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Acute sleep deprivation aggravates nitroglycerin-evoked hyperalgesia in mice

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Abstract

Sleep deprivation can trigger migraine, and migraineurs often choose to sleep to relieve headaches during acute migraine. This study aimed to explore the effect of acute sleep deprivation on hyperalgesia induced by nitroglycerin in mice. In part one, after either 6-h sleep deprivation or 6-h normal sleep, mice were intraperitoneally injected with nitroglycerin or saline. The mechanical pain threshold and withdrawal latency of the hindpaw were measured every 30 min for 6 h. Next, the same sleep deprivation and injection procedure was performed with new mice, and mice were sacrificed 4.5 h after injection. The trigeminal nucleus caudalis and upper cervical spinal segments were taken for immunofluorescence Fos staining. In part two, after injection of saline or nitroglycerin, the mice were either deprived of sleep for 6 h or allowed to sleep without interference. The mechanical and thermal pain threshold were measured after 6 h. In part three, we compared the sleep time of mice after intraperitoneal injection of saline or nitroglycerin without interference. Sleep deprivation for 6 h did not cause any changes in the baseline pain thresholds in mice. However, pretreatment with 6-h sleep deprivation significantly prolonged the duration of hyperalgesia induced by nitroglycerin. Additionally, the expression of Fos at 4.5 h was significantly higher in the 6-h sleep deprivation and nitroglycerin group than in the other three groups. When intraperitoneal injection was given first, the mechanical pain threshold of the hind paw was significantly lower in the group that received nitroglycerin with 6-h sleep deprivation than in the other groups. Compared to the saline injection, one-time nitroglycerin injection would result in a significant increase in sleep latency and decrease in sleep duration for the normal mice. Acute sleep deprivation significantly aggravated the hyperalgesia induced by nitroglycerin in mice, which highlights the importance of sleep disorders for migraine.

Keywords

migraine, sleep deprivation, nitroglycerin, hyperalgesia

Introduction

Epidemiologic studies have shown that the one-year prevalence rate of migraine is 15%–18% worldwide and 9.3% in China.[1,2] According to the WHO, migraine has now become the first disabling disease for people under 50, and it puts a heavy burden on patients financially and psychologically.[3,4] Sleep has the effect of maintaining homeostasis and optimizing the functions of multiple physiological systems. Insufficient sleep is increasingly recognized as a public health problem.[5] In the United States, there are 50 million to 70 million people troubled by sleep disorders, and 30% of employees do not sleep long enough on average to meet their basic needs.[6]

Sleep is closely associated with migraine clinically. In our previous work, patients with sleep disorders had a significantly higher risk of migraine.[7] It was also reported that approximately 85.9% of migraineurs have sleep quality that is worse than that of those without headache.[8] Currently, sleep disorders are regarded as risk factors for migraine chronification.[9,10] However, it remains unknown how sleep influences the phase of migraine.

Sleep deprivation (SD) is a kind of sleep disorder. In the clinic, SD is one of the common triggers of migraine.[11]

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During acute migraine, patients often choose to sleep for relief of the headaches.[12] Therefore, we wondered whether SD would influence the extent and duration of acute headaches.

Intravenous nitroglycerin (NTG) can reliably trigger headache in migraineurs and healthy human subjects.[13] NTG-evoked hyperalgesia in rodents has been widely used in basic research on migraine.[14] In the current study, NTG-evoked migraine-related hyperalgesia in mice was observed before and after acute SD. The effect of one-time NTG injection on the sleep time of normal mice was also explored. We found that acute SD significantly aggravated the hyperalgesia induced by NTG in mice.

Methods

Material

Male C57BL/6 mice, weighing between 20 and 30 g, were purchased from SipeiFu Biotechnology Co., Ltd. (Beijing). Mice were kept in the animal room at 22°C. Food and water were freely available. The light cycle was controlled, and lights were on for 12 h every day (lights on at 9:00 and lights off at 21:00).

The whole process of the experiment met the requirements of the ethics committee.[15] The whole experimental process maintained care for the animals and minimized the pain of the animals.

Acute sleep deprivation procedure

As previously reported, we continually provided novel objects, such as small paper balls, plastic balls, and gauze, into the home cages of the mice; the mice bit and chewed these objects rather than sleeping.[16,17] During this process, to minimize the stress, we did not touch the mice. On the test day, the experimenter constantly monitored the mice in their cages. When the mice were observed to be awake, no intervention was required. When a mouse remained stationary for more than 1 min, fresh objects were put into its cage without touching the mouse. When a mouse rested again for more than 1 min, new objects were provided. If the improved fresh material could not keep it awake, it was necessary to tap on the cage. The total duration of SD was 6 h. Food and water were available during those 6 h.

Behavioral test

Before the experiment day, mice were placed into the test cage for 30 min every day to habituate. To avoid expectation bias, all the behavioral tests were conducted by a researcher who was blinded to the groups.

Mechanical allodynia

The mice were placed in individual cages with wire mesh on the bottom. Von Frey filaments were applied to the sole of the hindpaw. The measurement was performed as previously described.[18,19] The experimenter bent the von Frey filaments into a C shape, maintained contact for 6–8 s, and observed whether the mouse withdrew or licked its hind paw. Then, 5 stimulations were repeated with an interval of 20 s each time. If withdraw or licking occurred 3 out of 5 times, it was considered positive. The scale from small to large was applied until a positive result appeared. This was how the mechanical pain threshold of the mice was determined.

Thermal hyperalgesia

The hot plate produced by Shenzhen Reward Technology Life Co., Ltd. was used to assess the thermal pain threshold of the mice. Before the test, the experimenter turned on the hot plate and adjusted the temperature to 52°C. When the temperature remained stable, the mice were put on the hot plate, and the button on the instrument was pressed to start timing. After the behaviors of hind paw withdrawal or licking were observed, the timing was stopped, and the mice were immediately removed from the hot plate. The withdrawal latency was regarded as the thermal pain threshold of the mice.

Groups

The test consisted of three parts (Figure 1). NTG-evoked migraine-related hyperalgesia in mice was observed both before and after acute SD. Besides, the sleep time of mice after intraperitoneal injection of saline or NTG without interference was compared.

In part one, SD was started at 9 a.m. on the test day. The control groups were kept under the same conditions with the lights on without any interference. 6 hours later, a behavioral test was performed, and the baseline level was recorded. Then, the mice in each group were injected with NTG (5 mg/kg) or saline (5 mg/kg).[20] Behavioral tests were performed every 30 min after the injection.

In part two, mice in each group were injected with NTG (5 mg/kg) or saline (5 mg/kg) after the behavioral tests at 9 a.m., and then, SD started. 6 hours later, behavioral tests were conducted again.

In part three, mice were deeply anesthetized with avertin (20 ul/g intraperitoneally) and mounted on a stereotaxic instrument. After head and neck was shaved, the electrodes of electroencephalogram (EEG) were inserted into the skulls, and the electrodes of electromyogram (EMG) were fixed into the nuchal muscles. Subsequently, the mice were housed separately for a week. On the test day, mice were put into a clean cage for 2-h habituation after 9 am. Then, recordings were continued for 6 h since saline was injected. The mice were kept without any interference throughout the test. On the second day, the same mice were put into the cage at the time point as previous day for habituation. The 6-h recordings were sustained after NTG was injected. Polysomnographic signals were collected by the data acquisition system

(Pinnacle Technology Inc.), and data of sleep was analyzed by the software (MATLAB 2020b).

Tissue preparation and immunohistochemistry

The mice were euthanized with an i.p. injection of pentobarbitone (200 mg/kg) and transcardially perfused with cold, fresh saline, followed by 4% formaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for fixation. The brain and upper cervical spinal cord were dissected out.

The trigeminocervical complex (TCC) comprises the trigeminal nucleus caudalis (TNC) and the upper cervical spinal cord (C1). The expression of Fos in the TNC and C1 cervical spinal cord was analyzed. The fixed samples were frozen in OCT compound (Sakura Finetek, Torrance, CA, USA) and cut into 20-μm, free-floating sections. The sections were incubated for an hour with buffer consisting of 0.2% Triton X-100, 10% goat serum (ZLI-9005; ZSGB-BIO), and 0.01 M phosphate-buffered saline (PBS pH 7.4) and then incubated overnight at 4°C with an antibody against Fos protein (1: 2000, ab190289). After washing with PBS, the sections were incubated with Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (1:2000, A-11034; Thermo Fisher, Waltham, MA, USA) for 2 h at room temperature in the dark. The number of neurons containing Fos immunoreactivity in TCC was quantified under a microscope (DP73; Olympus, Tokyo, Japan) using a ×20 objective by a single observer blinded to the experimental groups. Three randomly selected images were obtained for each brain region, as determined by coordinates from the atlas by Paxinos and Watson,[32] per sample.

Date analysis

The statistical analyses were performed using SPSS version 19.0. One-way ANOVA with LSD test as a multiple comparison method was used to compare differences in among groups. Paired-Samples T test was used to compare the sleep time and sleep latency between groups intraperitoneal injection of saline and NTG in part three. Statistical significance was set at $p < .05$.

Results

Sleep deprivation for 6 h did not cause any change in the pain threshold of the hind paws in the normal mice

In part one, behavioral tests were conducted immediately after 6 h of SD. The results showed that there was no significant difference in the baseline mechanical pain threshold and hind paw thermal pain threshold between the SD and control (SD + saline compared to CON + saline) groups (Figure 2). Additionally, there were no significant differences in mechanical pain threshold or withdrawal latency between these two groups at any time point

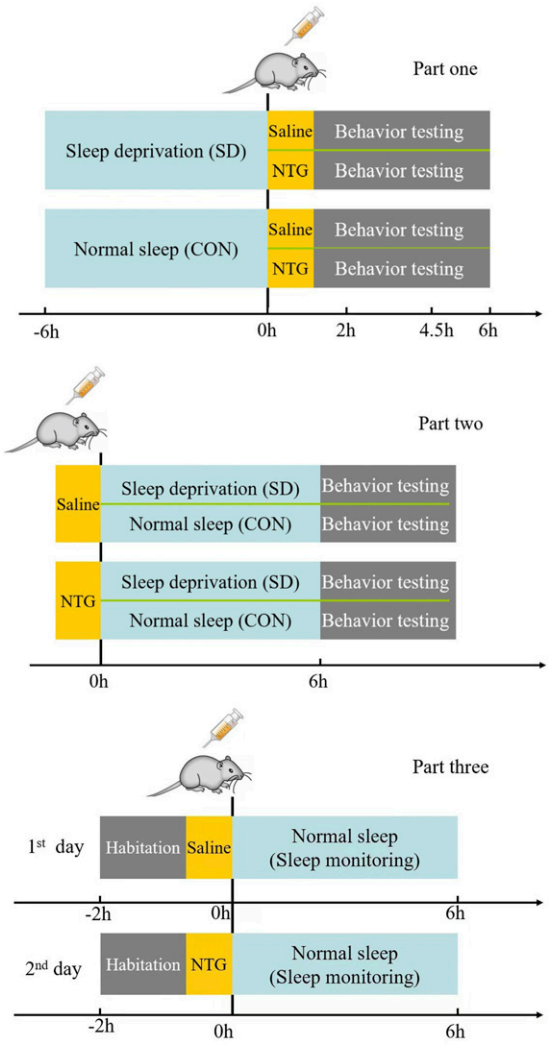

Figure 1. Flow chart of the experiment.

(Figure 2). Therefore, it could be concluded that 6-h SD did not cause significant changes in the pain threshold of the normal mice.

Intraperitoneal injection of NTG caused hyperalgesia of the hind paws

After the mice were given an intraperitoneal injection of NTG or saline, behavioral testing was performed every half hour. Compared with the saline group, the mechanical pain threshold and thermal pain threshold of the mice treated with NTG started to decrease significantly at 30 min (Figure 2). At 60 min and 90 min, the mechanical pain threshold reached the lowest value, and then, it gradually increased, returning to the baseline level in approximately 4.5 h. The change in the thermal pain threshold was similar to that of the mechanical pain threshold. A single intraperitoneal injection of NTG caused reversible changes in the pain threshold of mice.

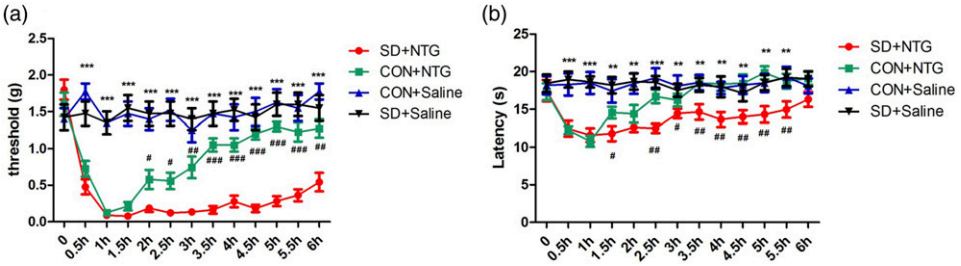

Figure 2. The mechanical and thermal pain threshold of the hind paw in each group in part one of the experiment. (a): mechanical pain threshold ($n = 8$ per group); (b): thermal pain threshold ($n = 8$ per group). $p < .5$ *, $p < .01$ **, $p < .001$ *** for One-way ANOVA test among four groups; $p < .5$ #, $p < .01$ ##, $p < .001$### for the CON + NTG vs SD + NTG.

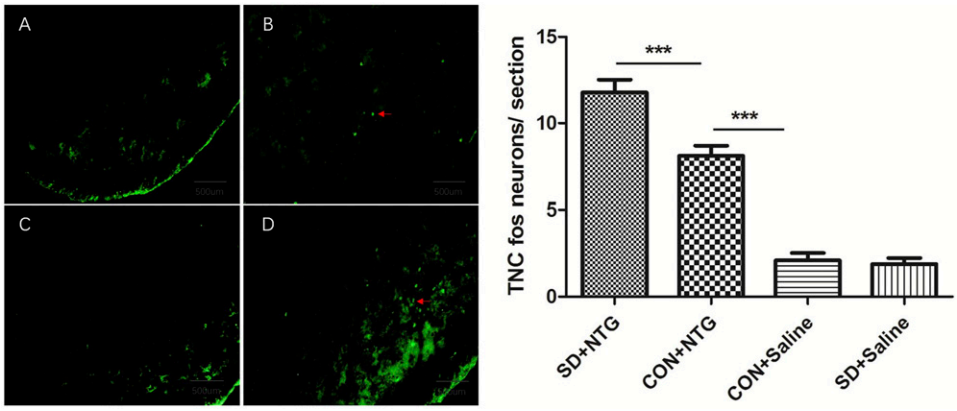

Figure 3. FOS expression in the TNC area. A: CON + Saline; B: CON + NTG; C: SD + Saline; D: SD + NTG. ($p < .5$ *, $p < .01$ **, $p < .001$ ***).

Pretreatment with acute SD aggravated the hyperalgesia induced by NTG in mice

The group that underwent 6 h of SD before NTG injection exhibited lower mechanical pain thresholds at 2 h ($p < .05$), 2.5 h ($p < .05$), 3 h ($p < .001$), 3.5 h ($p < .001$), 4 h ($p < .001$), 4.5 h ($p < .001$), 5 h ($p < .001$), 5.5 h ($p < .001$) and 6 h ($p < .01$) than the control group with normal sleep before NTG injection (Figure 2(a)). Similarly, there were significant differences between these two groups in withdrawal latency that occurred at 1.5 h ($p < .05$), 2.5 h ($p < .01$), 3 h ($p < .05$), 3.5 h ($p < .01$), 4 h ($p < .01$), and 4.5 h ($p < .01$) (Figure 2(b)). Pretreatment with acute SD prolonged the hyperalgesia induced by NTG in mice.

The result of Fos immunofluorescence staining in the TNC and upper cervical spinal area

The above results showed that the mechanical and thermal pain threshold of the group with normal sleep before NTG injection returned to baseline at 4.5 h. At that same time, significant hyperalgesia existed in the group with acute SD before NTG injection. The mice in all groups were deeply anesthetized, and tissues from the brain and spinal cord were obtained. Significantly increased FOS expression in the TNC and upper cervical spinal area was observed in the group that was acutely sleep deprived before NTG injection (Figures 3 and 4).

Acute SD after NTG injection induced prolonged hyperalgesia

Initially, there were no significant differences in mechanical pain threshold or withdrawal latency among the four groups. The group with 6-h SD after NTG injection exhibited a lower mechanical pain threshold than the group with normal sleep after NTG injection (Figure 5(a)). There was no significant difference in thermal pain threshold between these two groups (Figure 5(b)).

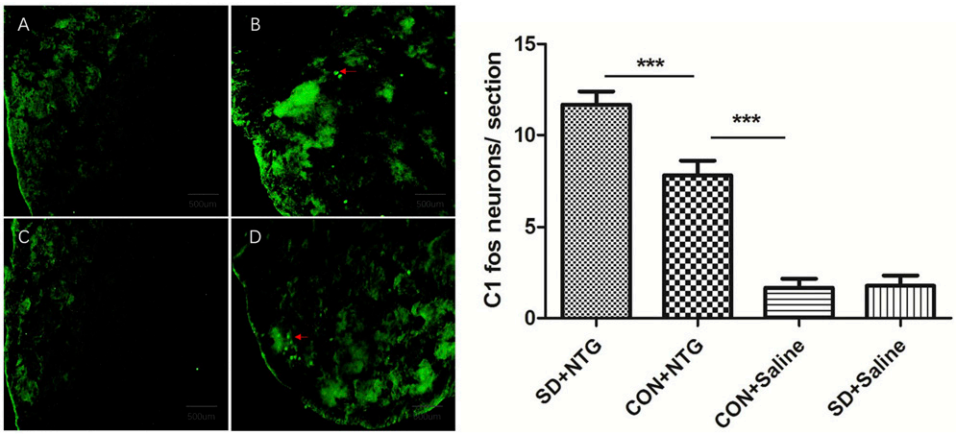

Figure 4. FOS expression in the upper cervical spinal area. A: CON + Saline; B: CON + NTG; C: SD + Saline; D: SD + NTG. ($p < .5$ *, $p < .01$ **, $p < .001$ ***).

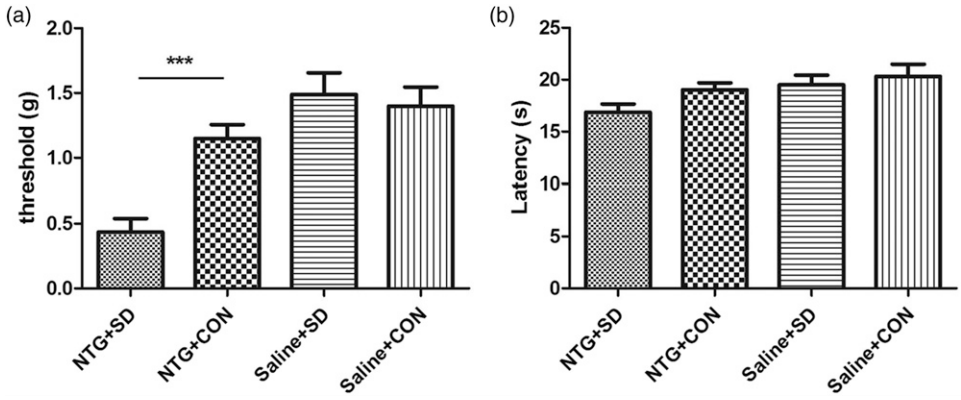

Figure 5. Mechanical and thermal pain threshold of the hind paw in each group in part two of the experiment. (a): mechanical pain threshold ($n = 8$ per group); (b): thermal pain threshold ($n = 8$ per group). ($p < .5$ *, $p < .01$ **, $p < .001$ ***).

One-time NTG injection affected the sleep time of the normal mice

The sleep condition of mice after intraperitoneal injection of saline or NTG without interference were recorded by EEG and EMG. The current study indicated that one-time NTG injection would result in a significant increase in sleep latency (Figure 6(c)) and decrease in sleep du ration (Figure 6(b)) for the normal mice.

Discussion

Our studies indicated that 6-h SD before or after NTG injection aggravated the hyperalgesia induced by NTG in mice.

Diverse methods have been reported to deprive rodents of sleep.[21,22] However, the confounding effects of stress in some protocols made it difficult to interpret the results related to the change in pain threshold. A nonstressful method to deprive mice of sleep was applied in this study. It has been indicated that stress-related behaviors would not increase through this method.[16] We found that 6-h acute SD did not cause any significant change in the mechanical and thermal pain thresholds of the normal mice, which was consistent with previous literature. Thus, before saline or NTG injection, all groups were comparable to the baseline levels.

A single intraperitoneal injection of NTG caused a reversible decrease in the pain threshold of the hind paws of mice, as previously reported.[19,23] The hyperalgesia could be attenuated by antimigraine drugs sumatriptan, and it was considered as a biomarker to assess the effect of potential treatments to alleviate migraine in acute NTG mice model.[19,20,33,34] It has been reported that NTG could reliably evoke hyperalgesia in mice for 4 h. The underlying mechanism may be that NTG releases nitric oxide (NO) in vivo, and NO can activate intracranial pain-sensitive structures such as the dura mater and arterial vessel wall.[24,25] Activation of the trigeminal nerve vascular system was associated with

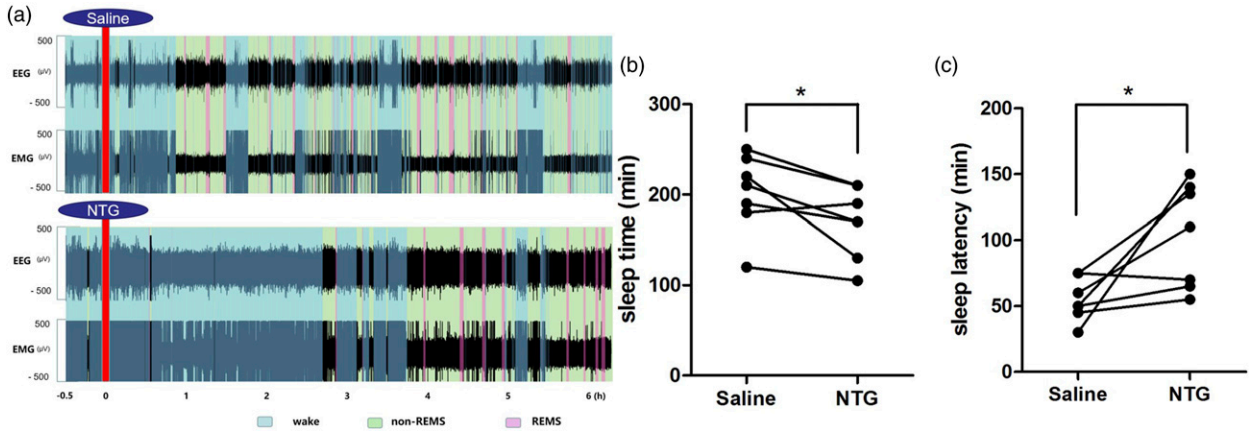

Figure 6. Sleep latency and sleep duration of mice after intraperitoneal injection of saline or NTG without interference. (a) data of a mouse after intraperitoneal injection of saline or NTG. REMS : rapid eye movement sleep; (b) sleep time of mice (n = 7 per group); (c) sleep latency of mice (n = 7 per group) (p < .5*).

the occurrence of migraine. In the current study, for the group with normal sleep and NTG injection, the lowest mechanical pain threshold occurred from 1 h to 1.5 h, and then, the threshold began to increase.

Pretreatment with 6 h of SD prolonged the duration of the lowest pain threshold and overall hyperalgesia induced by NTG in comparison to the control mice without SD. In the clinic, cutaneous allodynia was associated with acute migraine, and a decreased pain threshold in non-cranial regions of the body was also detected in patients during acute migraine.[26] With the relief of migraine, the hyperalgesia would recover.[26] The degree of hyperalgesia is thought to be positively correlated with the severity and chronification of migraine.[27,28]

The above behavioral findings were confirmed by histology. At 4.5 h after NTG injection, FOS expression in the TNC and upper cervical spinal area was significantly increased in the group with sleep deprivation. At that time point, this group exhibited more sensitivity to mechanical and thermal stimulation. Activation of the trigeminovascular system is considered an essential factor for migraine onset.[29] Fos protein is a marker of cellular activation, and Fos expression in the TCC induced by intervention has been regarded as a standard to reflect the degree of activation of the trigeminal vascular system.[30,31] In the clinic, SD is one of the most common triggers of migraine, and over half of reported migraine attacks are followed by daytime sleepiness, which implies that SD may be involved in the beginning of migraine.[11,12] Our research mimicked acute SD prior to the migraine-related stimulation onset, and the results suggested that SD might also affect the transmission and maintenance of nociceptive stimulus signals in the trigeminal nervous system, which highlights the importance of sleep disorders for migraine.

Our study also simulated SD after the beginning of migraine-related stimulation and revealed that acute SD after NTG injection would prevent the recovery of hyperalgesia in mice, which provides proof that ongoing SD may also influence the transmission of noxious stimuli induced by NTG. We believe this finding is an important supplement to the clinical fact that migraineurs often prefer to sleep looking for relief from their headache.[12] This study may provide evidence for migraineurs to formulate a more reasonable lifestyle schedule.

According to the current results, acute SD influenced the process of migraine-related hyperalgesia evoked by NTG. The underlying mechanism remains unclear. We postulate that it may be associated with increased levels of the excitatory neurotransmitter glutamate in the cerebral cortex, but further research is still needed. In addition, we compared the sleep time of mice after intraperitoneal injection of saline or NTG without interference. It showed that one-time NTG injection would induce a significant increase in sleep latency and decrease in sleep duration for the normal mice, which implied that the NTG affected the process of sleep in acute NTG mice model. Taken together, this study may help us to have a better understanding of the association between sleep and migraine.

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Author contributions

The study was conceived and designed by SYY, RZL and ZD. ZY, BZL and WJT performed the part one and part two of the experiments. The part three was conducted by BZL. ZY wrote the manuscript. All the authors read and approved the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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