Disruption of circadian clocks promote progression of Alzheimer's disease in diabetic mice

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Research Article

Keywords: Circadian rhythm, Light, Alzheimer's disease, Type 2 diabetes mellitus

Posted Date: February 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-235837/v1

Abstract

The circadian clock is an endogenous system designed to anticipate and adapt to daily changes in the environment. Alzheimer's disease (AD) is a progressive neurodegenerative disease, which is more popular in patients with type 2 diabetes mellitus (T2DM). However, the effect of circadian disorder on mental and physical health for T2DM patients are not yet fully understood, even though circadian disruption has been confirmed to promote the progression of AD in population. By housing db/db mice on a disrupted (6:18 light/dark cycle) circadian rhythm, we assessed the circadian gene expression, body weight, cognitive ability and AD-related pathophysiology. Our results indicated housing in these conditions had disrupted diurnal circadian rhythms in hippocampus and contributed to weight gain. In the brain, circadian-disrupted db/db mice showed a decreased cognitive ability and an increased hyperphosphorylation of tau protein, even though no difference was found in Aβ deposition. We also found that the hyperphosphorylated tau protein exhibited more disruptive daily oscillations in db/db mice hippocampus under 6:18 light/dark cycle. circadian alterations could promote the development of AD in T2DM.

Introduction

Almost all plants and animals exhibit inherent 24-hour oscillations to adapt the Earth's rotation [1]. The main function of circadian rhythm was to prepare an organism for the potential outer opportunities and challenges. The circadian timing system was composed of central clock located in the hypothalamic suprachiasmatic nucleus (SCN) and multiple peripheral clocks. SCN received photic signals and synchronized the physiology of the organism to external environmental change. The light was regarded as the most important zeitgeber. The peripheral clocks mainly get entrained timing signal from SCN. The central molecular mechanism of circadian rhythm is the transcriptional–translational feedback loop (TTFL)[2]. In this circadian TTFL, the typical "clock genes" mainly include circadian locomotor output cycleskaput (*Clock*), brain muscle ARNT-like protein 1 (*Bmal1*), period *(Per1, Per2 and Per3*), cryptochrome (*Cry1 and Cry2*) and Reverb (*Reverbα/Reverbβ*)[3]. This molecular clock maintained the rhythmic signal output approximately a 24 h period. Many biological processes were related to circadian control, such as sleep-wake cycle, hormone secretion and blood pressure.

Type 2 diabetes mellitus (T2DM) is one of the most popular age-related diseases characterized by hyperglycemia and insulin resistance. The main organs of energy metabolism (such as liver, adipose tissue and pancreas) contain an autonomous clock and show a metabolic rhythm[4]. The activity of thecortisol secretion presented a diurnal rhythm, which has a great effect on glucose and insulin levels[5, 6]. Therefore, a close association between diabetes and circadian clock was raised. Disruption of circadian clock has been confirmed to affect glucose metabolism. Loss of Clock was easily developed into metabolic syndrome in mice [7]. Knockdown of circadian Bmal1 will lead to glucose intolerance, fasting and diurnal hyperglycemia as well as impair glucose-stimulated insulin secretion [8, 9]. Circadian disruption plays a key role in glucose control.

Alzheimer's disease (AD) is a popular progressive neurodegenerative disease. The typical pathophysiological changes of AD were the deposition of extracellular amyloid β (Aβ) plaques and the accumulation of intracellular neurofibrillary tangles due to hyper-phosphorylated tau proteins[10]. Recent evidence holds that circadian disruption can present in the early stage of the disease [11]. Mice with chronic circadian disruption showed a decrease in cognitive flexibility and a loss of dendritic length [12]. Circadian clock regulates Aβ oscillations in the hippocampus and has an impact on amyloid plaque deposition in AD models [13], which proved that circadian rhythm could directly influence AD pathogenesis. In Tg4510 mice (a model of tauopathy), the expression of PER2 and BMAL1 is evidently disrupted in the hippocampus [14]. Thus, circadian disruptions are considered as a cause of AD [15].

T2DM is the risk factor in the progression of AD. There is nearly 2.8 folds increased risk of developing AD in patients with T2DM[16]. In modern life, patients with T2DM are more easily exposed to environmental circadian disorders, such as staying up late and transmeridian flight. We would like to confirm whether patients with T2DM under circadian disorder could accelerate the progression of AD.

In the present study, we imitated the circadian disruption condition of modern industry. Disruption was induced by housing db/db mice in 6:18 light/dark (LD) cycles, whereas controls were maintained in normal 12:12 LD cycles. We evaluated the circadian gene expression, body weight, cognitive ability and AD-related pathophysiology to study the association between T2DM and AD after circadian disruption.

Materials And Methods

Animals and house

The five-week-old db/db mice (BKS- *Lepr*^{em2Cd479}*/Gpt*, strain number: T002407) were purchased from the GemPharmatech Co., Ltd (Nanjing, China). Mice were given 1 week to habituate to the facility and allowed ad libitum access to normal chow and water. For circadian disruption, one group (n=16) was kept on 6:18 light/dark cycle (6:18 LD cycle). The lights were switched at 7 am and off at 1 pm, which were regarded as zeitgeber time (ZT) 0 and ZT6 respectively. The control group (n=16) remained in a 12:12 light/dark cycle (12:12 LD cycle), with lights on at 7 am (ZT0) and off at 7 pm (ZT12). All animal experiments were approved by Animal Care and Use Committee of Tongji Hospital in accordance to the Public Health Service Policy on Human Care and Use of Laboratory animals.

At the age of 14 weeks, behavioral tasks were performed in db/db mice to assess their cognitive ability. Then they were euthanized every six hours in one day (ZT0, ZT6, ZT12 and ZT18) to evaluate the effect of light on diurnal rhythmicity. Their brains were removed and sagittally bisected. The right hemisphere was fixed in 4% paraformaldehyde for immunohistochemical analysis; the hippocampus was separated from the left hemisphere and frozen at −80 °C for later western blotting.

Behavioral assessment:

Open field test

For all behavior tests, we adapted the protocols from Volmar et al [17]. The open field test was performed to test the locomotor activity of db/db mice. Briefly, the open field arena was made up of a standard clear plastic box (45 ×45 × 45 cm) placed in a quiet, well-lit room. Animals were individually put in the center of the box for 10 min. The tracks of mice were recorded using a computerized Ethovision detection system (Noldus, Netherlands). Total distance as well as average speed each mouse traveled during that time were automatically recorded. Arena was clean by 70% ethanol between different tests.

Novel object recognition test

A novel object recognition test aims to assess hippocampal associated contextual learning in rodents. This test was performed in the same box where the open field test was conducted. First, each mouse was allowed to explore two identical objects in the arena for 5 min and then it was sent back to home cage. After 30 min interval, this mouse was exposed to two different objects (one was the previous object and another was a novel object with different shape and color) in the arena. The frequency and time the mouse explored the novel object were used to evaluate the use of learning and recognition memory. Frequency related memory index was defined as [(frequency of novel object investigation)/(total frequency of investigation of both objects)*100] and time related index was calculated as [(novel object investigation time)/(total investigation time of both objects)*100] [17] .

Barnes maze test

A Barnes maze test was used as a test for spatial learning and memory assessment. Simplified Barnes maze contained “Acquisition” and “ Probe” trials.

Barnes maze Acquisition trial. We used a dark grey PVC circular platform (100 cm in diameter, elevated 90 cm above the floor) with 20 holes (5 cm in diameter) as the Barnes maze. An escape box was hidden under one of the holes. The aversive cues (the bright light and buzzer sound) were set up in the surroundings to stimulate mice to escape in 5 min. When the mice were exposed to these conditions, they were allowed to escape or were gently guided to the box. From day 1 to day 3, we trained animals for twice with an inter-trial interval of 30 min. On day 4, the mice had a rest for 24 h. Then, on the final day, we conducted the probe trial.

Barnes maze Probe trial. In Probe trial, the escape box was removed from holes. Target zone was the goal hole where the escape box was previously located in the acquisition test. The errors the mouse made before finding the target zone was used as an evaluation of spatial memory retention.

RNA isolation and RT-qPCR

Hippocampus samples were collected for RNA isolation at the different ZTs. Total RNA was extracted by TRIzol reagent according to the manufacturer’s instructions (Takara Bio, Japan). cDNA was synthesized using the Hifair II Reverse Transcription System following the manufacturer’s instructions (Yeasen Biotech Co. Ltd, Shanghai, China). Real-time reverse transciptase–PCR was conducted using the

QuantStudio^{TM} 1 system (Thermo Fisher Scientific biosystem) and SYBR Green qPCR Master Mix (Yeasen Biotech Co. Ltd, Shanghai, China). Gene expression was normalized to housekeeping genes (glyceraldehyde 3-phosphate Dehydrogenase, Gapdh). The primer sequences were referred to previous study[3]. Comparative CT method ($2^{-\Delta CT}$) was used to calculated the relative gene expression.

Western blots

The total protein was obtained with the cell lysis buffer for western and IP containing a protease and phosphatase inhibitor (Beyotime Inc., Shanghai, China). We collected the supernatants after centrifugation and then measured the concentration via a bicinchoninic acid (BCA) assay (Boster Biological Technology co.ltd., Wuhan, China).

The prepared protein samples were separated in 10% Bis-Tris SDS-polyacrylamide gels and then transferred to a nylon-PVDF Immobilon-PSQ membranes at 200 mA. The membranes were blocked in blocking buffer for 1 h at room temperature, and then were incubated overnight with primary antibody at 4C. The primary antibodies were included Tau5 (1:5000, Abcam, Cat # ab80579), p-Ser199 (1:5000, Abcam, Cat # ab81268), p-Ser396(1:5000, Abcam, Cat # ab109390), p-Thr231(1:5000, Abcam, Cat # ab151559) and β-actin(1:10000, Proteintech, Cat # 66009-1-Ig). After washing for several times, the membrane was incubated with secondary antibodies (1:7500; Proteintech, Cat # SA00001-1 and SA00001-2) for 2 h. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection (Biosharp, Beijing, China), and were scanned using GelView 6000 Pro (antpedia, China). Band density was measured using Image J software.

Immunohistochemical analysis

The immunohistochemistry analysis was detected as previous study [18]. The immunoreactivities of the CA1 hippocampal region were measured with specific primary antibodies against anti-beta Amyloid 1-42 antibody (1:200, Abcam, ab201061). We used the streptavidin–horseradish peroxidase method and the reaction was visualized with the diaminobenzidine (DAB) detection process. The Aβ 1–42 immunoreactivities were calculated by counting blindly the numbers of Aβ plaques in CA1 regions under 10x, 20x and 40x magnification. Data were collected from four random fields in every section (n=3-5) for statistical analysis.

Statistical analysis

All statistical analyses were assessed by *Student's t test*, one-way and two-way ANOVAs using SPSS (version 24.0 for Windows). Figures were plotted with GraphPad Prism 8. We used post hoc with *Bonferroni's* correction when appropriate. Results were regarded as significance at $P < 0.05$.

Results

Altered light cycles contributed to disrupted circadian rhythms in hippocampus in db/db mice.

Light from environment was the important zeitgeber to coordinate the oscillation between outside and body rhythm. In contrast to human, the rodents always are active at night and take a rest at the day. We prolonged the dark duration of mice to simulate the overexposed to artificial light in this industrial society. The diurnal expression of key clock genes in hippocampus were measured to confirm the effect of light on hippocampal rhythmicity. Our results found that the rhythms of *Bmal1* and *Reverba* expression were significantly disrupted in the 6:18 LD cycle (Figure1A and 1C), which indicated that the hippocampus was susceptible to light-induced rhythm disorders. While gene *Per2* expression did not change compared to 12:12 LD cycle (Figure 1B).

Circadian disruption contributed to weight gain

Mice in both groups showed a gradually weight gain since the first week (Figure 2A). On the 2nd week, the increase in body weight of the mice fed in 6:18 LD cycle was significantly. On the 8th week, the db/db mice displayed a decrease in weight gain probably because their pancreas function was in failure. We also tested their blood glucose and found that they both consistently exhibited hyperglycemia during experiment (Figure 2B).

Circadian disrupted animals showed decreased cognitive ability

We further examined the effects of circadian disruption on locomotor behavior. We observed no significant difference between 12:12 LD cycle group and 6:18 LD cycle group for total distance traveled or average velocity (Figure 3A and 3B). In the next day, we measured the novel object recognition in the same arena. In this test, circadian disrupted mice showed significantly more poor performance than control mice in novel object exploration frequency (Figure 3C) and duration (Figure 3D).

We subsequently assessed spatial memory of mice using a Barnes maze. More errors the treated mice made in acquisition trials 3 and 5 compared with controls (Figure 3E). After 24 h of rest, the mice of the 6:18 LD cycle committed more errors than 12:12 LD cycle in the probe trial (Fig. 3F), indicating reduced spatial memory in mice with disrupted circadian rhythms.

Circadian disruption increased the hyperphosphorylation of tau protein in hippocampus

Accumulation of tau protein was the typical pathophysiological characteristic of AD. To investigate the influence of circadian rhythm on hippocampal tauopathy, we further detected the phosphorylation levels of tau protein. The AD-related tau protein sites (Ser199, Ser396 and Thr231) in the hippocampus were examined. The levels of tau protein were significantly hyperphosphorylated at the Ser199, Ser396 and Thr231 site in the 6:18 LD cycle group (Figure 4).

We also observed the deposition of Aβ plaques in CA1 regions under 10x, 20x and 40x magnification (Figure 5). However, no significance between two groups was found.

The hyperphosphorylation of tau protein exhibited more disruptive daily oscillations in animals with disrupted circadian rhythms.

In order to explore the effects of circadian disorder on tau phosphorylation, we compared the phosphorylation levels of tau protein at different time points between two groups. The results indicated that phosphorylation of tau protein showed daily oscillations. At ZT0 and ZT12 time points, no difference of tau phosphorylation was discovered between the 6:18 LD cycle and the 12:12 LD cycle (Figure 6). At ZT6 and ZT18 time points, the phosphorylation levels were significantly increased in disrupted group among at Ser199, Ser396 and Thr231 sites (Figure 6). The loss of central circadian rhythms leads to disruption of daily hippocampal phosphorylation of tau protein oscillations.

Discussion

In the present work, we demonstrated a number of findings: 1) Altered light/dark cycles disrupted circadian rhythms in hippocampus of db/db mice. 2) Circadian disorder led to body weight gain. 3) Decreased cognitive ability was more obvious in circadian disrupted mice. 4) Circadian disruption increased the hyperphosphorylation of tau protein in hippocampus. 5) The hyperphosphorylation of tau protein exhibited more disruptive daily oscillations in animals with disrupted circadian rhythms. These findings indicated that disorders of circadian rhythms could influence weight and result in cognitive changes, demonstrating the role of circadian rhythms mattered in both mental and physical health.

First, we disrupted the circadian clock of db/db mice by changing the light duration, with the control group exposed to a normal 24-h day (12:12 h LD cycle), and the disrupted group exposed to a longer dark (6:18h LD cycle) to imitate the stay-up in human. It is of note that, circadian rhythm disturbance that one day of 24-h is shortened into 20-h has been confirmed to have adversely effect on cardiac function [19] and impair memory [12]. Our method of changing light duration has been applied in rodents to investigate the association between chronic disruption in light/dark cycle and brain trace element concentrations [20]. Here, we explored the progression of AD in db/db mice under similar circadian rhythm disorder.

The Findings linking circadian disorders and obesity are becoming more and more popular[21]. Our findings suggested that altered light/dark cycle resulted in the weight gain in db/db mice. The rodents usually are active during the night and sleep at daytime. When we did not restrict their access to food, the mice exposed to longer dark duration had longer feeding time. In human, it was like eating midnight snack when we stayed up. Some reports have also observed similar changes in weight in *Clock* mutant mice [7]. A longitudinal study has found that long term exposure to night-shift work can promote weight gain and obesity in a nurses cohort [22]. We confirmed that the circadian disorder might accelerate weight gain in patients with T2DM.

In addition to body weight, we demonstrated behavior changes in disruptive animals. Our behavioral results indicated that there was no difference between two groups in open field test, suggesting they performed similar locomotor activity in this arena. The mice of 6:18 LD cycle showed a decreased ability in exploring the novel object, indicating contextual learning was impaired under disrupted circadian clock. In a spatial memory task, the animals of disruptive circadian rhythm made more errors in finding

targeting area, which confirmed that circadian disorder is harmful for longer-term learning in patients with T2DM. Previous work in humans indicated that flight crews of short-recovery have decreased performance on hippocampal memory and spatial cognitive deficits [23]. Our finding further confirmed that circadian disruption could accelerate the cognitive impairment in patients with T2DM.

Tau hyperphosphorylation, which leads to intracellular NFT accumulation, results in the destruction of cytoskeleton structural and microtubule dysfunction to promote cognitive disorder [24]. One research showed that phosphorylated tau levels are significantly increased in sleep-deprived mice [25]. Similarly, we found that the circadian rhythm disruption aggravated the hyperphosphorylation levels of tau protein in db/db mice. Interestingly, we observed that the difference between two groups varies at different time points. At ZT0 and ZT12, no significance was found in two groups while the 6:18 LD cycle has more increased hyperphosphorylation of tau protein than controls at ZT6 and ZT18. The loss of central circadian rhythms leads to more disruptive daily oscillations in phosphorylation of tau protein in 6:18 LD cycle. These findings may provide an effective view to target tau-related therapy in AD. However, there are few reports on the rhythmicity of tau pathology, and further researches are needed to investigate this issue. We did not observe the significant difference between two groups in brain Aβ burden. We suspected that the age of db/db mice maybe one reason. The obvious Aβ burden was measured in the older db/db mice in a previous study [26] while the mice in our study was only 14 weeks old. Moreover, the tau protein may be more susceptible to circadian disruption in db/db mice than Aβ deposition.

In present work, we have some advantages. First, we found that weight gain and decreased cognitions after circadian disruption in diabetic model. Second, the phosphorylation of tau protein has rhythmicity, which will promote the new target of AD treatment. This research also has limitations. We have not explored the potential mechanism how circadian disorder promote AD in diabetic mice. The Aβ deposition should be detected in older db/db mice.

In conclusion, our study suggested that the disruption of circadian rhythm can promote the progression of AD in db/db mice. Prevention from circadian disruption may slow down the progression of AD in patients with T2DM.

Declarations

Funding

This work was supported by the National Natural Science Foundation of China (Grants 81670754, 81800686 and 81974114) and funds of Jie Chu Jing Ying foundation (Grants 2018076).

Conflicts of interest

The authors declare that they have no conflicts of interest, financial or otherwise.

Availability of data and material

Data will made be available on reasonable quest.

Author's contributions

YY and JH conceived and designed the study and wrote the manuscript. YY, XS , KD and XY secured the study's funding. JH, XP, RF, KD, XS, SZand XY acquired and analyzed the data. All authors revised the article and approved its final version.

Acknowledgments

We thank all patients for making this study possible. We thank Danpei Li and Li Huang from Huazhong University of Science and Technology for technical advice and assistance with this study.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

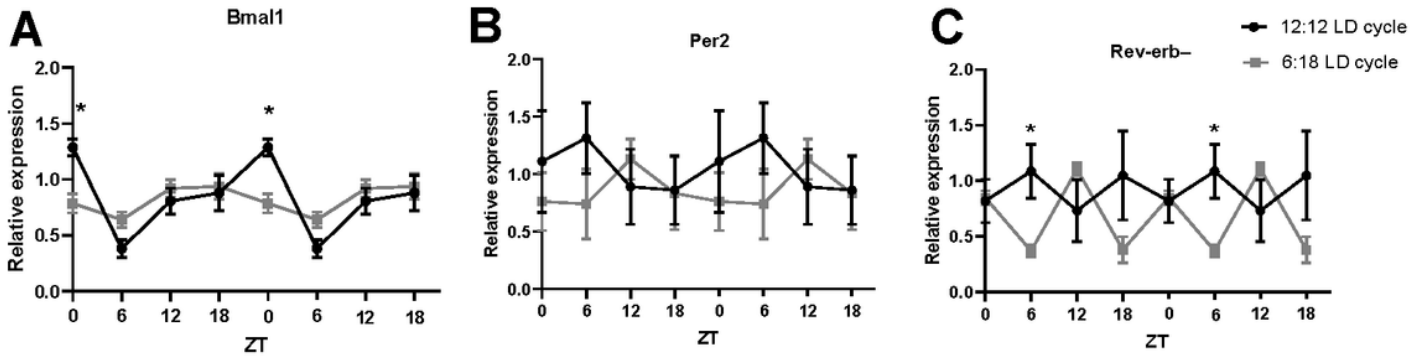

Figure 1

Altered light/dark cycle induces hippocampal rhythm disruption Bmal1 (A) and Reverbα (C) expression were significantly disrupted in the 6:18 LD cycle (n=3-4). While gene Per2 expression showed no

difference compared to controls (B, n=4). ZT, zeitgeber; data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

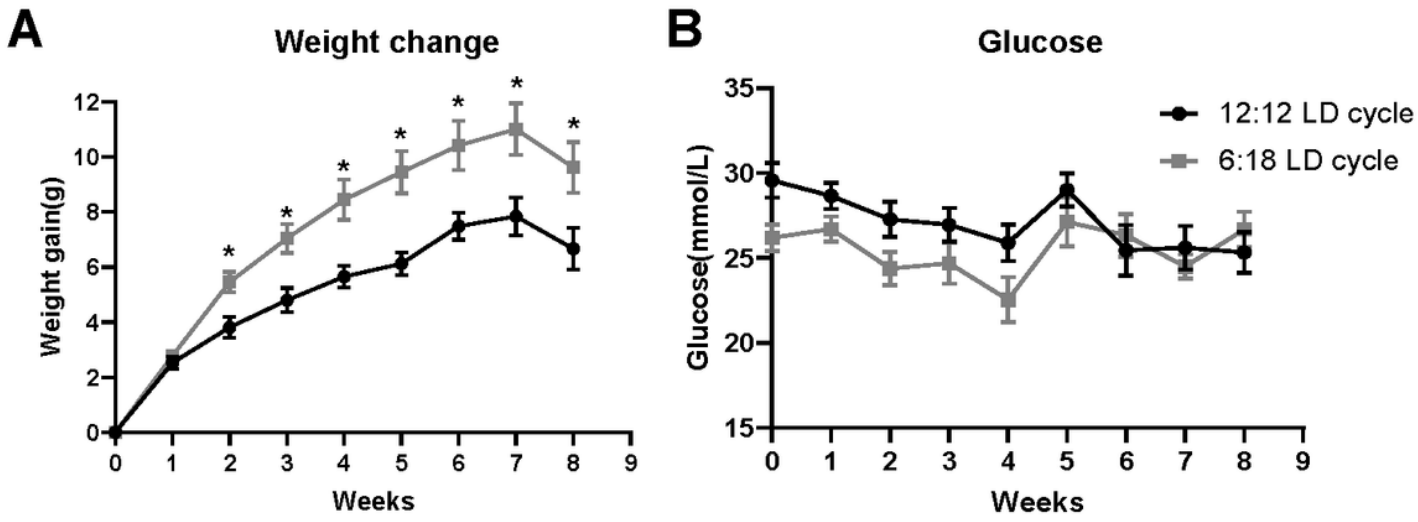

Figure 2

Circadian disruption results in weight gain The body weight gain of the mice fed in 6:18 LD cycle was significantly higher than controls (A, n=15 in each group). The blood glucose between two groups showed no difference (B, n-15 in each group). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

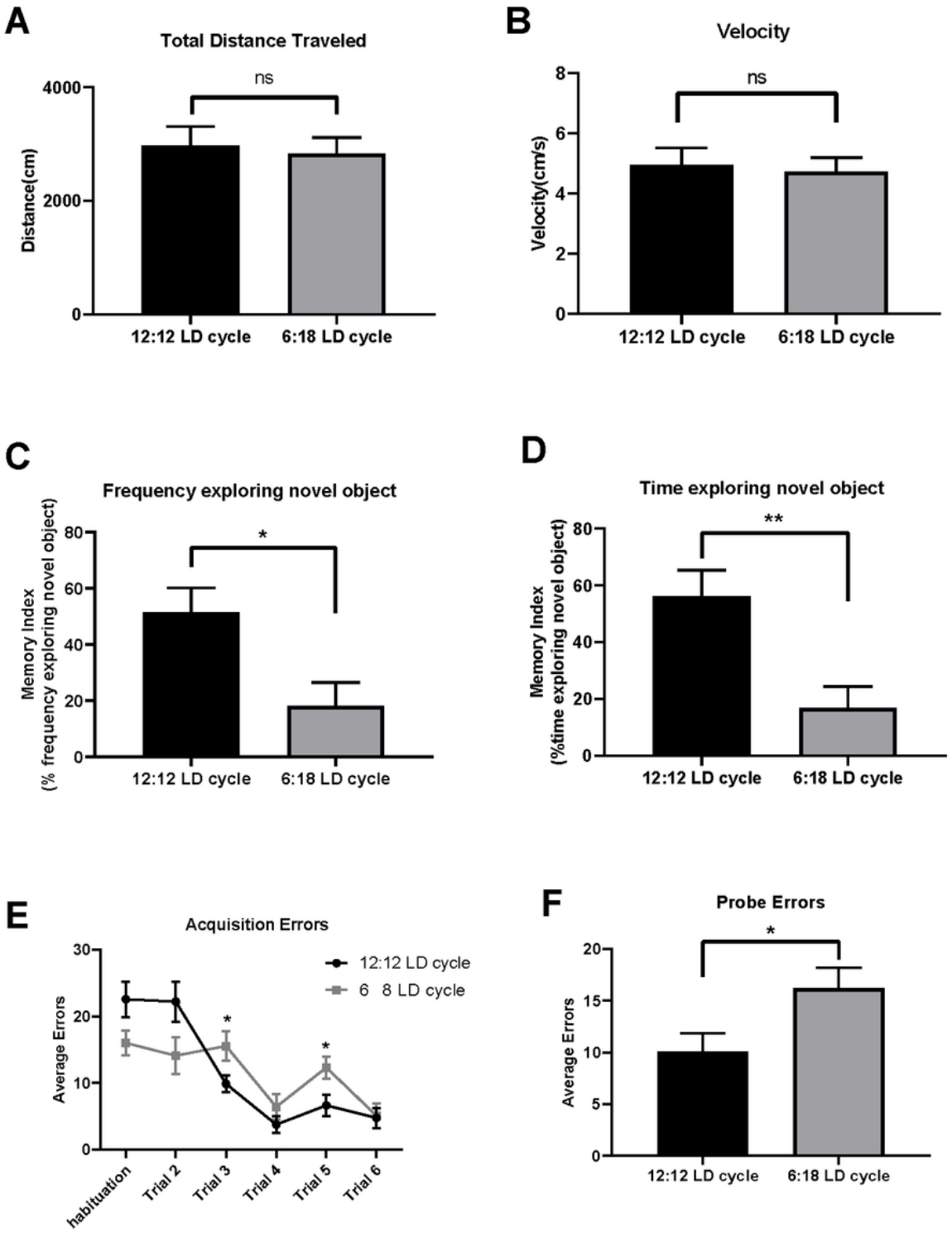

Figure 3

Circadian disruption leads to decreased cognitive ability No significant difference between 12:12 LD cycle group and 6:18 LD cycle group for total distance traveled (A) or average velocity (B) were observed. The novel object recognition in the same open field in the next day. Circadian disrupted mice showed significantly poor performance than control mice in novel object exploration frequency (C) and duration (D).We subsequently assessed spatial memory of mice using a Barnes maze. More errors the treated

mice made in acquisition trials 3 and 5 compared with controls (E). After 24 h of rest, the mice of the 6:18 LD cycle committed more errors than 12:12 LD cycle in the probe trial (F), indicating reduced spatial memory in disrupted mice. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, n=15 in each group.

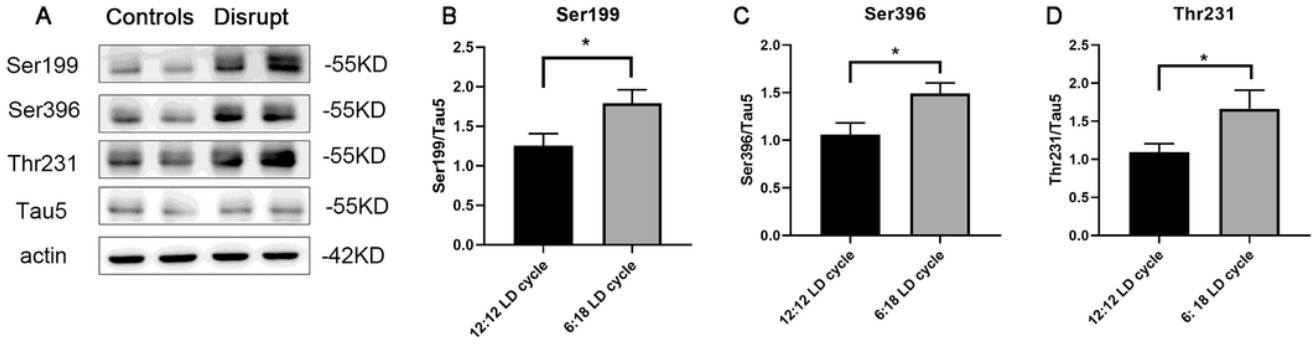

Figure 4

Circadian disruption increased the hyperphosphorylation of tau protein in hippocampus The phosphorylation levels of tau protein were detected in two groups (n=15 in each group). The AD-related tau protein sites (Ser199, Ser396 and Thr231) in the hippocampus were examined (A). The levels of tau protein were significantly hyperphosphorylated at the Ser199 (B), Ser396 (C) and Thr231 (D)site in the 6:18 LD cycle group. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

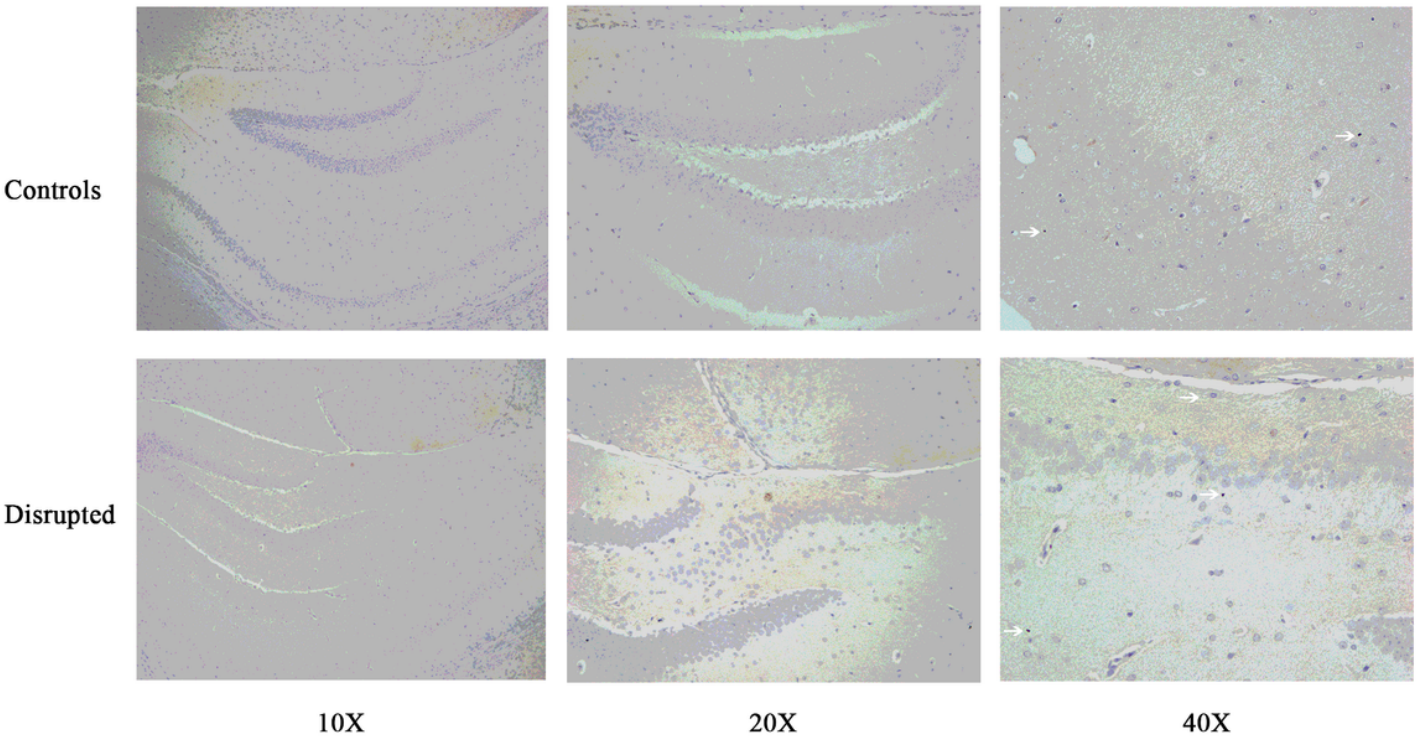

Figure 5

Immunostaining for Aβ 1–42 plaques in the CA1 region of the hippocampus The deposition of Aβ plaques in CA1 regions under 10x, 20x and 40x magnification were observed (n=3-5). However, no significance between two groups was found. *p < 0.05, **p < 0.01, ***p < 0.001.

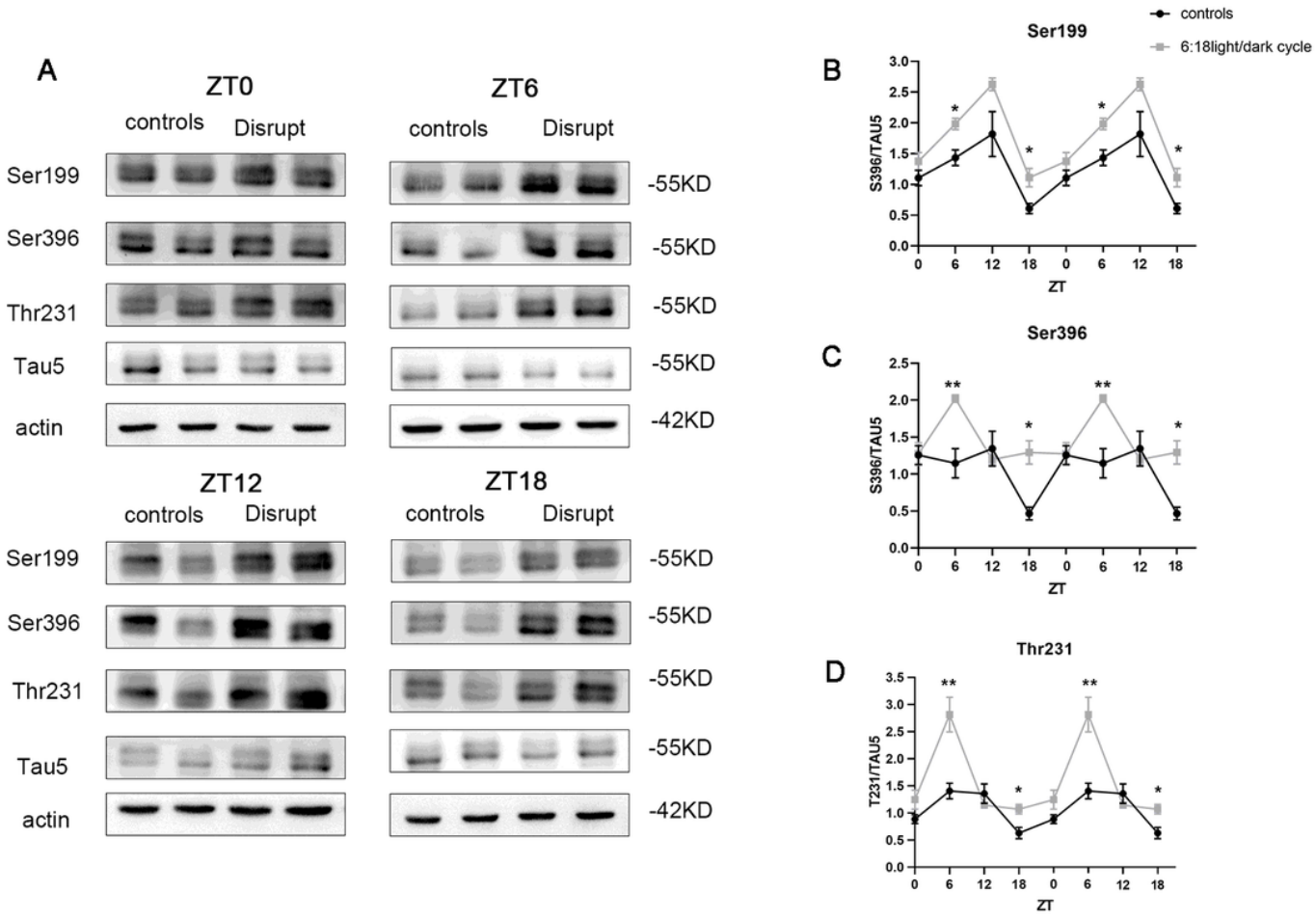

Figure 6

The phosphorylation of tau protein was regulated by circadian rhythm. The phosphorylation levels of tau protein at different time points (ZT0, ZT6, ZT12 and ZT18, n=3-4 in each group at different time points) between two groups were compared. At ZT0 and ZT12 time points, no difference of tau phosphorylation was discovered between the 6:18 LD cycle and the 12:12 LD cycle (A). At ZT6 and ZT18 time points, the phosphorylation levels was significantly increased in disrupted group among at Ser199 (B), Ser396 (C) and Thr231 (D) sites. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.