. The histograms show the number of DCX-immunoreactive (ir) cells in the DG (B). The results are shown as mean ± sem, $n = 5$. * indicates $p < 0.05$.

that experienced either weekly advances or delays for 8 weeks (Fig. 2). Following 8 weeks of phase shifts, the difference between the advance and delay groups was again evident; the number of DCX neurons in the advance group was significantly lower than that in the delay group (Fig. 2B, unpaired t-test, $t = -2.7$, $p = 0.03$).

4. Discussion

The results show that long-term exposure to a shifting LD schedule, particularly in the advance direction, has a negative impact on hippocampal neurogenesis. After 4 weeks of the shifts, the weekly advance group showed a ~20% reduction in the number of DCX-ir cells compared to the 4th delay or the non-shift groups. The difference between the treatment of phase delays and advances was further confirmed in a different cohort of animals that were phase shifted for 8 weeks, with the number of DCX cells in the advance group decreasing by almost 50% compared to that for the delay group.

The difference in DCX expression between the delay and advance groups is intriguing and warrants further investigation. Different responses of the circadian system to phase delays and advances have been well described in rodent species at behavioral and molecular levels. Following a 6 h shift in the LD schedule, the stable re-entrainment of mouse behavioral rhythms takes at least 7 days for advances and only 3 days for delays, with accompanied different temporal profiles in clock-genes expression [30]. Furthermore, clock-gene expression in the rat SCN shows a higher degree of dissociation between the SCN sub regions following a 6 h advance compared to a 6 h delay [27]. Although the underlying mechanisms are still not well understood, a rich body of literature documents distinct signaling cascades corresponding to phase delays or advances that are activated downstream from light-induced NMDA receptor activation in the retinorecipient neurons of the SCN (reviewed in [3]). For example, ryanodine receptors are critical for phase delays while PKG signaling is specifically involved in phase advances [3]. In addition to the intracellular signaling cascades, the spatial and temporal patterns of light-induced clock-gene expression also differ between phase delays and advances (reviewed in [36]). The divergent mechanisms involved may contribute to the different responses observed in circadian and other body functions associated with phase delays and advances, including the present results.

Davidson et al. has reported that when aged animals are subjected to the shifting paradigms of the present study, the weekly advance groups displayed higher mortality rate compared to the delay groups [6]. The impact of weekly phase shifts on the time keeping function of the SCN has been described [36]; the results reveal that at the end of 4 weekly 6 h phase shifts, the nocturnal pattern of locomotor activity and the daily rhythm of clock protein PER1 expression in the SCN were re-established. However, the peak level expression of PER1 in the SCN was attenuated, and the effect was more profound in the phase advance group compared to the delay group. Furthermore, the reduction in the peak level of PER1 expression was more substantial at the end of 8th shift compared to 4th shift. The directional aspect of these findings, namely that phase advances are more disruptive than phase delay in affecting the expression of clock genes, mirrors the findings of the present study on the number of neuron precursors in the DG. The results on PER1 expression in the SCN and DCX expression in the DG collectively point to a link between the function of the circadian clock and hippocampal neurogenesis.

The mechanisms through which the circadian rhythm disruption affects hippocampal neurogenesis are not well understood and likely involve multiple interconnected pathways [7]. Gibson et al. has shown that following several advances of LD cycle, the suppression in hippocampal cell proliferation is associated with elevated level of corticosterone [12], which is a well-known detrimental factor for hippocampal neurogenesis [21]. In addition to stress, chronic shifts in the ambient LD cycle can disrupt the sleep–wake cycle, which has also been implicated in proliferation and survival of new neurons [19,22] and apparently can do so independently of the effects of stress hormones. A recent study showed that a procedure for REM-sleep deprivation suppressed cell proliferation in adrenalectomized rats with low-dose corticosterone clamp, suggesting that sleep and rhythm disruption can inhibit hippocampal cell proliferation independent from elevated level of stress hormone [25,26]. A circadian regulation of the process of neurogenesis has been suggested by the observation of significant time of day effects in the survival of newborn hippocampal cells [33], and in the exercise-promoted increase in hippocampal cell proliferation [14]. The circadian system can potentially influence hippocampal neurogenesis through multiple pathways. For instance, the cell cycle is controlled by circadian clocks [17,24]. Circadian clocks are located in hippocampus where the expressions of clock genes show circadian oscillation [11]. Furthermore, the expressions of neurotrophic factors i.e. brain-derived neurotrophic factor (BDNF) and its receptor are also under the direct control of clock mechanisms [4,8,29,32]. Therefore, it is reasonable to speculate that during chronic jet-lag, when the internal circadian clock is desynchronized with the ambient LD cycle, the disturbance in the clock function can trigger disruptive responses in those potential downstream pathways that ultimately contribute to a reduction in hippocampal neurogenesis.

It should be noted that the present study only examined one marker, DCX, which labels the immature neurons [9,13].

Although the results clearly revealed a reduction in the number of DCX-containing cells, we cannot distinguish whether the reduction occurred at the stage of cell proliferation, differentiation, or survival. Using multiple markers targeting different stages of neurogenesis combined with more quantitative methods, i.e. western blot in addition to ICC, will likely provide a more detailed picture. Another limitation of the present study is that there was not an age-matched non-shift group for the 8th shifts groups. Although the results confirmed the different impact between phase delays and advances on the number of DCX cells in the DG, it is unclear whether the level of neurogenesis in the delay group would be below or comparable to that of undisturbed animals. The distribution of the data in 8th delay group, which were considerably variable (SEM = 9.6) compared to other groups (SEMs $\leq$ 4.3), suggests that there might be reduced neurogenesis in at least some individuals in that group.

In summary, the results of the present study show that long-term perturbation of circadian rhythms inhibits hippocampal neurogenesis. The severity of the perturbation in the time keeping function of the SCN (e.g. advances vs. delays) is associated with the degree of reduction in neurogenesis. Adult neurogenesis has been implicated in cognitive functions such as learning and memory [2]. Decreased hippocampal neurogenesis and long-term cognitive deficits have been reported in animals that experienced repetitive phase advances [12]. It would be of interest to compare the phase-delayed and -advanced animals in the learning and memory tasks in future studies, to determine whether the differences in the number of DCX-labeled immature neurons are translated into actual cognitive capacity. Another important question that needs to be addressed in future research is whether the impairments in hippocampal neurogenesis are reversible, and what factors contribute to the recovery of the hippocampal functions. A better understanding of these questions will bring insight into the neural mechanisms underlying the cognitive impairments associated with circadian rhythm perturbation displayed in humans [23], and contribute to the development of effective strategies for intervention and prevention.

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