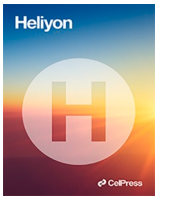
Heliyon 9 (2023) e21743

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Cherry leaf decoction inhibits NMDAR expression and thereby ameliorates CUMS- induced depression-like behaviors through downregulation of α2δ-1

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ARTICLE INFO

Keywords:
CLD
Depression
Behavioral tests
HPA
α2δ-1
NMDAR

ABSTRACT

Depression is a complex and prevalent mental illness. Cherry leaf is a traditional Chinese herbal medicine, which has confirmed to exert a certain antidepressant effect, but its potential neural regulation mechanism is not clear. This paper aims to investigate the improved action of cherry leaf decoction (CLD) on chronic unpredictable mild stress (CUMS) rats and its potential neural regulation mechanism by verifying the role and function of NMDAR regulatory target α2δ-1 in depression due to CUMS. Male SD rats were subjected to random stressors persisting for 5 weeks to establish the CUMS depression rat model. CLD could effectively alleviate depression-like behaviors of CUMS rats in behavioral tests including sucrose preference test, forced swimming test, tail suspension test and open field test. After the administration of the CLD, the expression of corticotropic-releasing hormone (CRH) in the hypothalamus was inhibited. Moreover, the levels of CRH, adrenal cortical hormone (ACTH) and corticosterone (CORT) in serum also decreased significantly. CUMS upregulated the expressions of α2δ-1, N-methyl-*d*-aspartate receptor 1 (NR1), NR2A and NR2B, and enhanced the binding ability to of α2δ-1 and NR1, which were reversed by CLD. The results demonstrated that CLD could ameliorate depression-like behaviors due to CUMS, which was related to the fact that CLD down-regulated α2δ-1 level and interfered with α2δ-1 binding to NR1, thereby reducing NMDAR expression and ultimately inhibiting HPA axis activity.

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https://doi.org/10.1016/j.heliyon.2023.e21743
Received 4 July 2023; Received in revised form 31 August 2023; Accepted 26 October 2023
Available online 7 November 2023

1. Introduction

Depression is a multiple psychiatric disorder, with clinical manifestations of mood disorders, weakness of will, passive pessimism and impaired cognition, which can lead to serious social dysfunction and affect mental health [1]. The annual number of suicides due to depression worldwide has reached 800,000, with hundreds of millions affected [2]. The World Health Organization forecasts that depression will be the most influencing factor in the burden of disease by 2030. Depression has become a dangerous and unstable reason for social and economic development [3].

The occurrence of depression is the result of the interaction of several factors, including psychological burden, environmental stress and epigenetic factors [4]. Previous research established that the risk of depression is proportional to the number and intensity of stressful events that occur [5]. The emergence and evolution of depression involves multiple neuromodulatory mechanisms, among which dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis has received widespread attention as the important endocrine pathway triggering depression [6]. The HPA axis is a key factor linking stress and depression [7]. Stress stimulation will cause impaired function of the HPA axis, manifested mainly by increased activity and expression of hypothalamic CRH neurons, resulting in increased release of CRH, which in turn stimulates increased secretion of downstream ACTH and CORT, eventually leading to HPA axis hyperactivity [8]. Stress-induced imbalance in the HPA axis is an important pathological manifestation of mood disorders and is also closely associated with the frequent occurrence of suicidal behaviors [9]. In addition, the abnormal stress response of the HPA axis has been one of the most extensive and promising bio-systems in research on the pathogenesis of depression [10].

The increased activity of PVN-CRH neurons induced by chronic stress is mainly driven by the neuroplasticity of the afferent fibers of glutamatergic neurons [11]. The research paradigm of depression prevention and treatment is slowly changing, and the glutamate (Glu)-based synaptic plasticity theory is gaining more attention and development [12]. Glu is an excitatory neurotransmitter of the amino acid class that is highly expressed in the central nervous system and widely found in mammals; it is involved in mediating cognitive memory function and synaptic plasticity regulation [13]. Previous research demonstrated that depression is often associated with Glu metabolism disorder, and its level can reflect the severity of depression, which is closely related to NMDAR [14]. NMDAR is one of the receptor types for Glu, which is distributed in 70 % of the brain's synapses [15]. Stress can cause excessive release of glutamate, which can lead to excessive activation of extrasynaptic NMDAR, causing cytotoxicity and neurodegeneration and inducing multiple mental diseases, including depression [16]. Adaptive changes in NMDA receptors have emerged as the ultimate common pathway in the mechanism of neural modulation of antidepressant action [17]. The rapid antidepressant effect of NMDAR antagonist ketamine has been widely verified in clinical practice, which also proves the key role of NMDAR in the treatment of depression [18]. However, the molecular mechanism of increased NMDAR activity and excitatory synaptic transmission during CUMS-induced depression remains unclear.

α2δ-1, a regulatory subunit of the voltage-gated calcium channels (VGCCs), widely distributed in the nervous system, is a classic target of action for drugs such as gabapentin (α2δ-1 inhibitor) used to treat neuropathic pain [19]. Numerous studies have reported that the expression of α2δ-1 is significantly increased in both the brain and spinal cord of animals in the case of neurogenic hypertension, ischemic brain injury and other neurological injury diseases of the brain [20]. α2δ-1 can also interact with NMDAR through the *C*-side structured domain to form the protein complex, thereby enhancing NMDAR activity and facilitating its synaptic transport [21]. Inhibition of α2δ-1 expression or interference with α2δ-1 binding to NMDAR counteracts the enhancement of hypothalamic presynaptic and postsynaptic NMDAR activity by angiotensin [22]. Nevertheless, it is not yet known whether α2δ-1 is involved in regulating depression-causing processes in CUMS and what changes occur in the complex of α2δ-1 and NMDAR in depressive states, which has important implications for whether α2δ-1 will be a novel therapeutic channel for depression.

Cherry leaf *(Prunus pseudocerasus Lindl. [Rosaceae])* is a widely distributed traditional Chinese medicine, with multiple effects of warming the stomach, strengthening the spleen, stopping coughing and bleeding, which can be used to treat food accumulation, diarrhea and cough, etc [23]. It is included in the '***Chinese Materia Medica***' compiled under the auspices of the State Administration of Traditional Chinese Medicine of China [24]. Modern studies have found that cherry leaves have various pharmacological actions, including inhibition of inflammatory response, reduction of oxidative stress levels and being hypotensive [25,26]. However, there are relatively few reports on its antidepressant effect. In our previous study, we detect a variety of antidepressant active ingredients in CLD by HPLC, including kaempferol, rutin, quercetin and chlorogenic acid. In addition, we also find that CLD can improve the depression-like behavior of CUMS depressed rats in FST, which provides an experimental basis for the development of this study. Meanwhile, the potential neural regulatory mechanism of its antidepressant effect needs to be further explored.

The antidepressants currently used in clinical practice are mainly selective serotonin reuptake inhibitors and tricyclic antidepressants, mostly based on the 'monoamine neurotransmitter hypothesis' [27], and the side effects and risks they pose are becoming increasingly prominent [28]. The exploration of Chinese medicine may provide new options for the prevention and treatment of clinical depression.

2. Materials and methods

2.1. Animals

Fifty-four healthy adult Sprague-Dawley (SD) rats (SPF-grade; male; 140–180 g) were purchased from Changsheng Biotechnology Co., Ltd. (Liaoning; licenses no. SCXK (Liao) 2020–0001). The rats were raised in cages with standard conditions (Temperature, 20–25 °C; Humidity, 50–65 %), maintaining a 12-h day/night cycle. Prior to the CUMS procedure, the rats were provided with

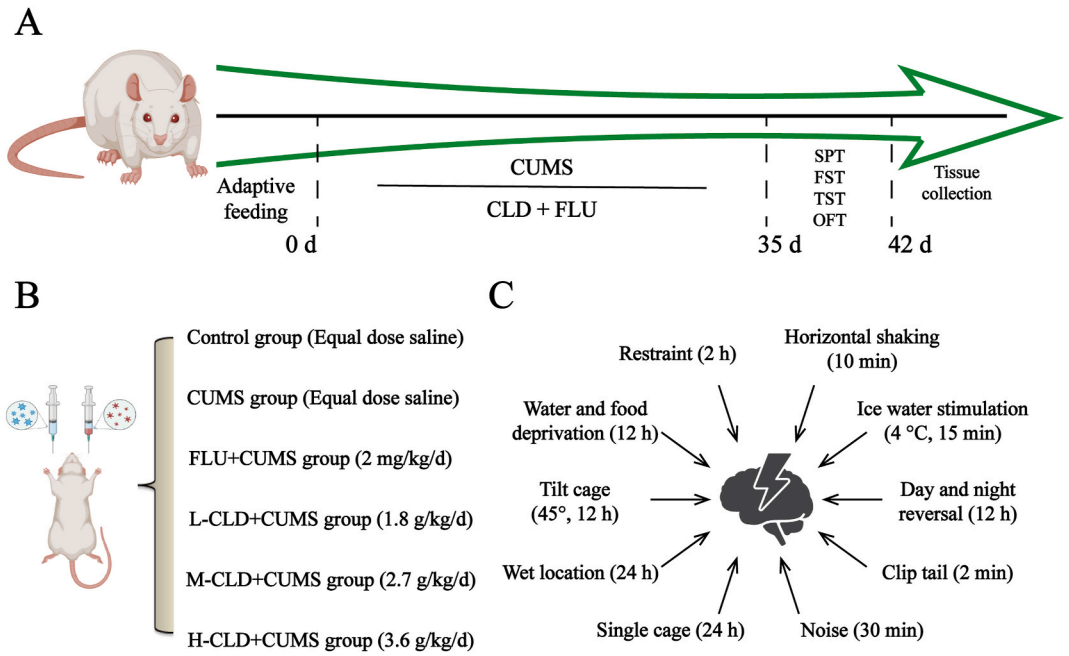

Fig. 1. Flow diagram of our experimental plan. A, Timeline of study execution; B, Grouping and medication administration; C, Stressors used in the CUMS program.

unfettered access to food and water for a week.

The animal experiments in this research strictly adhered to the Codes of Animal Experiments from the Committee of Medical Ethics, National Health Department of China, which had been ratified by the Ethics Committee for Animal Experiments of Hebei University of Chinese Medicine. Reference number DWLL202202010.

2.2. Drugs

Cherry leaves were acquired from professional planting base of Shijiazhuang (Hebei Province, China) and had been authenticated and approved by Professor Yuping Yan, an expert in the identification of traditional Chinese medicine (College of Pharmacy, Hebei University of Chinese Medicine). The specimen had been preserved at the Herbarium of Hebei University of Chinese Medicine. The preparation of CLD was made with reference to previous research and pre-experiments, which had minor modifications [29]. The cherry leaves were washed, dried (55 °C, 12 h), crushed, soaked (5 h) in turn and then boiled (20 min) to gain the filtrates. The filtered drugs were put into distilled water and decocted again. The filtrates were blended to prepare the concentrated solution, which was stored at 4 °C for backup. The concentration of CLD was 3.6 g/ml.

Fluoxetine (FLU) was supplied by Aladdin Biochemical Technology Co., Ltd. (Shanghai, China; F131623) as the positive control drug, which had been widely used in the treatment of clinical depression. FLU was dissolved in normal saline (2 mg/ml) and administered by gavage.

2.3. Groups and drug administration

At the end of the acclimatization feeding, all rats were weighed, numbered and randomly divided into 6 groups of 9 rats each, with the control (CON) group, CUMS group, FLU group, low-dose CLD (L-CLD) group, medium-dose CLD (*M*-CLD) group and high-dose CLD (H-CLD) group. The doses administered in the FLU and treatment groups were 2 mg/kg/d, 1.8 g/kg/d, 2.7 g/kg/d and 3.6 g/kg/d respectively, while others were given equal doses of saline. The CLD, FLU and saline were administered by gavage once a day for 35 days. The drug dose was defined with reference to the preceding research and pre-experiments, which had been adjusted appropriately [30,31]. The group and drug treatment was presented in Fig. 1.

2.4. CUMS procedure

The design and execution of the CUMS procedure referred to preceding studies with suitable changes [32,33]. During the CUMS procedure, except for the CON group, the rats were exposed to stressors, consisting of water and food deprivation (12 h), restraint (2 h), tilt cage (45°, 12 h), day and night reversal (Light off/on 12 h), wet location (24 h), horizontal shaking (10 min), clip tail (2 min), single cage (24 h), noise (30 min) and ice water stimulation (4 °C, 15 min), which were illustrated in Fig. 1. Two stressors were randomly

provided daily. In addition, each stressor must not appear continuously but at least twice a week. The CUMS procedure lasted 35 d.

2.5. Body weight

The body weight of rats was measured weekly before and during the CUMS procedure.

2.6. Behavioral tests

Behavioral tests were completed on days 36–42, during which time appropriate stress measures and medication continued to be administered to prevent adaptive changes in the rats. The relevant experimental methods were obtained from previous studies and adjusted appropriately [34,35].

2.6.1. Sucrose preference test (SPT)

The SPT was applied to appraise the situation of anhedonia in the rats. Each rat was housed individually and given sucrose water (1 %) and tap water lasting 24 h, during which time their locations were changed at regular intervals. Subsequently, all rats were deprived of water and food lasting 24 h. Ultimately, each rat was given sugar water (100 ml; 1 %) and tap water (100 ml) lasting 2 h. Their consumption in 2 h was recorded to calculate the sucrose preference index. The calculation formula was as follows:

SPT % = sucrose water consumption (ml)/[(sucrose water consumption (ml) + tap water consumption (ml)]*100 %.

2.6.2. Forced swimming test (FST)

The FST was applied to appraise the level of despair in the rats. The rats were placed in a cylindrical container (30 cm × 60 cm) whose water depth was kept at 30 cm and whose temperature was kept at about 24 °C. The entire experiment took 6 min. Except for the first 2 min of FST, the immobility time of the rats was counted. The whole experiment would be recorded through the video system. After the experiment, the immobility time was recorded by three members of the research group who did not participate in the experiment to reduce the experimental error. During this time, the environment was required to remain quiet. It is worth noting that the water in the container was cleaned and replaced timely.

2.6.3. Tail suspension test (TST)

The TST was used to assess the desperation and anxiety of the rats. The tails of the rats (2 cm from the caudal tip) were suspended on the stand with tape, with the head kept 10 cm away from the platform. The experiment lasted 6 min. Except for the first 2 min in TST, the immobility time of the rats was counted. The whole experiment would be recorded through the video system. After the experiment, the immobility time was recorded by three members of the research group who did not participate in the experiment to reduce the experimental error. During this time, everyone was required to remain quiet. The immobile state included the passive swaying of the body and the motionlessness of the limbs and trunk.

2.6.4. Open-field test (OFT)

The OFT reflected the free exploration ability and tension of the rats. The experiment was carried out in a black box (80 cm × 80 cm × 30 cm), which was divided into central and peripheral areas. All rats were allowed to move freely for 5 min, starting from the center of the box. The recorded indicators were as follows: the total distance, the distance in center, the time in center, crossing numbers, rearing numbers and stretching numbers. The active areas of the rats were cleaned with alcohol to remove the odor during the experimental interval. The experiment process was recorded and analyzed by a software and image system (SMART v3.0).

2.7. Sample collection

At the end of the behavioral tests, all the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (1 %, 50 mg/kg). Blood samples were taken from the abdominal aorta. Subsequently, the hypothalamus of the rats was speedily stripped on ice. Some tissues were placed in paraformaldehyde reagent (4 %) for tissue fixation, while the rest was stored in a −80 °C refrigerator.

2.8. Elisa

After the blood sample was obtained, the tubes containing the sample were placed symmetrically into the centrifuge to collect the supernatant, which was set to 3000 rpm, lasting 15 min. The concentrations of relevant serum markers, including CRH, ACTH and CORT, were measured using ELISA kits purchased from Senbeijia Biological Technology Co., Ltd. (Nanjing, China). All experiments were conducted according to the instructions.

2.9. Immunofluorescence staining

The fixed tissues were divided into sections, which were subsequently dewaxed, repaired (EDTA; pH 9.0; 5 min), cleaned and soaked in PBS (1 min). Then, the slices were incubated with closed sheep serum (30 min; 37 °C). After the serum was removed, the slices were separately hatched by applying diluted CACNA2D1 Polyclonal antibody (27453-1-AP) and CRH/CRF Polyclonal antibody (10944-1-AP) overnight at 4 °C, and then washed with PBS (15 min). Polyclonal antibodies were obtained from Proteintech Biological

Table 1
The primer sequences of α2δ-1, NR1, NR2A, NR2B and β-actin.

Gene	Primer sequence	Primer length
α2δ-1	Forward: CTGGTGGACGTGAGTGGAAGT Reverse: ACGAAATCATCATCGGAAAGG	98bp
NR1	Forward: ATGCCCGTAGGAAGCAGATG Reverse: CCCTATGACGGGAACACAGC	285bp
NR2A	Forward: ACGGCTTGGTGTTTGGAGAT Reverse: GAACGTGGATGTCGGATCCT	151bp
NR2B	Forward: CTATGATAATGGCGGATAAGGA Reverse: AGTAGGTGGTGACGATGGAAA	135bp
β-actin	Forward: CGTTGACATCCGTAAAGAC Reverse: TAGGAGCCAGGGCAGTA	110bp

Technology Co., Ltd. (Rosemont, IL, USA). The slices were separately hatched by applying diluted Goat anti-rabbit IgGs (H + L) (ZF-0511; ZF-0516), which was obtained from Zhongshan Jinqiao Biological Technology Co., Ltd. (Beijing, China), and the above cleaning process was repeated. The sections were added to DAPI (AR1177; Boster, CA, USA) and incubated (10 min; 37 °C) without light. Ultimately, the sections were sealed with distilled water and observed with fluorescence microscopy (Olympus IX71 fluorescence inverted microscope, Japan) after being washed with distilled water. The images were acquired through an imaging software acquisition system (IS Capture). The experimental results were evaluated according to positive expression area and integral optical density value.

2.10. In situ hybridization

The fixed tissues were made into wax blocks and then sectioned. The slices were washed with H_2O_2 (30 %) and distilled water (1:10; 37 °C) (15 min). Subsequently, the sections were dripped with pepsin (3 % citric acid diluted) to expose the mRNA nucleic acid fragments (37 °C, 30 min). After that, the sections were incubated in the hybridization solution and washed with SSC, then blocked (30 min, 37 °C). The slices were sequentially dripped with biotinylated mouse anti-digoxin and SABC biotinylated peroxidase and then cleaned using PBS. The slices were stained by applying DAB dropwise. Then, after termination of color development, the slices were re-dyed using hematoxylin and cleaned with water. Finally, the slices were sequentially dehydrated, made transparent and sealed. Images were acquired and viewed with a specialized microscope, and the expressions of target proteins appeared brownish-yellow or brown. The images were acquired through an imaging software acquisition system (IS Capture). The experimental results were evaluated according to positive expression rate and mean optical density value.

2.11. Western blotting

The frozen tissues were weighed, ground and added to RIPA lysis solution (R0020; Solarbio, Beijing, China) overnight. The tissue homogenate was centrifuged to obtain the supernatant and then the protein concentration was detected by a protein quantifier (22,331 hamburg; Eppendorf, Germany). The protein samples were denatured and added to the gel pores of DS-PAGE for electrophoresis (80 V) until the target band reached the position of two-thirds of the separating glue. Then, the target bands on the gel were transferred (170 mA) to the PVDF membrane (IPVH00010; Massachusetts, USA) for 60 min and sealed by applying 5 % non-fat milk (1172- GR500; BioFroxx, Germany) lasting 2 h. The closed membranes were incubated with CACNA2D1 Polyclonal antibody (27453-1-AP), NR1 Polyclonal antibody (27676-1-AP), NR2A Polyclonal antibody (28571-1-AP), NR2B Polyclonal antibody (21920-1-AP) and GAPDH Polyclonal antibody (10494-1-AP) overnight at 4 °C and then cleaned using PBST. The primary antibodies in this experiment were acquired from Proteintech Biotechnology Co., Ltd. (USA). After that, they were incubated using HRP-labeled goat anti-rabbit IgG (ZB-2301; Zhongshan Jinqiao, Beijing, China) and cleaned. Subsequently, the membranes were cleaned as above. The reaction membranes were laid flat with reagents prepared in the ECL kit (sc-2048; Zhongshan Jinqiao, Beijing, China) to be developed, exposed and made into films. The films were scanned to determine the grayscale values by software (Tanon Gis) for analysis.

2.12. RT-PCR

The RNAs of tissues were obtained by applying Trizol (15,596,018; Ambion, TX, USA). The concentration and mass of RNA were detected using a UV spectrophotometer (BioPhotometer, Eppendorf). Based on the manuals, the reverse transcriptions were conducted using the HiFiScript gDNARemoval cDNA Synthesis Kit (CW2582 M; ComWin, Beijing, China). The relative quantification of the data was performed using the fluorescent quantitative PCR instrument (CFX connectTM, BIO-RAD) and calculated using the $2^{-\Delta\Delta CT}$ method. The amplification was respectively performed with the target gene primer and the internal reference gene primer. Simultaneously, the dissolution curve was analyzed at 65–95 °C. Information about the primer sequences were shown in Table 1.

2.13. Co-immunoprecipitation assays (CO-IP)

The protein of the sample was extracted and tested using a BCA kit (MD913053; Medical Discovery Leader, Hebei, China). 10 μl of

Table 2
The body weight of rats measured before and during CUMS procedure.
Note: The statistical outcomes were manifested as mean ± SEM. Contrast to CON group: **P < 0.01; Contrast to CUMS group: #P < 0.05,##P < 0.01; N·S: Not significant.

Groups	weight					
	0 week	1st week	2 nd week	3rd week	4th week	5th week
Control	205.00 ± 2.72	242.78 ± 2.13	273.89 ± 3.11	304.44 ± 3.47	337.89 ± 2.71	369.56 ± 3.09
CUMS	199.33 ± 2.66	224.00 ± 1.89**	240.78 ± 3.44**	261.00 ± 3.14**	280.56 ± 2.22**	301.89 ± 3.15**
FLU	208.33 ± 2.29	229.78 ± 2.47N·S.	261.67 ± 2.88##	286.22 ± 2.28##	310.44 ± 3.04##	334.22 ± 2.58##
L-CLD	199.56 ± 2.26	225.33 ± 1.60N·S.	245.67 ± 2.89N·S.	268.67 ± 3.88N·S.	291.67 ± 2.60N·S.	313.44 ± 2.26#
M-CLD	204.11 ± 2.61	229.11 ± 2.41N·S.	252.56 ± 3.34N·S.	275.89 ± 4.02#	298.78 ± 3.41##	324.11 ± 1.89##
H-CLD	203.33 ± 2.39	228.33 ± 2.14N·S.	260.78 ± 3.78##	287.67 ± 2.49##	312.22 ± 2.83##	335.67 ± 2.42##

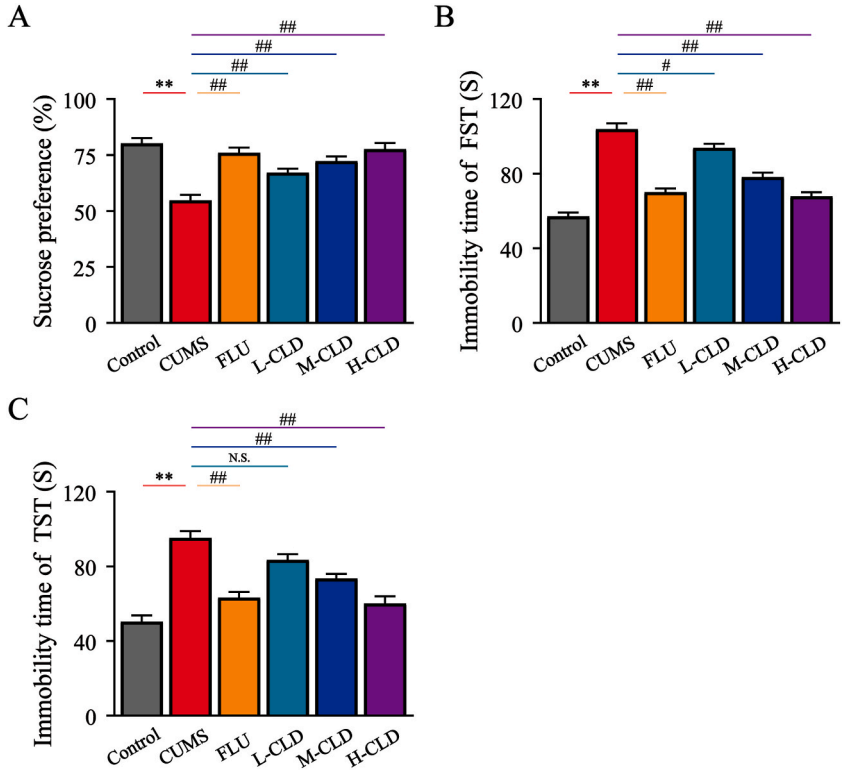

Fig. 2. The effect of CLD on SPT, FST and TST of CUMS rats. A shows the differences in sucrose preference among groups of rats. B–C display the duration of rat static state in FST and TST. The statistical outcomes were manifested as mean ± SEM (n = 9). The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: **P < 0.01; Contrast to CUMS group: #P < 0.05, ##P < 0.01; N·S: Not significant.

whole protein was used as input for western blotting analysis. The rest of the samples were divided into 4 portions and spiked with 1 μg of the CACNA2D1 and NR1 Polyclonal antibodies, which were incubated overnight at 4 °C in a shaker. Protein A agarose beads (MD7125, Medical Discovery Leader) were cleaned by applying lysis buffer (P0013; Beyotime, Shanghai, China) and centrifuged (3000 rpm) lasting 4 min. The pretreated agarose beads were added to the protein extract containing antibodies and incubated in a shaker to couple the antibodies to the agarose beads. Subsequently, the agarose beads were centrifuged (4 °C, 3000 rpm) and the supernatant was removed. The agarose beads were cleaned by reapplying lysis buffer and adding SDS (15 μ, 2 ×) loading buffer (MD6619, Medical Discovery Leader), which was boiled for 5 min. The interacting proteins were analyzed using the western blotting method.

2.14. Statistical analysis

SPSS v 22.0 system was applied to arrange experimental data and implement data analysis. The experimental results were presented as the mean ± standard error of mean (S.E.M.). The data was tested for normality using Shapiro-Wilk Test. After meeting the normal distribution, the data was tested for variance homogeneity by Levene's Test. One-way ANOVA followed by Tukey's test was

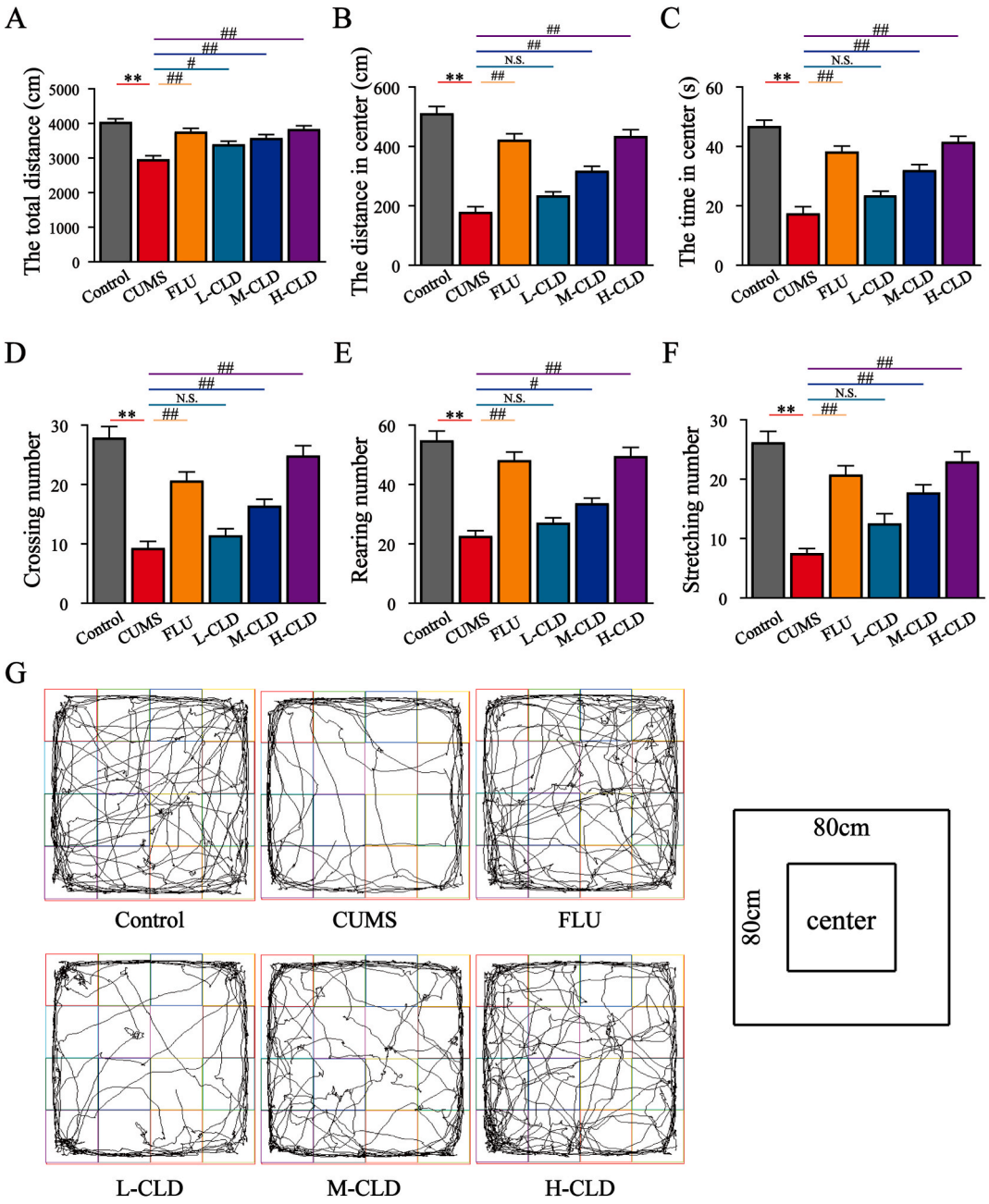

Fig. 3. The effect of CLD on the autonomous activity ability of CUMS rats. An indicates the total activity distance that the rats moved. B-D exhibit the distance, time and crossing number of rats in the center. *E*-F exhibit the somatic movements (rear and stretch); G is the representative trajectory of rats in the experimental area. The statistical outcomes were manifested as mean ± SEM (n = 9). The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: $^{**}P < 0.01$; Contrast to CUMS group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; N·S: Not significant.

employed to contrast the differences in data between groups. The significance standards were defined as $P < 0.05$ or $P < 0.01$.

3. Results

3.1. CLD inhibited weight loss caused by CUMS

The weight of rats between groups was significantly different in the first week ($F_{(5, 48)} = 9.94$, $P < 0.01$), second week ($F_{(5, 48)} = 13.67$, $P < 0.01$), third week ($F_{(5, 48)} = 22.31$, $P < 0.01$), fourth week ($F_{(5, 48)} = 49.59$, $P < 0.01$) and fifth week ($F_{(5, 48)} = 80.18$, $P < 0.01$), as revealed in Table 2. After the rats were exposed to CUMS stressors, their weight significantly decreased ($P < 0.01$), which was

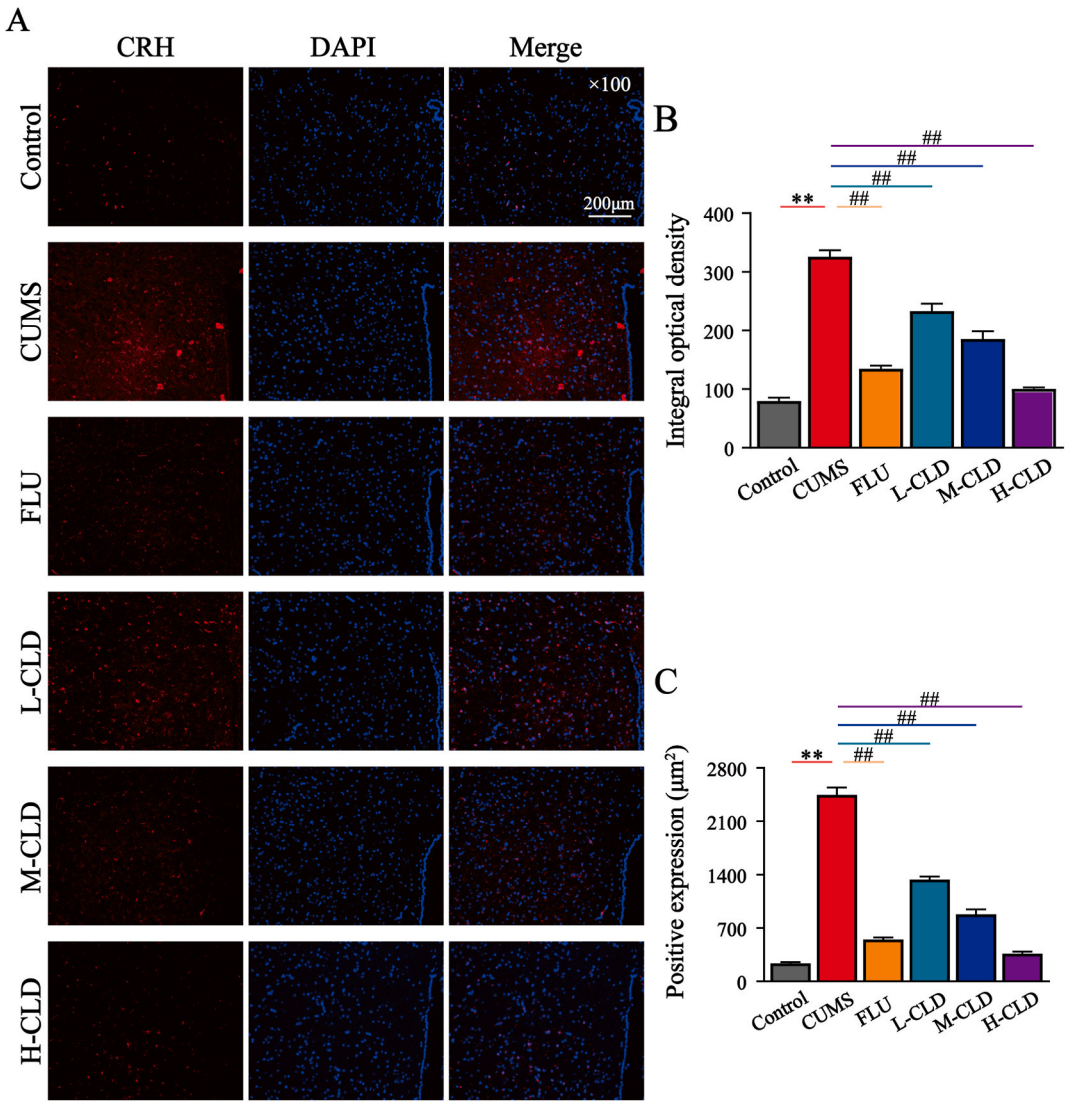

Fig. 4. The effect of CLD on CRH in the hypothalamus. A is the typical image of CRH expression and distribution in the hypothalamus (immunofluorescence; × 100). The integral optical density and positive expression area of hypothalamic CRH can be observed in B–C. The statistical outcomes were manifested as mean ± SEM (n = 3). The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: **P < 0.01; Contrast to CUMS group: ##P < 0.01.

ameliorated after treatment with FLU (P < 0.01). H-CLD treatment could also inhibit the trend of weight loss in rats (P < 0.01), except for the first week (P > 0.05). Meanwhile, the weight of the *M*-CLD group rose remarkably (P < 0.05 or P < 0.01), except for the first two weeks (P > 0.05), in contrast to the CUMS group. The weight of the L-CLD group was not statistically significant in the first four weeks (P > 0.05) until 5 weeks, when a significant increase was observed (P < 0.05).

3.2. CLD alleviated CUMS-induced depression-like behaviors

3.2.1. Effect of CLD on SPT

In this experiment, the intake of sucrose water was different for each group ($F_{(5, 48)} = 15.80$, P < 0.01). Related data was exhibited in Fig. 2A. The relative intake of sucrose water was significantly reduced in rats after CUMS stimulation (P < 0.01), which provided the basis for the smooth establishment of our model. After the intervention of FLU, L-CLD, *M*-CLD and H-CLD, the sucrose preference index was obviously improved (P < 0.01).

3.2.2. Effect of CLD on FST

The duration of the static state of rats in each group in this experiment was remarkably different ($F_{(5, 48)} = 54.62$, P < 0.01). Related

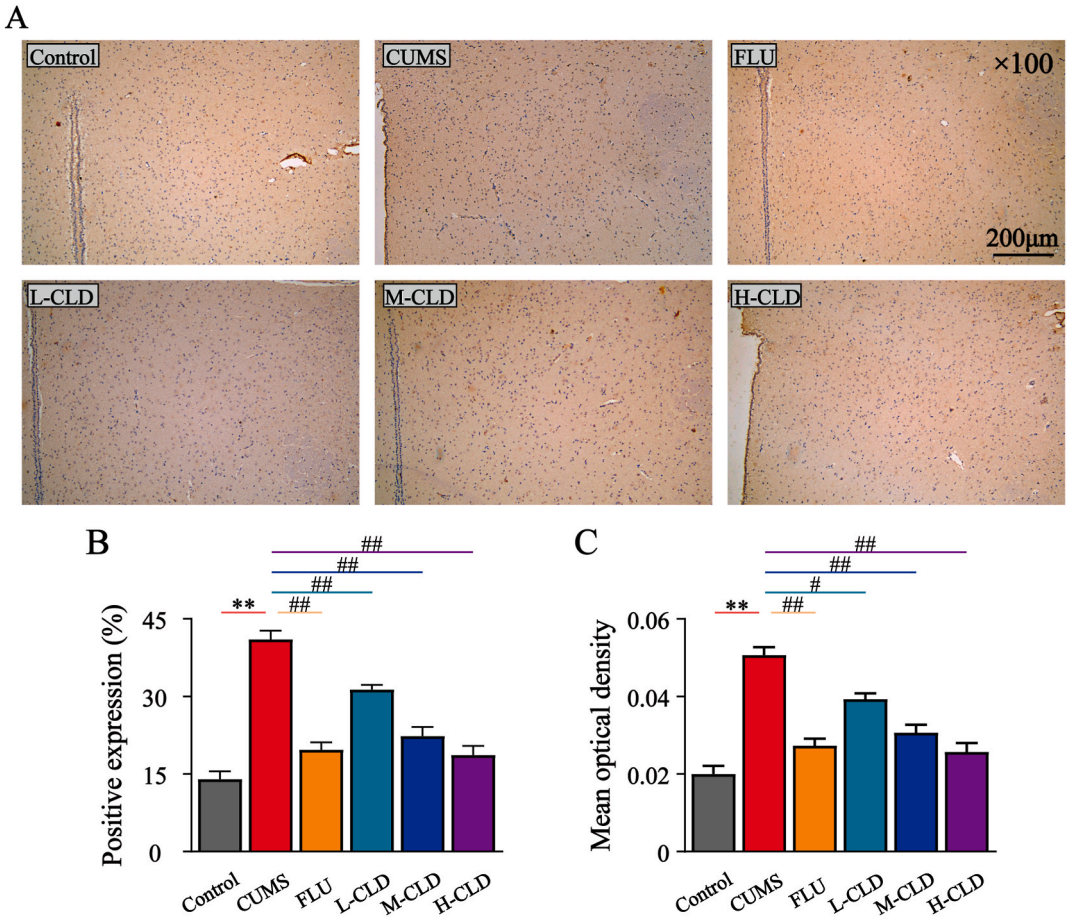

Fig. 5. The effect of CLD on CRH in the hypothalamus. A is the typical image of CRH expression and distribution in the hypothalamus (in situ hybridization; × 100). The positive expression rate and mean optical density of hypothalamic CRH can be observed in B–C. The statistical outcomes were manifested as mean ± SEM (n = 3). The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: **P < 0.01; Contrast to CUMS group: $^{\#}$P < 0.05, $^{\#\#}$P < 0.01.

data was exhibited in Fig. 2B. The duration of quiescence in CUMS rats was markedly increased (P < 0.01), then normalized after FLU and CLD interventions (P < 0.05 or P < 0.01).

3.2.3. *Effect of CLD on TST*

The duration of the static state of rats in TST ($F_{(5, 48)} = 24.98$, P < 0.01) was revealed in Fig. 2C. We found that rats had obviously less physical activity and more rest time under stress (P < 0.01). After treatment with FLU, *M*-CLD and H-CLD, the immobility time decreased markedly (P < 0.01). Furthermore, the duration of limb rest in rats in the L-CLD group did not experience a noticeable reduction due to drug intervention (P > 0.05).

3.2.4. *Effect of CLD on OFT*

In OFT, the results for total distance ($F_{(5, 48)} = 16.17$, P < 0.01), distance in center ($F_{(5, 48)} = 48.33$, P < 0.01), time in center ($F_{(5, 48)} = 35.93$, P < 0.01), crossing number ($F_{(5, 48)} = 27.48$, P < 0.01), rearing number ($F_{(5, 48)} = 30.80$, P < 0.01) and stretching number ($F_{(5, 48)} = 21.81$, P < 0.01) were summarized, as exhibited in Fig. 3.

Compared to the CON group, the total distance, rearing number and stretching number of CUMS rats were remarkably diminished (P < 0.01); some activities of rats in the central area, including distance, time and crossing number, were also obviously lessened (P < 0.01) in contrast to the CON group, indicating that the autonomic activity ability and environmental exploration ability of CUMS rats had been weakened. After administration of FLU, *M*-CLD and H-CLD, CUMS rats showed a remarkable increase in mobility and exploration. The total distance in center, time in center, crossing number, rearing number and stretching number increased significantly (P < 0.05 or P < 0.01). The rats of the L-CLD group experienced no notable therapeutic effect in most aspects (P > 0.05) except for total distance (P < 0.05).

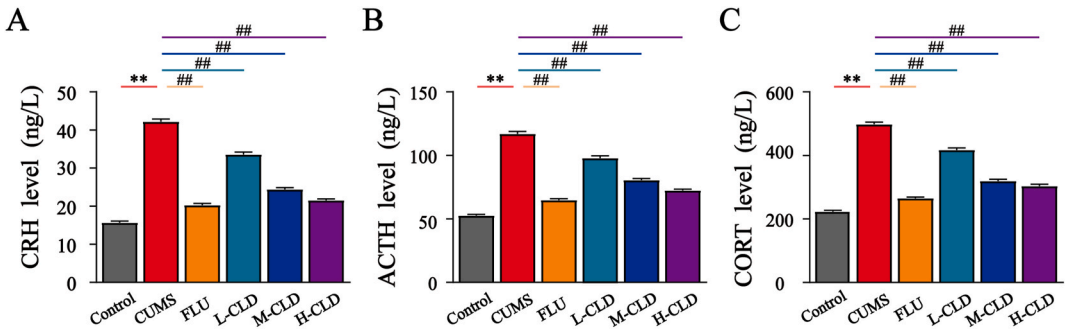

Fig. 6. The effect of CLD on the concentration of CRH (A), ACTH (B) and CORT (C) in serum. The statistical outcomes were manifested as mean ± SEM (n = 9). The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: **P < 0.01; Contrast to CUMS group: ##P < 0.01.

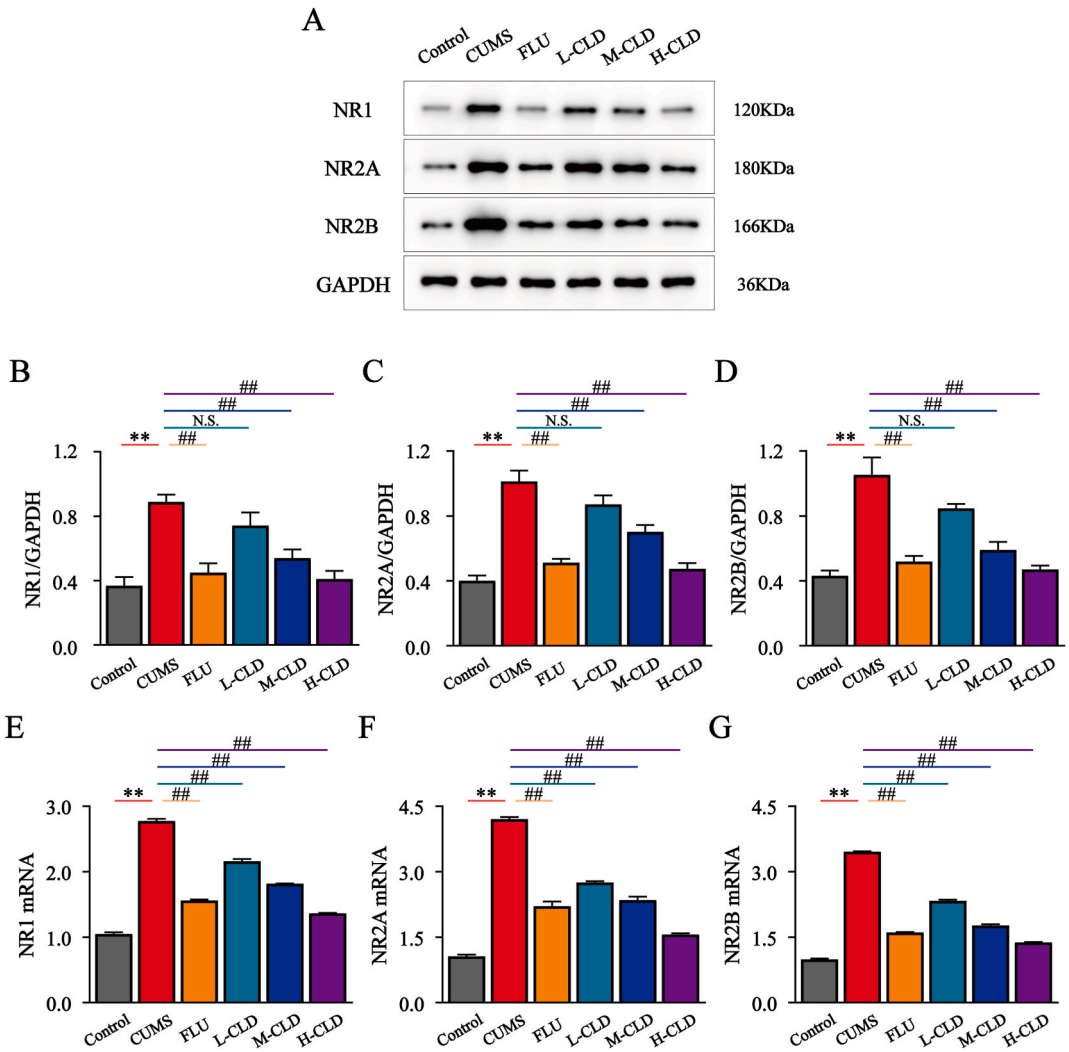

Fig. 7. The effect of CLD on the expression levels of NR1, NR2A and NR2B of the hypothalamus. A is the representative blot of NR1, NR2A, NR2B and GAPDH. B-D reveal the ratios of protein expression of NR1, NR2A and NR2B to GAPDH (n = 4). *E*-G reveal the levels of mRNA of NR1, NR2A and NR2B (n = 3). The statistical outcomes were manifested as mean ± SEM. The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: **P < 0.01; Contrast to CUMS group: #P < 0.05, ##P < 0.01; N·S: Not significant.

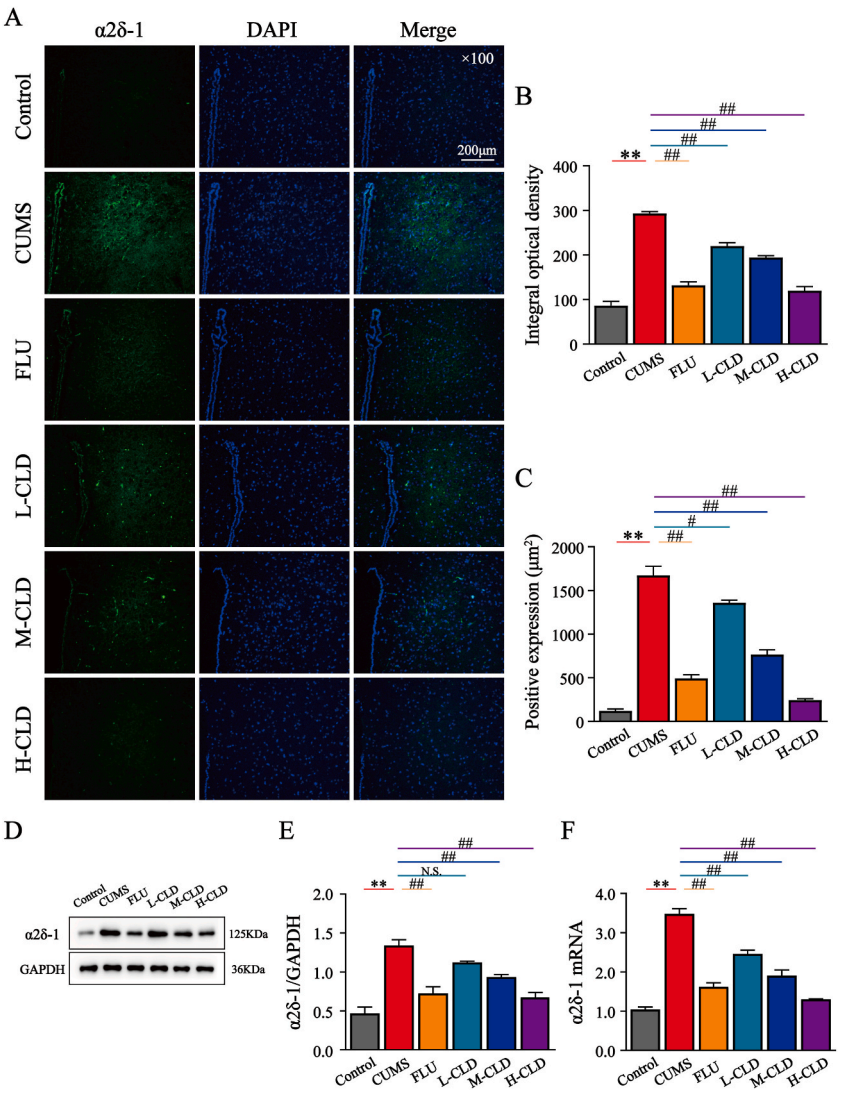

Fig. 8. The effect of CLD on the expression level of α2δ-1. A is the typical image of the expression and distribution of CRH in the hypothalamus (immunofluorescence; × 100). The integral optical density and expression area of hypothalamic α2δ-1 can be observed in B–C (n = 3). D is the representative blot of α2δ-1 and GAPDH. *E*-F reveal the ratio of protein expression of α2δ-1 to GAPDH (n = 4) and the mRNA level of α2δ-1 (n = 3). The statistical outcomes were manifested as mean ± SEM. The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: $^{**}P < 0.01$; Contrast to CUMS group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

3.3. CLD downgraded the expressions of CRH levels in the hypothalamus

The expression levels of CRH in the hypothalamus were detected by immunofluorescence methods including integral optical density (CRH, $F_{(5, 12)} = 100.72$, $P < 0.01$) and positive expression (CRH, $F_{(5, 12)} = 243.21$, $P < 0.01$), as exhibited in Fig. 4. The integral optical density and positive expression of CRH in CUMS rats notably increased in contrast to the CON group ($P < 0.01$). Due to the intervention of FLU, L-CLD, *M*-CLD and H-CLD in rats, the integral optical density and positive expression were significantly reduced ($P < 0.01$).

Additionally, CRH was identified and detected by in situ hybridization, including positive cell rate ($F_{(5, 12)} = 40.81$, $P < 0.01$) and mean optical density ($F_{(5, 12)} = 31.49$, $P < 0.01$). Related data appeared in Fig. 5. Compared to the CON group, the levels of positive cell rate and mean optical density in CUMS group rats were notably raised ($P < 0.01$). However, the expressions in FLU, L-CLD, *M*-CLD and H-CLD groups were obviously reduced with medication ($P < 0.05$ or $P < 0.01$).

3.4. CLD reduced the levels of CRH, ACTH and CORT in serum

The serum levels of CRH ($F_{(5, 48)} = 371.54$, $P < 0.01$), ACTH ($F_{(5, 48)} = 285.37$, $P < 0.01$) and CORT ($F_{(5, 48)} = 336.12$, $P < 0.01$) in

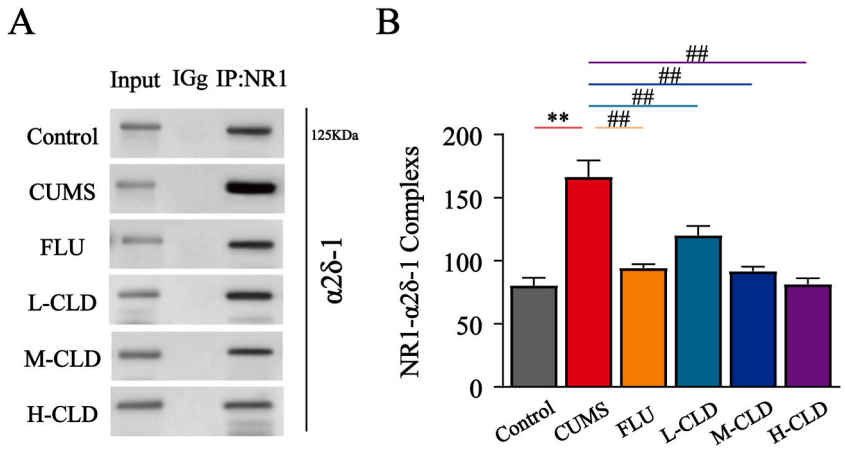

Fig. 9. The effect of CLD on the protein complexes of α2δ-1 and NR1. A shows the representative blots of α2δ-1 and NR1 protein complexes from CO-IP; B exhibits the expression level of the protein complex of α2δ-1 and NR1. The statistical outcomes were manifested as mean ± SEM (n = 3). The data was analyzed with One-way ANOVA followed by Tukey's test. Contrast to CON group: **P < 0.01; Contrast to CUMS group: $^{\#\#}$P < 0.01.

rats had significant differences, as exhibited in Fig. 6. CUMS could significantly increase the concentrations on CRH, ACTH and CORT in serum (P < 0.01); this was reversed with treatment of FLU, L-CLD, *M*-CLD and H-CLD (P < 0.01).

3.5. CLD downgraded the protein levels of NR1, NR2A and NR2B in the hypothalamus

The protein expression levels of NR1 ($F_{(5, 12)} = 12.37$, P < 0.01), NR2A ($F_{(5, 12)} = 28.83$, P < 0.01) and NR2B ($F_{(5, 12)} = 20.69$, P < 0.01) in the hypothalamus of each group were remarkably different, as exhibited in Fig. 7. In contrast to the CON group, the protein levels of NR1, NR2A and NR2B in the CUMS group were notably increased (P < 0.01). And the expressions of the above proteins in FLU, *M*-CLD and H-CLD group were significantly lower than that of the CUMS group (P < 0.01). However, the rats of the L-CLD group had no statistical differences in the expression of all proteins (P > 0.05).

3.6. CLD reduced the mRNA levels of NR1, NR2A and NR2B in the hypothalamus

The mRNA expression levels of NR1 ($F_{(5, 12)} = 682.47$, P < 0.01), NR2A ($F_{(5, 12)} = 298.07$, P < 0.01) and NR2B ($F_{(5, 12)} = 2159.02$, P < 0.01) in the hypothalamus of each group were remarkably different, as exhibited in Fig. 7. CUMS could lead to a remarkable increase in the mRNA levels of NR1, NR2A and NR2B (P < 0.01). Due to the intervention with FLU, L-CLD, *M*-CLD and H-CLD, the mRNA expression levels gradually tended to be normal (P < 0.01).

3.7. CLD downregulated the expression level of α2δ-1 in the hypothalamus

We detected α2δ-1 in hypothalamus of rats by immunofluorescence, Western blotting ($F_{(5, 12)} = 25.08$, P < 0.01) and RT-PCR ($F_{(5, 12)} = 76.32$, P < 0.01), as shown in Fig. 8. Among them, we used the positive expression area ($F_{(5, 12)} = 134.64$, P < 0.01) and integral optical density ($F_{(5, 12)} = 113.15$, P < 0.01) to evaluate its expression in immunofluorescence. Compared with the CON group, the integral optical density, positive expression area, protein and mRNA were significantly increased in CUMS rats (P < 0.01). After FLU and CLD treatment, the expression levels of the above indicators obviously decreased (P < 0.05 or P < 0.01), except for the protein expression of α2δ-1 in the L-CLD group (P > 0.05).

3.8. CLD decreased the complexes of α2δ-1/NR1 in the hypothalamus

The effect of CLD on α2δ-1 and NR1 interactions was examined using CO-IP, as shown in Fig. 9. The complexes of α2δ-1 and NR1 were significantly different in each group ($F_{(5, 12)} = 21.92$, P < 0.01). In contrast to the CON group, the protein level of complexes of CUMS rats was notably elevated (P < 0.01). However, the protein expression levels of the complexes were significantly reduced in rats after administration of FLU and CLD by gavage (P < 0.01).

4. Discussion

This research indicated that CLD could improve depression-like behaviors caused by CUMS, as reflected mainly in a raised index for sucrose preference, reduced immobile time in FST and TST, enhanced autonomic motor ability in OPT of CUMS rats. CLD also inhibited HPA axis hyperactivity in a depressed state, reducing CRH, ACTH and CORT levels. In addition, CLD could downregulate α2δ-1 and NMDAR expression in the hypothalamus and reduce the protein level of α2δ-1/NMDAR complexes.

Chronic exposure to uncontrollable and unpredictable life stressors was often considered a major contributing factor to the development of depression [36]. It was also an important basis for the use of the CUMS model in our study. Previous research had shown that CUMS-induced depression in rat models was accompanied by other disorders, but it had been constantly improved [37]. The CUMS model could simulate the main core symptoms of depressed patients and was extensively used in related research fields, which had extremely high authority and recognition [38,39].

We evaluated the effect of the CUMS depression model and the effect of CLD by weight, SPT, FST, TST and OFT. The weight change caused by loss of appetite and eating imbalance was one of the criteria for depression [40]. During the CUMS procedure, the weight of CUMS rats was lower than that of other groups. Preceding research confirmed that CUMS stimulation led to weight loss or slow growth due to loss of appetite in rats [41]; this was suppressed after CLD treatment in our study, especially in the H-CLD group. SPT was the primary choice for evaluating anhedonia, which was a significant clinical characteristic of depressed patients [42]. The FST and TST were used to detect behavioral despair states in models of depression; they had become more professional and widely used experimental methods in related research fields [43]. OFT could reflect the animal's ability to move autonomously in an unfamiliar environment, the desire to explore the central area and the sense of mental anxiety in addition to quantitatively evaluating the depressive state of rats [44]. CUMS reduced the rate of sucrose preference in SPT in rats [45]; the same performance was obtained after we exposed rats randomly to the stressor. We also found that the duration of inactivity in rats was significantly prolonged in FST and TST performed at the end of the CUMS procedure. The autonomous activity distance and exploration of CUMS rats in the focal area were noticeably reduced in OFT; this was the same as in preceding reports and suggested that the CUMS model was successfully established [46,47]. And the decrease of autonomic activity in OFT of CUMS rats might also be one of the important reasons for the prolongation of immobility time in FST and TST. By analyzing and organizing the experimental data, we found that after the intervention with CLD, the performance of CUMS rats in the above behavioral experiments was reversed, which also confirmed that CLD had a relatively significant ameliorating effect on depression-like behaviors. At the same time, this improvement effect was drug concentration-dependent.

The hypothalamus was the key brain region regulating mood disorders, and the HPA axis under its influence was essential for maintaining endocrine homeostasis and the feedback device for the body to reply to stress [48,49]. Abnormal HPA-axis activity was an inevitable reaction of the organism to CUMS provocation, which was involved in the pathophysiological changes of depression (Wang et al., 2020). The elevated levels of CRH and its regulated ACTH and CORT were important markers of HPA axis hyperactivity [50]. Previous research confirmed that the excessive release of CRH was an important cause of depressive symptoms, which mediates the regulation of mood disorders and stress responses [51]. We similarly found overexpression of CRH in the serum and hypothalamus. After our administration of CLD, there was a significant reduction in CRH expression. Implementation of CRH knockout could inhibit the synthesis of excitatory transmitters including catecholamine induced by stress [52]. Therefore, we speculate that CLD had the potential to inhibit excitatory neurotransmitters. In addition, ACTH and CORT downstream of CRH were often used in the assessment and analysis of clinical depression in patients with the condition [53]. Clinical reports suggest that ACTH levels could predict severity and even persistent symptoms in patients with depression [54]. Over-injection of CORT can also cause neuronal damage and induce depression-like behaviors in mice [55]. Our experimental data proved that CUMS did upregulate the serum ACTH and CORT levels of rats, which was identical to the preceding report [56]. Also, the serum levels in CLD-administered rats were just the opposite, which suggested that CLD could improve the abnormalities of the HPA axis caused by CUMS. From the above findings, we speculated that the HPA axis might be one of the important pathways through which CLD ameliorated CUMS-induced depression-like behaviors.

Sustained hyperactivity of the HPA axis under stress could lead to the release of Glu-based excitatory neurotransmitters [57]. The large accumulation of Glu in the synaptic gap would stimulate the over-activation of NMDAR, resulting in the inward flow of calcium ions, which induced neurotoxicity and neuronal dysfunction [58]. NMDAR participated in the adjustment of the excitability of the central nervous system by mediating Glu input, which acted as a key step during the depression-causing process of CUMS [59]. The improvement of neurological dysfunction caused by the imbalance of Glu expression was achieved mainly through the modulation of NMDAR [60]. NMDAR possesses several functional subunits, including NR1, NR2 and NR3, of which NR1 and NR2 were considered to be the basic structures of the functional organization of NMDAR and often used as binding sites for the receptor in physiological and pharmacological studies [61]. A prior study had shown that ethanol could induce conformational changes in the amino-terminal structural domain of NR1, which in turn affected NMDAR function and produced antidepressant traits [62]. Phosphorylation sites on the NR2A subunit could affect NMDA receptor-mediated downstream signaling and depression-related behavior [63]. NR2B, an important target for protecting brain cell function, could also mediate stroke-induced brain tissue damage [64].

NMDAR-targeted blockers were emerging agents in the clinical treatment of depression [65]. In our research, we detected a significantly elevated expression of NR1, NR2A and NR2B in the hypothalamus of CUMS rats, which was in perfect agreement with the prior findings [66]. We also confirmed that CLD inhibited the CUMS-caused abnormal increase in the above key subunit proteins, possessing a function similar to that of NMDAR antagonists. Previous research had proven that the activity of the HPA axis could be regulated through the expression of NMDAR under stress conditions [67]. These manifestations also indicated that the effect on NMDAR might be the significant pathway for CLD to suppress HPA axis hyperactivity in CUMS rats. However, the pathway through which CLD achieved regulation of NMDAR expression and activity was not yet known.

α2δ-1 was abundantly expressed in several brain regions, such as the hypothalamus and hippocampus, with a functional bias toward excitatory neurons [68]. Previous research had shown that the upregulation of α2δ-1 could increase Glu secretion by affecting NMDAR in neurons [69]. Also, the overexpression of α2δ-1 was necessary for NMDAR hyperactivity in the hypothalamic paraventricular nucleus in the sympathetic vasodilatory activity of hypertension [70]. Neuronal damage and apoptosis caused by NMDAR overexpression were effectively alleviated after knockdown of α2δ-1 [71]. In our study, we found that the distribution and expression of α2δ-1 in the hypothalamus were significantly increased in the depressed state. Therefore, we conjectured that the modulatory effect

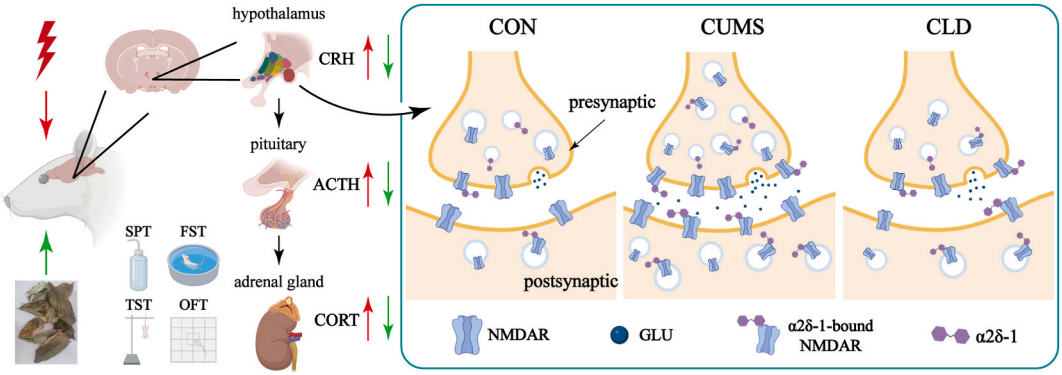

Fig. 10. The effect of CLD on CUMS-induced HPA axis hyperactivity and depression-like behaviors based on the regulation of α2δ-1 and NMDAR expression. CLD can reduce NMDAR expression by downregulating α2δ-1 as well as its level in complex with NR1, thereby suppressing HPA axis hyperactivity and alleviating CUMS-induced depression-like behaviors.. (Some of the materials in the figure are from *app. biorender.com*.)

of α2δ-1 on NMDAR expression and activity was also applicable to depressive states.

In chemotherapy-induced neuropathic pain, increased α2δ-1 and NMDA complexes promoted Glu input in spinal dorsal horn neurons [72]. The complex of α2δ-1 and NMDAR also participated in the adjustment of cognitive function [73]. The α2δ-1-NMDAR complex mediated by α2δ-1 became an important target for regulating NMDAR activity and expression. Our experimental data indicated that CUMS could lead to an increase in the complex of α2δ-1 with NR1. Therefore, we presumed that CUMS could increase the binding capacity of α2δ-1 and NR1 and that the regulation of NMDAR by α2δ-1 might also be achieved by affecting the combination of both in depressed states to some extent. We verified this hypothesis in the next study using α2δ-1 knockout rats. Yet, we also found that the α2δ-1 and NR2 complexes did not show significant upregulation in the depressed state in the pre-experiment, which suggested that the binding capacity of α2δ-1 and NR2 was not enhanced in the depressed state, while this was in contrast to their expression in neuropathic pain [74]. Therefore, we also speculated that NR1 was the main target of α2δ-1-mediated NMDAR in the depressed state, but not NR2, which we would explore in depth again, adopting more technical methods.

Furthermore, after our intervention with CLD administration, the expressions of α2δ-1 and its complex with NR1 were reversed. Therefore, we inferred that CLD might interfere with the binding of α2δ-1 to NR1 by down-regulating the expression of α2δ-1, thereby inhibiting the expression and activity of NMDAR. Previous research reported that antagonism α2δ-1 expression could inhibit the activity of NMDAR without impairing its physiological function, which was superior to pure unidirectional antagonists of NMDAR [75]. From this perspective, CLD seemed to be more advantageous in the clinical management of depression, and antidepressants targeting α2δ-1 might be a new direction for the clinical treatment of depression.

5. Conclusions

In summary, we found that CLD could reduce CUMS-induced depression-like behaviors by inhibiting the hyper-excitability of HPA. We also presumed that this effect might be related to the fact that CLD could downregulate α2δ-1 and the complexes of α2δ-1 to NR1, ultimately inhibiting the activity and expression of NMDAR, as shown in Fig. 10. The above experimental discoveries were still in the preliminary exploration stage, and we would further verify and discuss it utilizing more experimental means in the next study. We believed that these findings could provide new prevention and treatment strategies for clinical research on depression, as well as new targets and research directions for the development of antidepressant drugs.

Ethics statement

The animal experiments in this research strictly adhered to the Codes of Animal Experiments from the Committee of Medical Ethics, National Health Department of China and had been ratified by the Ethics Committee for Animal Experiments of Hebei University of Chinese Medicine. Reference number **DWLL202202010.**

Statement

There are no funds, grants and no conflicts of interest or anything that could be considered a potential conflict of interest for this study.

CRediT authorship contribution statement

Chuan Jiang: Writing – original draft, Methodology, Conceptualization. **Chaonan Wang:** Validation, Investigation, Formal analysis. **Weizhong Qu:** Visualization, Software. **Yuanyuan Wang:** Validation, Methodology. **Hua Wang:** Investigation. **Xin Wei:**

Software. **Mingyan Wang:** Methodology. **Qianqian He:** Formal analysis. **Yihan Wang:** Investigation. **Lirong Yuan:** Supervision, Resources, Project administration, Data curation. **Yonggang Gao:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funded by key project of Traditional Chinese Medicine Joint Fund of Hebei Natural Science Foundation (H2022423381), the Project of Hebei Natural Science Foundation (H2020423004), the Project of advancement of Science and Technology Capability of Hebei University of Chinese Medicine (KTY2019051), the Special Project of Basic Scientific Research Operation Fee for Provincial Colleges and Universities of Hebei University of Chinese Medicine (JCYJ2021001), the Science and Technology Project of Hebei Education Department (QN2022070; QN2022006) and the National Innovative Training Program for College Students (202214432007).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21743.

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Abbreviations

ACTH: adrenal cortical hormone
CLD: cherry leaf decoction
CORT: corticosterone
CO-IP: co-immunoprecipitation
CRH: corticotropin releasing hormone
CUMS: chronic unpredictable mild stress
FLU: Fluoxetine;
FST: forced swimming test
Glu: glutamate
HPA: hypothalamic pituitary adrenal
NMDAR: n-methyl-*d*-aspartate receptor
OFT: open field test
RT-PCR: reverse transcription-polymerase chain reaction
SPT: sucrose preference test
TST: tail suspension test
VGCCs: voltage-gated calcium channels