Understanding how sleep disturbance affects clock functions in the hippocampus and its consequences

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Background of research:

Alzheimer's disease (AD) is the most common age-dependent neurodegenerative disease and the leading cause of dementia (over 75% of all cases) worldwide(1). In 2010, the number of AD and dementia patients in China was 5.69 million and 9.19 million, respectively(2). According to the report from the Ministry of Civil Affairs of the People's Republic of China (PRC), in 2014, the population aged 60 years and over, who have a higher susceptibility to developing AD and dementia, was about 10% of the total population (~212 million), and this ratio is expected even to be higher over time due to decreased death rate(3). This suggests that the number of AD and dementia patients in China and worldwide will keep increasing due to the growing population over 60.

In the early AD stage, neurodegeneration starts in the hippocampus, resulting in functional disconnection of the hippocampus from other parts of the brain(4). The hippocampus located in the temporal lobe is divided into the hippocampal cornu ammonis (CA), dentate gyrus (DG), subiculum, and entorhinal area (EC). Inputs from the supramammillary nucleus of the hypothalamus move to EC and then to DG →CA3 → CA1 → subiculum → the rest of the brain(5). Neural circuits connecting these hippocampal areas perform the primary functions that are cognition and memory formation(5, 6). Therefore, hippocampal neurodegeneration in AD results in cognitive impairment and memory loss(4).

AD patients also show non-cognitive disabilities like sleep disturbance(7, 8). Recent studies have reported that sleep disturbance precedes AD pathology and influences the disease onset and progression. A potential mechanism to explain how is that the brain may remove toxic metabolic waste like amyloid-β (Aβ)40/42 more efficiently during sleep. So, inadequate sleep quality accumulates insults in the brain, accelerating the disease onset and progression(9, 10). While other studies reported that Aβ accumulation causes sleep disturbance in AD because they observed that the hypothalamus, where controls sleep cycles, and hippocampus were vulnerable brain areas for Aβ42 accumulation—these areas are liable to be injured in the early stage of AD(11). Although further studies are needed to understand their relationship better, these studies support that sleep is associated with AD and hippocampus function.

Sleep patterns are controlled by the suprachiasmatic nucleus (SCN) in the hypothalamus(12). The SCN is the pacemaker of circadian rhythms—all daily rhythms in biological processes(13). Circadian rhythms are autonomously generated by internal molecular machinery called circadian clocks, built on a transcriptional-translational negative feedback loop(14-16). The circadian transcription factor BMAL1 is a core of circadian clocks. It forms a heterodimer with CLOCK to transcribe thousands of target genes (clock genes), including own inhibitors Period (*Per*) and Cryptochrome (*Cry*)(17, 18). After PER/CRY complexes suppress BMAL1/CLOCK activity, PER/CRY turnover ends the negative feedback loop of the cycle and allows the initiation of a new cycle(19). Thus, physiological processes operated by clock gene products, including the sleep-wake cycle, show circadian rhythms in their activities based on gene expression patterns, thereby maintaining homeostasis(20).

The SCN mainly generates and synchronizes circadian rhythmic sleep patterns, but altered sleep patterns also affect SCN functions(21). In other words, sleep disturbance interrupts synchronization between L/D cycles and SCN clocks. This desynchronization alters circadian rhythmic activity of SCN clocks, thereby subsequently changing non-SCN clocks in the body. Although underlying mechanisms are still not clear, recent studies reported evidence that sleep disturbance altered not only SCN neuron activity but also reduced circadian amplitude of clock gene expression in peripheral tissues like blood(21, 22). This means that SCN clocks and sleep patterns reciprocally affect each other, resulting in further changes in non-SCN clocks in the brain and body.

This project will study the mechanisms of how sleep disturbance affects circadian clocks in the hippocampus and its consequences. Why do we focus on circadian clocks and sleep disturbance effects in the hippocampus? Abnormal circadian phenotypes from molecular clocks to sleep patterns and hippocampal damage are detected from the early AD stage(4, 7, 8). Without understanding

mechanisms, many clinical trials tried to restore abnormal circadian phenotypes as physiological rhythms by light therapy and melatonin administration (a well-known circadian hormone) to improve disease symptoms. However, most of them ended with no significant effects(23). These failures suggest that abnormal circadian phenotypes result from multiple underlying mechanisms with complex roles. This means that their targeting must be more specific than restoring all clocks as physiological rhythms. For this, we need to know the following. 1) Among altered clocks and sleep disturbance, which one is a cause/mediator of neurodegeneration? Or are both of them simply consequences of neurodegeneration? 2) Are circadian clocks in which cell types affected, and what are the consequences of their alterations? 3) Do they (altered clocks and sleep disturbance) have unexpected roles in the damaged hippocampus? Unfortunately, current studies cannot answer these questions due to the knowledge gap. Thus, we propose this project to understand the relationships between circadian clocks, sleep, and hippocampus functions in neurodegenerative conditions, answering the above questions. Finally, completing this project will provide more precise targets to modulate specific clock functions to protect or repair the damaged hippocampus with its functional recovery.

Preliminary Results:

Neurodegeneration reduces BMAL1 levels in hippocampal neurons with different consequences.

The hippocampus is responsible for learning and memory and is damaged at the early stages of AD(4, 6). To mimic this condition, we induced neurodegeneration in the hippocampus by glutamate excitotoxicity. Note that glutamate excitotoxicity (exceeding amounts of the neurotransmitter glutamate damage neurons) is observed in various neurodegenerative diseases, including AD. We then confirmed well-known features of neurodegeneration in this condition—reduced neurons and increased reactive astrocytes and activated microglia (Figure 1).

To study roles of circadian clocks in neurodegeneration, we first checked the circadian rhythm in the hippocampus and found the core circadian transcription factor *Bmal1* expression peaked at circadian time (CT) 10 (Figure 2A). Thus, we decided to study BMAL1 in the hippocampus at CT10 as a representative of circadian clock activity—all experiments in this project will be performed at CT10.

Since we observed significantly reduced neurons in CA1 and DG areas in Figure 1, we first checked cell death by TUNEL assay. Unexpectedly, we observed TUNEL+ dead neurons only in the CA1 but not in the DG (Figure 2B). We next asked if BMAL1 also behaves in this unexpected way. We examined BMAL1 expression patterns in the same experimental conditions and found BMAL1 levels were reduced in the CA1 and DG both. This shows that neurodegeneration reduces BMAL1 (altered circadian clocks) in CA1 and DG areas, but BMAL1 is involved in different cellular processes—cell death in the CA but non-cell death-related roles in the DG. Based on this observation, we hypothesized that BMAL1 plays cell type specific roles in the hippocampus. Since BMAL1 in the DG is expected to play non-cell death-related roles, we wanted to further study this.

Granule cells with reduced BMAL1 increase NSC numbers in the DG by reducing BMAL1 in NSCs.

The DG is divided into the granule cell layer (GCL) with granule cells/neurons (GCs) and the subgranular zone (SGZ) with adult neural stem cells (NSCs)(24). Since NSCs maintain stemness, mostly they are not affected by glutamate excitotoxicity. Glutamates act on NMDA receptor (NMDAR) that are highly expressed in neurons but not in NSCs(25). Thus, GCs in excitotoxic DG lesions are the major source affecting NSCs. It is well-known that clocks in all cell types in the body directly or indirectly communicate with each other to synchronize, thereby orchestrating physiological processes. Thus, we tested whether reduced BMAL1 in GCs (GC-BMAL1) affects BMAL1 in NSCs (NSC-BMAL1) in neurodegenerative conditions. For this, neurodegeneration was induced in the hippocampus of Nestin-EGFP mice expressing enhanced green fluorescent protein (EGFP) in NSCs and progenitors to easily monitor NSCs in the SGZ. We observed increasing $EGFP^+$

NSCs in neurodegenerative conditions compared to the control (Figures 3A-3B). Then, BMAL1 levels were examined. In control, most $EGFP^+$ cells expressed BMAL1 ($BMAL1^+/EGFP^+$). However, in neurodegeneration, BMAL1 was significantly reduced in $EGFP^+$ NSCs ($BMAL1^-/EGFP^+$, Figures 3A and 3C). This suggests that reduced NSC-BMAL1 is involved in NSC regulation, such as proliferation, to increase cell numbers in neurodegenerative conditions.

We next asked whether reduced BMAL1 in EGFP+ NSCs is sufficient to increase cell numbers through proliferation. To answer this, we examined NSC states in *Nestin-Cre:Bmal1$^{f/f}$* mice, of which the Cre-lox system deletes *Bmal1* in Nestin-expressing cells. In other words, lower BMAL1 levels in both GCs and NSCs of *Nestin-Cre:Bmal1$^{f/f}$* mice mimic BMAL1 patterns in neurodegenerative DG. We examined cell proliferation (Ki67) and NSC (NESTIN) markers in *Nestin-Cre:Bmal1$^{f/f}$* mice. Compared to the control, significantly increased NESTIN $^+/Ki67^+$ cells were detected in *Nestin-Cre:Bmal1$^{f/f}$* mice (Figures 3F-3H). This is a similar result to what we observed in neurodegenerative DG. This means reduced NSC-BMAL1 is sufficient to increase NSC proliferation, and it can be a target to regulate NSC numbers in the hippocampus.

Sleep disturbance reduces BMAL1 in CA1 neurons and GCs.

Circadian clocks and sleep disturbance play reciprocal actions(21), and patients with neurodegenerative diseases like AD exhibit both phenotypes, clock alterations and sleep disturbance, as early symptoms(7, 8). However, we still do not know which one is a cause or consequence of neurodegeneration and whether sleep disturbance is involved in neurodegeneration through clock alterations or clock-independent manner. Thus, we decide to study the following. 1) Sleep disturbance effects on hippocampal circadian clocks—does sleep disturbance alter hippocampal clocks as similar to neurodegenerative conditions? From answering this question, we will know if sleep disturbance induces neurodegenerative conditions in the hippocampus via circadian clocks. 2) If hippocampal clocks are altered by sleep disturbance, which hippocampal cell types are affected and its consequences. 3) Examine whether altered clocks by sleep disturbance can be targets to improve hippocampus functions. To study this, we induced sleep disturbance for at least 6h during the daytime for 3 consecutive days in adult Nestin-EGFP mice, followed by immunostaining with anti-BMAL1 antibodies. Figures 4A-4B show that sleep disturbance itself (SD-Ctrl) reduced BMAL1 levels in CA1 neurons and GCs compared to the normal sleep cycle control (NS-Ctrl)—a similar pattern to neurodegeneration (NS-Excito-3D). However, the number of $EGFP^+$ NSCs was not increased in SD-Ctrl—no difference with NS-Ctrl (Figures 4A and 4C). This suggests that sleep disturbance mainly affects hippocampal neuronal clocks to mimic neurodegenerative conditions but may not have significant effects on NSCs. Based on this, we hypothesize that sleep disturbance reduces BMAL1 in hippocampal neurons to mimic neurodegenerative conditions, and this negatively affects hippocampal functions, cognition and memory formation. We will test this major hypothesis in this proposed project and understand underlying mechanisms.

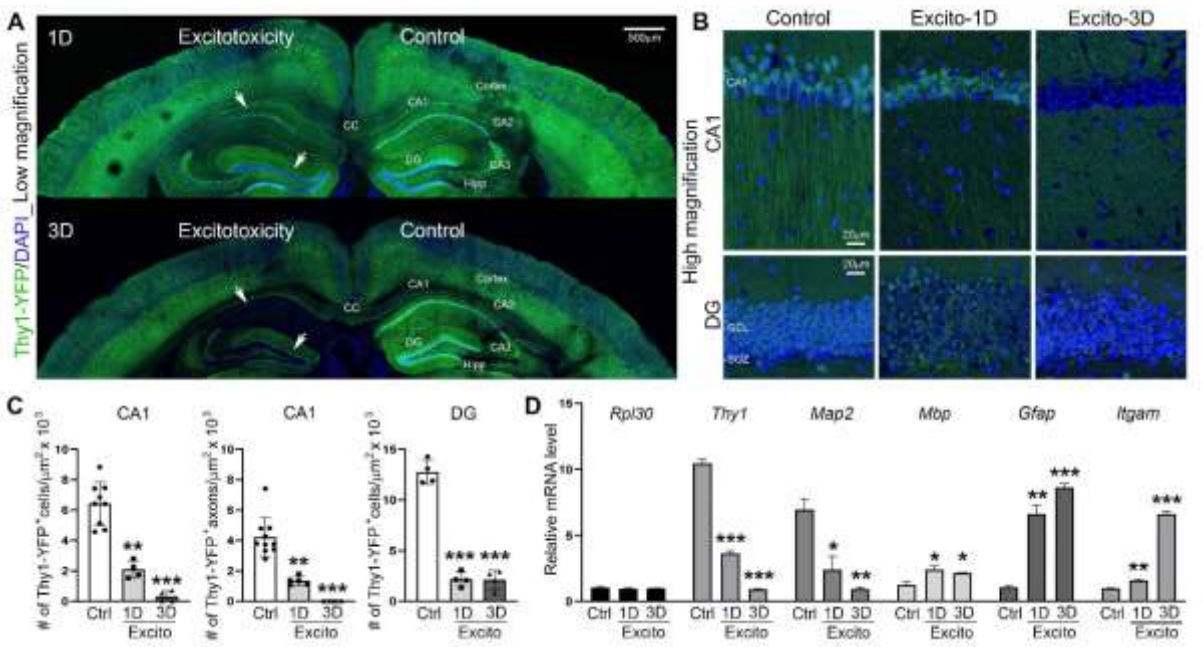

Figure 1. Excitoxocity-induced neurodegenerative conditions in the adult hippocampus. Reduced YFP+ neurons in the CA1 and DG of neruodegenerative hippocampus. (A) Confocal image of Thy1-YFP transgenic mouse brains in control and neurodegeneration on day 1 (ND-1D) and day 3 (ND-3D). (B) Cropped and amplified images of (A). DAPI (blue) was used as a nuclear counterstain. (C) Quantification of (B). (D) Effects of neurodegeneration on the transcription of neural cell markers (*Thy1*, *Map2*, *Mbp*, *Gfap*, and *Itgam*) and a control gene (*Rpl30*) in the hippocampus. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

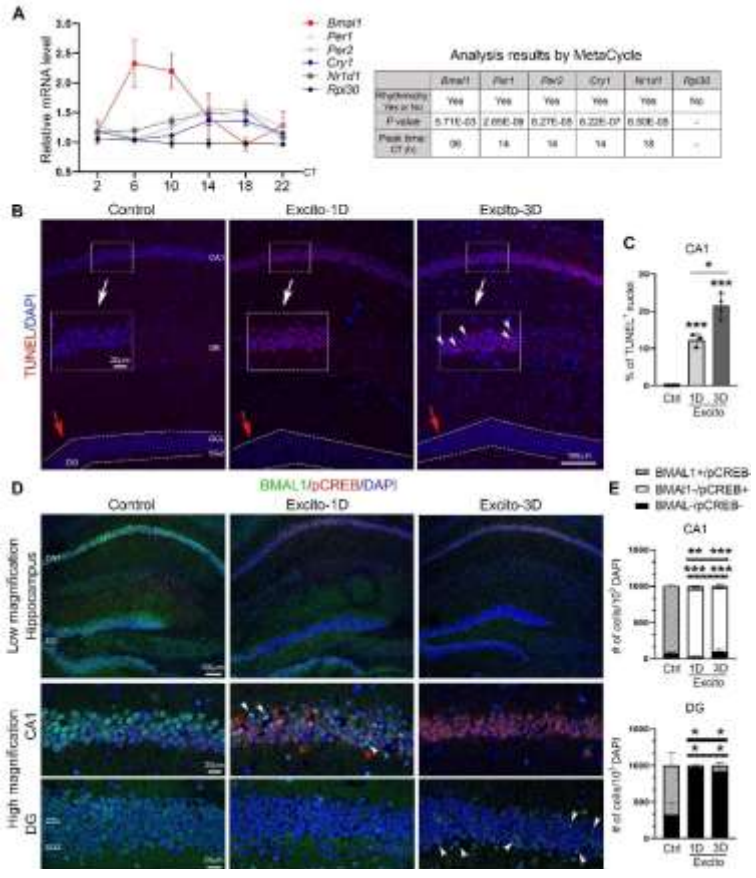

Figure 2. Neurodegeneration reduces BMAL1 levels in hippocampal neurons with different consequences. Neurodegeneration reduced BMAL1 in CA1 and DG cells but induce apoptosis only in CA1 neurons. (A) The transcription of clock genes in the hippocampus across the circadian cycle. (B) Confocal micrographs of control and neurodegenerative hippocampus (ND-1D and 3D), stained with TUNEL (red). (C) Quantification of (B). (D) Confocal images of BMAL1 (green) and pCREB (red) in the control and ND. (E) Quantification of (D). * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

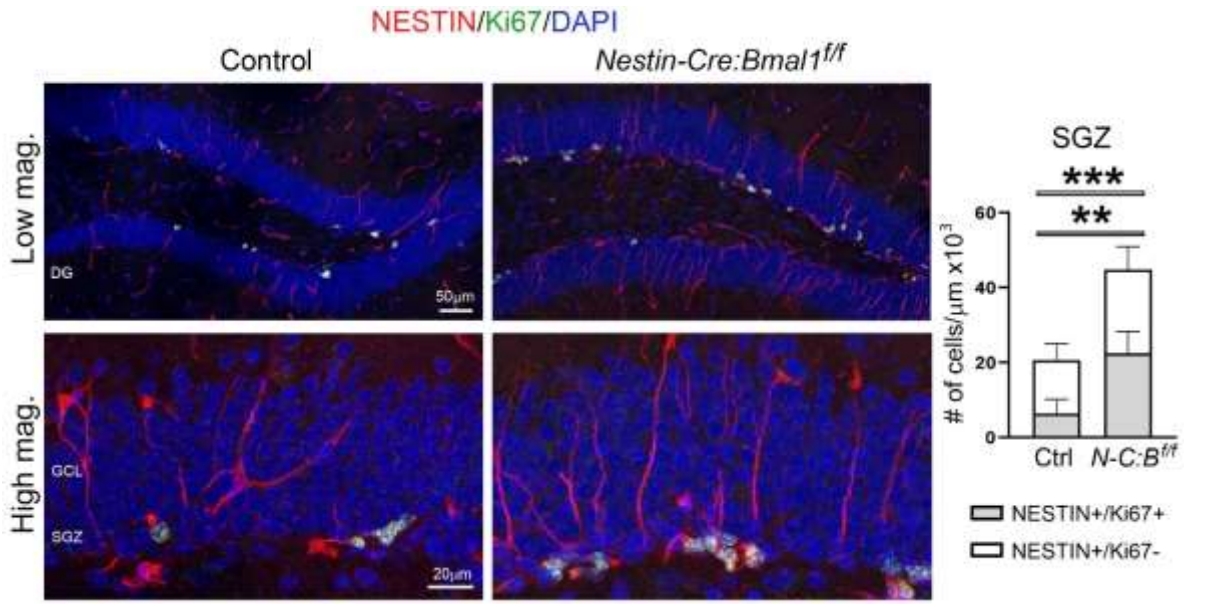

Figure 3. Granular cells with reduced BMAL1 subsequently reduce NSC-BMAL1 to increase NSC numbers in the neurodegenerative DG. (A) Confocal images of Nestin-EGFP transgenic mice in control and neurodegeneration (ND-1D and 3D), stained with anti-BMAL1 (green) antibodies. The white arrowhead in control indicates $BMAL1^{+}/EGFP^{+}$ cells. White arrowheads in ND-1D indicate $BMAL1^{-}/EGFP^{+}$ cells. (B-C) Quantification of (A). (D) Confocal images of the DG of control and *Nestin-Cre:Bmal1$^{f/f}$* (*N-C:B$^{f/f}$*) mice, stained with anti-NESTIN (red) and Ki67 (green) antibodies. Reduced BMAL1 in GCs and NSCs induces NSC proliferation. (E) Quantification of (D). * $P < 0.05$; ** $P < 0.01$; * $P < 0.0001$.

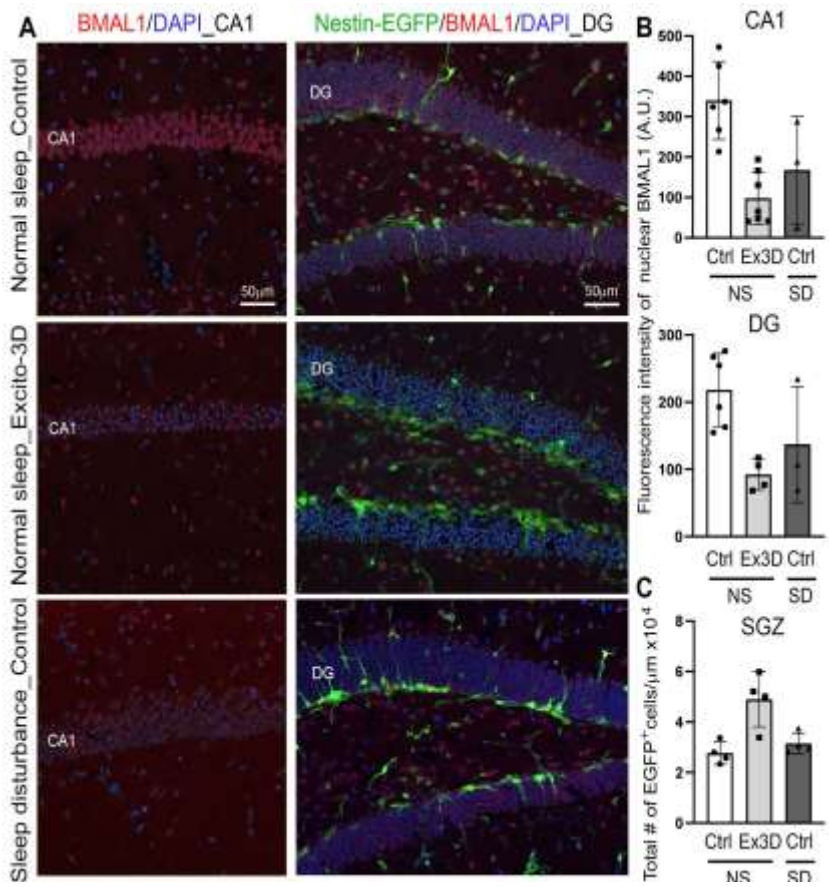

Figure 4. Sleep disturbance is sufficient to reduce BMAL1 in the hippocampal neurons, similar to neurodegenerative conditions. (A) Confocal images of the CA1 and DG of control and Nestin-EGFP mice, stained with anti-BAML1 (red) antibody. Sleep disturbance (SD) mimics neurodegenerative conditions in the hippocampus by reducing BMAL1 levels in CA1 neurons and GCs, although the effects are less significant than excitotoxicity-induced neurodegeneration (ND-3D). (B-C) Quantification of (A). NS, Normal sleep. * $P < 0.05$.