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Original Article

Phase-delay in the light–dark cycle impairs clock gene expression and levels of serotonin, norepinephrine, and their metabolites in the mouse hippocampus and amygdala

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ABSTRACT

Objective: A number of animal studies have implicated circadian clock genes in the regulation of mood, anxiety, and reward. However, the effect of misalignment of the environmental light–dark and internal circadian clock on the monoamine system is not fully understood. In the present study, we examined whether an abnormal light–dark schedule would affect behavior-, circadian clock–, and monoamine-related gene expressions, along with monoamine contents in the amygdala and hippocampus of mice.
Methods: Mice were subjected to an 8-hour phase delay in the light–dark cycle (Shift) every two days for four weeks, and locomotor activity was continuously measured. We examined the circadian expression of clock genes (*Per1*, *Per2*, and *Bmal1*) and genes of the NE/5HT uptake transporters (*Net* and *Sert*). In addition, the levels of NE/5HT and their metabolites MHPG/5HIAA were analyzed in the amygdala and hippocampus.
Results: Locomotor activity showed a free-running phenotype with a longer period (>24 hours) and showed misalignment between the light–dark and inactive–active cycles. The amplitude of the day–night fluctuation of *Bmal1* expression was reduced in the amygdala and hippocampus of light–dark–shifted mice. *Net* gene expression in the Shift group showed different profiles compared with the Control group. In addition, NE and 5HT levels in the amygdala of the Shift group increased during the active period.
Conclusions: The present results suggest that misalignment of the internal and external clocks by continuous shifting of the light–dark cycle affects the circadian clocks and monoamine metabolism in the amygdala and hippocampus of mice.

1. Introduction

In mammals, the circadian rhythm refers to an approximately 24-hour internal oscillation that controls physiological and behavioral rhythms. Circadian oscillators are located in the suprachiasmatic nucleus (SCN) and peripheral organs, which are referred to as the central clock and peripheral clock, respectively [1]. Circadian oscillators are regulated by endogenous circadian clock genes such as *Period1 (Per1)* and *Period2 (Per2)*, which act as negative factors, and *Clock* and *Brain and Muscle ARNT-like protein 1* (*Bmal1*), which are positive factors [1]. Long-term circadian rhythm impairments have been reported in various physical and mental disorders. Many animal studies have implicated individual circadian genes in the regulation of mood, anxiety, and reward via modulation of the neurochemical system in the brain [2]. Several studies have reported that photoperiod change, such as long vs. short days, affects the monoamine system [3,4]. However, there are no reports examining daily changes in monoamine metabolism under shifts of the light–dark cycle. Previous studies reported that phase delays are less disrupted than phase advances in the SCN. For example, one study showed that chronic phase advances induce higher mortality rates than chronic phase delays, and another study showed that it is easier for the SCN to readjust to phase delays than phase advances [5,6]. Therefore, we investigated the effect of chronic phase delay of the light–dark cycle on the circadian clocks and monoamine neuronal system in the present study.

It is widely known that monoamines, including serotonin (5HT) and norepinephrine (NE), play important roles in the neurochemical system of the brain. Monoamine fluctuation is related to changes in mood, anxiety, and reward in the neurochemical system in the brain. In addition, these changes play important roles in peripheral tissues, including the cardiovascular system and intestinal tract system [7,8]. Therefore, the monoamine neural system is an important factor in regulating the central and peripheral neural systems.

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The amygdala and hippocampus are thought to be important brain regions for mood regulation [9,10]. These brain structures receive input from monoamine-transmitting neurons that originate in the brainstem. Moreover, they are regulated by a biological clock with input from the SCN [11]. Therefore, these brain areas are suitable for elucidating clock function and monoamine metabolism. Monoamine dynamism is an important factor in the condition of the system, and it is sensitive to internal and external conditions. Therefore, monoamines might be influenced by abnormal light–dark cycle conditions.

2. Methods

2.1. Animals

Six-week-old male C57BL/6JKwl mice were obtained from Tokyo Laboratory Animals Science Co., Ltd (Tokyo, Japan). Animals were maintained in the laboratory for one week before the start of the experiment for adaptation to laboratory conditions, which included a 12/12-hour light–dark cycle, a temperature of 22 ± 2 °C, humidity of 60% ± 5%, and food and water ad libitum. In the 24-hour cycle, Zeitgeber time 0 (ZT0) was designated as the time when the lights came on, and ZT12 was designated as the time when the lights went off. All procedures were carried out with permission (2013-A058, 2013-A071) from the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University and the Law and Notification Department of the Japanese Government. The body weights of the mice were measured at the beginning of the study and after euthanization.

2.2. Chronic phase delay schedule

After one week of acclimation, the mice were either exposed to a normal light–dark cycle for four weeks (Control group) or an 8-hour delay in the time of lights-on and lights-off every two days for one week (the normal LD cycle was reinstated on Monday and Tuesday) (Fig. 1). The delayed shift was continued for four weeks (Shift group) (Fig. 1). The delays were accomplished by extending the dark period for eight hours on the delay days. On the day after the 4-week experimental period, mice in the Control group were sacrificed at ZT0, ZT6, ZT12, or ZT18 (Fig. 1C), and mice in the Shift group were sacrificed at Circadian time (CT) 0, CT6, CT12, or CT18 (CT12 was defined as activity onset in the Shift condition by locomotor activity monitoring) (Fig. 1D).

2.3. Analysis of locomotor activity rhythmicity

General locomotor activity was recorded with an infrared radiation sensor (F5B; Omron, Tokyo, Japan). Double-plotted actograms of locomotor activity are shown in 6-minute epochs. Circadian rhythmicity of activity onset and activity counts was evaluated using CLOCKLAB software (Actimetrics, Wilmette, IL, USA).

2.4. Reverse transcription polymerase chain reaction

Each experimental group (Control and Shift) contained 12 mice per time point (ZT0, ZT6, ZT12, ZT18, CT0, CT6, CT12, and CT18; total $n = 96$). Mice were anesthetized with midazolam/xylazine and their brains were removed. Frontal brain slices (2-mm thick) that

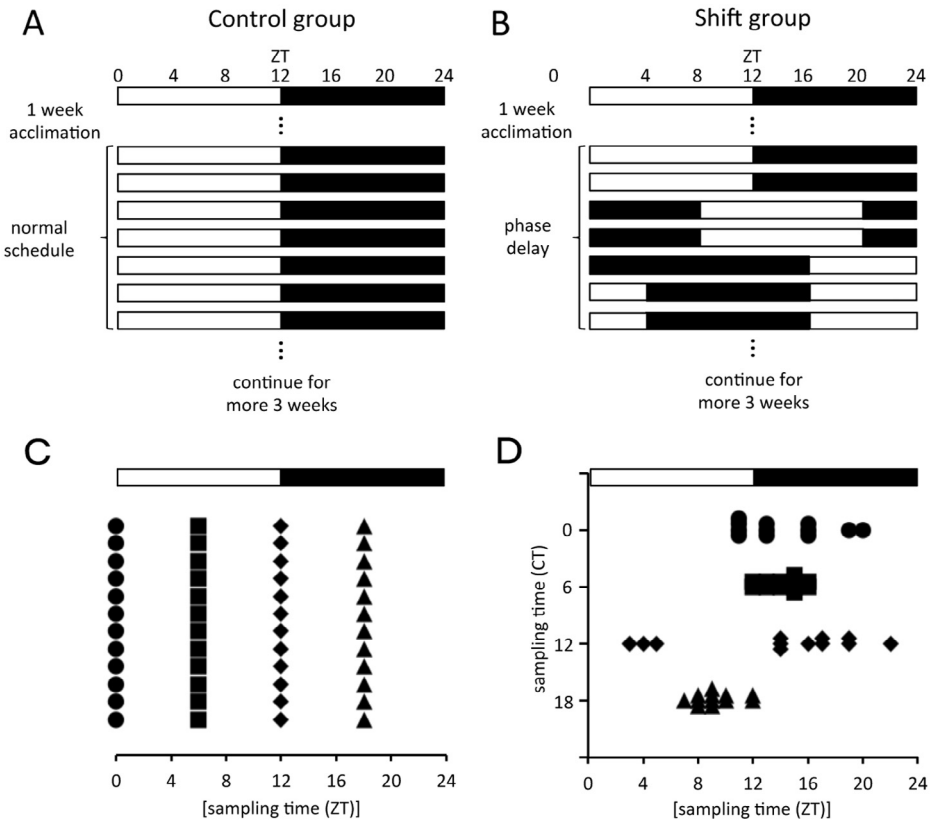

Fig. 1. Experimental design. (A) Control group. (B) Shift group. White bar indicates light period; black bar indicates dark period. After four weeks of the respective light–dark schedule, mice were sacrificed at Zeitgeber time (ZT)0, ZT6, ZT12, and ZT18 for the Control group, and at Circadian time (CT)0, CT6, CT12, and CT18 for the Shift group. (C) Sampling time in mice and ZT distribution for the Control group. (D) Sampling time in mice and ZT distribution for the Shift group. Circle denotes ZT(CT)0; square, ZT(CT)6; diamond, ZT(CT)12; triangle, ZT(CT)18.