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TITLE:

A Chronic Sleep Fragmentation Model Using Vibrating Orbital Rotor to Induce Cognitive Deficit and Anxiety-Like Behavior in Young Wild-Type Mice

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KEYWORDS:

chronic sleep fragmentation, orbital rotor, cognitive deficit, anxiety-like behavior, obstructive sleep apnea, neurodegenerative diseases

SUMMARY:

Presented here is a protocol for chronic sleep fragmentation (CSF) model achieved by an electrically controlled orbital rotor, which could induce confirmed cognitive deficit and anxiety-like behavior in young wild-type mice. This model can be applied to explore the pathogenesis of chronic sleep disturbance and related disorders.

ABSTRACT:

Sleep disturbance is generally common in populations as a chronic disease or a complained event. Chronic sleep disturbance is proposed to be closely linked to the pathogenesis of diseases, especially neurodegenerative diseases. We recently found that 2 months of sleep fragmentation initiated Alzheimer's disease (AD)-like behavioral and pathological changes in young wild-type mice. Herein, we present a standardized protocol to achieve chronic sleep fragmentation (CSF). Briefly, CSF was induced by an orbital rotor vibrating at 110 rpm and

operating with a repetitive cycle of 10 s-on, 110 s-off, during light-ON phase (8:00 AM–8:00 PM) continuously for up to 2 months. Impairments of spatial learning and memory, anxiety-like but not depression-like behavior in mice as consequences of CSF modeling, were evaluated with Morris water maze (MWM), Novel object recognition (NOR), Open field test (OFT) and Forced swimming test (FST). In comparison with other sleep manipulations, this protocol minimizes the handling labors and maximizes the modeling efficiency. It produces stable phenotypes in young wild-type mice and can be potentially generated for a variety of research purposes.

INTRODUCTION:

Sleep disturbance is increasingly common both in patients with sleep-disturbing conditions and healthy people with sleep-disturbing events. It has been observed that, patients with neurodegenerative diseases, chronic pain, emotional stress, respiratory system diseases, urinary system diseases, etc., usually complain about unpleasant sleep experiences[1-5]. Obstructive sleep apnea (OSA), periodic limb movements in sleep (PLMS), sleep maintenance insomnia among other sleep disorders are the most common causes, which induce sleep fragmentation[6,7]. In developed countries, OSA has over 5% to 9% prevalence in adult population and 2% in child population[8-10]. Meanwhile, there is an increasing proportion of the healthy population experiencing sleep disturbance due to the overuse of smart phones, irregular sleep habits, annoying noises, and work duties, such as night shifts for caregivers. Sleep is acknowledged to be important for brain waste clearance[11,12], memory consolidation[13,14], metabolic balance[15,16], among many other physiological processes. Yet, it still remains largely unknown whether long-term sleep disturbance gives rise to irreversible pathogenesis alterations in healthy human beings, and whether it is the etiology or a contributing factor of developing central nervous system diseases, such as neurodegenerative diseases in a couple of years down the road. Our goal is to report an experimental model that generates stable and evident cognitive deficit and anxiety-like behavior in young wild-type mice after a 2-month sleep fragmentation treatment. This model would be applied for answering the scientific questions listed above.

Sleep disturbance is listed as a potential risk factor for developing Alzheimer's disease (AD) or dementia. Kang et al. first found and described the exacerbation of AD pathology by 6 h acute sleep deprivation[17]. Thereafter, many other studies reported that sleep deprivation or fragmentation could aggravate pathogenesis in transgenic AD mice models[18-20]. However, very few researchers have studied the consequence of sleep disturbance in young wild type mice – whether sleep disturbance gives rise to AD-like behavior or pathological changes in young wild-type mice. In our recent publication, we reported that 2 months of sleep fragmentation induced evident spatial memory deficit and anxiety-like behavior, as well as increased intracellular Amyloid-β (Aβ) accumulation both in cortex and hippocampus in 2–3 month-old wild-type mice[21]. We also observed altered expression levels of endosome-autophagosome-lysosome pathway markers and microglia activation, which was similar to the pathological changes reported in APP/PS1 mice[21,22].

This presented sleep fragmentation (SF) protocol was validated by Sinton et al.[23] and modified by Li et al.[24]. In brief, an orbital rotor vibrating at 110 rpm interrupts sleep for 10 s every 2 min during light-ON phase (8:00 AM–8:00 PM). Sleep structure alteration in this model was

previously characterized with electrophysiological sleep recordings and reported by Sigrid et al. [24], indicating a significant increase in the wake time and decrease in rapid eye movement (REM) sleep during the light-ON phase, with the total sleep and wake times (in 24 hour) unaffected after more than 4 weeks' modeling[24]. Currently, total sleep or partial sleep deprivation are the most commonly used sleep manipulation models. Total sleep deprivation is usually performed by sustained gentle handling or exposing the animal to novel objects, alternatively by continuously rotating a bar or a running treadmill[25-29]. Due to ethical reasons, total sleep deprivation is usually shorter than 24 h. The most commonly applied partial sleep deprivation model is the water platform method, which primarily ablating REM sleep[30-32]. Other approaches using either a treadmill or a bar that sweeps along the bottom of the cage, could induce sleep fragmentation when set on at fixed intervals[33-38]. It is noteworthy that SF interrupts sleep and intermittently causes arousals across all sleep stages[24]. One of the prominent advantages of this CSF model applying orbital rotor is that it can be performed continuously for months automatically controlled by machines, which avoids frequent processing labor daily except for regular monitoring. Furthermore, the apparatus would allow to simultaneously model multiple cages of mice under uniformed interventions. During entire modeling sessions, mice are housed in their home cages with usual bedding and nesting materials, while some other methods require exposure to diversified environments and inevitable stress.

Sleep fragmentation was previously characterized by the sleep manipulation method, which mimics frequent arousals during the sleep phase and substantial sleep rebound during the wake phase. In some literatures, CSF was regarded as the animal model for OSA[39,40]. In this study, the rationale of the chosen frequency of arousal to be 30 times per hour is based on the observation of arousal indices in patients with moderate-to-severe sleep apnea. It was observed that 4 weeks' sleep fragmentation significantly increased hypercapnic arousal latency and the tactile arousal threshold, which could at least last 2 weeks after recovery[24]. This phenotype was explained by revealing *c-fos* activation reduction in noradrenergic, orexinergic, histaminergic, and cholinergic wake-active neurons in response to hypercapnia, as well as reduced catecholaminergic and orexinergic projections into the cingulate cortex[24]. However, it is necessary to note that the most important feature in OSA is hypoxia caused by airway obstruction, which results in sleep disruption[41,42]. Sleep disturbance and repetitive hypoxia reciprocally interact with each other in OSA pathogenesis. Therefore, sleep fragmentation alone might not be able to fully demonstrate all key features of OSA in mice.

Herein, we present a standardized protocol to model chronic sleep fragmentation in young wild-type mice. Cognitive deficit and anxiety-like as well as depression-like behaviors after CSF treatment were evaluated by Morris water maze, Novel object recognition, Open field test, and Forced swimming test. It is important to note that this model should be taken as a whole that generates phenotypes of dysregulated sleep pattern, cognitive deficit, and anxiety-like behavior. The current model could potentially be applied, but not be limited, to the following purposes: 1) Further investigating the functional or molecular pathogenesis mechanisms induced by chronic sleep disturbance in young mice without genetic predisposition, 2) Identifying the direct pathway leading to neurodegeneration initiated by sleep disturbance, 3) Exploring the therapeutics for improving phenotypes induced by chronic sleep disturbance, 4) Studying the

intrinsic protective/compensatory mechanisms in wild-type mice upon chronic sleep disturbance, 5) To be applied for studying sleep-wake regulation and state-transition mechanisms.

PROTOCOL:

This protocol was approved by the Institutional Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

1. Mice screening and preparation for the experiment

1.1 Select wild-type adult (8–10 weeks old) male mice with weight of 20–28 g for the whole experiment.

NOTE: Wild-type C57BL/6 mice are obtained from the Hubei Research Center for Laboratory Animals, Hubei, China.

1.2 Randomly assign all mice to the CSF and the control group. House 3–5 mice in each cage to avoid social isolation stress. The number of mice housed in the control cages is matched with that housed in the paired CSF cages.

NOTE: Mice in the same group cages are pooled to perform follow-up behavioral experiments.

1.3 Locate the control cages in the same room with the CSF cages, to keep the surrounding environment and labor effects identical.

1.4 Number and mark the mice in each group on their ears using an ear tag for monitoring purposes.

1.5 Maintain ambient temperature and humidity between 21–23 °C and 35%–60 %.

1.6 Maintain the ambient environment in 12-hour light-dark cycle (8:00 AM–8:00 PM light-ON, 8:00 PM–8:00 AM light-OFF), to avoid biased effect on normal sleep rhythm in mice.

1.7 Minimize the noise and interference while the researcher is present in the modeling room.

1.8 Provide mice with sufficient food and water. Use long nozzles with ball valve tips on water bottles, to prevent water leakage upon the platform movements. Fasten the water bottle on top of the cage with a spring to avoid the dislocation of the bottle during rotor running.

2. Preparation and setting of the orbital rotor

2.1 Prepare an electrically controlled orbital rotor with enlarged platform (67 cm x 110 cm), on which 10 cages can be placed at most.

2.2 Set the orbital rotor on during light-ON phase (8:00 AM–8:00 PM) controlled by a program timer, which is the time when mice exhibit the majority of their daily sleep.

2.3 Set the orbital rotor with a speed of 110 rpm and a repetitive cycle of 10 s-on, 110 s-off controlled with a solid-state timer.

NOTE: The load capacity of the platform is 50 kg. The fixed amplitude of the rotor horizon vibrating is 2.5 cm.

2.4 Fasten the CSF cages on top of the rotor platform by thick springs to prevent dislocation of cages upon platform rotations.

3. Chronic sleep fragmentation modeling and monitoring

3.1 Place cages of the CSF and the control mice into the modeling room for one week prior to experiments, to let mice adapt to the ambient environment.

3.2 At the beginning of the modeling, ensure that all mice have free access to food and water during orbital rotations.

3.3 At the beginning of the modeling, observe at least for 1 h to ensure the orbital rotor operating in gear.

3.4 During the period of modeling, check that the orbital rotor is operating properly and mice conditions every 2 days to ensure mice have enough food and water. Change beddings of cages weekly.

3.5 During the period of modeling, weigh the mice weekly at 8:00 AM when changing the bedding. Remove the mice with significant weight loss from the modeling and, also the experimental groups.

NOTE: Significant weight loss is defined as weighing less than 20 g lasting for 2 weeks.

3.6 During the entire modeling sessions, remove the aggressor, if any, from the cage and, also from the experimental groups.

3.7 After the termination of modeling, continue to maintain and feed the mice in the original room.

4. Morris water maze (MWM) test

4.1 Preparation for the test

4.1.1. Prepare the apparatus of a circular tank filled with warm water (20–23 °C).

4.1.2 Suspend four signs with different shapes and colors on the curtain surrounding the tank in four quadrant directions as the distant vision reference. Make the water to appear opaque by the addition of powdered milk.

4.1.3 Locate a platform in the middle of the southwest quadrant.

4.2. The training test

4.2.1 Subject mice to four consecutive trials between 8:00 AM and 12:00 AM each day over a 5-day training period.

4.2.2 Release each mouse into the water facing the sidewall at one of four quadrants in four trials. In each trial, allow the mouse to swim for 60 s to find the platform. If the mouse is unable to arrive at the platform within 60 s, guide it to the platform and to remain there for 15 s.

4.2.3 Use a video tracking system to automatically record the escape latency of mice to find the hidden platform.

4.3. The probe test

4.3.1. Conduct the probe test on the sixth day after 5 training days.

4.3.2. Remove the platform. Release each mouse from the northeast quadrant and allow it to swim for 60 s

4.3.3. Use a video tracking system to automatically record the track data of mice.

5. Novel object recognition (NOR) test

5.1. The familiar phase

5.1.1 Place mice in a tank (length 30 cm, width 28 cm, height 35 cm) in sequence, which contains two copies of objects (A1 and A2). Allow the mice to explore freely (10 min per trial).

5.1.2 Use a video tracking system to automatically record the track data of mice.

5.2. The test phase

5.2.1 Conduct the test trial after a 1 h delay of the familiar phase. Replace one of the original objects by a novel object (“novel”) in the tank keeping the other one unchanged. Return the mice to the tank and allow it to explore for 5 min per trial.

5.2.2 Use a video tracking system to automatically record the time spent in exploration of each object by each mouse.

NOTE: The exploration of the object is determined by licking, sniffing, chewing, or moving vibrissae while orienting the nose toward and less than 1 cm from the object. The Discrimination Index (DI) is calculated with the equation (TN − TF)/(TN + TF), where TN = time spent exploring the “novel” object and TF = time spent exploring the “familiar” object.

6. Open field test (OFT)

6.1 Prepare the apparatus of a tank (30 cm x 28 cm x 35 cm).

6.2 During the test, place each mouse into the center of the tank and allow it to explore freely for 5 min. Clean the tank with 75% ethanol after each trial to avoid the leftover effects of the previous mouse.

6.3 Use a video tracking system to automatically record the track data of mice.

7. Forced swimming test (FST)

7.1 Prepare the apparatus of an open cylindrical vessel, which contains water (20–23 °C) that is 15 cm deep.

7.2 During the test, place each mouse into the cylinder and allow it to remain there for 6 min.

7.3 Use a video track system to automatically record the immobility time during the last 4 min of the test by each mouse.

NOTE: The mouse is determined to be immobile when it stops struggling and floats in the water, making only movements which are necessary to keep its head above water.

8. Data Analysis

8.1 Analyze data using statistical analysis software (e.g., GraphPad Prism 6.0).

8.2 Express all data as the mean ± SEM.

8.3 Compare the escape latency in MWM test between two groups using two-way ANOVA with repeated measures followed by Bonferroni posttests. Other comparisons between the CSF and the control groups are determined by unpaired t tests.

8.4 Consider differences significant if $P < 0.05$ in all tests.

REPRESENTATIVE RESULTS:

All the representative results and figures were reproduced from our recent publication[21]. The reuse of the figures was permitted by the original journal.

The entire experimental design is illustrated in the order of time, which indicates the timing of CSF modeling, behavioral tests of MWM, NOR, OFT, and FST (**Figure 1A**). We obtained weights of mice every week from the CSF and the control groups, to monitor their general conditions during the modeling sessions. No evident difference was found in the weight increase in mice between two groups during the modeling (**Figure 1B**).

To evaluate the effects of CSF on spatial learning and memory performance, we conducted MWM behavioral trial[43,44]. The CSF group displayed poorer escape capacities to find the platform throughout 5 training days in comparison with the control group (**Figure 2A**). In the probe test, the CSF mice spent significantly less time proportion in the targeted quadrant and crossed the previous platform location by fewer times (**Figure 2B,C**), without swimming speed difference (**Figure 2D**). These above results indicated that the spatial learning and memory retrieval capabilities of mice were impaired after CSF.

We also conducted NOR test to assess object recognition and short-term working memory after CSF[45]. In the familiar phase, there was no significant difference in the total exploration time between the CSF and the control group (**Figure 3A**). Correspondingly, no differences were found in the exploration time between objects A1 and A2, respectively in two groups (**Figure 3B**). The above results guaranteed that there were no differences in the mice's abilities for exploration and preferences for location. In the test phase, the Discrimination Index (DI) of the CSF mice was significantly reduced versus controls (**Figure 3C**), which evidently indicated deficits in object recognition and short-term working memory after CSF.

We further performed OFT and FST, respectively to examine anxiety-like and depression-like behaviors of mice[46,47]. Interestingly, in the OFT, it was found that the CSF group spent less time in the central zone than the control group (**Figure 4A**), which illustrated that sleep fragmentation could induce anxiety-like behavior to a certain extent. Additionally, CSF mice exhibited longer total distance moved in the tank (**Figure 4B**), suggesting increased spontaneous activity after modeling. Nevertheless, this CSF modeling could not induce depression-like behavior, verified by non-significant difference in the immobility time between two groups subjected to the FST (**Figure 4C**).

FIGURE LEGENDS:

Figure 1 The flowchart of experimental design procedure. (**A**) The experimental design procedure indicating the timing of CSF modeling and behavioral tests (i.e., MWM, NOR, OFT, and FST). (**B**) Body weight curves of the CSF and the control mice during the first month after the CSF model was established. This figure has been modified from Xie et al.[21]

Figure 2 CSF impaired spatial learning and memory abilities evaluated by MWM test. (**A**) The

CSF mice performed longer escape latency compared to the control mice during the 5-day training test. **p < 0.01. (**B**) In the probe test, the CSF mice exhibited less percentage time spent in the platform quadrant in contrast with the control mice. Upper panel shows representative tracings of two groups. ****p < 0.0001. (**C**) In the probe test, the CSF group performed less times of crossing the platform location when compared to the control group. *p < 0.05. (**D**) The swimming speed of two groups in the probe test. n.s. indicates changes between different groups were not significant. Data were all presented as mean ± SEM. n = 10 per group. This figure has been modified from Xie et al.[21]

Figure 3 CSF impaired object recognition and short time working memory evaluated by NOR test. (**A**) The total exploration time between the CSF and the control mice in the familiar phase, n.s. indicates no significant changes between different groups. (**B**) The exploration time for objects A1 and A2 respectively between two groups in the familiar phase. n.s. indicates no significant changes between different groups. (**C**) In the test phase, the Discrimination Index (DI) of the CSF group was significantly decreased compared to that of the control group. *p < 0.05. Data were all presented as mean ± SEM. n = 10 per group. This figure has been modified from Xie et al.[21]

Figure 4 CSF exacerbated anxiety-like but not depression-like behavior evaluated by OFT and FST. (**A**) The CSF mice spent less time in the central zone during the observed 5 min compared with the control mice in OFT. *p < 0.05. (**B**) The CSF group displayed longer total distance moved in the tank versus the control group in OFT. *p < 0.05. (**C**) The immobility time between the CSF and the control groups in FST. n.s. indicates no significant changes between different groups. Data were all presented as mean ± SEM. n = 10 per group. This figure has been modified from Xie et al.[21]

DISCUSSION:

Critical steps in the current protocol include setting up sleep fragmentation machines with the optimized parameters according to the study purpose and maintaining the mice in comfortable and quiet living environment throughout the entire modeling sessions. It is also crucial to decide the proper timing to interrupt or stop sleep fragmentation and arrange behavioral tests for those mice. Like other sleep manipulation models, it is important to perform the protocol in a dedicated room with controlled light cycles and void of all possible unnecessary interferences. Efforts should be taken to avoid inducing noises and minimize the operating time conducted by the researchers for checking, refilling food, and water supply, changing the beddings, etc. In rare occasions, there are aggressors attacking the littermates, especially at the initiation of uncomfortable sleep disruption sessions. The aggressor when present should be removed out of the home cages as well as the experimental groups. Most of the experimental animals except for a few to our experience, would adapt to the treatment and manage to access the water and food as needed. Mice with intrinsic problems, such as deformed teeth, underweight and skin wounds might cause weight loss or weakness. They also need to be avoided being used for the modeling. As this protocol could potentially induce chronic stress and metabolic dysregulation, it is essential to use mice screened with uniformed criteria, such as body weight, for modeling and experiments.

In the described protocol, the orbital rotor would be automatically turned on during 8:00 AM–8:00 PM (light-ON) daily, which is the time when mice exhibit most of their daily life. The rotor was set running on a repetitive cycle of 10 s-on, 110 s-off during light-ON phase to induce frequent arousals. Various modeling durations would give rise to different phenotypes. Acute sleep fragmentation could result in absolute reduction in sleep duration, increased sympathetic nervous system activities, such as elevated cortisone levels and impaired insulin sensitivity[23,24]. However, chronic sleep fragmentation showed unaffected cortisone levels, and balanced total sleep time[24]. Any modifications based of the current protocol, such as light cycles, matched vibrating settings (speed, amplitude, repetitive cycle, etc.) and modeling durations, could potentially alter the phenotypes. It is required to conduct sleep recording and sleep structure analysis under different modeling settings to identify the sleep phenotypes. It might also result in distinctive behavioral and pathological changes. As we explored the cognitive deficit after long-term rather than one-night sleep fragmentation and tended to avoid the biased effects of intermittent sleep fragmentation on mice behaviors in MWM and NOR, we performed these two behavioral tests after terminating the CSF protocol on day 60. However, inevitably, the effect of recovery sleep in mice might have confounded the results for MWM and NOR shown.

Although this model is entitled with sleep fragmentation model, it is actually composed of fragmented sleep patterns during the light-ON phase, dysregulation of circadian rhythm, and compensatory sleep rebound during the light-OFF phase. This protocol could induce not only sleep pattern alterations, but also substantial neuroinflammation, metabolic imbalance, immune system disturbance, etc[21,23,24]. All these pathological processes may interact with each other and mediate phenotypes like an orchestra. This model should be taken as a whole to generate the mice with phenotypes of dysregulated sleep pattern, cognitive deficit, and anxiety-like behavior in young wild-type mice. As mentioned in the previous section, this model is not exactly mirroring OSA due to lack of repetitive hypoxia. Another limitation is that it is difficult to generate accurate pathological changes and sleep phenotypes in the same mice. The widely applied EEG/EMG electrode implantation for sleep recording unavoidably induced severe gliosis in the cortex[48]. In recent years, video monitoring and image analysis techniques based on artificial intelligence were applied in sleep studies, which would collect precise sleep information without invasive electrode implantation[49-51].

The significances of this CSF method in comparison with existing methods include: 1) Different from sleep deprivation protocols that usually are performed for hours or days, the current protocol better mimics long-term sleep disturbance in healthy human beings. The compensatory sleep rebound in sleep fragmented mice perfectly mirrors the daytime somnolence and retardant working performance in people with poor sleep quality during the night[52,53]. 2) It is so far the only chronic sleep fragmentation model in young wild-type mice with confirmed cognitive deficit and anxiety-like but not depression-like behavior phenotypes, as well as evident molecular pathological changes in brain tissue. 3) This treatment causes milder irritations to mice so that the modeling could last for months, even with the possibility to be performed in longer periods of time. 4) With proper settings, this model can generate stable phenotypes of sleep disturbance, cognitive deficit, and anxiety-like behavior, which can be used

either as disease models or interventions for different study designs. 5) Some sleep deprivation models require full session interference by researchers to apply gentle handling or novel objects. Except for regular monitoring, this method minimizes handling labors, which also eliminates the artificial bias.

This CSF protocol provides the opportunity to answer a number of key scientific questions, such as, is chronic sleep disturbance the cause or consequence of neurodegenerative diseases? Is chronic sleep disturbance induced pathogenesis during young age reversible? Do the compensatory mechanisms upon chronic sleep disturbance vary between the young and elder people, healthy people, and patients? This protocol can also be applied to explore therapeutics by assessing the severity and improvement of the behavioral and molecular phenotypes. It would also be applied to model the mice with chronic craniectomy, optic fiber implantation preparations for functional recordings. Moreover, it can possibly be used as interventional strategy to induce or aggravate phenotypes on top of pre-existing conditions. Finally, it can be used for studying sleep-wake state transitional mechanisms. Interestingly, the current CSF model could induce anxiety-like rather than depression-like behavior in mice, which is in line with the clinical observation that the sleep disturbance in patients would likely be associated much more with anxiety than with depression[54,55]. It provides a practical model to study emotional disorders in rodents.

In summary, we present the protocol of modeling chronic sleep fragmentation by use of a vibrating orbital rotor, which could produce stable phenotypes in young wild-type mice and minimize the modeling labors with high efficiency. It can be potentially generated for a variety of research purposes.

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The authors declare that they have no competing financial interests.

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Figure.1

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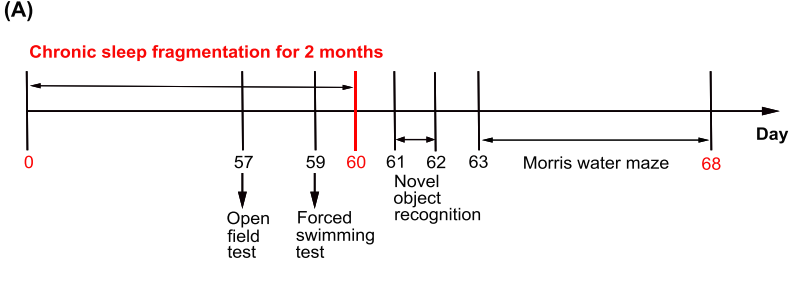

Figure.2

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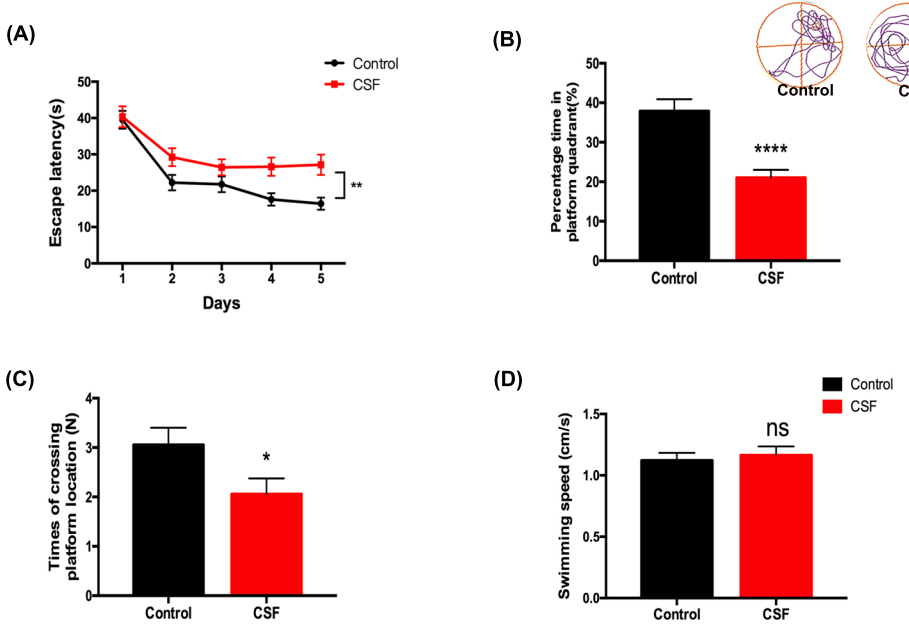

Figure.3

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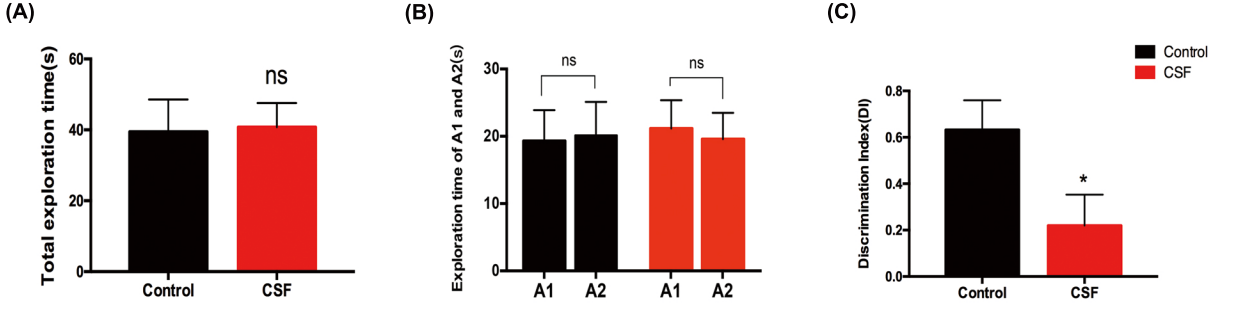

Figure.4

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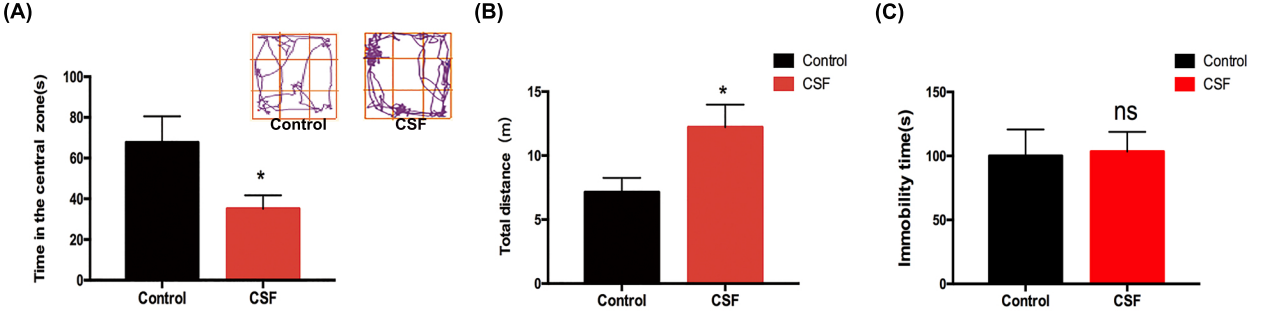

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Any-maze behavior tracking system	Stoelting,Inc,USA	-	A video-tracking system which was used to record the behavior track of mice.
C57BL/6J mice	Hubei Research Center for Laboratory Animals, Hubei, China.	-	healthy male C57BL/6J mice aged 10-12 weeks were purchased from Hubei Research Center for Laboratory Animals
Graphpad Prism 6.0 Software	Graphpad Software,Inc.USA	-	Graphpad Prism 6.0 software was used to draw statistical graphs.
Morris water maze system	Shanghai XinRuan Information Technology Co.,Ltd,China	XR-XM101	The system was used to perform Morris water maze test
Orbial rotor	Shanghai ShiPing Laboratory Equipment Co.,Ltd,China	SPH-331	The orbital rotor was used to establish the chronic sleep fragmentation model
Solid state timer	OMRON Corporation, Kyoto, Japan	H3CR-F8-300	The solid state time was used to control the frequency and time of the rotor running
Wooden Lusterless Tank	-	-	length 30 cm, width 28 cm, height 35 cm The tank was used to perform open field test and novel object recognition test

Dear Dr. Bajaj,

Thank you so much for offering us the opportunity for us to revise our manuscript, entitled "*The chronic sleep fragmentation model inducing cognitive deficit and anxiety-like behavior in mice*" (ID: JoVE61531R1). We really appreciate your insightful editing advices and the efforts in gathering all the reviewers' comments. It really helped us improve our manuscript substantially after making all the suggested edits.

The point-to-point answers towards all the critiques or comments are listed below. We also addressed in detail how and where the text was modified, with the corresponding amendments highlighted in red in the revised manuscript. This revision has been approved by all the co-authors for the resubmission.

Thank you for all your support.

Sincerely,

Fengfei Ding, M.D.
Aug 1st, 2020,

Editor

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

   Response: Thanks. We would retain and use the attached file for revision.

2. Please address all the specific comments marked in the manuscript.

   Response: Thanks for the kind reminder. We have addressed all the specific comments marked in the manuscript.

3. The manuscript needs thorough proofreading. Please use professional copyediting services if available.

   Response: Thanks for the comment. We have performed thorough proofreading and made corresponding improvements to the manuscript. We can pursue professional editing service if necessary.

4. Please ensure that the highlight is no more than 2.75 pages including headings and spacings.

   Response: Thanks for the kind reminder. We have checked and ensured that the highlight is no more than 2.75 pages including headings and spacings.

Reviewer#2

1. In Comment #4 from Reviewer#2 for the initial review process, this reviewer asked why Morris water maze (MWM) and the novel object recognition test (NOR) were performed after terminating the CSF protocol at day 60. This was

because the results presented in Figures 3 (NOR) and 4 (MWM) may be confounded by the effect of recovery sleep instead of chronic sleep fragmentation alone. For example, why there were no significant differences between the two groups in Figures 3A, 3B, and 4C may be because the CSF group had partially recovered from CSF effect. The statement of the authors on this point in the rebuttal letter is not clear enough. Although they described that "Open field test and Forced swimming test could be finished within a few hours, so that we performed these tests a few days prior to the termination of SF," the training phase or the habituation phase for MWM and NOR can also be finished within a few hours. Why cannot the mice be put back to the CSF protocol each day after the training/habituation for MWM and NOR? The authors should discuss in the manuscript whether it would be possible/better to perform MWM and NOR during CSF protocol to avoid the effect of recovery sleep. In addition, especially if it is not possible/recommended for some reason to perform MWM and NOR during the CSF protocol, the authors should discuss in the manuscript that the effect of recovery sleep might have confounded the results for MWM and NOR shown in Figures 3 and 4.

Response: Thanks for the insightful and helpful comments. We agree that conducting the behavioral tests after termination of sleep fragmentation treatment might involve the effect of sleep recovery. We included the discussion about the effect of sleep recovery for NOR and MWM based on the current protocol in manuscript. (**page 15, line 349** to **page 16, line 353**). Further studies can be conducted to compare the difference between protocols setting the sleep fragmentation termination time point before or after the behavioral tests. Based on the current protocol and experimental results, it suggests that the cognitive impairment as well as pathological changes resulted from chronic sleep fragmentation persists for at least 8 days after termination of the treatment. We also encourage to conduct the NOR and MWM tests in parallel instead of in sequential order to minimize the interference from the preceding test.

2. There are still minor grammatical errors in the manuscript, although in a much smaller number than in the original manuscript.

   Response: Thanks for the advice. We have performed thorough proofreading and improved the manuscript to our best.

Reviewer#3

1. Lines 73-74 "…modified by Sigrid [24]" - but [24] listed in the REFERENCES on line 511 is an article by Li et al (The last author is Sigrid Veasey.)

   Response: Thanks for the comments. We have revised this sentence in **(page3,line 75-76)**

2. Lines 166-167 and line 388 "…normally the sleep phase for mice." This is not correct. Mice typically exhibit ~60-70 percent of their daily sleep during the light phase, and the remainder during the dark phase. This should be correct to read "the time when mice exhibit the majority of their daily sleep."

   Response: Thanks for the advice. We have corrected the sentence in **(page 15,line 338-339)**

3. Line 197 "underweight of 20 g…" should read "weighing less than 20 g"

   Response: Thanks for the advice. We have corrected the sentence in **(page8, line 176)**

4. Line 374 "settled light cycle" could be replace with "controlled light cycles"

   Response: Thanks for the advice. We have replaced the "settled light cycle" with "controlled light cycles" **(page 14,line 324)**

Reviewer#4

1. In the introduction section, sentence "Cognitive deficit and anxiety-like but not depression-like behavior after CSF treatment were evaluated by Morris water maze, novel object recognition, open field test and forced swimming test." should be changed. Namely, authors evaluated anxiety as well as depression like behavior, but results showed presence of anxiety and not depression like behavior (Fig 4). Please correct this statement.

   Response: Thanks for the helpful advice. We are sorry for the confusion. We have corrected this statement as suggested in **(page 5,line 113-115)**

Re:RE: Asking for a letter

"Fengfei Ding" <francesding2016@163.com>

收件人："Zhou, Buddy" <bzhou@wiley.com>

时　间：2020-5-18 2:18:59

附　件：　image001.jpg　　下载附件

Dear Dr. Zhou,

Thank you so much for confirmation.

We will upload this email as supplementary element at our re-submission, which was required by the journal of JoVE .

Thank you so much for your support.

Best,

Fengfei

At 2020-05-12 07:57:30, "Zhou, Buddy" <bzhou@wiley.com> wrote:

> Dear Dr. Ding,
>
>
>
> Regards,

Buddy

Buddy Zhou

Managing Editor, CNS Neuroscience & Therapeutics

http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1755-5949

E-mail: bzhou@wiley.com

WILEY

From: Fengfei Ding <francesding2016@163.com>
Sent: 2020年5月12日 2:35
To: CNSNT <CNSNT@wiley.com>
Subject: Asking for a letter

This is an external email.

Dear Dr. Zhou,

It has been our great honor to be able to publish in your journal, CNS Neuroscience & Therapeutics. We really appreciate your tremendous support for our recent publication with you. https://onlinelibrary.wiley.com/doi/full/10.1111/cns.13218

We are delighted to tell you that this paper was well accepted by the readers and we got good feedbacks. The journal of JoVE recently invited us to publish a methodology paper related with our recent paper in your journal. The manuscript is now under review with JoVE editorial board. It reports the method details of modeling chronic sleep fragmentation and behavioral tests we performed. We were asked to inquire for the permission from you to let us present a few figures published in CNSNT. We are wondering if it is possible for you to email JoVE editorial board to give them the permission.

Otherwise, please let us know which would be the better way to proceed our potential publication in JoVE which would closely link with the our recent publication in CNSNT.

We regret if this matter brings you extra work. We sincerely appreciate your kind consideration and time in advance.

Thanks,

Fengfei