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Compound Chaijin Jieyu Tablets ameliorating insomnia complicated with depression by improving synaptic plasticity via regulating orexin A, melatonin, and acetylcholine contents

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ABSTRACT

Objective To investigate the efficacy and mechanism of action of Compound Chaijin Jieyu Tablets (复方柴金解郁片, CCJJYT) in rats with insomnia complicated with depression.

Methods Seventy-two Sprague-Dawley rats were randomly assigned into eight groups: the control, chronic unpredictable mild stress (CUMS), sleep deprivation (SD), CUMS + SD, positive drug (venlafaxine hydrochloride + diazepam), CCJJYT high-dose (CCJJYT-2×), medium-dose (CCJJYT-1 ×), and low-dose (CCJJYT-0.5 ×) groups, with nine rats in each group. Depression-like behavior was evaluated by body weight, food intake, and behavioral tests such as the sucrose preference test (SPT), open field test (OFT), forced swimming test (FST), and pentobarbital-induced sleep test (PST). Hematoxylin-eosin (HE) staining and Golgi-Cox staining were used to observe changes in pathological tissue and synaptic morphology, respectively. Enzyme-linked immunosorbent assay (ELISA) was used to detect the contents of orexin-A and acetylcholine. The expression levels of orexin receptor 1 (OXR1), melatonin receptor 1 (MT1A), melatonin receptor 2 (MT1B), acetylcholinesterase (AChE), and choline acetyltransferase (ChAT) were detected by immunohistochemistry and Western blot.

Results In the present study, rats in the model group showed significant behavioral changes as well as a reduction in hippocampal dendritic branch length and synaptic number, along with increasing the content of orexin A and acetylcholine ($P < 0.05$), and altered expression levels of OX1R, MT1A, MT1B, ChAT, and AChE in the hippocampus and prefrontal cortex after modeling ($P < 0.05$). CCJJYT can improve depressive insomnia behavior and synaptic plasticity of rats ($P < 0.05$), which is similar to that of the positive drug group. It can also decrease the content of orexin A and acetylcholine, and reduce the expression levels of OXR1 and ChAT in hippocampus and prefrontal cortex ($P < 0.05$), and increase the expression levels of MT1A, MT1B, and AChE proteins ($P < 0.05$).

Conclusion CCJJYT has good antidepressant and insomnia effects, probably through the regulation of orexin-A, melatonin, and acetylcholine content in hippocampus and prefrontal cortex of rats, improving synaptic plasticity and thus exerting antidepressant and insomnia effects.

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1 Introduction

According to the recent data, more than 350 million people worldwide suffer from depression, which has become the fourth most common disease and is growing rapidly. The World Health Organization (WHO) also predicts that depression will be the top one disease burden throughout the world by 2030 [1]. Insomnia, an independent risk factor for many psychiatric disorders such as depressive disorders, is not only a symptom of depression, but also a cyclical and continuous progression of a common disease state [2, 3]. About 80% of people with depression suffer from severe sleep disorders [4]. Clinically, sleep disorders precede depressive symptoms, and are associated with an increase in the prevalence of depression [5]. The co-morbidity mechanism between insomnia complicated with depression is still unclear, but based on similar or even identical clinical manifestations and risk factors of insomnia complicated with depression, both are considered to be closely related yet different. The causal or subordinate relationship between them has attracted widespread attention [6, 7]. It has been suggested that insomnia might contribute to depression through negative effects on signaling pathways involved in synaptic plasticity. Insomnia has been reported to negatively affect hippocampal functions, and might cause symptoms of depression and cognitive dysfunctions [8].

Sleep disturbance and melatonin secretion disorder due to circadian rhythm disturbance are major features of depression [9]. Hypocretin, also known as orexin, is synthesized and secreted by the hypothalamus, and can regulate the sleep-wake cycle [10]. Orexin receptor antagonists can exert anti-insomnia effects, and orexin-related drugs are mainly used in sleep disorders, depression, anxiety, and drug addiction [11]. A recent study has suggested melatonin receptors act as novel targets for antidepressant action and agomelatine, an agonist of melatonin 1a receptor (MT1A) and melatonin 1b receptor (MT1B), for treating insomnia complicated with depression symptoms in neurological disorders [12]. Acetylcholine (ACh) is an important neurotransmitter of the limbic dopamine system in the midbrain, and literature shows that ACh levels in depressive patients are higher than in normal people [13]. Blockade of acetylcholinesterase (AChE) has been shown to cause symptoms of depression in human subjects [14]. A recent study reported that co-ordinated ACh release in the prefrontal cortex and hippocampus was associated with arousal and reward over time, and showed that ACh might be involved in the sleep-wake mechanism [15].

Compound Chaijin Jieyu Tablets (复方柴金解郁片, CCJJYT) were developed based on the classical antidepressant compound Xiao Yao San (逍遥散, XYS). Our previous study found that CCJJYT could significantly improve the depressive behaviors and sleep quality of patients, and was associated with few adverse events and high safety profile [16]. Further studies showed that the mechanism of CCJJYT for improving insomnia complicated with depression might be related to the regulation of the Glu/GABA ratio imbalance in the hippocampus and hypothalamus [17].

Our recent study indicated that CCJJYT could exert antidepressant effects by improving synaptic plasticity [18]. In this study, a model of insomnia complicated with depression in rats was established. Behavioral and molecular biological experiments were performed to assess whether CCJJYT plays a role in insomnia complicated with depression by regulating appetite, melatonin, and acetylcholine to improve synaptic plasticity.

2 Materials and methods

2.1 Drugs, reagents, and instruments

The CCJJYT consists of 9 g Chaihu (Bupleuri Radix), 9 g Jianghuang (Curcumae Radix), 9 g Baishao (Paeoniae Radix Alba), 6 g Guanyejinsitao (Hyperici Perforati Herba), 12 g Renshen (Ginseng Radix et Rhizoma), 6 g Zhimu (Anemarrhenae Rhizoma), 6 g Shichangpu (Acori Tatarinowii Rhizome), 9 g Fuling (Poria), and 6 g Yuanzhi (Polygalae Radix).

The drugs and reagents used in this study mainly include: venlafaxine hydrochloride sustained release capsule (Beijing Wansheng Pharmaceutical Co., Ltd., China), diazepam tablets (Shanxi Angsheng Pharmaceutical Co., Ltd., China), orexin-A elisa kit (Jingtian Company, China), trizol (The American Amion Company, USA), cDNA synthesis kit (Thermo, USA), the rabbit polyclonal antibodies to OXR1 and glyceraldehyde-3-phosphate dehydrogenase (Changsha Lefeng Biotechnology Co., Ltd., China), rabbit polyclonal antibodies to AChE, MT1A, MT1B and sheep anti-rabbit secondary antibodies (Beijing Boaosen Company, China). CCJJYT (Laboratory preparation, China).

The instruments used in this study mainly include: Microplate reader (Thermo Scientific, MK3), barnstead (ELGA, PURELAB Chorus 2 +), high-speed cryogenic centrifuge (Eppendorf, 5415R), ultra micro accounting protein concentration tester (Bio Drop, μLite +), automatic homogenizer (IKA, T25), analytical balance (Shimadzu, AP135W).

2.2 Experimental animals

In this study, 72 male Sprague-Dawley rats, weighing 180 – 200 g, were used. All rats were provided by Hunan SJA Laboratory Animal Co., Ltd. [SCXK (Xiang) 2019-0004],

and raised in specified pathogens free (SPF) experimental animal center [SYXK (Xiang) 2020-0004] at room temperature (25 ± 2) °C under relative humidity (40% – 50%) and light/dark cycle (12 h/12 h). The whole experiment was approved by the Institutional Animal Care and Welfare Ethics Committee of Hunan University of Chinese medicine (LLBH-202010130004).

2.3 Experimental groups and drug administration

After five days of adaptation feeding, 72 rats were randomly assigned to eight groups (n = 9): the control, CUMS, sleep deprivation (SD), CUMS + SD, the positive drug (venlafaxine hydrochloride + diazepam), CCJJYT high dose (CCJJYT-2×), medium dose (CCJJYT-1×), and low dose (CCJJYT-0.5×) groups. The day of grouping was recorded as day zero. CUMS model and SD model were established on days 0 to 35 and 14 to 35, respectively, while the control group was fed normally. The CUMS +SD, positive drug, CCJJYT-2×, CCJJYT-1×, and CCJJYT-0.5 × groups established the CUMS + SD composite model on days 0 to 35. The schematic diagram of the experiment and schedules are shown in Figure 1.

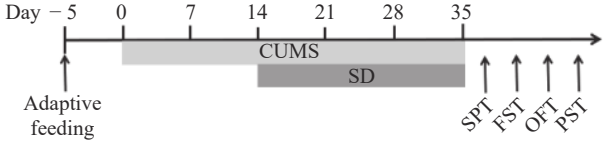

Figure 1 Schematic overview and timeline of the experimental approach

CUMS model was established based on the following conditions: tilting of the cage (45°, 12 h), ice water swimming (4 °C, 4 min), electric shock (2 mA, 1 min), noise (4 h), tail pinch (1 min), damp bedding (200 mL/cage, 24 h), reversed light/dark cycle (12 h/12 h), and water and food deprivation (24 h). With 1 – 2 types of stimulation per day, the same stimuli could not be given continuously. SD model was established based on our previous modeling method [17]. Chronic sleep deprivation was induced, and an 18 h sleep deprivation was performed at 15:00 – 9:00 (+ 1) every day for 21 consecutive days.

The dose of CCJJYT used in the high dose group (CCJJYT-2×) was two times the clinical human dose (5.68 g/kg). For the medium dose group (CCJJYT-1×), it was equivalent to the clinical human dose (2.84 g/kg), and for the low dose group (CCJJYT-0.5×), it was 0.5 times the clinical human dose (1.42 g/kg). The clinical human dose of venlafaxine hydrochloride extended-release capsules was 75 mg/d, and the clinical human dose of diazepam for sleep aid was 5 mg/d. Venlafaxine hydrochloride (13.5 mg/kg) was given on days 0 – 14 in the positive drug group and was combined with diazepam on days 15 – 35 with a dose of 0.9 mg/kg in rats. CCJJYT was administered in the groups by gavage.

2.4 Changes in body weight and food intake

Starting from day 0 and every seven days, the body weight of the rats was recorded until the end of day 35. The change in body weight was calculated using the following formula:

$$\text{Rate of weight change (\%)} = \frac{W_{7n} - W_0}{W_0} \times 100\%$$

(n = 1, 2, 3, 4, 5)

The body weight on day 0 was recorded as W_0, and the body weight on day 7 was recorded as W_7. The weight of added feed was recorded in each group of rats from day 0, and the intake was counted on days 7, 14, 21, 28, and 35 to observe the average daily intake of rats.

2.5 Animal behavior tests

2.5.1 Sucrose preference test (SPT) The SPT was performed on days 0, 14, and 35. On the first day, 1% sucrose solution was placed on the left and right sides of the rat cage. The next day, 1% sucrose solution and water were added to the left and right sides of the rat cage, respectively. Food and water were deprived, and SPT was employed after 24 h. The whole test period was four hours, and 1% sucrose water and water were placed on the left and right sides of the rat cage, respectively. After two hours, the left and right positions were exchanged. During the whole experimental period, the rats consumed 1% of the weight of sucrose water. The sucrose preference rate of rats was calculated by the following formula: sugar water preference rate (%) = sugar water consumption/(sugar water + water) consumption × 100%.

2.5.2 Open field test (OFT) The OFT was performed on days 14 and 35 in a quiet and dark room. Rat moved in a cube open box with a height of 0.4 m and an area of 0.64 m^2. The bottom area of the box was divided into 16 equal parts. After the rats were acclimatized for 1min, their horizontal and vertical movements within three minutes were recorded. Struggling in the water was recorded. Before the start of the experiment, the rat was acclimatized for 30 s, and the accumulated time for the rats to remain motionless within five minutes was recorded.

2.5.3 Forced-swimming test (FST) The FST was performed on days 14 and 35. A transparent cylindrical water tank (20 cm in diameter) was filled with water at a depth of 30 cm. The rat was placed in a water tank filled with water, and the time when the rat gave up struggling in the water was recorded. Before the start of the experiment, the rat was acclimatized for 30 s, and the accumulated time for the rats to remain motionless within five minutes was recorded.

2.5.4 Pentobarbital sodium test (PST) The suprathreshold dose observation of pentobarbital sodium was 35 mg/kg. The disappearance and recovery of righting reflex in each group was observed, and the injection time of pentobarbital sodium, sleeping time, and awakening time was recorded. The sleep latency and sleep duration were calculated using the following equations: (1) sleep latency =sleeping time – injection time of pentobarbital sodium, and (2) sleep duration = awakening time – sleeping time. The subliminal dose observation of pentobarbital sodium was 18 mg/kg. The number of sleeping rats in each group was recorded.

2.6 Hematoxylin-eosin (HE) stain evaluating pathological changes in brain regions

Isolated brain tissues were immersed overnight in 4% paraformaldehyde. After the brain tissues were dehydrated and buried in wax, they were cut at 4 μm thickness, and the paraffin sections were dewaxed and hydrated, stained in hematoxylin for 5 – 20 min, and incubated in differentiation solution for 30 s. The slices were washed with warm water, and put into an eosin dye solution for rewashing, followed by soaking, dehydration, and sealing with a neutral adhesive and observation under a microscope.

2.7 Golgi-Cox stain evaluating synaptic plasticity

Golgi-Cox staining was adopted to observe synaptic morphology. The brain tissues were cut into small pieces, soaked in a mordant dye solution, and placed in the dark for 3 d. Subsequently, silver plating was immersed in silver nitrate aqueous solution, and placed in the dark for 3 d, followed by rinsing, gradient ethanol dehydration, glue xylene sealing, drying and examination under a microscope for neuronal synapse morphology observation.

2.8 Enzyme-linked immunosorbent assay (ELISA) detecting levels of orexin-A and ACh

Homogenates from the hippocampus and prefrontal cortex were collected after the behavior test. An ELISA kit was employed to measure the levels of orexin-A and ACh.

2.9 Immunohistochemistry detecting the expression levels of MT1A and MT1B

The paraffin-embedded tissue was soaked in xylene solution for dewaxing. After two times of high-temperature treatment, each time lasted for 1 h, at 56 °C and 90 °C, respectively. During this period, gradient ethanol and citrate buffer with pH 6 were used to loosen the tissue structure, promote the infiltration of digestive juice and remove a constant amount of xylene and formaldehyde from tissues. The slides were sealed with 3% BSA for 1 h at room temperature, and incubated overnight at 4 °C with a primary antibody (anti-MT1A, 1 : 2 000; anti-MT1B, 1 : 2 000). After repeated washing, a secondary antibody (HRP-labeled) was applied the next day for 1 h. After dyeing with hematoxylin for 3 min, it was dehydrated and covered, and the images were collected and analyzed.

2.10 Western blot detecting the expression levels of OX1R, MT1A, MT1B, ChAT, and AChE

The total protein of the prefrontal cortex and hippocampus was at 12 000 rpm for 15 min, and the concentration of protein was determined by a bichloroacetic acid (BCA) protein assay kit. The target protein was isolated on 10% gel and transferred to the polyvinylidene fluoride (PVDF) membrane. The membrane was sealed with 5% skim milk for 1 h, and the primary antibodies against AChE (1 : 2 000), ChAT (1 : 2 000), MT1A (1 : 2 000), MT1B (1 : 2 000), OXR1 (1 : 2 000), and GAPDH (1 : 10 000). After washing with TBST buffer, it was detected by a single antibody.

2.11 Statistical analysis

Graph Prism 8.0.2 software was used for statistical analysis of all data. Measures are expressed as means ± standard deviation. One-Way ANOVA analysis was used for comparison between multiple groups. The difference was considered statistically significant when $P < 0.05$.

3 Results

3.1 Effects of CCJJYT on body weight and food intake

The data of day 35 showed the bodyweight growth rate of rats in the positive drug group was significantly greater than that of the CUMS + SD group ($P < 0.01$) (Figure 2A). The food intake in CUMS + SD group significantly decreased compared with the control group on day 7 ($P < 0.001$). Further, the food intake of the CCJJYT-2× group ($P < 0.01$) and positive drug group ($P < 0.001$) on day 35 were higher than those of the CUMS + SD group. These results indicated that CCJJYT could increase the weight and food intake of rats with insomnia complicated with depression (Figure 2B).

3.2 Effects of CCJJYT on depressive-like and insomnia behaviors

3.2.1 Sucrose preference in different groups of rats SPT is a classical approach to detecting the loss of pleasure caused by depression, with a decrease in sucrose preference indicating depression in the animals [19]. After modeling for 14 d, the rats in the CUMS + SD group showed a significantly lower sucrose preference rate than that in

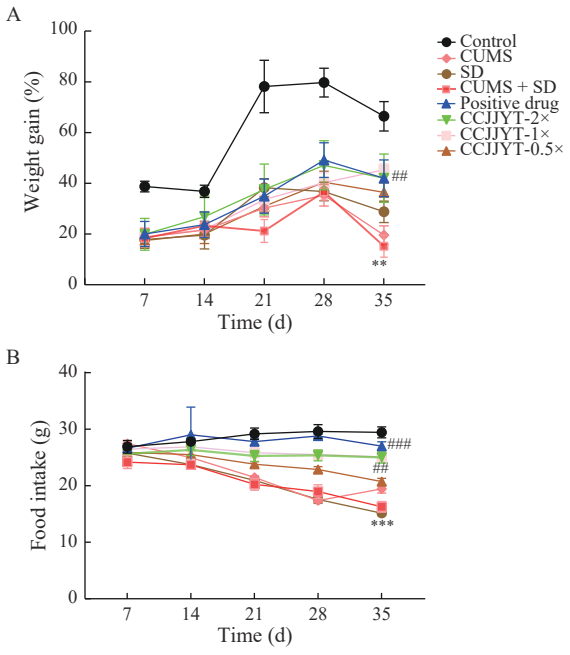

Figure 2 Effects of CCJJYT on body weight and food intake of rats with insomnia complicated with depression

A, body weight. B, food intake. Data are expressed as $\bar{x} \pm s$ (n = 9), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with the CUMS + SD group.

the control group ($P < 0.05$). Compared with the CUMS + SD group, the sugar-water preference results in the positive drug, CCJJYT-2×, and CCJJYT-1× groups significantly increased ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively). After modeling for 35 d, the CUMS + SD group significantly lower sucrose preference than that of the control group ($P < 0.01$), and the sugar-water preference results of the positive drug, CCJJYT-2 × , and CCJJYT-1× groups significantly increased compared with the CUMS + SD group ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) (Figure 3A).

3.2.2 Exploratory behaviors in different groups of rats OFT represents the independent and exploratory behaviors of rats in a strange environment. After modeling for 14 d, the horizontal movement distance of rats in the CUMS and CUMS + SD groups were significantly shorter than that of the control group ($P < 0.01$, and $P < 0.05$, respectively). The positive drug, CCJJYT-2×, CCJJYT-1×, and CCJJYT-0.5× groups demonstrated superior OFT results compared with the CUMS + SD group ($P < 0.01$, $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively). On day 35, the positive drug, CCJJYT-2×, and CCJJYT-1×groups demonstrated greater horizontal movement than the CUMS + SD group ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) (Figure 3B).

3.2.3 Swimming immobility time in different groups of rats FST is a behavioral test to assess depressive-like behaviors in animal models. On day 14, the immobility time of rats in SD group was longer than that in the control group ($P < 0.001$, Figure 3C). After 35 days of modeling, the immobility time of forced swimming in the CUMS + SD group was significantly longer than that in the control group ($P < 0.01$). However, the immobility time of the positive drug and CCJJYT-0.5× groups rats was significantly shorter compared with the CUMS + SD group ($P < 0.05$).

3.2.4 Sleep and awakening time in different groups of rats The PST behavioral method was used to assess whether CCJJYT produced sedative-hypnotic effects. The sleep latency of rats in CUMS group was longer than that in the control group ($P < 0.05$, Figure 3D). The sleep duration was shortened and changed significantly in rats of the CUMS and SD groups ($P < 0.05$). The positive drug group had longer sleep duration than that of the CUMS + SD group ($P < 0.05$, Figure 3E). In addition, at subthreshold doses of pentobarbital sodium, the amount of sleep of rats in CUMS, SD, and CUMS + SD groups decreased significantly ($P < 0.05$), but the CCJJYT-2×, CCJJYT-1×, CCJJYT-0.5×, and positive drug groups could reverse this effect significantly ($P < 0.05$, $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) (Figure 3F).

3.3 Effects of CCJJYT on pathological changes and synaptic plasticity in prefrontal cortex and hippocampus

Pathological changes in the prefrontal cortex and hippocampus were observed by HE staining (Figure 4A). The synaptic morphology of neurons in these regions was observed by Golgi-Cox staining (Figure 4B). Our results showed that the cone and granule cells in the prefrontal cortex and hippocampus of the control rats had regular morphology and neat and dense arrangement, without obvious inflammatory changes. Neuronal dendritic spines were also neatly arranged, densely packed, and clustered into a network. In contrast, in CUMS, SD, and CUMS + SD groups, the pyramidal and granule cells were absent, while the pyramidal cells and nuclei were crinkled and irregularly arranged, with marked inflammatory infiltration. Meanwhile, the hippocampal neuron dendritic spines were broken, blurred, or even disappeared, and did not form networks. The positive drug, CCJJYT2 ×, CCJJYT-1×, and CCJJYT-0.5× groups had narrowed nuclei, regular arrangement, and a small amount of inflammatory cell infiltration. The degree of expansion of nerve cells and perivascular gaps was lesser than that in the model group. The hippocampal dendritic spines of rats in the CCJJYT-2× and CCJJYT-1× groups were neatly arranged, had newly developed dendritic spines, and constructed neat network connections. It has been shown that CCJJYT could repair damaged prefrontal cortex, hippocampal pyramidal cells and granule cells in the CUMS + SD group under stress, thus repairing the damage to neural dendritic spines, and improving learning, memory, and cognitive dysfunction.

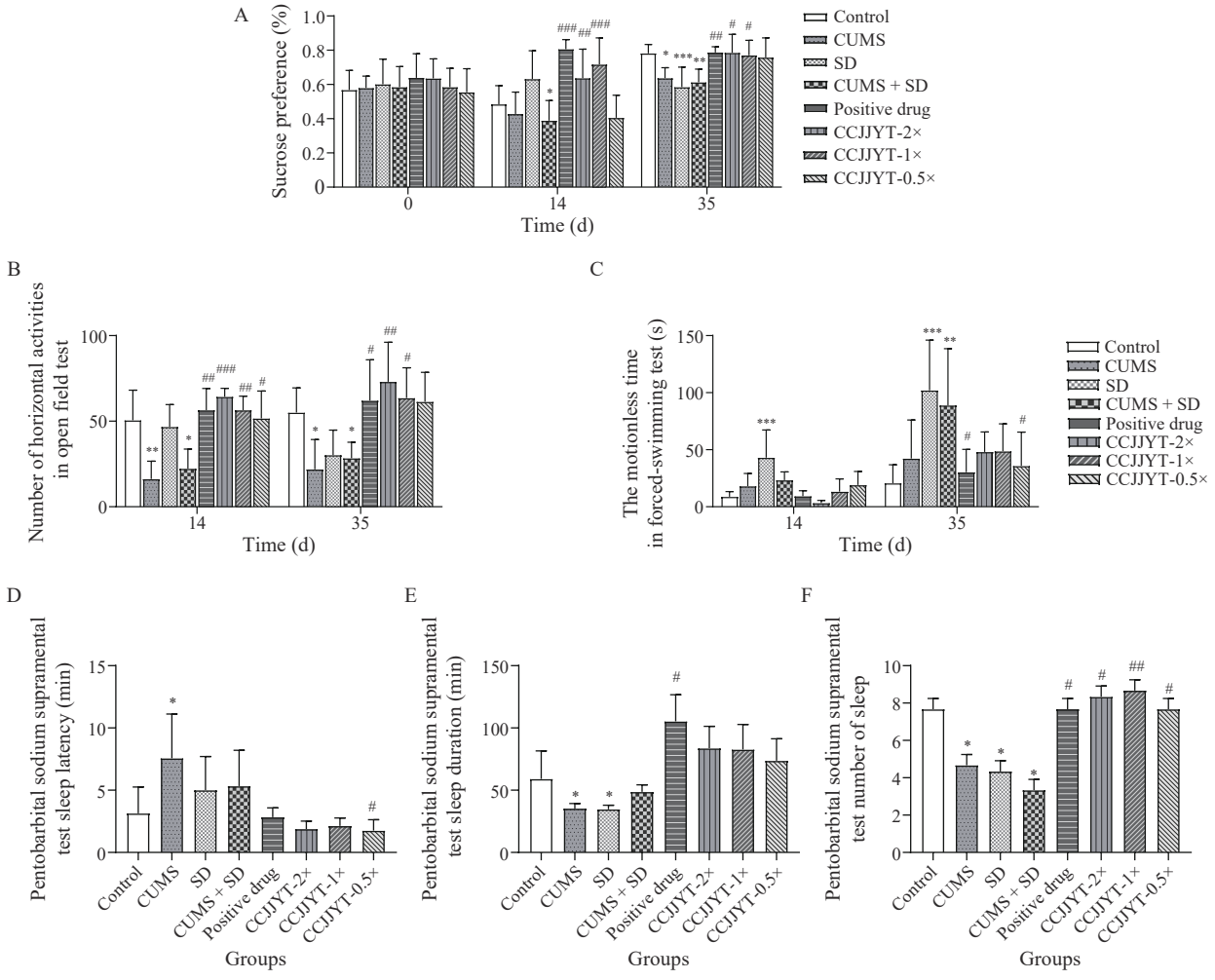

Figure 3 Effects of CCJJYT on the behavior of rats with insomnia complicated with depression

A, the SPT of rats in each group. B, the horizontal movement distance of rats in each group based on OFTs. C, the immobility time of the rat in each group based on FSTs. D–F, effects of CCJJYT on pentobarbital-induced sleep in depressive insomnia rats. Data are expressed as $\bar{x} \pm s$ ($n = 9$), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with the CUMS + SD group.

3.4 Effects of CCJJYT on the expression levels of orexin-A and its receptor (OXR1) in the prefrontal cortex and hippocampus

The content of orexin-A in the prefrontal cortex was detected by ELISA (Figure 5A). The results showed that the content of orexin-A in the prefrontal cortex of rats was higher in the CUMS , SD, and CUMS + SD groups than that in the control group ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively). The content of orexin-A in the positive drug and CCJJYT-2×groups rats was lower than that in the CUMS + SD group ($P < 0.05$). The expression level of the OXR1 was significantly higher in the prefrontal cortex and hippocampus of rats in the CUMS, SD, and CUMS + SD groups than that in the control group ($P < 0.001$, Figure 5B - 5E). In the prefrontal cortex, OXR1 content was significantly lower in the CCJJYT-1× and CCJJYT-0.5× groups than that in the CUMS + SD group ($P < 0.001$). In the hippocampus, OXR1 content was significantly reduced in the positive drug, CCJJYT-1×, and CCJJYT-0.5×groups ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively).

3.5 Effects of CCJJYT on the expression levels of MT1A and MT1B in the prefrontal cortex and hippocampus

As shown in Figure 6A - 6C, MT1A protein expression level in the prefrontal cortex was significantly increased in the CUMS, SD, and CUMS + SD groups compared with the contronl group ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respevtively). MT1B protein expression level significantly decreased in the CUMS + SD and CUMS groups ($P < 0.05$). The expression levels of MT1A and MT1B significantly increased in the CCJJY-2×, CCJJYT-1×, and CCJJYT-0.5× groups ($P < 0.001$). The expression level of MT1A protein in hippocampus was significantly lower in the CUMS, SD, and CUMS + SD groups than that in the contronl group ($P < 0.05$, Figures 6D - 6F). Compared with the contronl group, the expression level of MT1B protein

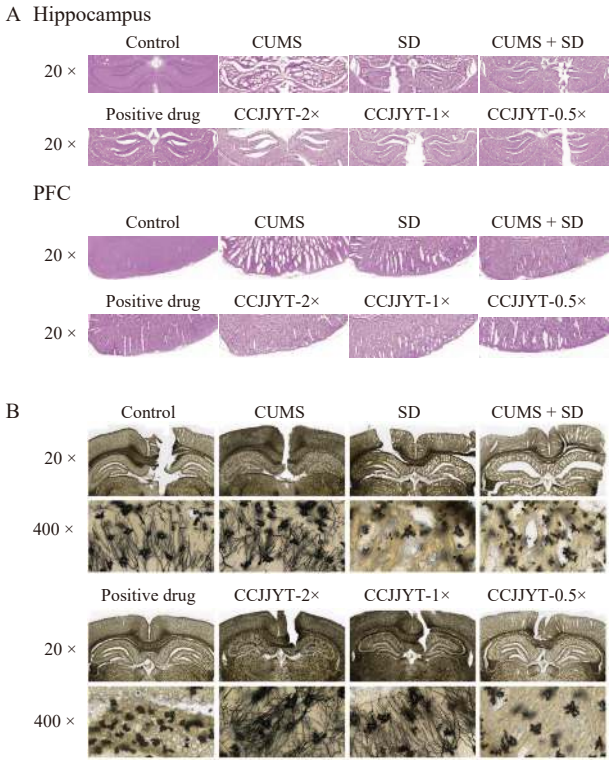

Figure 4 Effects of CCJJYT on pathological changes and synaptic plasticity in prefrontal cortex and hippocampus of rats with insomnia complicated with depression

A, pathological changes. B, changes of synaptic plasticity ($n = 3$).

was lower in the CUMS, SD, and CUMS + SD groups ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively). However, compared with the CUMS + SD group, the hippocampal MT1A protein expression level significantly increased in the positive drug, CCJJYT-2×, CCJJYT-1×, and CCJJYT-0.5× groups ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.01$, respevtively). The expression level of MT1B protein in the positive drug, CCJJYT-0.5×, CCJJYT-1×, and CCJJYT-2× groups significantly decreased ($P < 0.01$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respevtively).

Here, we investigated the expression levels of melatonin receptors (MTNR1A and MTNR1B) by immunohistochemical staining. The results showed a significant reduction in expression level of MTNR1A in the hippocampus and prefrontal cortex of rats in the CUMS + SD group ($P < 0.05$ and $P < 0.001$, respectively) (Figure 6G - 6I). The MTNR1A expression level was significantly higher in the hippocampus and prefrontal cortex in the CCJJYT-2× group than that in the CUMS + SD group ($P < 0.001$ and $P < 0.05$, respectively). The MTNR1A expression level significantly increased in the hippocampus and prefrontal cortex in the CCJJYT-1× group ($P < 0.001$ and $P < 0.05$, respectively). Further, a significant reduction in the MTNR1B expression levels of the hippocampus and prefrontal cortex of rats was observed in the CUMS + SD group ($P < 0.01$, Figure 6J – 6L). The MTNR1B expression level in both the hippocampus and prefrontal cortex was significantly higher in the CCJJYT-0.5× group than that in the CUMS + SD group ($P < 0.01$). These results indicated that CCJJYT could exert antidepressant and sleep-improving effects by targeting MTNR1A and MTNR1B.

3.6 Effects of CCJJYT on the expression level of ACh in the prefrontal cortex and hippocampus in rats with depressive insomnia

The content of ACh in the prefrontal cortex and hippocampus was detected by ELISA (Figure 7A and 7B). The

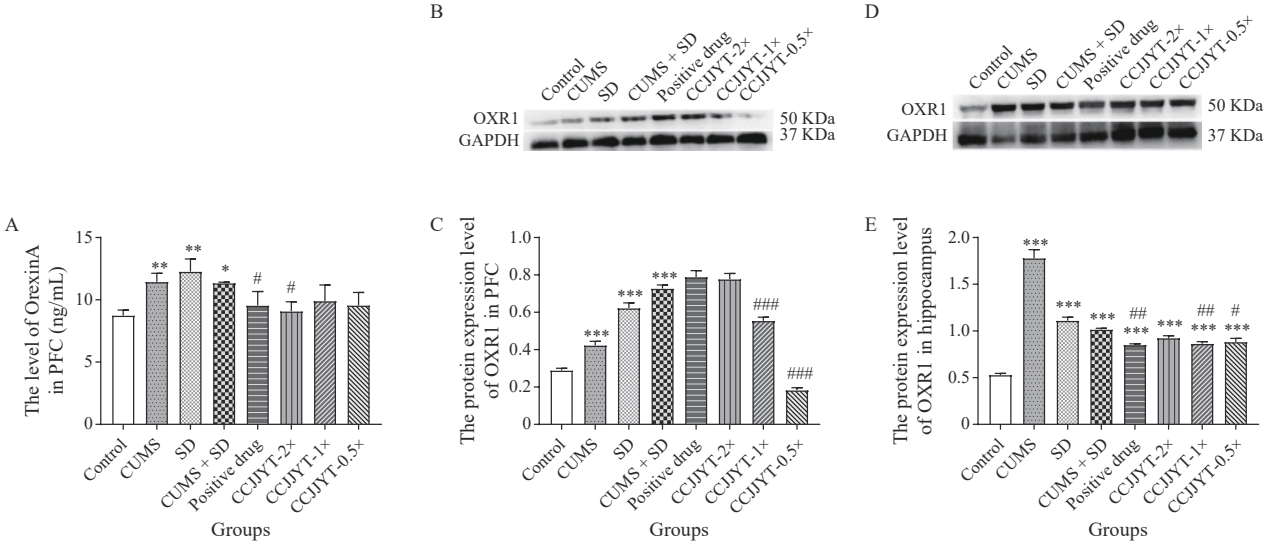

Figure 5 Effects of CCJJYT on the expression levels of orexin-A and OXR1 in prefrontal cortex and hippocampus of rats with insomnia complicated with depression

A, contents of orexin-A in the prefrontal cortex. B and C, protein expression level of OXR1 in the prefrontal cortex. D and E, protein expression level of OXR1 in the hippocampus. The data are expressed as $\bar{x} \pm s$ ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with the CUMS + SD group.

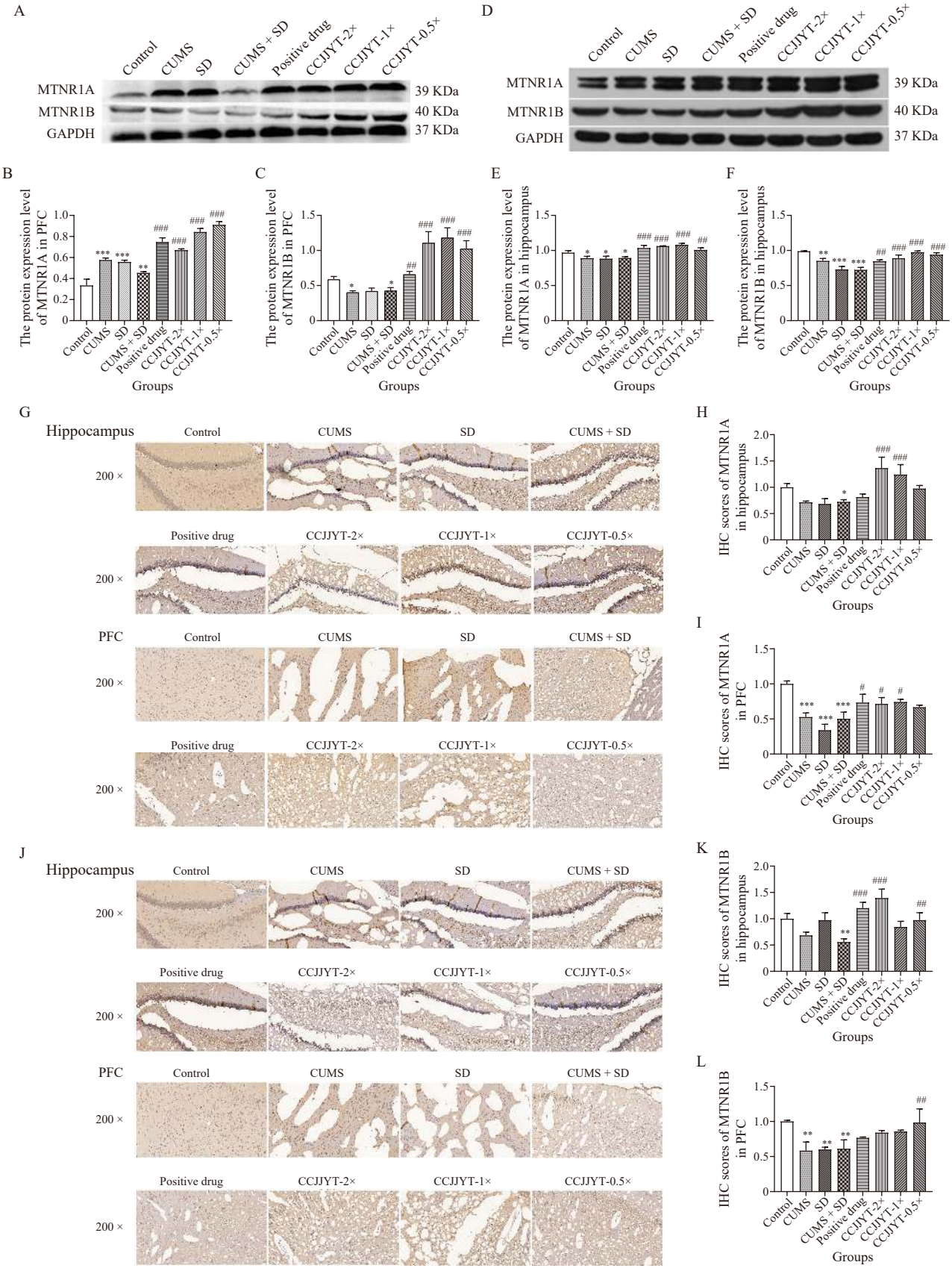

Figure 6 Effects of CCJJYT on expression levels of MT1A and MT1B in the prefrontal cortex and hippocampus in rats with insomnia complicated with depression

A – C,protein expression levels of MT1A and MT1B in the prefrontal cortex. D – F, protein expression levels of MT1A and MT1B in the hippocampus. G – L, immunohistochemistry showing the expression levels of melatonin receptors (MTNR1A and MTNR1B) in the prefrontal cortex and hippocampus, respectively. The data are expressed as $\bar{x} \pm s$ ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with the CUMS + SD group.

content of ACh in the hippocampus and prefrontal cortex of the CUMS + SD group significantly increased (P < 0.001 and P < 0.01, respectively). The content of ACh in the positive drug group and the CCJJYT-2× group significantly decreased (P < 0.01). The protein expression levels of AChE and ChAT in the prefrontal cortex and hippocampus of the rats was detected by Western blot. The expression level of AChE in the prefrontal cortex in CUMS, SD and CUMS + SD model groups was significantly lower than that in the control group (P < 0.05). However, the protein expression level of AChE significantly increased in the prefrontal cortex of rats in the CCJJYT-2×, CCJJYT-1×, and CCJJYT-0.5× groups (P < 0.001). Further, a significant increase in the expression level of AChE protein was observed in the hippocampus of the rats in the CCJJYT-2× and CCJJYT-1× groups (P < 0.001), and in the positive drug group (P < 0.01). In addition, hippocampal ChAT protein expression levels significantly increased in the CUMS + SD group compared with the control group (P < 0.001), whereas pharmacological intervention in the positive drug, CCJJYT-1×, and CCJJYT-2× groups reversed the CUMS + SD induced changes significantly in ChAT protein expression levels (P < 0.01, P < 0.01, and P < 0.001, respectively) (Figure 7C - 7G).

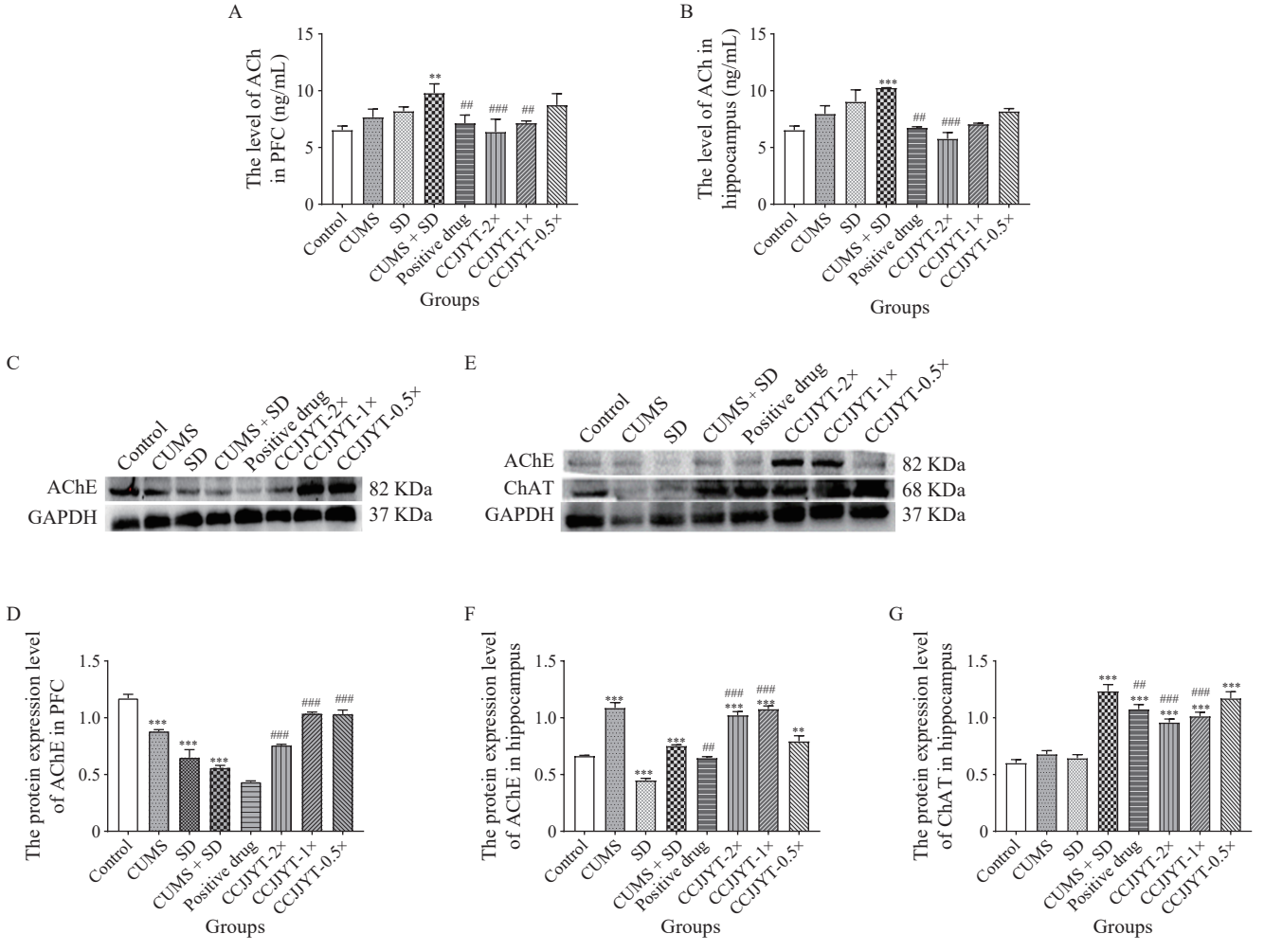

Figure 7 Effects of CCJJYT on the expression levels of AChE and ChAT in prefrontal cortex and hippocampus in rats with insomnia complicated with depression

A and B, the content of ACh in the prefrontal cortex or hippocampus. C and D, expression of AChE in the prefrontal cortex. E – G, expression of AChE and ChAT in the hippocampus . The data are expressed as $\bar{x} \pm s$ (n = 3), $^{*}P$ < 0.05, $^{**}P$ < 0.01, and $^{***}P$ < 0.01, compared with the control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, and $^{\#\#\#}P$ < 0.001, compared with the CUMS + SD group.

4 Discussion

Currently, the elevated incidence of insomnia complicated with depression is alarming and affecting the physical and mental health of many people. It is well known that there is a close relationship between sleep and depression. Poor sleep quality or short duration has been shown to be associated with increased depressive symptoms [3]. Traditional Chinese medicine has the advantages of being multi-target and having fewer side effects and can effectively improve the sleep quality and ameliorate depression conditions of the patients [20]. This study was designed to investigate innovative therapeutic drugs against insomnia complicated with depression by assessing the

efficacy of CCJJYT in improving insomnia complicated with depression induced by CUMS combined with sleep deprivation. In a previous study, the changes in weight, food intake, depressive behavior, insomnia behavior, and sleep were assessed by our group, a model that successfully established a model of chronic unpredictable moderate stress combined with 21 days of sleep deprivation was proposed [21]. The results of our drug intervention experiment showed that CCJJYT ameliorated the reduction in body weight and food intake of rats under stress, and increased their sugar and water consumption.

Synaptic plasticity refers to the adaptive changes of synapses under continuous stimulation of brain nerve cells, mainly manifested as changes in the number, structure, and function of synapses, thereby affecting the nerve functions of the brain. Continuous stress or depression can result in brain tissue atrophy and damage synaptic plasticity. Brain imaging studies showed a significant reduction in the neural connections between the prefrontal cortex, hippocampus, and other related brain regions in patients with depression and that the degree of neuronal atrophy was correlated with the duration of depression and therapeutic effects [22]. Golgi-Cox staining showed that the density of hippocampal dendritic spines in insomnia complicated with depression rats was significantly reduced, and the CCJJYT-2× and CCJJYT-1× groups had a significant reduction in hippocampal damage. Since changes in dendritic spine density can directly affect synaptic transmission functions, and a decrease in synaptic plasticity caused by a decrease in dendritic spine density in the hippocampus is closely related to the occurrence of insomnia complicated with depression [23, 24], that the alleviation of CCJJYT depression-like and insomnia behaviors in rats could be related to its regulation of synaptic plasticity was speculated.

Previous studies found changes in the cholinergic system involving cholinesterase, acetyltransferase, and cholinergic receptors in depression patients and animal models [25]. It is reported that ACh has a key role in regulating neuronal excitability of the whole brain, which is released by the ChAT transporter. ACh in the synaptic cleft could be degraded by AChE, the key enzyme to regulate the extracellular ACh level [26]. Interestingly, *in vivo* experiments showed that cholinergic activation could modulate bidirectional synaptic plasticity [27, 28]. ZANNONE et al. [29] reported that acetylcholine could affect the synaptic plasticity of the hippocampus, and then caused depression. Dopamine can reverse this situation. Our study found that the levels of orexin-A and acetylcholine were upregulated while that of melatonin was down-regulated in the prefrontal cortex and hippocampus of rats with insomnia complicated with depression. However, CCJJYT could effectively reverse these effects. Thus, these findings indicated that CCJJYT might improve synaptic plasticity by regulating the changes of orexin-A, melatonin, and acetylcholine in the prefrontal cortex and hippocampus, thereby demonstrating antidepression and insomnia effects.

Unlike chemical drugs, which have a clear quantitative-effect relationship, the quantitative-effect relationship of herbal compounding is more complex and has more influencing factors. Although a proportion of herbal compound prescriptions do have a positive relationship between dose and efficacy, there are many other herbal compound prescriptions for which the positive correlation between dose and efficacy remains unclear. It is speculated that this situation may arise because the dose administered is too high and the drug concentration is excreted without absorption in the gastrointestinal tract of rats so the drug cannot work effectively.

5 Conclusion

This study successfully established an insomnia complicated with depression rat model and showed a reduced expression levels of acetylcholinesterase, acetyltransferase, and cholinergic hyperactivation, and abnormal expression of appetite receptors and melatonin receptors in the brain of the modle rats, which might be closely related to the pathogenesis of insomnia complicated with depression. Intervention with herbal compound preparations increased the expression levels of AChE and ChAT, downregulated the orexin-A/OXR1 signaling pathway, and upregulated the expression levels of MT1A and MT1B in the prefrontal cortex and hippocampus of the rats. To sum up, the results showed that CCJJYT could improve synaptic plasticity, regulate appetite hormone, melatonin, and acetylcholine levels, and serve as an effective antidepressant treatment.

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Competing interests

The authors declare no conflict of interest.

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复方柴金解郁片通过调节食欲素 A、褪黑素和乙酰胆碱含量改善突触可塑性发挥抗抑郁失眠作用

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【摘要】目的 探讨复方柴金解郁片（CCJJYT）对失眠并发抑郁症模型大鼠的疗效及作用机制。**方法** 将 72 只 SD 大鼠随机分为 8 组，分别为对照组、慢性不可预测轻度应激（CUMS）组、睡眠剥夺（SD）组、慢性不可预测轻度应激合并睡眠剥夺（CUMS + SD）组、阳性药物（盐酸文拉法辛 + 地西泮）组、CCJJYT 高剂量（CCJJYT-2×）组、中剂量（CCJJYT-1×）组、低剂量（CCJJYT-0.5×）组，每组 9 只。通过体重、摄食量和行为测试 [蔗糖偏好试验（SPT）、旷场试验（OFT）、强迫游泳试验（FST) 和戊巴比妥诱导睡眠试验（PST）] 来评价抑郁样行为。苏木精-伊红（HE）染色和 Golgi-Cox 染色分别观察病理组织和突触形态的变化。采用酶联免疫吸附法（ELISA）检测食欲素 A 和乙酰胆碱的含量。采用免疫组化和 Western blot 检测食欲素受体 1（OXR1）、褪黑素受体 1（MT1A）、褪黑素受体 2（MT1B）、酰胆碱酯酶（AChE）和胆碱乙酰转移酶（ChAT）的蛋白表达。**结果** 模型组大鼠出现明显的行为学改变以及海马树突棘分支长度和突触数量减少，同时造模成功后食欲素 A 和乙酰胆碱的含量增加（$P < 0.05$），海马和前额叶皮层 OXR1、MT1A、MT1B、ChAT 和 AChE 表达发生改变（$P < 0.05$）。CCJJYT 可改善大鼠抑郁性失眠行为 ($P < 0.05$) 和突触可塑性，与阳性药物组疗效相似。CCJJYT 可减少食欲素 A 和乙酰胆碱的含量（$P < 0.05$），降低海马和前额叶皮层中 OXR1 和 ChAT 的表达（$P < 0.05$），提高 MT1A、MT1B 和 AChE 蛋白的表达 ($P < 0.05$)。**结论** CCJJYT 具有较好的抗抑郁和改善失眠的作用，其机制可能是通过调节大鼠海马和前额叶皮层的食欲素 A、褪黑素和乙酰胆碱含量从而改善突触可塑性，进而发挥抗抑郁失眠作用。

【关键词】 复方柴金解郁片；抑郁；失眠；食欲素 A；褪黑素；乙酰胆碱；突触可塑性