Establishment of a chronic insomnia rat model of sleep fragmentation using unstable platforms surrounded by water

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Abstract. Chronic fragmented sleep is a very common type of insomnia that affects the daily lives of numerous people around the world. However, its pathogenesis is not very clear and a corresponding rat model has not been reported for this purpose at present. The present study aimed to establish a rat model of chronic insomnia with sleep fragmentation using self-made multiple strings of unstable platforms surrounded by shallow water. During the establishment of the models, changes in body weight and differences in food and water intake in the daytime and at night were acquired. The rat models were assessed using several tests, including the Morris water maze test, pentobarbital sodium-induced sleep, infrared monitoring and electroencephalogram/electromyography during sleep. The expression levels of certain inflammatory factors and orexin A were detected in the serum and brain tissues using ELISAs, immunohistochemistry and immunofluorescence. The expression levels of orexin 1 receptor (orexin 1r) were also detected in the brain. Polysomnography indicated that the model rats were successfully prepared with reduced non-rapid eye movement (non-REM) sleep in the daytime, which was increased at night, and considerably lower REM duration during the day and night. The number of instances of sleep arousals were also increased in the day and at night, and the average duration of each sleep bout was decreased in the daytime. The body weights of the model rats increased at a normal rate. However, the reduction of body weight in the daytime and increased in body weight at night were significantly less than those of the control rats. The daytime food and water consumption of the model rats increased significantly compared with that of the control rats, but was similar to that of the control group at night. The Morris water maze test indicated that the model rats were slow to learn to escape the platforms and performed a lower number of target crossings. The pentobarbital-induced sleep experiment confirmed that the model rats exhibited a longer sleep latency and shorter sleep time. The serum IL-1β, IL-6, TNF-α and orexin A levels of the model rats were significantly increased, whereas their serum IL-10 levels were significantly decreased compared with those of the control rats. The expression levels of IL-1β, IL-6, orexin A and orexin 1r in the brain tissues of the model rats were also significantly increased. In conclusion, these data indicate that learning and memory function, sleep time, arousal times, diurnal and nocturnal body weight changes, food and water intake, and expression levels of the specific inflammatory factors orexin A and orexin 1r were altered in the model rats. This suggests the chronic insomnia rat model with sleep fragmentation was successfully established using multiple strings of unstable platforms surrounded by water.

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Introduction

Insomnia is a very common disease, and chronic sleep fragmentation is a frequently occurring type of insomnia (1). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders published by the American Psychiatric Association (2) and the 3rd edition of the International Classification of Sleep Disorders (3), chronic insomnia is defined as symptoms of difficulty in the initiation of sleep, the maintenance of sleep due to frequent arousal or difficulty in sleeping again after arousal, or early waking, with daytime function impairment occurring ≥3 times per week and lasting for ≥3 months with adequate sleep opportunity (time in bed ≥7.5 h) (1). Chronic sleep difficulty is essentially chronic sleep fragmentation caused by frequent arousal, which affects normal rest, daytime physical activity and social function in the same manner as any other type of insomnia (4,5). A cross-sectional national online survey in Australia found that 41.5% of females and 35.3% of males suffered from chronic difficulties in the initiation and maintenance of sleep, as well as daytime symptoms (6). Chronic insomnia with sleep fragmentation is also a social and financial burden (1,4,5). Therefore, the establishment of chronic insomnia models to investigate this type of insomnia is of considerable importance.

Modified multiple platforms surrounded by water have been frequently been utilized to deprive rats or mice of rapid eye movement (REM) sleep for 24-120 h (7-11). This method is mainly suitable for the preparation of acute insomnia models with 24 h sleep deprivation per day (12,13). It is also suitable for the preparation of a chronic sleep restriction model with 8-18 h sleep deprivation (13,14). However, a ≥6-h rest may provide sufficient time for the rats to sleep, which may result in failure to establish the insomnia model. Since rats often begin to die following several days of 24 h/day sleep deprivation, a functional 24 h/day rat model of chronic insomnia using a modified multiple platform device for >10 days has not yet been reported.

In the clinic, the body weights of patients with chronic sleep fragmentation do not usually decrease significantly and are often higher than those of normal subjects (15,16). However, in animal experiments, the body weights of the rats used to establish insomnia models have often been reported to be decreased (17), which indicates that the modeling methods used require reappraisal.

Clinically, sleep monitoring using polysomnography is considered the gold standard for the measurement of sleep duration. When the duration of sleep is <6 h (6-8 h is the normal sleep duration) and daytime dysfunction presents, a diagnosis of insomnia can be made (4,5). In rat models, sleep is often monitored using electroencephalogram (EEG)/electromyography (EMG) methods, particularly with a 2 EEG/1 EMG biosensor (18-21). However, at present, no specific time standard has been established for use when evaluating the success of REM sleep deprivation in model rats. The method of comparing REM sleep time with that in the control group is usually adopted.

Insomnia is accompanied by daytime dysfunction in learning, memory, driving and communication, with sleepiness and napping. The Morris water maze training and test experiment is a method used to evaluate the learning and memory function of rats (10). Pentobarbital sodium-induced sleep is often used in the evaluation of animal sleep (22-24).

It has been reported that the levels of inflammatory cytokines, namely IL-1, IL-6, TNF-α and IL-10, are affected by insomnia (25). The abnormal levels of these cytokines further affect brain function, such as learning and memory (26,27). Furthermore, orexin A and its receptors play important roles in the regulation of food intake, circadian rhythms, arousal and sleep (28-32).

In the current study, a rat model with sleep fragmentation was established using multiple strings of unstable platforms surrounded by shallow water. The performance of the model was evaluated using the Morris water maze test and pentobarbital sodium-induced sleep assessment. The duration of non-REM (NREM) and REM sleep, frequency of arousal, frequency of daytime eating and drinking, and body weight changes during the daytime and at night were evaluated. The effects on the modeling process on the expression levels of IL-1, IL-6, TNF-α, IL-10, orexin A and orexin 1 receptor (orexin 1r) were also investigated. The aim of the study was to provide evidence for further research on insomnia. The protocol of the experimental model used in the present study is presented in Fig. 1.

Materials and methods

Reagents. Pentobarbital sodium (cat. no. P11011) was purchased from Merck KGaA. The ELISA kits used for the detection of IL-1β (cat. no. RAB0277), IL-6 (cat. no. RAB0311) and TNF-α (cat. no. RAB0479) were purchased from Sigma-Aldrich (Merck KGaA). The rat IL-10 ELISA kit (cat. no. ab214566) was purchased from Abcam and the orexin A ELISA kit (cat. no. YX-E28754) was purchased from Sinobestbio. The primary antibodies for IL-1β (cat. no. bs-0812R), IL-6 (cat. no. bs-4539R), orexin A (cat. no. bs-15509R) and orexin 1r (cat. no. bs-18029R) and the secondary antibody kit (containing H_2O_2, antigen blocking fluid, secondary antibody, horseradish peroxidase; cat. no. SP-2003) were purchased from BIOSS. The labeled antibody horseradish peroxidase-IgG (cat. no. RCA015) and the dyes TYR-CY3 (red light; cat. no. RCA019) and TCR-488 (green light; cat. no. RCF020) were purchased from Recordbio. Anti-fluorescence attenuation sealing agent (cat. no. s2100) was used in the immunofluorescence assay and was bought from Beijing Solarbio Science & Technology Co., Ltd. Neutral balsam (cat. no. G8590) were purchased from Beijing Solarbio Science & Technology Co., Ltd. DAB (cat. no. ZLI-9018) was purchased from Origene Technologies, Inc.

Instruments. The following instruments were used in the current study: Morris water maze device (WMT-100; Chengdu Taimeng Science and Technology Co., Ltd.), low-speed refrigerated centrifuge (KDC-2044; Zonkia Scientific Instruments Co., Ltd.), ultracentrifuge (Himac; Eppendorf), fluorescence microscope (DMi8; Leica Microsystems, Inc.), microplate reader (Multiskan™ GO; Thermo Fisher Scientific, Inc.), stereotaxic instrument (digital brain stereotaxic instrument company), sleep monitoring instrument (2 EEG/1 EMG headmount; Pinnacle Technology, Inc.) and infrared monitor (TP-LINK; TP-Link Technologies Co., Ltd.). Immunohistochemical analysis software was also used (Image-Pro Plus 6.0; Media Cybernetics, Inc.).

Animals. A total of 24 male Sprague Dawley rats (body weight, 200±240 g; age, 8-9 weeks) were obtained from the Animal Experiment Center of Xinjiang Medical University [animal certificate: 650007000940; production license: SYXK(X) 2016-0003]. The animals were raised in the animal center situated in the experimental building of the University. The room temperature was set to 23±2˚C with 12-h light/dark cycles. The relative humidity was 40-60%. Food and water were provided *ad libitum*. The animal experimental protocol was approved by the Ethics Committee of Xinjiang Medical University Animal Experiment Center (IACUC-20210115-07) for The Graduate Innovation and Entrepreneurship Project of Xinjiang Medical University (grant no. CXCY2021023) and The First Affiliated Hospital of Xinjiang Medical University (IACUC-20150225-118) for the project of National Natural Science Foundation of China (grant no. 81560762). The protocol adhered to the guidelines of China Experimental Animals Administration Legislation to minimize their suffering.

Animal grouping and modeling. The rats were fed for 3 days to adapt to the environment and were randomly distributed into control and model groups (n=12/group). The rats were housed in a cage (length, 50 cm; width, 35 cm; height, 20 cm) with 3 rats/cage. Each cage comprised three strings of platforms

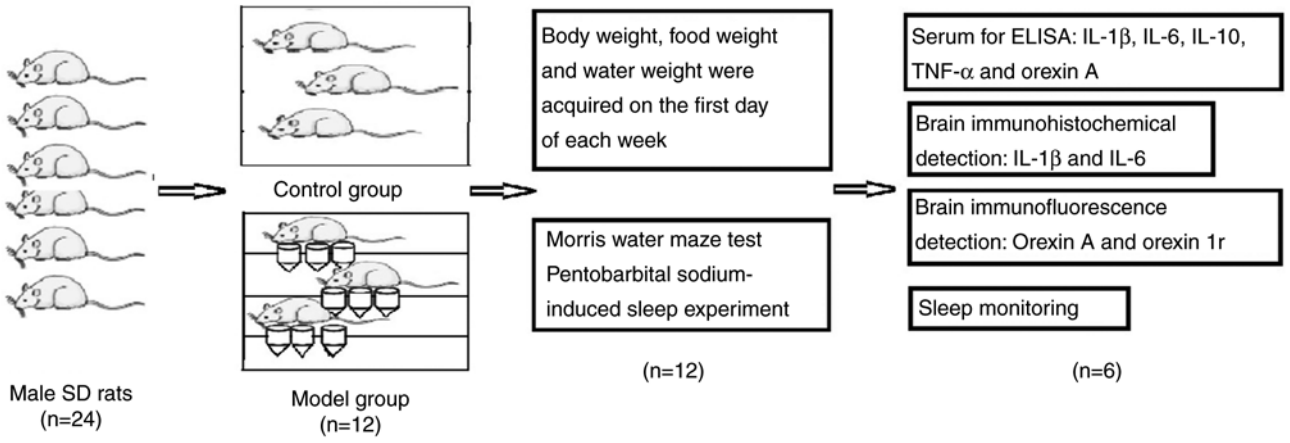

Figure 1. Schematic illustration of the study. SD, Sprague-Dawley; orexin 1r, orexin 1 receptor.

surrounded by shallow water (depth, 1.5 cm). Each string of platforms comprised 3 or 4 bullet-shaped platforms, which were composed of a 2-cm-high cylinder (diameter, 5 cm) with a hole parallel to the plane of the platform and a 1-cm-high cone touching the bottom of the cage. The platforms were penetrated by a stainless-steel wire (diameter, 3 mm) to form a string of platforms for a rat to sleep on. The steel wires were fixed to the sidewalls of the cage in a manner that enabled the platforms to rotate in a vertical plane perpendicular to the steel wire when the rats caused an imbalance in the two sides of the small platforms due to the loss of muscle tone during REM sleep. This caused the rats fall into the water, thereby resulting in REM sleep deprivation. The model rats were raised for 6 weeks in cages with platforms surrounded by water, with the exception of the first day of each week. The cages of the model group were washed and fresh 25˚C water was added every morning and evening. The rats of the control group were raised normally.

Changes in bodyweight and food and water intake during the day and night. On the first day of each week, the model rats were transferred to normal cages. The bodyweights of the rats and the weights of available food and water were weighed at 6:00 and 18:00 on that day, and at 6:00 on the following day to estimate the body weight changes of the rats and their food and water intake in the daytime and at night, separately.

Learning and memory function assessment using the Morris water maze. The Morris water maze training and test experiment was carried out in the evening on days 37-41 according to a method described in a previous study (33). The latency in approaching the platform and the number of times the target was crossed were recorded.

Pentobarbital sodium-induced sleep experiment. The experiments were carried out on day 43 following the intraperitoneal injection of 35 mg/kg pentobarbital sodium dissolved in normal saline. The sleep latency and sleep time were recorded as previously described (22-24).

Sleep monitoring. On day 44, 6 rats from each group were randomly selected for sleep monitoring. Using the stereotaxic instrument, the 2EEG/1 EMG head mounts were fixed to the parietal bones of the heads (n=6) with four special screws and dental cement under anesthesia. On day 46, the head mounts were connected to an amplifier to monitor the sleep from 6:00 to 18:00 and from 18:00 to 6:00 on the following day. The monitoring was performed by the 2EEG/1 EMG mount with concomitant use of an infrared monitor. The researchers were able to monitor the rats at any time using a mobile phone. The frequency of waking up and sleep duration were estimated from the polysomnography recordings by two independent experts, who aided the monitoring process with infrared video cameras. The duration of REM sleep being lower in the model group than in the control group was regarded as the time standard for the successful preparation of a REM sleep deprivation model in the present study.

Sample collection. On the morning of day 44, the remaining 6 non-sleep-monitored rats (smallest body weight, 298 g) in each group were anesthetized with 35 mg pentobarbital sodium, and 8 ml blood was collected from the abdominal aorta. Following blood collection, the rats were decapitated using tissue scissors while still under anesthesia. Blood samples and brains of the sleep-monitored rats were acquired in the same manner following sleep monitoring. The serum was acquired following centrifugation at 4˚C and 3,000 x g for 15 min. The coronal middle sections of the brain ~0.4-cm thick were acquired and placed into 4% paraformaldehyde in phosphate-buffered saline for fixation at room temperature for 3 days. Slices 4-μm thick were made and stained with hematoxylin and eosin at room temperature for 8 min, then photographed under a light microscope (Eclipse-Ni-U; Nikon Corporation). This staining was performed in order to identify the areas of interest for comparison in the immunohistochemical and immunofluorescence detection analyses (Fig. 2).

ELISA. The serum levels of IL-1β, IL-6, IL-10, TNF-α and orexin A were detected with the aforementioned ELISA kits according to the manufacturer's instructions.

Immunohistochemical detection. The expression levels of IL-1β and IL-6 were detected in the brain by immunohistochemical analysis according to the instructions provided

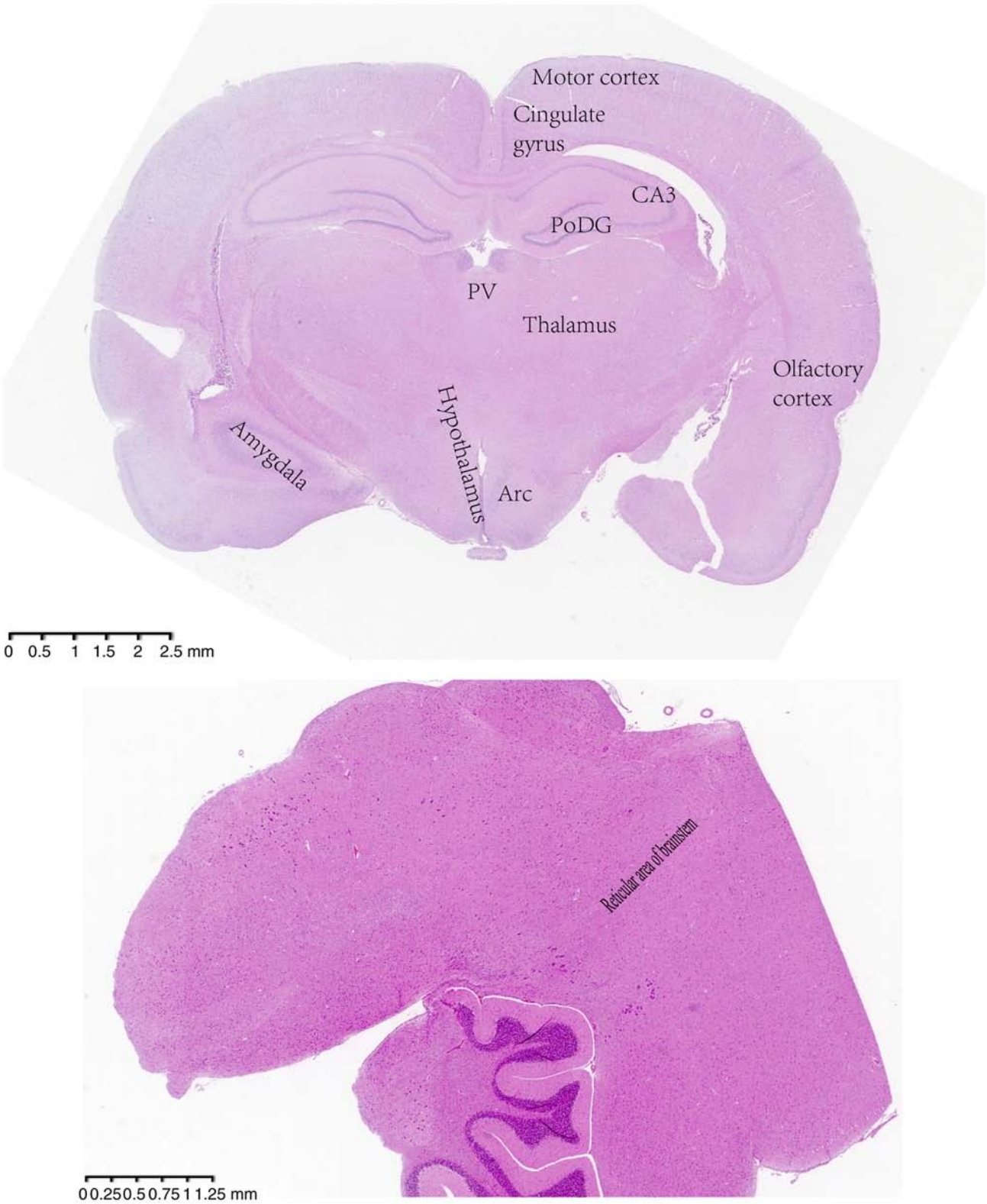

Figure 2. Hematoxylin and eosin staining to indicate areas of the brain for comparison between groups in the immunohistochemistry and immunofluorescence staining experiments (magnification, x10). Upper panel shows the total brain (scale bar, 2.5 mm) and lower panel shows the reticular area of the brainstem (scale bar, 1.25 mm). Amygdala, amygdala location within the limbic system; arc, arcuate nucleus of the hypothalamus; CA3, CA3 area of the hippocampus; motor cortex, motor area of the cerebral cortex; PoDG, polymorph layer of the dentate gyrus of the hippocampus; PV, paraventricular nucleus of the thalamus.

by the antibody manufacturer (BIOSS). Incubation was performed with primary antibodies against IL-1β (diluted at 1:100) and IL-6 (diluted at 1:100) at 4˚C overnight. Finally, the stained tissues were sealed with neutral balsam. The stained slices were imaged using a light microscope (Eclipse-Ni-U; Nikon Corporation). The images were analyzed with Image Pro Plus 6.0 software to acquire the integrated optical density.

Immunofluorescence detection. Brain orexin A and orexin 1r were detected by immunofluorescence analysis. Paraffin sections were put into xylene I for 15 min, then into xylene II for 15 min to dewax the section, and then into anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, 95% ethanol I for 5 min, 95% ethanol II for 5 min, 85% ethanol I for 5 min, 85% ethanol II for 5 min and 70% ethanol for 5 min to remove the xylene, followed by rinsing under tap water for 5 min and under double distilled water for 5 min (3 times). The hydrated sections were put into alkaline antigen repair solution EDTA (pH 9.0) at 95˚C (water bath for 20 min) to repair the antigen on the sections, then washed with pH 7.4 PBS for 5 min (3 times) on a decoloring shaker. 3% hydrogen peroxide was added to the sections, which were placed into a dark box to avoid the light for 15 min. The sections were then washed with PBS for 5 min (3 times) on a decoloring shaker. A circle was drawn with an immunohistochemistry pen around the tissues on the sections. Antigen blocking fluid was dripped onto the tissues of the sections to block the antigen for 30 min at room temperature. After the blocking solution was shaken off, the primary antibody of orexin A (1:200) was incubated with the sections at 4˚C overnight. The sections were washed with PBS for 5 min (3 times) on a decoloring shaker. The labeled antibody horseradish peroxidase-IgG was incubated with the sections for 50 min in the dark at room temperature. The sections were washed with PBS for 5 min (3 times) on a decoloring shaker. TYR-CY3 was added in the dark for 10 min at room temperature. The sections were washed again with PBS for 5 min (3 times) on a decoloring shaker. The process from antigen repair to TYR-CY3 addition (10 min) and PBS wash was repeated with the primary antibody orexin 1r (1:200) to replace orexin A, and with TCR-488 to replace TYR-550. The slices were sealed with anti-fluorescence attenuation sealing agent. The stained tissues were imaged using a fluorescence microscope and analyzed with Image Pro Plus 6.0 software.

Statistical analysis. SPSS software (version 18.0; IBM Corp.) was used for statistical analysis. One-way ANOVA was used to compare the indices of the two groups at a $P>0.05$ following homogeneity of variance and Shapiro-Wilk normality tests. $P<0.05$ was considered to indicate a statistically significant difference. Data are shown as the mean ± standard deviation.

Results

Changes in the general condition of the model rats. The body weights of the model rats were comparable with those in the control group (Fig. 3A). However, it was noted that the fur of the model rats gradually became earth-yellow. The daytime activity of the model rats, including their eating and drinking behavior was increased and the night-time activity was decreased compared with that in the control group as determined using an infrared monitor. These data indicate that the rats are behaving similarly to insomnious subjects with fragmented sleep who are exhausted and often nap in the daytime and eat additional meals at night.

Body weight changes of the model rats are significantly decreased. The absolute values of the body weight changes of the model rats were decreased compared with those of the control group in the daytime and at night (Fig. 3B and C).

Food and water consumption of the model rats is increased. The food and water consumption of the model group demonstrated a marked and significant increase during the period from 6:00 to 18:00 compared with that of the control group. However, the food and water consumption from 18:00 to 6:00 the following morning was similar to that of the control rats (Fig. 3D-G).

Development of learning and memory dysfunction in the model rats. The results of the Morris water maze training and testing experiments indicated that the model rats had longer latency to approach the platform in the total course of the training and test periods; they also crossed over the target regions fewer times than the control rats, which indicated that their learning and memory ability was significantly reduced (Fig. 3H and I).

Model rats are resistant to sleep induction. The pentobarbital sodium-induced sleep experiment indicated that the model rats exhibited significantly longer sleep latency and shorter sleep time than the control rats, which indicated that the model rats were somewhat resistant to the sleep-inducing drug (Fig. 3J and K).

Model rats suffer from sleep fragmentation insomnia. The model rats demonstrated a greater duration of NREM at night, reduced NREM and REM sleep in the daytime, and reduced REM sleep at night (Fig. 3L and M). The model rats were awake more times during the day and at night (Fig. 3N). Therefore, they had a reduced time period per bout of sleep (Fig. 3O).

Immune and endocrine disorders occur in model rats. The serum levels of IL-1β, IL-6, TNF-α and orexin A were increased, whereas those of IL-10 were decreased in the model rats compared with the controls (Fig. 4). The expression levels of IL-1β were increased in certain brain regions of the model rats, including the arcuate nucleus of the hypothalamus, the paraventricular nucleus of the thalamus, the dentate gyrus of the hippocampus, and the cingulate gyrus of the cerebral cortex (Fig. 5 and Table I). IL-6 expression also increased in various brain regions of the model rats, including the brain stem, amygdala, motor cortex and olfactory cortex (Fig. 6 and Table II).

Expression levels of orexin A in serum and in the brain tissues are increased in model rats. The serum levels of orexin A were detected by ELISA (Fig. 4B) and the expression levels of this peptide in the brain were also detected (Figs. 7 and 8). Specifically, orexin A levels were determined by immunofluorescence in the CA3 region of the hippocampus (Fig. 7), the motor area of the cortex (Fig. 7), the hypothalamus (Fig. 8) and the thalamus (Fig. 8); the quantified results indicate that the orexin A levels in all four regions were significantly increased in the model group compared with the control group.

Orexin 1r levels of the model rats are increased in the majority of the brain areas. The results reveal a significant increase in the levels of orexin 1r in the model group in the motor area of

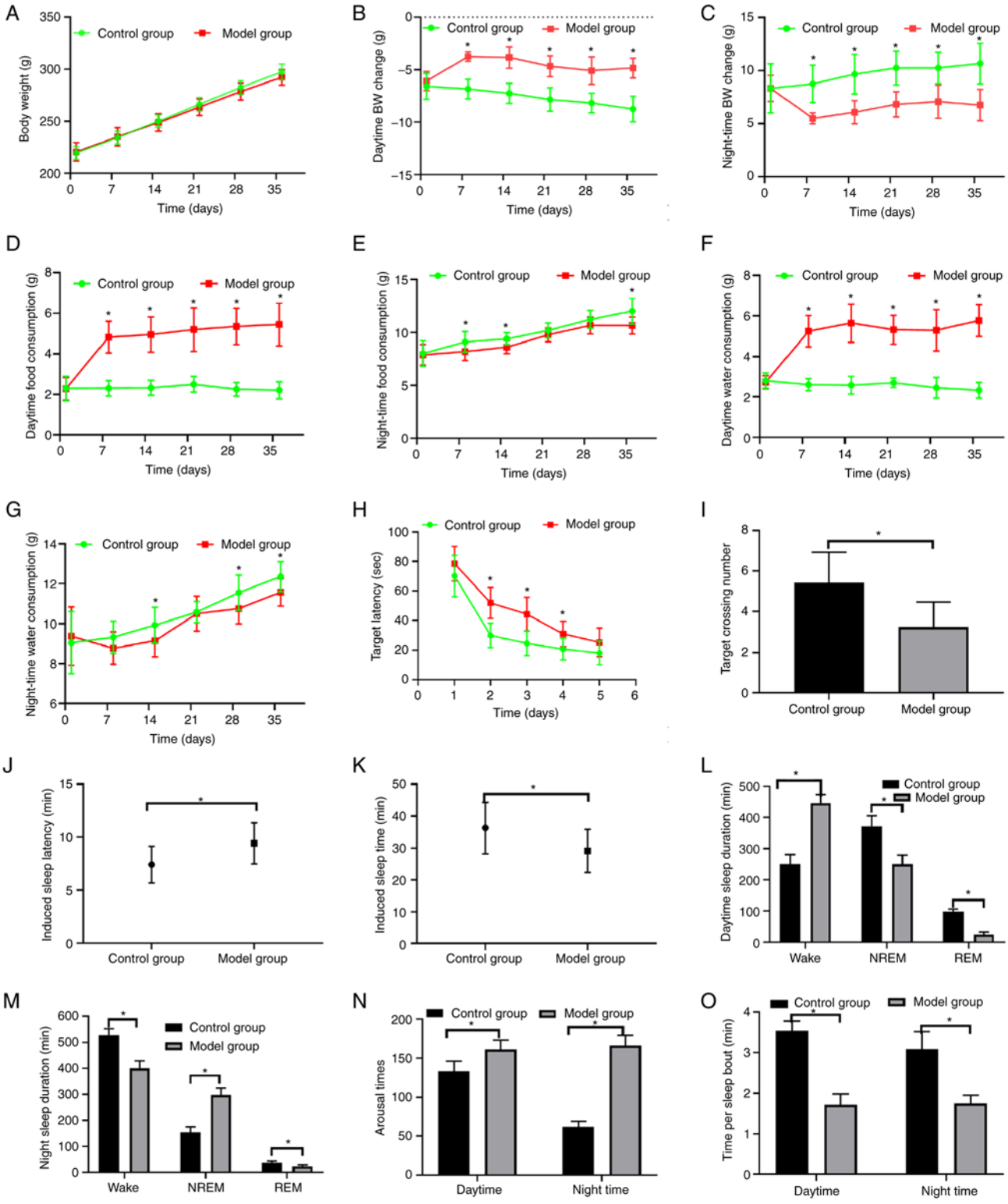

Figure 3. Comparison of body weights, food and water intake, Morris water maze indices, pentobarbital-induced sleep experiment and sleep monitoring results between the two groups. (A) Body weights, (B) daytime body weight changes and (C) night-time body weight changes. Comparison of (D) daytime and (E) night-time food consumption and (F) daytime and (G) night-time water consumption. (H) Target latency (escape latency) and (I) the number of times the target was crossed in the Morris water maze test. (J) Induced sleep latency and (K) induced sleep time in the pentobarbital-induced sleep experiment. Comparison of (L) daytime sleep duration, (M) night-time sleep duration, (N) numbers of arousal episodes and (O) time per sleep bout in the model and control groups. *P<0.05 compared with the control group (A-K, n=12; L-O, n=6). BW, body weight; REM, rapid eye movement; NREM, non-REM.

the cortex (Fig. 7), the hypothalamus (Fig. 8) and the thalamus (Fig. 8). However, no significant difference was noted in the expression levels of this marker in the hippocampus between the model and control groups (Fig. 7).

Discussion

In the clinic, numerous subjects suffer from chronic insomnia. In order to investigate this condition, modified multiple

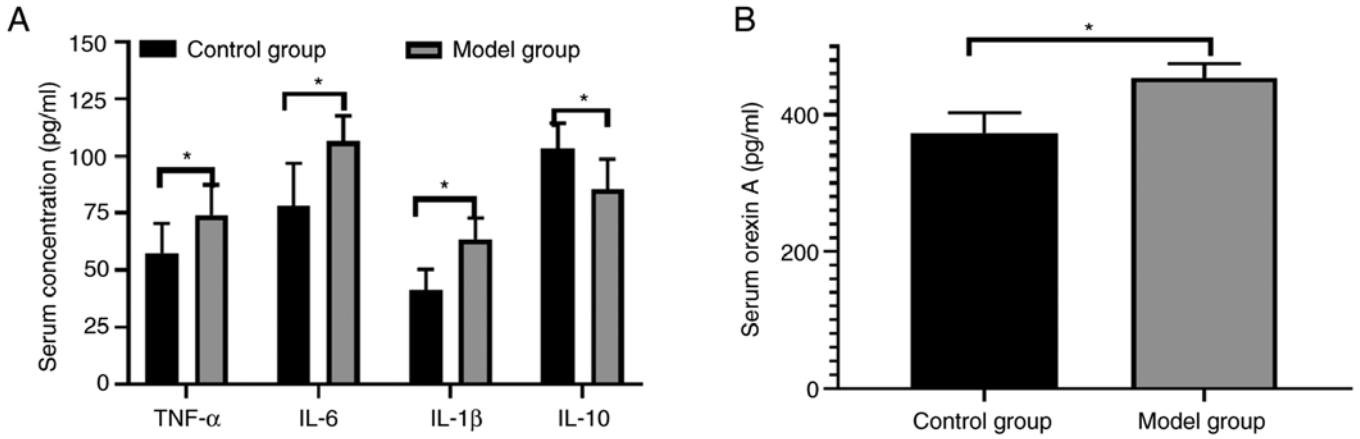

Figure 4. Serum TNF-α, IL-1β, IL-6, IL-10 and orexin A levels in the model and control groups. Concentrations of (A) TNF-α, IL-1β, IL-6 and IL-10 and (B) orexin A. *P<0.05 compared with the control group (n=6).

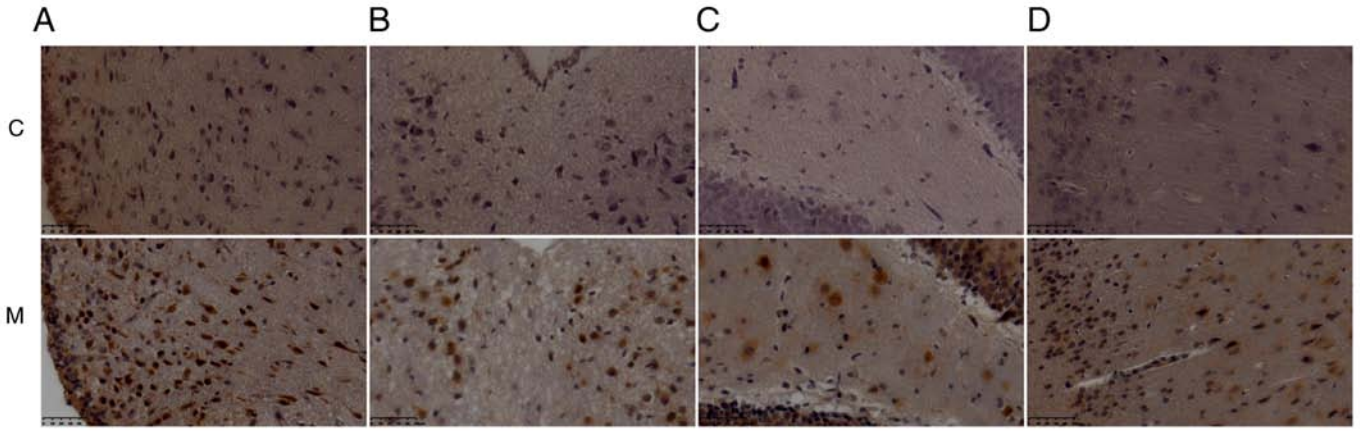

Figure 5. Expression of IL-β in various regions of brain of the model and control groups. (A) arcuate nucleus of the hypothalamus, (B) paraventricular nucleus of the thalamus, (C) dentate gyrus of the hippocampus and (D) cingulate gyrus of the cortex. Representative images are shown (n=6; magnification, x200; total scale bar, 50 μm). C, control group; M, model group.

separate platforms surrounded by water have been used to prepare acute sleep deprivation or chronic sleep restriction models in various studies (7-11). However, to the best of our knowledge, chronic sleep deprivation models involving modified multiple platforms have not previously been reported. In the present study, self-made multiple strings of unstable platforms were used. This novel device has several advantages. Firstly, the low-strung (3 cm high) platforms are very convenient for the rats to stand up, walk or sleep on. The model rats can easily lie down on their backs on the short-strung platforms in order to sleep or change their positions. Therefore, NREM can be retained to a certain degree by the rats adapting flexible postures, rather than the rats only being allowed to sit on the platforms. Secondly, unlike the deep-water design used for multi-platform devices in previous studies (34), the shallow water allows the rats to walk freely and to communicate or play with each other without getting most of their body wet, which can protect them from developing depression or soaking-associated skin diseases. Thirdly, although the platforms allow the rats to sleep lightly, they may rotate around the steel wire when the balance between the two sides of the platform is distorted during REM sleep. This is due to loss of muscle tone, the dreaming activities of the animals and the very low level of consciousness in REM sleep, and may result in the rats entering the water. Furthermore, in the present study, three strings of platforms were present and three rats were housed in each cage, which allowed them to influence each other's sleep.

Abnormalities were noted in the model rats in the daytime and at night with regard to body weight, food and water intake, response to the Morris water maze training and testing experiment, pentobarbital sodium-induced sleep experiment, infrared monitoring and 2 EEG/1 EMG polysomnography.

Normally, the body weight of rats decreases in the daytime, while it increases at night. This process is regulated by orexin/orexin receptors (30-32). In the present study, the body weight of the model rats decreased by a lower extent than that of the controls during the daytime, which can be explained by their higher food and water intake during the day. Secondly, the monitoring indicated that the model rats spent more time eating and drinking when the control rats were sleeping. Furthermore, molecular analysis indicated that orexin A levels were increased in the serum and brain tissues, whereas orexin 1r was increased in the brain in the morning, which may be an

Table I. Comparison of IL-1β expression in different brain regions.

	Integrated optical density (x10^6)			
Group (n=6)	Hypothalamus	Thalamus	Hippocampus	Cerebral cortex
Control	3.55±1.05	4.14±0.78	2.33±0.47	2.95±0.90
Model	5.74±1.31[a]	5.82±1.30[a]	3.70±0.84[a]	8.81±0.78[a]

[a]P<0.05 compared with the control group.

Table II. Comparison of IL-6 expression in different brain regions.

	Integrated optical density (x10^6)			
Group (n=6)	Brain stem	Amygdala	Motor cortex	Olfactory cortex
Control	0.94±0.33	4.91±0.45	0.94±0.33	2.34±0.29
Model	2.92±0.46[a]	6.05±0.36[a]	2.92±0.46[a]	3.79±0.47[a]

[a]P<0.05 compared with the control group.

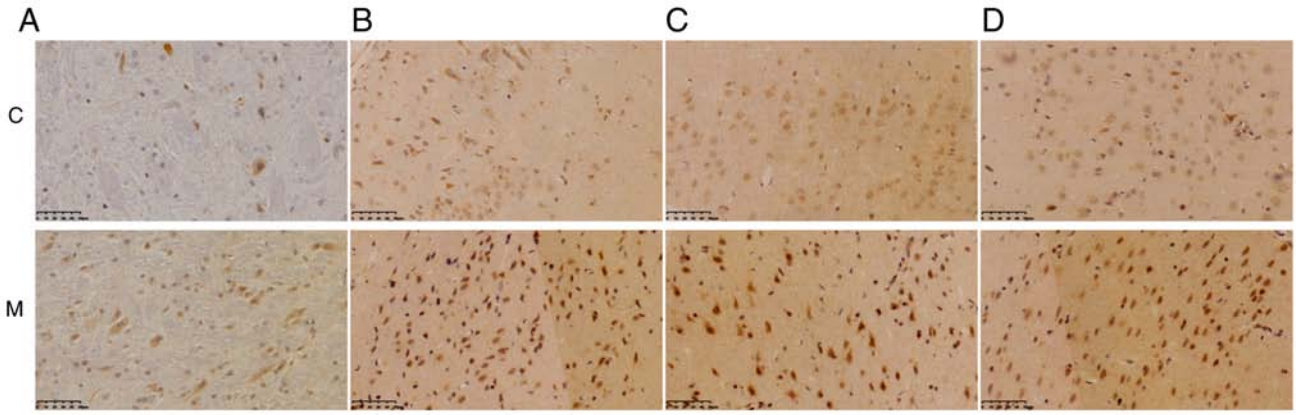

Figure 6. Expression of IL-6 in various regions of brain of the model and control groups. (A) Reticular structure of the brain stem, (B) amygdala of the limbic system, (C) motor cortex and (D) olfactory cortex. Representative images are presented (n=6; magnification, x200; total scale bar, 50 μm). C, control group; M, model group.

internal mechanism to which the food intake increase in the daytime period can be attributed. Since the sleep deprived rats spent more time awake, they consumed more energy. The body weights of the model rats were basically normal, as they were comparable to those in the control group. These results differ from those reported in a previous study (17).

In contrast to human subjects, rats sleep more time during the day and carry out more activities during the night. In the present study, the rats became highly alert and influenced each other's sleep. They also spent more time eating and drinking, and fell into the water several times. Therefore, their sleep in the daytime became shorter and the NREM sleep at night became longer as compensation. However, the duration of REM sleep was shorter in the model rats in the daytime and at night due to the REM sleep deprivation associated with the platforms surrounded by water (28). The average duration of each bout of sleep was also decreased due to the reduced time spent sleeping and a higher number of arousals. In order to compensate for the lost and non-restorative sleep, the rats had to reduce their night-time activities in order to sleep due to physical exhaustion.

The Morris water maze test can be used to study dysfunction of learning and memory ability (35-40). Increased expression levels of pro-inflammatory factors contribute to dysfunction of learning and memory ability (26,27). In the present study, the night-time Morris water maze training and test experiment indicated that the memory dysfunction in the model rats was accompanied by a longer escape latency and reduced number of times crossing the target regions. The night-time period was selected because rats tend to sleep more in the daytime and to be more active at night. The results of serum analysis indicated an increase in the expression levels of certain pro-inflammatory factors, namely TNF-α, IL-1β and IL-6, and a reduction in the expression

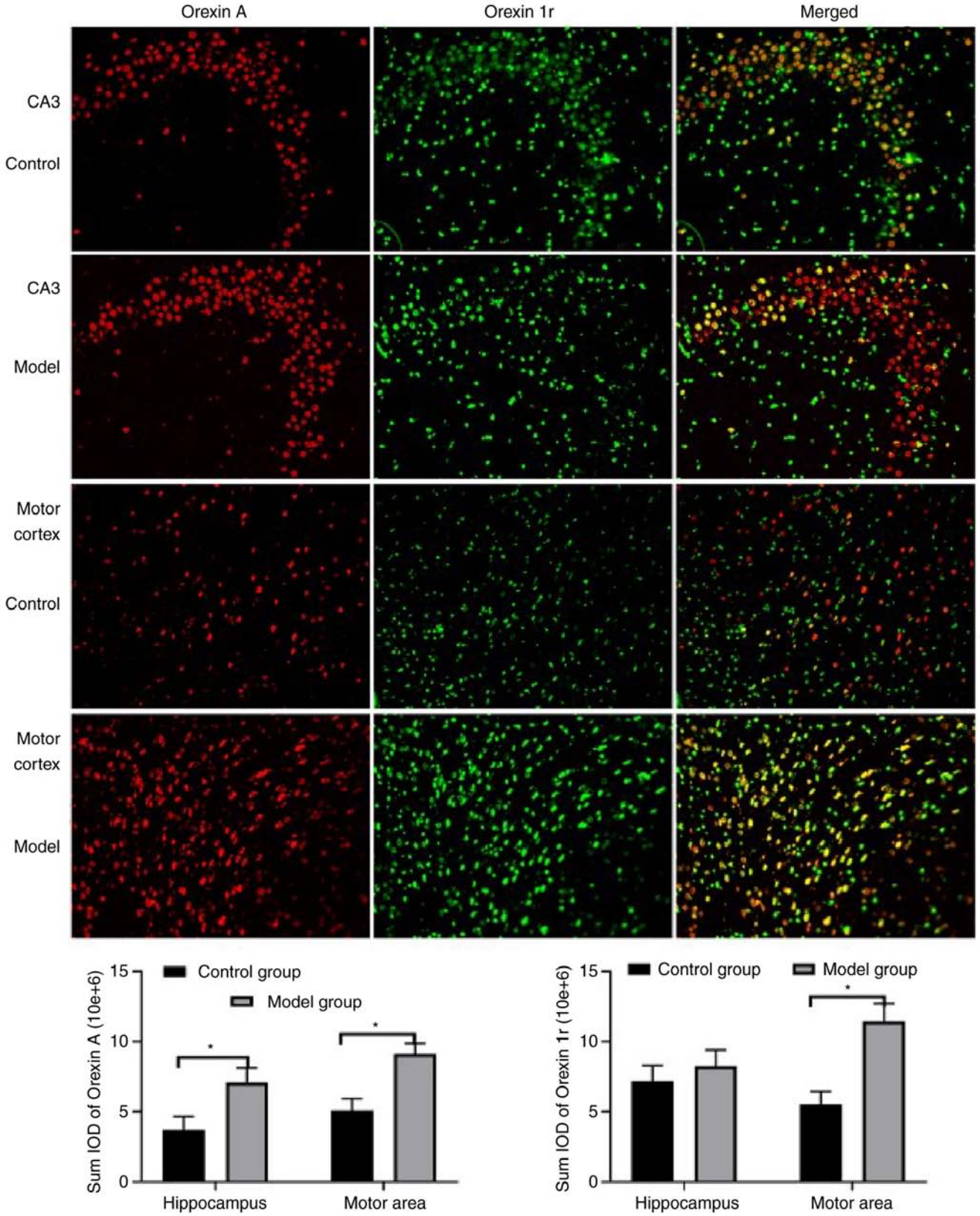

Figure 7. Comparison of orexin A and orexin 1r expression in the CA3 region of the hippocampus and the motor cortex of the model and control groups. Representative fluorescent images (magnification, x100) and quantified data are presented. *P<0.05 compared with the control group (n=6). Orexin 1r, orexin 1 receptor; IOD, integrated optical density.

levels of the anti-inflammatory factor IL-10 (41), which may be a contributing factor to the impairment of learning and memory ability.

Induction of sleep by pentobarbital sodium is often used in the evaluation of the sleep function of model rats (23,24,42). In the present study, the pentobarbital sodium-induced sleep

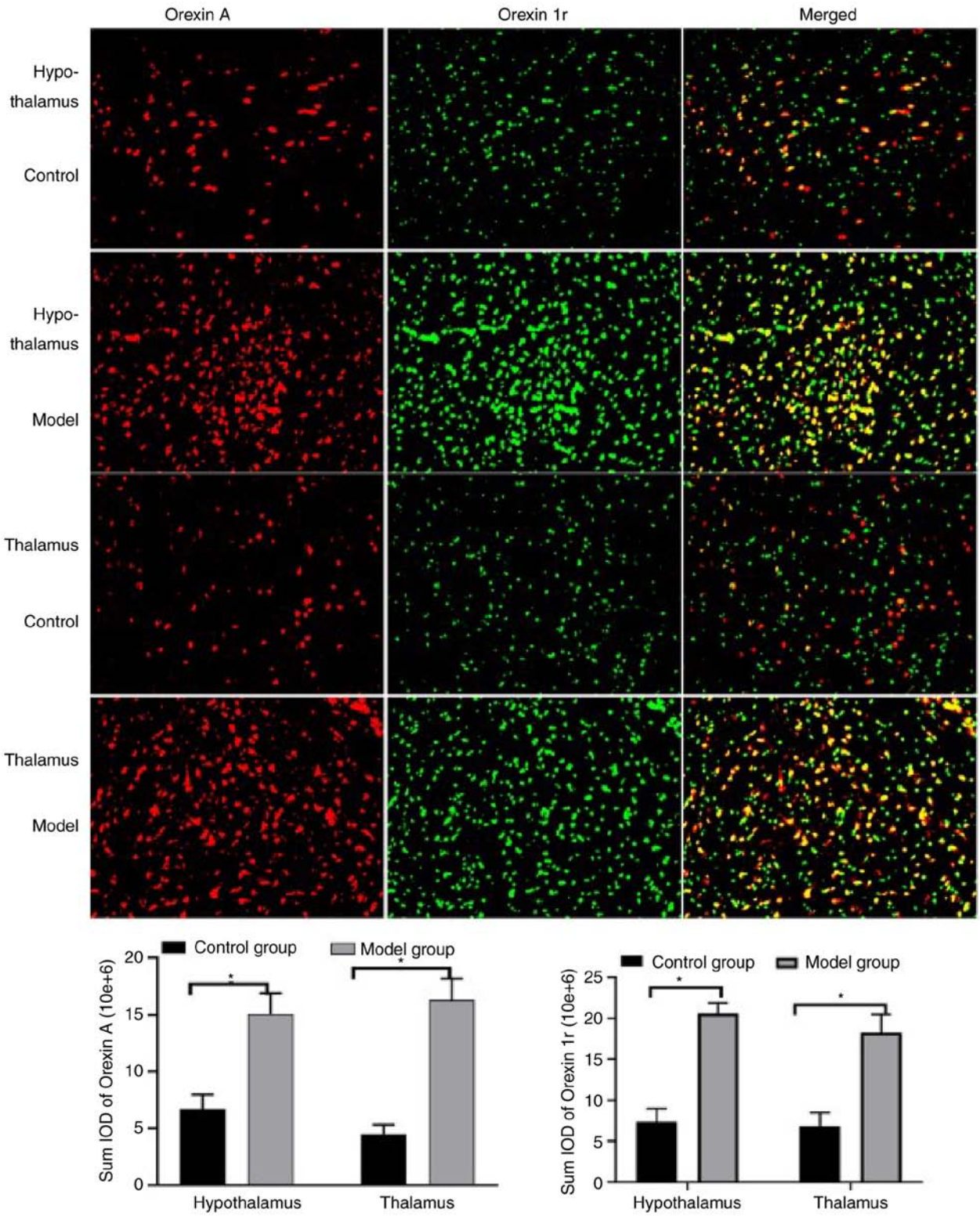

Figure 8. Comparison of orexin A and orexin 1r expression in the hypothalamus and thalamus of the model and control groups. Representative fluorescent images (magnification, x100) and quantified data are presented. *P<0.05 compared with the control group (n=6). Orexin 1r, orexin 1 receptor; IOD, integrated optical density.

experiment demonstrated sleep resistance of the model rats due to a longer sleep latency and shorter time spent sleeping. However, the mechanism of action for this is as yet unknown.

Insomnia promotes pathophysiological changes of an immunological nature, and the expression levels of TNF-α, IL-1β, IL-6 and IL-10 in serum or in tissues reflect the functional state of immunity (41). In the present study, IL-1β and IL-6 levels were increased in serum and brain tissues, whereas the serum levels of TNF-α were increased and those of IL-10 were decreased in the model rats. These findings are consistent with those reported in previous clinical studies (43-45), indicating the presence of immune disorders in the model rats.

Insomnia promotes an increase in the expression levels of feeding and arousal regulators. Orexin is a hormone/neurotransmitter synthesized and secreted by the hypothalamus, which can stabilize REM sleep (46,47). Since REM sleep is reduced when rats are kept on platforms surrounded by water, orexin expression increases as a compensatory mechanism. However, the increase in orexin A levels promotes arousal and feeding through orexin 1r (48). In the present study, serum orexin A levels were increased, and the expression levels of orexin and orexin 1r in the brain tissues were increased in the model rats, which is consistent with the results reported in previous studies (48,49).

In conclusion, the present study established a chronic insomnia rat model with sleep fragmentation using self-made multiple strings of platforms surrounded by water. The changes induced in the model rats with regard to sleep duration, sleep-inducing drug resistance, diurnal and nocturnal body weight changes, and cognitive function were evaluated. Furthermore, the expression levels of inflammatory factors and neurohumoral regulators involved in feeding behavior, arousal and sleep were assessed. The results indicate that the present chronic insomnia rat model is suitable for use in studying the mechanism of action of chronic insomnia with sleep fragmentation.

However, the present study has several limitations. Firstly, the sample size was very small, which should be expanded in future studies. Secondly, a positive control group was not included for confirmatory purposes. Thirdly, quantitative histopathology for specific analysis is lacking, which requires further investigation. Furthermore, several mechanisms involved in changes of the parameters measured in the model rats are not clear and merit investigation in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DQY designed the study and wrote the manuscript. DQY, ND, WHZ, TL, QWY, ZPT, KKW, GYW and ZTL performed experiments and analyzed the data. XPZ designed the study and performed experiments. XPZ and DQY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were conducted in accordance with China Experimental Animals Administration Legislation and were approved by the Ethics Committee of Xinjiang Medical University (approval no. IACUC-20150225-118 Urumqi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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