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PSYCHIATRICS • ORIGINAL ARTICLE

Hypnotic activities of Zao Ren An Shen capsule, a traditional Chinese medicine, in an anxiety-like mouse model

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Abstract

Purpose Zao Ren An Shen capsule (ZRASC) which is composed of three kinds of traditional Chinese herbs is a popular Chinese medicine for the treatment of insomnia. This study investigated the hypnotic effect of ZRASC in an anxiety-like mouse model.

Methods We determined the role of ZRASC in anxiety and co-morbid insomnia using electroencephalogram and electromyogram recordings. Anxiety-like behaviors were tested by using the open-field, light/dark box, or elevated plus-maze in mice. Immunohistochemical techniques were employed to reveal the mechanism by which ZRASC regulated anxiety and insomnia.

Results ZRASC at 680 mg/kg prolonged the time spent in the central area, open arms area, and light box by 1.9, 2.3, and 1.7-fold respectively, compared with the vehicle control group in immobilization stress (IMS) mice. ZRASC at 680 mg/kg given at 08:00 h increased the amount of non-rapid eye movement sleep by 1.4-fold in a 2-h period after dosing in IMS mice. However, it did not alter the sleep-wake behaviors in normal mice. Immunohistochemistry showed that IMS increased c-Fos expression in the neurons of the stria terminalis and tuberomammillary nucleus by 1.8 and 1.6-fold, respectively. In addition, ZRASC (680 mg/kg) reversed the IMS-induced c-Fos expression.

Conclusions Our results suggest that ZRASC is an effective therapeutic strategy for both anxiety disorder and sleep disturbances in an anxiety-like mouse model.

Keywords Sleep disturbance · Zao Ren An Shen capsule · Anxiety · c-Fos · Immobilization stress

Introduction

Anxiety disorders are some of the most widespread mental disorders [1]. According to large population-based surveys, more than 33.7% of the population is affected by an anxiety disorder which seriously affects human quality of life [2]. Sleep disturbances are commonly observed in individuals with anxiety and related disorders [3], and this link is found across all ages [4]. Sleep disturbances negatively affect the quality of life of patients with anxiety. More than 30% of patients complain of a sleep disorder, frequently associated with an increase in sleep latency or reduction in slow-wave sleep [5]. Mounting evidence indicates that restricted sleep will aggravate anxiety symptoms, beginning a vicious cycle [6]. However, the most commonly used anxiolytics and sedatives have limitations. Benzodiazepine and buspirone are the most commonly used agents to treat anxiety disorder, but they have side effects such as dizziness, headache, drowsiness, and ataxia [7]. Furthermore, tolerance and dependence are undesirable side-effects in long-term exposure. Therefore, developing more effective sleep-promoting and anxiolytic drugs with fewer side effects remains a challenge for the scientific community.

It has been reported that many classic traditional Chinese formulae have good clinical effects in the treatment of neuropsychiatric disease [8–10]. Zao Ren An Shen capsules (ZRASC) are comprised of the following three Chinese herbs: *Ziziphus jujuba var. spinosa (Bunge) Hu ex H.F.Chow*, *Salvia miltiorrhiza Bunge*, and *Schisandra*

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chinensis (*Turcz.*) *Baill*. Previous clinical studies have shown that ZRASC is an effective sedative-hypnotic agent [11]. ZRASC combined with trazodone has improved sleep dysfunction rating scale scores in patients with insomnia, and the effects were superior to trazodone alone [12]. Ideal agents should effectively improve both anxious symptoms and sleep disturbances without impairing the patient's daytime life. There is a lack of evidence for hypnotic properties of ZRASC in anxiety-induced insomnia, and it is unclear if therapeutic doses of ZRASC influence motor coordination and drowsiness.

In this study, the effects of ZRASC on sleep disturbance were evaluated in a mouse immobilization stress (IMS) anxiety model [13]. The bed nucleus of the stria terminalis (BNST) is a key region for an extended amygdala stress response for fear and anxiety [14, 15]. Recent studies have revealed that the BNST involves the transition of sleep-wake behavior [16]. The tuberomammillary nucleus (TMN), located in the posterior hypothalamus, plays an important role in promoting wakefulness [17]. Beyond this, lesion of TMN histaminergic neurons can induce anxiolytic-like effects in rats [18]. These findings suggest that both BNST and TMN are involved in the regulation of sleep-wake behavior and anxiety symptoms. Therefore, we speculated that BNST and TMN might be important targets of ZRASC in treating the sleep disturbance caused by anxiety. To test this hypothesis, we proposed measuring the number of c-Fos positive neurons using immunohistochemistry after ZRASC administration. The c-Fos is an immediate early gene. The detection of its product, the c-Fos protein, is classically used to identify the activity of neurons involved in stimulation responses [19]. We futher proposed measuring the c-Fos protein.

Materials and methods

Animals

Male SPF inbred C57BL/6J mice, 10–12 weeks old and weighing 20–26 g, were purchased from the Laboratory Animal Center at the Chinese Academy of Sciences (Shanghai, China). Animals were housed individually at an ambient temperature of 22 ± 0.5 °C, with relative humidity of 60 ± 2%, under an automatically controlled 12 h light/12 h dark cycle (lights on at 07:00), and with free access to food and water. Experimental protocols were approved by the Medical Experimental Animal Administrative Committee of Fudan University. Every effort was made to minimize potential distress, pain, or discomfort to the animals throughout all experiments.

Chemicals

Zao Ren An Shen capsules (lot no. 150603) were obtained from Sinopharm Group Tongjitang Pharmaceutical Co., Ltd. (Guizhou, China). ZRASC was made of three Chinese medicinal materials, *Ziziphus jujuba var. spinosa* (*Bunge*) *Hu ex H.F.Chow*, *Salvia miltiorrhiza Bunge*, and *Schisandra chinensis* (*Turcz.*) *Baill* processed by vinegar according to the ratio of 5:1:1. In the production process, the three Chinese medicinal materials were extracted by absolute ethanol, and the remaining solid residues were extracted by purified water. Then, these two extracts were concentrated, mixed with excipients, and dried to obtain solid particles and filled into the capsules. Each ZRASC prepared by this method contains less than 0.4 mg spinosin, 0.24 mg tanshinone II A and 0.3 mg schisandrin A. Diazepam was purchased from the Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). All drugs were dissolved in 0.9% sterile saline just before the experiment. Rabbit polyclonal anti-c-Fos antibody were purchased from Millipore (Boston, MA, USA), and biotinylated donkey anti-rabbit IgG, avidin–biotin-peroxidase were purchased from Vector Laboratories (Burlingame, CA, USA). 3,3′-diaminobenzidine tetrahydrochloride (DAB) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Polygraphic recordings and vigilance state analysis

Under 5% chloral hydrate (360 mg/kg, i.p.) anesthesia, mice were implanted with electrodes for polysomnographic electroencephalogram (EEG) and electromyogram (EMG) recordings. Two stainless steel screws (1 mm in diameter) were inserted through the skull (antero-posterior, + 1.0 mm; left-right, − 1.5 mm from the bregma or lambda) according to the mouse brain atlas; these screws served as EEG electrodes. Two teflon-coated, insulated stainless steel wires were placed bilaterally into trapezius muscles, and these served as the EMG electrodes. All electrodes were attached to a microconnector and fixed onto the skull using dental cement [20, 21].

Immobilization stress anxiety model in mice

Animals were immobilized for 10 min by taping their four limbs to a board after placing them on their backs using zinc oxide hospital tape [13]. The tape was unraveled after moistening with ethanol in order to minimize pain or discomfort.

Elevated plus maze test

The elevated plus maze consisted of two open arms (30 cm × 5 cm, length × width) and two closed arms (30 cm × 5 cm × 15 cm, length × width × height) connected by an open central

platform (6 cm × 6 cm, length × width), and the apparatus was elevated 45 cm above the floor. The plus maze was illuminated by two yellow lights (25 W) placed above the device and provided 100 lx illumination. A digital video camera was mounted directly above the maze to record the activity of the mice during testing. Mice were individually placed on the platform with head toward the open arms, and allowed to freely explore for 5 min. Then, the distance traversed and time spent in the open arms were recorded by computer. Between trials, the maze was cleaned with 75% ethanol to remove odor cues [22].

Open field test

In the open field (OF) test, mice were individually placed in the center of a square area (50 cm × 50 cm) with Plexiglas walls (35 cm) around. This apparatus was illuminated with two yellow lights (25 W) fluorescent lamps placed 120 cm above the center of the square area. At the beginning of the experiment, the mice were placed in the center of open field and allowing it to move freely for 10 min. [23]. A video motion tracking system could record the distance and time spent in the central area. Finally, open field trials were conducted 60 min after ZRASC treatment. Between trials, the maze was cleaned with 75% ethanol to remove odor cues.

Light/dark box test

After the open field test, the light/dark box test was performed immediately. The apparatus consisted of two compartments with lid divided by a connection door (3.5 cm × 3.5 cm). The light compartment (24 cm × 20 cm × 20 cm) was illuminated by a single 60 W (300 lx) fluorescent lamp which was positioned 30 cm above the compartment. The dark compartment (12 cm × 20 cm × 20 cm) was entirely black. Each mouse was individually placed in the light compartment facing the door and monitored for 5 min [24]. The number of transfers between the two compartments and the time spent in white compartments was recorded by a video motion tracking system. Between trials, the maze was cleaned with 75% ethanol to remove odor cues.

Pharmacological treatments

To study the effect of ZRASC on sleep disturbance under anxiety-like conditions, 170, 340, or 680 mg/kg ZRASC, 6 mg/kg diazepam, or vehicle (saline) was administered intragastrically (i.g.) at 08:00 h on the day of the experiment in IMS mice. A 680 mg/kg dose of ZRASC was administered i.g. at 21:00 h to investigate possible drowsiness effects on the active stage of normal mice.

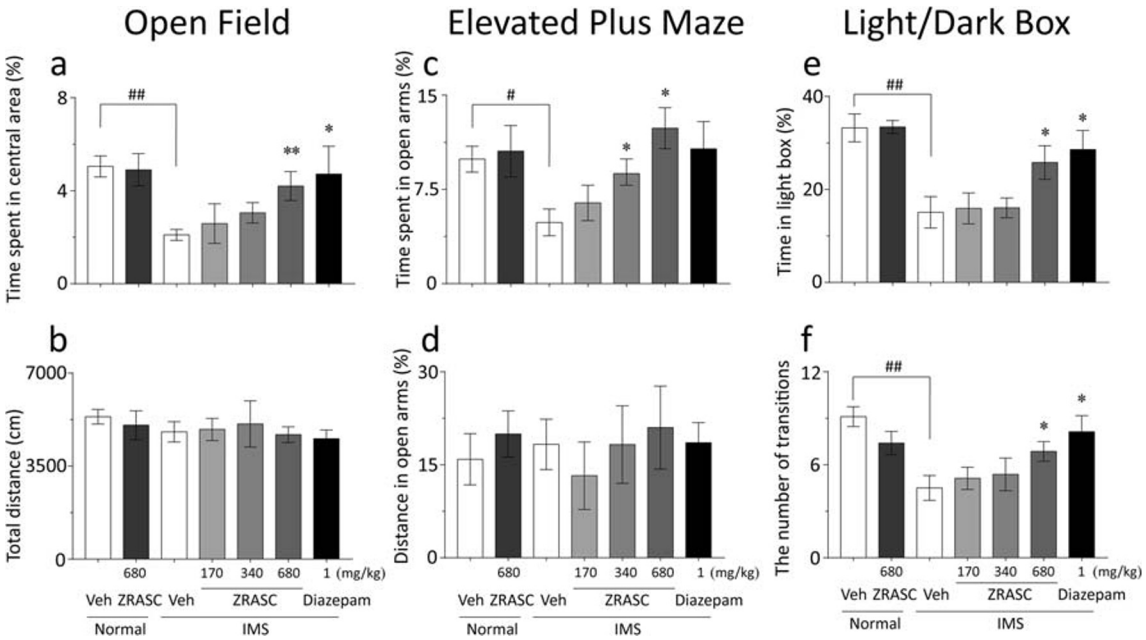
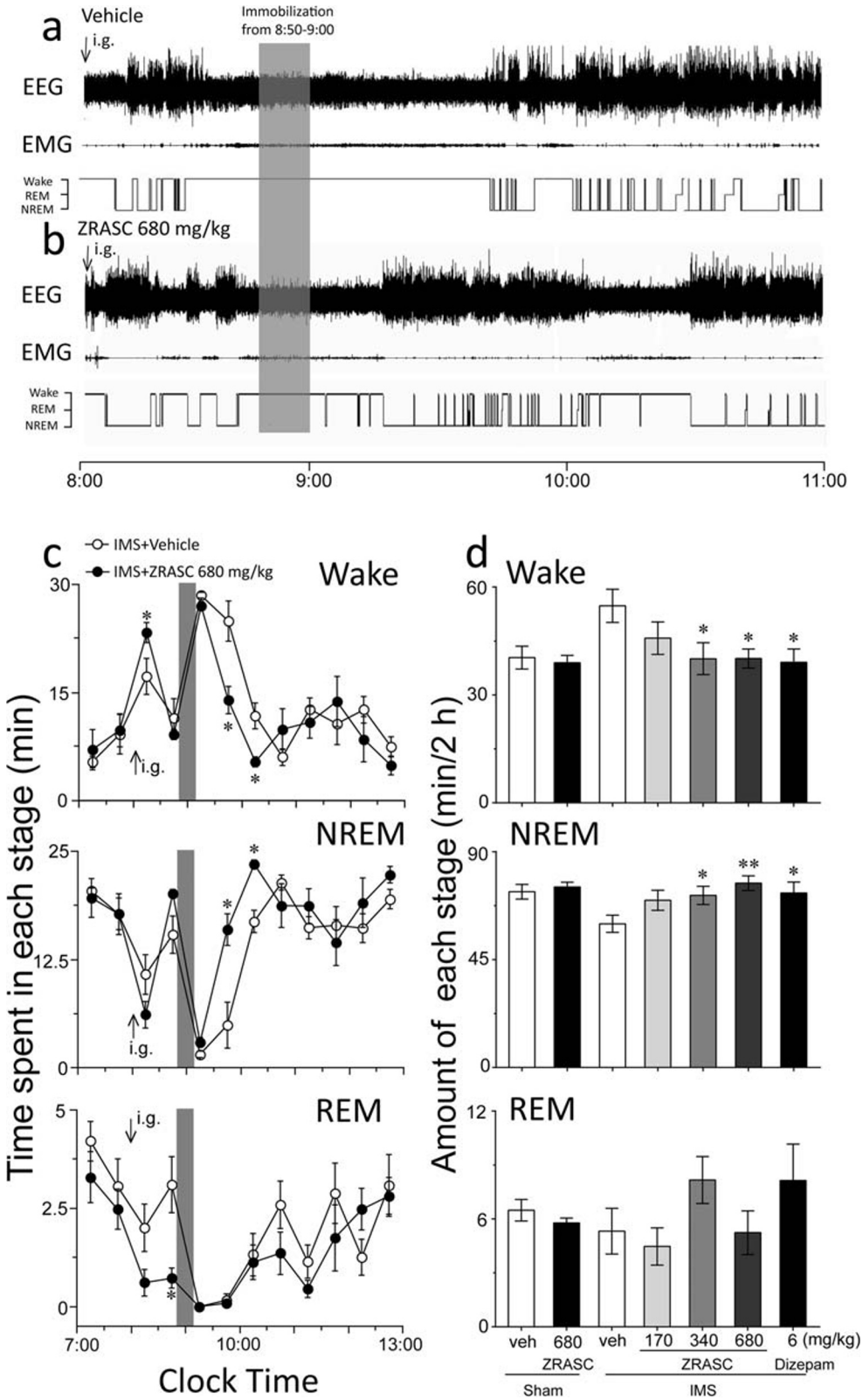


Fig. 1 ZRASC exerted anxiolytic effects in IMS-induced anxiety model mice. The percentage of time spent in the central area (**a**) and the total distance (**b**) were measured for 10 min in the open field test. The percentage of time spent in the open arms (**c**) and the distance in the open arms (**d**) were measured for 5 min in the elevated plus maze test. The percentage of time spent in the light box (**e**) and the number of transitions (**f**) were measured for 5 min in the light/dark box test. Mice were treated with vehicle or ZRASC 1 h before the beginning of the behavior test. Values are the means ± SEM ($n = 8$–9). $^{*}P < 0.05$ or $^{**}P < 0.01$ indicates significant differences from the vehicle group as assessed by a two-tailed unpaired Student's t test. $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ indicates significant difference from the vehicle value in the normal group



To study the anxiolytic effect of ZRASC in IMS mice, ZRASC (170, 340, 680 mg/kg), 1 mg/kg diazepam [25] was administered i.g. 1 h before performing the anxiety-like behavioral testing.

c-Fos immunohistochemistry

The experimental animals were randomly divided into the following four groups: IMS + vehicle, IMS + 680 mg/kg

◀ **Fig. 2** Sleep–wake profiles produced by administration of ZRASC in IMS mice. **a**, **b** Typical examples of polygraphic recordings and corresponding hypnograms in IMS mice treated with vehicle (top panel) or ZRASC (bottom panel) at a dose of 680 mg/kg. **c** Changes in NREM, REM sleep, and wakefulness over time in IMS mice treated with ZRASC. Each circle represents the half-hourly mean amount of each stage. Open and closed circles represent profiles of vehicle and ZRASC treatments, respectively. Values are means ± SEM ($n = 6$–8). *$P < 0.05$ or **$P < 0.01$ indicates significant differences compared to the vehicle control group as assessed by repeated measures ANOVA followed by the PLSD test. **d** Dose–response effect on total time spent in NREM sleep, REM sleep, and wakefulness for 2 h after immobilization. Open and filled bars show the profiles of vehicle and ZRASC or diazepam treatments, respectively. Values are the means ± SEM ($n = 6$–8). *$P < 0.05$ or **$P < 0.01$ indicates significant differences from the vehicle value in the IMS group assessed by one-way ANOVA followed by the PLSD test

ZRASC, sham + vehicle, and sham +680 mg/kg ZRASC. Each group was pretreated with either vehicle or ZRASC (i.g.) at 08:00 h. After 50 min, the IMS groups were subjected to immobilization for 10 min, while the sham groups were subjected to mouse grabbing. An hour later, all mice were then sacrificed for immunohistochemical assessments as described previously [26].

Statistical analysis

All results were expressed as means ± standard errors of the means (SEM). The time courses of the half-hourly amounts measured at each stage in IMS mice treated with vehicle or ZRASC were compared using repeated measures ANOVA followed by Fisher's probable least-squares difference (PLSD) test. The amounts of sleep and wakefulness, the number of transitions between sleep and wakefulness, and the number and duration of bouts of sleep and wakefulness in the ZRASC and vehicle groups were assessed using the non-paired, two-tailed Student's *t* test. The anxiety-like behavioral analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls post hoc test. For the number of c-Fos immunoreactive neurons, one-way ANOVA followed by the PLSD test was used. A *P* value under 0.05 was considered to be statistically significant.

Results

ZRASC exerted anxiolytic effects in IMS-induced anxiety model mice

To verify IMS-induced anxiety-like behaviors, mice were evaluated in the open field, elevated plus maze, and light/dark box tests 30 min after IMS. As shown in Fig. 1a–e, the IMS group spent 58.4% ($P < 0.01$), 46.0% ($P < 0.05$), and 54.2% ($P < 0.01$) less time in the central area, open arms, and light box, respectively, compared with the vehicle normal control. In addition, in the light/dark box test, the IMS group had 50.5% fewer transitions in the light box ($P < 0.01$) (Fig. 1f). Taken together, these results indicate that IMS causes anxiety-like effects in mice.

To evaluate the anxiolytic effect of ZRASC, we performed three types of classic anxiety tests: the open field, dark/light box, and elevated plus maze tests after administration ZRASC 1 h in IMS mice. High-dose ZRASC (680 mg/kg) acute treatment increased the percentage of time spent in the central area 1.9-fold ($P < 0.01$) in the open field test (Fig. 1a) and increased the percentage of time spent in the open arms 2.3-fold ($P < 0.05$) in the elevated plus maze test (Fig. 1c), compared with vehicle controls. ZRASC also improved the percentage of time spent in the light box 1.7-fold ($P < 0.05$) and the number of transitions 1.5-fold ($P < 0.01$) in light/dark box test (Fig. 1 e and f). ZRASC given at 340 mg/kg only increased the time spent in the open arms 1.6-fold ($P < 0.05$) compared with the vehicle control. Furthermore, ZRASC did not affect locomotor activity (Fig. 1b). The anxiolytic effects of ZRASC at 680 mg/kg were no different from the effects of diazepam at 1 mg/kg. The above results confirm that single administration of ZRASC exerts anxiolytic effects in IMS mice.

Hypnotic effect of ZRASC in IMS mice during the light phase

To investigate whether ZRASC exerted a hypnotic effect in the anxiety-like state in light (inactive) period, a gradient of ZRASC doses (170, 340, or 680 mg/kg) was administered i.g. at 08:00 h before the immobilization (8:50–9:00 h). Diazepam at 6 mg/kg was as a positive control. Typical examples of polygraphic recording in IMS mice treated with vehicle or ZRASC at 680 mg/kg are shown in Fig. 2a–b. Mice administered ZRASC spent more time in NREM sleep, compared with the control group. Changes in time courses revealed that pretreatment with ZRASC at 680 mg/kg caused a significant increase in NREM sleep and a decrease in wakefulness in IMS mice, compared with vehicle-treated mice. ZRASC improved NREM sleep time of IMS mice by 3.0- ($P < 0.05$) and 1.4-fold ($P < 0.05$) at the second and third half-hour after immobilization, respectively. Correspondingly, 75% ($P < 0.05$) and 55.5% ($P < 0.05$) decreases in wakefulness were found. ZRASC did not affect the amount of REM sleep (Fig. 2c). Figure 2d summarizes the total amounts of time spent in NREM sleep, REM sleep, and wakefulness in the first 2 h after immobilization. Compared with the vehicle, pretreatment of IMS mice with ZRASC at 680 mg/kg or positive controls with diazepam at 6 mg/kg increased NREM sleep 1.35- ($P < 0.05$) and 1.48-fold ($P < 0.05$) and decreased wakefulness by 20.4% ($P < 0.05$) and 35.4% ($P < 0.05$), respectively. ZRASC at 340 mg/kg increased NREM sleep 1.3-fold ($P < 0.05$). The duration of REM sleep did not change in the

IMS group. Our results suggested that ZRASC increased NREM sleep in IMS mice.

Changes in the mean duration of episodes, number of bouts, stage transition, and NREM sleep latency induced by ZRASC in IMS mice

To further investigate the sleep-wake architecture change caused by ZRASC, the distribution of bouts of NREM sleep and wakefulness was determined as a function of the duration of the bout or episode. ZRASC at 680 mg/kg increased the number of bouts of NREM sleep that had durations of 128–256 and 256–512 s (Fig. 3a, left panel). Correspondingly, ZRASC decreased the number of wakefulness bouts with durations greater than 2048 s (Fig. 3a, right panel). As shown in Fig. 3b, the total number of episodes of wakefulness, NREM sleep, and REM sleep showed no difference between the ZRASC treatment group and control group during the 2 h after immobilization. However, ZRASC (680 mg/kg) decreased the mean duration of wakefulness by 37.2% ($P < 0.05$) and increased the mean duration of NREM sleep by 63.4% ($P < 0.05$), although there was no difference in the mean duration of REM sleep (Fig. 3c). No difference in the number of transitions either from wakefulness to NREM sleep or from REM sleep to wakefulness was observed (Fig. 3d).

As shown in Fig. 3e, administration of ZRASC shortened NREM and REM sleep latency remarkably in IMS mice. NREM sleep latency is defined as the time from the end of immobilization to the appearance of the first NREM or REM sleep episode lasting for at least 20 s. The latency-to-NREM sleep period in mice treated with ZRASC 360 mg/kg was 17.9 min. This was significantly shorter than the 28.8-min latency in the vehicle group. The ZRASC also decreased the latency-to-REM sleep period from 51.7 to 28.6 min. The short sleep latency of the ZRASC-treated mice clearly indicates that ZRASC accelerated the initiation of NREM and REM sleep in IMS mice.

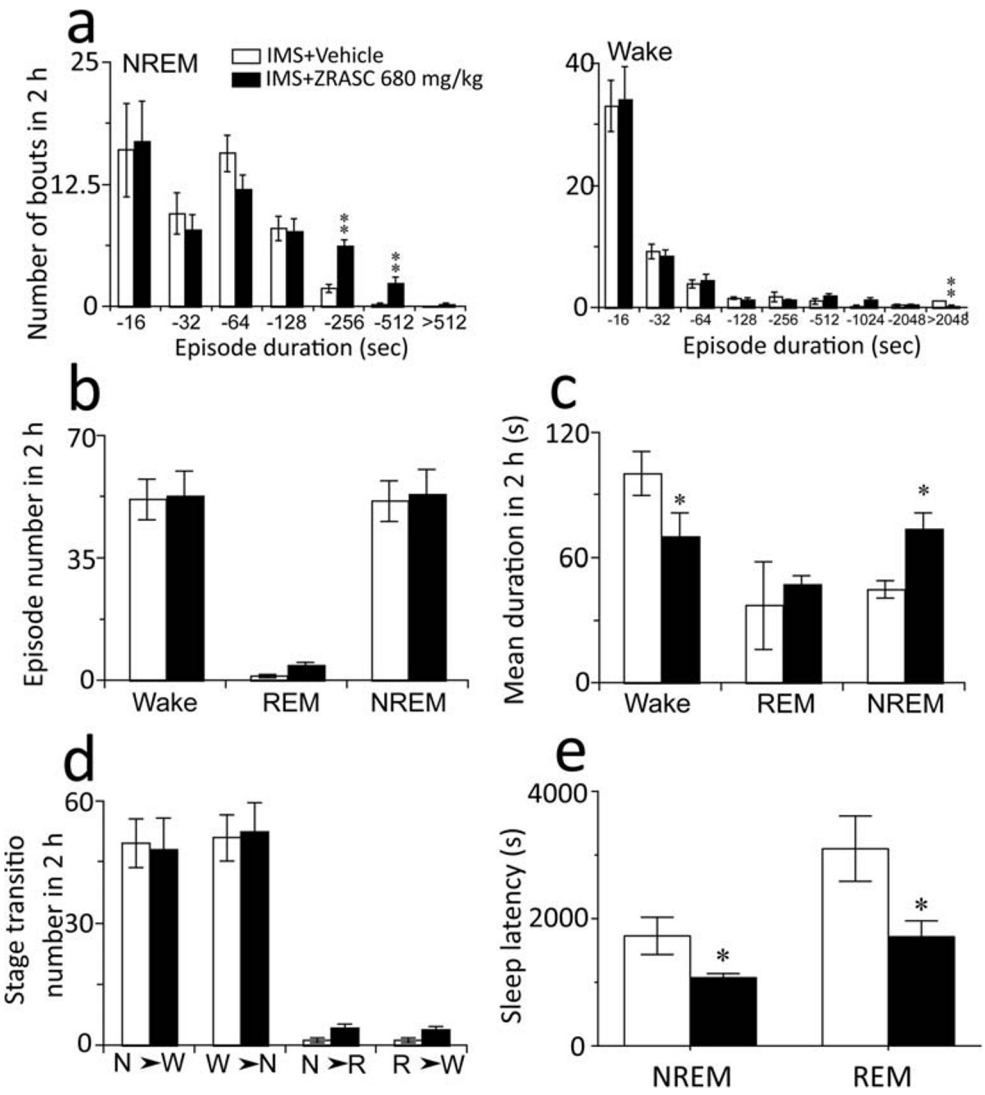


Fig. 3 Characteristics of sleep–wake episodes and transitions during the 2 h after immobilization. **a** Number of NREM sleep and wake bouts, **b** total number of episodes, **c** mean durations, **d** stage transitions, **e** NREM and REM sleep latency. Open and filled bars show the profiles of vehicle and ZRASC treatments, respectively. Values are the means ± SEM ($n = 6–8$). *$P < 0.05$ or **$P < 0.01$ indicates significant differences from vehicle values in the IMS group, assessed by a two-tailed unpaired Student's *t* test

ZRASC did not induce drowsiness effect in normal mice

In order to explore the effect of ZRASC on sleep–wake profiles in night (active) period, ZRASC at 680 mg/kg or vehicle was administered i.g. to normal mice at 21:00 h, when mice spent most of their time awake. We found that ZRASC did not affect sleep–wake behavior (Fig. 4). The results confirmed that ZRASC did not elicit drowsiness in the active phase in normal mice.

Effects of ZRASC-induced c-Fos expression in the BNST and TMN of normal and IMS model mice

To further study the involvement of the BNST and TMN in the anxiolytic effects of ZRASC, we observed the changes in the number of c-Fos positive neurons in the BNST and TMN after ZRASC administration. Representative photomicrographs (Figs. 5a–d and 6a–d) of c-Fos expression are demonstrated for each group. Analysis of the number of c-Fos positive neurons showed that IMS increased the expression of c-Fos in the BNST 1.8-fold ($F_{3,16} = 5.19$, $P < 0.05$) relative to the vehicle group, and ZRASC decreased the number of c-Fos positive neurons in the BNST of IMS mice by 38% ($P < 0.05$) (Fig. 5f). In addition, IMS increased the expression of c-Fos in the TMN 1.6-fold ($F_{3,16} = 3.25$, $P < 0.05$) compared with the vehicle group, and ZRASC decreased the number of c-Fos positive neurons in the TMN of anxiety model mice by 38% ($P < 0.05$) (Fig. 6f). No significant effect of ZRASC on the expression of c-Fos in the BNST and TMN regions of normal mice was observed.

Discussion

In this study, ZRASC improved anxiety-like behaviors in IMS mice and did not impair motor function. In addition, ZRASC shortened sleep latency and increased the amounts of NREM sleep in a mouse model of anxiety. Immunohistochemistry showed that ZRASC reverted the overexpression of c-Fos in the BNST and TMN caused by IMS. These results clearly suggest that ZRASC exerts anti-anxiety and hypnotic effects in an anxiety-like mouse model.

Anxiety is a negative emotion thought to be caused by many kinds of stress, such as restraint, social defeat, forced swim, and inescapable footshock [27]. In the present study, IMS caused marked anxiety-like behavior. Our results are consistent with the findings of a previous report that acute immobilization stress caused severe anxiety in rodents [11]. Recent clinical research also suggests that anxiety is closely related to the development of insomnia [12]. We found that IMS induced anxiety symptoms and insomnia in mice. These results provide a hint that the IMS mice can simulate clinical symptoms of anxiety and insomnia in patients. In this study, 680 mg/kg ZRASC exerted anxiolytic effects in IMS mice but did not affect the anxiety behaviors in normal mice. This suggested that the anxiolytic effects of ZRASC are effective against anxious states. ZRASC at a dose of 680 mg/kg did not alter locomotion activity in an open field test, indicating that ZRASC can effectively improve anxiety symptoms without impairing motor function.

Diazepam and clonazepam are classical benzodiazepines used to treat anxiety disorder. They have been shown to relieve anxiety and induce sleepiness, torpor, and relaxation in patients [8, 28]. This is the desired effect when the medication is taken as a hypnotic at night to induce sleep, but is an unnecessary adverse effects when administered during the day for generalized anxiety disorder. Furthermore, benzodiazepines have been shown to impair psychomotor functions such as speed and accuracy [14]. Buspirone, which acts as a partial agonist of the serotonin 5-HT_{1A} receptor, has also been used to treat patients with generalized anxiety disorder [15]. However, buspirone increases wakefulness and suppresses sleep [16, 18]. A recent study found that *Semen ziziphi spinosae* and *Salvia miltiorrhiza*, two crucial components of ZRASC, can shorten sleep latency, prolong sleeping time and movement convalescence time induced by sodium pentobarbital in mice. Intriguingly, researchers also found that

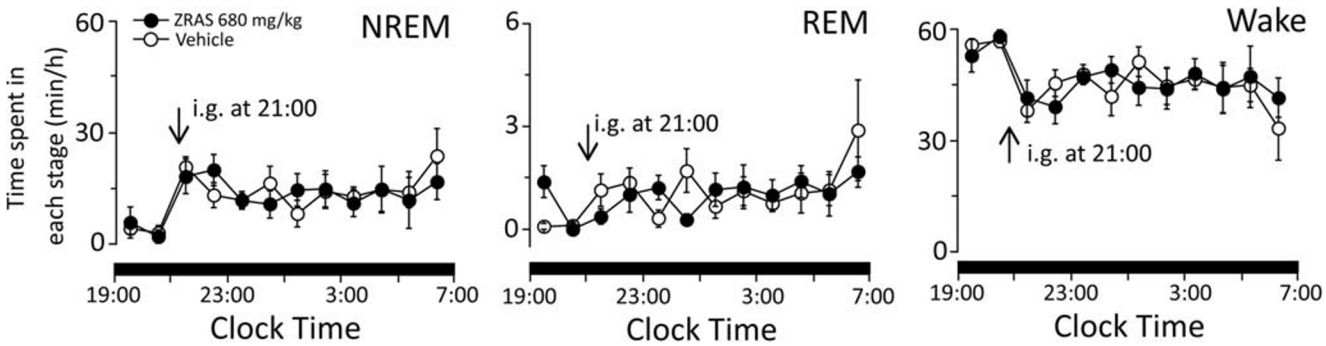


Fig. 4 Changes in NREM and REM sleep and wakefulness in normal mice treated with ZRASC. Each circle shows the hourly mean duration of each stage. Open and closed circles show the profiles of vehicle and ZRASC treatments, respectively. There was no essential difference in NREM and REM sleep or wakefulness between ZRASC treatment and vehicle control

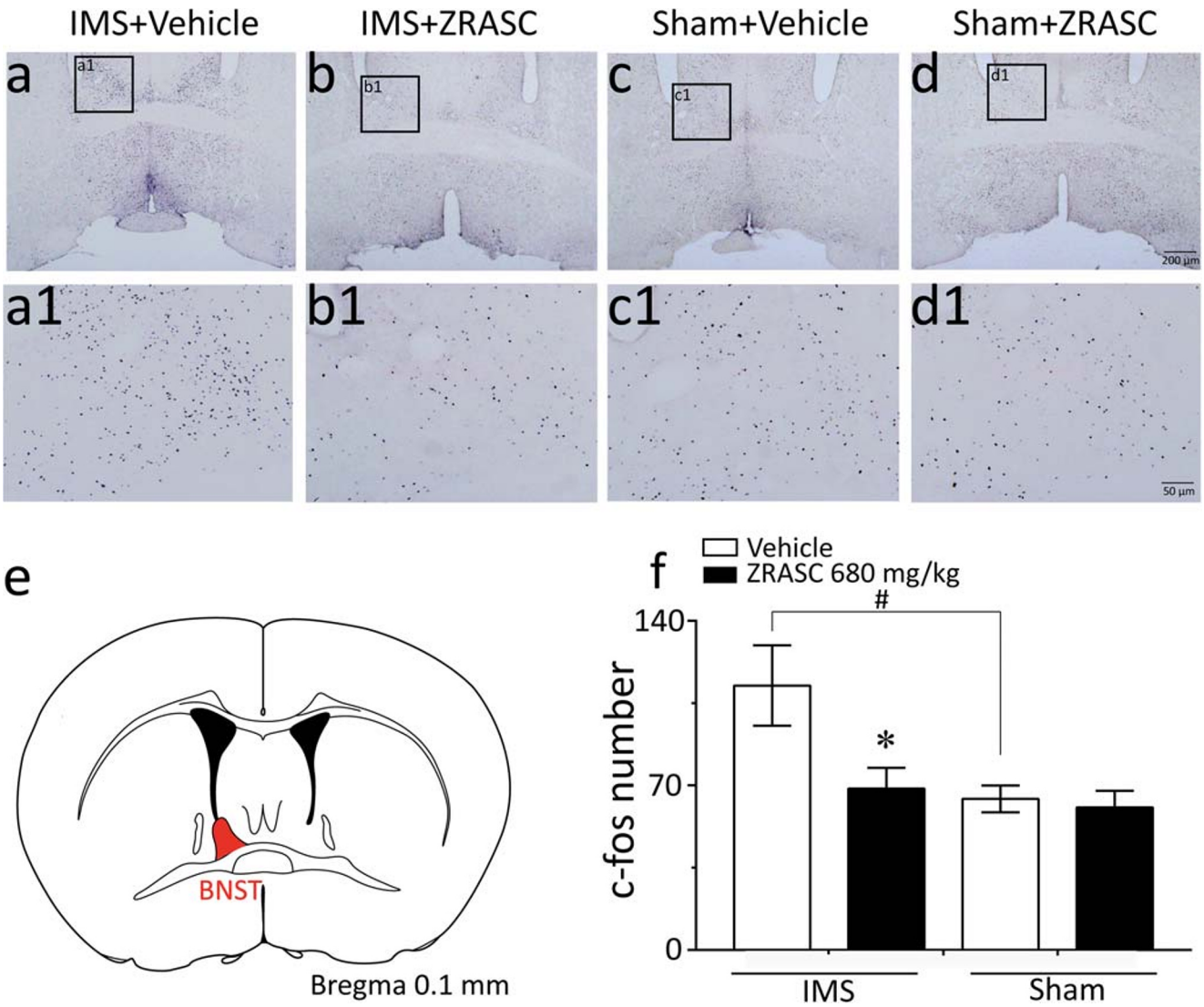


Fig. 5 Effects of ZRASC-induced c-Fos expression in the BNST of normal and IMS model mice. Representative photomicrographs (**a**–**d**, a1–d1) of c-Fos expression in the BNST are demonstrated for IMS + vehicle mice (**a**, a1), 680 mg/kg ZRASC-treated IMS mice (**b**, b1), sham + vehicle mice (**c**, c1), and 680 mg/kg ZRASC-treated sham mice (**d**, d1). Scale bars in **a**–**d**, 200 μm; scale bars in (a1–d1), 50 μm. **e** Schematic drawings of the BSNT in the coronal mouse brain atlas. **f** Amount of the number of c-Fos positive neurons in the BNST of mice at 90 min after treatment with vehicle or ZRASC. Values represent mean ± SEM ($n = 5$). $^{*}P < 0.05$ indicates significant difference from the IMS + vehicle group, $^{\#}P < 0.05$ indicates significant difference from the vehicle value in the sham group, assessed by one-way ANOVA followed by the PLSD test

combination of *Semen ziziphi spinosae* and *Salvia miltiorrhiza* showed significant synergistic effect in the treatment of insomnia [29]. These results suggest that the ZRASC formula is more effective on sedation than the single ingredient. Another major component, *Schisandra chinensis*, has been demonstrated to reverse stress-induced anxiety-like behavior and changes in cortex monoamine transmitters and plasma corticosterone [30]. The current study revealed that ZRASC (680 mg/kg) obviously improved sleep disturbance after immobilization during the light phase, the "sleep period" for mice. Both the total time and the number of long duration bouts of NREM sleep (128–256 s and 256–512 s) in IMS mice were increased significantly during the first 2 h after ZRASC administration. In addition, the sleep latencies of NREM and REM sleep were decreased. These data suggest that ZRASC lessened difficulty in falling asleep and sleep fragmentation in the IMS mice. Interestingly, the data also indicated that ZRASC did not affect the sleep patterns during the dark, active period for mice. These results suggest that ZRASC can mitigate sleep disturbance without causing drowsiness or ataxia during the active phase.

Fos expression can be utilized as biomarker of neural activation by ZRASC. The bed nucleus of the stria terminals is a constituent of the extended amygdala. Neuroimaging studies have found that the activity of BNST area significantly increases during anxiety-like behavior [31]. Deisseroth et al.

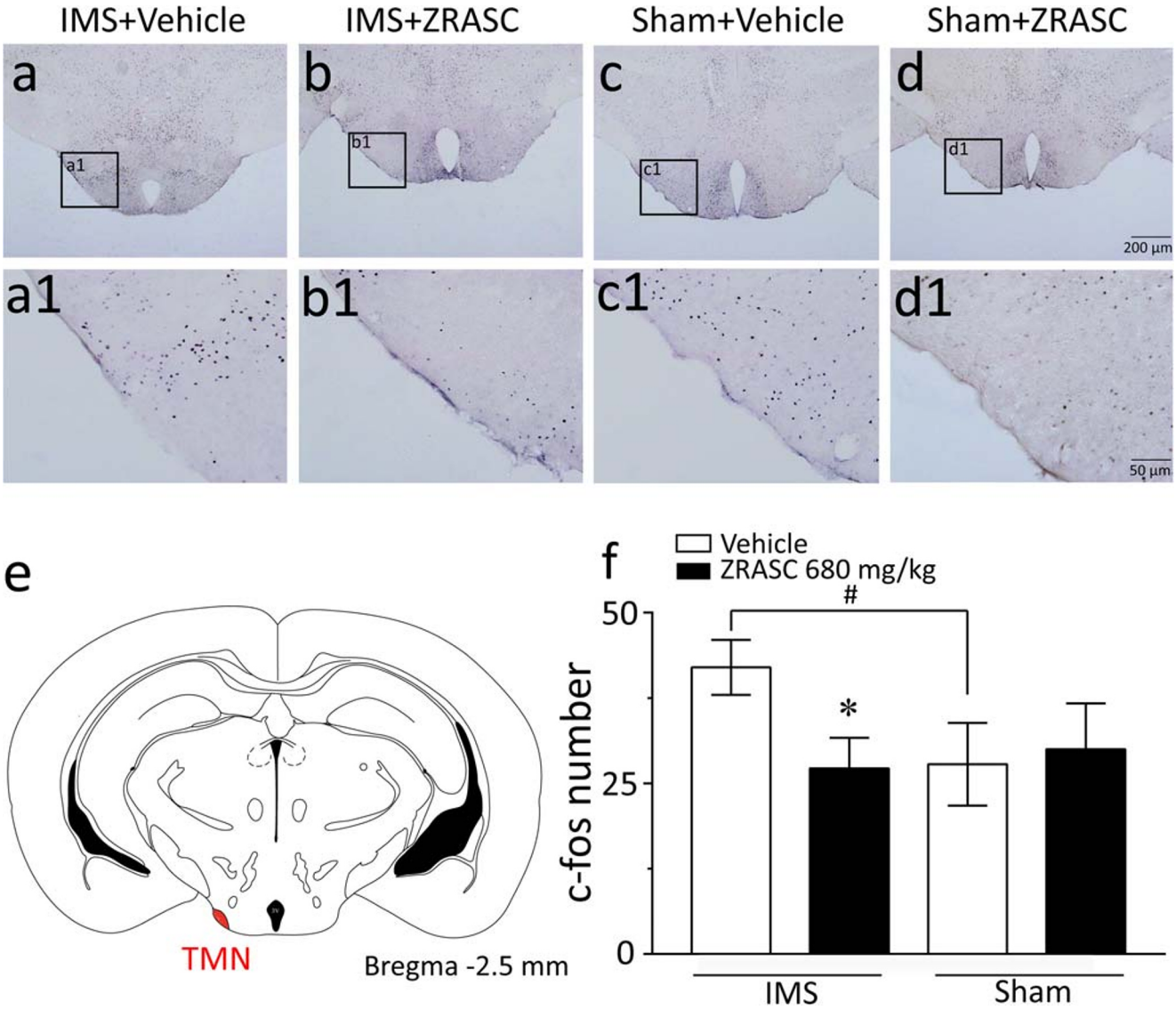


Fig. 6 Effects of ZRASC-induced c-Fos expression in the TMN of normal and IMS model mice. Representative photomicrographs (**a**–**d**, a1–d1) of c-Fos expression in the TMN are demonstrated for IMS + vehicle mice (**a**, a1), 680 mg/kg ZRASC-treated IMS mice (**b**, b1), sham + vehicle mice (**c**, c1), and 680 mg/kg ZRASC-treated sham mice (**d**, d1). Scale bars in **a**–**d**, 200 μm; scale bars in (a1–d1), 50 μm. **e** Schematic drawings of the TMN in the coronal mouse brain atlas. **f** Amount of the number of c-Fos positive neurons in the TMN of mice at 90 min after treatment with vehicle or ZRASC. Values represent mean ± SEM ($n = 5$). $^{*}P < 0.05$ indicates significant difference from the IMS + vehicle group; $^{\#}P < 0.05$ indicates significant difference from the vehicle value in the sham group, assessed by one-way ANOVA followed by the PLSD test

confirmed that BNST projection fibers innervating the parabrachial nuclear complex, ventral tegmental area, and lateral hypothalamus. Notably, these brain regions are highly associated with anxiety. They used optogenetics to selectively activate the BNST neurons, which can regulate the different characteristics of anxiety-like behavior, such as respiratory frequency, subjective preference, and dangerous escape. In contrast, inhibition of the BNST can reverse these behaviors [32]. Pharmacological studies have shown that anxiogenic drugs (FG-7142, yohimbine, mCPP, caffeine) increased the expression of c-Fos in the BNST region [33]. Anxiolytic drugs (diazepam, imipramine) can reverse the increase of c-Fos in the BNST region caused by immobilization stress [8]. Taken together, previous results confirm that BNST region is a critical area for regulating anxiety-related behaviors. Histaminergic output from the TMN is thought to play an important role in the mediation of forebrain arousal. Inhibition of these neurons may play a major role in causing sleep. In the present study, we observed that ZRASC (680 mg/kg i.g.) decreased c-Fos protein expression in the BNST and TMN in IMS mice, indicating that inhibition of BNST and TMN may play crucial roles in the anxiolytic and hypnotic effects of ZRASC.

In conclusion, this study demonstrated that ZRASC exerted anxiolytic effects, increased NREM sleep during the first 2 h after administration in an anxiety-like mouse model, suggesting potential applications in the treatment of insomnia, especially for patients with an objective sleep disorder and anxiety.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics of animal experiments All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Fudan at which the studies were conducted.

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