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CLM Treats Sleep-Deprived Rats Via Modulating Serotonin Receptor Protein 1A and Serotonin Receptor Protein 2A

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Authors' Contribution
NWZ presented the concept. XLL, NWZ, JZ, MYL planned methodology. XLL managed funding acquisition and administered the project. LHL, XLL and NWZ arranged resources. LC, XLL and NWZ supervised the project. MYL, JZ and LHL curated data. NWZ, MYL, HQD and YFC did formal analysis. MYL, JZ, HQD, LHL, LC and YFC did investigation and software. MYL and LC validated the study. XLL and NWZ wrote and edited the manuscript.

Key words
Chaihu-jia-Longgu-Muli-tang, Serotonin receptor protein 1A, Serotonin receptor protein 2A, Sleep deprivation, 5-HT2AR, Brain-derived neurotrophic factor

ABSTRACT

The aim of this study was to explore the treatment of rats with sleep deprivation (SD) with CLM (Chaihu-jia-Longgu-Muli-tang) via modulating Serotonin receptor protein 1A (5-HT1AR) and serotonin receptor protein 2A (5-HT2AR). A rat model of intermittent rapid eye movement SD was established. 5-HT1AR, 5-HT2AR, serotonin (5-HT), adrenaline (NE), brain-derived neurotrophic factor (BDNF), interleukin (IL)-6, Tumor necrosis factor-α (TNF-α), and gamma-aminobutyric acid (GABA) and GLU in the serum of SD rats were measured. The impacts of CLM on the sleep condition and negative emotional reactions of SD rats were investigated. Finally, pathological conditions of the brain tissue of SD rats were detected. We found that 5-HT1AR expression was reduced and 5-HT2AR expression was elevated in the serum of SD rats. CLM enhanced 5-HT1AR but repressed 5-HT2AR expression. The insomnia and antidepressant effects of CLM on SD rats were similar to those of Valium and Celexa. In the serum of SD rats, 5-HT, NE, BDNF, and GABA levels were decreased, but IL-6, TNF-α, and GLU levels were elevated. The levels of these indicators were reversed by CLM. Further, CLM mitigated the pathological conditions of the brain tissue in SD rats. To conclude CLM is available to mitigate insomnia and adverse emotional reactions in SD rats via modulating 5-HT1AR and 5-HT2AR.

INTRODUCTION

Sleep is an extremely momentous lifestyle and consist of two stages: Non-rapid eye movement (NREM) and rapid eye movement (REM) (Ocalan *et al*., 2019). It has always been generally believed that the quality and quantity of sleep are linked with the individual's physical and mental health and homeostasis (Shigiyama *et al*., 2018). Obviously, good sleep quality is beneficial for health, and vice versa (Wang *et al*., 2019). Sleep deprivation (SD) can impair neurobehavior, resulting in anxiety, depressive symptoms, and impaired judgment (Alzoubi *et al*., 2019). This cognitive dysfunction is associated with elevated oxidative stress and inflammatory factors in the brain, and is particularly prone to negative effects on the brain's hippocampus (Moon *et al*., 2018). REM SD can cause hippocampus-dependent learning and memory impairment.

Chaihu-jia-Longgu-Muli-tang (CLM), a traditional Chinese medicine prescription, has certain impacts on depression, dementia, insomnia, and other mental diseases (Tsujimura *et al*., 2011). Saponin SCLM extracted from CLM has antidepressant impacts on unpredictable chronic mild stress rats. SCLM up-regulates brain-derived neurotrophic factor (BDNF) via restoring monoamine neurotransmitters to reverse depressive behaviours of UCMS rats (Li *et al*., 2012). In the meantime, CLM can restore hypothalamic-pituitary-adrenal system dysfunction by preventing the reduction of dopamine and serotonin (5-HT) transmission in the prefrontal cortex (Mizoguchi *et al*., 2003). Although the mechanism of action of CLM in the treatment of insomnia has been elucidated, the potential molecular mechanism of CLM in SD remains to be further explored.

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Serotonin receptor protein 1A (5-HT1AR) and serotonin receptor protein 2A (5-HT2AR) are both 5-HT receptors, and 5-HT, a neurotransmitter and vasoactive amines, takes part in the modulation of physiological functions (Li *et al*., 2015). 5-HT1AR and 5-HT2AR are extensively expressed in the human brain (Chang *et al*., 2009). For instance, 5-HT2AR, a major postsynaptic receptor of 5-HT2 in the brain, is involved in neuropsychiatric and neurological disorders associated with memory deficits. In the absence of 5-HT2AR signal, 5-HT harms recognition memory retrieval by activating 5-HT1AR, that is, 5-HT1AR and 5-HT2AR interact in the medial prefrontal cortex of mice and participate in the recognition memory process (Morici *et al*., 2015). 5-HT1AR and 5-HT2AR have become research hotspots in recent years due to their extensive manifestations in the brain and completely opposite functional effects, as well as extensive evidence of their involvement in mental disorders and treatments.

In this study, CLM, 5-HT1AR, and 5-HT2AR were selected as research objects, their characteristics in SD were analyzed, and the potential molecular mechanism of CLM treatment in SD rats was identified by activating 5-HT1AR and 5-HT2AR.

MATERIALS AND METHODS

Experimental animals and model preparation

Specific pathogen-free grade healthy Sprague Dawley male rats, with the weight of (200±20) g were fed for 1 week. One week later, rats were grouped in line with their body weight. During the whole experiment, the rats had free access to eat and drink and were kept at 21±2℃ with 60~70% relative humidity and 12-h light exposure.

A rat model of intermittent REM SD was prepared. A multi-platform water environment method was employed. An SD box was made of an acrylic plastic sheet and was 41 cm long, 33 cm wide and 25 cm high. Four cylindrical platforms with a diameter of 6.5 cm and a height of 8.0 cm were fixed at the bottom of the SD box. The vertical spacing of the platform was 8 cm, the horizontal spacing was 12 cm, and the height of the water anomaly platform in the box was 1.0 cm. The top of the box was covered with stainless steel mesh to prevent rats from escaping. Enough food and water were put in the box. From 6:50 in the morning to 18:50 in the evening, 40 W fluorescent light was given. The water temperature was maintained at 23°C ~25°C, and the water was changed every day and kept clean. The rats were put on the platform of the SD box to move freely, eat and drink. After entering REM sleep, the rat hung its head as the muscle tone decreased and the muscles relaxed throughout the body and therefore touched or fell into the water to keep awake. During the adaptive feeding period of rats, except for the Sham group, the other groups of rats were trained in the SD box for 1 h/time/day. On the 8th day, the model was constructed, and the rats were placed in the SD box once a day for 12 h each time for 3 weeks.

Evaluation method of SD rat model

(1) General condition: During and after the model establishment, the mental status, circadian activity degree, response to external stimuli, and changes in fur color of rats were observed. (2) Pentobarbital sodium sleep synergy experiment: After modeling, rats were given intraperitoneal injection with pentobarbital sodium at 30 mg/kg. The rats were placed belly up so that their bodies were placed vertically on the plate. Holding this position for more than 60 s meant that the righting reflex disappeared. The time from intraperitoneal injection to the disappearance of the righting reflex was recorded as the sleep latency time. The recovery of righting reflex occurred when rats turned over twice or more within 60 s. The time from when the righting reflex disappears to when it recovers is sleep time. IBM SPSS Statistics 23 statistical software was utilized for statistical analysis of sleep latency time and sleep duration to evaluate whether the modeling was successful.

Animal grouping

Rats were assigned into the following 7 groups. The rats were routinely fed, and then given 5 ml of normal saline or different drugs (calculated as 6.3 times the adult dose, dissolved in 5 ml of normal saline, and heated). Sham/Model group (5 mL normal saline), Valium group (diazepam tablets suspension, 0.45 mg/[kg-d]), Low/Medium/High dose group (CLM granule agent, [5.8/11.6/23.2 g]/[kg-d]), Celexa group (escitalopram oxalate suspension, 0.9 mg/[kg-d]).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

QIAzol Lysis Reagent (Qiagen, Inc.) was employed for the extraction of total RNA, with TaqMan Reverse Transcription kit (Guangzhou RiboBio) for reverse transcription of total RNA into complementary DNA in line with the manufacturer's protocol. Subsequently, PCR was performed with a PCR machine (Bedford, UK). The primer sequence was manifested in Table I. 5-HT1AR and 5-HT2AR were measured by SsoFast EvaGreen Supermix Kit (Bio-Rad, Hercules, CA, USA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Data analysis was done with $2^{-\Delta\Delta Ct}$.

Table I. Primer sequence.

Genes	Primer sequence
5-HT1AR	F: 5'-CGTGCACCATCAGCAAGGA-3'
	R: 5'-CTGAAGATGCGCCCGTAGAGA-3'
5-HT2AR	F: 5'-ACCGCTATGTCGCCATCCA-3'
	R: 5'-GACCTTCGAATCATCCTGTAGTCCA-3'
GAPDH	F: 5'-GACATGCCGCCTGGAGAAAC -3'
	R: 5'-AGCCCAGGATGCCCTTTAGT -3'

Western blot

Total tissue protein was extracted with Radio-Immunoprecipitation assay lysis buffer (Beyotime Biotechnology, Shanghai, China) consisting of protease inhibitors and quantified by bicinchoninic acid. The total protein (50 μg/lane) was separated by 10% sulfate-polyacrylamide gel electrophoresis, electro-blotted onto a nitrocellulose membrane, blocked with 5% skimmed milk, and incubated with primary antibodies 5-HT1AR, 5-HT2AR and GAPDH (1:1000, cell signaling technology), and horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by Pierce electrogenerated chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). GADPH served as a loading control. Gel Pro Analyzer Software 4.5 (Beijing Zhongsheng Tiancheng Technology Co., Ltd.) was utilized for analyzing optical density values of protein bands.

Behavioral observation

Sucrose water preference test

After 2 weeks of continuous drug intervention, rats in each group were subjected to a sucrose water preference test. Before the experiment, the rats in the Low/Medium/High dose, Valium, and Celexa groups were given two bottles of water: one bottle of 1% sucrose water and one bottle of pure water. After 30 min, the position of the sucrose water and pure water was changed. After 60 min, two bottles were weighed. Sucrose water preference rate = sucrose water consumption/total water consumption × 100%.

Open field test

After 2 weeks of continuous intervention with drugs, an open field test was carried out. The rats were placed in the center square of an open box (100 cm length × 100 cm width × 40 cm height). The number of grids each rat traverses horizontally in a 5-min period (This is the horizontal movement score, and when all four paws go into the square, it counts as 1 point) and the number of times of the hind limbs erect (This is the vertical movement score, and 1 point is scored when the two front paws are in the air or climbing on a wall) were manually counted. The score in the open field test was the sum of the two. This test was completed by two experimenters who did not know the grouping of experimental rats beforehand, and the average and recorded scores of the two experimenters were the final scores. After one rat completed the test, the box was thoroughly dried and cleaned with 75% ethanol, and the next rat was observed to avoid odor affecting the results.

Forced swimming test

At room temperature, a transparent plastic cylinder with a diameter of 30 cm and a height of 50 cm was filled with water at a depth of 35 cm. The rats were put in the cylinder without touching the bottom with their tail or hind limbs. Before the experiment, the rats were allowed to swim for 15 min to adapt to the new environment. The rats were then forced to swim for 5 min. Behavior during this time was recorded via high-altitude cameras, including rest and escape times, as well as the number of climbs and swims. After the experiment, the rats were dried with a towel and returned to their cages. The water in the cylinder between tests was changed. During the last 5 min of the total swim time, a timer was utilized to accurately record the rest time. The evaluation was carried out through observers in a blind manner. When rats float passively on the surface of the water, not struggling or swimming, but just keeping their heads above water, they are considered to be at rest.

Enzyme-linked immunosorbent assay (ELISA)

Samples were collected the morning after completion of all experimental items. After anesthesia, at least 5 ml blood from the abdominal aorta of each rat was collected in 10 mL vacuum blood collection tubes. The tubes were placed vertically for 30 min and centrifuged at 2000 r/min (centrifugation radius of 15 cm and centrifugation time of 15 min). The extracted serum was stored in the refrigerator at -80°C. 5-HT, adrenaline (NE), BDNF, interleukin (IL)-6, tumor necrosis factor-α (TNF-α), gamma-aminobutyric acid (GABA), and GLU in the serum were measured by ELISA.

Rat brain tissue preparation

After blood collection, rats in each group were injected intraperitoneally with ketamine and xylazine. The rats were given cardiac perfusion with 150 mL normal saline, and brain tissues were resected and fixed with 200 mL 4% paraformaldehyde. Brain tissue pieces were taken from 2 mm before to 2 mm behind the chiasm. The tissues were then embedded in paraffin and prepared into coronal continuous sections of 3 μm thickness.

Hematoxylin-eosin (HE) staining

The brain tissues were conventionally dewaxed with xylene, washed with gradient alcohol, and stained with hematoxylin. After differentiation with hydrochloric acid and alcohol, the brain tissues were treated with ammonium hydroxide, stained with eosin, conventionally dehydrated, cleared, and sealed with neutral gum. Morphological changes in neurons were observed under a light microscope.

Nissl staining

The brain tissues were washed with pure water, stained with tar violet solution, immersed in pure water, and differentiated with 70%-80%-95% alcohol (10 sec each time). After dehydration with absolute ethanol, the tissues were cleared with xylene, fixed with neutral resin, and observed under a microscope (Nikon, Japan).

TUNEL staining

Cell apoptosis in the cortex was observed by TUNEL staining using the in situ Cell death Detection kit (Roche, Germany) according to the manufacturer's instructions. In brief, paraffin sections were dehydrated, incubated with 3% H_2O_2 at room temperature for 15-20 min, digested with protease K (Sigma-Aldrich Chemical Company, St Louis, MO, USA) at 37℃ for 20 min, added with citrate for 30 min, and cultured with TUNEL reaction mixture in an incubator at 37°C for 60 min. TUNEL-positive cells were observed and counted by a fluorescence microscope (Nikon, Japan) (Vahidinia *et al*., 2017).

Data processing and statistical analysis

IBM SPSS statistics 23 software was applied for data sorting and analysis. Normally distributed measurement data were manifested as mean±standard deviation (SD). The comparison of two independent samples was conducted by independent sample t test, while that among multiple groups was by homogeneity test of variances. One-way analysis of variance (ANOVA) was applied for analysis of equal variance. Pairwise comparison was conducted by least significant difference (LSD) method, and nonparametric test was put into use in non-uniform variance. Measurement data of skewed distribution were clarified as the median (interquartile range) (Median (1QR)). The two-group comparison was done with rank sum test of two independent sample comparison, and the comparison among multiple groups was done with rank sum test of multiple independent sample comparison. Enumeration data were shown as numbers and evaluated by Chi-square test. Metastats analysis was used for analysis of differences in species composition of groups, and Spearman rank correlation test was employed for correlation analysis. $P < 0.05$ emphasized obvious differences.

RESULTS

Success of the SD rat model

The weight of SD rats was reduced. In the process of SD modeling, the fur of the model rats was discolored and discolored, and the alertness and reactivity of the rats to the stimulation of the surrounding environment were enhanced, and irritability and aggression were enhanced. The tail and PAWS of some rats were damaged, and the stool was dry and irregular.

After 3 weeks of intermittent SD, pentobarbital sodium sleep synergy test was conducted. A total of 50 rats underwent SD. Among them, 14 rats were injected with pentobarbital sodium according to body weight, which showed mental atrophy such as slow movement, closed eyes, and napping, but the right reflex did not disappear. These rats accounted for 28% of the total number of model rats, and individual physical differences could also lead to drug resistance. Considering that the sleep disorder of these 14 rats was more severe than that of other model rats, and repeated injections of pentobarbital sodium was likely to be fatal, 14 rats did not receive repeated injection of pentobarbital sodium. The sleep latency of the remaining 36 SD rats was longer, and the sleep duration was shorter. Combined with the general observation, it was concluded that insomnia occurred in SD rats and the modeling was successful. The specific results are shown in Table II.

Table II. Pentobarbital sodium sleep synergy test to evaluate sleep of REM SD rats (Median (IQR)/mean ± SD).

Groups	Cases	Sleep latency (min)	Sleep duration (min)
Blank rats	6	4.00(1.00)	68.50±17.19
SD rats	36	6.00(3.00)	46.70±14.35

5-HT1AR and 5-HT2AR expression in SD rats

To determine whether CLM regulates 5-HT1AR and 5-HT2AR in SD rats, 5-HT1AR and 5-HT2AR were detected. 5-HT1AR expression was reduced but 5-HT2AR expression was elevated in the serum of rats in the Model group. After treatment, 5-HT1AR expression was enhanced and 5-HT2AR expression was suppressed, among which the changes in 5-HT1AR and 5-HT2AR in rats of the medium-dose group of CLM were the most apparent (Fig. 1A, B).

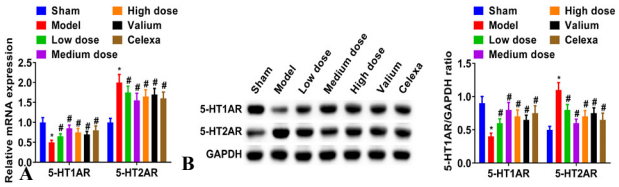

Fig. 1. Effect of valium, celexes low, medium and high dose of CLM on 5-HT1AR and 5-HT2AR in SD rats.
A, B: RT-qPCR and Western blot detection of serum 5-HT1AR and 5-HT2AR in SD rats. The data in the figure were all measurement data and shown as mean ± SD.* vs the Sham, $P < 0.05$; # vs the Model, $P < 0.05$.

Impacts of CLM on the sleep of rats in each group

Sleep latency was longer in the Model group versus the Sham group, and that in each treatment group was shorter versus the Model group. Sleep latency of the rats in the CLM medium-dose was the most shortened. Sleep duration in the Model group was shorter versus the Sham group, and that in each treatment group was longer than the Model group. Among them, the sleep duration of the rats in the CLM medium-dose was the most prolonged. The specific results are manifested in Table III. All in all, the effect of CLM on insomnia in SD rats was similar to that of Valium and Celexa.

Table III. Effect of valium, celexes low, medium and high dose of CLM on sleep of rats in each group (Median (IQR)/mean ± SD).

Groups	Sleep latency (min) (n=6)	Sleep duration (min) (n=6)
Sham	4.50(2.00)	70.00±12.54
Model	6.00(3.50)	53.20±11.52
Low dose	4.50(3.00)	67.80±12.40
Medium dose	4.00(2.00)	79.50±25.49
High dose	4.50(3.00)	73.70±17.63
Valium	3.50(4.00)	63.63±15.11
Celexa	4.00(2.00)	69.20±8.77

Impacts of CLM on negative emotion reaction of rats in each group

Sucrose consumption test was applied to evaluate the antidepressant effect of CLM. The experimental results showed that after 2 weeks of continuous administration, the sucrose water preference rate of the rats in the Model group was decreased, while that in each treatment group was increased. The sucrose water preference rate of the CLM medium-dose was the most elevated (Fig. 2A). Moreover, open field test discovered that the time and distance of rats in the Model group in the central area of the open field were reduced, while those of rats in each treatment group were increased, and the time and distance of the rats in the central area of the open field were the most increased in the CLM middle-dose group (Fig. 2B, C). Additionally, in the forced swimming test, the rats in the Model group had increased resting time, while CLM decreased resting time and increased swimming time of SD rats (Fig. 2D). All in all, CLM had similar antidepressant impacts to Valium and Celexa in SD rats.

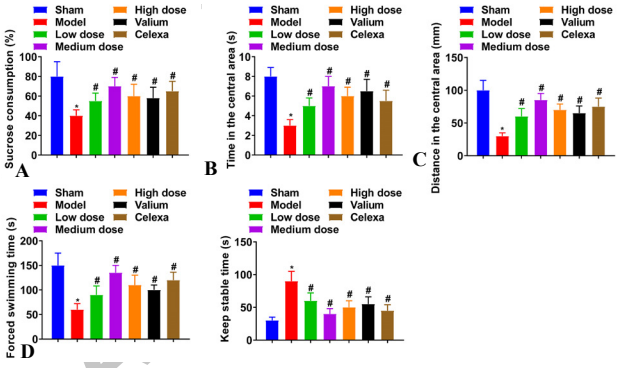

Fig. 2. Effect of valium, celexes low, medium and high dose of CLM on impacts of CLM on negative emotion reaction of rats in each group.
A: Sucrose preference test; B/C: Open field test; D: Forced swimming test. The data in the figure were all measurement data and shown as mean ± SD.* vs the Sham, $P < 0.05$; # vs the Model, $P < 0.05$.

Table IV. Effect of valium, celexes low, medium and high dose of CLM on serum 5-HT and NE of rats in each group (Median (IQR)).

Groups	5-HT (pg/mL) (n=6)	NE (pg/mL) (n=6)
Sham	1809.20(59.90)	1267.50(61.10)
Model	112.90(94.80)	81.46(32.30)
CLM low dose	209.10(33.00)	124.57(23.60)
CLM medium dose	1180.60(153.40)	818.50(109.60)
CLM high dose	552.65(272.20)	369.96(194.40)
Valium	994.40(117.80)	685.50(84.10)
Celexa	1062.40(160.90)	734.07(114.90)

Effects of CLM on serum 5-HT, NE, BDNF, IL-6, TNF-α, GABA, and GLU in rats of each group

After continuous administration for 2 weeks, serum 5-HT, NE, BDNF, and GABA of rats in the Model group were repressed, but IL-6, TNF-α, and GLU were enhanced. After treatment, 5-HT, NE, BDNF, and GABA were elevated, and IL-6, TNF-α, and GLU were repressed. The changes in the factors were most obvious in the rats of the CLM medium-dose group (Table IV and Fig. 3A-E).

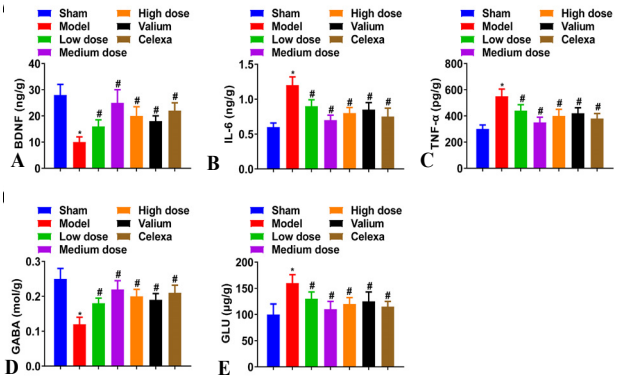

Fig. 3. Effect of valium, celexes low, medium and high dose of CLM on effects of CLM on serum BDNF, IL-6, TNF-α, GABA and GLU in rats of each group.
E: ELISA examination of serum BDNF, IL-6, TNF-α, GABA, and GLU in rats. The data in the figure were all measurement data and shown as mean ± SD.* vs the Sham, $P < 0.05$; # vs the Model, $P < 0.05$.

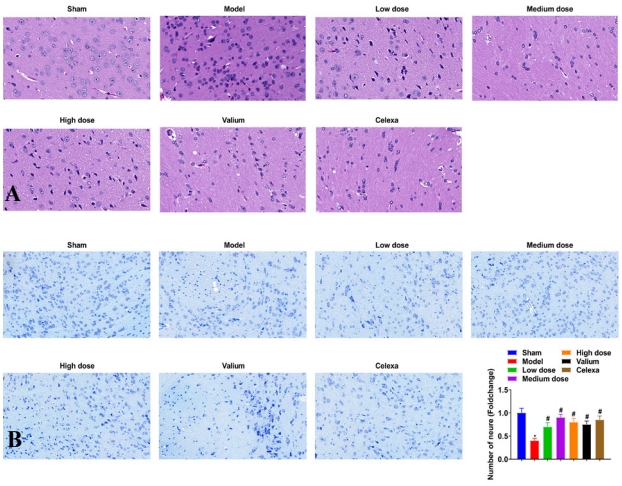

Fig. 4. Effect of valium, celexes low, medium and high dose of CLM on pathological condition of rat brain tissue in each group. A: HE staining; B: Nissl staining. The data in the figure were all measurement data and shown as mean ± SD.* vs the Sham, $P < 0.05$; # vs the Model, $P < 0.05$.

Impacts of CLM on pathological condition of rat brain tissue in each group

Cells in the brain tissue of the Sham group were neatly arranged and compact, with abundant cell cytoplasm clear nuclear membrane and visible nucleoli. The cell structure in the brain tissue of rats in the Model group was disordered, with local hemorrhagic changes and a few inflammatory cells. The pathological condition of the brain tissue of rats in each treatment group was alleviated (Fig. 4A). The neuronal cytopathological evaluation showed that the neuronal loss in the brain tissue of the Model group was significantly increased, and the neuronal loss in the brain tissue of the rats in each treatment group was reduced (Fig. 4B). TUNEL-positive cells were occasionally seen in the brain tissue of the Sham group, and the number of TUNEL-positive cells in the Model group was clearly augmented, while that in each treatment group was reduced (Fig. 5).

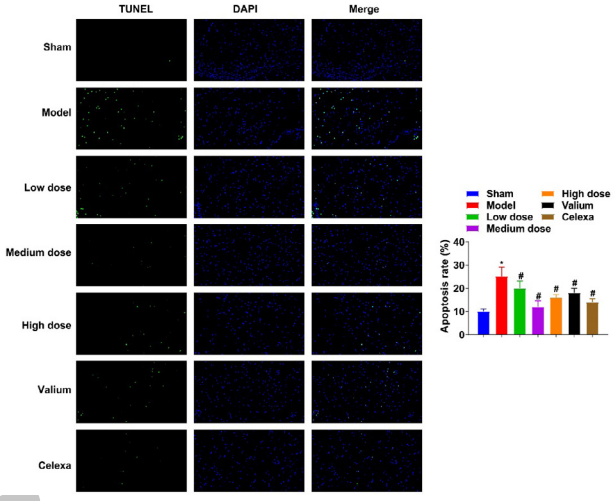

Fig. 5. Effect of valium, celexes low, medium and high dose of CLM on apoptosis of rat brain tissue in each group. Cell apoptosis in rat brain was detected by TUNEL staining. The data in the figure were all measurement data and shown as mean ± SD.* vs the Sham, $P < 0.05$; # vs the Model, $P < 0.05$.

DISCUSSION

Behaviors like cognitive impairment, mania, depression, etc. are often linked with insomnia (Alzoubi *et al.*, 2018). In the meantime, neurological diseases, like Alzheimer's disease (AD) (Mander *et al.*, 2016), multiple sclerosis (Sahraian *et al.*, 2017), Parkinson's disease (Lee *et al.*, 2021), etc. are all accompanied by insomnia. There are some lesser-known links between SD, immunity, and neurodegeneration. However, there is currently no reliable research on their mechanism of action, and there is not enough evidence to suggest that under normal circumstances, sleep affects immune areas of the brain (Korin *et al.*, 2020). In China, traditional Chinese medicine has long been employed to cure insomnia, like compound-sweet dream oral liquid (Su *et al.*, 2016), modified Sini San decoction (Han *et al.*, 2018) and CLM (Lin *et al.*, 2020). However, the latent mechanism of Chinese herbal medicines is still uncertain (Zou *et al.*, 2017). In this study, CLM treatment mitigated insomnia symptoms and depression in SD rats, reduced 5-HT1AR, IL-6, TNF-α,

and GLU, but elevated 5-HT2AR, 5-HT, NE, BDNF, and GABA, indicating that CLM treatment was beneficial to SD rats.

A study has discovered that the latent mechanisms of depression consist of monoamine neurotransmitters (norepinephrine, dopamine and 5-HT) and reduced nutritional factors in brain-derived nerves in the hippocampus and prefrontal cortex (Aleksovski *et al*., 2018). The present therapeutic approach for depression is mainly oral medication, including selective serotonin reuptake inhibitors (Stewart *et al*., 2018) and paroxetine (Yeh *et al*., 2016). CLM or saponins extracted from CLM also have antidepressant impacts on rats. For instance, a single dose of CLM immediately leads to an antidepressant-like effect in OB mice, indicating that CLM has an antidepressant effect by rescuing the NMDA-Akt-mTOR-AMPA pathway (Wang *et al*., 2018). Saponins extracted from CLM also have antidepressant-like impacts on rats by elevating the concentration of 5-HT and inducing BDNF in the prefrontal cortex and hippocampus (Liu *et al*., 2010). CLM was investigated in this study to understand its latent molecular mechanism in the treatment of REM SD rats. It was found that CLM prolonged rat's sleep duration dose-dependently, increased sucrose preference rate, and improved behavioral functions. These results affirmed that CLM had insomnia and antidepressant effects on SD rats. Meanwhile, it was also found that CLM impacted some factors and neurotransmitters in SD rats, including inflammatory factors, 5-HT, NE, BDNF, and GABA. CLM elevated serum 5-HT, NE, BDNF, and GABA dose-dependently, but repressed IL-6, TNF-α, and GLU, which is consistent with the results of a previous study (Wang *et al*., 2019). Interestingly, it was discovered for the first time that CLM was linked with 5-HT1AR and 5-HT2AR.

5-HT1AR and 5-HT2AR are two receptors of 5-HT (Leem *et al*., 2019). It has been reported that abnormal expression of 5-HT receptors is associated with certain diseases, such as AD (Giil *et al*., 2017), chronic mild stress eczema and allergic contact dermatitis (Wetterberg *et al*., 2011). Meanwhile, 5-HT receptors have also been found to be aberrantly expressed in tumors (Naimi-Akbar *et al*., 2010). Recent studies have found that certain drugs can control 5-HT1AR and 5-HT2AR (Wang *et al*., 2016; Cai *et al*., 2019). In this research, it was found that 5-HT1AR in SD rats was reduced, but 5-HT2AR was elevated, and CLM could enhance 5-HT1AR but repress 5-HT2AR expression. The above results indicated that CLM was available to treat insomnia and depression in SD rats via controlling 5-HT1AR and 5-HT2AR.

In addition, CLM treatment can alleviate brain tissue pathology, which was manifested as a reduction of brain tissue cell structure disorder, improvement of local bleeding, reduction of inflammatory cell infiltration, and reduction of brain tissue neuron loss. Moreover, CLM treatment was available to repress brain cell apoptosis. These results further confirm the idea that CLM can treat SD rats. In future studies, we hope to further understand the psychological and behavioral effects of 5-HT1AR and 5-HT2AR on SD rats. Previous studies have suggested that 5-HT1AR and 5-HT2AR mediate the activation of the ERK1/2 pathway, and its downstream targets and regulatory mechanisms deserve further investigation.

CONCLUSION

In conclusion, the study demonstrates for the first time that CLM treats insomnia and depression in SD rats dose-dependently. The study clarifies a molecular mechanism for insomnia, that is, CLM mitigates insomnia and adverse emotional response of SD rats via elevating 5-HT1AR and depressing 5-HT2AR and pro-inflammatory cytokines. The results manifest that there may be a connection between SD and immune response, and CLM also functions in relieving inflammation to a certain extent. Moreover, CLM treatment mitigates the pathology of the brain tissue of SD rats. The results will offer a theoretical basis for CLM as a new medicine for the cure of insomnia and depression.

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Ethical statement and IRB approval

The present study was approved by the Animal experiments were approved by The Third Affiliated Hospital of Beijing University of Chinese Medicine Animal Experimental Ethics Committee. and all procedures complied with the National Institutes of Health Guide for the Use of Laboratory Animals (201806BJ029).

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Aleksovski, B., Novotni, A., Vujović, V., Rendevski,

V., Manusheva, N., Neceva, V., Filipce, A., Risteski, A., Aleksovski, V. and Gjorgoski, I., 2018. Evaluation of peripheral serotonin content and α (2)-adrenergic receptor function as potential markers for life-long recurrent depressive disorder by using methodological improvements. *Int. J. Psych. clin. Pract.,* **22**: 215-224. https://doi.org/10.1080/13651501.2017.1411516

Alzoubi, K.H., Al-Mosabih, H.S. and Mahasneh, A.F., 2019. The protective effect of edaravone on memory impairment induced by chronic sleep deprivation. *Psych. Res.,* **281**: 112577. https://doi.org/10.1016/j.psychres.2019.112577

Alzoubi, K.H., Malkawi, B.S., Khabour, O.F., El-Elimat, T. and Alali, F.Q., 2018. *Arbutus andrachne* L. reverses sleep deprivation-induced memory impairments in rats. *Mol. Neurobiol.,* **55**: 1150-1156. https://doi.org/10.1007/s12035-017-0387-8

Cai, M., Zhou, L., Liao, J., Huang, Q., Xia, Z. and Shang, J., 2019. IFN-γ inhibits 5-HT-induced melanin biosynthesis via downregulation of 5-HT receptors *in vivo/in vitro*. *J. Pharmacol. Sci.,* **141**: 1-8. https://doi.org/10.1016/j.jphs.2019.05.005

Chang, C.W., Poteet, E., Schetz, J.A., Gümüş, Z.H. and Weinstein, H., 2009. Towards a quantitative representation of the cell signaling mechanisms of hallucinogens: Measurement and mathematical modeling of 5-HT1A and 5-HT2A receptor-mediated ERK1/2 activation. *Neuropharmacology,* **56 (Suppl 1)**: 213-225. https://doi.org/10.1016/j.neuropharm.2008.07.049

Giil, L.M., Vedeler, C.A., Kristoffersen, E.K., Nordrehaug, J.E., Heidecke, H., Dechend, R., Schulze-Forster, K., Muller, D.N., von Goetze, V.S., Cabral-Marques, O., Riemekasten, G., Vogelsang, P., Nygaard, S., Lund, A. and Aarsland, D., 2017. Antibodies to signaling molecules and receptors in alzheimer's disease are associated with psychomotor slowing, depression, and poor visuospatial function. *J. Alzheimers Dis.,* **59**: 929-939. https://doi.org/10.3233/JAD-170245

Han, C., Li, F., Liu, Y., Ma, J., Yu, X., Wu, X., Zhang, W., Li, D., Chen, D., Dai, N., Lin, B., Wu, F. and Mao, M., 2018. Modified Si-Ni-San decoction ameliorates central fatigue by improving mitochondrial biogenesis in the rat hippocampus. *Evid. Based Complement. Altern. Med.,* **2018**: 9452127. https://doi.org/10.1155/2018/9452127

Korin, B., Avraham, S., Azulay-Debby, H., Farfara, D., Hakim, F. and Rolls, A., 2020. Short-term sleep deprivation in mice induces B cell migration to the brain compartment. *Sleep,* **43**. https://doi.org/10.1093/sleep/zsz222

Lee, H.M., Han, K.D., Suh, J.D. and Cho, J.H., 2021. Uvulopalatopharyngoplasty may decrease the incidence of Parkinson's disease associated with obstructive sleep apnea. *Sci. Rep.,* **11**: 9608. https://doi.org/10.1038/s41598-021-89205-4

Leem, Y.H., Jang, J.H., Park, J.S. and Kim, H.S., 2019. Exercise exerts an anxiolytic effect against repeated restraint stress through 5-HT(2A)-mediated suppression of the adenosine A(2A) receptor in the basolateral amygdala. *Psychoneuroendocrinology*, **108**: 182-189. https://doi.org/10.1016/j.psyneuen.2019.06.005

Li, H.F., Liu, J.F., Zhang, K. and Feng, Y., 2015. Expression of serotonin receptors in human lower esophageal sphincter. *Exp. Ther. Med.,* **9**: 49-54. https://doi.org/10.3892/etm.2014.2050

Li, L.F., Lu, J., Li, X.M., Xu, C.L., Yang, J., Qu, R. and Ma, S.P., 2012. Antidepressant-like effects of the saponins extracted from Chaihu-jia-longgu-muli-tang in a rat unpredictable chronic mild stress model. *Fitoterapia,* **83**: 93-103. https://doi.org/10.1016/j.fitote.2011.09.017

Lin, C.H., Chiu, H.E., Wu, S.Y., Tseng, S.T., Wu, T.C., Hung, Y.C., Hsu, C.Y., Chen, H.J., Hsu, S.F., Kuo, C.E. and Hu, W.L., 2020. Chinese herbal products for non-motor symptoms of parkinson's disease in Taiwan: A population-based study. *Front. Pharmacol.,* **11**: 615657. https://doi.org/10.3389/fphar.2020.615657

Liu, Y., Ma, S. and Qu, R., 2010. SCLM, total saponins extracted from Chaihu-jia-longgu-muli-tang, reduces chronic mild stress-induced apoptosis in the hippocampus in mice. *Pharm. Biol.,* **48**: 840-848. https://doi.org/10.3109/13880200903296154

Mander, B.A., Winer, J.R., Jagust, W.J. and Walker, M.P., 2016. Sleep: A novel mechanistic pathway, biomarker, and treatment target in the pathology of Alzheimer's disease? *Trends Neurosci.,* **39**: 552-566. https://doi.org/10.1016/j.tins.2016.05.002

Mizoguchi, K., Yuzurihara, M., Ishige, A., Aburada, M. and Tabira, T., 2003. Saiko-ka-ryukotsu-borei-to, a herbal medicine, ameliorates chronic stress-induced depressive state in rotarod performance. *Pharmacol. Biochem. Behav.,* **75**: 419-425. https://doi.org/10.1016/S0091-3057(03)00131-X

Moon, E.J., Ko, I.G., Kim, S.E., Jin, J.J., Hwang, L., Kim, C.J., An, H., Lee, B.J. and Yi, J.W., 2018. Dexmedetomidine ameliorates sleep deprivation-induced depressive behaviors in mice. *Int. Neurourol. J.,* **22(Suppl 3)**: S139-146. https://doi.org/10.5213/inj.1836228.114

Morici, J.F., Ciccia, L., Malleret, G., Gingrich, J.A., Bekinschtein, P. and Weisstaub, N.V., 2015. Serotonin 2a receptor and serotonin 1a receptor interact within the medial prefrontal cortex during recognition memory in mice. *Front. Pharmacol.,* **6**: 298. https://doi.org/10.3389/fphar.2015.00298

Naimi-Akbar, C., Ritter, M., Demel, S., El-Nour, H., Hedblad, M.A., Azmitia, E.C. and Nordlind, K., 2010. Different serotonergic expression in nevomelanocytic tumors. *Cancers* (Basel), **2**: 1166-1177. https://doi.org/10.3390/cancers2021166

Ocalan, B., Cakir, A., Koc, C., Suyen, G.G. and Kahveci, N., 2019. Uridine treatment prevents REM sleep deprivation-induced learning and memory impairment. *Neurosci. Res.,* **148**: 42-48. https://doi.org/10.1016/j.neures.2019.01.003

Sahraian, M.A., Rezaali, S., Hosseiny, M., Doosti, R., Tajik, A. and Naser Moghadasi, A., 2017. Sleep disorder as a triggering factor for relapse in multiple sclerosis. *Eur. Neurol.,* **77**: 258-261. https://doi.org/10.1159/000470904

Shigiyama, F., Kumashiro, N., Tsuneoka, Y., Igarashi, H., Yoshikawa, F., Kakehi, S., Funato, H. and Hirose, T., 2018. Mechanisms of sleep deprivation-induced hepatic steatosis and insulin resistance in mice. *Am. J. Physiol. Endocrinol. Metab.,* **315**: E848-e858. https://doi.org/10.1152/ajpendo.00072.2018

Stewart, R.M., Hood, S.D., Rao, P., Moore, J.K., Runions, K.C., Murphy, S.E., Wong, J.W.Y. and Zepf, F.D., 2018. Using acute tryptophan depletion to investigate predictors of treatment response in adolescents with major depressive disorder: Study protocol for a randomised controlled trial. *Trials,* **19**: 434. https://doi.org/10.1186/s13063-018-2791-4

Su, X., Wang, C., Wang, X., Han, F., Lv, C. and Zhang, X., 2016. Sweet dream liquid Chinese medicine ameliorates learning and memory deficit in a rat model of paradoxical sleep deprivation through the ERK/CREB signaling pathway. *J. Med. Fd.,* **19**: 472-480. https://doi.org/10.1089/jmf.2015.3530

Tsujimura, A., Miyagawa, Y., Okuda, H., Yamamoto, K., Fukuhara, S., Nakayama, J., Takao, T., Nonomura, N. and Okuyama, A., 2011. Change in cytokine levels after administration of saikokaryuukotsuboreito or testosterone in patients with symptoms of late-onset hypogonadism. *Aging Male,* **14**: 76-81. https://doi.org/10.3109/13685538.2010.502268

Vahidinia, Z., Alipour, N., Atlasi, M.A., Naderian, H., Beyer, C. and Azami, T.A., 2017. Gonadal steroids block the calpain-1-dependent intrinsic pathway of apoptosis in an experimental rat stroke model. *Neurol. Res.,* **39**: 54-64. https://doi.org/10.1080/01616412.2016.1250459

Wang, L., Gu, Y., Zhang, J. and Gong, L., 2019. Effects of sleep deprivation (SD) on rats via ERK1/2 signaling pathway. *Med. Sci. Monit.,* **25**: 2886-2895. https://doi.org/10.12659/MSM.913839

Wang, W., Cui, G., Jin, B., Wang, K., Chen, X., Sun, Y., Qin, L. and Bai, W., 2016. Estradiol valerate and remifemin ameliorate ovariectomy-induced decrease in a serotonin dorsal raphe-preoptic hypothalamus pathway in rats. *Annls Anat.,* **208**: 31-39. https://doi.org/10.1016/j.aanat.2016.08.001

Wang, X., Chen, J., Zhang, H., Huang, Z., Zou, Z., Chen, Y., Sheng, L., Xue, W., Tang, J., Wu, H., Liu, H. and Chen, G., 2019. Immediate and persistent antidepressant-like effects of Chaihu-jia-Longgu-Muli-tang are associated with instantly up-regulated BDNF in the hippocampus of mice. *Biosci. Rep.,* **39**. https://doi.org/10.1042/BSR20181539

Wang, X., Zou, Z., Shen, Q., Huang, Z., Chen, J., Tang, J., Xue, W., Tao, W., Wu, H., Wang, D. and Chen, G., 2018. Involvement of NMDA-AKT-mTOR signaling in rapid antidepressant-like activity of chaihu-jia-longgu-muli-tang on olfactory bulbectomized mice. *Front. Pharmacol.,* **9**: 1537. https://doi.org/10.3389/fphar.2018.01537

Wetterberg, J., Taher, C., Azmitia, E.C. and El-Nour, H., 2011. Time-dependent modulation of serotonin and its receptors 1A and 2A expression in allergic contact dermatitis. *J. Eur. Acad. Dermatol. Venereol.,* **25**: 1200-1205. https://doi.org/10.1111/j.1468-3083.2010.03952.x

Yeh, T.C., Kao, L.C., Tzeng, N.S., Kuo, T.B., Huang, S.Y., Chang, C.C. and Chang, H.A., 2016. Heart rate variability in major depressive disorder and after antidepressant treatment with agomelatine and paroxetine: Findings from the Taiwan Study of Depression and Anxiety (TAISDA). *Prog. Neuropsychopharmacol. Biol. Psych.,* **64**: 60-67. https://doi.org/10.1016/j.pnpbp.2015.07.007

Zou, X., Huang, W., Lu, F., Fang, K., Wang, D., Zhao, S., Jia, J., Xu, L., Wang, K., Wang, N. and Dong, H., 2017. The effects of Jiao-Tai-Wan on sleep, inflammation and insulin resistance in obesity-resistant rats with chronic partial sleep deprivation. *BMC Complement. Altern. Med.,* **17**: 165. https://doi.org/10.1186/s12906-017-1648-9