STRESS INDUCED NEURAL CHANGES IN EMOTIONAL DISORDERS

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Editorial: Stress Induced Neural Changes in Emotional Disorders

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Editorial on the Research Topic

Stress Induced Neural Changes in Emotional Disorders

Psychological processes include two equally important aspects of mental processes: cognition and emotion, emotional disorders might account for more than 90% of mental disorders. For example, major depression is a prevalent emotional disorder that affects more than 1/5 of the populations worldwide, making it one of the most prevalent health-related causes of human suffering. Moreover, it is a leading risk factor for the estimated one million deaths by suicide per year world-wide. Despite that emotion is critically important to us, emotion is one of the least studied biological phenomena of the brain, the mechanism of the emotional disorders is not clear. Thus, the treatment is not as effective as expected, which might be effective in only a subset of patients and acts slowly (1).

Stress has been regarded as a critical causing factor for emotional disorders. Stress is an evolutionarily adaptive response to deal with situations that impact threat to the organism and require rapid "flight or fight" responses (2). Stress is evolutionarily important for survival and benefits to all lives, however, overwhelming stress is considered to one of the main risk factors for the development of many emotional disorders such and anxiety, depression. For example, the onset of major depression are often correlated with stressful events in earlier lives, many studies reported significant correlation between the onset of major depression and the number of traumatic events within 3 months before the onset of the disease. In addition, stress can happen very long ago, for example, early life stress can induce emotional depression in adult lives (3). This means stress can induce long term changes in the body to induce emotional disorders.

The stress induced neural changes are very complicated. There is compelling evidence of a causal link between chronic stress and the sympathetic system as well as the HPA axis (hypothalamus-pituitary-adrenal axis) and emotional disorders. Stress causes elevation of corticosteroids and other stress hormones, such as CRF (corticotropic-releasing factor), the known stress hormone. HPA dysregulation may be both a causative factor and a consequence of emotional disorders. In addition to hypothalamus, the locus Coerleus (central norepinephrine) also plays an important role in stress. The major function of norepinephrine (NE) system has been known as "fight or flight," or "fear or anger" emotions (4). As we reported before, everything around us happens in an anticipated way (expected) or not anticipated way (surprising). If it happens as expected, people feel calm; if it happens unexpected, people will feel scared or angry (5). Therefore, chronic unpredictable stresses are most often used in animal models of depression. When the animals are faced with stress, they often respond with fear or anger emotions, and "fight or flight" responses trying to get rid of these stressors. After a long term of failure to deal with these stressors, they learned the "helplessness" and try to live with these stressors sadly. We say that these animals are depressed and the depression model is set up.

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Furthermore, stress can induce long term changes in the endocrine systems, which in turn induce behavior changes that habitually deal with the stressful situations, thus cytokine is also regarded as a causing mechanism for depression. Lymphocytes, mast cells and other immune system cells contain adrenoreceptors that respond to the peripheral release of NE. The central NE system innervates many vital parts of the immune system- the lymph organs, spleen, thymus. It is reported that lymphocyte activity was dramatically reduced in the depressed patients. There is evidence that sympathetic nervous, immune and endocrine systems are linked. Moreover, some epigenetic changes or neural changes induced by early life stress might be involved in the emotional disorders. The expression of all receptors for previous neuromodulators, neurotransmitters, cytokines can be affected by epigenetics. These changes have been suggested to be involved in the emotional diseases.

In 2017, we proposed one topic "Stress Induced Neuroplasticity and Mental Disorders" for Neural Plasticity and got 41 submissions. In 2018, we proposed one topic titled "Early life stress and depression" for Frontiers in Psychiatry and got 42 submissions. This is a fast developing topic, therefore, here we continue to propose a similar topic to collect recent studies about the mechanisms of stress inducing emotional disorders, such as phobia, depression and anxiety. In this special issue, we received 27 submissions and got 18 papers accepted.

In the review paper, titled "The Locus Coeruleus-Norepinephrine System in Arousal: Unraveling Historical, Current and Future Perspectives," the authors Ross and Van Bockstaele from Drexel University gave a detailed review about the arousal function of locus-coeruleus (LC)-NE system. They discussed technological advancements that chronologically led to our current understanding of the arousal system. The paper was reviewed by professors Leszek Kubin from University of Pennsylvania, and Shahzad Khan from Stanford University, who gave a high praise for this paper: "A strength of this review is its detailed and thorough overview of stress and arousal research." "To cover this broad topic based on several subfields of neuroscience that, traditionally, developed along their own separate paths is an ambitious undertaking. The authors have largely succeeded in producing a text that has superior educational value."

In an experimental paper, author Yang et al. from Zhejiang University, one of most famous universities in China, identified some specific genetic targets for the diagnosis and treatment of depression. They screened 325 differentially expressed genes, with 42 genes down-regulated, while 283 genes up-regulated in hippocampal tissue. The results showed a significant change in Wfs1 after chronic stress stimulation, and they suggested that Wfs1 can be used as a prognosis and treatment target for depression. The paper is titled "Wfs1 and Related Molecules as crucial Biomarkers in the Hippocampus of Depression."

In the experimental paper, titled "Childhood maltreatment and depression in adulthood in Chinese female college students: the mediating effect of coping style," Zheng et al. investigated the mediating effect of coping style in depression in adulthood among Chinese female college students. They used self-report questionnaires assessing childhood maltreatment, depression and coping style among 738 participants, and the results illustrated that childhood maltreatment could influence adult depression through the mediating role of coping style.

In the review paper titled "Autophagy-based hypothesis on the role of brain catecholamine response during stress," the authors Limanaqi et al. investigated stress induced changes in the homeostatic balance of the catecholamine brain systems, including norepinephrine (NE)- and dopamine (DA)-containing neurons within the locus coeruleus (LC) and ventral tegmental area (VTA), which are readily and similarly activated by psychostimulants. They found that brain catecholamine response to stress results in time-dependent regulatory processes involving mesocorticolimbic circuits and networks, where LC-NE neurons respond more readily than VTA-DA neurons. In this mini-review, they also discussed the role of autophagy in brain catecholamine response to stress and those factors which may lead to their dysregulation. They suggested that autophagy plays a crucial role in the response and adaptation of LC-NE and VTA-DA systems to stress.

In the experimental paper "Electroacupuncture alleviates cerebral ischemia-reperfusion injury in rats by histone H4 lysine 16 acetylation-mediated autophagy," the authors Xu et al. used acupuncture to treat animals, and found that acupuncture can change the autophagy via the reduction in lysine 16 of histone H4 acetylation. Their finding offer some epigenetic suggestion for stress induced depression, that regulating histone H4 lysine 16 acetylation-mediated autophagy may be a key mechanism for stress induced depression.

Maternal separation (MS) has been commonly used as a rodent model to identify the developmental effects of child neglect. In the paper "Early life neglect alters emotional and cognitive behavior in a sex-dependent manner and reduces glutamatergic neuronal excitability in the prefrontal cortex," Sun et al. examined the impact of early-life stress on glutamatergic neuronal excitability in the prefrontal cortex. Rats were separated from the mom for 4 h per day on postnatal days 2–5 and for 8 h per day on postnatal days 6–16 and then weaned on day 17. The results showed that maternal separation reduced the number of glutamatergic neuron activities in male rats, and induced anxiety-like behavior, a passive coping strategy and increased fear memory in male rats and decreased locomotor activity in both sexes. In addition, maternal separation also slightly impaired working memory during non-stressful situations in female rats but did not change spatial reference memory or associative learning under stressful circumstances in either sex.

In another paper, which was titled "The effect of early maternal separation combined with adolescence chronic unpredictable mild stress on behavior and synaptic plasticity in adult female rat," the authors Huang et al. evaluated the depression model of early MS combined with CUMS in female adult SD rats, and measured synaptophysin (SYN), postsynaptic density-95 (PSD-95) and growth-associated protein-43 (GAP-43) expressions in hippocampus were detected by western blot. The results showed that rats in MS+CUMS group presented more serious depression-like and anxiety-like behavior than in MS group. In addition, few Nissl bodies in the hippocampus CA1 and

DG regions, less percentage of SYN-positive cells and down-regulated expressions of SYN, PSD-95, and GAP43 were found in hippocampus of rats in MS+CUMS group. They concluded that adult female rats that had undergone MS and CUMS performed more critical depression-like and anxiety-like behaviors, and this process may be resulted from synaptic plasticity impairing.

An experimental animal study, which was titled as "The differential role of cytokines on stress responses in ovariectomized female rats," investigated the mechanisms for menopause induced anxiety and depression. The authors Park et al. applied stress to ovariectomized rats, and found that ovariectomy and stress combine to increase the depressive like behaviors and neuro-inflammatory responses. In addition, they show neuroinflammation as a potential contributor to depressive like symptoms during menopausal transition.

In the experimental paper, Zhang et al. studied another kind of sex related depression, premenstrual dysphoric disorder, which is a common mental health disturbance associated with several periodic psychological symptoms in women. The paper was titled "Paeonol at Certain Doses Alleviates Aggressive and Anxiety-like Behaviors in Two Premenstrual Dysphoric Disorder Rat Models." Selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment for PMS/PMDD patients; however, side effects are inevitable, especially in long-term treatment. In this study, they used a kind of natural compound paeonol, and found it to be effective in central nervous system disorders with its anti-inflammatory, anti-oxidant, and neuroprotective effects.

In the review paper, "Gender differences in depression: evidence from genetics," Zhao et al. compared gender difference for depression in aspect of behavioral genetics. They found that gender differences exist in heritability and the gene associated with depression after reviewing relevant research. Both genes and gene-environment interactions contribute to the risk of depression in a gender-specific manner. They talked about the relationships between serotonin transporter gene-linked promoter region (5-HTTLPR) and depression in great detail.

The experimental paper, title "Perceived stress and life satisfaction among Chinese clinical nursing teachers: A moderated mediation model of burnout and emotion regulation," investigated the underlying mechanisms of how and when perceived stress increased the risk of burnout and decreased life satisfaction among clinical teaching nurses. They used questionnaires about perceived stress, burnout, emotion regulation and life satisfaction were self-administered among 1,372 teaching nurses from eight tertiary military hospitals in China. The results revealed that perceived stress had direct and indirect impacts on life satisfaction, with the principal element of burnout- emotional exhaustion acting as a mediator. Moreover, the association between perceived stress and emotional exhaustion was moderated by emotion suppression- a key emotion regulation strategy.

In the interesting study, the authors Song et al. investigated the effects of a kind of Chinese herb on depression. The paper is titled "Effects of Xiaoyaosan on Depressive-Like Behaviors in Rats with Chronic Unpredictable Mild Stress through HPA Axis Induced Astrocytic Activities." They investigated the behavioral changes of chronic unpredictable mild stress (CUMS), the expression of corticosterone of the HPA axis, the expression of Glutamate receptor and astrocytes glial fibrillary acidic protein in the hippocampus. The study proved that this kind of Chinese herb affects depression by affecting the HPA axis and glutamate recycling.

In another paper, "Effect of Jian-Pi-Zhi-Dong Decoction on the amino acid neurotransmitters in a rat model of Tourette Syndrome and Comorbid Anxiety Disorder," the authors Zhang et al.studied another kind of Chinese herb with depression. They observed mitochondrial changes with transmission electron microscopy, and they also measured the content of Glutamate and γ-aminobutyric acid (GABA) both in the serum and striatum and the expression of their receptors by western blot and real-time polymerase chain reaction. The study revealed that the Chinese herb was effective in ameliorating the severity of behavioral symptoms of anxiety via increase in GABA levels and decreases in glutamate levels in the serum.

In one more paper, which was titled "Effect of the ZiBuPiYin recipe on diabetes-associated cognitive decline in Zucker diabetic fatty rats after chronic psychological stress," Bi et al. examined the effect of another kind of Chinese herb ZiBuPiYin recipe on Zucker diabetic fatty rats and explored the impact of chronic stress on altered β-amyloid (Aβ) metabolism through insulin receptor substrate (IRS) 1/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway after the induction of chronic psychological stress. They found that the herb treatment significantly decreased anxious-like behaviors and plasma corticosterone levels, and ameliorated learning and memory impairments of the rats after chronic psychological stress. So they suggested that the herb may be a potential therapeutic application for the treatment of depression induced by chronic psychological stress.

Inflammation has recently been found to be a causing factor for depression, in the experimental paper "Changes of serum melatonin, interleukin-6, homosysteine, complement C3 and C4 levels in patients with depression," Tao et al. investigated the changes in serum concentrations of melatonin, interleukin-6 (IL-6), homocysteine, complement C3, and C4 in depression patients and relationships of them with depression activity. The results suggest that higher serum MT, IL-6, and hcy levels were correlated with pathogenesis of depression.

Similarly, in another paper, titled "Susceptibility to hyperglycemia in rats with stress- induced depressive-like behavior: involvement of IL-6 mediated glucose homeostasis signaling," Li et al. also studied the role of inflammation in the pathogenesis of depression and insulin resistance. The results showed that CUMS exposure resulted in the depression-like behavior at various time points in rats. Moreover, the rats exhibited increased peripheral glucose levels, impaired hepatocytes and hippocampal neurons, and decreased hypothalamic GLUT4 levels after 6 week of CUMS exposure. The experiment also showed that 6-week CUMS stimulation also induced susceptibility to hyperglycemia, which is associated with IL-6-mediated inhibition of glucose homeostasis signaling in the hypothalamus.

In the paper "SGK1 (serum-and glucocorticoid-regulated kinase 1): into a straight line for the major depressive disorder,"

Dattilo et al. reviewed the expression of both the brain-derived neurotrophic factor (BDNF) and the vascular endothelial growth factor (VEGF), and their effects in neurogenic activity. In addition, they reviewed studies about SGK1 on the expression of BDNF and VEGF, trying to depict SGK1 as molecular junction of the complex mechanisms underlying the MDD, in the effort to suggest the kinase as a potential biomarker and strategic target in a modern molecular antidepressant therapy.

In the experimental paper "Repeated nitrous oxide exposure exerts antidepressant-like effects through neuronal nitric oxide synthase activation in the medial prefrontal cortex," the authors Liu et al. also investigated the neurogenesis effects of N_2O on BDNF, and suggested that the antidepressant-like effects of N_2O work through enhancing BDNF expression levels, which might underlie the pharmacological mechanism of the antidepressant-like effects of N_2O exposure.

Taken together, several pathogenic hypotheses, such as monoamines deficiency and neurobiological alterations in the stress-responsive system, including the HPA axis, monoamine system and the immune system, have been proposed for MDD, recently, many studies point to neurogenesis (6). In this special issue, these 18 papers investigated stress induced depression-related behaviors, offering some new mechanisms, such as VEGF changes, BDNF changes, which might be modulated by N_2O or SGK1. We hope these papers will shed some new light in the field of stress and emotional studies.

AUTHOR CONTRIBUTIONS

FW and JH did the writing. FP and YT helped with the revision. All authors contributed to the article and approved the submitted version.

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Paeonol at Certain Doses Alleviates Aggressive and Anxiety-Like Behaviours in Two Premenstrual Dysphoric Disorder Rat Models

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Premenstrual dysphoric disorder (PMDD) is a severe form of premenstrual syndrome (PMS), a common mental health disturbance associated with several periodic psychological symptoms in women. Selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment for PMS/PMDD patients; however, side effects are inevitable, especially in long-term treatment. In previous studies, the natural compound paeonol in Moutan Cortex was found to play effective roles in central nervous system disorders with its anti-inflammatory, anti-oxidant, and neuroprotective effects. Consequently, we assume that paeonol might produce positive effects in the treatment of PMS/PMDD. In this study, the open-field test (OFT) and elevated plus maze (EPM) and light dark box (LDB) tests were performed in mice to determine the optimal dose of paeonol for treating anxiety. Then, paeonol was used to treat the progesterone withdrawal (PWD) and resident intruder paradigm (RIP) rat models of PMDD. Using these two reliable models, the OFT and EPM, LDB, and composite aggressive tests were performed to evaluate the effect of the drug on behavioural symptoms of PMDD. From the dosage screening results, the optimal anti-anxiety dose of paeonol was identified as 17.5 mg/kg/d for 7 days. With regard to the effect of paeonol on PMDD rat models, a significantly improvement was found in the behavioural symptoms, but the effective dose varied in different models. For the PWD model rats, treatment with 6.05 mg/kg paeonol could significantly improve anxiety and irritability, while that with 24.23 mg/kg paeonol resulted in anxiety-like effects in behavioural tests. In RIP model rats, treatment with 12.11 mg/kg paeonol demonstrated excellent effects in improving anxiety, particularly irritable emotional behaviour. In conclusion, our study indicates that paeonol is a potential therapeutic compound for PMS/PMDD; it is a drug option that helps establish dosage guidance for treatment of this condition.

Keywords: paeonol, premenstrual dysphoric disorder, rat models, resident intruder, progesterone withdrawal

INTRODUCTION

Women experiencing premenstrual syndrome (PMS), one of the most common mental health disturbances, have several characteristic psychological symptoms including anger, irritability, depressed mood, and anxiety (1). A severe form of PMS, afflicting up to 5–8% of patients, was termed as premenstrual dysphoric disorder (PMDD) in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders. In women with PMDD, symptoms occur during the luteal phase of each menstrual cycle and disappear by the end of menstruation.

In clinical trials, the first-line treatment for PMS/PMDD patients is antidepressants in the form of selective serotonin reuptake inhibitors (SSRIs), such as clomipramine, escitalopram, fluoxetine, sertraline, and paroxetine (2, 3). SSRIs have played a significant role in reducing both mood symptoms and somatic complaints; thus, they have been considered as the gold standard in the treatment of this disorder (4, 5). However, a re-evaluation published in a journal, *CNS Drugs*, in 2006 found that although SSRIs were more effective in PMDD than a placebo, the response rate was less than 60%, which was far from satisfactory (6). In addition, side effects like nausea and headache are common, particularly in long-term treatment (7). A recent report (8) indicated that short-term low dose fluoxetine could prevent anxiety-like behaviour related to the oestrous cycle in female rats; however, evaluation of its effect on depression and irritability is lacking. Therefore, there is an urgent need to develop new drug treatment methods to improve the therapeutic management of PMS/PMDD. Traditional Chinese medicine has shown remarkable efficacy in the treatment of PMDD (9). The natural compound paeonol is an active constituent of Moutan Cortex. It has been reported that paeonol has a neuroprotective effect by the inflammatory processes mediated by microglial activation (10, 11) and plays a role in central nervous system disorders including depression (12, 13). Based on this evidence, we assume that paeonol might produce positive effects in the treatment of PMS/PMDD.

As reported, long-term exposure to exogenous progesterone along with its abrupt withdrawal has been a reliable method for establishing a rodent PMDD model. The use of progesterone withdrawal (PWD) protocols sufficiently induces depression and a negative mood during the luteal phase (14–17). Besides, resident intruder paradigm (RIP) is a common method used to establish a PMDD rat model. By performing ovariectomy and providing exogenous hormone supplementation, the normal cyclical release of hormones was mimicked. The cycle-dependent aggressive behaviours (irritability and anger) were elicited by introducing an intruder rat into a resident rat cage, which has been confirmed in several reports (18–20).

In this study, the open-field test (OFT) and elevated plus maze (EPM) and light dark box (LDB) tests were initially performed in mice to determine the optimal dose of paeonol for treatment of anxiety. Then, paeonol was used to treat rats subjected to PWD and RIP stress. To evaluate the drug effect precisely using these two recognised and reliable PMDD models, a series of behavioural assessment tests was performed including the OFT and EPM, LDB, and composite aggressive tests.

Abbreviations: CE, closed arm entry times; CMC-Na, sodium carboxymethyl cellulose; CT, time in the closed arm; EPM, elevated plus maze; LDB, light dark box; OE, open-arm entry times; OFT, open-field test; OT, time in the open arm; PMDD, premenstrual dysphoric disorder; PMS, premenstrual syndrome; PWD, progesterone withdrawal; RIP, resident intruder paradigm; SSRIs, selective serotonin reuptake inhibitors.

MATERIALS AND METHODS

Ethical Approval

All experiments were approved by the ethics review board of Shandong University of Traditional Chinese Medicine (No. DWSY201703013) and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Subjects

To determine the optimal dose of paeonol for the treatment of anxiety, C57BL/6J male mice (6–8 weeks old, weighing 18–22 g) were used in the OFT and EPM and LDB tests. Then, the PMDD-PWD model and the resident intruder model of PMDD were established to evaluate the drug effect on female Wistar rats (6–7 weeks old, weighing 140–160 g). All animals were purchased from the Vital River Laboratories (Beijing, China) and housed at 21 ± 1°C and 55% relative humidity under a 12:12 h light/dark cycle with food and water available *ad libitum*. The animals were habituated to maintenance conditions for 1 week and handled daily to eliminate the human factor.

Experimental Design and Drug Treatment Regimen

In the dosage screening against anxiety, mice were randomly divided into seven groups: one control group (received saline with 0.5% sodium carboxymethyl cellulose [CMC-Na]), five paeonol groups (received 8.75, 17.5, 35, 70, and 140 mg/kg/d of paeonol, respectively), and one positive control group (received 2 mg/kg/d of diazepam). Paeonol (purity > 98%, from the pharmaceutical laboratory of Shandong University of Traditional Chinese Medicine) and diazepam (Jichuan Pharmaceutical Group Co., Ltd., H32021083) used for the positive control group were dissolved in saline-0.5% CMC-Na. All drugs were administered through gavage once daily at approximately 9:00 am for 7 consecutive days. The behavioural tests were conducted after the final administration. To make the results more accurate, each mouse was used for only one behavioural test, and the other studies were conducted in separate cohorts.

In order to determine the optimal dose of paeonol for the treatment of anxiety, the PMDD-PWD model and the resident intruder model of PMDD were used in the pharmacodynamic evaluation. The rats were divided into six groups: one control group (normal rats received saline), one PMDD group (PMDD rats received saline), three paeonol groups (each PMDD rat received low, moderate, and high doses of paeonol), and one

positive control group (PMDD rat received 2.7 mg/kg/d of fluoxetine). As fluoxetine is a first-line regimen used in the clinical treatment for PMDD, which is different from the anti-anxiety drug, diazepam used in the dosage screening experiment as previously mentioned, we used fluoxetine in the positive control group to be consistent with the clinical treatment. Based on the optimal dose of paeonol against anxiety in mice, the dose used in PMDD rats was calculated as follows according to the third edition of the Pharmacologic Experimental Methodology (Editor-in-chief: Shuyun, People's Medical Publishing House, Beijing 2002) and dissolved in saline with 0.5% CMC-Na: moderate dose=optimal dose in mice/9.1×6.3; low dose=moderate dose/2; high dose=moderate dose×2. Fluoxetine (Lilly Suzhou Pharmaceutical Co. Ltd., J20160029) was dissolved in saline-0.5% CMC-Na. All drugs were administered intragastrically once daily at approximately 9:00 am, for 16 consecutive days in the PMDD-PWD rat model and for 12 consecutive days in the resident intruder model of PMDD.

PMDD-PWD Rat Model

To induce the PMDD-PWD model (**Figure 1**), the rats were administered intraperitoneal progesterone (Sigma, 101327062) injections (6 mg/d for each rat, dissolved in saline) for 21 consecutive days. Then, they were subjected to PWD as it could reliably increase depression-like behaviour according to a previous study (16). The drug treatment (fluoxetine or paeonol) was started from the 8th day after the first progesterone administration to the day of the behavioural tests. The behavioural tests were conducted 48–72 h after the final progesterone administration. Each rat was used for only one behavioural test, and the other studies were conducted in separate cohorts.

Resident Intruder Paradigm Model of PMDD (PMDD-RIP)

A typical rat oestrus is divided into four phases: non-receptive phase (3 days, including metoestrus, dioestrus I, and dioestrus II) and receptive phase (1-day, pro-oestrus/oestrus). Ovulation occurs in the receptive phase. In order to bring the subjects into consistent oestrus, the ovary was removed and exogenous oestrogen was supplemented to induce the periodic oestrus (**Figure 2**). In the ovariectomy procedure, the rats were anaesthetised using an intraperitoneal injection of 2% pentobarbital sodium (60 mg/kg) and immobilised on the rat board under aseptic conditions. Then, bilateral ligation of uterine tubes and removal of ovaries were performed, and the incision was sutured. The subjects were allowed 7 days to recover from the surgery.

Seven days after the surgery, exogenous oestrogen was administered to induce the oestrous cycle according to the following regimen (18): at 11:30 am on the 8th day, 0.5 ug oestradiol benzoate (McLean Shanghai trading Co. Ltd., 10042617; dissolved in 0.1 ml saline) was injected subcutaneously; 32 h later, 0.5 ug oestradiol (McLean Shanghai trading Co. Ltd., 10006920; dissolved in 0.1 ml saline) was injected subcutaneously; 44 h later, 0.5 mg progesterone (dissolved in 0.1 ml saline) was injected subcutaneously. The exogenous oestrogen injection regimen was repeated five times with a 1-day interval. The first, second, and third days of exogenous oestrogen injection corresponded to the dioestrus I, dioestrus II, and receptive phase, respectively.

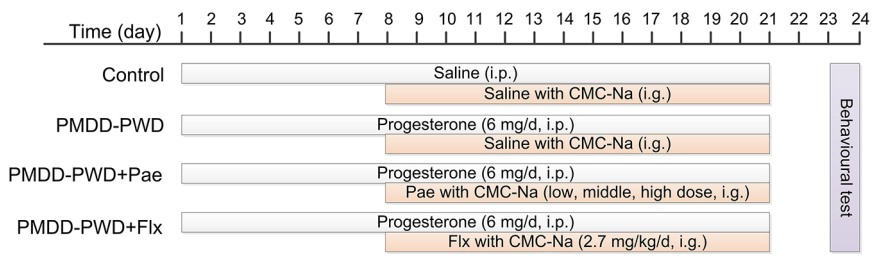

FIGURE 1 | Schedule for the progesterone withdrawal protocol, drug administration, and behavioural testing. PMDD-PWD, premenstrual dysphoric disorder-progesterone withdrawal; i.p., intraperitoneal injection; i.g., intragastric administration; CMC-Na, sodium carboxymethyl cellulose; Pae, paeonol; Flx, fluoxetine.

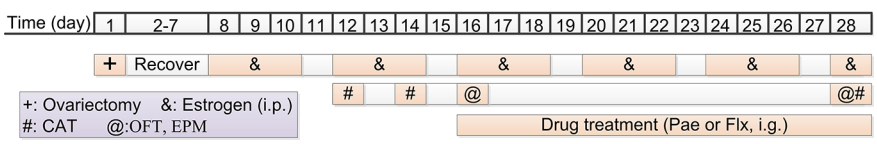

FIGURE 2 | Schedule for the resident intruder model of premenstrual dysphoric disorder (PMDD) drug administration and behavioural testing. i.p., intraperitoneal injection; i.g., intragastric administration; Pae, paeonol; Flx, fluoxetine; CAT, composite aggressive test; OFT, open-field test; EPM, elevated plus maze.

During the second injection of exogenous oestrogen, a composite aggressive test was performed during the receptive and dioestrus I (18, 21) phase. The test was conducted at 12:30–15:30 pm under a dim (< 2 lux) lighting condition. After 15 min of habituation of the resident rat, an ovariectomised stimulus female intruder was introduced into the resident's cage and this was video recorded for 10 min. During this period, we scored the aggressive behaviour (attacking, biting, and jumping at the intruder) (20). The composite aggressive score was defined as the attacking instances + 0.2×attacking time (s) + biting instances + 0.2×jumping time (s). Then, the animals were divided into different groups according to the value of the composite aggressive score in dioestrus I (12^{th} day) minus the score in the receptive phase (14^{th} day). The animals were sorted by their scores from high to low, and the first 30% were divided into the PMDD model groups (the PMDD group and paeonol groups) and the last 30% were divided into the control group. The rest of the animals were eliminated (20).

Open-Field Test

The animals were placed in a square apparatus (50 cm × 50 cm for mice and 100 cm × 100 cm for rats), with the arena divided into nine equal squares for 6 min. Each mouse or rat was placed individually into the centre and permitted free exploration. With the XR-Super Maze tracking system (Shanghai Xinsoft Information Technology Co. Ltd.), the total distance, central area distance, time at central area, and the instances of standing on the hind legs (rearings) were recorded during the test time (22). The arena was cleaned with 70% ethanol after every trial. Anxiety-like behaviour was defined when the animal spent more time near the edges of the box than at the centre.

Elevated Plus Maze

The EPM test was conducted using the XR-Super Maze tracking system and a polypropylene plastic cruciform apparatus that was elevated 76 cm above the floor. The cruciform box consisted of two open arms, two closed arms (30 cm × 5 cm for mice and 50 cm × 10 cm for rats), and a central platform (the junction area, 5 cm × 5 cm for mice and 10 cm × 10 cm for rats). Each animal was placed on the central platform with the head towards the open arm, and the behaviour was recorded within 5 min, including the open-arm entry times (OE), closed arm entry times (CE), time in the open arm (OT, s), time in the closed arm (CT, s), total distance, and distance in the closed arm. Then, the OE% and OT% were calculated as follows: OE%=OE/(OE+CE)×100%, OT%=OT/(OT+CT)×100%. Higher anxiety was indicated by a lower frequency of entry into the open arms and lesser time of stay (23). The arena was cleaned with 70% ethanol after every trial.

Light Dark Box

The light dark box consisted of two chambers of the same size (25 cm × 25 cm × 30 cm), with the dark and light compartments separated by a door (6.5 cm × 6.5 cm). The animals were placed in the centre of the light box and allowed to move freely between the two chambers within 5 min. A video tracking system (XR-Super Maze) was used to record the total distance, time spent in the light box, light box entry times, and the distance in the light box (24). As the rodents have an innate aversion to light areas and were allowed spontaneous exploration, the indexes previously mentioned could indicate their anxiety-like behaviour.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 7.0.4 software (GraphPad Software, Inc., San Diego, CA, USA). First, the Kolmogorov-Smirnov and the Levene tests were performed to test for normal distribution and homogeneity of variance. One-tailed unpaired *t*-tests were performed for two-group comparison, while one-way analysis of variance followed by Bonferroni's test was used to compare the differences among three or more groups. Data were presented as mean ± standard error of the mean. For all analyses, P values <0.05 were considered significant, and the level of significance was described as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

RESULTS

Dosage Screening of Paeonol Against Anxiety

To determine the appropriate paeonol dosage for the pharmacodynamic evaluation of PMDD animals, we first designed the experiment using mice treated with different dosages of paeonol. Specifically, in the OFT, as shown in **Figures 3A–D**, diazepam intervention decreased the time in the central area (p=0.0319) and rearing times (p=0.0004) compared with the control group, and 140 mg/kg/d of paeonol decreased the central area distance (p=0.0298) and time in the central area (p=0.0365). Then, in the EPM test (**Figures 3E–G**), the OE% of mice was increased by diazepam (p=0.0348) and 17.5 mg/kg/d paeonol (p=0.0363); diazepam also increased the total distance (p=0.0180). Lastly, in the LDB test (**Figures 3H–K**), diazepam increased the light entries (p=0.0248), light distance (p=0.0048), and total distance (p=0.0064), which confirmed its anti-anxiety effect; 17.5 mg/kg/d paeonol also increased the time in the light box (p=0.0450), light entries (p=0.0442), and light distance (p=0.0483). Furthermore, 35 mg/kg/d paeonol could increase the light distance (p=0.0376). Using the three classical anti-anxiety behavioural tests including the OFT, EPM, and LDB, the effects of the different paeonol dosages were evaluated, and the optimal anti-anxiety dosage was confirmed as 17.5 mg/kg/d.

Behavioural Identification of the PMDD-PWD Rat Model

In a manner similar to that of the dosage screening experiment previously mentioned, behavioural tests including the OFT, EPM, and LDB were also used in the behavioural identification of the PMDD-PWD rat model. In **Figure 4**, compared with control rats (n=12), PMDD-PWD rats showed decreased time in the central area (n=12, p=0.0326) and rearing number (p=0.0478) in the OFT (**Figures 4A–F**). In the EPM test,

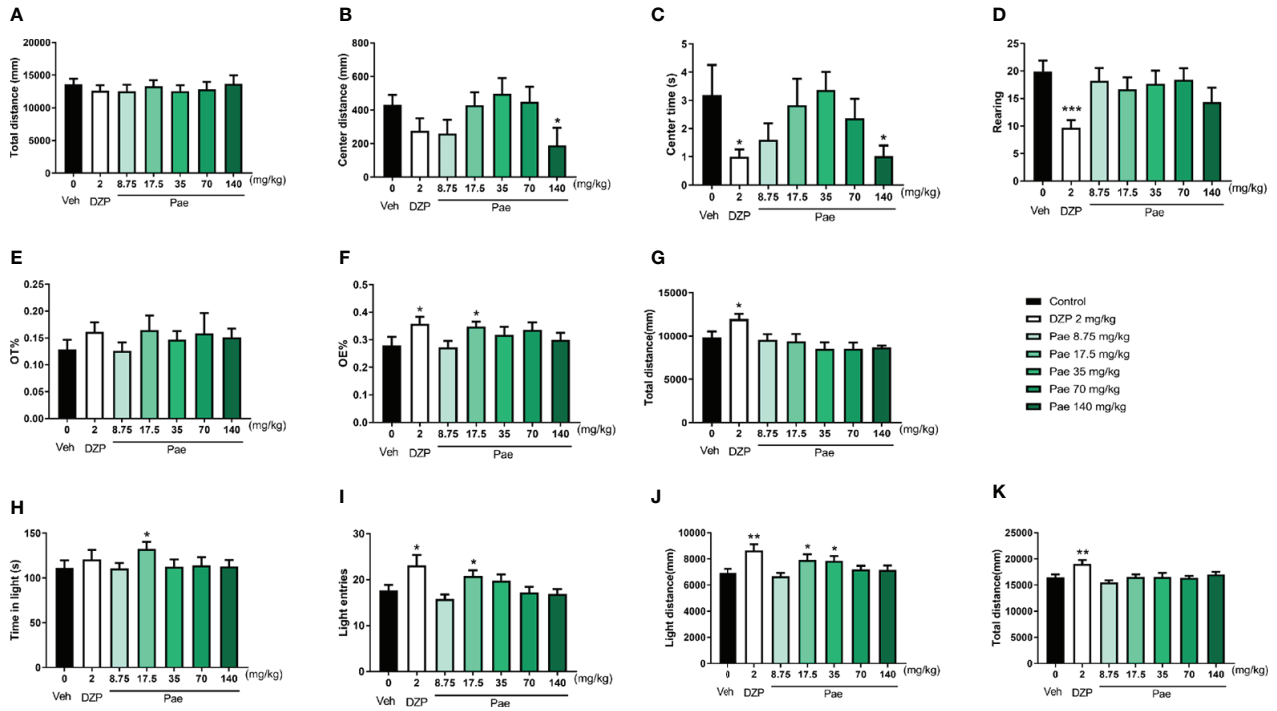

FIGURE 3 | Results of the dosage screening of paeonol against anxiety. **(A)** Total distance in the open-field test (OFT). **(B)** Central area distance in the OFT. **(C)** Time in the central area in the OFT. **(D)** Instances of standing on the hind legs (rearings) in the OFT. **(E)** Percentage of time in the open arm in the elevated plus maze (EPM) test. **(F)** Percentage of open-arm entry times in the EPM test. **(G)** Total distance in the EPM test. **(H)** Time spent in the light compartment in the light dark box (LDB) test. **(I)** Light compartment entry times in the LDB test. **(J)** Distance in the light compartment in the LDB test. **(K)** Total distance in the LDB test. Veh, vehicle; Dzp, diazepam; Pae, paeonol; OFT, open-field test; EPM, elevated plus maze; LDB, light dark box; *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group. Nine animals were used in each group.

PMDD-PWD rats tended to stay in the closed arms, thereby decreasing the OT% (p=0.0054) and OE% (p=0.0061) (**Figures 4G–I**). In addition, PMDD-PWD rats also showed significantly increased distance in the dark area (p=0.0485) in the LDB test (**Figures 4J–L**). These results showed that the PMDD-PWD model was established successfully because the animals significantly demonstrated anxiety-like behaviour.

Behavioural Identification of the Resident Intruder Model of PMDD

The composite aggressive test, OFT, and EPM test were designed to evaluate the behavioural phenotypes of PMDD-RIP animals. In the composite aggressive test (**Figures 5A, B**), the PMDD-RIP rats (n=12) showed a significantly higher composite aggressive score than that of the control rats (n=12, p < 0.0001). PMDD-RIP rats also showed a shorter distance in the central area (p=0.0058) and reduced time (p=0.0465) in the central area in the OFT (**Figures 5C–F**); however, the total distance remained unchanged. In the EPM test (**Figures 5G–J**), the OT% (p=0.0003) and OE% (p=0.0301) were all decreased in PMDD-RIP rats. All these results indicated that we established a successful and reliable resident intruder model of PMDD because of the significant anxiety-like behaviour.

Paeonol Improved the PMDD-Like Behaviour in Rat Models

The Effect of Paeonol on the PMDD-PWD Rat Model

According to the dosage screening results of paeonol against anxiety, the dose of paeonol in rats was determined as follows: moderate dose=12.11 mg/kg/d, low dose=6.05 mg/kg/d, and high dose=24.23 mg/kg/d. In the OFT results as shown in **Figure 6**, the drug effect was not very obvious, except that 24.23 mg/kg paeonol decreased the rearing number (p=0.0478) and the peripheral area distance (p=0.0017) compared with PMDD-PWD rats.

In the EPM test (**Figure 7**), 6.05 mg/kg paeonol showed significant anti-anxiety effects: the distance in the closed arm (p=0.0076), CE (p=0.0059), total entry times (p=0.0205), OE% (p=0.0024), and OT% (p=0.0059) were all reversed to normal level compared with PMDD-PWD rats receiving fluoxetine treatment (p=0.0026, 0.0121, 0.0484, 0.0091, and 0.0048, respectively). Likewise, 24.23 mg/kg paeonol also reversed the distance in the closed arm (p=0.0097).

In the LDB test shown in **Figure 8**, the total distance was not affected by the PMDD model or drug treatment. However, with respect to the light distance, 6.05 mg/kg paeonol increased the light distance (p=0.0223) compared with the PMDD-PWD

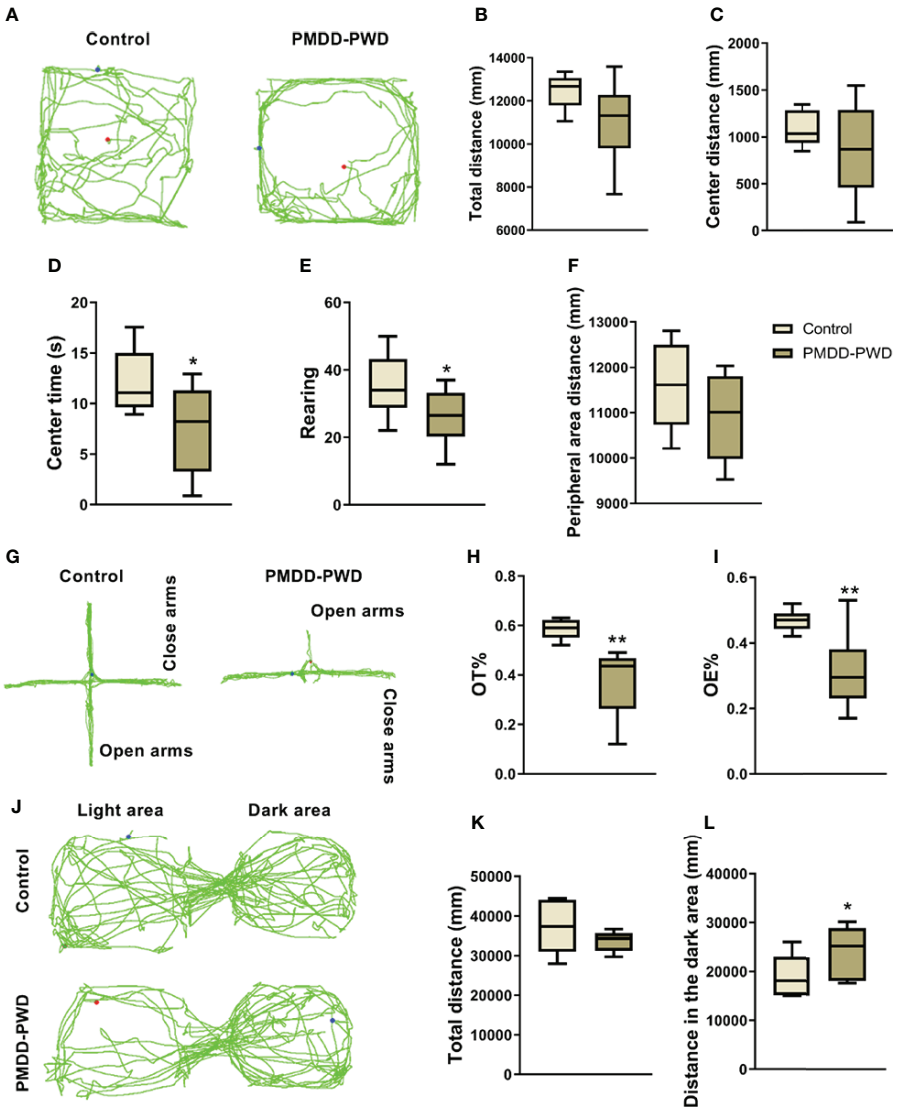

FIGURE 4 | Results of the behavioural identification in the premenstrual dysphoric disorder-progesterone withdrawal (PMDD-PWD) rat model. **(A)** The representative trajectory diagram of control and PMDD-PWD rats in the open-field test (OFT). **(B)** Total distance in the OFT. **(C)** Central area distance in the OFT. **(D)** Time in the central area in the OFT. **(E)** Instances of standing on the hind legs (rearings) in the OFT. **(F)** Peripheral area distance in the OFT. **(G)** The representative trajectory diagram of control and PMDD-PWD rats in the elevated plus maze (EPM) test. **(H)** Percentage of time in the open arm (OT%) in the EPM test. **(I)** Percentage of open-arm entry times (OE) in the EPM test. **(J)** The representative trajectory diagram of control and PMDD-PWD rats in the light dark box (LDB) test. **(K)** Total distance in the LDB test. **(L)** Distance in the dark compartment in the LDB test. *$p < 0.05$, **$p < 0.01$ compared with the control group. PMDD-PWD, premenstrual dysphoric disorder-progesterone withdrawal; OFT, open-field test; EPM, elevated plus maze; LDB, light dark box; OE, open-arm entry times; OT, time in the open arm.

group, while 24.23 mg/kg paeonol decreased this distance (p=0.0078); 6.05 mg/kg paeonol also increased the light time (p=0.0298) and decreased the dark time (p=0.0283). With respect to the dark distance, the fluoxetine group (p=0.0373), 6.05 mg/kg paeonol group (p=0.0109), and 12.11 mg/kg paeonol group (p=0.0225) yielded lower values compared with the PMDD-PWD group. The light entry times were increased with 6.05 mg/kg paeonol (p=0.0146) and decreased with 24.23 mg/kg paeonol (p=0.0009). Conversely, the dark entry times were decreased with 6.05 mg/kg paeonol treatment (p=0.0005) and increased with 24.23 mg/kg paeonol (p=0.0440).

The Effect of Paeonol on the Resident Intruder Model of PMDD

To determine the effect of paeonol on PMDD-RIP rats, the OFT and EPM and composite aggressive tests were performed in each group after the final drug administration (on the 28th day after ovariectomy). In the OFT (**Figures 9A–C**), paeonol did not show

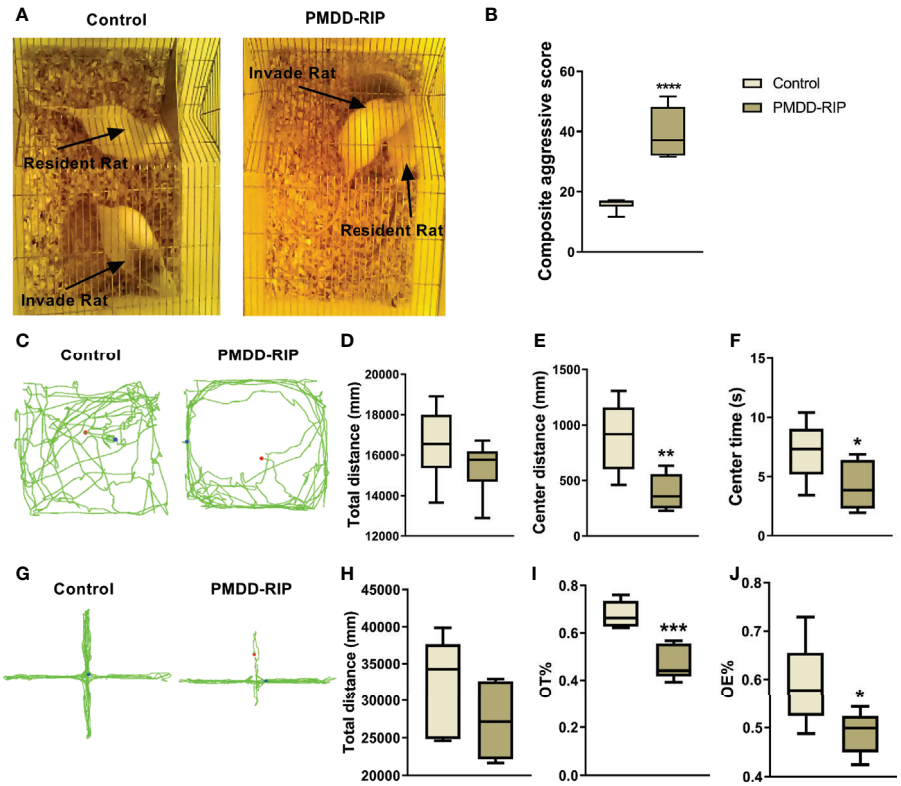

FIGURE 5 | Results of the behavioural identification in the resident intruder model of premenstrual dysphoric disorder (PMDD). **(A)** The representative photograph of control and PMDD-RIP rats in the composite aggressive test. **(B)** Composite aggressive score in the dioestrus I phase. **(C)** The representative trajectory diagram of control and PMDD-RIP rats in the OFT. **(D)** Total distance in the OFT. **(E)** Central area distance in the OFT. **(F)** Time in the central area in the OFT. **(G)** The representative trajectory diagram of control and PMDD-RIP rats in the EPM test. **(H)** Total distance in the EPM test. **(I)** Percentage of time in the open arm (OT%) in the EPM test. **(J)** Percentage of open-arm entry times (OE) in the EPM test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with the control group. PMDD-RIP, premenstrual dysphoric disorder-resident intruder paradigm; OFT, open-field test; EPM, elevated plus maze; OE, open-arm entry times; OT, time in the open arm.

any significant effect, while fluoxetine reversed the central area time (p=0.0358) compared with PMDD-RIP rats. In the EPM test (**Figures 9D–F**), 12.11 mg/kg paeonol and fluoxetine both significantly increased the OT% (p=0.0007 and 0.0090) and OE% (p=0.0202 and 0.0425), indicating an obvious anti-anxiety effect. Moreover, the composite aggressive scores before (16^{th} day after ovariectomy) and after administration (28^{th} day after ovariectomy) are shown in **Figures 9G, H**. The results showed that drug treatment including fluoxetine (p=0.0020), 6.05 mg/kg paeonol (p < 0.0001), 12.11 mg/kg paeonol (p < 0.0001), and 24.23 mg/kg paeonol (p=0.0007) reversed the alterative composite aggressive score in PMDD-RIP rats.

DISCUSSION

Based on the above mentioned results, we conclude that in the dosage screening experiment, the optimal anti-anxiety dose of paeonol was identified as 17.5 mg/kg/d for 7 days. Paeonol could significantly improve the behavioural symptoms in PMDD rat models; however, the effective dose varied in different models, suggesting a dose-dependent effect. In PMDD-PWD model rats, 6.05 mg/kg paeonol treatment could significantly improve the anxiety and irritability, while treatment with 24.23 mg/kg paeonol showed anxiety-like effects in behavioural tests. In PMDD-RIP model rats, 12.11 mg/kg paeonol showed a superior effect in improving anxiety, particularly irritable emotional behaviour.

17.5 mg/kg Paeonol Is the Optimal Dosage Against Anxiety

Classical behavioural tests including the OFT, EPM, and LDB are simple and fast, and they are widely used in the initial screening of anti-anxiety drugs. Therefore, in this study, we selected these three methods to evaluate the drug effect and screen the optimal dosage of paeonol. The OFT is a method for evaluating the ability of autonomous movement and exploration of animals in a strange environment (25). On one hand, animals mainly move around the peripheral area with less activity in the central area because of the fear of the new environment. On the other hand,

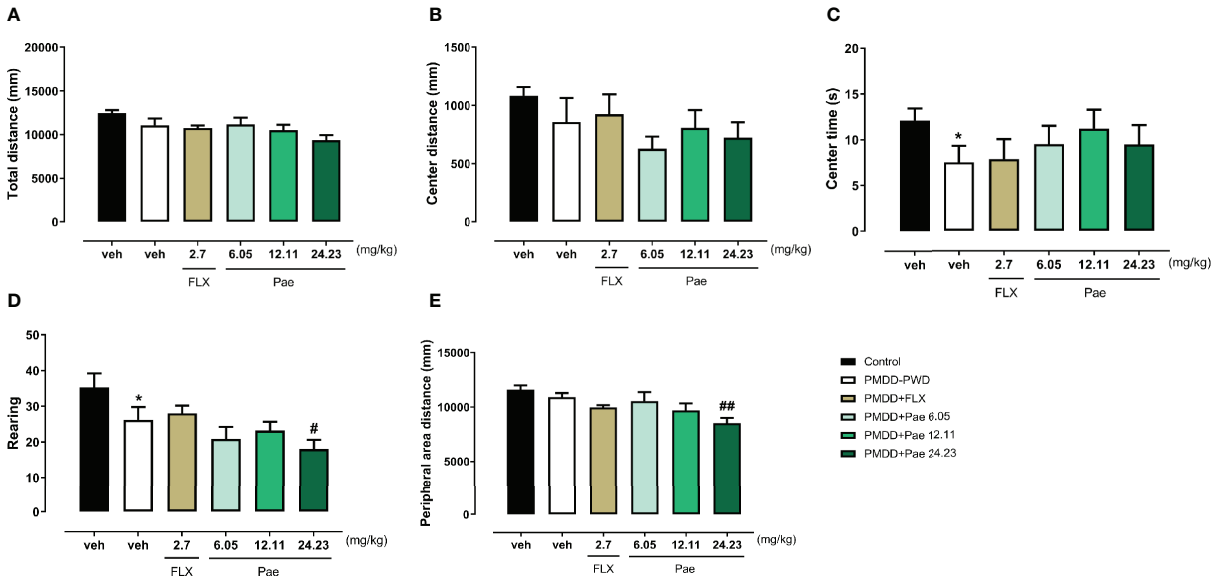

FIGURE 6 | Results of the effect of paeonol on the premenstrual dysphoric disorder-progesterone withdrawal (PMDD-PWD) rat model in the OFT. **(A)** Total distance in the OFT. **(B)** Central area distance in the OFT. **(C)** Time in the central area in the OFT. **(D)** Instances of standing on the hind legs (rearings) in the OFT. **(E)** Peripheral area distance in the OFT. PMDD-PWD, premenstrual dysphoric disorder-progesterone withdrawal; OFT, open-field test; Flx, fluoxetine; Pae, paeonol; *$p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the PMDD-PWD group; $^{\#\#}p < 0.01$ compared with the PMDD-PWD group. Twelve animals were used in each group.

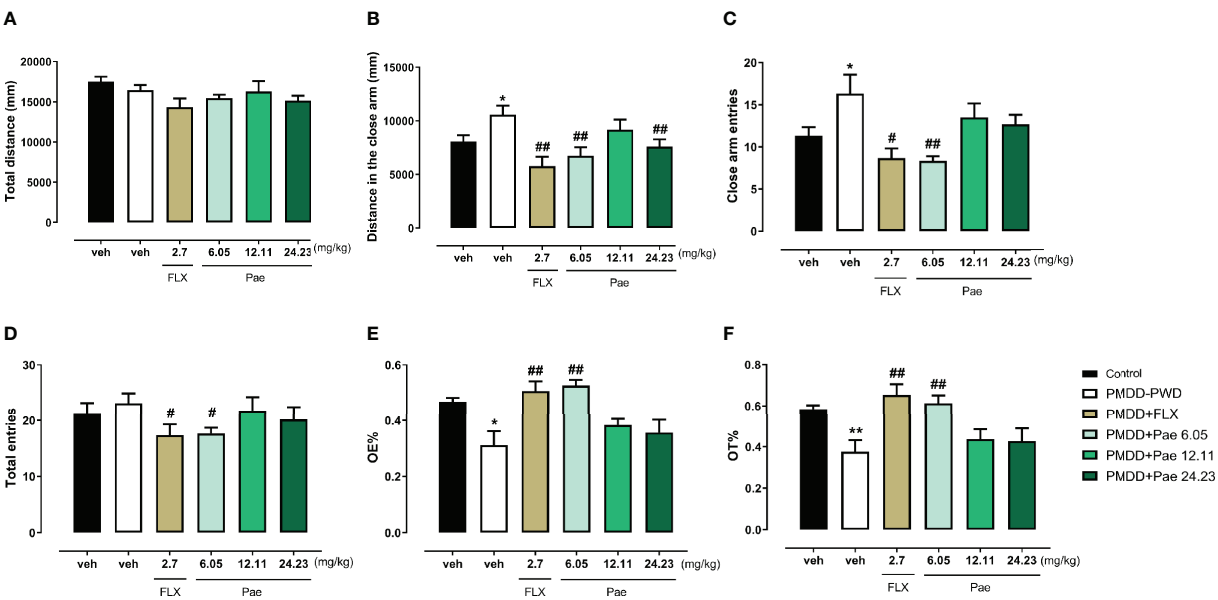

FIGURE 7 | Results of the effect of paeonol on the premenstrual dysphoric disorder-progesterone withdrawal (PMDD-PWD) rat model in the elevated plus maze (EPM) test. **(A)** Total distance in the EPM test. **(B)** Distance in the closed arm in the EPM test. **(C)** Closed arm entry times in the EPM test. **(D)** Total entry times in the EPM test. **(E)** Percentage of time in the open arm in the EPM test. **(F)** Percentage of open-arm entry times in the EPM test. PMDD-PWD, premenstrual dysphoric disorder-progesterone withdrawal; EPM, elevated plus maze; Flx, fluoxetine; Pae, paeonol; *$p < 0.05$ compared with the control group; **$p < 0.01$ compared with the control group; $^{\#}p < 0.05$ compared with the PMDD-PWD group; $^{\#\#}p < 0.01$ compared with the PMDD-PWD group. Twelve animals were used in each group.

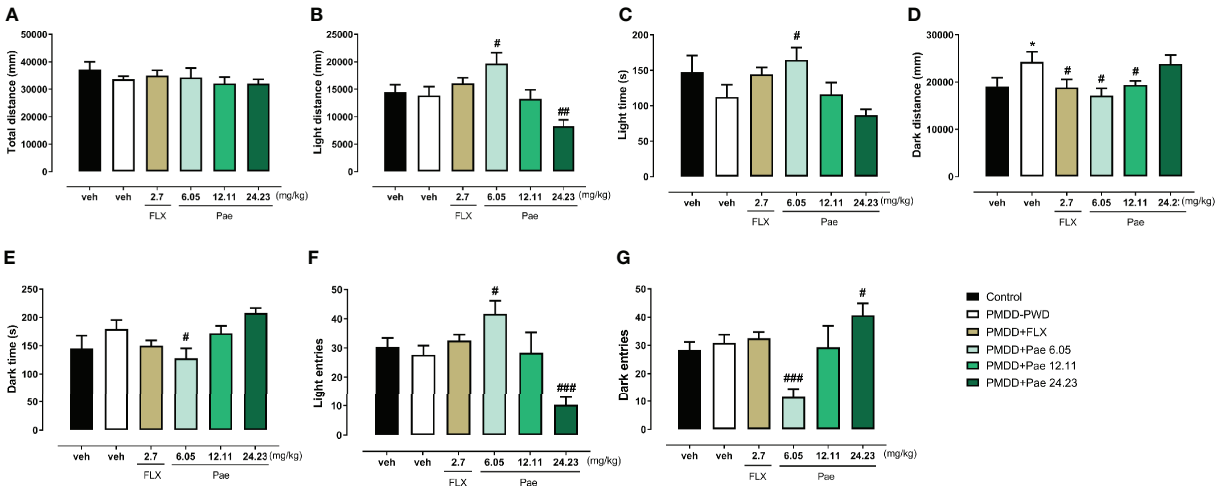

FIGURE 8 | Results of the effect of paeonol on the premenstrual dysphoric disorder-progesterone withdrawal (PMDD-PWD) rat model in the light dark box (LDB) test. **(A)** Total distance in the LDB test. **(B)** Distance in the light box in the LDB test. **(C)** Time in the light box in the LDB test. **(D)** Distance in the dark box in the LDB test. **(E)** Time in the dark box in the LDB test. **(F)** Entry times to the light box in the LDB test. **(G)** Entry times to the dark box in the LDB test. PMDD-PWD, premenstrual dysphoric disorder-progesterone withdrawal; LDB, light dark box; Flx, fluoxetine; Pae, paeonol; *$p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with the PMDD-PWD group; $^{\#\#}p < 0.01$ compared with the PMDD-PWD group; $^{\#\#\#}p < 0.001$ compared with the PMDD-PWD group. Twelve animals were used in each group.

the inquisitive nature of animals leads them to attempt entry into the central area. Therefore, the anxiety state of animals could be evaluated (26). The EPM model is based on the spontaneous behaviour of animals, which has the advantages of simplicity and good reproducibility (27, 28). The conflicts of animals' fear, exploration, and avoidance abilities were used to evaluate the anti-anxiety effects of the drugs. The LDB experiment was designed based on the fact that animals prefer darkness to light. Normally, rodents tend to stay in the dark, but their exploratory behaviour drives them to the light box. Therefore, the increase in the number and time of activities in the light box can reflect a reduction in animal anxiety (29, 30). Using the reliable behavioural evaluation system mentioned previously, we effectively screened 17.5 mg/kg as the optimal anti-anxiety effective intervention dose in mice treated with different doses of paeonol. Furthermore, in the OFT, we found that a 140 mg/kg paeonol treatment for 7 days showed significant anxiety-inducing effects, indicating that the anti-anxiety effect of paeonol was dose-dependent.

The anxiolytic-like effect of paeonol in mice has been confirmed previously in Xiao et al.'s study (31), wherein they found that the motor ability in animals were significantly limited with an increase in the dosage of diazepam. In our results, diazepam demonstrated an anxiety-causing effect in the OFT but an anxiolytic-like effect in the EPM and LDB tests. This conflicting phenomenon is possibly related to the drug dose. A previous report indicated that at a low dose, diazepam produced an anti-anxiety effect and improved the animals' exploration ability in the central region, but at a high dose, it demonstrated a sedative effect (32). However, our research showed that a high dose of paeonol had no effect on the animals' motor ability, which could be concluded from all kinds of behavioural tests. This indicates that the side effect of paeonol is less than that of diazepam, which provides a new option for clinical anti-anxiety applications.

The Reasonability of Modelling Methods and Validity Analysis of Animal Models

Through clinical trials, Lotta Andréen et al. found that after long-term exposure to progesterone followed by withdrawal, the progesterone metabolite 3α-5α-tetrahydroprogesterone decreased γ-aminobutyric acid (GABA)-gated current by increasing the $GABA_AR$ α4 subunit, resulting in a change in the composition of the $GABA_AR$ subunit, thus reducing the inhibition of GABA in the central nervous system (33). Yan Li et al. confirmed that a sudden progesterone withdrawal could change the expression of $GABA_AR$, increase the excitability of GABA in the central nervous system, and induce anxiety-like behaviour during a period of 24–72 h after stopping exogenous hormone injections (16). Because of the pathogenesis homology between the anxiety-like behaviour of the PMDD-PWD animal models and the anxiety symptoms in clinical PMDD patients, this study adopted the progesterone withdrawal paradigm to create a PMDD rat model for exploring the possible effects of paeonol. We found that rats showed a typical anxiety-like behaviour after the withdrawal of long-term progesterone injection, while fluoxetine, a first-line treatment for PMDD, alleviated the abnormal behavioural changes related to the oestrous cycle in model rats, which was consistent with the report of Machado Figueiredo et al. (8). Furthermore, in the 6.05 mg/kg dose group, paeonol could also significantly improve the anxiety and restlessness-like mood in PMDD-PWD model rats.

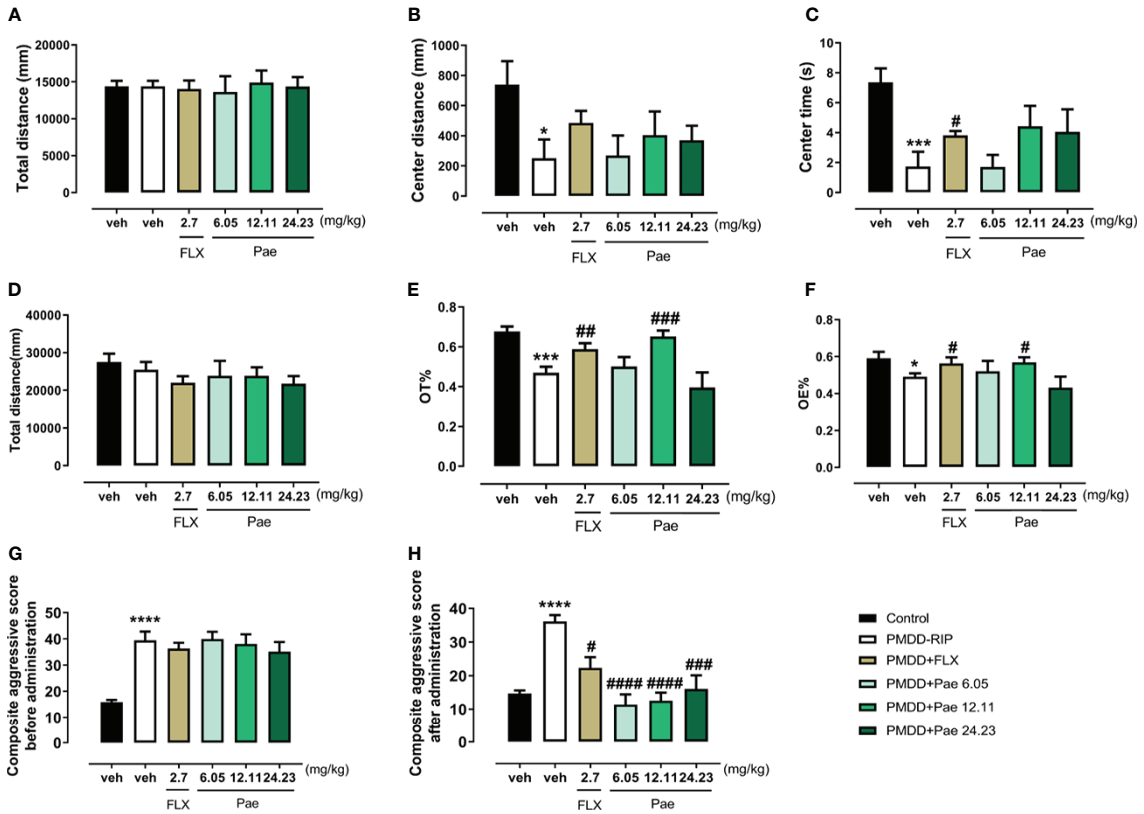

FIGURE 9 | Results of the effect of paeonol on the resident intruder model of premenstrual dysphoric disorder (PMDD) in the open-field test (OFT) and elevated plus maze (EPM) and composite aggressive tests. **(A)** Total distance in the OFT. **(B)** Distance in the central area in the OFT. **(C)** Time in the central area in the OFT. **(D)** Total distance in the EPM test. **(E)** Percentage of time in the open arm in the EPM test. **(F)** Percentage of open-arm entry times in the EPM test. **(G)** Composite aggressive score before the drug administration. **(H)** Composite aggressive score after the drug administration. PMDD-RIP, premenstrual dysphoric disorder-resident intruder paradigm; OFT, open-field test; EPM, elevated plus maze; Flx, fluoxetine; Pae, paeonol; *$p < 0.05$ compared with the control group; ***$p < 0.001$ compared with control group; $^{\#}p < 0.05$ compared with the PMDD-RIP group; $^{\#\#}p < 0.01$ compared with the PMDD-RIP group; ###$p < 0.001$ compared with the PMDD-RIP group; $^{\#\#\#\#}p < 0.0001$ compared with the PMDD-RIP group. Twelve animals were used in each group.

All the evidence mentioned above show that a PMDD-PWD rat model has good surface validity and predictive validity.

RIP makes use of the territorial behaviour of resident rats in their home cages. When facing the invasion of exotic animals, resident animals face inexplicable pressure and show irritability and defensive behaviour. The psychology of this defensive behaviour in rodents is related to human anxiety, which simulates the irritable emotional expression in patients with clinical PMDD (34). In the aggressive behavioural test, the animals in the model group showed cycle-dependent changes in the composite aggressive score during the non-receptive period, while there were no significant changes in the composite aggressive score of animals in the control group before and after administration, indicating that the PMDD rat model was reliable and solid, which reproduced and verified the findings of Hoi-Por Ho et al. (18). Fluoxetine could significantly reduce the aggressive behaviour in animals, which proved that a first-line treatment of PMDD could improve the irritable mood of PMDD (21). In addition, each dose group of paeonol can significantly improve the irritable mood in PMDD animals after treatment, which enriches the performance of paeonol in the treatment of emotional diseases.

Paeonol Could Improve the Behavioural Symptoms of PMDD Rat Models

In previous studies, paeonol was proven to play remarkable roles in numerous central nervous system disorders including diabetic encephalopathy, cerebral ischaemic injury, Alzheimer's disease, Parkinson's disease, ageing, and depression (35). The neuroprotective effect of paeonol may be related to the reduced release of neurotoxic and proinflammatory factors and the inhibitory effects of neuroinflammation (10, 11, 36, 37). However, in the treatment of PMS/PMDD, the pharmacodynamic evaluation of paeonol has not yet been reported. To address this issue, we established two classical and recognised PMDD models and observed the effect of paeonol on the behavioural symptoms.

As the anxiety-like emotional behaviour in the animal model of PWD is similar to the pathogenesis of anxiety in patients with

clinical PMDD, in this study, PWD was used to establish the PMDD-PWD model (16). We found that 6.05 mg/kg paeonol could significantly improve the anxiety and irritability in PMDD-PWD model rats, while a dosage of 24.23 mg/kg demonstrated anxiety-like effects in the behavioural tests. Thus, the dose-dependent effect of paeonol was reconfirmed.

For the clinical diagnosis criteria of PMDD, a regular menstrual cycle is required with symptoms of irritability occurring over at least three menstrual cycles (1). However, in experimental research, animals with three consecutive oestrous cycles are rare, and the emotional cycle of animals is easily affected by the environment, diet, sleep, emotion, and other factors. In order to eliminate this challenge in the study, along with previous research (18), exogenous hormone supplementation was administered to induce the normal oestrous cycle after the ovaries were removed. Then, the PMDD-RIP model was established using a resident intruder to simulate the clinical performance of irritable emotional symptoms. Firstly, we found that the symptoms in the PMDD-RIP model rats were oestrous cycle-dependent. Irritability was obvious during the dioestrus I phase (non-receptive phase), which is consistent with the performance of clinical PMDD patients in the premenstrual period. Secondly, for these typical symptoms, such as the irritable mood symptoms of PMDD-RIP rats, 12.11 mg/kg paeonol showed an effective improvement.

Thus, with these two rat models, the anti-PMDD property of paeonol and its few side effects were confirmed in our study. As the first-line treatment in PMS/PMDD patients, the antidepressant, SSRI, is associated with obvious side effects. Thus, there is an urgent need to develop new drug treatment methods to improve the therapeutic measures for PMS/PMDD (5). Traditional herbal medicine has been used widely, and studies have shown that many traditional Chinese medicines or its components have anti-depressant or anti-anxiety effects (38–41). However, for PMS/PMDD treatment, reports on the effect of traditional Chinese medicine are rare. In addition to the anti-inflammatory, anti-oxidant, and neuroprotective properties of paeonol confirmed in previous studies (10, 35), the anti-PMDD effect of paeonol in this study may be concluded.

The Effective Dose of Paeonol Varied in Different PMDD Models

Based on the abovementioned conclusions, paeonol could evidently improve the behavioural symptoms in both PMDD-PWD and PMDD-RIP rat models, but the effective dose varied. For PMDD-PWD rats, 6.05 mg/kg paeonol showed a significant treatment effect on PMDD-like symptoms, while for PMDD-RIP rats, 12.11 mg/kg was the optimal dosage. As the two PMDD models correspond to different pathogeneses, the mechanism by which paeonol works may vary. The underlying mechanism of this phenomenon remains to be further explored. Currently, the most popular hypothesis for interpreting the PMDD pathogenesis is associated with the GABA-A receptor; however, this is still being debated. Determining and confirming the pathophysiological alterations in PMS/PMDD is critical in this area, which can probably help in the interpretation of our findings. Moreover, paeonol has a variety of pharmacodynamic effects including anti-inflammatory, anti-tumour, neuroprotective, and anti-cardiovascular disease effects (35). The specific mechanism by which paeonol administration affects PMDD is still unclear, warranting further research for confirmation.

The Effect of Paeonol Was Dose-Dependent

In the anti-anxiety effect experiment with mice or in the effect of paeonol on PMDD rats, it was easy to determine that a relatively high dose could cause anxiety-like behaviour, and conversely, an appropriate dose could relieve anxiety significantly. As a type of benzodiazepine, the potential target of paeonol are the benzodiazepine sites that bind to GABA-A receptors, and then, the Cl^- current of GABA-A receptor is mediated to increase the release of GABA (42). Different doses of paeonol may change the GABA-A receptor-gated current differently owing to the varied metabolism in the central nervous system. In this study, we only preliminarily confirmed the improvement effect of paeonol on the behavioural symptoms of PMDD, failing to provide a complete chain of evidence to interpret the underlying mechanism.

Other issues that were not investigated, which might affect the integrity of this study should be mentioned. First of all, this study failed to further explore the possible underlying mechanisms of drug intervention in model animals, which is necessary to understand and explain the occurrence of disease and drug effects. In fact, we also detected changes in hormones and neurotransmitters in the brain and peripheral blood, and failed to obtain valuable positive results. This finding is consistent with the finding that no valuable biomarkers were found in clinical patients and animal models with PMDD (43, 44). This is one of the puzzling problems in this field. The key to this problem may be the subtyping of PMDD (45–47). Different subtypes of PMDD may be mixed together, resulting in no valuable molecular clues. The animal models used in this article are typically symptom-mixed. This suggests that we should perform more in-depth and detailed studies on the heterogeneity identification of PMDD animal models in the future. Secondly, there is adequate evidence to suggest that the key mechanism of PMDD is the sensitivity of specific emotional regulation of the brain regions to the fluctuation of progesterone metabolites in different periods of the menstrual cycle (oestrous cycle) (7, 48), which has been revealed to some extent in previous studies (21), but not in-depth. In our follow-up work, we will use paeonol, an active monomer of traditional Chinese medicine that has been proved to be effective against PMDD, to interfere with model animals, and then observe the changes in brain sensitivity, to explore and confirm the possible central mechanism of paeonol.

CONCLUSIONS

In this study, first, the anti-anxiety effect of paeonol was verified by using comprehensive behavioural tests, and the optimal dose was identified as 17.5 mg/kg/d for 7 days. Secondly, using two different PMDD rat models (PMDD-PWD and PMDD-RIP), the

anti-PMDD effect of paeonol was concluded as follows: (i) paeonol could significantly improve the anxious and agitated emotion-like behaviour in PMDD rat models, but the effective dose varied in different models. (ii) The effect of paeonol on PMDD is dose-dependent; a high dose could cause anxiety-like behaviour, while an appropriate dose could relieve anxiety. Although additional investigation is still needed, our study indicates that paeonol is a potential therapeutic compound for PMS/PMDD and a new drug option that can help establish treatment dosage guidance.

DATA AVAILABILITY STATEMENT

PS, SW and MQ are responsible for presenting data supporting the results reported in the current study when required.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Review Board of Shandong University of Traditional Chinese Medicine (No. DWSY201703013).

AUTHOR CONTRIBUTIONS

HZ and XG performed all experiments. ZL, YL, KX, HW and JX provided key assistance. PS, SW and MQ directed the project, performed statistics, interpreted data and finished the manuscript.

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Effect of the ZiBuPiYin Recipe on Diabetes-Associated Cognitive Decline in Zucker Diabetic Fatty Rats After Chronic Psychological Stress

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Background: Cognitive impairment is a complication of type 2 diabetes mellitus (T2DM) that affects the central nervous system (CNS). Studies have shown that chronic psychological stress may promote the development of T2DM into diabetes-associated cognitive decline (DACD). Previously, cognitive impairment in T2DM was correlated predominantly with insulin resistance in the medial prefrontal cortex (mPFC).

Aims: We examined the effect of the ZiBuPiYin recipe (ZBPYR) on Zucker diabetic fatty (ZDF) rats and explored the impact of chronic stress on altered β-amyloid (Aβ) metabolism through insulin receptor substrate (IRS) 1/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway after the induction of chronic psychological stress.

Main Methods: After chronic psychological stress and drug treatment, cognitive function was observed *via* behavioral experiments. The activation of the hypothalamus-pituitary-adrenal (HPA) axis and levels of Aβ were detected by enzyme-linked immunosorbent assay, and the expression of related proteins was evaluated by Western blotting.

Key Findings: ZBPYR treatment significantly decreased anxious-like behaviors and plasma corticosterone (CORT) levels, and ameliorated learning and memory impairments of ZDF rats after chronic psychological stress. ZBPYR also reduced the deposition of Aβ in the mPFC, improved brain insulin resistance, and modulated the mTOR-autophagy pathway.

Significance: ZBPYR may be a potential therapeutic application for the treatment of DACD induced by chronic psychological stress.

Keywords: diabetes-associated cognitive decline, chronic psychological stress, ZiBuPiYin recipe, β-amyloid, IRS-1/AKT/mTOR signaling pathway

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia, increased insulin resistance over time, and progressive failure of pancreatic insulin secretion (1). Epidemiologic studies have reported that the worldwide prevalence of diabetes mellitus is expected to increase from 425 million in 2017 to an estimated 629 million in 2045 (2). Both cross-sectional and longitudinal studies reported that T2DM is associated with Alzheimer's disease (AD) (3), mild cognitive impairment (MCI) (4), anxiety, and depression (5). Diabetes-associated cognitive decline (DACD), a series of slight cognitive decrements over time, is generally thought to be a central nervous system (CNS) complication of T2DM (6, 7).

The development and progression of DACD is a complex process that likely involves synergistic effects of genetic susceptibility and environmental factors (8, 9). Previous studies have shown both cerebral insulin resistance and altered β-amyloid (Aβ) metabolism could affect cognitive function of DACD (10–12). In addition, insulin receptor substrate (IRS) 1/ protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathways play a critical role in insulin signaling. Insulin recruits and activates the IRS and activates the downstream AKT cascade, which then activates the mTOR-autophagy pathway and affects Aβ deposition. Hence, interference with the IRS1/AKT/mTOR pathway may lead to cognitive impairment.

Recent reports have shown that changes in lifestyle could increase the risk for DACD (13, 14). In particular, there is persuasive evidence that psychological stress contributes to both T2DM and DACD (14). Although immediate psychological stress is not considered an issue, chronic activation of psychological stress is a potent pathogenic factor in these disorders (15). Chronic psychological stress makes it increasingly difficult for the body to function, which not only impacts mental health, but can also predispose a person to various metabolic disorders (16, 17). Thus far, the molecular, physiological, and behavioral pathways involved in chronic psychological stress-induced DACD and the link between chronic psychological stress and changes in the brain that accompany DACD have not been clarified. Because chronic psychological stress adversely influences quality of life T2DM patients, developing a strategy to avoid DACD is important.

ZiBuPiYin recipe (ZBPYR), a traditional formula of Chinese medicine documented in the book of Bujuji written by Wu Cheng in the Qing dynasty, is derived from Zicheng Decoction and used for the treatment of cognitive impairment. Early reports have shown that ZBPYR improves the learning and memory process in high-fat diet combined with low-dose streptozotocin (STZ)-induced diabetic rats, and regulates the deposition of Aβ in the brain (18). Our recent data also demonstrated that chronic psychological stress impairs glucose intolerance and decreases insulin sensitivity in Zucker diabetic fatty (ZDF) rats, suggesting that chronic psychological stress can contribute to the development of insulin resistance in T2DM (19). In the present work, we used spontaneous ZDF rats as a T2DM model and examined the hypothesis that ZBPYR could reverse the impairment of chronic psychological stress leading to DACD, mainly by reducing Aβ deposition through the IRS-1/ AKT/mTOR signaling pathways in the medial prefrontal cortex (mPFC).

MATERIALS AND METHODS

Reagents and Antibodies

Antibody against amyloid-β protein precursor (APP) was acquired from the Sigma-Aldrich (St Louis, MO, USA). The Inhibitors Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail and the antibodies against Phospho-IRS1 (Ser307), AKT, phospho-AKT (Ser473), Phospho-mTOR (Ser2448), phospho-P70S6K (Thr389), LC3A/B, β-actin and secondary antibody horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG, and goat-anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against IRS-1, GSK3β, Phospho-GSK3β (Ser9), mTOR, and P70S6K were acquired from Abcam (Cambridge, MA, USA). Western blotting material was obtained from Bio-Rad (Hercules, CA, USA). All other experimental supplies and reagents were purchased from Biosharp (Shanghai, China) and Solarbio (Beijing, China).

Animal Model

Male 6-week-old ZDF (*fa/fa*) rats were purchased from Vital River Laboratories (VRL) (Beijing, China) and housed in a specific pathogen-free (SPF) animal experiment center at Nanjing University of Chinese Medicine. The ZDF strain has a homozygous leptin receptor mutation pre-disposing the rats to T2DM. When inbred ZDF males are fed the Purina 5008 diet (protein = 23.6%, nitrogen-free extract [by difference] = 50.3%, fiber [crude] = 3.3%, ash = 6.1%, fat [ether extract] = 6.7%, and fat [acid hydrolysis] = 8.1%; Vital River Laboratories, Beijing, China], which is high in carbohydrates and fats, they exhibit hyperinsulinemia, hyperglycemia, hypercholesterolemia, and hypertriglyceridemia, mimicking a type 2 diabetic state.

All rats were provided food and water ad libitum. They were maintained at 23°C ± 2°C with 65% ± 5% humidity on a 12-h light/dark cycle and were allowed to acclimatize to their environment for one week prior to drug or ultrapure water administration. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals at Nanjing University of Chinese Medicine (Nanjing, China), and were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (approval no. ACU170606).

Preparation and Administration of ZBPYR

ZBPYR were purchased from the Sanyue Chinese Traditional Medicine Co., Ltd. (Nantong, China), as shown in **Table 1**. Voucher specimens were certified by the company, and quality inspection reports were provided. The administration and the exact compounds/chemicals of ZBPYR were provided in previous studies (20, 21): the mixtures were soaked in 8 volume per weight

TABLE 1 | The ingredients of ZBPYR.

Herbal name	Botanical Latin name	Place of origin	Part used	Amount used (g)
Hong-Shen	*Panax ginseng* C. A. Mey.	Jilin	Root	30
Shan-Yao	*Dioscorea polystachya* Turcz.	Henan	Rhizome	15
Fu-Shen	*Poria cocos* (Schw.) Wolf	Anhui	Root	15
Bai-Shao	*Paeonia lactiflora* Pall.	Anhui	Root	15
Dan-Shen	*Salvia miltiorrhiza* Bunge	Shandong	Root	12
Bai-Bian-Dou	*Lablab purpureus* (L.) Sweet	Zhejiang	Bean	15
Lian-Zi	*Nelumbo nucifera* Gaertn.	Hunan	Seed	20
Shi-Chang-Pu	*Acorus gramineus* Sol. ex Aiton	Zhejiang	Rhizome	10
Yuan-Zhi	*Polygala tenuifolia* Willd.	Hebei	Root	10
Tan-Xiang	*Santalum album* L.	Guangdong	Sandalwood	4.5
Ju-Hong	*Citrus maxima* "Tomentosa"	Sichuan	Epicarp	9
Gan-Cao	*Glycyrrhiza uralensis* Fisch. ex DC.	Neimenggu	Root	9
Total amount				164.5

(1:8, w/v) of distilled water for 30 min and subsequently boiled for 90 min, then Tan-Xiang was added and boiled for 30 min. The mixtures were extracted twice. The decoction was filtered and concentrated to a final density of 3.29 g/ml.

Experimental Design

After 1 week of adaptive feeding, rats were randomly divided into 3 groups (n=6 each): ZDF model (ZDF group), chronic psychological stress-induced DACD (PSD group), and chronic psychological stress combined with ZBPYR administration (PDZ group). The PSD group and PDZ groups were subjected to two stress simulations for 6 weeks: restriction and rotation. During the restriction simulation, rats were put in open bottles. The size of the bottle did not allow the rat to turn around. The experimental time was limited to 2 h and was employed every other day. In the rotation simulation, the rats were placed in the homemade rotating device rotating for 15 min at 30 rpm with an interval ranging from 40 to 150 min. Four cycles were performed in each experiment every other day. It should be noted that rotation stress and restriction stress tests were not carried out on the same day. During the stress simulations, ZDF group simultaneous removal food and water.

During a period of 8 weeks, ZBPYR was administered by oral gavage daily at a dose of 32.9 g/kg body weight in the PDZ group, while other groups were orally administered an equal dose of ultrapure water instead of ZBPYR (Milli-Q Integral Water Purification System, Millipore Corporation, Billerica, MA, USA). The experimental process is shown in **Figure 1**.

Measurement of Plasma Corticosterone Levels

We measured plasma corticosterone (CORT) levels to determine whether the hypothalamic pituitary adrenal (HPA) axis was activated. After the end of chronic psychological stress, 100 μL of blood was collected from the tail vein of each rat. Blood samples were centrifuged at 1,000 × g for 10 min at 4°C. The supernatant plasma was collected and stored at −80°C until required. The concentration of CORT levels in plasma were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions and calculated based on the standard curve.

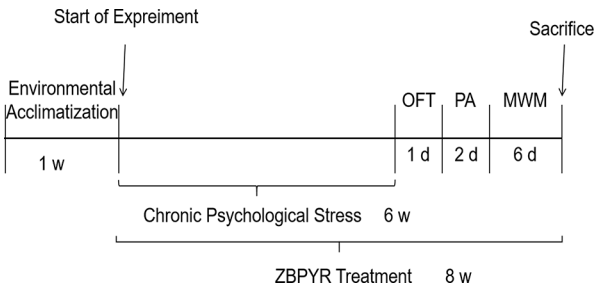

FIGURE 1 | Experimental process. OFT, open field test; PA, passive avoidance test; MWM, the Morris water maze test.

Behavioral Tests

Behavioral tests, including an open field test (OFT), a passive avoidance (PA) test, and the Morris water maze test (MWM), were performed during the last 9 days of treatment, according to the following schedule: day 1, OFT; days 2–3, PA test; and days 4–9 MWM test.

OFT

The OFT assessed the effects of chronic psychological stress on anxious behavior. Briefly, rats were placed in the test environment for 1 h prior to testing. After the start of the test, rats were placed in the center of a dimly illuminated open field chamber (50 cm × 50 cm × 50 cm) and allowed to explore freely for 5 min. The square arena was divided into a center zone (10 cm × 10 cm) and an outer zone. Behavioral patterns measured in the chamber included time in the center %, entries in the center, total distance, and velocity. All animals were used only once, and the locomotion was recorded by video camera and registered in the computer. After each trial, the square arena was cleaned with 70% alcohol to prevent the influence of the odors present in the urine and feces of the previous rat.

PA

In the PA test, animals learned to avoid an environment in which an adverse stimulus (foot shock) was applied. The PA apparatus consisted of two identical compartments separated by a guillotine

opaque door. One compartment was illuminated (safe), and the other was dark. The procedure was done on two consecutive days. On the first day, animals were individually placed into the safe compartment, allowed to freely explore for 5 s, and allowed to move into the dark compartment by opening the door. Training was done by closing the door and applying a mild electrical foot shock (1 mA for 5 s) upon entry of the rat into the dark compartment. Memory test was conducted 24 h after training. The procedure was performed the same as training without any shock. Each rat was again positioned in the safe compartment backing to the door. The time to enter the dark compartment was recorded as the error number. The latency period to enter the dark compartment was acquired during a maximum of 300 s.

MWM

Spatial learning and memory performance of the rats were evaluated by the MWM test, which consisted of 5 days of training (invisible plat training sessions and a probe trial), and a visible platform training session on day 6. The apparatus consisted of a circular pool (160 cm in diameter, 50 cm in height, and filled to a depth of 40 cm with water maintained at 24°C ± 1°C) and a platform (12 cm in diameter and 29 cm in height), which was hidden under water. On the first day, rats were permitted to swim freely in the tank for 120 s without the platform to adapt to the new condition. Over the following 4 days, rats were trained with 4 trials per day at intervals of 120 s until they reached the platform and were allowed to rest for 60 s. If the rat failed to reach the platform within 120 s, it was gently guided to the platform and allowed to stay for 60 s. The escape latency taken to reach the platform was measured automatically. For the probe trial test, the platform was removed. Rats had to swim for 120 s, and time searching for the original platform and time across the platform area were recorded. On day 6, a visible platform test was performed. The platform was located 2 cm over the water surface and placed in a position different from the previous test. During this test, the escape latency was recorded. All data were measured by a camera and automated analysis system.

Brain Sample Collection

Rats were deeply anesthetized with isoflurane and decapitated. Brains were removed, dissected, and the mPFC was isolated on ice and rapidly frozen in liquid nitrogen. All dissociated tissues were stored at −80°C until required.

ELISA Analyses

Frozen mPFC samples were homogenized in liquid nitrogen and extracted with buffer (1× TBS containing 5 mM EDTA and 2 mM 1,10-phenanthroline). The homogenates were kept at 4°C for 15 min and then centrifuged at 500,000×*g* for 1 h at 4°C. The supernatants were collected, and soluble Aβ40 and Aβ42 levels were measured using ELISA kits. Afterward, the pellets were homogenized in 70% formic acid (FA) solutions and centrifuged at 500,000×*g* for 1 h at 4°C. The FA-containing supernatants were neutralized *via* a 1:20 dilution into 1 M Tris-base (pH=11), and insoluble Aβ40 and Aβ42 levels were then measured using ELISA kits. All experiments were performed per the manufacturer's instructions. The optical density of each well was read at 450 nm using a microplate spectrophotometer.

Western Blotting

Frozen mPFC samples were lysed for 30 min in an ice-cold radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) containing 100× Inhibitors Protease Inhibitor Cocktail and 100× Phosphatase Inhibitor Cocktail to extract total proteins. Following centrifugation (14,800 rpm, 10 min), the supernatants were harvested, and protein concentrations were measured using a Minim Spectrophotometer. Subsequent to boiling and denaturing, the same amounts of protein samples (25 μg extract/lane) were loaded and separated by 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis. Then the proteins were electro-transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline Tween-20 (TBST, 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.1% Tween-20) for 2 h at room temperature, the membranes were incubated overnight with targeted primary antibodies in TBST at 4°C as follows: p-IRS1 (1:1000), IRS1 (1:1000), p-AKT (1:1000), AKT (1:1000), p-GSK3β (1:1000), GSK3β (1:1000), p-mTOR (1:1000), mTOR (1:1000), p-P70S6K (1:1000), P70S6K (1:1000), and LC3A/B (1:1000). After washing, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. After washing with TBST 3 times for 5 min, the membranes were incubated with secondary antibodies conjugated to HRP (1:2000) for 60 min at room temperature. The washings were then repeated. For visualization, the immunoreactive bands were treated with a chemiluminescence solution (ECL, Tanon, Shanghai, China) and detected with X-ray films. The optical density values of the target protein bands were quantified with ImageQuant TL 1D (GE Healthcare, USA) and normalized to β-actin (1:1000) loading control. The results were expressed as the means of three experiments.

Statistical Analysis

All data were obtained from at least three independent experiments and expressed as means ± standard error of means (SEM). Statistical significance was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Fisher's LSD. All statistical analyses were performed using SPSS 19.0 (IBM, Chicago, IL, USA). P values < 0.05 were considered significant.

RESULTS

Impact of ZBPYR on Anxious-Like Behaviors and Plasma CORT Levels

Rats exposed to chronic psychological stress exhibited a series of abnormal behaviors, such as piloerection, indolence, and fidgeting, which were typical anxiety-like behaviors. In the OFT, the PSD group was in the center for a shorter time (**Figure 2A**; $P < 0.01$) and entered the center less frequently (**Figure 2B**; $P < 0.01$) compared with ZDF group. The behavior of rats in the PDZ group returned to normal levels after 8 weeks

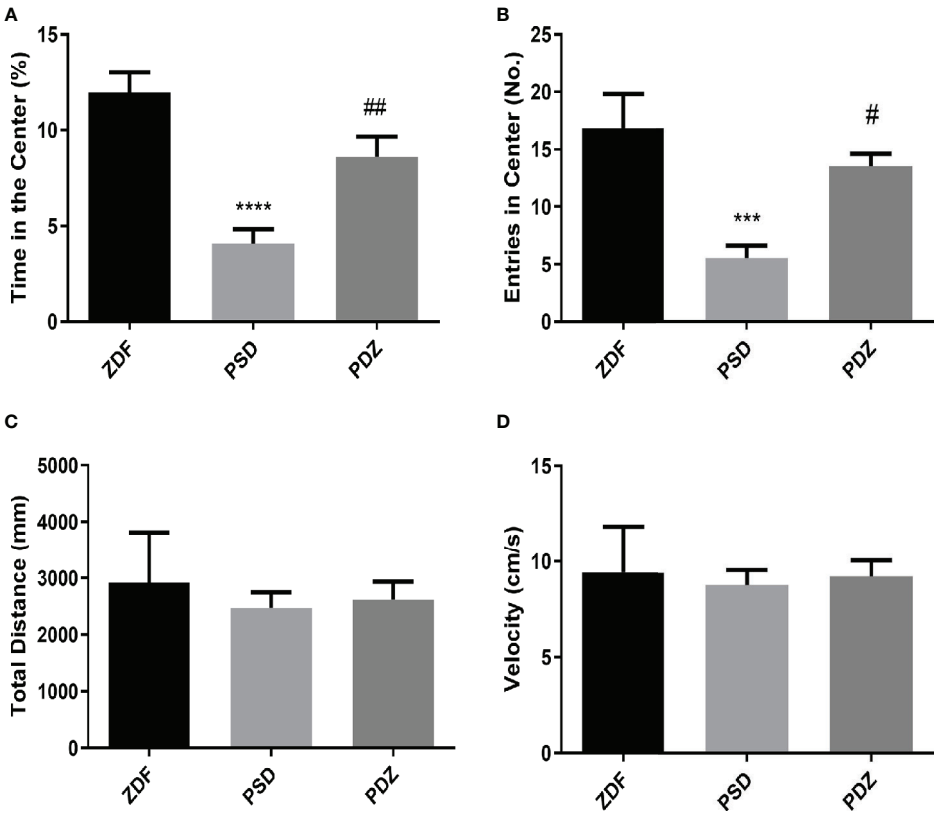

FIGURE 2 | Effect ZBPYR on activities in the open-field test. Performance of ZDF rats in each group was measured, including time in the center (%) **(A)**, entries in the center **(B)**, total distance **(C)** and velocity **(D)**. Bars represent the mean ± SEM of 6 rats per group. ***$P < 0.001$, ****$P < 0.0001$, PSD group *vs.* ZDF group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, PDZ group *vs.* PSD group.

of treatment of ZBPYR. ZDF rats gained weight from 8 to 15 weeks of age (22), but the behavioral experiments involved in our study did not find an effect of body weight on the experimental results, as shown by the total distance and velocity of movement of rats. There were no significant changes in physical activity in the three groups (**Figures 2C, D**).

We measured plasma CORT hormone levels in all rats to determine whether the HPA axis was activated CORT levels in the PSD group were significantly higher than in the ZDF group, while the groups that received ZBPYR showed significantly decreased CORT levels (**Figure 3**).

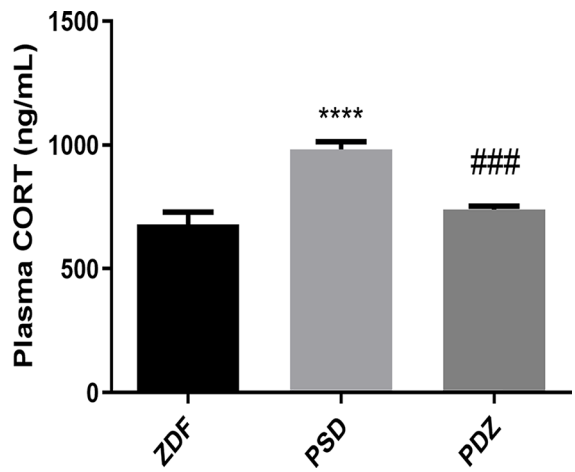

FIGURE 3 | Effect of ZBPYR on plasma corticosterone (CORT) levels. Bars represent the mean ± SEM of 6 rats per group. ****$P < 0.0001$, PSD group *vs.* ZDF group; $^{\#\#\#}P < 0.001$, PDZ group *vs.* PSD group.

Impact of ZBPYR on Cognitive Impairment

To investigate whether chronic psychological stress affects cognitive function, we evaluated the passive avoidance memory and spatial learning abilities in different groups by PA test and MWM test. In the PA test, the PSD group had a significantly lower latency and a greater error number than the ZDF group. However, cognitive impairment was significantly reversed after 8 weeks of ZBPYR administration (**Figures 4A, B**; $P < 0.05$; $P < 0.01$). MWM results showed that the time to find the hidden

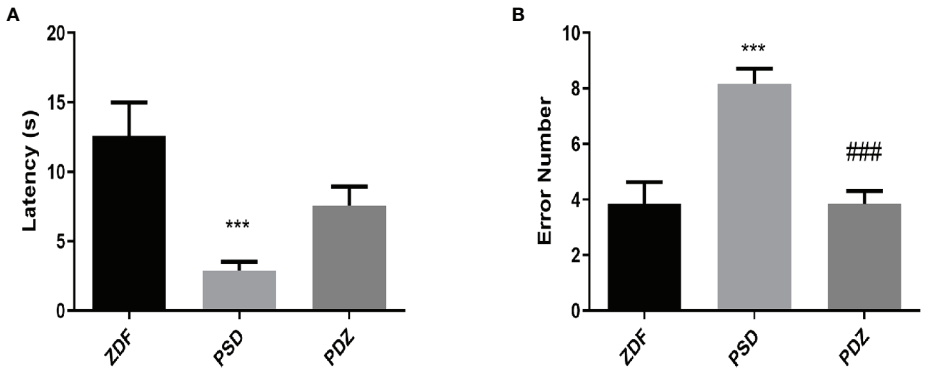

FIGURE 4 | Effect of ZBPYR on cognitive impairment in the passive avoidance test. The latency **(A)** and error number **(B)** were assessed. Bars represent the mean ± SEM of 6 rats per group. $^{***}P < 0.001$, PSD group *vs.* ZDF group; $^{###}P < 0.001$, PDZ group *vs.* PSD group.

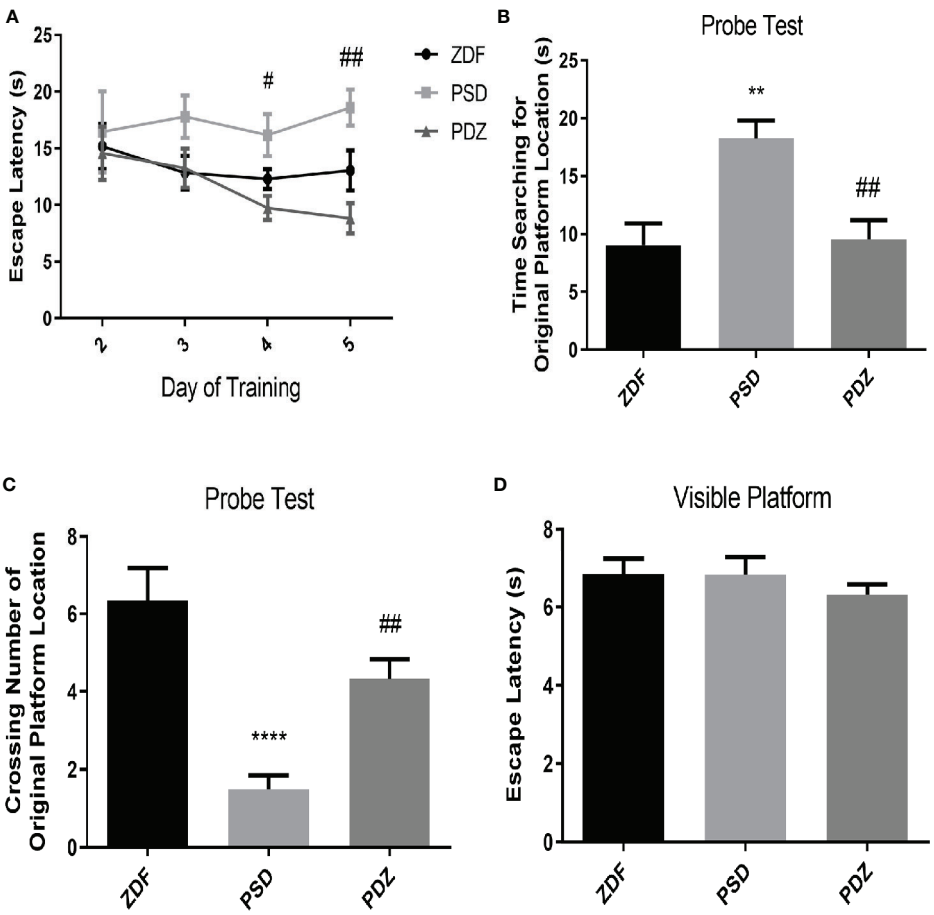

FIGURE 5 | Effect of ZBPYR on cognitive impairment in the Morris water maze (MWM) test. The escape latencies of the animals were analyzed in the training trials **(A)**. Performance in the probe test was analyzed, including time searching for the original platform location **(B)** and number of crossings over the original platform location **(C)**. Escape latency in the visible platform version of the MWM test was also analyzed **(D)**. Bars represent the mean ± SEM of 6 rats per group. $^{**}P < 0.01$, $^{****}P < 0.0001$, PSD group *vs.* ZDF group; $^{#}P < 0.05$, $^{##}P < 0.01$, PDZ group *vs.* PSD group.

platform progressively decreased in the PDZ group (**Figure 5A**, $P < 0.05$). In the invisible plat training test, the escape latency of the PSD group was significantly longer than that of the ZDF group. In contrast, the PDZ group showed a shorter escape latency compared with PSD group. In the probe trial test, we found that the PSD group cost more seconds during the test, and number of times across the original platform area were significantly lower than that of ZDF group (**Figures 5B, C**, $P < 0.05$; $P < 0.01$). Performance in the visible platform version was similar across groups in terms of escape latency (**Figure 5D**). All these findings suggest that ZBPYR could attenuate the impairment of learning and memory impairments caused by chronic psychological stress in ZDF rats.

Impact of ZBPYR on Aβ Deposition

Aβ is the main component of amyloid plaques. To determine whether cognitive impairments were associated with Aβ deposition, we assessed both soluble and insoluble Aβ40 and Aβ42 levels in the mPFC of ZDF rats. The soluble Aβ40 and Aβ42 levels in the PSD group were significantly higher compared with the ZDF group, whereas in the PDZ group, levels were further downregulated after ZBPYR-treatment (**Figures 6A, B**, $P < 0.001$). Similarly, the insoluble Aβ40 and Aβ42 levels in the PSD group were significantly increased compared with the ZDF group, whereas the insoluble Aβ42 levels were not affected by ZBPYR in the PDZ group (**Figures 6C, D**, $P < 0.001$).

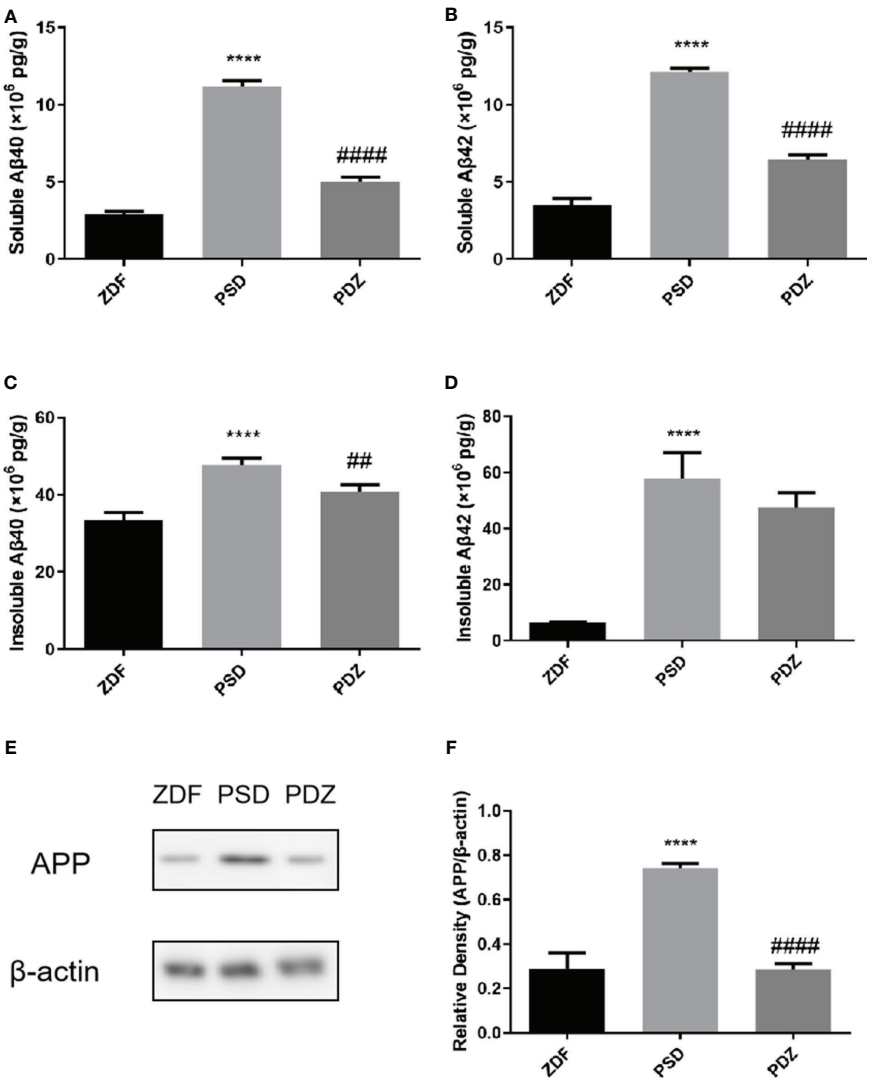

FIGURE 6 | Effect of ZBPYR on generation and deposition of β-amyloid in the medial prefrontal cortex (mPFC) of ZDF rats. Levels of soluble and insoluble Aβ40 and Aβ42 in the mPFC of each group were measured using enzyme-linked immunosorbent assay **(A–D)**. Protein expression of APP in the mPFC of each group were detected by western blotting **(E, F)**. Bars represent the mean ± SEM. $^{****}P < 0.0001$, PSD group vs. ZDF group; $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$, PDZ group vs. PSD group.

We further detected the expression patterns of APP, where cerebral Aβ production originates. Consistent with the ELISA results, the protein expression levels of APP were markedly significantly increased in the PSD group, and reduced after administration of ZBPYR (**Figures 6E, F**, $P < 0.001$), indicating that ZBPYR reduces Aβ generation and deposition in ZDF rats after chronic psychological stress.

Impact of ZBPYR on Brain Insulin Resistance

The IRS1/AKT/GSK-3β pathway was pivotal to maintenance of the neuronal network and cognitive ability. To evaluate the effect of ZBPYR on insulin signaling in ZDF rats after chronic psychological stress, we detected the expression of insulin signaling factors IRS1 and p-IRS1, AKT, and p-AKT in mPFC by western blotting analysis. No significant changes in total levels of IRS1 and AKT were seen in the three groups (**Figures 7B, E**). However, we observed a significant increase in p-IRS1 levels and a significant down-regulation of p-AKT levels in the PSD group. In the PDZ group, the expression of p-IRS1 was significantly down-regulated and p-AKT was significantly elevated compared to the PSD group (**Figures 7A, C, D, F**, $P < 0.01$). These results indicate that ZBPYR ameliorate cerebral insulin resistance after chronic psychological stress.

GSK3β was a well-known down-stream target of insulin signaling, and we tested the expression of GSK3β in ZDF rats. There was no change in the total level of GSK3β in the mPFC. However, the p-GSK3β expression level was down-regulated in

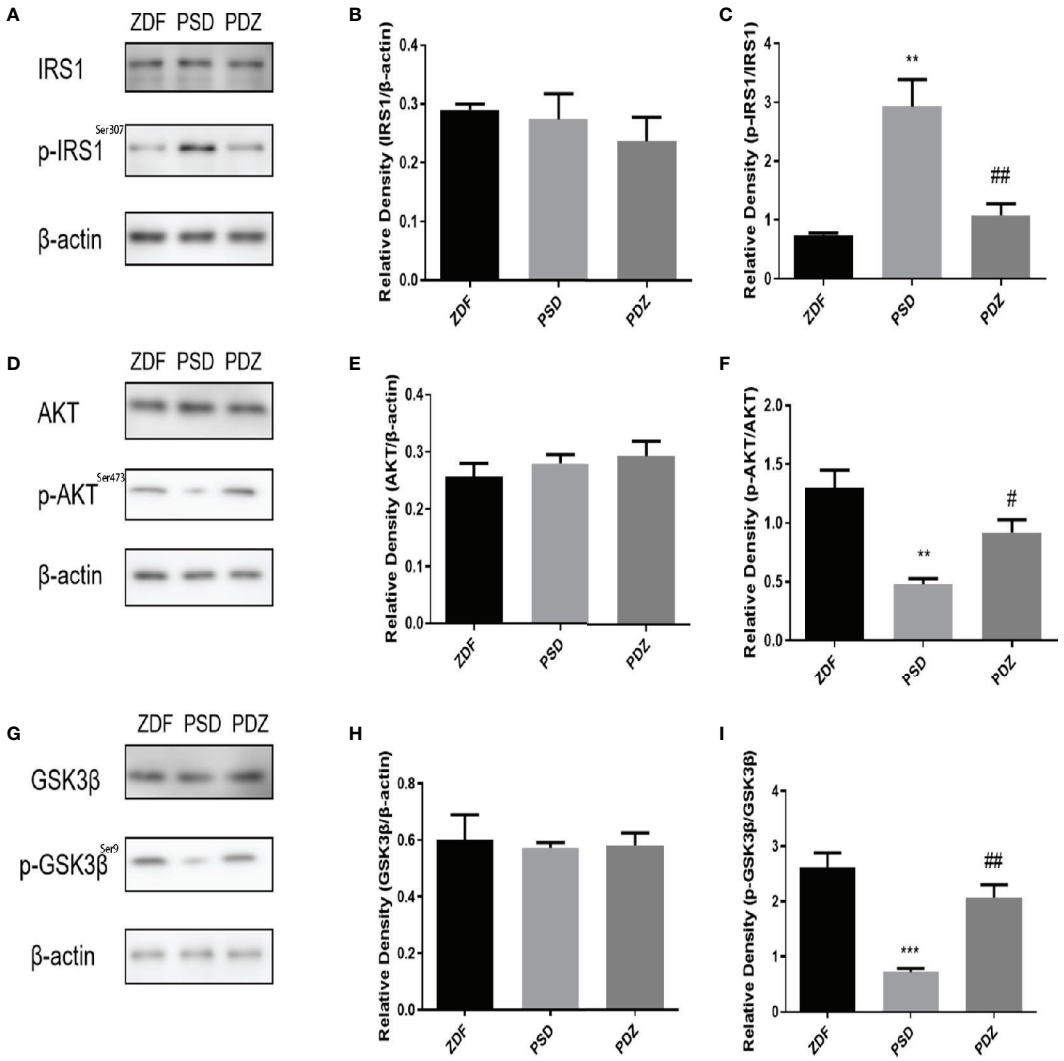

FIGURE 7 | Effect of ZBPYR on cerebral insulin signaling and GSK3β in the mPFC of ZDF rats. Protein expression of IRS1 and p-IRS1 in the mPFC of each group **(A–C)**. Protein expression of AKT and p-AKT in the mPFC of each group **(D–F)**. Protein expression of GSK3β and p-GSK3β in the mPFC of each group **(G–I)**. Bars represent the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, PSD group *vs.* ZDF group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, PDZ group *vs.* PSD group.

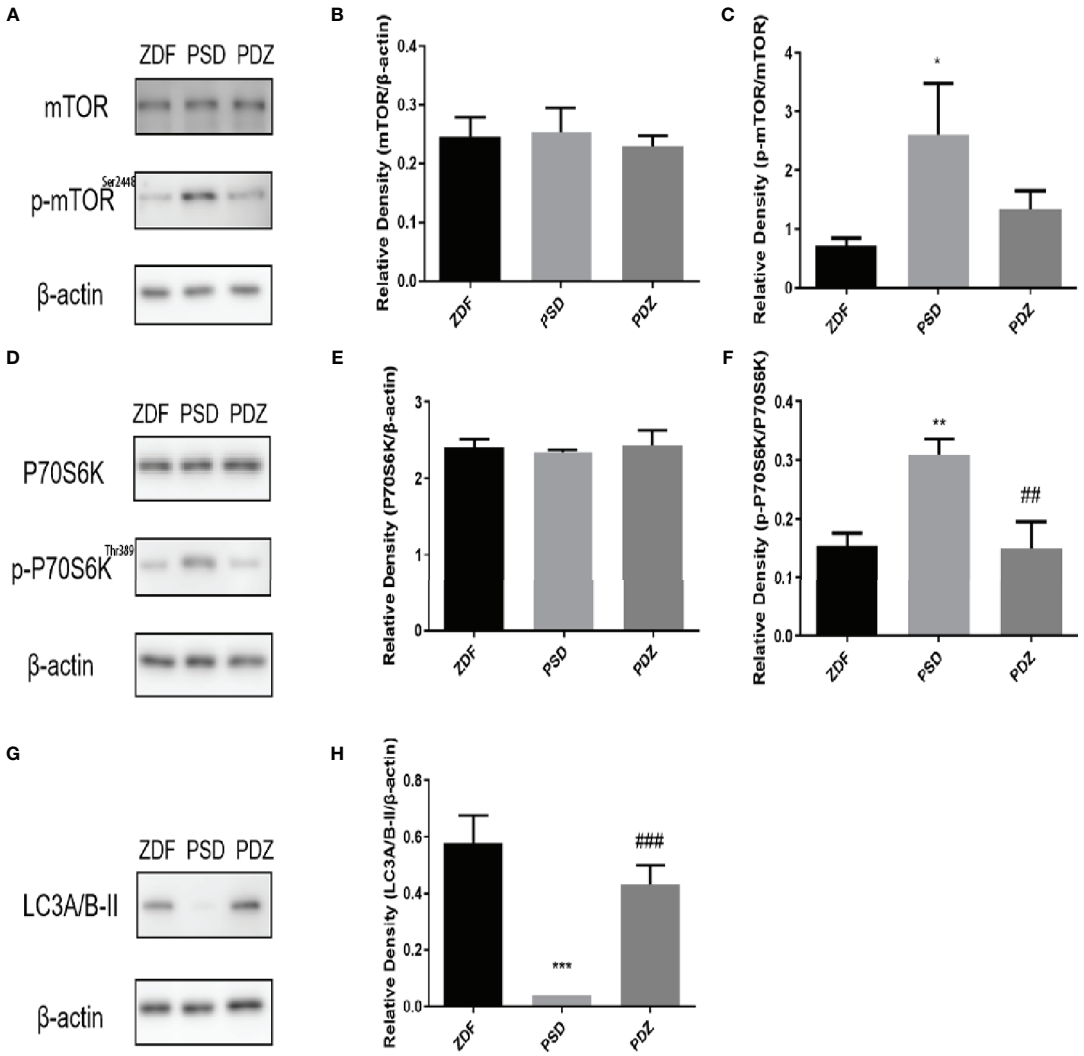

FIGURE 8 | Effect of ZBPYR on the mTOR-autophagy pathway in the mPFC of ZDF rats. Protein expression of mTOR and p-mTOR in the mPFC of each group **(A–C)**. Protein expression of P70S6K and p-P70S6K in the mPFC of each group **(D–F)**. Protein expression of LC3A/B in the mPFC of each group **(G–H)**. Bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, PSD group *vs.* ZDF group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, PDZ group *vs.* PSD group.

the PSD group, and ZBPYR increased p-GSK3β levels compared with PSD group (**Figures 7G–I**, $P < 0.01$).

Impact of ZBPYR on the mTOR-Autophagy Pathway

The mammalian target of rapamycin (mTOR) was an atypical serine/threonine protein kinase that regulated autophagy. mTOR was mainly regulated by IRS1/Akt/mTOR signaling pathway, which played important roles in inhibition of cell apoptosis, promotion of cell proliferation, cell survival, and enhancing cognition. Given the involvement of mTOR -autophagy pathway in the clearance of Aβ, we evaluated the molecular mechanism of chronic psychological stress affected cognitive impairments. As expected, a significant increase in the expression of p-mTOR and p-P70S6K was seen in the PSD group (**Figures 8A, C, D, F**, $P < 0.01$), whereas a decrease in the expression of the autophagosome marker LC3A/B was noted (**Figures 8G, H**, $P<0.001$). ZBPYR treatment inhibited phosphorylation of mTOR and P70S6K and enhanced the expression of LC3A/B. There were no significant changes in total levels of mTOR and P70S6K before and after ZBPYR treatment in the three groups (**Figures 8B, E**).

DISCUSSION

Based on the rapidly increasing prevalence of T2DM and its potential relationship to cognitive decline and dementia, understanding how chronic psychological stress contributes to DACD is of critical public health importance. This

understanding is necessary to determine appropriate clinical interventions that will mitigate the risk for cognitive decline in this population. The high efficiency and low toxicity of traditional Chinese medicine (TCM), which has the advantage of providing multiple therapeutic effects on multiple targets, suggest the feasibility of treatment for DACD. ZBPYR, a traditional Chinese medicine, has been shown to be effective for the treatment of T2DM and DACD according to our previous studies (23, 24). The individual herbs used in ZBPYR have different pharmacologic effects, and some of these compounds may contribute to the effects of ZBPYR in preventing or treating diabetes or providing neuroprotection (25–28). However, the mechanism of ZBPYR in regulating DACD induced by chronic psychological stress is unknown.

Stress refers to a physiological and psychological response to environmental changes and noxious stimuli. Compared with acute stress, chronic psychological stress mainly affects the central sympathetic system (norepinephrine system), and continually and repeatedly destroys homeostasis *via* psychological and physiological reactions to environmental changes and regulation of emotions (29). During chronic psychological stress responses, the hypothalamus-pituitary-adrenal (HPA) axis is activated through the secretion of corticotropin-releasing hormone (CRH) and vasopressin from the hypothalamus. The subsequent release of adrenocorticotropin (ACTH) hormone from the pituitary gland initiates glucocorticoid secretion from the adrenal cortex (30). The major glucocorticoid is cortisol in humans and CORT in rodents. CORT can feed back to the brain and affect subsequent behavioral changes (31). Exogenous glucocorticoids also increase human habits-based learning and memory (32). In vitro experiments have also confirmed that elevated CORT can change neuronal cell function and excitability (33). It is believed that CORT is the most important regulator of stress response, as a key node involved in glucose metabolism of T2DM individuals and regulates islet function, and participates in the negative feedback regulation of HPA axis (34). Therefore, we chose CORT as a functional indicator for HPA axis detection. The activity of the HPA axis, a marker of stress, was increased in the present study, as shown by elevated plasma CORT levels. This finding implies that the procedures used to induce chronic psychological stress in this study were enough to cause anxiety. The mPFC, one of the most affected regions in the brain, modulates the expression of emotion processing (35, 36). Moreover, memory-related brain circuits in the mPFC were shown to be impaired in T2DM and prefrontal atrophy who had been diagnosed (37, 38). According to one study, insulin action in the brain, especially in the mPFC, is selectively impaired in T2DM (39). In the present study, mPFC changed synchronously with chronic psychological stress in this study, which indicates a connection between the stress-related cognitive circuits in this region.

Aβ was reported to be the primary pathogenic factor of AD, and Aβ42 deposition is more obvious in the brain of subjects with cognitive decline (40, 41). Relatively small changes in brain Aβ levels early in life appear to be sufficient to lead to early AD pathology in humans, suggesting that an increase in brain Aβ levels over several years in patients with T2DM may lead to cognitive decline (42, 43). Lesions in the mPFC associated with chronic psychological stress could be attributed to the generation of some signaling pathway, such as impaired Aβ plaque and insulin receptor signaling. The APP gene is best known as the precursor molecule whose proteolysis generates Aβ (44). It was reasonable that we observed incremental increases in levels of Aβ and APP in the mPFC after chronic psychological stress because these types of increases also appeared in monkeys with T2DM (45). The present study showed that Aβ levels significantly increased after chronic psychological stress. One potential mechanism is that the chronic psychological stress experienced by rats in this study increased APP levels *via* the inhibition of APP degradation, thus promoting Aβ generation. ZBPYR decreased Aβ and APP levels, thereby partially improving cognitive function. Unfortunately, we have not been able to detect APP hydrolysis intermediates and secretases, though this may be important to explain the underlying mechanisms of changes in terms of Aβ and APP levels, which will be explored in the future as we continue our in-depth research.

The insulin signaling pathway is considered as the major mechanism that involved in the pathogenesis of T2DM and DACD. Recent epidemiologic evidence suggests that CNS insulin resistance is a risk factor for cognitive decline (46, 47). Downstream targets of the insulin signaling pathway, for example, insulin receptor substrate serine phosphorylation 1, were up-regulated in the brains of T2DM rats (48). AKT, a key marker protein of insulin signaling, mediates the effect of insulin *via* important intracellular signaling cascades including the IRS-1/AKT pathway (49). In the study, we identified an increase in p-IRS1 and a decrease in p-AKT in the mPFC of rats undergoing chronic psychological stress, suggesting that central insulin signaling was impaired. ZBPYR may correct CNS insulin resistance by regulating p-IRS1 and p-AKT. In addition, GSK3β is a downstream substrate of AKT and is the bridge connecting insulin signaling and Aβ (39, 50). This study found that GSK3β activity was improved after ZBPYR treatment, indicating that altered GSK3β was mediated by reduced signaling in the IRS-1/AKT pathway. Moreover, our results indicate that the changes in the brains that are consistent with changes in insulin signaling, as well as changes in Aβ deposition, are likely to contribute to the risk of developing AD pathology and clinical manifestations of the disease over time.

In fact, it is well recognized that mTOR signaling is associated with T2DM and AD pathology in several ways, suggesting its potential role in energy imbalance, learning and memory impairment, and Aβ deposition (51, 52). The upstream signal components, which can interact and modulate the mTOR activity, are phosphoinositide 3-kinase (PI3K)/AKT signaling pathways and glycogen synthase kinase 3 (GSK-3) (53). The major downstream target for mTOR is phosphorylate ribosomal protein S6 kinase (P70S6K). Its phosphorylation level is the parameter used to evaluate mTOR activity (54). Our study showed that chronic psychological stress is able to regulate the P70S6K activation response *via* the mTOR pathway, particularly in the brain of ZDF rats. In addition, autophagy is an intracellular

degradation process, which is essential for cell growth, survival, differentiation, development, and protein homeostasis. A number of mTOR-autophagy modulators have been shown to have positive effects on cognitive decline. The mTOR inhibition triggers autophagy to decrease Aβ and improve T2DM and AD memory impairment (55). LC3, which is associated with the autophagosome from its formation to its maturation into autolysosome, serves as a marker for autophagy (56). LC3-II is the critical form that needs to be focused on. The present study demonstrated that the expression of phosphorylated mTOR in the cortex region after chronic psychological stress was reduced by ZBPYR treatment, which may be one reason ZBPYR decreased the accumulation of Aβ in ZDF rats. Combined with our *in vitro* research results (data not shown), we speculate that the regulation effect of ZBPYR on chronic psychological stress may be achieved by inhibiting mTOR up-regulation, enhancing autophagy, and then promoting Aβ clearance. Taken together, the present findings provide molecular biological evidence for the preventive effects of ZBPYR on DACD caused by chronic psychological stress in rats.

CONCLUSION

In summary, we describe a novel relationship between insulin signaling and Aβ production and accumulation in ZDF rats after chronic psychological stress. Our data suggest that interventions that regulate insulin signaling and Aβ production and accumulation in the brain may provide novel opportunities for treating DACD induced by chronic psychological stress. However, unfortunately, the lack of the negative control group was included to analyze the specific correlation between chronic psychological stress and T2DM disease progression. In particular, future studies should include negative control studies for a better explanation the effects of chronic psychological stress and the role of ZPBYR. In addition, further human studies are required to determine whether Aβ level changes or other variations in the brain could affect insulin secretion or insulin effects, and whether the insulin signaling function in the CNS could modulate insulin effectiveness in peripheral tissues.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

LZ conceived the idea, directed the project, designed the experiments, and involved in modifying the manuscript. TB, WZ, and HS performed the experiments, obtained the samples, and acquired the data. TB conducted the statistical analysis and wrote the manuscript. LZ, WZ, and HS edited the manuscript. All authors contributed to and had approved the final manuscript.

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Effect of Jian-Pi-Zhi-Dong Decoction on the Amino Acid Neurotransmitters in a Rat Model of Tourette Syndrome and Comorbid Anxiety Disorder

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Amino acid neurotransmitters have been shown to correlate with Tourette syndrome (TS) and its comorbidities. In this study, we investigated the effects of Jian-Pi-Zhi-Dong Decoction (JPZDD), a formula containing 10 different Chinese medical herbs, on amino acid neurotransmitters in rats. We established a rat model of Tourette syndrome and comorbid anxiety with an iminodipropionitrile injection plus uncertain empty water bottle stimulation for 3 weeks. Then the rats were randomly divided into four groups: control group and model group were gavaged with saline, while the remaining two treatment groups were gavaged with fluoxetine hydrochloride or JPZDD for four consecutive weeks. We recorded the behaviors of the rats with TS and comorbid anxiety by stereotypy recording, open field test, and elevated plus maze. We observed mitochondrial changes with transmission electron microscopy. We measured the content of glutamate (GLU) and γ-aminobutyric acid (GABA) both in the serum and striatum and the expression of their receptors by Western blot and real-time polymerase chain reaction. The study revealed that JPZDD was effective in alleviating the behavioral symptoms of both tic and anxiety in the rat model groups. These results might be associated with the increase in GABA levels and decrease in GLU levels in the serum, as well as an increase in striatal GABA level by the activation of GABA receptors Type A (GABAAR). JPZDD treatment also reversed the mitochondrial dysfunction both in the striatum and cortex in affected animals.

Keywords: Tourette syndrome, comorbidity, Jian-Pi-Zhi-Dong Decoction, Traditional Chinese Medicine, glutamate, γ-aminobutyric acid, N-methyl-D-aspartate receptor, GABA receptors Type A

Abbreviations: TS, Tourette syndrome; ADHD, attention deficit hyperactivity disorder; JPZDD, Jian-Pi-Zhi-Dong decoction; FLX, fluoxetine hydrochloride; GLU, Glutamate; GABA, γ-aminobutyric acid; GABAAR, GABA receptors Type A; CSTC, cortico-striato-thalamo-cortical; OFT, open field test; EPM, elevated plus maze; IDPN, 3,3-iminodipropionitrile; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene fluoride; RNA, ribonucleic acid; mRNA, messenger ribonucleic acid.

INTRODUCTION

Tourette syndrome (TS) is a neuropsychiatric condition with the manifestation of multiple, spontaneous movements and vocalizations called tics. Normally, TS is diagnosed by the onset of multiple motor tics and vocal tics lasting at least 1 year in clinic. The different tics do not need to present concurrently, but manifest at some point throughout the course of the illness (1). The disorder is usually self-limiting, but may continue into adulthood. TS is also characterized by high rates of psychiatric comorbidities. The prevalence of comorbidities among TS patients is 85.7% (2). Among the comorbidities, around two thirds of patients had attention deficit hyperactivity disorder (ADHD) or obsessive compulsive disorder (OCD), and around one-third of patients had different kinds of mood disorders, such as anxiety, and disruptive behavior (3). The clinical impact of co-occurring conditions may be greater and compromise an extra burden much more than the tics (2). The comorbidities represent greater impairment. Treatment of comorbidities is crucial for all patients with TS, but few studies have fully characterized these comorbidities. The purpose of this study was to determine if Chinese traditional medicine could affect neurotransmitters and mitochondrial function in model rats with TS and comorbid anxiety.

The prevalence of anxiety in TS patients changes greatly depending on the different age level and methodologies used (4). For example, the ratio of TS comorbid generalized anxiety disorder has ranged from 19% to 80%, with high incidence rates in children and youth (5). There is a positive correlation between the degree of anxiety and the degree of the tic; a higher degree of anxiety was associated with more severe tic symptoms (6). Cortico-striato-thalamo-cortical (CSTC) circuits link specific regions in the frontal cortex to subcortical structures (including the basal ganglia). Lower white matter volume in prefrontal cortex can be seen in TS (7). Evidence supports that the involvement of CSTC circuits could provide the feasibility for understanding TS (8). Several neurotransmitters, including dopamine, Glutamate (GLU), and γ-aminobutyric acid (GABA) are active within CSTC circuit and neurotransmitter abnormality has been considered to be relevant in the pathophysiology of tics (9). For example, abnormalities of GABA and GLU have been found to be closely associated with TS (9). When GLU or GABA is released into the synaptic cleft, it binds its receptors in the postsynaptic membrane to exert biological effects. GLU, which is the primary excitatory neurotransmitter, is an essential component of the CSTC pathway implicated in TS. In postmortem examination of TS patients, reduced GLU levels in the brain could evidence the involvement of GLU system dysfunction within CSTC pathways (10). The N-methyl-D-aspartate receptor (NMDAR) is a main but non-exclusive receptor of GLU. Targeting selective subunits of the NMDAR or release-modulating GLU autoreceptors provides a new method to modulate the dysfunction of GLU neurotransmission in TS patients (11). One proposed hypotheses in this study is that the enhancement of neurotransmission at the NMDAR would be beneficial in TS (12). GABA is the primary inhibitory neurotransmitter located in both the striatum and cortex. Once the GLU/GABA balance within the striatum is disrupted, tic-like behavior can cause. So GABA signaling is thought to play a key role in the inhibition control of TS (13). GABA regulates brain excitability *via* its $GABA_A$ receptors. Postmortem examination of a brain with TS has demonstrated that altered GABA receptors Type A (GABAAR) binding within the striatum is involved in the pathogenesis of TS (14). In fact, not only GABA and GLU neurotransmitter but also their various receptors are supposed to potential therapeutic targets of TS and its comorbidities. Major psychiatric illnesses have traditionally been viewed as "neurochemical diseases," these disorders are associated with mitochondrial disorders. The mitochondria are essential for not only energy metabolism but also neurotransmission. The function of mitochondria influences neurotransmission mainly by promoting short- and long-term neuronal plasticity, adjusting cellular resilience to stress and behavioral adaptation (15). New research reports that TS also involves a mitochondrial disorder (16).

Traditional Chinese medicine (TCM) is applied to the treatment of TS and its comorbidities in Chinese clinics. The Jian-Pi-Zhi-Dong Decoction (JPZDD) is derived from a modification of Liu-Jun-Zi-Tang (LJZT) and Xie-Qing-Wan (XQW), which contains 10 ingredients. JPZDD has displayed not only anti-tic properties, but also properties that help treat mood disorders in clinic (17). Although early reports have demonstrated JPZDD could modulate the balance of excitatory and inhibitory neurotransmission in TS rats (18), we have been unable to find any study that investigates the potential effect of JPZDD on mitochondrial function and the neurotransmitters in a rat model with TS and comorbid anxiety. In the present work, our model used 3,3-iminodipropionitrile (IDPN) injection combined with uncertain empty bottle stimulation (19), aiming to verify that the beneficial effects of JPZDD on both tics and anxiety mainly through alleviating brain mitochondrial dysfunction and keeping the balance of neurotransmitters by its receptors.

MATERIALS AND METHODS

Experimental Animals

Male Sprague Dawley rats (n = 48, 3 weeks old, 50 ± 10 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, People's Republic of China; No SCXK 2012-0001). All the experimental animal procedures conformed to the guidelines of the Beijing University of Chinese Medicine Animal Care and Use Committee. All experimental protocols were reviewed and approved by the Animal Experimentation Ethics Committee at the Beijing University of Chinese Medicine (No: BUCM-4-2019042503-2098). All the work tried to minimize suffering. The animals were maintained at 21°C ± 1°C in a standard 12-h light/dark cycle with their environment maintained at the relative humidity of 30% to 40%.

TS and Comorbid Anxiety Model

The rats were provided food and water freely 7 days before the beginning of the experiment. The rats were randomly assigned to the saline group (control group) (n = 12) or the TS and comorbid anxiety model group (model group) (n = 36).The model group was induced by injecting with IDPN (250 mg kg^{-1}) once daily for seven consecutive days (18). The saline group was injected with an equal volume of 0.9% saline (15 ml kg^{-1}) by intraperitoneal injection. The rats in the model group were provided water at regular times (9:00 AM to 9:10 AM, and 9:00 PM to 9:10 PM) simultaneously during the 7 days. The rats in the model group were given an empty water bottle stimulation randomly each day during the watering periods to induce the emotional stress for 14 consecutive days, while rats in the control group were allowed to get purified water freely (20). Twenty-one days later, The TS and comorbid anxiety model group was further divided into three groups by evaluating grades of stereotypy (21): the model group (n = 12), the fluoxetine hydrochloride (FLX) group (n = 12), and the JPZDD group (n = 12). Then, all the rats were fed *via* gavage for four consecutive weeks. Series of behavioral tests were conducted by stereotypy test, open field test, and elevated plus maze. At last, the rats were euthanized.

Drugs and Reagents

JPZDD granules were provided by the Pharmacy Department of the Third Affiliated Hospital of Beijing University of Chinese Medicine identified by Shu Lu. Ten different Chinese medical herbs were included in JPZDD (**Table 1**). A pair of JPZDD granule was dissolved in 50 ml of distilled water, after well-mixed, the solution was stored at 4°C before use. The control and model groups received gavage with 0.9% saline (10 ml kg^{-1}), the FLX-treated group received gavage with FLX (4.2 mg kg^{-1} d^{-1}) (0943A; Patheon France, Jiangsu, People's Republic of China), and the JPZDD group was fed *via* gavage with JPZDD (16 g kg^{-1} d^{-1}) once daily during a period of 4 weeks.

Behavioral Studies

Stereotypy Recording

Stereotypy assessment was evaluated by two trained observers as previously described (21). The observers were blinded to the group conditions, and they recorded the stereotypy data after observation of the rat's behavior. For evaluating the stereotypy, each rat was observed for 2 min after IDPN injection and drug administration at the end of 1, 2, and 4 weeks. We took each observation of the rat from each observer and calculated the mean as the average score and using that as the objective indicator of behavioral alterations.

Elevated Plus Maze

The elevated plus maze (EPM) test was conducted 1, 2, 4 weeks after oral administration. The rats were tested for EPM as described previously (20, 22). The paradigm consisted of a cross-shaped plastic apparatus, elevated 100 cm above the ground, with two opposite open arms (50 × 10 cm^2) and two closed arms (50 × 10 cm^2) surrounded by a black plastic wall, 15 cm tall. During the trial, the rat was placed in the middle of the maze and was allowed to freely explore the new environment for 3 min. The ratio of open arm entries and total arm entries was counted during the test.

Open Field Test

The rats' general locomotor and rearing activity were measured by an open field test (OFT) as described in Zhang et al. (20, 23). It is used to test the anxiety-related behavior. The apparatus was made of a wooden box (75 cm long × 75 cm wide × 40 cm high) with black walls. The floor of the box was divided into 25 equal squares with 1 cm wide black lines. Once the rat was placed at the centre, both locomotor activity (number of line crosses) and rearing activity (standing upright) were manually recorded over a 5-min period. Each rat was tested individually, and only once at the end of 1, 2, and 4 weeks.

Ultrastructural Examination by Transmission Electron Microscopy

Sample preparation was carried out as previously described (24). First, the striatum and the prefrontal cortex tissues (n = 3) were cut into approximately 1-mm^3 pieces and then the samples were fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) overnight at 4°C and osmicated in 1% osmium tetroxide for 2 h at 20°C. The sample was embedded in Epon812 and sectioned using a Leica UC7 ultramicrotome after dehydration using the published methods (24). The photographs of the sections were viewed with Transmission Electron Microscopy (TEM) using (Tecnai G 20 TWIN, FEI) at 200 kV.

TABLE 1 | Contents of JPZDD.

Chinese name	Common name	Family	Weight(g)	Part used
Tai Zi Shen	Pseudostellaria heterophylla	Caryophyllaceae	10	root
Bai Zhu	Atractylodes macrocephala Koidz	Compositae	10	root
Fu Ling	Poria cocos Wolf	Polyporaceae	10	sclerotium
Ban Xia	Pinellia ternata Breit	Araceae	6	tuber
Chen Pi	Citrus reticulata Blanco	Rutaceae	6	peel
Fang Feng	Saposhnikovia divaricate Schischk	Umbelliferae	6	root
Long Dan Cao	Gentiana scabra Bge	Gentianaceae	3	root
Dang Gui	Angelica sinensis Diels	Umbelliferae	10	root
Chuan Xiong	Ligusticum chuanxiong Hort	Umbelliferae	6	root
Gou Teng	Uncaria rhynchopylla Jacks	Rubiaceae	10	stem

Enzyme-Linked Immunosorbent Assays

After the animals were euthanized, the blood was collected from the abdominal aorta and placed in Ethylene Diamine Tetraacetic Acid (EDTA)-coated tubes, kept on ice, and centrifuged at 3000 × *g* for 15 min at 4°C. The plasma was separated from the whole blood and spit into small tubes to store at −70°C. The GABA and GLU levels in plasma were determined by enzyme-linked immunosorbent assays (ELISA) using a commercially available assay kit from Rui-Bo-Ge (Beijing, China). All procedures were performed strictly according to the instructions provided in the kit.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was performed using ESA 5600A HPLC with model 5600A CoulArray Detector-8, and ESA MD-15 column (3.2 × 150 mm, 5 μm). Data were collected and analyzed with ESA Software Version. Separation of analysis was performed using a Waters XterraTM MS column (3.0 × 50 mm, 2.5 μm, Part 186000598), preceded by a pre-column filter (Shiseido, Guard Cartridge, Capcell C18 MG S-5, 4.0 × 10 mm). The mobile phase consisted of methanol (20%), acetonitrile (3.5%), disodium hydrogen phosphate (100 mM) (pH 6.7, adjusted by phosphoric acid). Woking solutions of GABA (G1251; Sigma, St. Louis, MO, USA) and GLU (5835, sigma, St. Louis, MO, USA) (40, 20, 10, 5, 2.5, and 1.25 μg/ml) were used. The column temperature was maintained at an ambient temperature of 40°C. The flow rate was set to 0.6 ml min^{-1}.

Protein Extraction and Western Blot for GABAAR and NMDAR

The left striatum and cortex of rats (n = 3, per group) were homogenized in an ice-cold radio immunoprecipitation assay buffer (RIPA) (C1053; Applygen, Beijing, People's Republic of China) and protease inhibitor (4693124001; Hoffman-La Roche Ltd., Basel, Switzerland), followed by being centrifuged at 10,000*g* for 10 min at 4°C.Then the supernatants was collected. Bicinchoninic acid (P1511; Applygen) was used to quantify the protein concentrations in the supernatants. Proteins (40 μg) were separated with 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After washing with Tris-buffered saline, the PVDF membranes were blocked with 5% skimmed milk powder for 1 h, and incubated overnight at 4°C with the appropriate primary antibody (ab193311 and ab52177; Abcam, Cambridge, UK) at 1:1,000 and 1:3,000 dilution recommended by the supplier. The membrane was then washed three times in Tris-buffered saline with Tween 20, and primary antibodies were detected with secondary antibodies (goat anti mouse IgG, ZB-2305; goat anti rabbit IgG, ZB-2301; ZSGB-BIO, Beijing, People's Republic of China) at a dilution of 1:1,000 and 1:2,000 conjugated to horseradish peroxidase for 1 h at room temperature. The protein bands were visualized and analyzed with Super ECL Plus Detection Reagent (sc-2048, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Real-Time Polymerase Chain Reaction for GABAAR and NMDAR

The total ribonucleic acid (RNA) from the striatum and cortex (n = 6, per group) were isolated with Trizol reagent according to the manufacturer protocol (15596018; Thermo Fisher Scientific, Waltham, MA, USA). GABAAR α2 gene primers were as follows: forward primer, 5′- TGGCTGAACAGAGAATCGGT-3′ and reverse primer, 5′- GGGAAGGGAATTTCGAGCAC-3′. NMDAR1 primers were as follows: forward primer, 5′-TCCGTGGACATCTACTTCCG-3′, and reverse primer, 5′-AGATAAAGGCGTGCAGCTTG-3′. GAPDH primers were as follows: forward primer, 5′- CAACTCCCTCAAGATTGTCAGCAA-3′ and reverse primer, 5′- GGCATGGACTGTGGTCATGA-3′. The basic protocol for real-time polymerase chain reaction (RT-PCR) was carried out as previous described (18). After initial denaturation at 94°C for 10 min, 45 cycles of amplification followed. The cycles were conducted at 94°C for 15 s and at 60°C for 60 s in order to cDNA amplification. At last, the final elongation was at 72°C for 10 min. Then a RT-PCR machine (ABI7500; Thermo Fisher Scientific) was used to detect the SYBR green signal. The PCR products were analyzed by gel electrophoresis and melting curve analysis to confirm specific amplifications. Messenger ribonucleic acids (mRNA) expressions were normalized to GAPDH. Transcript levels were quantified using the 2-ΔΔCt-value method.

Statistical Analysis

Data shown in the article were expressed as the mean ± standard deviation and analyses performed using IBM SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using either one-way analysis of variance (ANOVA) (equal variance) or a Welch's ANOVA (unequal variance) test. Differences were considered to be statistically significant for $p < 0.05$.

RESULTS

Effects of JPZDD on TS and Comorbid Anxiety Models

Stereotypy Recording

According to the criterior of stereotypy observation, the score of rats in the control group were zero. They took on normal activity (21). Compared with the control group, rats in the model group showed abnormal stereotypy behaviors before the treatment ($P < 0.05$). There was no difference between the model group and the treatment groups at week 1. Two weeks after treatment, the stereotypy scores for the JPZDD group showed a significant decrease compared with the model group from week 2 to week 4. ($P < 0.05$, $P < 0.01$). The Stereotypy score for the JPZDD group decreased significantly compared with the FLX group ($P < 0.05$) at week 4 (**Figure 1**).

Elevated Plus Maze Test

The ratio of open arm entries and total arm entries (OE/TE) in the model group decreased significantly compared with the control group from week 1 to week 4 ($P < 0.01$). After the treatment, the JPZDD group showed increased OE/TE compared

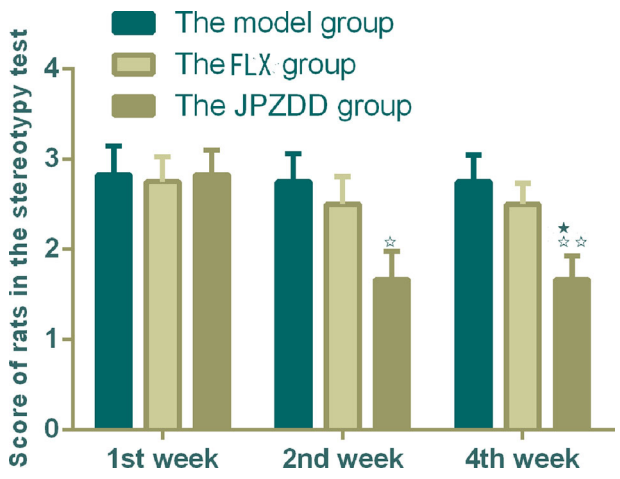

FIGURE 1 | Effect of treatments on the stereotypy behavior score of TS and comorbid anxiety rats. Data are presented as mean ± standard deviation. (n = 12; 1 week: F = 0.03, P = 0.97; 2 weeks: F = 3.36, P = 0.05; 4 weeks: F = 4.56, P = 0.02); ☆☆P < 0.01, ☆P < 0.05 vs. the model group; ★P < 0.05 vs. the FLX group; Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

with the model group at each of the three points ($P < 0.01$). At week 1 and week 4 time points, the ratio of OE/TE increased significantly in the JPZDD group compared with the FLX group ($P < 0.05$) (**Figure 2**).

Open Field Test

The OFT showed that the locomotion/exploratory behavior of the rats in the model group decreased significantly compared with the control group at week 1 and week 4 ($P < 0.01$, $P < 0.05$). The locomotion behavior in both the JPZDD group and the FLX group increased after the treatment, and there was a statistical difference between them at week 2 and week 4 (both $P < 0.05$) (**Figure 3**).

Effects of JPZDD on Mitochondrial Ultrastructure Changes in the Cortex and Striatum of the Brain

The representative ultrastructural micrographs of the prefrontal cortex for each group are shown in **Figure 4**. The organelles were rich. The membrane of the mitochondria in the control group seemed smoothly and clearly; the mitochondrial cristae could be seen clearly and was properly ordered (A1, A2). By contrast, in the model group, the boundary of the nuclear membrane was unclear, the cytoplasm was swollen, and mitochondrial dysfunction could be seen (such as swollen cristae), there were numerous irregular mitochondria, and part of the mitochondrial membrane was absent. Also, the axonal alignment was disordered and swelling could be seen (B1, B2). All treatments reversed these alterations (C1, C2, D1, D2).

The representative ultrastructural micrographs of striatum for each group are shown in **Figure 5**. In the control group, the neuron was rich, the structure normal, and the mitochondrial cristae was clearly visible and properly ordered (A1, A2). In the model group, significantly higher numbers of mitochondrial abnormalities were consistently observed, and the rupture of the double membrane and the destruction of the internal cristae could be seen (B1, B2). The FLX group (C1, C2) and the JPZDD group (D1, D2) experienced a reverse in these alternations after treatment.

Effects of JPZDD on the Expression of Amino Acid Neurotransmitters in Serum

Serum concentrations of GLU and GABA were confirmed by ELISA. The serum levels of GABA in the model group decreased than the control group ($p < 0.01$). The content of GABA in the

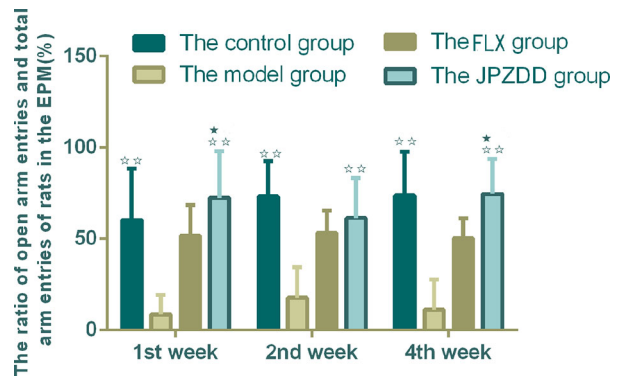

FIGURE 2 | Effect of treatments on the elevated plus maze score of TS and comorbid anxiety rats. Data are presented as mean ± standard deviation. (n = 12; 1 week: F = 6.32, P = 0.00; 2 weeks: F = 7.2, P = 0.00; 4 weeks: F = 7.28, P = 0.00) ☆☆ P < 0.01 vs. the model group; ★ P < 0.05 vs. the FLX group; Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

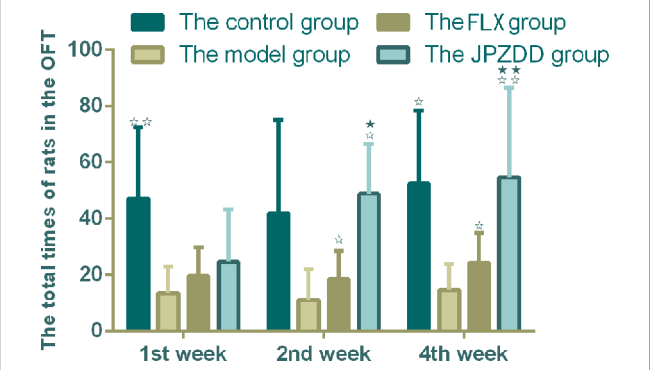

FIGURE 3 | Effect of treatments on the OFT of TS and comorbid anxiety rats. Data are presented as mean ± standard deviation. (n = 12; 1 week: F = 18.06, P = 0.00; 2 weeks: F = 2.08, P = 0.04; 4 weeks: F = 19.38, P = 0.00) ☆☆ P < 0.01, ☆P < 0.05 vs. the model group; ★★ P < 0.01, ★ P < 0.05 vs. the FLX group; Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

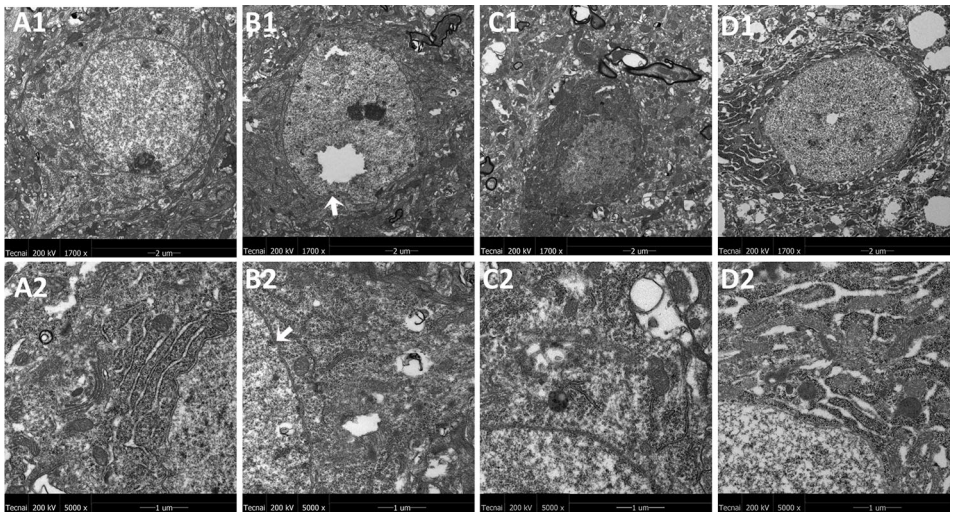

FIGURE 4 | Mitochondria abnormalities identified in the frontal cortex region by transmission electron microscope. n = 3 for each group. Representative image of each group was viewed at 1,700 × (scale bar = 2 μm) and 5,000 × (scale bar = 1 μm) magnifications to detect changes in the frontal cortex mitochondria. A1: Representative image of low magnification (1,700×) image from control group. B1: Representative image of low magnification (1,700×) image from model group; The white arrow indicates mitochondria abnormalities. C1: Representative image of low magnification (1,700×) image from FLX group; D1: Representative image of low magnification (1,700×) image from JPZDD group; A2: Representative image of high magnification (5,000×) image from control group. B2: Representative image of high magnification (5,000×) image from model group; Abnormal mitochondrion is shown by the white arrow. C2: Representative image of high magnification (5,000×) image from FLX group; D2: Representative image of high magnification (5,000×) image from JPZDD group.

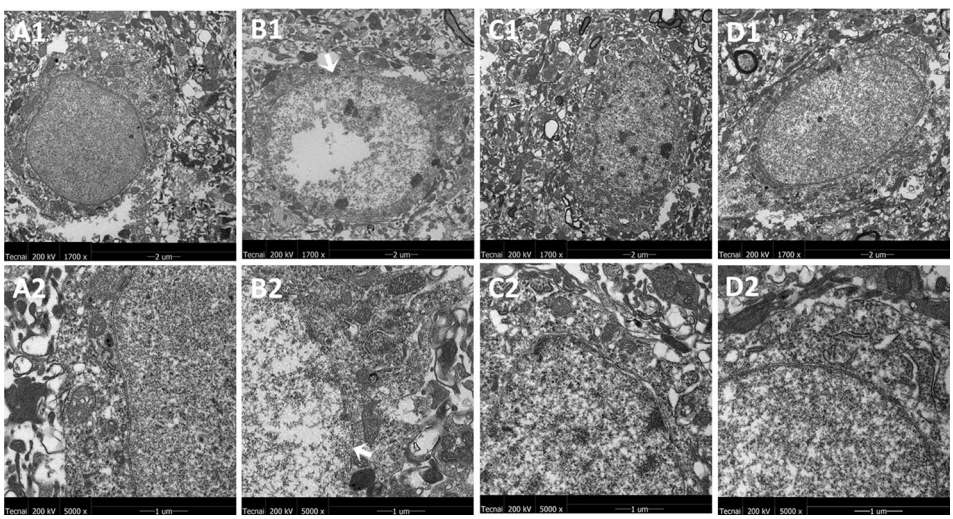

FIGURE 5 | Mitochondria abnormalities identified in the striatum region by transmission electron microscope. n = 3 for each group. Representative image of each group was viewed at 1,700× (scale bar = 2 μm) and 5,000× (scale bar = 1 μm) magnifications to detect changes in the striatum mitochondria. Abbreviations: A1: Representative image of low magnification (1,700×) image from control group. B1: Representative image of low magnification (1,700×) image from model group; The white arrow indicates the rupture of the double membrane. C1: Representative image of low magnification (1,700×) image from FLX group; D1: Representative image of low magnification (1,700×) image from JPZDD group. A2: Representative image of high magnification (5,000×) image from control group. B2: Representative image of high magnification (5,000×) image from model group; "Disrupted" membrane is shown by the white arrow. C2: Representative image of high magnification (5,000×) image from FLX group; D2: Representative image of high magnification (5,000×) image from JPZDD group.

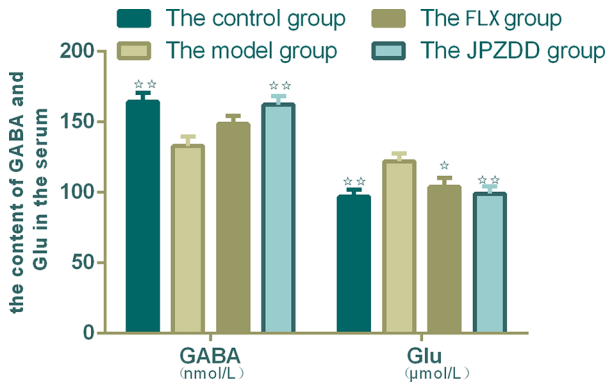

FIGURE 6 | Effect of JPZDD on expression of GABA and GLU in the serum. Data are presented as mean ± standard deviation. n = 10 for the control group, FLX group, and JPZDD group; n = 12 for the model group (GABA F = 5.785, P < 0.01; GLU F = 4.4.54, P < 0.01). ☆☆ P < 0.01, ☆ P < 0.05 vs. the model group; Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

JPZDD group increased significantly than in the model group ($p < 0.01$). GLU concentration in the model group was higher than the control group, and GLU levels in both the FLX group and JPZDD group decreased compared with the model group ($p < 0.05$, $p < 0.01$) (**Figure 6**).

Effects of JPZDD on the Expression of Amino Acid Neurotransmitters in the Striatum

The concentrations of GLU and GABA of the striatum were also confirmed by HPLC. GLU and GABA were well separated with different peak value. Thus, we convinced that this method was specific credible for the measurement of two amino acids. GABA levels in the model group (451.89 ± 77.80) decreased compared with the control group (561.12 ± 92.75, p < 0.05). The GABA levels in the FLX and JPZDD groups showed an elevated trend compared with the model group, but there was no significant difference. And there was no statistical difference in GLU levels among the groups (**Figure 7**).

Effects of JPZDD on the Expressions of GABAAR and NMDAR

The protein expressions of GABAR and NMDAR in the cortex and striatum were confirmed by Western blot (WB) analysis. In the cortex, the expressions of GABAAR and NMDAR in the control group were 1.00 ± 0.00. The level of GABAAR and NMDAR in the model group were lower than the control group (0.47 ± 0.15 and 0.59 ± 0.05, both $P < 0.01$). The JPZDD group demonstrated the attenuated dissipation of GABAAR (0.69 ± 0.14, $P < 0.05$) and NMDAR (0.75 ± 0.08, $P < 0.01$). The difference between the JPZDD group and the FLX group was found in NMDAR test (**Figure 8**). In the striatum, the expression of GABAAR and NMDAR were lower in the model group (0.54 ± 0.02, 0.54 ± 0.07) than the control group (1.00 ± 0.00, 1.00 ± 0.00, $P < 0.01$). After treatment, the expression of GABAAR and NMDAR increased significantly in the JPZDD group (0.91 ± 0.11, 1.02 ± 0.43), compared with the model group ($P < 0.01$, $P < 0.05$). GABAAR expression in the JPZDD group was higher than the FLX group (0.62 ± 0.08, $P < 0.01$) (**Figure 9**).

GABAAR and NMDAR mRNA transcript expressions in the cortex and the striatum were measured by RT-PCR. Standard curves were drawn for the genes. No primer dimers or other PCR products were generated. There was no statistical difference among the groups in the cortex (**Figure 8**). The same trends can be seen in the striatum, but there was still no statistical difference among the groups (**Figure 9**).

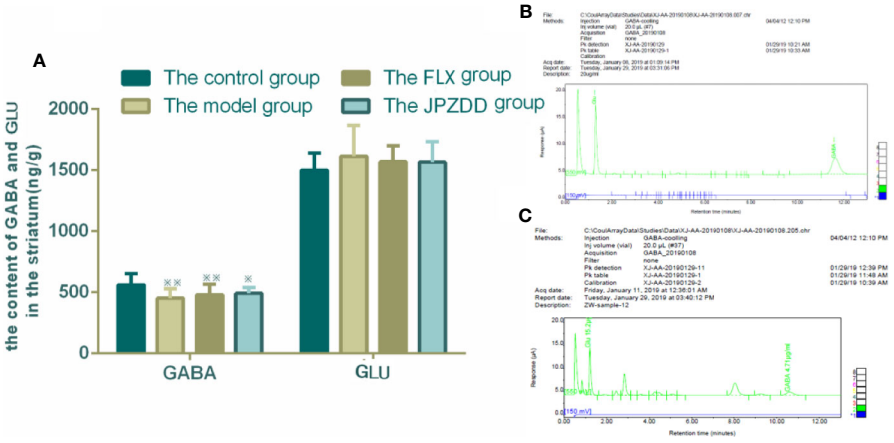

FIGURE 7 | Effect of JPZDD on expression of GABA and GLU in the striatum. Data are presented as mean ± standard deviation. n = 12 for each group (GABA F = 4.19, P < 0.01; GLU F = 0.81, P > 0.05). ※※ P < 0.01,※ P< 0.05 vs. the control group; **(A)** The content of GABA and GLU in the striatum **(B)** Mixed amino acid standards **(C)** A sample of amino acids in brain striatum. Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

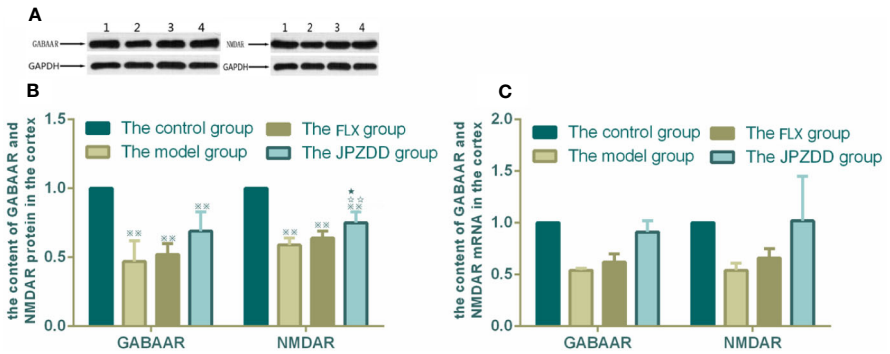

FIGURE 8 | Effects of treatments on the expression levels of GABAAR and NMDAR in the cortex of TS and comorbid anxiety rats. WB and RT-PCR were performed to investigate changes in the expression of GABAAR and NMDAR and the effects of JPZDD on those levels in the cortex. ※※ $P < 0.01$ vs. the control group; ☆☆ $P < 0.01$ vs. the model group; ★ $P < 0.05$ vs. the FLX group. **(A)** representative immunoblots showing the effects of FLX and JPZDD on the expression of GABAAR and NMDAR in the cortex. 1, control group; 2, model group; 3, FLX group; 4, JPZDD group; **(B)** the content of GABAAR and NMDAR protein in the cortex. (n = 3 per group, $F = 36.45$, P=0.01; $F = 34.6$, $P = 0.00$). **(C)** GABAAR and NMDAR mRNA expression in the cortex (n = 6 per group, $F = 0.348$, $P = 0.791$; $F = 0.499$, $P = 0.687$). Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride-treated group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

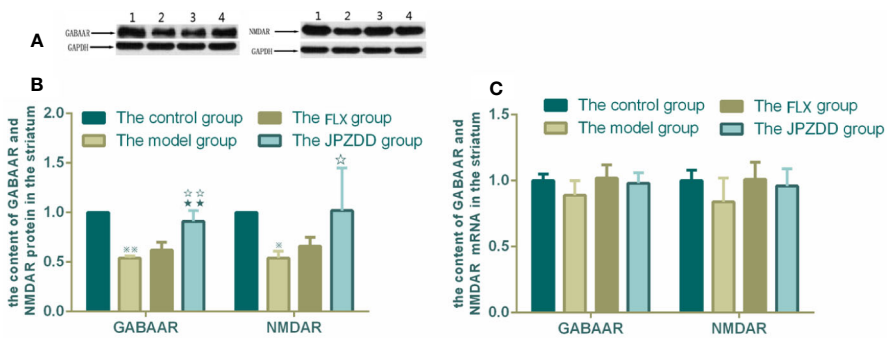

FIGURE 9 | Effects of treatments on the expression levels of GABAAR and NMDAR in the striatum of TS and comorbid anxiety rats. WB and RT-PCR were performed to measure changes in the expression of GABAAR and NMDAR and the effects of JPZDD on those levels in the striatum. ※※ $P < 0.01$, ※ $P < 0.05$ vs. the control group; ☆☆ $P < 0.01$, ☆ $P < 0.05$ vs. the model group; ★★ $P < 0.01$, vs. the FLX group. **(A)** representative immunoblots showing the effects of FLX and JPZDD on the expression of GABAAR and NMDAR in the striatum. 1, control group; 2, model group; 3, FLX group; 4, JPZDD group; **(B)** the content of GABAAR and NMDAR protein in the striatum (n = 3 per group, $F = 30.74$, $P = 0.00$; $F = 39.39$, $P = 0.00$). **(C)** GABAAR and NMDAR mRNA expression (n = 6 per group, $F = 2.62$, $P = 0.08$; $F = 2.07$, $P = 014$). Model group, TS and comorbid anxiety model group; FLX group, fluoxetine hydrochloride-treated group; JPZDD group, Jian-Pi-Zhi-Dong Decoction group.

DISCUSSION

Normally motor and phonic tics are the typical symptoms of TS. Apart from the tics, most patients with TS have associated neuropsychiatric comorbidities. Treatment of comorbid conditions is essential for all patients with TS, but until now, there has been a lack of studies on efficient therapeutic regimens. FLX is selected as the first-line drug to treat TS and comorbid anxiety disorders (25). In addition to being effective for the anxiety, there may be an indirect reduction in tic severity (26). But the adverse effect of FLX was more obvious in childhood, such as the symptoms of decreased food appetite, thirsty, insomnia, et al. Treatment with TCM can provide multiple therapeutic effects on multiple targets, which suggests its feasibility in the treatment of TS and comorbid anxiety.

According to traditional Chinese medicine, the heart houses the mind (shen) and the pathogenesis of TS and comorbid anxiety involves spleen Qi deficiency, liver wind stirring, and heart shen lost (27). Based on this theory, JPZDD was created to reinforce spleen Qi, smooth liver wind, and house heart shen. JPZDD is evolved by two ancient formulas of LJZT and XQW. The main function of LJZT is strengthening spleen Qi while XQW smoothing liver wind and housing heart shen. The compounds in the JPZDD may contribute to the effects of JPZDD in preventing and treating TS and comorbid anxiety. *Gentiana scabra* Bge could clear away the liver wind, and *Pseudostellaria heterophylla* could invigorate the spleen Qi, and they

are the chief drugs. *Atractylodes macrocephala* Koidz, *Poria cocos* Wolf, *Pinellia ternata* Breit, and *Citrus reticulate* Blanco are adjuvant herbs. *Saposhnikovia divaricate* Schischk and *Uncaria rhynchopylla* Jacks, *Angelica sinensis* Diels, and *Ligusticum chuanxiong* Hort are assistant herbs. JPZDD took good effect in both anti-tics and improving the mood condition of patients with TS and comorbid anxiety in clinics. In previous studies, efforts were made to reveal the appropriate dose and the separated formula of JPZDD (28). We tried to find the target of JPZDD. We found that JPZDD played a crucial role in the abnormal behaviors of TS rats by keeping the balance of neurotransmitters in the CSTC circuit (18, 29), but the therapeutic mechanism underlying the activity of JPZDD in treating TS and comorbid anxiety is yet unknown. So in this study, we tried to investigate the function of the protective mechanism and the related mechanisms of JPZDD in model rats with TS and comorbid anxiety.

In our study, we established a TS and comorbid anxiety rat model by IDPN intraperitoneal injection combined with an uncertain empty bottle stimulation. Surface validity and structural validity were verified, and it could be used as an animal model for studying TS and comorbid anxiety (19). Behavioral tests, such as the stereotypy test, OFT, and EPM, were conducted. Stereotypy test is widely used in the evaluation of tic-like behavior, while EPM and OFT are reliable tests for investigation of anxiety-like behaviors in rodents. We found that the severity of stereotypy behavior in the JPZDD group alleviated significantly compared with the FLX group at week 4. This indicated that JPZDD positively correlated with a reduced number of tics. In the OFT test, the locomotion/exploratory behavior of the rats in the model group decreased significantly, while in the EPM test, the OE/TE also decreased in the model group. These indicated the rats in the model group showed anxiety. Our findings indicated that JPZDD significantly reversed the anxiety behavior of TS and comorbid anxiety rats in the OFT and EPM tests. From the results of those behavioral tests, we found JPZDD had a positive effect on both alleviating the degree of tic and anxiety. However, further studies should explore the multi-effect mechanism of JPZDD.

The exact pathophysiology between TS and comorbid anxiety is still unknown; however, they are thought to be partly related to dysfunction in the CSTC (2). The basal ganglia (caudate, striatum, and globus pallidus) play important role in the control of voluntary movements and abnormalities affecting these areas, and they can suffer from a variety of movement disorders, including TS (2). Different cortex areas can produce multiple effects, simple tics can be caused by the abnormal activation of the motor cortex, while complex tics can be associated with the abnormal activation of the premotor cortex. Structural changes in neurons of the striatum can be seen in neuropathological studies. Ablation of fast-spiking interneuron in the dorsal striatum in Tourette syndrome can produce anxiety (30). Several studies suggest psychiatric illnesses can be caused by mitochondrial dysfunction (31). Psychological stress can cause mitochondrial injuries in rats (32). The present study observed the ultrastructure changes of the rat's cortex and striatum. Subtle but significant changes in TEM demonstrated three things: the boundary of the nuclear membrane was unclear, the cytoplasm swelling, and mitochondrial dysfunction (such as irregular mitochondria and imperfect of the mitochondrial membrane) could be seen both in the cortex and striatum of the model group, and both FLX and JPZDD could reverse these alternations. The mitochondrial dysfunction not only impairs energy production, but also affects other key cellular processes. Impaired mitochondrial function might disrupt normal neural plasticity and reduce cellular resilience. And this could promote the development or progression of mood and psychiatric disorders (31). Although the cause of dysfunction in the basal ganglia structures is still obscure, enhancing mitochondrial function may represent a new approach for the development of treatment method for these complex disorders.

Moreover, abnormalities in CSTC circuit with an imbalance of excitatory and inhibitory neurotransmitters seem to have a significant effect on the pathogenesis of TS and its comorbidities (33). Neurotransmitters, including dopamine, GLU, serotonin, and acetylcholine, located within these pathways, are relevant in the pathogenesis of TS and comorbid disorders (8). The influences of dopamine and GLU are widely recognized in TS; however, inhibitory GABA loads which counterbalance to GLU transmission in the central nervous system may also play an influential role in the etiology of TS and comorbid anxiety. The interneurons of GABA are located in both the striatum and the cortex. Our research also pays more attention to the change of GABA in the striatum and prefrontal cortex in mood regulation. Abnormalities in the GABA pathway are postulated to cause the disinhibitive behavior in TS patients. High-anxiety rats had severe disturbances in response to stress owing to changes of neurotransmitters including GABA (34). GLU, the primary excitatory neurotransmitter, is the neurotransmitter of cortical and thalamic projection neurons and the subthalamic nucleus. Arguments supporting the role of the GLU system in TS include its essential role in CSTC pathways and extensive interaction between the glutamatergic and dopaminergic systems. A change of one neurotransmitter often influences the function of other interconnected transmitters significantly. The dynamic change between excitation and inhibition of the excitatory neuron mediates its excitatory neuronal plasticity, firing pattern and excitability, so the change of the neurotransmitters may be a dynamic indicator in the pathophysiology of tics.

Whether TS is associated with a hyper- or hypo-glutamatergic state is still controversial for lack of data support, therapeutic trials must consider both of these possibilities for this reason. Reduced levels of GLU have been identified in TS patients compared with controls. However, using 7T magnetic resonance spectroscopy, GLU level in children with TS was higher than the control group within the striatum and premotor cortex (9). The pathogenesis of TS is complicated including metabolic disturbances in excitatory and inhibitory neurotransmitters (35), and that this imbalance may result in changes of GLU and GABA serum levels. Both GLU and GABA are considered as biomarkers of TS (36). Is the change of amino-acid neurotransmitters in the serum consistent with the change of amino-acid neurotransmitters in the brain? So in this research, we examined the contents of GLU and GABA both in the serum and striatum and tried to reveal the truth. Our studies showed that an IDPN injection caused a significant decrease in GABA levels both in the striatum and serum of the model rats when

compared with the control group. The GLU levels in the serum were higher in the model group than the control group. The neurotoxicity of GLU is mainly expressed in the following ways: damaging the cell membrane ion channels and causing cell edema, reducing mitochondrial functions, disturbing energy metabolism, and causing apoptosis in some cells (37). The alleviation of symptoms of tics and anxiety in TS and comorbid anxiety rats treated with JPZDD could be seen by the trend of increases of GABA levels in the striatum and the serum. The relative increase in GLU levels of the TS and comorbid anxiety exerted a neurotoxic effect, causing an excitatory state, while increased GABA may contribute to a tonic extra synaptic inhibition, interfering with the excitatory/inhibitory balance (18, 38) and improved the injury of the cerebral ultrastructure.

GABA is produced by GABAergic neurons and released at synapses, GABA mediate its brain excitability by the activation of its receptors. Benzodiazepines (which enhance the effects of GABA), are one kind of modulators of GABA A receptors, and are used widely in clinical studies (39). In the studies of TS patients, a reduced number of GABAergic interneurons in the striatum (14) and a decreased binding of $GABA_A$ receptors in postmortem brains (40) indicated TS was associated with a reduced level of brain GABA by its receptors. In rodent and primate models, once the injection of GABA type A receptor antagonists disrupt the GABAergic connectivity, tic-like behaviors can be seen (38, 41). This work identified a decrease of GABAAR in the cortex and striatum of model rats, suggesting the GABA transmission was impaired. In addition, JPZDD exhibited stronger activity in the activation of GABAAR compared with the FLX group, especially in the striatum. A glutamatergic excitatory drive *via* the activation of *N*-methyl-D-aspartate receptor (NMDA) receptors (42). The presence of NMDA receptors on dopaminergic neurons are thought to underlie memory and habit learning (43). Or rather, damaging NMDA receptors did not prevent goal-directed but habitual learning (44). In a transmission disequilibrium study, NMDA receptors were associated with the pathogenesis of TS in Chinese Han trios (39). In this study, we also found that NMDAR was involved in the occurrence of TS and comorbid anxiety. The number of NMDAR available decreased in the model group both in the cortex and striatum. After treatment, the number of NMDAR in the JPZDD group increased significantly differently from the model group both in the cortex and striatum. The number of NAMDAR increased significantly in the cortex among the JPZDD group when compared to the FLX group. In this study, we proposed a hypothesis that the activation of neurotransmission at the NMDAR would be beneficial to alleviate TS symptoms and TS-associated conditions (12). Unfortunately, no change was found in the GLU levels in the striatum. But evidence provides support for the use of GLU modulators (45). Binding neurotransmitters to receptors is important for the activation of a downstream signaling pathway. Therefore, the reason for JPZDD treatment can alter the neurotransmitter levels mainly through the changes of the GABAAR or NMDAR in the striatum or cortex. However, the present study still has some limitations. First, these studies in animal models mainly focused on limited neurotransmitters and its one kind of receptor, other neurotransmitters and receptors remains to be known. Second, JPZDD is a compound prescription, the exact mechanism of each Chinese herb in JPZDD should be validated by fingerprint of traditional Chinese medicine or pharmacokinetics in the future. Third, GLU and GABA are released by distinct neurons, so the structural and functional integrity of neurons is the basis of neurotransmitter release and regulation. Observing the ultrastructure of neurons by immunofluorescence would have important significance. We will continue our research in the future.

In brief, our present results provided new data for the preventive mechanism of JPZDD on TS and comorbid anxiety by molecular, biological methods. We also observed the mitochondrial changes in the rat brain. We found JPZDD exerted favorable effects on the rat model with TS and comorbid anxiety. We concluded that the beneficial effect of JPZDD was associated with keeping the balance of GLU and GABA neurotransmission by regulating its receptor expression and reversing mitochondrial ultrastructure changes. However, the exact function of mitochondria and the contributions of other signaling pathways and inhibitors related to the neuroprotective effects of JPZDD are the subject of ongoing studies.

CONCLUSIONS

Comorbidity, or the presence of more than one disorder in an individual, is a prevalent condition affecting of the global population with TS. The purpose of our study was to determine if traditional Chinese medicine, specifically the compound JPZDD, could ameliorate or lower the presence of physical and phonic tics and comorbid anxiety. The findings of this study suggest that JPZDD can alleviate not only the stereotypy behavior but also anxiety behavior in rats with TS and comorbid anxiety. The related mechanisms might be associated with increases in the GABA level and decreases in the GLU level in the serum, as well as an increase in the striatal GABA level by activating GABAAR. JPZDD treatment also reverses mitochondrial dysfunction, both in the striatum and cortex in affected animals. In summary, the finding of this study provides new ideas into the pharmacological potential for using JPZDD in the treatment of TS and comorbid anxiety.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All the experimental animal procedures conformed to the guidelines of the Beijing University of Chinese Medicine Animal Care and Use Committee. Experimental protocols were approved by the Animal Experimentation Ethics Committee at the Beijing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

WZ contributed to the interpretation of results and writing of manuscript. WY contributed to the interpretation of results and data analysis. XL participated in the study design. QW and XB conducted the experiments. XC and SW reviewed and approved the manuscript.

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Susceptibility to Hyperglycemia in Rats With Stress-Induced Depressive-Like Behavior: Involvement of IL-6 Mediated Glucose Homeostasis Signaling

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Depression is a common psychiatric disorder comorbid with diabetes and may lead to high morbidity, disability, and mortality. However, the underlying mechanism behind their association remains unknown. Cytokine-mediated inflammation in brain may play important roles in the pathogenesis of depression and insulin resistance. In the present study, we subjected the rats to chronic unpredictable mild stress (CUMS) for 3 to 8 weeks. The tests to ascertain depression-like behaviors including open field test (OFT) and forced swimming test (FST) were performed, and levels of morning fasting blood glucose, triglyceride (TG), total cholesterol (CHOL), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C), body weight, food intake, histopathological examinations of liver, adipose tissues and hypothalamus, hypothalamic GLUT4 as well as the IL-6-mediated glucose homeostasis signaling pathway were measured. The results showed that CUMS exposure resulted in the depression-like behavior at various time points in rats. Moreover, the rats exhibited increased peripheral glucose levels, impaired hepatocytes and hippocampal neurons, and decreased hypothalamic GLUT4 levels after 6 weeks of CUMS exposure. Meanwhile, activated IL-6 but suppressed IL-6-mediated glucose homeostasis signaling was observed in the hypothalamus. Markers of lipid metabolism including TG, CHOL, HDL-C and LDL-C were dysregulated, and body weight and food intake were decreased in the CUMS-exposed rats. Our results show that depressed rats induced by 6-week CUMS stimulation display susceptibility to hyperglycemia, which is associated with IL-6-mediated inhibition of glucose homeostasis signaling in the hypothalamus.

Keywords: depression, hyperglycemia, interleukin 6, glucose transporter 4, hypothalamus

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INTRODUCTION

Stress is commonly defined as a real or perceived threat to one's safety (1). It can be categorized as "good stress", "tolerable stress", and "toxic stress". Toxic stress refers to a situation in which a person chronically confronts adverse events that exceed his or her ability to cope with them effectively, resulting in adverse effects on the behavior and physiology of the person (2). Chronic stress generally evokes certain emotional and physiological reactions, and is one of the most important factors responsible for mental disorders in human beings (3). Unfortunately, depression is a highly prevalent chronic stress-induced psychiatric disorder but with limited treatment options and poorly understood pathophysiology. Behavioral impairment due to chronic stress also affects the systemic physiology and has been linked to the metabolic disorders such as diabetes and cardiovascular diseases (4). Emerging evidence demonstrated that depression could be an independent risk factor for the development of diabetes (5). Clinical data have also reported that one out of every four people suffering from type 2 diabetes mellitus (T2DM) also suffers from some extent of depression. In addition, depression also increases the risks of hyperglycemia, insulin resistance, and micro- and macrovascular complications (6). Although the impact of psychosocial stress on energy metabolism is increasingly being recognized, whether the depression-like behaviors induced by long-term chronic stress mediate an individual's susceptibility or resilience to glucose homeostasis remains unknown and the molecular mechanisms underlying the relationship between chronic stress, depression, and glucose homeostasis are yet to be elucidated.

Chronic unpredictable mild stress (CUMS) is highly prevalent in several neuropsychiatric disorders such as depressive disorder in rodent models. CUMS-induced behavioral changes are intended to be homologous to depression, and thus can be used as an experimental tool in understanding the pathology of depression (7). When rats or mice are exposed to chronically mild but unpredictable stressors, several obvious behavioral changes such as decreased response to rewards, increased response to hopelessness, and decreased response to a novelty environment are observed. These behavioral changes correlate with the core symptoms of depression such as anhedonia, despair, and loss of interest, respectively. The CUMS model is commonly used for assessment of antidepressant effects of various therapeutic interventions (8–10). Notably, emerging evidence indicates that animals exposed to chronic stress exhibit metabolic abnormalities including insulin resistance, glucose intolerance, and hyperlipidemia (11), which is in agreement with the clinical reports that stress related depression is highly comorbid with diabetes. However, the effect of CUMS on peripheral glucose levels and its potential mechanism have largely been unexplored.

Abbreviations: T2DM, Type 2 diabetes mellitus; CUMS, Chronic unpredictable mild stress; HPA axis, Hypothalamic–pituitary–adrenal axis; IL-6, Interleukin 6; JAK2, Janus kinase 2; STAT3, Signal transducer and activator of transcription protein 3; IRS-1, Insulin receptor substrates 1; PI3K, Phosphoinositide 3-kinase; GLUT4, Glucose transporter 4; OFT, Open field test; FST, Forced swim test; TG, Triglyceride; CHOL, Total cholesterol; HDL-C, High density lipoprotein; LDL-C, Low density lipoprotein; WB, Western blotting; RT-qPCR, Real-time fluorescence quantitative PCR; SD, Standard deviation.

Compared with individuals without depression, patients with depression have a considerably higher risk of the morbidity and mortality of diabetes, and are greatly affected by diabetes (6, 12). This linkage suggests shared potential biological mechanisms underlying depression and diabetes. It has been proposed that some pathological changes that participate in the process include abnormal hypothalamic-pituitary-adrenal axis (HPA axis) function, inflammation, environmental factors, and autonomic and neurohormonal dysregulation (13). Among these, the overactivation of innate immune system leading to a cytokine-mediated inflammatory response could target the brain, resulting in the increased risk of development of both depression and diabetes (14). Interleukin 6 (IL-6), a cytokine with wide immunological implications, was originally identified as a B cell differentiation factor (15). The interruption of IL-6 signaling plays an important role in the process of insulin resistance and the pathogenesis of T2DM (16). However, the role of IL-6 in insulin resistance seems to be more complex. Many studies have demonstrated that excessive IL-6 is involved in impaired insulin action on the liver and skeletal muscle of mice (17). In contrast, IL-6-deficient mice (IL-6$^{-/-}$) showed that absence of IL-6 leads to the development of inflammation and insulin resistance in the liver (18), indicating that IL-6 might also play beneficial roles in the improvement of insulin sensitivity. Therefore, the role of IL-6 in the development of insulin resistance remains controversial, and might be tissue and activation phase dependent (19). Interestingly, emerging studies have shown that IL-6 is also involved in the regulation of energy metabolism in the central nervous system (20). Several mechanisms of IL-6-induced disruption of insulin signaling have been suggested. Particularly, IL-6 signaling could mediate JAK2-dependent regulation of signal transducer and activator of transcription protein 3 (STAT3). Insulin-induced phosphorylation of insulin receptor substrates 1 (IRS-1)/phosphoinositide 3-kinase (PI3K)-Akt cascades have been shown to be involved in the regulation of the glucose transporter 4 (GLUT4), thus participating in the process of glucose uptake and transport (21). However, whether depression susceptibility to hyperglycemia is associated with IL-6-mediated disruption of glucose homeostasis, is not clear.

Hence, it was hypothesized that stress-induced depressive disorder is likely to contribute to imbalance of glucose metabolism, and that cytokine IL-6-mediated disruption of glucose homeostasis signaling may participate in the process. In the current study, we first investigated the consequences of CUMS on depression-like behaviors. Further, the changes in the energy metabolism including peripheral glucose and lipid metabolism, body weight, and food intake were determined. Finally, we explored the mechanism of susceptibility of CUMS-induced depressive disorder to glucose metabolic disorder in hypothalamus based on IL-6-mediated glucose homeostasis signaling.

MATERIALS AND METHODS

Animals

A total of 80 male Sprague–Dawley rats (SCXK 2012-0001) weighing 180–200 g were obtained from Vital River Laboratory Animal Technology Limited Company (Beijing, China). The animals were maintained under standard laboratory conditions at room

temperature of 25 ± 2°C and humidity 65 ± 5%. The standard 12 h light/dark cycle (light phase 6:00–18:00) was changed only in the course of the stress regime. Food and water were freely available to the rats except when food and water deprivation were applied as a stressor. After an acclimatization period of 7 days, an equal number of rats were randomly allocated to the Control group (n = 40) and CUMS group (n = 40). All experiments and animal care were approved by the Ethics Committee of China Academy of Chinese Medicine Sciences (No. 2016-0012) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The experimental design is shown in detail in **Figure 1**.

CUMS Procedure

The rats were exposed to a CUMS as previously described with modifications (7). The rats were either exposed to CUMS for 3 to 8 weeks or kept as controls. The weekly stress regime consisted of food deprivation for 18 h followed by 1 h of restricted access to food, water deprivation for 18 h followed by 1 h exposure to an empty bottle, swimming in ice-cold water for 5 min, heat stress at 45°C for 5 min, white noise (85 dB) for 5 h, reversed light/dark cycle for 24 h, physical restraint for 3 h, and soiled bedding (200 ml water in 100 g sawdust bedding) for 17 h. The above stress regimes were randomized ensuring that each stressor was not repeated for two consecutive days.

Behavioral Tests

Open Field Test (OFT)

OFT is a well-validated and commonly performed test for general locomotion and exploratory behavior. All animals were placed in the center of an open-field apparatus one by one to explore freely for 5 min before acclimatizing to the new environment. The activities of all the rats in the field were recorded by a video camera mounted above the arena. The total distance travelled and the time spent in the center of the field were recorded and analyzed using the Observer 5.0 software (Noldus, Netherlands) and EthoVision 14.0 software (Noldus, Netherlands).

Forced Swim Test (FST).

The animals were individually placed in a Plexiglas cylinder (50 cm height and 20 cm diameter) filled with water (30 cm depth and 20–25 °C) and allowed to swim for 15 min. Each rat was removed after 15 min, gently dried with a towel, and returned to its home cage. After 24 h, the rat was again placed in the cylinder filled with water and allowed to swim for 5 min. The immobility time, i.e., the time spent by the animal in floating in the water without struggling and making only movements necessary to keep its head above the water level, was recorded and analyzed.

Measurement of Peripheral Glucose, Triglyceride (TG), Total Cholesterol (CHOL), High- and Low-Density Lipoprotein (HDL-C, LDL-C), Body Weight, and Food Intake

Morning fasting blood glucose, TG, CHOL, HDL-C, and LDL-C were determined using an automatic biochemical analyzer (Mindray, China).

The body weight during the acclimatization period was measured prior to the experiment as baseline weight and subsequently measured every week throughout the study. Food intake was monitored every 24 h and determined by subtracting the amount of remaining food including the spilled food at the bottom of the cage from their respective amount on the previous day.

Hematoxylin–Eosin (HE) Staining and Immunohistochemical Analysis

The rats were anesthetized with 3% sodium pentobarbital by intraperitoneal injection and then fixed with 4% paraformaldehyde *via* transcardial perfusion. The liver, abdominal adipose tissues and hypothalamus were isolated and embedded in paraffin. Coronal sections of 5-μm thickness were cut using a rotary microtome (Leica, Wetzlar, Germany). The sections were stained with HE and the pathological changes were observed under a light microscope (Olympus, Tokyo, Japan).

The slices were first dewaxed and retrieved the antigens. Then, the endogenous peroxidases and the nonspecific staining in the tissues were successively blocked using a solution of 3% hydrogen peroxide for 10 min and 5% normal goat serum for 30 min at room temperature, respectively. Afterwards, the sections were incubated with anti-GLUT4 primary antibody (CST, #2213, diluted 1: 100) overnight at 4°C. The sections were next incubated in horseradish peroxidase-conjugated secondary antibody (diluted 1:1,000) for 2 h at room temperature and subsequently incubated in a DAB solution for 6 min. The images of the GLUT4-positive staining were analyzed using the Image-Pro Plus 6.0 software.

Western Blotting (WB) Analysis

The rats were anesthetized with 3% sodium pentobarbital by intraperitoneal injection and their brains were rapidly removed on ice. Subsequently, the hypothalamic tissues were isolated from the brain in ice. All samples were immediately kept in liquid nitrogen and stored at −80°C until further analysis.

Total proteins were extracted from the rat hypothalamus tissues for the western blot analysis. A total of 30 μg protein was loaded on 10% SDS polyacrylamide gel and the resolved protein bands were subsequently transferred onto polyvinylidene difluoride membrane using a standard wet transfer system. The membranes were blocked with 5% nonfat milk at room temperature for 1 h, and subsequently incubated with corresponding primary antibodies [GLUT4, CST, #2213; IL-6, Abcam, #ab9324; P-STAT3 (Tyr705), CST, #9131; STAT3, ProteinTech, #10253-2-AP; P-IRS-1 (Ser307), CST, #2381; IRS-1, CST, #2390; P-PI3K (Tyr458/Tyr199), CST, #4228; PI3K, CST, #4249; Akt, ProteinTech, #60203-2-Ig; β-actin, ProteinTech, #66009-1-Ig] overnight at 4°C. Thereafter, the membranes were washed with TBST for 10 min (three times), incubated with appropriate Horse Radish Peroxidase (HRP) conjugated secondary antibodies at room temperature for 1 h, and then re-washed with TBST for 10 min (three times). The membranes were visualized with an enhanced chemiluminescence reagent (Bio-Rad, USA) on

the ChemiDoc™ Imaging System (Bio-Rad, USA). The relative quantitation was calculated by normalization to β-actin.

Real-Time Fluorescence Quantitative PCR (RT-qPCR) Analysis

Total RNA was isolated using Trizol reagent (Life Technologies, USA) according to a standard protocol. Reverse transcription was performed using a PrimeScript™ RT Master Mix (Takara, Cat.# RR036A) for cDNA synthesis on a Mastercycler® nexus gradient (Eppendorf, Germany) according to the manufacturer's instructions. All the reverse transcription reaction products were amplified with TB Green™ Advantage® qPCR Premix kit (Takara, Cat. #639676) in a total volume of 25 μl on a CFX96 Real-time PCR System (Bio-Rad, USA) according to the two-step cycling parameters: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. The amplification reactions were performed in triplicates. The sequence of the GLUT4, IL-6, STAT3, IRS-1, PI3K, Akt, and GAPDH primers are listed in **Table 1**. Data were collected and expressed as values of threshold cycle. The relative expressions of the target genes were calculated by normalization to GAPDH expression.

Statistical Analysis

All data were analyzed using SPSS 20.0 software and expressed as mean ± standard deviation (SD). One-sample t test and repeated measures ANOVA were used when appropriate. The body weight data and food intake data were analyzed using Repeated-measures ANOVA to determine significant differences considering as the time and stress. A p-value <0.05 was considered statistically significant. All data were analyzed using GraphPad Prism 7.0.

RESULTS

Provoked Depression-Like Behaviors After Exposure to CUMS in Rats

To assess the impact of CUMS on depression behaviors of rats, we measured the motor function, exploration activity, and immobility times of the CUMS group and the control group rats (16). As shown in **Figures 2A–C**, total distance travelled was significantly shorter and the time spent in the center was remarkably reduced in CUMS group as compared to the control group ($p < 0.01$).

In the FST, rats exposed to CUMS stimulation for different time periods showed longer immobility time than rats in the control group (**Figure 2D**, $p < 0.01$).

Impaired Glucose and Lipid Metabolism and Decreased Body Weight and Food Intake in CUMS Exposed Rats

Peripheral glucose and lipid metabolism, body weight, and food intake were compared between the CUMS group and the control group rats. As shown in **Figure 3A**, during CUMS lasting for 3 weeks, glucose levels increased slightly. However, the increase was statistically significant at week 6 when compared to the timed controls ($p < 0.01$), however, a dramatical drop in glucose levels was reported after 6 weeks of consecutive CUMS stimulation.

As shown in **Table 2**, the impacts of our CUMS paradigm on lipid metabolism are contradictory and somewhat confusing. The TG levels were lower in animals with 3-week exposure to CUMS, however, a significant difference was observed only at 4-week CUMS exposure ($p < 0.05$). Similarly, CHOL levels were significantly lower in CUMS-rats when compared to the control rats ($p < 0.05$). The HDL-C levels in the CUMS group were lower than those, on the contrary, LDL-C levels were lower in 3-week group as compared with those of the control rats.

The body weight and food intake were affected throughout as well as after the stress period. The body weight of control rats gradually increased with time, but the body weights of 2-week CUMS-exposed rats statistically decreased when compared with the control group (**Figure 3B**, $p < 0.05$ at 2 weeks and $p < 0.01$ at 3, 4, 5, 6, 7, 8 weeks). Consistently, CUMS made the animals consume less food weekly than corresponding control rats (**Figure 3C**, $p < 0.05$ or $p < 0.01$).

Impaired Hepatocytes and Hypothalamic Neurons but Unaffected Adipocytes in CUMS Exposed Rats

To evaluate the influence of CUMS on the function of hepatocytes, the rat liver tissues were stained using H&E staining. The results of liver histology are demonstrated in

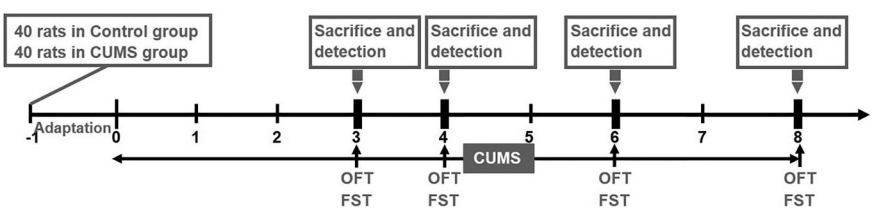

FIGURE 1 | Experimental schedule. Prior to the experiment, a total of 80 rats (n = 40 in control group, n = 40 in CUMS group) were allowed a one-week adaptation period. Rats in the CUMS group received a daily CUMS stimulation, and behavior tests including OFT and FST were performed at various time points (3, 4, 6, 8 weeks). Subsequently, 10 rats from each of the two groups were sacrificed to detect the levels of blood glucose, blood lipids, proteins, and genes at the above time points.

TABLE 1 | Sequence of oligonucleotides used for RT-qPCR.

Gene	Forward primer	Reverse primer	Annealing temperatures
GLUT4	AGCCAGCCTACGCCACCATAG	CAGCAGAGCCACCGTCATCAAG	62°C
IL-6	AGGAGTGGCTAAGGACCAAGACC	TGCCGAGTAGACCTCATAGTGACC	60°C
STAT3	CCAGTCGTGGTGATCTCCAACATC	CAGGTTCCAATCGGAGGCTTAGTG	60°C
IRS-1	AGCAACAGCAGCAGCAGTCTTC	ACTCTTCCGAGCCAGTCTCTTCTC	60°C
PI3K	AACTCGCCTCATAGCAGAGCAATG	TGGCACGCAGTCATGGTTGATC	59°C
Akt	GGCAGGAGGAGGAGACGATGG	TTCATGGTCACACGGTGCTTGG	60°C
GAPDH	CCATTCTTCCACCTTTGAT	TGGTCCAGGGTTTCTTACT	58°C

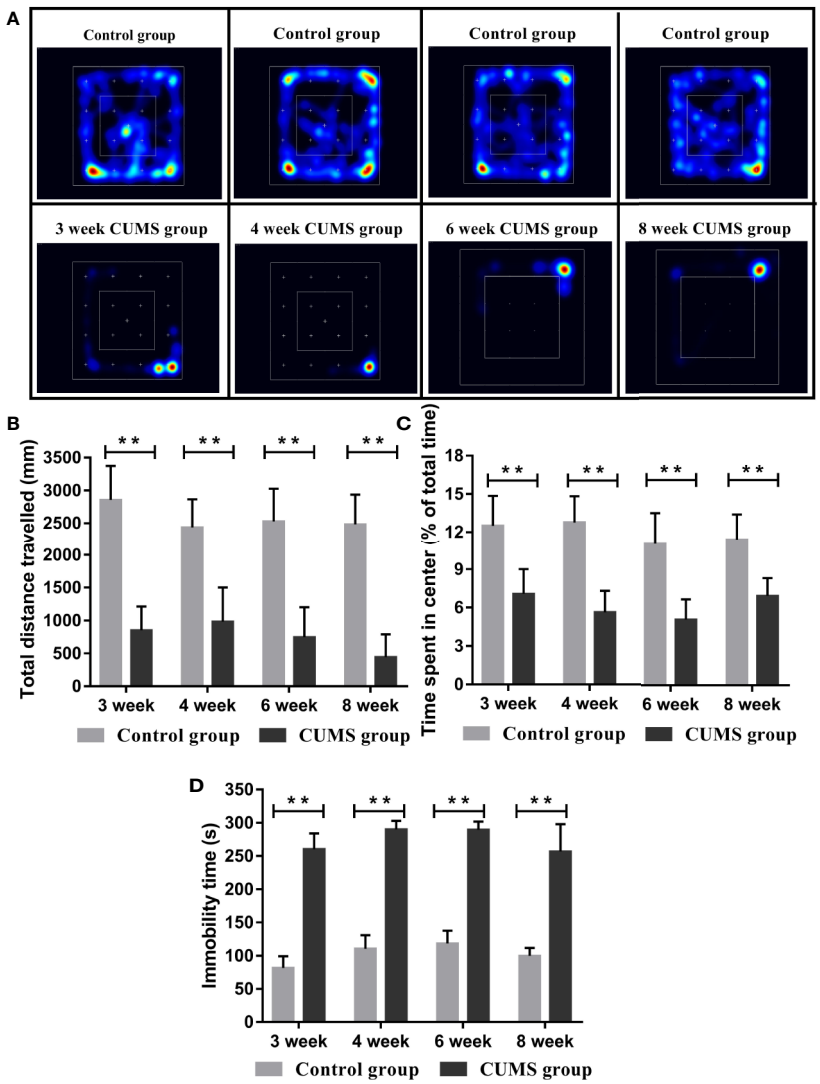

FIGURE 2 | Effect of CUMS exposure on the depression-like behavior of rats. **(A)** Heat maps of the OFT. **(B)** Total distance travelled in the OFT. **(C)** Time spent in the center in OFT. **(D)** Immobility time in the FST. Data are presented as mean ± SD, n = 10; **p < 0.01 vs. Control group.

Figure 4A. In the control group, the hepatocytes showed well-preserved cytoplasm and clear nuclei, which were closely packed and regularly structured. The well-arranged hepatocytes with center nuclei were also seen in the CUMS group; however, enlarged cellular bodies and transparent cytoplasm with ballooning degeneration were also observed.

To confirm whether CUMS affected the size of adipocytes, the rat adipose tissues were stained using H&E staining. The

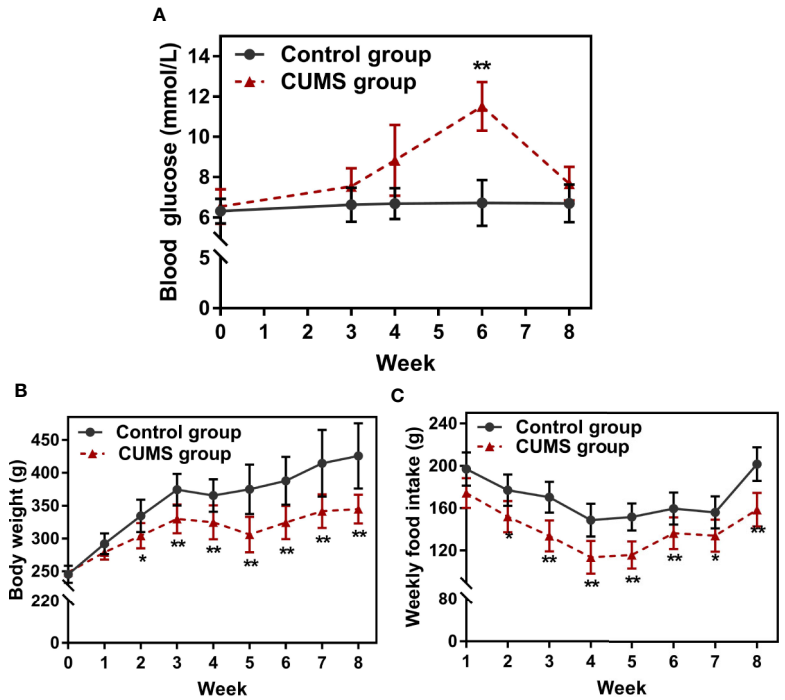

FIGURE 3 | Effect of CUMS exposure on peripheral glucose, body weight, and food intake. **(A)** Fasting blood glucose. **(B)** Body weight. **(C)** Weekly food intake. Data are presented as mean ± SD, n = 10; *p < 0.05, **p < 0.01 vs. Control group.

histological analysis of adipose tissue is shown in **Figure 4B**. Our results showed that the rats exposed to CUMS displayed normal sized-adipocytes similar to that of the control group.

The effects of CUMS on the neuronal injury in arcuate nucleus (ARC) of hypothalamus were also determined using HE staining, as shown in **Figure 5A**. The normal neurons were stained in the control group, which displayed large, round cells with identifiable cell membranes, nuclei and discrete nucleoli. But, the CUMS-induced neurons exhibited cell shrinkage and pyknosis, small-sized and condensed nuclei, even or lack of nucleolus, indicating CUMS damaged the neurons in the ARC of hypothalamus.

Reduced GLUT4 Expression in the Hypothalamus of Rats After 6-Week Exposure to CUMS

To study the effects of CUMS on glucose transport proteins in the brain, we measured GLUT4 protein and mRNA levels in rat hypothalamus. As shown in **Figures 5B, C**, a significant decrease in GLUT4-positive neurons was observed in the ARC of hypothalamus in rats after 6-week CUMS compared to those in the control group (P <0.05), whereas this change was no found in other stressed groups. Moreover, the results of GLUT4 proteins by WB analysis showed a consistent with the increased blood glucose levels by CUMS stimulation. As shown in **Figure 6A**, animals displayed a corresponding decrease in GLUT4 protein expression after CUMS exposure. Then, a reversed trend was observed following 8-week CUMS exposure. But, a statistically significant difference in GLUT4 protein expression was found only between 6-week CUMS-exposed rats compared with the control rats (p <0.01).

Similarly, rats exposed to CUMS for 6-weeks exhibited lower GLUT4 mRNA levels. However, a higher GLUT4 transcript levels were observed in rats exposed to CUMS for 8 weeks. Moreover, the data in 6-week CUMS group alone showed statistically significant difference (**Figure 6B**, p <0.001).

TABLE 2 | Effects of CUMS exposure on TG, CHOL, HDL-C and LDL-C.

Group		TG (mmol/L)	CHOL (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control group		0.318 ± 0.101	1.513 ± 0.240	1.159 ± 0.223	0.285 ± 0.049
CUMS group	3 weeks	0.255 ± 0.085	0.957 ± 0.239**	0.702 ± 0.170**	0.201 ± 0.058**
	4 weeks	0.222 ± 0.085*	1.241 ± 0.271**	0.888 ± 0.210**	0.254 ± 0.051
	6 weeks	0.295 ± 0.107	1.093 ± 0.198**	0.845 ± 0.145**	0.208 ± 0.046**
	8 weeks	0.343 ± 0.125	1.243 ± 0.290**	0.981 ± 0.231*	0.207 ± 0.042**

*Data are presented as the mean ± SD, n = 10; *p < 0.05, **p < 0.01 vs. Control group.*

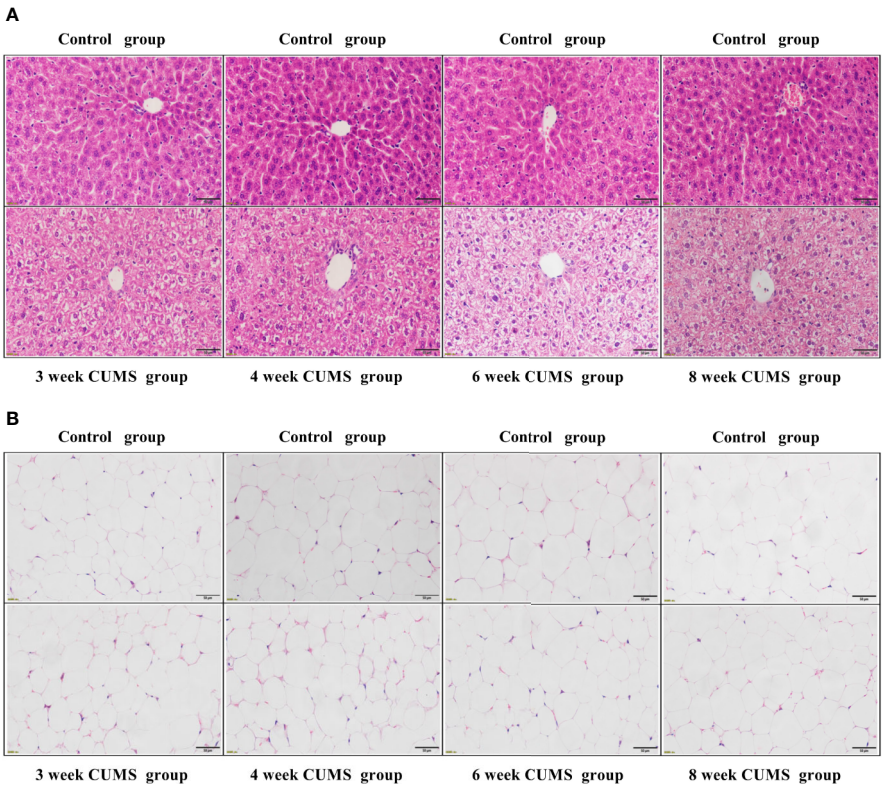

FIGURE 4 | The influence of CUMS on hepatocytes and adipocytes using H&E staining. **(A)** Representative micrographs of rat liver tissues under a light microscope (scale bar = 50 μm, 400× magnification). **(B)** Representative micrographs of abdominal adipose tissues under a light microscope (scale bar = 50 μm, 400× magnification).

Activated IL-6 and Impaired Insulin Signaling Pathway in the Hypothalamus of CUMS Rats

To study the possible mechanism underlying susceptibility of CUMS-induced depression to hyperglycemia, we then detected the hypothalamic inflammatory cytokine-IL-6 and the insulin signaling pathway. As shown in **Figures 7A, B**, the protein and mRNA levels of IL-6 were significantly increased in CUMS-exposed rats compared to the control group rats ($p < 0.01$), suggesting that the expression of IL-6 was activated in the rats in response to CUMS. The results of insulin signaling pathway in the hypothalamus affected by CUMS were displayed in **Figures 7C, D**. The rats exposed to 6-week CUMS showed significantly lower levels of p-STAT3 and STAT3 in hypothalamus than Control group rats ($p < 0.01$). In line with the trend of STAT3 signaling, impairment of insulin signaling was observed in the hypothalamus of CUMS-rats, with significant decrease in the ratio of p-IRS1 to IRS-1 protein levels ($p < 0.01$) and slight decrease in the mRNA levels of IRS-1 as compared to the control group (**Figures 8A, B**). Meanwhile, decreased expression of p-PI3K ($p < 0.01$), PI3K ($p < 0.01$), and Akt ($p < 0.05$) genes were observed in the 6-week CUMS group (**Figures 8C, E**). However, there were no statistical differences in the ratio of p-PI3K to PI3K protein levels and the mRNA levels of PI3K and Akt between the two groups ($p > 0.05$, **Figures 8D, F**).

DISCUSSION

Depression is a common mental health disorder with susceptibility to comorbid diabetes that has high morbidity, disability, and mortality. Accumulating evidences suggest that stress triggers neuroinflammation, which is most likely involved in the pathogenesis of depression comorbid with glucose intolerance (22). However, the roles of CUMS exposure, a common model to induce depression in rodents leading to glucose metabolic disorder, and of cytokine IL-6-mediated disruption of glucose homeostasis signaling in hypothalamus in the pathogenesis of depression comorbid with glucose intolerance, remain unknown. In the present study, rats exposed to CUMS for different periods of time showed susceptibility of depression-like behaviors to hyperglycemia. Importantly, the glucose metabolic disorder including high blood glucose and decreased GLUT4 levels in the hypothalamus were found only in rats exposed to 6-week CUMS stimulation. Furthermore, continuous exposure to CUMS for 6 weeks activated the inflammatory factor IL-6 in the hypothalamus of rats, resulting in the impairment of STAT3 and insulin signaling. This could have led to the reduction of GLUT4

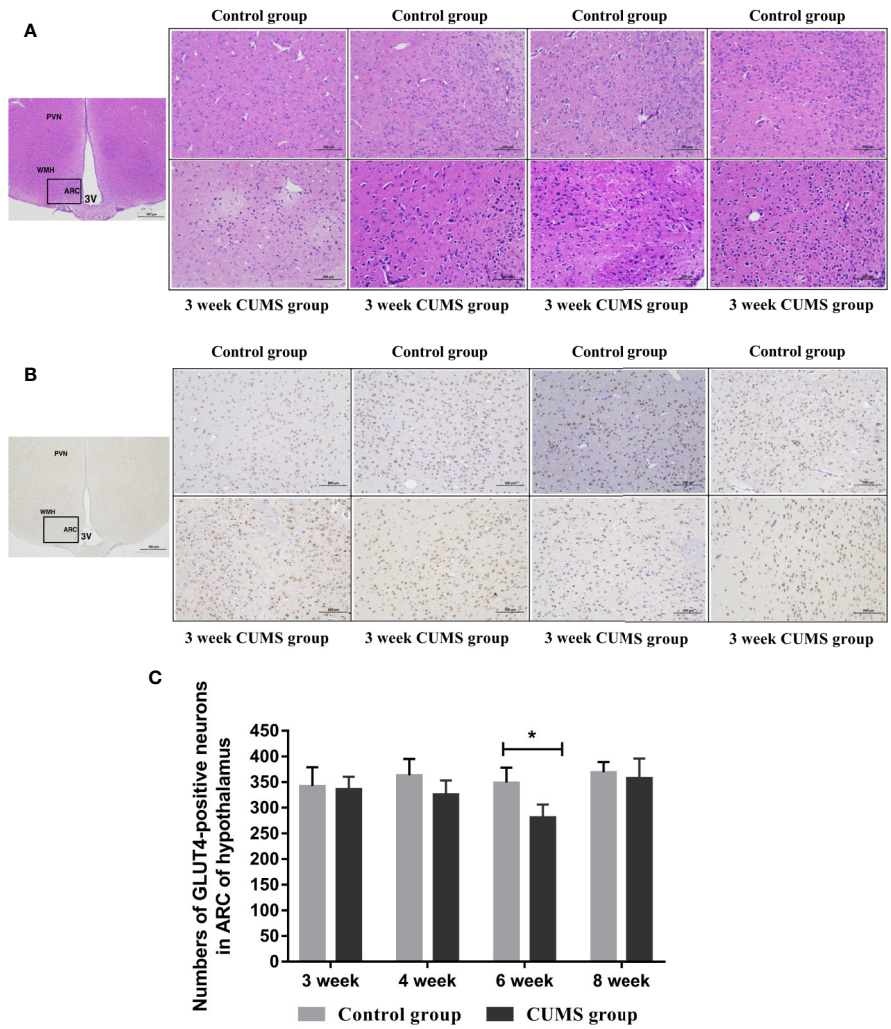

FIGURE 5 | The influence of CUMS on histomorphology and GLUT4-postive neurons in hypothalamus of rats. **(A)** Representative micrographs of neurons in hypothalamus. **(B)** Representative micrographs of immunohistochemical staining for the GLUT4 proteins in the hypothalamus. The first micrographs was captured at low magnification (scale bar = 500 μm, 40x magnification); the remaining micrographs were captured at higher magnification (scale bar = 100 μm, 200x magnification), and observed the ARC of hypothalamus. **(C)** Quantitative analysis of the numbers of GLUT4-poistive neurons in the ARC of hypothalamus. PVN, paraventricular nucleus; VMH, ventromedialnucleus; ARC, arcuate nucleus; 3V, third ventricle. Data are presented as mean ± SD, n = 4 in immunohistochemical analysis; *p < 0.05 vs. Control group.

in the hypothalamus of rats and consequently influence the peripheral glucose metabolism (**Figure 9**). Meanwhile, CUMS stimulation also provoked energy imbalance as evident by abnormal lipid metabolism and significant reduction in body weight and food intake with time.

Depression is a frequent comorbid condition in people with diabetes including both the major diabetes types. Clinical data has shown that one in every four people with T2DM are affected by depression (6). In turn, depression also increases the risk of the development of T2DM, and the subsequent risks of hyperglycemia and insulin resistance. Recent epidemiologic evidence showed that in both, types 1 and 2 diabetes, the depressive symptoms were associated with higher HbA1c, suggesting that depression was closely linked to hyperglycemia (23). In rodents, depression-like behavior could be induced by CUMS, and CUMS-induced depression comorbid glucose intolerant phenotypes were also reported in some studies (24, 25). On the contrary, another study reported lower blood glucose levels in depressive mice (26). Since the information available is inconsistent, this study attempted to reveal the association of CUMS-induced depression with changes in glucose levels. In line with previous reports (27), our study showed that CUMS stimulation for different time periods increased depression-like behaviors, which was shown by less exploration of a novel environment by the rats as evident by a decrease in the total distance travelled and time spent in the center of OFT, and increased response to hopelessness observed by a prolonged immobility time in the FST. Our results showed an initial gradual rise in blood glucose followed by a recovery trend with the extension

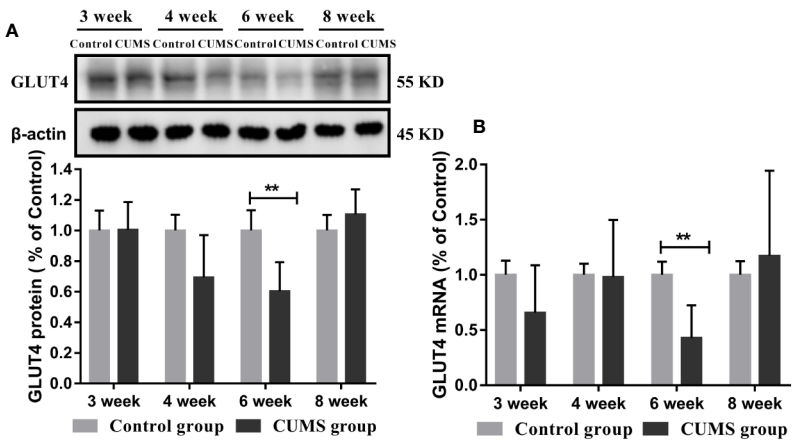

FIGURE 6 | Effect of CUMS exposure on expression of GLUT4 in the hypothalamus of rats. **(A)** Protein levels of GLUT4 by western blot, the representative images for immunoblots are shown in the top panels, and quantitative data are shown in the bottom panels. **(B)** mRNA levels of GLUT4 by RT-qPCR. Data are presented as mean ± SD, n = 6; **p < 0.01 vs. Control group.

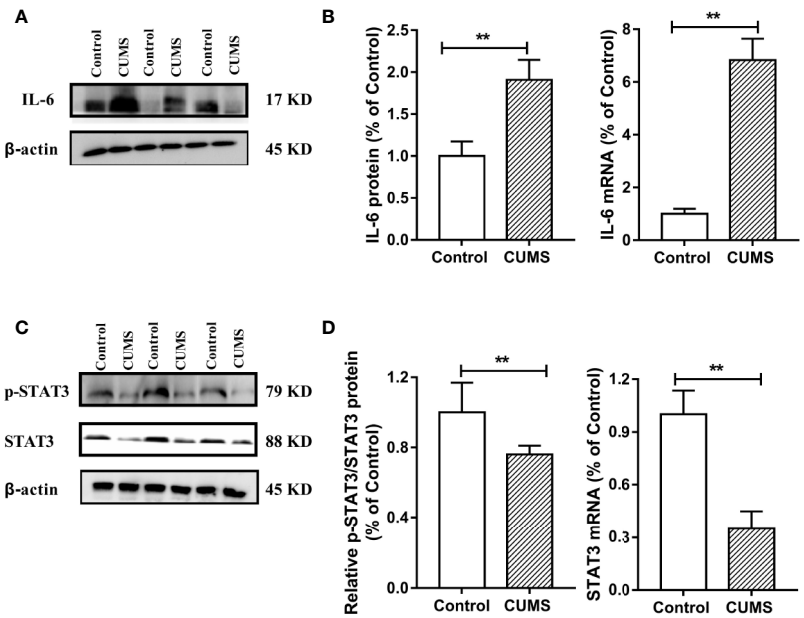

FIGURE 7 | Effect of CUMS exposure on IL-6-mediated STAT3 signaling in the hypothalamus of rats. **(A)** The IL-6 protein representative images for immunoblots. **(B)** Protein and mRNA levels of IL-6. **(C)** The p-STAT3 and STAT3 protein representative images for immunoblots. **(D)** Relative p-STAT3/STAT3 protein and mRNA levels of STAT3. Data are presented as mean ± SD, n = 6; **p < 0.01 vs. Control group.

of CUMS stimulation. However, a significant increase in blood glucose was shown only in 6-week CUMS-exposed rats. This phenomenon that the impact of stress on glucose levels in peripheral blood has been demonstrated in some parallel researches. The research found that glucose levels initially tended to drop for short-time stressed mice but were gradually increased with the prolonged stress time. Then, the peripheral hyperglycemia returned to baseline as the stress was continued (28). Furthermore, one of the primary reasons responsible for the trend may be related to the stress state, in which a temporary insulin resistance is driven by many complex factors such as counter-regulatory hormones like glucagon, cortisol, catecholamines, and the activation of pro-inflammatory factors like tumor necrosis factor-α (TNF-α), IL-6 (29, 30), consequently provoking the hyperglycemia. Therefore, the results indicated that the CUMS exposure, especially for 6 weeks, could induce depression-like behaviors and susceptibility to hyperglycemia in rats. However, unlike the clinical reports that have shown that an increase in the glucose intolerance is often

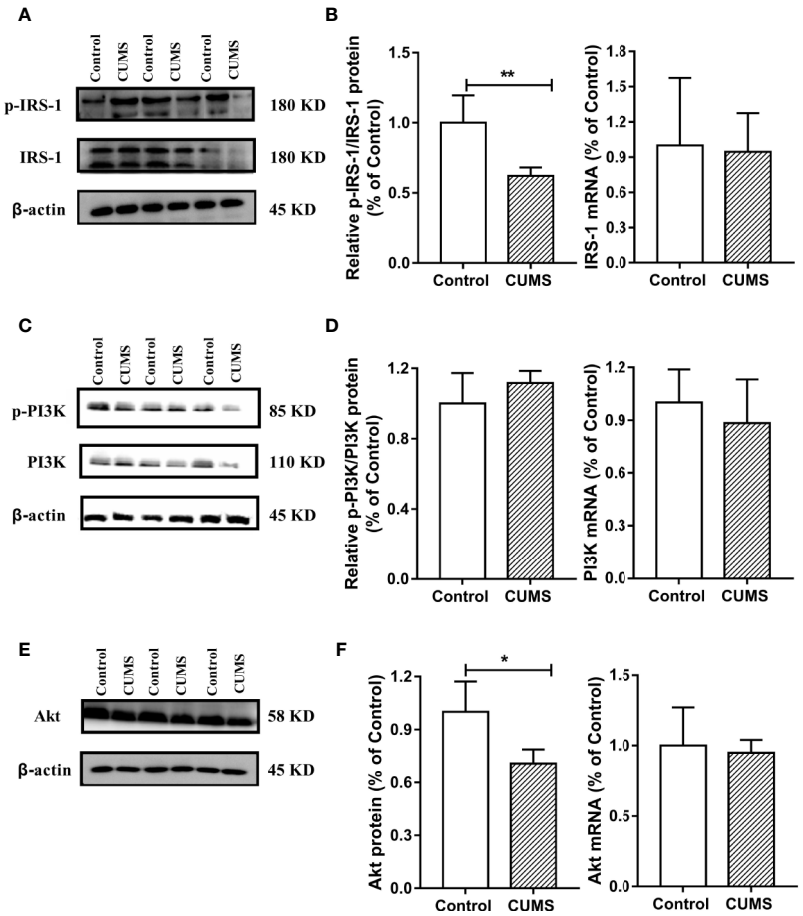

FIGURE 8 | Effect of CUMS exposure on IL-6-mediated insulin signaling in the hypothalamus of rats. **(A)** The p-IRS-1 and IRS-1 protein representative images for immunoblots. **(B)** Relative p-IRS-1/IRS-1 protein and mRNA levels of IRS-1. **(C)** The p-PI3K and PI3K protein representative images for immunoblots. **(D)** Relative p-PI3K/PI3K protein and mRNA levels of PI3K. **(E)** The Akt protein representative images for immunoblots. **(F)** Protein and mRNA levels of Akt. Data are presented as mean ± SD, n = 6; *p < 0.05, **p < 0.01 vs. Control group.

accompanied by abnormal blood lipid metabolism, we obtained inconsistent results for blood lipid metabolism. Although the low HDL-C in CUMS-exposed rats partly reflected the characteristic of hyperlipidemia, decrease in TG, CHOL, and LDL-C levels were inconsistent with hyperlipidemia, and was probably linked to the reduction in food intake and body weight. Additionally, we also found that CUMS impaired the normal hepatocytes, one of the major sites of blood glucose homeostasis. Overall, our results provide evidence for a close association between CUMS-induced depression and hyperglycemia.

The linkage of depression and diabetes reflect that they may share a common biological origin. Chronic cytokine-mediated inflammatory response and overactivation of innate immunity have been extensively studied in the development of depression and diabetes (14, 31). IL-6 is a central cytokine in the regulation of innate immunity produced by a variety of cell types, such as immune cells, skeletal and smooth muscle cells, fibroblasts, microglial cells, astrocytes, and islet β-cells (32). Due to its broad tissue distribution, IL-6 is also involved in non-immune events including pathogeneses of insulin resistance, diabetes, and depression. Numerous studies have provided evidence that IL-6, *via* its actions on insulin sensitive tissues like adipose tissue, liver, skeletal muscle, and pancreatic islets, plays a significant role in the regulation of glucose metabolism (33). Similarly, treatment with IL-6 has been shown to have an effect on insulin signaling and translocation of GLUT4 in the adipose tissue and skeletal muscle, thus influencing glucose metabolism (34). Moreover, accumulating evidence from rodent and human studies suggests that IL-6 mediates the communication between peripheral and central nervous system, thereby playing a key role in the pathophysiology of depression (35, 36). Scientific data has provided evidence that a targeted approach to selectively inhibit IL-6 signaling may offer antidepressant effects (37). Our study further found that CUMS-induced depression and comorbid hyperglycemia activated IL-6 in the hypothalamus, suggesting that overactivation of innate IL-6 could play a crucial role in the pathogenesis of depression susceptibility to hyperglycemia, which is consistent with several previous reports.

STAT3 is one of the transcription factors regulating the production of the cytokine IL-6, IL-10, TNF-α, and IL-1β, which have been shown to be involved in depression (38). Importantly, IL-

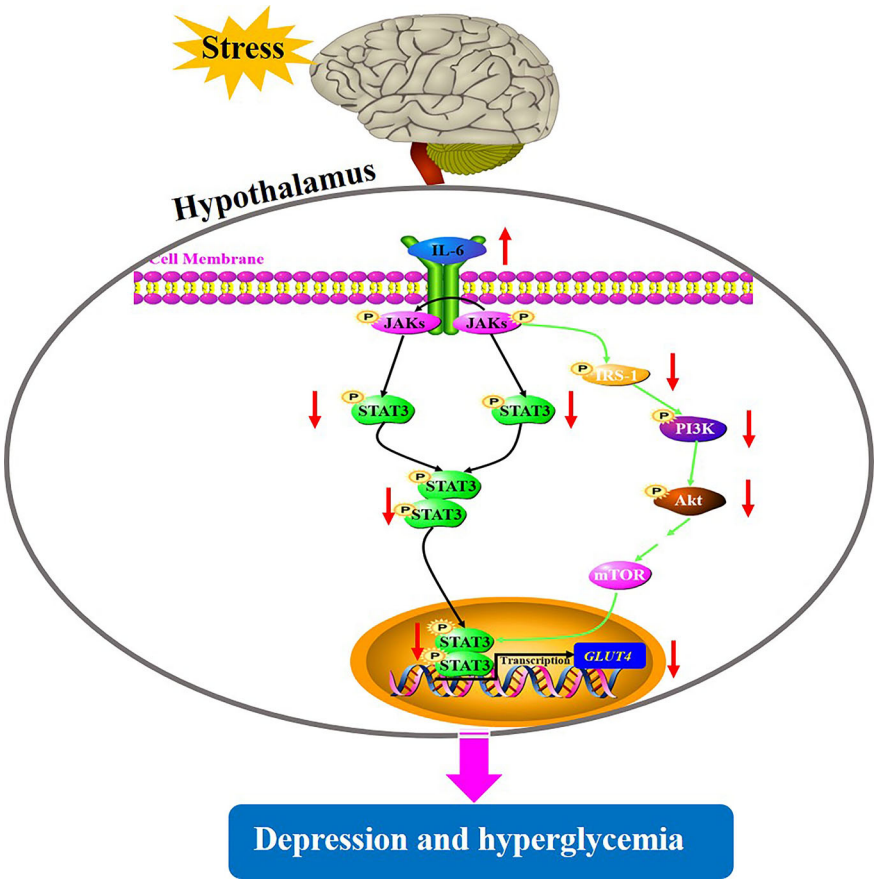

FIGURE 9 | A putative mechanism underlying depression-like behavior susceptibility to hyperglycemia in rats, which is likely to be associated with the activation of IL-6-mediated inhibition of glucose homeostasis signaling in the hypothalamus.

6/STAT3 signaling was shown to regulate the depressive behavior as well as the process of insulin resistance (39). Specifically, IL-6 binding to its receptor activates JAKs which subsequently phosphorylate STAT3, enabling its transport into the nucleus and downstream regulation of transcription of its target genes such as GLUT4, which are involved in glucose homeostasis. The insulin signaling pathway consisted of IRS-1/PI3K-Akt signaling which is a major mechanism underlying the development of diabetes (40). Activated JAK2 also binds and phosphorylates IRS-1, which regulates PI3K activity and subsequent phosphorylation of Akt, consequently participating in insulin signaling (41). Therefore, the dysfunction of glucose homeostasis signaling mediated by IL-6 eventually affects the transcription of GLUT4, partly elucidating the common biological mechanism of depression and diabetes. In this current study, GLUT4 mRNA and protein levels were significantly decreased by CUMS stimulation, possibly due to stimulated IL-6 expression, which induced the above signals. However, there are conflicting views on the influence of IL-6 on STAT3 in the regulation of depression as well as glucose homeostasis. For example, Sun-Ho Kwon and his colleagues knocked out STAT3 in CNS and reported that depression-related behaviors were regulated by cytokines; they further speculated that the inhibition of STAT3 could be a potential therapeutic strategy for depression (42). This result was consistent with many earlier findings (43, 44). On the other hand, conditional inactivation of IL-6 receptor or STAT3 has shown to prevent metabolic disturbance including obesity and insulin resistance (39). In contrast, it has been shown that the introduction of IL-6 improved obesity and decreased glucose tolerance by triggering the phosphorylation of STAT3 (19). These conflicts reports may be related to the characteristics of IL-6 depending on the tissue type. In our study, rats exposed to CUMS for 6 weeks displayed excessive activation of IL-6 and inhibition of STAT3 in the hypothalamus. Therefore, the suppression of STAT3 mediated by activated immune response could be closely associated with depression susceptibility to comorbid diabetes. Similarly, we also observed the IRS-1/PI3K-Akt insulin signal transduction in this study. Consistent with previous reports (45, 46), CUMS exposure inhibited the activation of IRS-1 and its cascade PI3K-Akt signaling, leading to reduced STAT3 in nucleus and consequently reduced transcription of GLUT4. These findings revealed that activation of IL-6 and suppression of its downstream signaling cascade including STAT3 and IRS-1/PI3K-Akt insulin signaling in the hypothalamus may be a potential shared mechanism underlying

depression susceptibility to comorbid diabetes. However, there must be a feedback mechanism to explain why the active IL-6, instead of activating, inhibits its downstream signal transduction. Therefore, further studies are necessary to identify the mechanisms that play important regulatory roles in the IL-6-mediated decreased glucose homeostasis signaling in the hypothalamus of rats.

CONCLUSION

In summary, to the best of our knowledge, this is the first study to show that exposure to CUMS for 6 weeks contributes to concurrent depression-like behaviors and hyperglycemia in rats. The mechanism underlying this model may be related to the activation of IL-6-mediated inhibition of glucose homeostasis signaling in the hypothalamus. Our data reported a decrease in IL-6 downstream signaling including STAT3 and IRS-1/PI3K-Akt insulin signaling in the hypothalamus. We believe that these findings will help to understand the pathology of depression comorbid diabetes from the view of neuroinflammation in brain and to develop novel therapeutic approaches.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of China Academy of Chinese Medicine Sciences (No. 2016-0012).

AUTHOR CONTRIBUTIONS

XL and WQ designed, conducted the experiment and wrote the manuscript. NL helped conduct the animal experiments and behavior tests. XD, QM, YH, TW, and MS helped to collect and analyze the data. JC supervised the experiments and contributed to the final draft of the paper. All authors contributed to the article and approved the submitted version

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Changes of Serum Melatonin, Interleukin-6, Homocysteine, and Complement C3 and C4 Levels in Patients With Depression

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Objectives: Cytokine activation and low complement levels are common in depression patients. This study is aimed at investigating the clinical significance of changes in serum concentrations of melatonin (MT), interleukin-6 (IL-6), homocysteine (hcy), and complement C3 and C4 in depression patients and relationships of them with depression activity.

Methods: A total of 95 depression patients, including first-episode group (n = 43) and recurrent group (n = 52), and 45 age- and gender-matched healthy controls (HC) were recruited. Serum levels of MT, IL-6, hcy, C3, and C4 in all samples were measured by using enzyme-linked immunosorbent assay (ELISA), chemiluminescence method, enzyme circulation method, and immuno-scatter turbidimetric assay, respectively.

Results: The serum MT, IL-6, and hcy levels in the first-episode group (113.08 ± 5.06 pg/ml, 2.06 ± 0.12 ng/L, and 13.87 ± 0.45 μmol/L), and recurrent group (117.63 ± 4.63 pg/ml, 2.20 ± 0.12 ng/L, and 13.61 ± 0.46 μmol/L) were significantly higher than those in the control group (89.50 ± 5.10 pg/ml, 1.57 ± 0.06 ng/L, and 11.34 ± 0.40 μmol/L). The serum levels of C3 in the first-episode group (0.95 ± 0.02 ng/L) were significantly lower than those in the recurrent group (1.05 ± 0.03 ng/L) and control group (1.12 ± 0.03 ng/L). There was no significant difference in serum C4 level between each group.

Conclusion: These results suggest that higher serum MT, IL-6, and hcy levels were correlated with pathogenesis of depression.

Keywords: depression, melatonin, interleukin-6, homocysteine, complement

INTRODUCTION

Depression, also known as depressive disorder, is a kind of mental and psychological disease characterized by listless, depression, and inferiority. Some depression patients may have self-harm or suicidal behavior, and the incidence rate of depression is rising toward younger age (World Health Organization, 2017). The pathological mechanisms of depression involve complicated physiological and psychological factors. Recent researches have reported that monoamine hormones, inflammatory cytokines, neurotrophic factors, and autoimmune markers are most likely to exist as biomarkers of depression (Miller and Raison, 2016), but further research is still needed. Melatonin (MT) is an amine hormone that is secreted from human pineal gland and released into blood. It can modulate the phase of circadian rhythms by binding to MT receptor in hypothalamic suprachiasmatic nucleus. The altered secretion mode of MT causes the disruption of circadian rhythms and then facilitates mood disorder in depression patients (Valdés-Tovar et al., 2018). Furthermore, MT can suppress the release of glutamate and decrease the inhibitory activity of glutamate on brain-derived neurotrophic factor to lower damage in the neurons of hippocampus, improving cognitive function of major depressive disorder (MDD) patients (Treadway et al., 2015). Moreover, a combination of anti-depressant drug buspirone with MT could improve the cognitive function of the major depression disorder patient when compared with the buspirone-alone group and the placebo group (Targum et al., 2015), suggesting the importance of MT in pathogenesis of depression.

However, interestingly, MT can inhibit the expression of IL-6 in hippocampus (Dong et al., 2016). IL-6 is a multifunctional pro-inflammatory cytokine that is produced in response to inflammatory stimuli and secreted by macrophages and monocytes, playing a key role in important biological pathways underlying stress and stress-induced depression (Anderson et al., 2013). Previous studies also have demonstrated that the serum levels of IL-6, interleukin-1β, TNF-α, and other pro-inflammatory cytokines were increased in depression patient under long-term chronic stress (Leonard and Maes, 2012). High level of IL-6 increases the risk of developing late-life depression (Setiawan et al., 2015) and is associated with treatment resistance of depression patients. Vogelzangs et al. (2014) further reported that measuring of plasma level of pro-inflammatory factor IL-6 can reflect the efficacy of antidepressant. Injection of IL-6 into rat amygdala and hippocampus could induce depression-like symptoms (Anderson et al., 2013). These results suggested that change of IL-6 level is important in development of depression and assessment of the efficacy of antidepressant.

Melatonin supplements also can reduce elevated plasma homocysteine (hcy) level. Hcy is a sulfurized amino acid derived from methionine and is crucial in mediating methylation and maintaining the biochemical balance within the central nervous system. A higher hcy level can enhance the oxidative stress, activation of *N*-methyl-D-aspartate (NMDA) glutamate receptor, and inhibition of sodium–potassium ATPase function, resulting in apoptosis of neurons to cause the death of hippocampal neurons in human brain (Lee et al., 2013). High serum hcy level is closely associated with high risk of depression, and lower hcy level can reduce the incidence of depression (Chellappa and Ramaraj, 2009). Hcy treatment could induce occurrence of depression (Ferlazzo et al., 2008). However, whether serum hcy level could be used to diagnose the occurrence and recurrence of depression remains unknown.

Circulating IL-6 can also promote production of acute phase protein such as C-reactive protein (CRP), which can trigger classical pathway of complement (Bilbo and Schwarz, 2012). Complement system plays an important role in innate immune system and inflammation, and alternation of complement components may contribute to the pathogenesis of depression by participating in inflammation (Wei et al., 2017). Complement C3 is the main activator in complement pathways and has a close correlation with brain development, plasticity, and brain dysfunction. Research data from human postmortem brain samples and animal studies have demonstrated an important role for C3 in mediating depressive behaviors (Lanfumey et al., 2013). More importantly, knockout of complement component 3a receptor in chronic stress-induced depression model mice attenuates chronic stress-induced monocyte infiltration and depressive-like behavior (Crider et al., 2018). Similarly, C4 was also found to be involved in the neural synapse elimination and to play a potential role in MDD either by participating in inflammation or regulating neural functions (Bialas and Stevens, 2013). However, whether the complement system could be used for the diagnosis of depression remains unclear. More guidance in the diagnosis and treatment of depression through monitoring the levels of complements is needed.

In this study, we detected five indicators (MT, IL-6, hcy, C3, and C4) in first-episode depression patients, recurrent depression patients, and the healthy group. The results showed that serum MT, IL-6, and hcy levels in the first-episode group and recurrent group were significantly higher than those in the control group, supporting that serum MT, IL-6, and hcy levels were correlated with pathogenesis of depression.

MATERIALS AND METHODS

Subjects

A total of 95 patients with depression were recruited from the Department of Psychiatry of the Second Xiangya Hospital of Central South University between November 2018 and March 2019. The patients were aged 16–65 years old, including 37 males and 58 females (**Table 1**). Meanwhile, 45 healthy controls (HC) were recruited from the health management center of the Second Xiangya Hospital of Central South University. As shown in the **Table 1**, the HC were 23–62 years old, including 17 males and 28 females, and had no mental illness and other family history of hereditary psychosis. The patients were divided into two subgroups: first-episode group (43 cases) and recurrent group (52 cases). These patients are in compliance with the fifth edition of Diagnostic Statistics of Neuropsychiatrics published by the American Psychiatric Association. The inclusion criteria for patients were as follows: (1) the scores from 24 Hamilton Depression Scale (HAMD) greater than or equal to 20 points; (2)

TABLE 1 | Demographic data for depression patients and control group.

	First-episode depression patients	Recurrent depression patients	Control group	t/χ^2	*P* value
Sex	*N* (%)	*N* (%)	*N* (%)	0.032	0.984
Male	15(34.9%)	22(42.3%)	17(37.8%)		
Female	28(65.1%)	30(57.7%)	28(62.2%)		
Total	43	52	45		
Age (mean ± SE)	34.13 ± 2.86	41.73 ± 2.53	40.41 ± 1.71	2.439	0.091

no other mental illnesses and neurological diseases, and there are no obvious abnormalities in examination results of three routine, biochemical, immune, and physical; (3) no history of alcohol abuse; and (4) no immunosuppressants or immunopotentiators used in the past 6 months. The exclusion criteria for patients were as follows: (1) severe physical illness within other tissue and organ; (2) unknown drug usage; (3) five or more drugs were taken in the short term; and (4) pregnant or lactating women. Our study was supported by the Ethics Committee of the Second Xiangya Hospital of Central South University. Informed consent was signed by all patients or their legal guardians and HC.

Sample Collection and Processing

Peripheral venous bloods of all patients and HC were collected from each participant at 08:00 a.m. after overnight fasting. Serum samples were stored at −80°C until analysis after centrifuging at 3000 rpm for 10 min. All samples were thawed to room temperature before testing. Serum MT levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (ArigoBiolaboratories, Taiwan, China). Serum IL-6 levels were measured with electrochemical luminescence in Cobas E602 (Roche, Germany). Serum hcy levels were measured with Hitachi 7600 automatic biochemical analyzer. Serum complements C3 and C4 levels were measured with immuno-scatter turbidimetric assay in automatic specific protein analyzer (Beckman Court).

Statistical Analysis

SPSS v21.0 (IBM) statistical software was used to perform statistical analysis. All values are presented as mean ± standard deviation. Two-group comparisons of the continuous variables were performed using unpaired two-tailed Student's *t* test. One-way analysis of variance (ANOVA) was used to perform multiple-group comparisons. *P* value < 0.05 was considered statistically significant.

RESULTS

Table 1 summarizes the demographic and clinical characteristics of the cases on enrollment. The mean age of the first-episode depression patients was 34.13 ± 2.86 years old, and 34.9% of patients were males. The mean age of the recurrent depression patients was 41.73 ± 2.53 years old, and 42.3% of patients were males. Similarly, the mean age of the control group was 40.41 ± 1.71 years old, and 37.8% were males. There was no significant difference in terms of age and gender between the three groups ($t = 2.439$, $P = 0.091$; $\chi^2 = 0.032$, and $P = 0.984$).

Changes of Serum MT Levels in the First-Episode, Recurrent, and Control Group

The serum MT levels in the first-episode group and recurrent group (113.08 ± 5.06 pg/ml, 117.43 ± 4.63 pg/ml) were significantly higher ($P = 0.027$; $P = 0.009$) than those in the control group (89.50 ± 5.10 pg/ml). However, there was no significant difference in serum MT levels between the first-episode group and recurrent group ($P = 0.644$; **Figure 1**).

Changes of Serum IL-6 Levels in the First-Episode, Recurrent, and Control Group

Serum IL-6 levels in the first-episode group and recurrent group (2.06 ± 0.12 ng/L, 2.20 ± 0.12 ng/L) were significantly higher ($P < 0.038$; $P = 0.008$) than those in the control group (1.57 ± 0.06 ng/L). However, there was no significant difference in serum IL-6 levels between the first-episode group and the recurrent group ($P = 0.508$; **Figure 1**).

Changes of Serum Hcy Levels in the First-Episode, Recurrent, and Control Group

Serum hcy levels in the first-episode group and the recurrent group (13.87 ± 0.45 μmol/L, 13.61 ± 0.46 μmol/L) were significantly higher ($P = 0.009$; $P = 0.019$) than those in the control group (11.34 ± 0.40 μmol/L). However, there was no significant difference in serum hcy levels between the first-episode group and the recurrent group ($P = 0.760$; **Figure 1**).

Changes of Serum Complement Factors C3 and C4 Levels in the First-Episode, Recurrent, and Control Group

Serum complement factor C3 levels in the first-episode group (0.95 ± 0.03 ng/L) was also significantly lower than those the in recurrent group (1.05 ± 0.03 ng/L) and control group (1.12 ± 0.03 ng/L; $P = 0.044$; and $P = 0.003$). However, there was no significant difference in serum complement factor C3 level between the recurrent group and the control group ($P = 0.215$; **Figure 1**). There was no significant difference in serum complement factor C4 level between the first-episode group, recurrent group, and control group (0.24 ± 0.01 ng/L, 0.24 ± 0.01 ng/L, and 0.27 ± 0.01 ng/L; $F = 1.526$, $P = 0.223$; **Figure 1**).

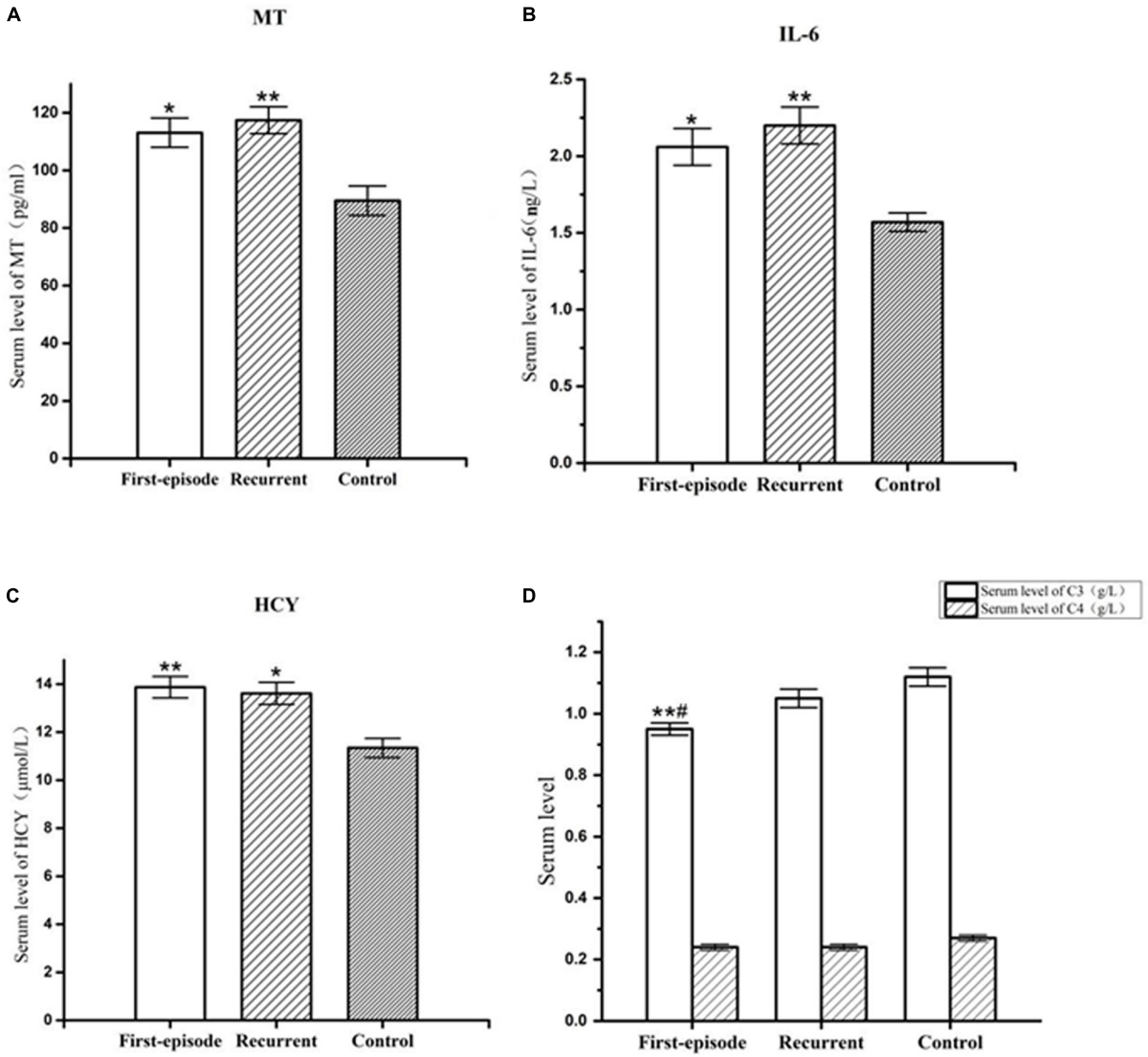

FIGURE 1 | Representative of the serum MT,IL-6, hcy, C3, and C4 levels in first-episode group,recurrent group, and control group. **(A)** Changes of serum MT concentration of patients with first-episode depression (*$P < 0.05$) and recurrent depression (**$P < 0.01$) in comparison to that of control group. **(B)** Changes of serum IL-6 concentration of patients with first-episode depression (*$P < 0.05$) and recurrent depression (**$P < 0.01$) in comparison to that of control group. **(C)** Changes of serum hcy concentration of patients with first-episode depression (**$P < 0.01$) and recurrent depression (*$P < 0.05$) in comparison to that of control group. **(D)** Changes of serum C3 concentration of patients with first-episode depression in comparison to recurrent depression ($^{\#}P < 0.05$) and control group (**$P < 0.01$). There was no significant difference in serum C4 concentration between each group.

DISCUSSION

In this study, we found that the serum levels of MT, IL-6, and hcy in the first-episode group and recurrent group were significantly higher than those in control group, and serum levels of C3 in the first-episode group were significantly lower than those in the recurrent group and control group. The metabolism of MT in peripheral blood could partially reflect depression disorder in the brain (Dmitrzak-Weglarz and Reszka, 2017). Rubin et al. (1992) have found that the concentration of nocturnal MT is significantly elevated in endogenous major depression patients when compared with that of the HC group. Srinivasan et al. also have reported that MT levels were increased in MDD patients. Here, our experiment results showed significantly higher serum MT levels in depression patients in comparison to those in the control group, which is consistent with previous reports. The higher serum MT levels in first-episode depression patients might be attributed to the pharmacological action of antidepressants (Srinivasan et al., 2012). However, Bumb et al. (2016) also found significantly reduced serum MT levels in major depressive patients. The different results may be related to the different time of serum collection or distinct method for measuring of serum MT levels (Bumb et al., 2016). In particular, the serum levels of MT in the recurrent group were significantly higher

than those in the control group. Previous studies reported that antidepressant drugs (fluvoxamine and imipramine) can cause significant elevation of urinary MT metabolites in treatment responders among major depression patients (Miller et al., 2001). In this study, the patients were treated with lexapro, seroquel, and sertraline. Therefore, we speculate that the increased serum MT levels in the recurrent depression patients may also be related to the pharmacological action of antidepressant drugs.

Interleukin-6 is a multifunctional pro-inflammatory cytokine produced by monocytes in periphery and secreted by microglia in the central nervous system (Rothaug et al., 2016) and can cross the blood–brain barrier-deficient areas. Eyre et al. (2016) found significantly higher concentrations of IL-6 in the blood of depressed subjects in comparison with HC. Dunjic-Kostic et al. (2013) also reported that serum IL-6 was significantly elevated in melancholic depressive patients compared to HC. Liu et al. (2012) reported that blood levels of IL-6 were significantly higher in MDD patients than those in HC. In this study, we found that serum IL-6 levels in depressive patients are significantly higher than those in the control group, which is consistent with the results reported by previous studies (Maes et al., 1993; Liu et al., 2012; Vogelzangs et al., 2016). Increased IL-6 activity in severe depression is related to hyperactivity of the HPA axis (Maes et al., 1993). Therefore, we speculate that the increased serum IL-6 levels in first-episode depression patients may be attributed to excessive activation of the HPA axis. Plasma pro-inflammatory factor IL-6 of depression patient who was treated with depressant drugs is higher than that in the control group (Vogelzangs et al., 2016). Therefore, higher serum IL-6 level in the recurrent group compared with that in control group may be due to the use of traditional antidepressant drugs.

Hyperhomocysteinemia can disturb normal neurological function in many ways like DNA damage, oxidative stress, and inflammation, inducing occurrence of depression. Bottiglieri et al. (2000) reported that 52% of severe DSM III depression patients have higher total plasma hcy than the HC group. Patients who were suffering from recurrent MDD have higher hcy levels compared to those in remission (Lok et al., 2014). Elevated serum hcy concentrations are associated with lifetime MDD and particularly with remitted MDD among men (Nabi et al., 2013). Our study found significantly higher serum hcy levels in patients with depression in comparison to control group, which is consistent with previous studies (Bottiglieri et al., 2000; Nabi et al., 2013; Lok et al., 2014). Folic acid and vitamin B12 were confirmed to be administered in depression treatment by decreasing the hcy level. Therefore, the high hcy level in first-episode and recurrent depression patients might be attributed to the deficiency of vitamin B12 and folic acid.

Complement C3 is the pivotal factor in connecting between classical and alternative pathway of complement system and is the most abundant complement factors in serum. Nguyen et al. (2016) reported lower complement expression in the periphery in conjunction with depressive symptoms post-stroke. Here, our results showed that serum C3 levels in the first-episode depression group are lower than those of the control group, which is consistent with the result reported by Nguyen et al. For the decreased serum C3 levels, we speculate that the reason might be that dysfunction of humoral immune, such as inflammation-induced enhancement of B cell function in patients with first-episode depression, led to increased clearance of immune complexes *in vivo*. However, change of C4 levels in our study is not obvious between first-episode depression patients and the control group. We can speculate that the three complement pathways cannot maintain balance in depression patients but the specific disorder mechanism is not detailed (Strenn et al., 2015). Besides, it is worth mentioning that the serum C3 level in the first-episode group is significantly lower than that in the recurrent group, which is presumed to be related to the relief of depressive symptoms after a period of anti-depressive drug treatment because C3 was significantly associated with response to antidepressants (Chan et al., 2016).

Besides, our results also demonstrated that there were no significant differences between first-episodic group and recurrent group in serum MT, IL-6, hcy, and C4 levels. The reasons may be complicated. Therefore, more studies are needed to clarify the underlying mechanism.

CONCLUSION

In summary, our results suggested that MT, IL-6, hcy, and complement factor C3 may be of great importance in pathogenesis process of depression. On the basis of the comparison of the change of serum MT levels in our study with that reported in previous research, we can infer that the disease stage may affect serum MT levels in depressed patients to a large extent. Furthermore, the changes of serum C3 levels between first-episode and recurrent patients may imply that more researches are needed to be done in order to deeply understand the detailed mechanism of complement disorder in patients with depression.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the Second Xiangya Hospital of Central South University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HT and YL were responsible for the design of the experiment, conducting the statistical analysis of the resulting data, and writing, revising, editing, and submitting the manuscript. XC and JF collected the clinical samples and data and conducted the statistical analysis of the resulting data. HZ and QY collected

the literature and wrote the draft. All authors contributed to the article and approved the submitted version.

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frontiers in Genetics

REVIEW
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The Emerging Role of SGK1 (Serum- and Glucocorticoid-Regulated Kinase 1) in Major Depressive Disorder: Hypothesis and Mechanisms

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Major depressive disorder (MDD) is a heterogeneous psychiatric disease characterized by persistent low mood, diminished interests, and impaired cognitive and social functions. The multifactorial etiology of MDD is still largely unknown because of the complex genetic and environmental interactions involved. Therefore, no established mechanism can explain all the aspects of the disease. In this light, an extensive research about the pathophysiology of MDD has been carried out. Several pathogenic hypotheses, such as monoamines deficiency and neurobiological alterations in the stress-responsive system, including the hypothalamic–pituitary–adrenal (HPA) axis and the immune system, have been proposed for MDD. Over time, remarkable studies, mainly on preclinical rodent models, linked the serum- and glucocorticoid-regulated kinase 1 (SGK1) to the main features of MDD. SGK1 is a serine/threonine kinase belonging to the AGK Kinase family. SGK1 is ubiquitously expressed, which plays a pivotal role in the hormonal regulation of several ion channels, carriers, pumps, and transcription factors or regulators. SGK1 expression is modulated by cell stress and hormones, including gluco- and mineralocorticoids. Compelling evidence suggests that increased SGK1 expression or function is related to the pathogenic stress hypothesis of major depression. Therefore, the first part of the present review highlights the putative role of SGK1 as a critical mediator in the dysregulation of the HPA axis, observed under chronic stress conditions, and its controversial role in the neuroinflammation as well. The second part depicts the negative regulation exerted by SGK1 in the expression of both the brain-derived neurotrophic factor (BDNF) and the vascular endothelial growth factor (VEGF), resulting in an anti-neurogenic activity. Finally, the review focuses on the antidepressant-like effects of anti-oxidative nutraceuticals in several preclinical model of depression, resulting from the restoration of the physiological expression and/or activity of SGK1, which leads to an increase in neurogenesis. In summary, the purpose of this review is a systematic analysis of literature depicting SGK1 as molecular junction of the complex mechanisms underlying the MDD in an effort to suggest the kinase as a potential biomarker and strategic target in modern molecular antidepressant therapy.

Keywords: major depressive disorder, SGK1, neurodevelopment, stress, inflammation, neurotrophins, neurogenesis, antidepressant

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INTRODUCTION

Major depressive disorder (MDD) is the most common psychiatric illness and a global public health problem (World Health Organization [WHO], 2020a). MDD is a symptomatically heterogeneous disease characterized by prominent and persistent low mood, loss of interest, low self-esteem, cognitive impairment, volitional decline, and vegetative symptoms, such as disturbed sleep or appetite. Due to these clinical symptoms and the high recurrence rate, MDD is the third leading cause of years lived with disability worldwide and a major contributor to suicide risk (James et al., 2018). It is estimated that about 50% of the 800,000 suicides per year worldwide occur among subjects with MDD, which presents a 20-fold more risk of dying by suicide compared to the general population (World Health Organization [WHO], 2020b; Chesney et al., 2014).

Parental and twin-based studies estimated a 37% chance of heritability, thus suggesting a genetic contribution to MDD (Flint and Kendler, 2014). Candidate gene association studies, widely used over the past 40 years in the genetic analysis of MDD, analyzed more the 100 candidate genes in order to identify the possible associations between their alleles and the risk of depression occurrence. This approach uses genes selected *a priori* based on their biological function and involvement in neurobiological mechanisms underlying MDD (Shadrina et al., 2018). Despite candidate gene association studies on MDD have revealed several suspected risk genes, the results obtained are conflicting. Indeed, even though a clear biological function of the gene may exist, this information may not always be complete, leading to incorrect assumptions about these functions and therefore not always reflecting in an associated outcome. Moreover, these studies do not consider indirect associations as the interactions between gene and environment (Verbeek et al., 2014). The advent of genome-wide genotyping techniques with DNA microchip technology gave the opportunity to conduct genome-wide associations studies (GWASs) with the aim to identify risk factors of depression onset independently from the starting hypotheses and using large sets of samples. However, since MDD is a complex disease arising from the combined effect of many small-size genetic variants, GWAS, identifying single nucleotide polymorphisms (SNPs) across the genome transmitted in linkage disequilibrium with a causative polymorphism, have had notable difficulties in identifying individual associated loci and in replicating significant findings (Bosker et al., 2011). More recent studies have proven moderately successful in the identification of several risk variants significantly associated with MDD, mainly located in genes involved in neurodevelopmental, inflammatory, and oxidative stress processes (Cai N. et al., 2015; Hyde et al., 2016; Okbay et al., 2016; Wray et al., 2018; Coleman et al., 2019; Howard et al., 2019). However, larger sample sizes or genetic isolates are needed to robustly detect specific risk loci given their effect sizes, thus overcoming the population heterogeneity. Furthermore, environmental factors, mainly sexual, physical, or emotional trauma during childhood, are strongly associated with an higher risk (from 2.66 to 3.73 times) of MDD occurrence in adulthood (Li et al., 2016; Nelson et al., 2017). Despite advances in the understanding of MDD pathophysiology, the causative mechanisms underpinning the interaction of environmental, genetic, and epigenetic factors are still far from being clarified. To date, several pathogenetic hypotheses of MDD have been proposed, centering around monoaminergic systems, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, inflammatory/immunological dysfunction, neuroplasticity, or neurogenesis alterations. Although they have been described as distinct pathogenetic hypotheses, they effectively appear to be linked in inducing the pathological phenotype. Haase and Brown (2015) reviewed a large body of published findings and proposed a model in which serotoninergic transmission and neurotrophins signaling are reciprocal interconnected in condition of inflammation-induced depression.

The monoamine-deficiency hypothesis was the first theory that has been proposed about the molecular mechanisms underlying MDD. Many of the antidepressant drugs that are currently used in the treatment of MDD exert their effects by increasing the availability of the monoamine neurotransmitters serotonin (or 5-hydroxytryptamine, 5-HT), noradrenaline (or norepinephrine, NE), and dopamine (DA) in the brain (Pitsillou et al., 2019). Although these drugs affect the neurotransmitter systems within hours after administration, the improvement of symptoms is often evident only after several weeks (4 to 6 weeks) of treatment, and this is a relevant problem in clinical practice (Liu B. et al., 2017).

The delayed efficacy of antidepressants can be explained by the neurotrophic hypothesis, a new molecular theory in the pathogenesis of MDD that does not rule out, but rather strengthens, the previous monoaminergic theory. The neurotrophic hypothesis arises from evidence that revealed an action of antidepressant drugs on neurotrophins, a class of small proteins supporting neural survival in embryonic development and promoting differentiation, enabling axonal growth, driving nerve-growth direction, preserving the survival of mature neurons, and accelerating neurogenesis (Levy et al., 2018). In particular, antidepressant drugs are known to enhance neuronal trophism by counteracting the reduction of axon growth and the abnormalities in dendritic arborization and spine density observed in animal models of depression. This improvement derives from the antidepressant-dependent increase in expression of genes encoding for the neural growth factor (NGF), and other neurotrophic factors, including the brain-derived neurotrophic factor (BDNF), the glial cell-derived neurotrophic factor (GDNF), and the vascular endothelial growth factor (VEGF) (Levy et al., 2018). However, the increase in neurotrophic factors proceeds hand-in-hand (some weeks) with the alleviation of symptoms under antidepressant treatment, thus suggesting that the onset of the pharmacological efficacy is not exclusively related to an increase in the level of monoamines, but may be the consequence of the restoration of a normal neuronal function, resulting from the action of neurotrophic factors (Neto et al., 2011). Moreover, the neurotrophic hypothesis is corroborated by the size restoration of different brain areas involved in controlling mood, such as hippocampus, prefrontal cortex, and nucleus accumbens, after long-term pharmacological treatment (Liu W. et al., 2017). However, post-mortem human studies are often

inconsistent and conflicting with the neurogenic theory. Reif et al. (2006) revealed no differences in the proliferation of hippocampal neural stem cells in brain samples of depressed patients compared to control subjects. Moreover, the proliferation rate appeared to be not modified by antidepressant drug treatment (Reif et al., 2006). On the other hand, some studies showed a significant decrease in hippocampal progenitor cell number of untreated depressed subjects (Lucassen et al., 2010), an effect counteracted by treatments with selective serotonin-reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) (Boldrini et al., 2009; Lucassen et al., 2014). Taken together, these conflicting findings denote that the role of neurogenesis in human depression remains elusive, thereby undermining the veracity of the neurogenic theory.

Another theory about the pathogenesis of MDD is the stress hypothesis based on the hypothalamic-pituitary-adrenal (HPA) axis dysregulation. The HPA axis is an important component of neuroendocrine system consisting of three parts: the hypothalamic area, the pituitary gland, and the adrenal cortex. Briefly, in response to environmental stimuli, neurons of hypothalamic area synthesize and release corticotropin-releasing hormone (CRH), thus promoting synthesis and release of adrenocorticotropic hormone (ACTH) by the pituitary gland, which, in turn, induces the adrenal cortex to produce and to secrete glucocorticoids (mainly cortisol). The HPA axis hyperactivity is an evident clinical manifestation in patients with MDD, showing increased secretion of CRH, ACTH, and glucocorticoids in the cerebrospinal fluid as well as elevated blood levels of glucocorticoids (Black, 2002; Zunszain et al., 2011). High concentration of glucocorticoids can play long-term negative effects, including the malfunction of negative feedback along the HPA axis and the excessive activation of glucocorticoids receptor (GR) in central nervous system (CNS) target cells. The aberrant GR activation leads to neuronal apoptosis and degeneration (Kino, 2015) through the reduction of both BDNF expression and cell proliferation (Chen et al., 2017; Odaka et al., 2017). Moreover, high levels of glucocorticoids are also able to enhance the GR-dependent expression of 5-HT transporter in the hippocampus, frontal cortex, amygdala, and other brain regions, thus inducing a decrease of 5-HT in the synaptic cleft and a further exacerbation of depressive symptoms (Cai S. et al., 2015). However, only approximately 50% of depressed patients exhibit disturbances of the HPA system, thus denoting the high heterogenicity of MDD (Keller et al., 2017).

Increasing evidence from animal studies as well as clinical observations in depressed patients support a role for inflammation and immune dysfunction in the occurrence of MDD. The rationale behind the inflammatory/immune hypothesis derives from the involvement of immune systems in the physiological stress-sensing pathways and its interaction with HPA axis, autonomic nervous system (ANS), and central nervous system (CNS) by a mutual regulation. Animal models of depression revealed how the cytokines, once transported through the blood brain barrier by specific endothelial cell transporter or diffusing through deficient barrier areas, can directly or indirectly affect brain circuits, behaviors, and mood, acting on neurons, astrocytes, and microglia (Kennedy and Silver, 2016; Ambrée et al., 2018). Furthermore, inflammatory signals can converge on CNS through the vagus nerve or by means of infiltrating peripheral immune cells. Animal models also revealed that, regardless of the signaling involved, inflammatory stimuli were able to alter the cellular expression pattern in CNS, thus affecting neurogenesis and plasticity (Hodes et al., 2015). In humans, severe infections as well as autoimmune diseases were described to be closely related with higher risk to develop MDD (Benros et al., 2013). In addition, meta-analyses displayed increased blood expression of pro-inflammatory cytokines and macromolecules such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and C reactive protein (CRP) in patients with MDD (Dowlati et al., 2010; Haapakoski et al., 2015). In a large-scale cohort study, genes belonging to IL-6 signaling have also been described to be increased in peripheral blood cells of patients with MDD compared with healthy controls (Jansen et al., 2016). Moreover, prospective studies showed that increased serum levels of IL-6 during childhood significantly increased the risk to develop MDD and psychosis in young adulthood (Khandaker et al., 2014). An elevated expression of TNF-α, IL-6, and other proteins related to innate immunity, such as interleukin 1β (IL-1β) and toll-like receptors 3 (TLR3) and 4 (TLR4), has been observed also in the brain of patients with MDD (Pandey et al., 2014, 2018). High levels of IL-1β, IL-2, and TNF-α can induce neuronal apoptosis, inhibit neuronal differentiation, abolish synaptic transmission, and counteract either the induction or maintenance of long-term potentiation, thus leading to an impairment of learning and further worsening of depression symptoms (Cai S. et al., 2015). Inflammatory cytokines also cause glucocorticoid resistance and reduction in BDNF expression by blocking the functions of GRs, thus interfering with negative feedback in the HPA axis. This results in a reduction in BDNF brain levels that leads to apoptosis or degeneration of neurons (Jin et al., 2019; Perrin et al., 2019). Moreover, non-depressed patients, treated with cytokines such as IL-2 or interferon-γ (IFNγ) as a part of their therapy for hepatitis virus infection or cancer, developed depressive symptoms (Myint et al., 2009). Conversely, the pharmacological blockade of inflammatory processes by the selective cyclooxygenase 2 inhibitor celecoxib or TNF-α inhibitors had positive effects on depressive symptoms (Köhler et al., 2014; Abbott et al., 2015). Another interesting target is represented by the potassium channel Kv1.3. Its role in glial neuroinflammation and in the pathogenesis of nigrostriatal lesion of Parkinson's disease, multiple sclerosis, and Alzheimer's disease has recently been determined (Wang et al., 2020). To date, its role in the depressive spectrum complex is less clear. However, due to its excitatory role and being an indirect target of SGK1 (Lang and Shumilina, 2013), further experimental evaluations in animal models could provide interesting evaluations of its possible involvement as a diagnostic and therapeutic target in MDD. Finally, additional evidence corroborating the link between inflammation and depression are provided by neuroimaging and brain post-mortem studies that indicated neuroinflammation and microglial activation in the CNS of MDD patients (Enache et al., 2019; Liu et al., 2019).

A new player with diversified genetic and functional roles emerges to bind, like a molecular string, the various pathological

and functional aspects within the intricate natural history of clinical manifestations, hidden below the definition of major depressive disorder (MDD): the serum- and glucocorticoid-regulated kinase 1 (SGK1).

Serum- and glucocorticoid-regulated kinase 1 is a serine/threonine kinase, part of the AGK Kinase family, that displays several homologies with AKT (Protein Kinase B), PKC (Protein Kinase C), and S6K (Ribosomal S6 Kinase) (Frödin et al., 2002; Mora et al., 2004; Bruhn et al., 2010). SGK1 is regulated via different mechanisms by insulin, cAMP (Perrotti et al., 2001; Boito et al., 2005; Menniti et al., 2005), IGF-1 (Insulin like growth factor-I) (Boini et al., 2008), steroids (Chen et al., 1999), IL-2 (Interleukin-2) (Amato et al., 2007), and TGFβ (Transforming growth factor-beta) (Lang and Voelkl, 2013) and has been depicted as a pivotal convergence point for peptide and steroid hormone regulation of ENaC-mediated Na^+ transport (Faletti et al., 2002). Interestingly, several SGK1 polymorphic variants are associated with type 2 diabetes, obesity, and increased blood pressure in Caucasian and African populations (Schwab et al., 2008), whereas a strong link between heart disease and depression, both of which are closely related to lifetime stress exposure, has been verified in Chinese Han patients (Han et al., 2019).

In terms of downstream effectors, SGK1 controls ENaC (amiloride-sensitive sodium channel), other ion channels (e.g., KCNE1/KCNQ1), carriers (such as NCC, NHE3, SGLT1), Na(+)/K(+)-ATPase, enzymes (including glycogen-synthase-kinase-3), and transcription factors or regulators (including FOXO3a, β-catenin, NF-kappaB, SP1, p27) (Lang and Görlach, 2010; Schmid et al., 2014; Voelkl et al., 2015). Ion channel physiology and pathophysiology of membrane polarization appear to play an important role in cell proliferation, although rigorous scientific demonstrations are still lacking (Zhang et al., 2014; Zhou et al., 2015b). Taken together, all these information underline that SGK1, originally studied as a kinase responsible for regulating several cellular ion channels and pumps (Wulff et al., 2002; Lang et al., 2011; Chraïbi and Renauld, 2014), can indeed have a role in oncology and immunology as well (Lang et al., 2006; Sobiesiak et al., 2009; Wu C. et al., 2013). SGK1 function is directly dependent on mTOR phosphorylation. Following the mTOR-dependent hydrophobic motif (H-motif) phosphorylation on serine 422 (García-Martínez and Alessi, 2008), the kinase changes into an open conformation for phosphorylation and complete activation by 3-phosphoinositide-dependent kinase-1 (PDK1) (Hong et al., 2008). Copy number variation, as well as an increase in the expression and/or activity of SGK1, has been found in several human tumors (Abbruzzese et al., 2012; Sommer et al., 2013; Stringer-Reasor et al., 2015; Talarico et al., 2015, 2016a,b; Ma et al., 2019). Currently, SGK1 expression is described as related to events of invasiveness and metastasization (Huang et al., 2015; Qin et al., 2015; Xiaobo et al., 2016). Conversely, SGK1 knock-out models have been shown to be strongly resistant to chemical carcinogenesis (Nasir et al., 2009). It has recently been demonstrated that SGK1 is a crucial step in mediating cell survival, proliferation, and differentiation via phosphorylation of Mouse Double Minutes 2 (MDM2), which controls p53 ubiquitylation and proteasomal degradation (Amato et al., 2009). SGK1 also influences mitotic stability by affecting the expression of RANBP1 (Ran-specific binding protein 1), the pivotal regulator of GTPase RAN (Amato et al., 2013; D'Antona et al., 2019). More recently, it has been shown that SGK1 through RANBP1/RAN strictly regulates the nucleocytoplasmic transport of pre-miRNAs, a necessary condition for miRNAs maturation, thus modulating the epigenomic framework of the cell (Dattilo et al., 2017). This mechanism, which is so important in oncological diseases, can be of considerable interest also in psychiatric diseases such as major depression, in which neuronal epigenetic and epigenomic reorganization is widely discussed (Pitsillou et al., 2019). Interestingly, a new inhibitor for SGK1, named SI113, has been developed. In cellular models as well as in murine models, SI113 has amply demonstrated potential antineoplastic capacity, showing a very low toxicity profile (Abbruzzese et al., 2017, 2019; Catalogna et al., 2017; Matteoni et al., 2019). Pharmacological ADME (absorption, distribution, metabolism, and excretion) studies have corroborated the ability of SI113 to cross the blood-brain barrier, thus opening the possibility of a rational use of the molecule, even in psychiatric and neurological diseases (Talarico et al., 2016b). Interestingly, the most important molecular interactors as well as the downstream targets of SGK1 and its upstream modulators are involved in various ways in the etiopathogenesis of depression, constituting a clear link between this signaling pathway and the depressive pathogenesis (**Table 1**). It is also interesting to note how the whole FOXO3a system and the complex of modulating cytokines also open up a new etiopathogenetic hypothesis on an autoimmune Th17-dependent basis. In this light, the purpose of this review is precisely to outline, in the current knowledge framework, the interconnections between SGK1 and its modulations with the causative mechanisms of MDD, eventually clarifying the theoretical problems for the potential definition of SGK1 as a therapeutic target.

SGK1 AND L-HPA AXIS ACTIVITY

In humans, cortisol is the stress hormone that acts through the Glucocorticoid Receptor (GR) and the Mineralocorticoid Receptor (MR), both of which can act as transcription factors. Low blood levels of endogenous glucocorticoids bind MR with high affinity, enhancing proliferation of human hippocampal progenitor cells and differentiation into astrocytes. Conversely, increased levels of stress-induced glucocorticoids bind GR with low affinity, resulting in a decrease of the neural stem/progenitor cells (NSPCs) proliferation and neural differentiation (de Kloet et al., 1998; Anacker et al., 2013a). This evidence indicates that glucocorticoids can affect neurogenesis through a dual activity: a positive function (via MR) and a negative function (via GR) (Bose et al., 2010; Samarasinghe et al., 2011; Raciti et al., 2016). Severely depressed patients as well as rodent stress models are characterized by increased glucocorticoid levels (Anacker et al., 2011). Indeed, the most common biological feature in patients with MDD consist of chronically elevated glucocorticoid levels after a prolonged exposure to stress (Numakawa et al., 2013).

TABLE 1 | Table illustrating the SGK1 signaling partners in the MDD.

Interaction/Upstream/Downstream effectors	Papers	References
RAN	Maternal Stress Predicts Altered Biogenesis and the Profile of Mitochondrial Proteins in the Frontal Cortex and Hippocampus of Adult Offspring Rats.	Głombik et al., 2015
NF-KappaB	BDNF/NF-κB Signaling in the Neurobiology of Depression.	Caviedes et al., 2017
SP1	Neurotrophic factor-α1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone.	Cheng et al., 2015
mTOR	The role of mTOR in depression and antidepressant responses.	Abelaira et al., 2014
IL-2, TGFβ and cytokines	Comorbidity between depression and inflammatory bowel disease explained by immune-inflammatory, oxidative, and nitrosative stress; tryptophan catabolite; and gut-brain pathways.	Martin-Subero et al., 2016
FOXO3a	IGF-1 defends against chronic-stress induced depression in rat models of chronic unpredictable mild stress through the PI3K/Akt/FoxO3a pathway. Th17 cells in depression.	Beurel and Lowell, 2018; Kuang et al., 2018

RAN, GTPase Ran; NF-KappaB, Nuclear Factor Kappa B; mTOR, mammalian Target of Rapamycin; IL-2, Interleukin 2; TGFβ, Transforming Growth Factor-beta; FOXO3a, Forkhead Box O3; BDNF, Brain-Derived Neurotrophic Factor; IFG-1, Insulin Like Growth Factor 1; PI3K, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; Akt, Protein Kinase B.

SGK1, an ubiquitously expressed serine/threonine kinase, is transcriptionally regulated by a wide variety of factors, including glucocorticoids (Faletti et al., 2002). SGK1 is a protein involved in cellular stress response with a relevant role in neuronal function (Lang et al., 2010). In particular, SGK1 regulates the neuronal activity, proliferation, and apoptosis, becoming then a key determinant of susceptibility to mental illness (Han et al., 2019). The brain expression of SGK1 is positively regulated by glucocorticoids (Sato et al., 2008; Sarabdjitsingh et al., 2010), motor hyperstimulation (Kalinichev et al., 2008), and administration of the antipsychotic drug clozapine (Robbins et al., 2008). Hippocampal SGK1 expression is regulated by fear stimulation and after enrichment training (Lang et al., 2006). SGK1 transcript levels are also promoted by the psychostimulant amphetamine, hallucinogenic drug lysergic acid dimethylamide (LSD), and neuronal injury (Lang et al., 2006). Hypothalamic SGK1 levels are increased by fasting in conditions of obesity (Nonogaki et al., 2006). Experimental evidence points to a role of SGK1 in long-term memory formation (Ma et al., 2006). More recently has been demonstrated that SGK1 stimulates dendrite growth, thus probably impacting on learning abilities (Lang et al., 2006). Moreover, SGK1 also participates in the establishment of the spatial memory and neuronal plasticity through the phosphorylation of IKKα with subsequent stimulation of NFκB, thus leading to the expression of genes, such as *NR2A* and *NR2B* (Tai et al., 2009). In addition, SGK1 could affect neuronal excitation by modulating Kv channels (Lang et al., 2006, 2009), which, in turn, are essential and limiting in the preservation of neuronal membrane potential (Pongs, 2007). Interestingly, SGK1 has been proposed as repressor of the neurogenic Hedgehog pathway via GR. In human hippocampal progenitor cell line HPC03A/07, the GSK650394, a SGK1-small molecule inhibitor, counteracted the cortisol-induced suppression of Hedgehog signaling, resulting in proliferation and neuronal differentiation. Moreover, SGK1 was able to potentiate and maintain GR activation following cortisol stimulation by allowing GR phosphorylation at the serine residues S203 and S211, thus favoring its nuclear translocation. This implies that SGK1 is not only a downstream target of GR signaling, but also exerts a positive feedback on GR activation by regulating the long-lasting effects of glucocorticoids (Anacker et al., 2013b). Luca et al. recently identified a single-nucleotide polymorphism (rs9493857) lying in the *SGK1* promoter region within an Oct1 binding site, a transcription factor cooperating with GR in the transactivation of target genes. This polymorphism showed marked allele frequency between populations of African and European ancestry, allowing the definition of an ancestral and a derived allele. The ancestral allele, more prevalent in African populations, binds the GR-Oct1 complex more efficiently than the derived allele and is associated with increased glucocorticoid-dependent gene expression when compared with the derived allele (Luca et al., 2009). The role of SGK1 in impairing hippocampal neurogenesis is suggested by the evidence obtained on two rodent stress models characterized by depressive behavior and decreased hippocampal neurogenesis. In details, in rat models of depression induced by unpredictable chronic mild stress (UCMS) or early life stress, a significant increase of the hippocampal *SGK1* mRNA level was observed, together with a reduction in Hedgehog signaling, as expected (Anacker et al., 2013b; Bockmühl et al., 2015). A similar trend toward an increase in SGK1 gene expression was detected in peripheral blood of drug-free depressed patients ($n = 25$ vs. $n = 14$ healthy controls), thus confirming the evidence observed in rodents (Anacker et al., 2013b). Furthermore, increased levels of SGK1 were observed in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) of *miR-17-92* KO mice, which exhibited reduced adult neurogenesis and anxiety- and depression-like behaviors. Otherwise, the direct targeting of SGK1 by miR-19a and miR-92a, belonging to the miR-17-92 cluster, over-expressed in *miR-17-92* over-expressing (OE) mice, resulted in the maintenance of proliferative neural progenitors and the generation of newborn neurons, producing anxiolytic and antidepression-like behaviors

in these mice. The results indicate that miR-17-92 cluster deletion or overexpression in mice results in elevated anxiety-like or antianxiety-like behaviors, respectively (Jin et al., 2016). This finding suggests that the effects of miR-17-92 deletion in hippocampal neurogenesis and mouse behaviors are, at least in part, mediated by increased SGK1. A significant increase in *SGK1* mRNA was also observed, by means of next-generation RNA sequencing and pathway analysis, in the periaqueductal gray (PAG) of two mouse models of neuropathic pain [spared nerve injury (SNI)] and depression [chronic unpredictable stress (CUS)], revealing that SGK1 expression may indeed link, at a molecular level, pain and chronic stress (Descalzi et al., 2017). Interestingly, a recent paper showed that SGK3, an isoform highly expressed in the brain sharing 80% amino acid sequence identity with SGK1 in the catalytic domain (Kobayashi et al., 1999), induced SGK3-mediated autophagic cell death (ACD), but no apoptosis of hippocampal neural stem cells (NSCs), thereby determining decline of adult hippocampal neurogenesis and cognitive deficits (Jung et al., 2019). Although most of evidence points to the enhancement of SGK1 expression under chronic stress conditions, few papers describe a decreased or unaffected SGK1 expression after acute stress exposures. Licznerski et al. (2015) showed a significant reduction of *SGK1* mRNA in a small cohort of post-mortem Prefrontal Cortex (PFC) of patients diagnosed with Post Traumatic Stress Disorder (PTSD, $n = 6$) compared to age-matched individuals without psychiatric diagnosis ($n = 6$). The down-regulation of SGK1 replicated also at protein level in the PFC of a subgroup of rats undergone to inescapable stress paradigm. Furthermore, rats infused in the PFC with an adeno-associated virus carrying a dominant negative form of SGK1 (S422A) caused helplessness- and anhedonic-like behaviors (Licznerski et al., 2015). A very recent study on adult rats, subjected to acute stress during adolescence, revealed no significant changes in *SGK1* mRNA levels in anxiety-related brain regions such as central amygdala, medial amygdala, ventral hippocampus, and paraventricular nucleus (Lovelock and Deak, 2019). Despite the high comorbidity between MDD and PTSD, the modulation of SGK1 expression could be mediated by distinct signaling pathways. Indeed, the expression of genes strictly related to HPA axis, such *FKBP5* and *NR3C1* (gene encoding glucocorticoids receptor), appeared not influenced under these conditions, thereby suggesting that HPA axis regulation results unaffected by acute stress exposure. Recent evidence supports the idea that plasticity in adult brain white matter structure and myelination arises from the activity-dependent modulation of oligodendrocyte function (Gibson et al., 2014; Pepper et al., 2018) in response to various experiential events, including new training on cognitive task (Fields, 2015). Although the molecular mechanism behind this process is not well-known, SGK1 appears to be a potential mediator of oligodendrocyte plasticity due to its rapid upregulation in adult rat and mouse brain white matter after acute stress (Miyata et al., 2011). Indeed, a dynamic glucocorticoid-dependent regulation of SGK1 mRNA as well as its kinase activity induction were observed in oligodendrocytes of corpus callosum of acute stressed adult male rats (Hinds et al., 2017) and chronic stress exposed mice (Miyata et al., 2015), respectively. Active SGK1 led to N-myc downstream-regulated gene 1 (NDRG1) phosphorylation and, as downstream effect, to the increase of N-cadherin, α-catenin, and β-catenin expression, both *in vitro* and *in vivo*. The abundance in catenin/cadherins expression resulted, in turn, in morphological changes in the oligodendrocytes of corpus callosum nerve fiber bundles. Remarkably, the recovery from chronic stress restored both the arborization of oligodendrocytes and the depression-like symptoms, confirming that morphological changes of these cells are conceivably related to depressive behaviors (Miyata et al., 2011). Similarly, chronic stress induced the SGK1-dependent up-regulation of *Dsg1* encoding for the cell adhesion molecule named desmoglein 1, a calcium-dependent desmosomal cadherin. This finding could suggest that also Dsg1, a target of SGK1, may be involved in molecular mechanisms responsible for the oligodendrocyte morphological changes in response to chronic stress exposure (Miyata et al., 2015). Finally, the hypothalamic expression of *SGK1* was described to be increased in a mouse model of stress induced by social isolation. Injection of a small interfering RNA (siRNA) oligonucleotide, specific for *SGK1*, into the third cerebral ventricle blocked the acute social isolation-induced reduction in body weight and increase in plasma active ghrelin (a hunger stimulating hormone) levels (Kaji and Nonogaki, 2010). Taken together, these pieces of evidences strongly propose the involvement, with a key role, of SGK1 in the pathogenetic stress hypothesis of major depression (**Table 2**).

SGK1, NEURODEVELOPMENT AND NEUROTROPHIC FACTORS

Decreased hippocampal volumes have been found in depressed human subjects exposed to chronic stress (Campbell and MacQueen, 2006; Schmidt et al., 2011). This finding supports the hypothesis that chronic stress can inhibit neurogenesis and retract dendritic processes, resulting in neuronal loss in the hippocampus (Schmidt and Duman, 2007). Neurotrophic factors (or neurotrophins) are key determinants in supporting neuronal survival and differentiation, playing an essential role in the process of regulating neuronal formation in neural networks (Meng et al., 2019).

Among neurotrophins, brain-derived neurotrophic factor (BDNF) has a leading role in the pathophysiology of depression (Numakawa et al., 2014; Zhang et al., 2017). Indeed, chronic stress has been described to decrease the BDNF blood level among MDD patients (Molendijk et al., 2011; Kreinin et al., 2015) as well as its expression in the hippocampal dentate gyrus of post-mortem brains of MDD subjects (Castrén and Rantamäki, 2010). Recently, BDNF was suggested to have a significance as predictive biomarker for the treatment of mood disorders (Polyakova et al., 2015). It should also be mentioned that BDNF and VEGF could play a synergistic role in the complex of mood disorders. VEGF is a multifunctional growth factor that stimulates not only angiogenesis, but also neurogenesis with neuroprotective properties (Carmeliet and De Almodovar, 2013).

Brain-derived neurotrophic factor is a neurotrophin highly expressed in the brain, especially in the hippocampus and cortical region, with a key role in the maintenance of neurons in the CNS.

TABLE 2 | Table illustrating the SGK1 involvement in humans, *in vitro* and *in vivo* models of stress.

Experimental model	Molecular features	Biological and clinical features	References
HPC03A/07 hippocampal progenitor cells exposed to cortisol	Hedgehog signaling suppression after SGK1 inhibition by GSK650394; Enhancing of GR nuclear translocation following its SGK1-dependent hyperphosphorylation at S203 and S211	Reduction in neural proliferation and differentiation	Anacker et al., 2013b
Drug-free depressed patients	Increased peripheral blood expression of SGK1	Depressive symptoms	Anacker et al., 2013b
Combination of population genetics	Increased GR/Oct1-dependent SGK1 expression in presence of rs9493857 polymorphism	Genetic variant associated with greater responsiveness of neuroendocrine signaling in response to environmental stressor stimuli, resulting in the predisposition of individuals to chronic disorder	Luca et al., 2009
UCMS-rats; Early life stressed rats	Increased hippocampal SGK1 mRNA levels; Reduction in Hedgehog signaling	Decreased hippocampal neurogenesis with depressive behaviors	Anacker et al., 2013b; Bockmühl et al., 2015
miR-17-92 KO mice	Increased SGK1 levels in the SGZ of hippocampal DG	Reduced adult neurogenesis with anxiety- and depressive-like behaviors	Jin et al., 2016
SNI and CUS mice	Increased SGK1 mRNA in the PAG	SGK1 as a linker between pain and chronic stress	Descalzi et al., 2017
Chronic stressed mice	SGK3-mediated autophagic cell death of hippocampal NSCs	Decline of hippocampal neurogenesis with cognitive deficits	Jung et al., 2019
Corpus callosum oligodendrocytes of chronic stress exposed mice	Increase in SGK1 mRNA and kinase activity; Increase in N-cadherin, α-catenin and β-catenin via SGK1/NDRG1 axis both *in vitro* and *in vivo*	Morphological changes in oligodendrocytes of corpus callosum nerve fiber bundles; Recovery from stress restored the normal arborization of oligodendrocytes as well as the depression-like symptoms	Miyata et al., 2011
Acute stressed adult rats	Increase in SGK1 mRNA in the corpus callosum	SGK1 as an important component of the interplay between oligodendrocytes and neuronal function including neuroplasticity	Hinds et al., 2017
Corpus callosum oligodendrocytes of chronic stress exposed mice; Primary oligodendrocytes exposed to dexamethasone	Increase in SGK1 expression/activity leading to Dsg1 up-regulation	Morphological changes in oligodendrocytes of corpus callosum	Miyata et al., 2015
Stressed mouse model by social isolation	Increase in hippocampal SGK1 expression	siRNA-mediated silencing of SGK1 inhibited the acute social isolation	Kaji and Nonogaki, 2010

SGK1, Serum- and Glucocorticoid-regulated Kinase 1; GR Glucocorticoids Receptor; Oct1, Octamer-binding protein 1; SGK3, Serum- and Glucocorticoid-regulated Kinase 3; NDRG1, N-Myc Downstream-Regulated Gene 1; Dsg1, Desmoglein 1; UCMS, Unpredictable Chronic Mild Stress; SNI, Spared Nerve Injury; CUS, Chronic Unpredictable Stress; SGZ, Subgranular Zone; DG, Dentate Gyrus; PAG, Periaqueductal Gray; NSCs, Neural Stem Cells.

BDNF exerts its functions binding with high affinity the receptor TRKB, which activates several downstream intracellular signaling pathways, leading to neuronal survival, synaptic plasticity, and neurogenesis (Numakawa et al., 2013). Since both BDNF/TRKB and GCs/GR systems are involved in neurogenesis, functional crosstalk between them has been a widely studied research topic. SGK1 presumably participates in the signaling of BDNF, which stimulates the PI3-kinase and thus probably SGK1. Since BDNF influences neuronal survival, mood, and long-lasting memory formation and intensifies synaptic plasticity with neuro-regenerative effects by weakening the processes of neuronal degeneration, SGK1 may contribute to several BDNF-dependent neuropsychiatric disorders including depression (Lang et al., 2010). However, the molecular mechanisms by which SGK1 could be a convergence point between glucocorticoids and BDNF in neurogenesis are still not completely clear. Current knowledge indicates that GCs/GR system appears to affect the intracellular signaling pathways involved in the BDNF/TRKB system (Numakawa et al., 2013). In rat cultured cortical neurons, the treatment with dexamethasone (DEX), a synthetic glucocorticoid, decreases the indirect association between TRKB and SHP2 (Src homology-2 domain-containing phosphatase 2), negatively modulating the ERK-mediated expression of synaptic proteins such as NR2A and synapsin I (Kumamaru et al., 2011). Furthermore, the chronic exposure to DEX or corticosterone, a natural glucocorticoid, inhibits the direct binding TRKB/GR

due to the decline in GR expression. Thus, the lack in the interaction of the two receptors leads to the suppression of BDNF-induced glutamate release by impairing the activation of the phospholipase C-gamma (PLCγ)/Ca^{2+} system in the above mentioned cellular model (Numakawa et al., 2009). Indeed, in the mouse hippocampal BZ cell line, GR is able to repress the transcription of *BDNF* gene, through binding an unidentified transcription factor at the gene promoter region (Chen et al., 2017).

Although the mechanisms underlying the pathophysiology of MDD are still not well elucidated, growing evidences converges to the neurotrophic hypothesis of depression (Duman and Li, 2012; Jaggar et al., 2019). In this context, BDNF and vascular endothelial growth factor (VEGF) reduced levels are closely linked with neuronal atrophy in some brain regions implicated in MDD by affecting the hippocampal volume and vascularization, and inducing cognitive decline (Duman and Monteggia, 2006; Duman et al., 2016). In particular, decreased levels of the neurotrophins and their respective receptors TRKB and FLK1, have been reported in the PFC and hippocampus in post-mortem studies of subjects with depression (n = 10 vs. n = 24 controls; n = 14 vs. n = 14 controls) (Karege et al., 2005; Qi et al., 2015) as well as in rodent models of chronic stress (Heine et al., 2005; Elfving et al., 2010; Howell et al., 2011). The synergistic actions of BDNF and VEGF on neurogenesis and their antidepressant-like effect is corroborated by other evidence, demonstrating that BDNF stimulates both expression and release of VEGF in neuroblastoma cells and rat primary cortical neurons (Deyama et al., 2019). Moreover, the BDNF-mediated induction of cortical neurons dendrite complexity is blocked by a selective VEGF–fetal liver kinase 1 receptor antagonist, indicating that BDNF neurotrophic and antidepressant-like actions require the downstream VEGF signaling (Deyama et al., 2019). The molecular mechanism according to which SGK1 may modulate VEGF levels has been elucidated through a viable *SGK1* knockout mouse model. The ablation of *SGK1* resulted in a robust decrease in phosphorylation of the target protein NDRG1 accompanied by down-regulation of two NF-κB inhibitory components: IκBα and NF-κB2/p100. The resulting enhancement of NF-κB signaling increased the expression of the downstream target protein, VEGF-A, thus disrupting the normal development and vessel formation (Zarrinpashneh et al., 2013). Based on these findings, SGK1 appears to exert, under chronic stress condition, its anti-neurogenic activity by negatively regulating the expression of both BDNF and VEGF neurotrophins.

SGK1, NEURODEVELOPMENT AND CYTOKINES

The role of neuro-inflammation in depression and consequently, the role of SGK1 in this specific phenotype is, in some ways, controversial. The inflammation resulting from physical stress has shown, at least in rats, to be associated with depression in presence of elevated levels of SGK1, both in blood and in hippocampal and amygdala neurons, within the framework of glucocorticoid-dependent depressive effects (Anacker et al., 2013b; Girgenti and Duman, 2018). Vice versa, the depression resulting from post-traumatic stress such as childhood and adolescent maltreatments is associated with a strong inflammatory component in the presence of decreased levels of SGK1, accompanied by reduced hippocampal neurogenesis (Pariante, 2017). Thus, it emerges that different depressive forms, although clinically part of the major depressive disorder, may have different molecular characterizations depending on the origin of the damage. The difference may be also the result of different cellular components involved. In depression with post-traumatic inflammation, it mainly involved the glial component (microglia and astrocytes), which plays a putative role of innate immunity in CNS (Inoue et al., 2016). Moreover, a novel line of research points to a possible role of SGK1 in mediating Th17-dependent inflammatory effects, in particular diets and stress conditions (Kleinewietfeld et al., 2013; Wu C. et al., 2013; Hernandez et al., 2015; Spagnuolo et al., 2018). In fact, high sodium chloride diets may drive inflammation and autoimmune disease by the induction of pathogenic Th17 cells through the expression of SGK1. It appears evident in this light that a reasonable identification of SGK1 as a pharmacological target requires a careful evaluation of the molecular pathways and cellular actors that are involved. Collectively, the causative role of SGK in MDD is made more confusing in presence of such large number of factors including altered distribution in distinct cell types, different levels of expression and protein interaction, and co-participation of different splicing variants (Lang et al., 2010). As the roles of SGKs may be dramatically divergent among different types of cells in brain, analysis of SGKs and its interactome in each type of cells is required.

SGK1, TARGET OF NEW ANTIDEPRESSANT-MIMICKING COMPOUNDS

The World Health Organization (WHO) estimates that around 350 million individuals suffer from depressive disorders worldwide and up to 40% of patients do not adequately respond to antidepressant medications (James et al., 2018). Over the past 30 years, the understanding of depression was mainly based on the monoamine-deficiency hypothesis, which identifies an association between the occurrence of depression and deficiencies of three major monoamine transmitters as 5-hydroxytryptamine (5-HT), norepinephrine (NE), and dopamine (DA). Therefore, the most commonly employed pharmacological treatments of MDD consist of monoamine oxidase inhibitors, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin/noradrenaline reuptake inhibitors (SNRIs), all triggering augmentation of synaptic monoamine levels (Otte et al., 2016). However, these antidepressant treatment have an efficiency of only 60 to 65% with a remission rate of ∼30% (Montes et al., 2004; Block and Nemeroff, 2014), and often require 14 to 21 days or longer for the onset of antidepressant efficacy. The elucidation of new molecular mechanisms in the pathogenesis of the MDD has accelerated the effort to develop novel and effective antidepressants.

In recent years, accumulating evidence has enforced the idea that medicinal herbs and constituents isolated from plant extracts possess psychotherapeutic activities in the treatment of psychiatric disorders, including depression (Zhang, 2004). Baicalin, a major active flavonoid compound extracted and purified from the dry roots of *Scutellaria baicalensis*, has been described to hold several biological properties, including antioxidant, anti-inflammatory, and neuro-protective actions (Zhou et al., 2015a; Shi et al., 2017). Interestingly, baicalin has been reported to overcome the brain-blood barrier (Liu et al., 2007) and exhibited antidepressant effects (Xiong et al., 2011). In a mouse model of depression, obtained by repeated exogenous corticosterone (CORT) injections, a 21-day treatment with baicalin (10 and 20 mg/kg) reversed CORT injection-induced depressive-like behaviors and restored serum corticosterone levels. Indeed, baicalin enhanced the mRNA and protein expression of glucocorticoid receptor (GR) and BDNF, whereas down-regulated SGK1 expression in the hippocampus (Li et al., 2015). Accordingly, in the same mouse model of depression, baicalin treatment (40, 80, or 160 mg/kg for 4 weeks) was reported to restore chronic CORT-induced suppression of hippocampal neurogenesis. In particular, baicalin restored the chronic CORT-induced decrease in GR protein levels, the rate in GR nuclear translocation, as well as the intensification of GR phosphorylation at Ser203 and Ser211. In addition, baicalin treatment also normalized the chronic CORT-induced increase of both FKBP5 protein levels and SGK1 phosphorylation at Ser422 and Thr256. Taken together, these findings suggest that baicalin counteracts anxiety/depression-like behaviors, promoting hippocampal neurogenesis through the regulation of SGK1- and FKBP5-dependent GR phosphorylation (Zhang et al., 2016). Although present findings elucidate the molecular mechanisms behind the antidepressant activity of baicalin, nevertheless, further studies are required to clarify the precise correlation between SGK1 and BDNF in neurogenesis. Recently, Icariin, another flavonoid isolated from *Herba Epimedii*, demonstrated to play potential antidepressant-like effects in several depression models (Pan et al., 2006, 2007, 2010). In a rat model of depression induced by unpredictable chronic mild stress (UCMS), the oral administration of icariin (20 and 40 mg/kg) for 35 days attenuated the development of depression-like behaviors. It was noteworthy that, similarly, to baicalin, icariin restored the UCMS-induced increases in the levels of cytosolic GR and SGK1 in both the hippocampus and the prefrontal cortex. Icariin also partially reversed the upregulation of FKBP5 and nuclear localization of GR in the hippocampus and in the prefrontal cortex, respectively (Wei et al., 2016). These data suggest that the antidepressant-like effects of icariin, similarly to the effects exerted by fluoxetine, a SSRI widely used in the psychopharmacology of MDD, can be explained by the restoration of the physiological negative feedback of the HPA axis and the canonical neurogenesis in related brain regions. Interestingly, antidepressant-like effects were also observed in mice model of CORT-induced depression treated with oleanolic acid, a triterpenoid compound present in natural plants. The antidepressant-like effect of the treatment with oleanolic acid (10 and 20 mg/kg for 21 days) appeared to be mediated by the down-regulation of the SGK1 and GR expression concomitantly to the upregulation of the hippocampal BDNF-AKT/mTOR signaling pathway (Dong et al., 2019). A recent *in vitro* study supported the hypothesis that SGK1 could play a key role in mediating the antidepressant effects of different natural compounds. In this study, leonurine, also called SCM-198 (4-guanidino-n-butyl syringate), a chemically synthesized compound based on a bioactive alkaloid extracted from the herbaceous perennial plant *Leonurus cardiaca*, has been demonstrated to increase cell viability of corticosterone-induced PC12 cells, with the maximal pro-survival effect at 60 μM. Indeed, the treatment with lenourine increased cell area, total neurite length, and maximum neurite length by inducing the expression of GR, BDNF, NT-3, and BCL-2, and inhibiting the SGK1 expression. Notably, the protective effect of leonurine against CORT-induced cell death, as well as its ability to increase the expression of the above-mentioned genes was potentiated by SGK1 inhibition through GSK650394 (20 μM) pre-treatment. These data suggest that leonurine may exert antidepressant effects, modulating the neurite outgrowth and neurotrophic activity as observed in CORT-cultured PC12 cells, and that this effect may depend on the negative modulation of GR/SGK1 signaling (Meng et al., 2019). In a different study, the ethanolic extract from the *Dipterocarpus alatus* leaf, containing flavonoids (luteolin-7-*O*-glucoside, kaempferol-3-glucoside, rutin) and phenolic acids (gallic acid, ferulic acid, and caffeic acid) as major constituents, showed an antidepressant activity, resulting in the attenuation of anhedonia (increased sucrose preference) and behavioral despair (decreased immobility time in tail suspension test and forced swimming test) in an UCMS mouse model of depression. Administration of the extract (100 and 500 mg/kg for 3 weeks) not only decreased the UCMS-induced elevation of serum CORT levels and the hyperactivation of the HPA axis, but also normalized, in a dose-dependent manner, the mRNA expression of SGK1, cyclic AMP-responsive element binding (CREB) and its downstream target BDNF in the frontal cortex and hippocampus, effects similar to those observed with imipramine (20 mg/kg) (Daodee et al., 2019). In addition, in an *in vitro* assay, the extract exerted also a partial selective inhibition on the enzyme monoamine oxidase (MAO)-A, whose abnormal activity is reported to play an important role in depressive disorders (Naoi et al., 2018). Although these findings are relevant for understanding the antidepressant mechanism of *Dipterocarpus alatus* leaf extract, further studies are necessary to clarify the specific mechanisms involved in the neurogenesis in dentate gyrus and in other signaling pathways involved in regulation of HPA axis.

Gypenoside, a very active saponin isolated from *Gynostemma pentaphyllum*, is known to have a variety of pharmacological properties, including anti-inflammatory (Liou et al., 2010), antioxidative (Schild et al., 2012), neuroprotective (Choi et al., 2010), and antidepressant-like activities (Mu et al., 2016). Gypenoside administration (100 mg/kg for 3 weeks) in chronic CORT-induced depressed mice significantly reverted the stress-dependent increase of hippocampal SGK1 protein levels as well as the decreased activation of CREB-BDNF signaling, suggesting

that the antidepressant-like effects of gypenosides are related to both the inhibition of SGK1 expression and the neurogenesis stimulation via GR-dependent pathway (Wu J. et al., 2013; Yi et al., 2018).

Recently, Moore A. et al. reviewed a fair amount of evidence showing that resveratrol, a polyphenol with antioxidant and anti-inflammatory properties that highly present in grape skins and therefore in red wine, abrogates depressive-like behavior and neuroinflammatory response, and enhances hippocampal neurogenesis in several animal model of depression. The neuroprotective effects exerted by resveratrol administration (10–80 mg/Kg/die) mainly arise from the enhancing of CREB/BDNF signaling as well as the regulation of HPA axis function (Moore et al., 2018). In addition, resveratrol has also been shown to have an *in vitro* inhibitory activity on SGK1 in HUH7 human hepatoma cells (Catalogna et al., 2019). The antidepressant effects of resveratrol are also being studied in humans, through a double-blind randomized clinical trial on 60 patients with depression, already in phase four of the experimentation U. S. Nation Library Of Medicine and ClinicalTrials.gov (2020). Although more studies elucidating the precise molecular mechanism are needed, taken together these finding suggest that the effects of resveratrol on the CNS are probably mediated, at least in part, by its action on SGK1.

Considering that oxidative stress is unequivocally associated with the advancement of depression, all this evidence suggests that nutraceuticals may help to reduce the symptoms of depression, notably via dietary supplementation with the minimization of depression risk due to their important anti-inflammatory and antioxidative natures. All these studies highlighted that the antidepressant effects of antioxidative compounds are related to the normalization of the "stress protein" SGK1 level in parallel with the restoration of neurogenesis (**Figure 1**); for this purpose, advanced investigations are needed to fully understand the mechanism of actions including neuroprotection, biotransformation of their metabolites in the body, and potential interactions with molecular target involved in the depression. Nowadays, except for resveratrol, the described studies on the above-mentioned nutraceuticals are still at a very early stage and further confirmations on their antidepressant effects in specific clinical trials are required. More recently, several studies focused on the role of ketamine and its derivatives in the treatment of treatment-resistant forms of depression (TRD) (Bratsos and Saleh, 2019;

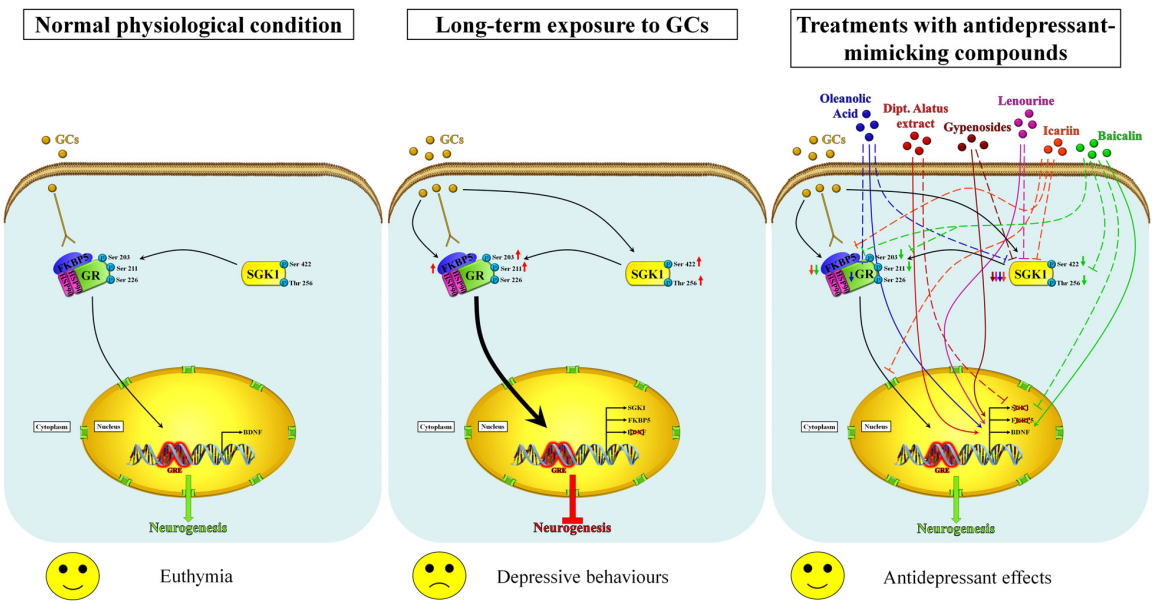

FIGURE 1 | Proposed mechanisms of action of nutraceuticals in the restoration of neurogenesis by modulating the GR/SGK1 signaling pathway. In the normal physiological state, the exposure of neuronal cells to basal levels of glucocorticoids activates GR and promotes its translocation into the nucleus, regulating thus neurogenesis **(left panel)**. Prolonged exposure to high concentrations of glucocorticoids increases the expression of FKBP5, which impairs cytosolic GR binding capability and therefore the ultra-short negative feedback loop on GR sensitivity, increasing thus the negative effects of glucocorticoids. Furthermore, high glucocorticoids levels also improve SGK1 activity by increasing the phosphorylation of SGK1 at Ser422 and Thr256, which in turn causes the phosphorylation of GR at Ser203 and Ser211. The hyperphosphorylation of GR stimulates its nuclear translocation (thick arrow) and, by binding unidentified co-repressor factors, down-regulates the BDNF expression that results in impaired neurogenesis and then in depressive-like behaviors **(middle panel)**. Nutraceuticals may counteract the effects of long-term exposure to glucocorticoids by restoring normal GR/SGK1 signaling through similar molecular mechanisms that lead to improved neurogenesis and antidepressant effects **(right panel)**. Inhibitory and enhancing actions are indicated by dashed lines and arrows, respectively. Baicalin (green) is able to counteract the GCs-induced effects by enhancing the BDNF expression, downregulating the expression of SGK1, decreasing the FKBP5 protein levels as well as the phosphorylation rate of both GR and SGK1. Icariin (orange) decreases the SGK1 and FKBP5 protein levels and affects the GR nuclear translocation. Lenourine (violet) stimulates the BDNF expression whereas negatively regulates the SGK1 expression. Gypenosides (brown) reduces the SGK1 protein levels and enhances the BDNF expression. Dipt. Alatus extract (red) normalizes the mRNA levels of SGK1 and stimulates the expression of BDNF. Oleanolic acid (blue) down-regulates the expression of both SGK1 and GR as well as up-regulates the expression of BDNF.

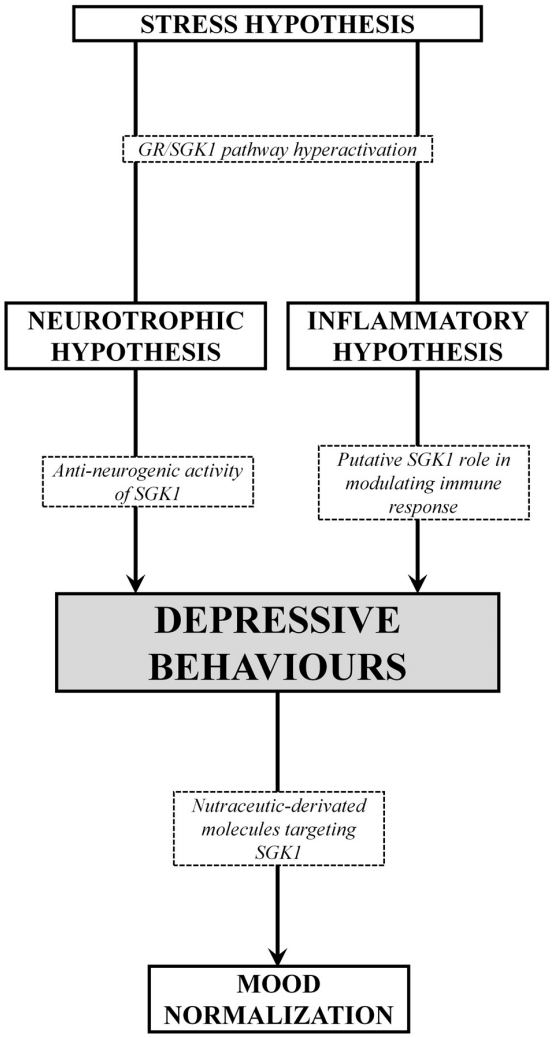

FIGURE 2 | Schematic overview of the SGK1 role in different pathogenetic hypothesis of MDD. Under stress conditions, the increase of glucocorticoids induce a dysregulation of the GR/SGK1 axis, thus resulting in depressive behaviors, presumably dependent on anti-neurogenic activity exerting a negative regulation in the expression of neurotrophins, and/or the immune/inflammatory response as well. On the other hand, nutraceuticals-derived molecules with antioxidant, anti-inflammatory and neuro-protective properties, exhibit antidepressant effects due, at least in part, to the negative modulation of SGK1.

Park et al., 2019; Kryst et al., 2020). Under these specific conditions, the trend of SGK1 appears paradoxical with respect to that observed in the classic stress-dependent forms of depression (Ficek et al., 2016; Qiao et al., 2019). The explanations for this paradoxical up-regulation of SGK1 in response to the ketamine treatment could lie in at least two key points: first, the neuronal model of glutamatergic activation differs from the canonical assessments of dopaminergic and serotoninergic neurons target for classical anti-depressants; second, it should be borne in mind that the rapid effect of ketamine is accompanied by a controlled dissociative state that could be related to high levels of anxiety-dependent stress probably with increase glucocorticoid release. It is interesting to note that in response to the treatment with ketamine and to the ketamine-dependent SGK1 activation, a deep modulation in the FOXO activity (Qiao et al., 2019) and presumably in its transcriptional functionality has been also noticed, which could, in turn, explain the rapidity of the antidepressant effects of ketamine. However, further studies will be necessary to understand the potential role of SGK1 as a pharmacological target for the ketamine and its derivatives in depressive conditions refractory to traditional therapies.

CONCLUSION

In the light of the above reviewed data, it is therefore clear that several hypotheses may be taken into account in the explanation of the pathogenesis of depression. At present, it appears more appropriate to refer to a depressive spectrum disease than to an organically structured depressive disease. Thus, for a correct clinical and molecular approach, it is necessary to first evaluate and subclassify the causes and symptomatic forms of the specific depressive disorder, and later to approach the more balanced therapeutic methodology, congruent with the most plausible pathophysiological hypothesis. The more resistant forms of MDD, which are refractory to traditional treatments, could offer a first experimentation frontier for new synthetic or nutraceutic-derivated molecules targeting SGK1. In fact, SGK1 could play as a neuronal resensitization factor and in any case a convergence point of different and often opposite signal pathways. In this light, by taking in account genetic heterogeneity and several environmental factors, it may be possible tailor therapeutical approaches to be more individualized, effective, and better tolerated. From a genetic point of view, the SGK1 kinase appears to be a clear piece for a unifying vision of the overall pathological forms examined (**Figure 2**). The role of gene mutations or functional dysregulations of SGK1 needs to be investigated in a well-designed clinical cohort of patients in order to ultimately define the role of SGK1 in the pathogenetic evolution of this pathological spectra. However, it is evident from what has been said so far that SGK1 is proposed as a therapeutic molecular target for a modern therapy of the depressive disorder and that efforts in this sense will increasingly clarify how much this new candidate gene can provide the explanations missing in the univocal vision of the disease.

AUTHOR CONTRIBUTIONS

VD and RA conducted the literature search, wrote the manuscript and prepared the tables and figures. NP and MG reviewed and edited drafts of the manuscript. All authors contributed to reading and approving the final version of the manuscript.

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Repeated Nitrous Oxide Exposure Exerts Antidepressant-Like Effects Through Neuronal Nitric Oxide Synthase Activation in the Medial Prefrontal Cortex

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Clinical studies have demonstrated that exposure to the inhalational general anesthetic nitrous oxide (N_2O) produces antidepressant effects in depressed patients. However, the mechanisms underlying the antidepressant effects of N_2O remain largely unknown. Neuronal nitric oxide synthase (nNOS)–mediated nitric oxide (NO) synthesis is essential for brain function and underlies the molecular mechanisms of many neuromodulators. We hypothesized that activation of the nNOS/NO pathway in the medial prefrontal cortex (mPFC) might mediate the antidepressant effects of N_2O. In this study, we revealed that repeated N_2O exposure produced antidepressant-like responses in mice. Our mechanistic exploration showed that repeated N_2O exposure increased burst firing activity and that the expression levels of BDNF with nNOS activation were dependent in the mPFC. In particular, the antidepressant-like effects of N_2O were also antagonized by local nNOS inhibition in the mPFC. In summary, our results indicated that N_2O exposure enhances BDNF expression levels and burst firing rates in an nNOS activation dependent manner, which might underlie the pharmacological mechanism of the antidepressant-like effects of N_2O exposure. The present study appears to provide further mechanistic evidence supporting the antidepressant effects of N_2O.

Keywords: nitrous oxide (N_2O), depression, mPFC, burst firing, neuronal nitric oxide synthase (nNOS), BDNF

INTRODUCTION

Major depressive disorder (MDD) is one of the most severe mental disorders in the world and accounts for most of the nonfatal burden of mental and substance use disorders (1). The currently the available antidepressants mostly target the monoaminergic system, but these classic antidepressants are associated with low remission rates and a lag in the onset of antidepressant action obtained (2–4),

highlighting an urgent and clear need for identifying a more efficacious and faster-acting therapeutic agents.

In recent decades, studies on the antidepressant effects of anesthetics have attracted considerable attention. Recent studies have suggested that some anesthetics show promise as therapeutics against MDD. Ketamine has attracted keen interest due to its remarkably rapid and sustained antidepressant effects (5–7). However, when administrated as a single intravenous dose, ketamine is associated with some unwanted side effects, including dissociation, headache, dizziness, elevated blood pressure, and blurred vision (8). Thus, the safety concerns of ketamine should be carefully considered, particularly after repeated dosing.

Nitrous oxide (N_2O) is a widely used inhalational general anesthetic (9). Recent clinical studies have shown that N_2O exerts rapid and marked antidepressant effects in patients with treatment-resistant major depression (TRMD), and the improvements can last for a full week (10). Importantly, compared with ketamine, N_2O does not have psychotomimetic or cognitive side effects (11). Hence, N_2O might be an attractive alternative for the development of mood disorder therapeutic. Because the improvements in TRMD obtained with N_2O are associated with relatively few safety concerns, studies of the mechanism of the N_2O-induced antidepressant effects will support a better understanding of the pharmacological treatment of depression.

N_2O influences brain functions through multiple mechanisms of action, and this agent is commonly used in dentistry and obstetrics because it is an effective analgesic and anxiolytic agent. The anxiolytic effect might involve the activation of $GABA_A$ receptors through the benzodiazepine-binding site (12). N_2O is also an antagonist of the N-methyl-D-aspartate (NMDA) receptor, which is a therapeutic target that might exert antidepressant effects (10, 11) similar to those obtained with ketamine. In addition, this agent has a wide range of other potential therapeutic targets, but the precise mechanism underlying the antidepressant effects of N_2O has been less well addressed and might involve other molecular and receptor systems.

Nitric oxide (NO), a widespread signaling molecule that can be regulated by N_2O, has diverse biological effects in the central nervous system (CNS) (13, 14). However, it remains unclear whether an increase in NO as a result of N_2O is part of the antidepressant mechanism of N_2O. Neuronal nitric oxide synthase (nNOS) is the key enzyme that mediates the NO signal transduction pathway in the brain (15), this enzyme participates in the regulation of learning, memory, and pathological conditions (16–19). Many shreds of evidence suggest that an imbalance of nNOS activity in the CNS implicated in the pathophysiology of MDD, and the nNOS system is proposed to be a potential therapeutic target for MDD (20). Thus, we hypothesized that the antidepressant effects of N_2O might be mediated by the activation of the nNOS/NO pathway in the mPFC.

In this study, we observed antidepressant-like effects in mice after repeated N_2O exposure. To address the underlying mechanism, we investigated the neuronal activity in the mPFC and further identified the functional roles of nNOS in the antidepressant-like effects of N_2O.

MATERIALS AND METHODS

Animals

Adult male CD-1 mice [weighing 25–35 g, postnatal day (PD) 42] were used in our experiments. The animals were housed in a limited-access rodent facility with four to five mice per cage in a room with a 12-h light/12-h dark cycle (cage size: 320 × 210 × 160 millimeters, lights on from 7:00 a.m. to 7:00 p.m.) and a constant temperature (22 ± 2°C). Sterilized drinking water and standard food were provided ad libitum. Before the experiments, the mice were habituated to these conditions for 2–3 days. In all experiments, animals were divided randomly into groups. Briefly, we numbered all animals and used Excel's random number generator to randomize the animals to groups. All the animal studies and experimental procedures were approved by the Animal Care Committees of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and the experiments were performed in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Stereotactic Surgery and Local Infusion

Adult CD-1 mice (aged 6–8 weeks) were anesthetized with pentobarbital sodium (80 mg/kg, i.p.) and fixed on an animal stereotaxic frame (RWD, 68016). Two 29-gauge guide cannulas were bilaterally placed 1 mm above the mPFC (anterior/posterior (A/P): +2.10 mm; medial/lateral (M/L): ± 0.35 mm; dorsal/ventral (D/V): -2.0 mm; **Supplementary Figure 3A**). A 39-gauge needle with a plastic cap on top was inserted into the guide cannula to prevent clogging during the recovery period. The cannulas were fixed to the skull using dental cement. After surgery, the mice were then allowed to recover for at least 1 week before the subsequent experiments. Drugs were microinjected with a 39-gauge injector cannula that was inserted into the guide cannula. One microliter of drug was infused (0.1 μl/min, 5 min total) into each side with a microsyringe. The injector cannulas were kept in place for an additional 5 min to minimize spread of the drug along the injection track. Finally, to confirm the accurate drug injection site and the spreading region of injected fluid, we have injected an equal volume of Chicago Sky Blue solution (2M NaCl and 0.5% Chicago Sky Blue) at the same coordinates in the mPFC (A/P: +2.10 mm; M/L: ± 0.35 mm; D/V: -3.0 mm). After behavioral tests, mice were sacrificed and each brain was sliced to examine the injection sites. Data were excluded where the injection site and the diffusion region were not located in the prelimbic subregion (PrL) of the mPFC.

Drugs and Drug Administration

Mice were randomly divided into four groups. (i) Mice in the control group were exposed to air in the chamber to exclude the

effects of gas exposures. In the vehicle (ii) and experimental *groups* (iii), mice were exposed to the 50% N_2 + 50% O_2 mixture and 50% N_2O + 50% O_2 mixture for one 2-h session per day for three consecutive days, respectively. The mice in the positive control (iv) group were exposed to ketamine (20 mg/kg) 24 h before behavioral test. The dose of 20 mg/kg is an available concentration to elicit antidepressant effects in rodents that was confirmed by previous reports (21, 22). For drug administration, the mice were temporarily placed in a transparent airtight chamber (35 cm × 25 cm × 15 cm) at room temperature for 10 min of acclimatization before the gas exposure sessions. The concentration of N_2O was monitored in real time. The gas flow was maintained at 2–3 L/min. At the end of the drug administration period, the mice were returned to their home cages, and 24 h after drug administration, the behavioral experiments of the mice were tested.

For local mPFC administration, the mice were fixed with gauze and allowed to recover for 1 week. The intracerebral drug deliveries were given in awake animals. *The* NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME, Abcam, ab120136) was dissolved in saline to a concentration of 10 μg/μL. Local L-NAME administration was performed 30 min prior to gas exposure. L-NAME was infused into the mPFC (0.1 μl/min, 5 min total).

For the electrophysiological experiments, L-NAME (20 mg/kg) was dissolved in saline and administered to the mice 30 min before every gas exposure through an intraperitoneal injection (i.p.).

Forced Swim Test (FST)

The apparatus used for the FST was a transparent plastic cylinder with size of 35 cm height × 10 cm diameter. During the FST, mice were individually placed in a cylinder of water with the depth of 20–26 cm to prevent the mice from touching the bottom with their limbs. The behavior tests were performed 24 h after the final N_2O exposure. The water temperature was maintained at 24–25°C. The mice were allowed to swim for 10 min at least 1 day before the normal experiments were performed. Twenty-four hours after drug administration, the mice were placed in a quiet behavioral testing room and left undisturbed for at least 1 h before initiation of the experiment. The behavioral test was performed under subdued light (the same condition controlled during the tail suspension and open field tests). During the experiments, the mice were allowed to swim for 6 min, and their activity was videotaped. The duration of immobility, which was defined as the time during which the mice were floating or remained motionless, was assessed during the last 4 min.

Tail Suspension Test (TST)

In the TST, the mice were suspended by affixing their tails to the edge of a shelf at a height of 80 cm above the floor, and each animal was subjected to a 6-min suspension session. The activity of the mice was videotaped. The duration of immobility, which was defined as the time during which the mice remained motionless, was assessed during the last 4 min.

Open Field Test (OFT)

The OFT is a standard method for profiling the exploratory behavior and general activity of mice. The apparatus was composed of opaque black plastic (35 cm × 35 cm × 40 cm) and was placed in a soundproof room. The mice were randomly placed in the center of the field and were allowed to explore the box for 10 min, and their activities were monitored online. The path and velocity of each animal were calculated offline.

Protein Extraction and Western Blotting

Twenty-four hours after drug administration, the mice were anesthetized with pentobarbital sodium (80 mg/kg, i.p.) and euthanized by decapitation. The bilateral mPFC regions were dissected immediately and homogenized in RIPA Lysis Buffer (Beyotime, P0013B) with 1 mM PMSF (Sigma, P7626), and dissociated by sonic disruption. The homogenate was centrifuged at 14,000 g for 15 min, and the total protein concentration was assessed. The primary and secondary antibodies used in the Western blotting assay were anti-nNOS (1:500, Abcam, ab76067), anti-iNOS (1:500, Abcam, ab15323), anti-BDNF (1:1,000, Abcam, ab108319), anti-β-tubulin (1:1,000, Cell Signaling Technology, 2128), and goat anti-rabbit IgG (1:5,000, Abcam, ab6721) antibodies. An enhanced chemiluminescence (ECL, Thermo, 32109) reaction solution was added. The samples were then recorded on X-ray film (Carestream, 8294985) for visualization, and the immune reactivity was quantified using ImageJ software.

Electrophysiological Techniques

The mice were anesthetized using pentobarbital sodium (80 mg/kg, i.p.) 24 h after drug administration, and then the electrophysiological experiment was performed. The recording lasted for 3–5 h, and during the recording period, the mice were maintained under anesthesia. The anesthetized mice were then mounted on a stereotaxic apparatus (Narishige, Japan) for extracellular recording. The body temperature was maintained at 36–37°C using a thermostatically controlled heating pad (ATC1000; WPI). In our study, we mainly recorded the spontaneous neuronal activities in the mPFC. The glass electrodes were filled with 2 M NaCl (Sigma, St Louis, MO, USA) and 0.5% Chicago sky blue (Sigma, C8679) solution. The electrodes were placed in the mPFC (coordinates: A/P: + 2.0 to +2.9 mm; M/L: 0 to ±0.90 mm; D/V: -2.5 to -3.5 mm) by using an electric-microdrive (Narishige). Neuronal firing was recorded by a preamplifier and bandpass filtered (Axon clamp, 900A). The data were analyzed with an oscilloscope (Nicolet, Model 2090-I, USA) and stored in a computer equipped with a Clampfit (10.2 Axon, USA) analysis system. To obtain stable firing, the first 2 min of firing were not included in the analysis, and the cells that fired for less than 5 min were excluded. After the recording, the location of the recorded neurons was histologically confirmed in the mPFC.

Statistics

The data analysis was performed in double-blinded manner. The data are expressed as the means ± SEMs. SPSS software (version 19 for Windows) was used for the statistical analyses. The statistical parameters, including the exact value of n, precision measures (means ± SEMs) and statistical significance, are presented in the figures. In single drug treatment experimental

design (**Figures 1**, **2**, **3A, B**, and **Supplementary Figures 1** and **3**), the data were evaluated by *one-way ANOVA* with a *post hoc least significant difference (LSD)* test. In the experimental design with an added NOS inhibitor (L-NAME, **Figures 3C, D–5** and **Supplementary Figure 2**), the data were analyzed using *two -way ANOVA* and *post hoc LSD tests*. $p < 0.05$ indicated statistical significance.

RESULTS

Repeated N_2O Exposure Exerted Antidepressant-Like Effects in Mice

In this study, *50%* N_2O mixed with 50% O_2 mixture was used based on the clinical study by Nagele et al. (10). First, we assessed whether N_2O exerted antidepressant-like effects in the male mice. A single dose of the N_2O mixture was administered for 2 h per day, and this exposure was repeated on three consecutive days (**Supplementary Figure 1**). To avoid stress response, three behaviors (FST, TST, and OFT) were tested once a day. The FST, TST, and OFT were performed 24, 48, and 72 h after the last exposure to N_2O, respectively. The timeline of the experiments and treatments is presented in **Figure 1A**. The results showed that both repeated N_2O exposure, and ketamine treatments significantly reduced the immobility duration in the FST and TST compared with those of the control and vehicle groups [**Figure 1B**, one-way ANOVA, $F(3, 34) = 2.896$, $p = 0.049$; **Figure 1C**, one-way ANOVA, $F(3, 33) = 2.923$, $p = 0.048$]. Total distance in the OFT was not significantly altered in the N_2O- and ketamine-treated groups [**Figure 1D**, one-way ANOVA, $F(3, 33) = 0.455$, $p = 0.715$], and this finding excluded the possibility that N_2O or ketamine-induced psychomotor changes and yielded false-positive results. Collectively, the results indicated that repeated exposure to N_2O induces antidepressant-like responses in mice.

N_2O Increased Neuronal Activity in the mPFC

To understand whether N_2O can affect neuronal activity, the spontaneous neuronal activity was assessed through *in vivo* extracellular electrophysiological recordings. The neural activity of the prelimbic subregion of the mPFC (PrL, A/P: +2.0 to +2.9 mm; M/L: 0 to ± 0.9 mm; D/V: -2.5 to -3.5 mm; **Supplementary Figure 3B**) was recorded. Representative samples of the spontaneous firing activities of different groups are shown (**Figure 2A**). A positive-skewed distribution in the inter-spike interval histogram (ISIH) (**Figure 2C**) indicated a burst firing pattern (**Figure 2B**, left). A single firing pattern was observed as the firing activity of neurons fired in irregular single spikes (**Figure 2B**, right). We recorded two discharge single firing patterns: one pattern was represented by a nearly symmetrical ISIH (**Figure**

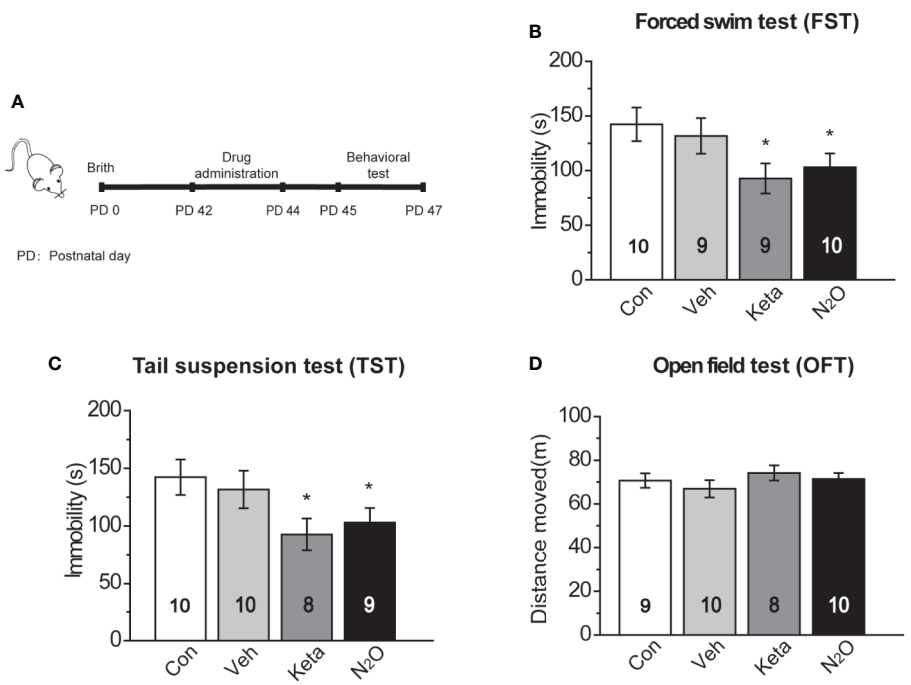

FIGURE 1 | Antidepressant-like effects induced by repeated N_2O exposure. **(A)** The timeline of the experiments and treatments is presented. Twenty-four hours after the final drug administration, the mice were subjected to forced swim test (FST) **(B)**, tail suspension test (TST) **(C)**, and open field test (OFT) **(D)**. The group details as follows: Control (Con, air exposure); Vehicle (Veh, 50% N_2 + 50% O_2 exposure); Ketamine (Keta, 20 mg/kg, i.p.); N_2O (50% N_2O + 50% O_2 exposure). All gas exposures were performed by administering a single dose for 2 h per day for three consecutive days. Ketamine was injected only once at the last day of drug exposure. The immobility time of the mice in the FST and TST was measured, and the total distance that the mice moved in the OFT was measured. The inserted number represents the number of animals in each group. *$p < 0.05$ compared with the control group.

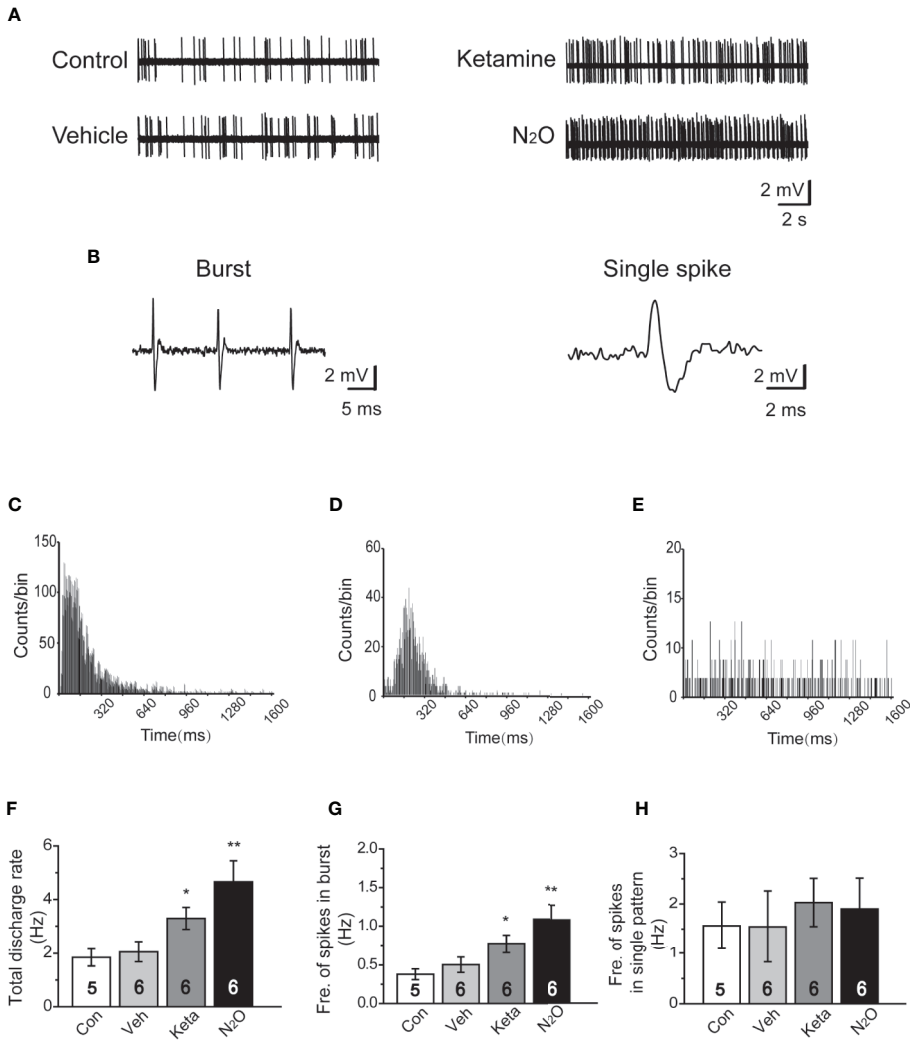

FIGURE 2 | N_2O enhanced the neuronal activity in the mPFC. In vivo extracellular electrophysiological recordings were performed 24 h after the final drug treatment. **(A)** Representative samples of the spontaneous firing activities of each group are shown. **(B)** Representative traces of the burst firing pattern (left) and single firing pattern (right) are shown. **(C)** The burst firing pattern is represented by a positive-skewed distribution of the inter-spike interval histogram (ISIH). The single firing patterns are represented by a nearly symmetrical ISIH **(D)**, and the other is a relatively straight ISIH **(E)**. The numbers of burst and single spikes were counted in the discharge rate. **(F)** The total discharge rates of the neurons in the mPFC of the different groups were counted. **(G)** The frequency of spikes in the burst firing patterns was shown for the different groups. **(H)** The frequency of spikes in the single firing patterns was shown for the different groups. The inserted number was the number of animals used for the electrophysiological recording. The group info is consistent with that in **Figure 1**. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

2D), and the other pattern was represented by a relatively straight ISIH (**Figure 2E**).

Because neurons often showed burst and single-spike firing activities within a single-unit recording, we calculated the discharge rate, which included the burst and single spikes. The results showed that N_2O can elicit a significant change in the discharge rate of neurons in the mPFC [**Figure 2F**, one-way ANOVA, $F\ (3, 64) = 7.637$, $p = 0.0001$]. Besides, the burst activity, including the frequency of spikes in the burst, obtained with N_2O was higher than that of the vehicle group [**Figure 2G**, one-way ANOVA, $F\ (3, 41) = 10.192$, $p = 0.00009$]. Furthermore, N_2O significantly increased the overall firing rate and shifted the cumulative frequency of inter-spike intervals (ISIs) toward those observed in a burst pattern. Thus, burst firing in the mPFC might contribute to the antidepressant-like effects of N_2O. We counted and analyzed the burst firing and single firing patterns, respectively, and found that approximately 65% of the recorded neurons exhibited burst firing and the remaining 35% of neurons showed single firing patterns. However, we found that single firing patterns did not cause significant changes in discharge in the different groups [**Figure 2H**, one-way ANOVA, $F\ (3, 19) = 0.173$, $p = 0.913$]. The increase in burst firing obtained after N_2O administration made major contributions to the enhancement in neuronal activities in the

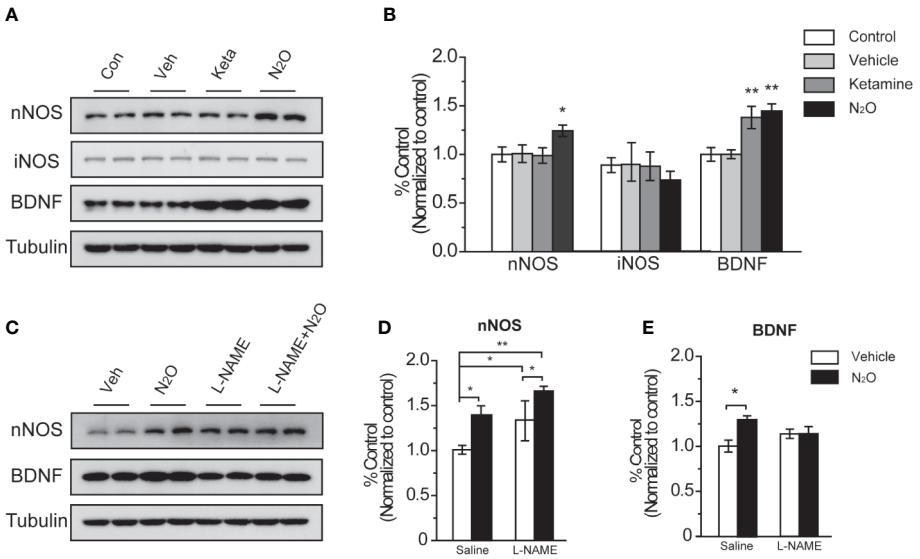

FIGURE 3 | N_2O enhanced the expression levels of nNOS and BDNF in the mPFC. All the mPFC tissues were extracted 24 h after the final drug administration. **(A**, **C)** Western blot images and **(B, D, E)** quantification analysis of nNOS, iNOS and BDNF expression levels in the mPFC. The β-tubulin was measured as the reference protein. For panels **(A, B)**, the group info is consistent with that in **Figures 1** and **2**. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group. For panels **(C–E)**, the mouse mPFCs were pre-infused with saline (5 μL/side) or L-NAME (5 μg/side) 30 min before every gas exposure. The groups were as follows: Vehicle (saline or L-NAME mPFC infusion before 50% N_2 + 50% O_2 exposure) and N_2O (saline or L-NAME mPFC infusion before 50% N_2O + 50% O_2 exposure). $^*p < 0.05$ and $^{**}p < 0.01$ compared with the vehicle group.

mPFC, which suggested that the burst firing pattern can be regarded as a predictor of the N_2O treatment response.

N_2O Enhanced the Expression Levels of nNOS and BDNF in the mPFC

To further examine the possible mechanisms underlying the antidepressant-like effects of N_2O treatment, we assessed the changes in protein expression by Western blotting.

NOS has three isoforms, namely, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), and functional deficits in nNOS or iNOS had been reported to associated with MDD. However, in our study, we found that repeated N_2O exposure increased the expression level of nNOS but not iNOS in the mPFC [**Figures 3A, B**, one-way ANOVA, $F(3, 20) = 5.803$, $p = 0.005$ for BDNF, one-way ANOVA, $F(3, 20) = 3.241$, $p = 0.044$ or nNOS; and one-way ANOVA, $F(3,12) = 1.551$, $p = 0.252$ for iNOS]. Furthermore, the level of BDNF, a critical neurotropic factor for neurogenesis and neuroplasticity, was also increased by the administration of N_2O (**Figures 3A, B**). BDNF plays important an role in neural development and the regulation of synaptic plasticity (23), which is diminished in depressive disorders (24).

To confirm whether BDNF expression is dependent on the activation of nNOS after N_2O administration, we applied NOS inhibitor, L-NAME (5 μg/side), which we locally infused into the mPFC before drug treatment to specifically block nNOS activity in the mPFC. The results showed that N_2O exposure didn't further increase BDNF levels in the mPFC when that region was pre-treated with L-NAME [**Figures 3C–E**, two-way ANOVA, $F(1, 19) = 3.766$, $p = 0.067$ for BDNF, and two-way ANOVA, $F(1, 20) = 1.325$, $p = 0.263$ for nNOS]. In addition, we unexpectedly observed that local L-NAME treatment increased nNOS expression levels without affecting BDNF expression levels (**Figures 3C–E**). Because BDNF is a major growth factor in the brain that might represent a potential therapeutic target in MDD, the increase nNOS activation dependent increase in BDNF might be involved in the antidepressant-like effects of N_2O.

N_2O Increases mPFC Neural Activity Mediated by the Activation of nNOS

The above results demonstrated that repeated N_2O exposure increased BDNF signaling in a nNOS activation-dependent manner. Therefore, we hypothesized that nNOS might play a key role in the N_2O-induced enhancement of neuronal activity in the mPFC.

To verify this hypothesis, we injected the mice with L-NAME (20 mg/kg, i.p.) in the mPFC 30 min before N_2O exposure. Twenty-four hours after the final N_2O exposure, extracellular electrophysiologyical recording was performed in the mPFC (**Figure 4A**). Interestingly, we found that the systemic injection of L-NAME could block the antidepressant-like effects induced by repeated N_2O exposure [**Supplementary Figure 2**, two-way ANOVA, $F(1, 35) = 3.259$, $p = 0.080$ for FST; two-way ANOVA, $F(1, 33) = 2.983$, $p = 0.93$ for TST; two-way ANOVA, $F(1,30) = 1.867$, $p = 0.182$ for OFT]. We further examined whether mPFC neuronal activity could also be inhibited by pretreatment with L-NAME. As expected, the N_2O-induced increases in the discharge rate and burst firing were blocked by pretreatment with L-

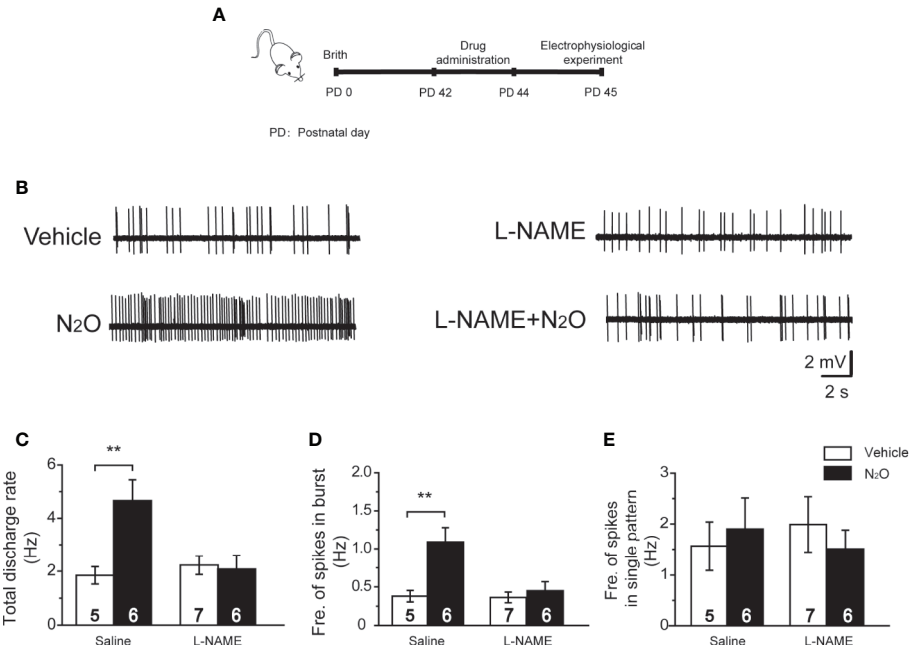

FIGURE 4 | A NOS inhibitor reversed the action of N_2O on the neuronal activity in the mPFC. **(A)** The timeline of the experimental process. In vivo extracellular electrophysiological recordings were performed 24 h after the final drug treatment. **(B)** Representative samples of the spontaneous firing activities of each group are shown. The numbers of burst and single spikes were both counted in the discharge rate. **(C)** The total discharge rates of the neurons in the mPFC were counted for the different groups. **(D)** The frequency of spikes in the burst firing patterns was shown for the different groups. **(E)** The frequency of spikes in the single firing patterns was shown for the different groups. The mice were pretreated with saline (10 mL/kg, i.p.) or L-NAME (20 mg/kg, *i.p.*) by intraperitoneal injection 30 min before the gas exposure. The groups were as follows: Vehicle (saline or L-NAME intraperitoneal injection before 50% N_2 + 50% O_2 exposure); N_2O (saline or L-NAME intraperitoneal injection before 50% N_2O + 50% O_2 exposure). The inserted number was the number of animals used for the electrophysiological recording. $^{**}p < 0.01$ compared with the vehicle group.

NAME [**Figures 4B, C**, two-way ANOVA, $F\ (1, 42) = 3.902$, $p = 0.055$; **Figure 4D;** two-way ANOVA, $F\ (1, 42) = 3.434$, $p = 0.071$]. The irregular firing did not significantly change after drug administration [**Figure 4E**, two-way ANOVA, $F\ (1, 24) = 1.485$, $p = 0.235$], as described above. These results suggested that the antidepressant-like effects of N_2O might be due to increased mPFC excitability followed by nNOS activation.

The Antidepressant-Like Effects of N_2O Were Dependent on NOS Activation in the mPFC

Based on the above results, we confirmed that N_2O increased neuronal activity and BDNF expression in the mPFC, and these processes were dependent on the activation of nNOS. Therefore, we further asked whether the antidepressant-like effects of N_2O were dependent on nNOS activation in the mPFC. To block the activity of nNOS in the mPFC, we pretreated the mPFC with L-NAME 30 min before N_2O exposure. The behavioral tests were performed 24 h after the final exposure of N_2O (**Figure 5A**). The results showed that repeated N_2O exposure decreased immobility duration in the FST and TST was reversed by pretreatment with L-NAME (5 μg/side) in the mPFC (PrL). L-NAME alone showed no significant effects on the FST and TST [**Figure 5B**, two-way ANOVA, $F\ (1, 32) = 1.768$, $p = 0.193$; **Figure 5C**, two-way ANOVA, $F\ (1, 29) = 0.391$, $p = 0.537$]. The total distance traveled in the open field did not show significant changes between the groups, which indicated that L-NAME and/or N_2O exposure did not affect the locomotor activity of the mice [**Figure 5D**, two-way ANOVA, $F\ (1, 21) = 0.092$, $p = 0.765$]. In summary, these results suggested that the antidepressant-like effects induced by repeated N_2O exposure were dependent on the activation of nNOS in the mPFC (PrL).

DISCUSSION

In our study, we investigated the antidepressant-like properties of N_2O through multiple behavioral tests. To address the underlying mechanism, we explored the neuronal activities in the mPFC and intracellular signal transduction pathways. The results showed that repeated N_2O exposure increased the neuronal burst-firing activity and upregulated the nNOS and BDNF expression levels in the mPFC. Furthermore, the antidepressant-like effects and enhanced neuronal activities induced by N_2O were blocked by the inhibition of nNOS. The results suggested that repeated N_2O exposure enhances BDNF expression level and burst firing rate in an nNOS activation-dependent manner in the mPFC, and these effects might underpin the molecular mechanisms underlying the antidepressant-like effects of N_2O.

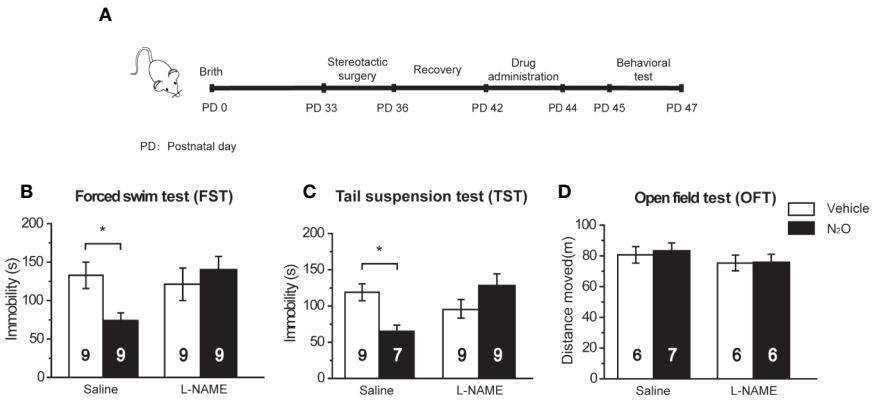

FIGURE 5 | The antidepressant-like effects of N2O were blocked by a NOS inhibitor. **(A)** The timeline of the experimental process. All the experiments were performed 24 h after the final drug administration, and the mice were then subjected to FST **(B)**, TST **(C)** and OFT **(D)**. The mPFC of the mice was pre-infused with saline (5 μL/side) or the NOS inhibitor L-NAME (5 μg/side) 30 min before the gas exposure. The group info is consistent with that in **Figures 3C–E**. The immobility time of the mice in the FST and TST was measured, and the total distance that the mice moved in the OFT was measured. The inserted number represents the number of animals in each group. *$p < 0.05$ compared with the vehicle group.

The concentration of general anesthetics is a critical impact factor for CNS function, and the affected sites of the CNS by general anesthetics are concentration dependent (25). The binding preference of general anesthetics for subunits of excitatory and inhibitory neurotransmitter receptors, such as NMDA receptors and $GABA_A$ receptors, is also concentration dependent (26). In addition, substantial studies have shown that subanesthetic concentrations prompt neuronal stem cell survival and neurogenesis, but high concentrations may cause neurotoxicity in the CNS (27). For N_2O, a clinical study by Nagele and colleagues applied short-term subanesthetic N_2O exposure (50% N_2O + 50% O_2 inhaled for 1 h in a single session), which showed antidepressant effects in TRMD. The subanesthetic dose of N_2O was selected based on the routine use for analgesia and mild sedation in anesthesiology and dentistry (10, 28). In the present study, the 50% N_2O concentration was selected, but unlike appearances in humans, repeated 2 h rather than a single 1-h subanesthetic N_2O exposure triggered antidepressant-like effects in mice. However, the underlying mechanism of this phenomenon is unclear. The optimal antidepressant dose of N2O should be further addressed in future studies.

NO is a gaseous neurotransmitter that governs multiple physiological functions in the CNS. NO is synthesized by NOS, which has three isoforms, namely, nNOS, iNOS, and eNOS, and all three NOS isoforms exist in the CNS and affect cell signaling in brain (29). Evidence has shown that deficits in NOS activity are associated with the neurobiology of MDD (30, 31). Especially for nNOS, studies have shown that dysfunction of nNOS in the paraventricular hypothalamic nucleus, locus coeruleus, mPFC, and hippocampus, is related to MDD (29, 32, 33). Besides, iNOS also is involved in stress-triggered depression, as iNOS derived NO and iNOS mRNA levels increased in the cortices of depression animal model (34). However, a lower number of studies reported the relationship between eNOS and depression and genetic studies suggested there is no correlation between eNOS and MDD (35). In the present study, we demonstrated that N_2O exposure significantly increased the expression level of nNOS rather than iNOS. As the major route for the production of NO in the brain is mediated by nNOS catalysis (29), our results suggest that nNOS might be a dominant molecule in mediating the antidepressant-like effects of N_2O.

Although abundant reports have emphasized the critical role of nNOS in the neuropathology of MDD, the conclusion is controversial (29). Some studies pointed to an increased activity of nNOS in the corticolimbic system in MDD, and NOS inhibitors showed antidepressant effects through indirect regulation of the monoaminergic system (29, 36). However, several other studies showed that stress exposure diminished nNOS activity in the hippocampus which further caused a deficit in learning and memory processes in animals (37, 38). Postmortem studies also revealed a decrease in nNOS expression in the anterior cingulate cortex (ACC) of MDD patients, which indicated weakened nNOS activity in the process of MDD (39). Therefore, there exists an imbalance but not simply an increase or decrease in nNOS activity in the pathophysiology of MDD. In the present study, we unexpectedly observed that local NOS inhibitor (L-NAME) treatment increased nNOS expression levels without affecting BDNF levels in the mPFC or the depressive behaviors of animals. For this paradoxical phenomenon, we infer that there may exist a feedback mechanism by which L-NAME, as a NOS inhibitor, inhibits the activity of nNOS and further elevates the expression level of nNOS as a result. Because the real activity of nNOS was low (inhibited by L-NAME), L-NAME induced higher expression levels of nNOS could not further activate BDNF signaling and trigger antidepressant-like effects. The concrete mechanisms need to be studied in the future.

BDNF is a developmentally expressed growth factor that regulates plasticity in the adult brain. Preclinical studies have shown that the function of BDNF could be decreased by several forms of stresses Chronic treatments with antidepressants

activate BDNF-mediated signaling (40). nNOS-derived NO could modulate BDNF signaling in stress adaptation, which further affects synaptic plasticity in emotionally related brain regions such as cerebral cortex, amygdala, hippocampus and striatum (29, 41). However, the interplay between NO and BDNF signaling has been elusive. Some reports point to that NO appears to negatively modulate BDNF function, because BDNF signaling is augmented by the NOS inhibitor L-NAME (42). However, evidence from cultured hippocampal neurons demonstrated that endogenous low levels of NO could facilitate BDNF signaling, indicating that BDNF signaling is regulated by NO in a concentration-dependent manner (43). In the present study, we observed that the BDNF expression level in the mPFC was upregulated by N_2O exposure, and the increase of BDNF was blocked by pre-administration of the NOS inhibitor L-NAME. Based on the above information, we suggest that N_2O exposure with a subanesthetic dose might increase intracellular NO, which activates BDNF-dependent synaptic plasticity. Our findings enhance the understanding of the role of nNOS activation induced increase of BDNF signaling in the antidepressant-like responses and support a promising research direction of N_2O as a potential antidepressant for clinical use.

Although N_2O and ketamine have similar antidepressant actions and mechanisms, the antidepressant efficacy of N_2O might not be robust as that of ketamine. Moreover, previous studies have indicated that N_2O exposure is associated with many unfavorable side-effects (e.g., euphoria, altered body awareness and image, altered time and perception, and dreamy, detached experiential states) (44) and prolonged N_2O administration interferes with the activity of vitamin B_{12}, which results in sensory ataxia problems (11, 45). Therefore, the efficacy, safety and tolerability of N_2O should be carefully evaluated in the future. More work needs to be done to investigate the effective initial dose and maintenance dose, the therapeutic drug duration and the potential risks of N_2O when given repeatedly over time (44).

A valid animal model of depression is necessary to identify the biological mechanisms of MDD and the features of antidepressants. Currently, stress-induced models are the most commonly used depression model, which might be based on the fact that initial episodes of depression are precipitated by adversity. In addition, the etiology-based animal model of depression that directly targeting the underlying biological causations, including the hypothalamus-pituitary-adrenal (HPA) axis, the neuroinflammation system and the neurotransmission, also recapitulating specific dysfunctions of depression (46). MDD is a heterogeneous disease, and diverse animal models are believed to recapitulate different aspects of symptomatic dimensions and neuropathology associated with depression (47). The antidepressant efficacies and pharmacology mechanisms of N_2O should be carefully detected in the future study.

Mood disorders are characterized by profound deficits in reward circuits of brain. Mesocorticolimbic dopaminergic system, as the most important reward circuits, is constructed by the ventral tegmental area (VTA) originated dopaminergic projections to many brain areas such as the mPFC, nucleus accumbens (NAc), amygdala, and hippocampus. Through highly complex inter-projections, these brain regions have been shown to be involved in emotion-related behaviors, especially in the encoding of stressful events (48). The antidepressants might have broad influences on the reward circuit system. According to literatures, ketamine rapidly increases the activity of reward-related brain regions, including the orbitofrontal cortex, ventral striatum, VTA, amygdala, insula, and the ACC, which accompanied with the fast remission of depressive symptoms by ketamine (49). Moreover, ketamine also have positive impacts on dopaminergic transmission (50, 51). In the present study, we depicted a specific antidepressant mechanism of N_2O by focusing on the mPFC. We believe that N_2O might also have diverse influences on other reward-related brain regions that similar to the effects of ketamine, which should be addressed in the future studies.

In conclusion, we verified that repeated N_2O exposure exerts antidepressant-like effects in animals. Our preliminary results suggested that repeated N_2O exposure can enhance BDNF expression level and burst firing rates in an nNOS activation dependent manner in the mPFC, which might underpin the pharmacological mechanism underlying the antidepressant-like effects of N_2O exposure. Collectively, the results obtained in the present study might provide some theoretical basis for developing a relatively promising and safe method for the treatment of MDD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by All the animal studies and experimental procedures were approved by the Animal Care Committees of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and the experiments were performed in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

WL, QL, and BY conducted the majority of the experiments, including stereotactic surgery, electrophysiological part and behavioral tests. HC, JL, and WD conducted the western blotting and stereotactic surgery. ZX, JL, and FS carried out the animal behavioral tests and electrophysiological part. TL and FG conducted the data analysis and brain regions detection. WL, QL, BY, BZ, and ZL. prepared the manuscript. WL, BZ, and ZL conceived and supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00837/full#supplementary-material

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Autophagy-Based Hypothesis on the Role of Brain Catecholamine Response During Stress

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Stressful events, similar to abused drugs, significantly affect the homeostatic balance of the catecholamine brain systems while activating compensation mechanisms to restore balance. In detail, norepinephrine (NE)- and dopamine (DA)-containing neurons within the locus coeruleus (LC) and ventral tegmental area (VTA), are readily and similarly activated by psychostimulants and stressful events involving neural processes related to perception, reward, cognitive evaluation, appraisal, and stress-dependent hormonal factors. Brain catecholamine response to stress results in time-dependent regulatory processes involving mesocorticolimbic circuits and networks, where LC-NE neurons respond more readily than VTA-DA neurons. LC-NE projections are dominant in controlling the forebrain DA-targeted areas, such as the nucleus accumbens (NAc) and medial pre-frontal cortex (mPFC). Heavy and persistent coping demand could lead to sustained LC-NE and VTA-DA neuronal activity, that, when persisting chronically, is supposed to alter LC-VTA synaptic connections. Increasing evidence has been provided indicating a role of autophagy in modulating DA neurotransmission and synaptic plasticity. This alters behavior, and emotional/cognitive experience in response to drug abuse and occasionally, to psychological stress. Thus, relevant information to address the role of stress and autophagy can be drawn from psychostimulants research. In the present mini-review we discuss the role of autophagy in brain catecholamine response to stress and its dysregulation. The findings here discussed suggest a crucial role of regulated autophagy in the response and adaptation of LC-NE and VTA-DA systems to stress.

Keywords: locus coeruleus, ventral tegmental area, dopamine, norepinephrine, drug addiction, sensitization, corticotrophin-releasing factor, brain-derived neurotrophic factor

INTRODUCTION

Stress is one consequence of challenges to the organism produced by events known as stressors that are usually identified with stimuli (or conditions) that, by definition, need to be unpredictable, uncontrollable and of forecasting uncertainty. These external or internal stimuli promote classic stress responses aimed at adaptation according to physiological and/or psychological compensation (1). Stress-associated adaptive changes may increase the resistance to pathological outcomes, thus

favoring resilience, at best, or, at worst, causing dysfunctional coping that increases "allostatic load" (1, 2), leading to a disease state instead. In mammals, including humans, the brain norepinephrine (NE) and dopamine (DA) systems, originating from the locus coeruleus (LC) and the ventral tegmental area (VTA) respectively, produce spread brain networks with cortical and subcortical projections (3). Both NE and DA brainstem neurons are targeted by stress hormones of the hypothalamus-pituitary axis (HPA) (4, 5). NE-LC and DA-VTA neurons are readily activated by stressful events involving neural processes related to perception, cognitive evaluation, appraisal, and stress-dependent hormonal factors. These systems operate in parallel and in synergism, allowing to implement neural adaptations and behavioral strategies aimed at supporting resilience and overcoming stressful events (6–9). In fact, both systems are crucially involved in reward and in efforts to support motivation and coping (3, 10–12).

Brain catecholamine response to stress results in time-dependent regulatory processes involving mesocorticolimbic circuits and networks (13–16). In this context, LC-NE neurons and their projections to the cortical, thalamic, basal forebrain, and brainstem regions, including the VTA, as well as forebrain DA-targeted areas, such as the medial prefrontal cortex (mpFC) and the nucleus accumbens (NAc), appear dominant in controlling DA-dependent responses to stress (2, 17–23). In fact, stressful stimuli activate LC-NE more readily than VTA-DA neurons, which is evident by the powerful release of NE within the mPFC surpassing at large that of DA, and by the increase in tyrosine hydroxylase (TH) and Fos expression occurring within LC but not VTA neurons (13, 14, 18, 24–28).

In detail, NE in the medial pre-frontal cortex (mPFC) may produce opposite effects on DA responses, inhibiting cortical DA transmission while increasing the accumbal DA outflow being induced by first exposure to motivationally salient stimuli (17, 18). Fluctuations of accumbal DA during novel uncontrollable/unavoidable stressful experiences are tightly controlled by the opposing influences of mPFC DA and NE. Enhanced DA release in the NAc is determined by NE release on pre-frontal cortical alpha 1-adrenoceptors (α1-ARs) in a condition of low mesocortical DA activation. Instead, inhibition of NAc DA release is promoted by the return of NE to basal levels and by a sustained increase of mesocortical DA release (18, 19), as occurs in long-lasting acute or in repeated/chronic stress (2, 29, 30). mPFC NE and DA might activate two different pathways to regulate mesoaccumbens DA release in opposite ways; an "activating pathway" provided by indirect glutamatergic (GLUT) projections onto VTA-DA cells (31) and an ''inhibitory pathway'' provided by prefrontal GLUT efferents to VTA-GABAergic interneurons or striato-mesencephalic neurons (32–34).

Again, stress-induced LC-NE over-activation, by potentiating DA outflow in the midline thalamus, leads to rapid and persistent decrease of $GABA_A$-mediated inhibitory transmission within the NAc-projecting neurons of the posterior paraventricular nucleus of the thalamus (pPVT) (23). This, in turn, promotes disinhibition of NAc-projecting neurons of the pPVT, which increases sensitivity to stress while potentiating DA efflux in the NAc shell (23, 35).

Sustained activity of LC neurons may directly potentiate the firing rate of VTA-DA neurons, mostly through α1-ARs (36–40). In addition to this direct excitation of VTA-DA neurons by LC-NE transmission acting on post-synaptic α1-ARs (36–40), an indirect stimulation is provided by inhibiting or stimulating VTA-GABA and VTA- GLUT terminals, respectively (41, 42). In fact, LC-NE may indirectly activate VTA-DA neurons by acting on pre-synaptic α1-ARs within VTA-GABA and VTA-GLUT terminals, and also on DA and GLUT terminals within the NAc (41–43). The regulation of VTA-DA neuronal activity by LC-NE inputs is quite complex. In fact, NE-induced excitation of VTA-DA neurons is followed by a long-lasting inhibition (36). This is in line with studies showing that selective lesion of LC may paradoxically increase the firing of VTA-DA neurons, though the underpinning mechanisms remain to be clarified (44). This may explain the apparent discrepancy which is present in experimental studies suggesting that stress-induced over-activity of LC-NE may exert either inhibitory or excitatory control of VTA-DA neurons (6, 45). In fact, LC-NE activity may reduce the vulnerability to emotional stress through opposite effects on VTA-DA neurons (6, 45), which may depend on the brain region and the time window of α1-AR stimulation. Such an issue remains under debate and needs experimental clarifications.

Noteworthy, stress is likely to affect catecholamine metabolism and neuroplasticity in a way which is reminiscent of the effects produced by abused substances (7, 46–48). In fact, stressful events are often reported to cause neuropsychiatric disorders going from depression to substance abuse, up to neurodegenerative insults where brain catecholamine-containing neurons and/or their projections are involved (4, 5). Chronic or heavy stress, similar to substance abuse, produces catecholamine-driven behavioral effects ranging from depression to addiction, and schizophrenia-like phenotypes. Such behavioral outcomes involving catecholamine systems are due to plastic phenomena underlying "neuronal sensitization" which in turn, is bound to alterations in the responsivity of type 1- or 2-like DA receptors (D1/D2-like DRs), and alpha/beta adrenergic receptors (α/β-ARs) (6, 16, 19, 49, 50). The occurrence of neural adaptation/maladaptation leads to specific stress-induced alterations of emotion, motivation, cognitive ability and coping.

In the latter decades, substantial attention has been paid to the role of the autophagy machinery in the physiology of catecholamine brain systems when insulted by pharmacological and neurotoxic agents (51–54). Autophagy has been recently connected with stress- and substance abuse-related disorders such as depression and addiction (51, 53, 55, 56). In keeping with this, the beneficial effects of several antidepressants and mood stabilizers are bound to autophagy activation (57–60) and autophagy inducers counteract behavioral sensitization induced by abused drugs (51, 56, 61). This wide and prolific research produced results that strongly suggest a crucial role of autophagy in response and adaptation of LC-NE and VTA-DA systems to stress. This enlightens the mechanisms by which the functional

balance of the nervous system and the related behavioral and cognitive capacities are guaranteed. A few studies directly explored the connection between stress and autophagy (62–64); most information can be drawn indirectly from psychostimulants research. In the present review we discuss the role of autophagy in brain catecholamine response to stress and those factors which may lead to dysregulation.

A BRIEF VIEW OF THE AUTOPHAGY MACHINERY: FROM DEGRADATION OF ALTERED INTRACELLULAR SUBSTRATES TO MODULATION OF SYNAPTIC PLASTICITY

Autophagy is a phylogenetically conserved eukaryotic cell-clearing system that plays a primordial role in cell homeostasis (65). It is generally distinguished into macroautophagy (hereafter referred to as "autophagy"), microautophagy, and chaperone-mediated autophagy, which all promote lysosome-dependent substrate degradation (66). Beyond removing altered protein substrates, autophagy targets mitochondria, pathogens, ribosomes, portions of endoplasmic reticulum or synaptic vesicles, which are conventionally designated as "mitophagy", "xenophagy", "ribophagy", "reticulophagy", and "vesiculophagy", respectively (66, 67). Moreover, autophagy modulates key cell functions ranging from synapse development to neurotransmitter release, and synaptic plasticity, as well as neuro-inflammation and –immunity (68, 69). A complex machinery including more than 30 autophagy-related-gene (Atg) products governs autophagy progression, starting from the biogenesis and maturation of autophagosomes up to their fusion with lysosomes. In particular, conversion of Atg8 (LC3 in mammals) into LC3-I, its ubiquitination-like enzymatic lipidation into LC3-II isoform, and eventually the incorporation of LC3-II into the phagophore membrane are critical for autophagosome assembly (59). In line with this, LC3-II is widely employed as a marker for monitoring autophagy at the morphological, ultrastructural, and biochemical level (52). However, since increased LC3-II levels may witness for either an increase or a decrease of the autophagy flux due to accumulation of stagnant vacuoles, assessment of LC3-II levels through semi-quantitative techniques can lead to results misinterpretation unless it is coupled with other autophagy markers or ultrastructural immune-labeling (52). Various additional Atg proteins ranging from Atg3 to Atg16 participate in autophagy progression *via* the processing and conjugation of Atg8/LC3 to the growing autophagosome membrane lipids (65). For instance, during Atg8 lipidation, Atg7 directly binds to and activates Atg8 fostering its transfer to the E2 enzyme Atg3. At the same time, Atg7 binds to Atg12 fostering its binding to Atg5. This leads to the formation of the Atg12-Atg5 conjugate complex, which then recruits Atg16 (65). The Atg12-Atg5/Atg16 complex localizes to the expanding phagopore where its acts as an E3 ligase mediating the final transfer of Atg8 to its lipid target phosphotidylethanolamine (PE).

The best-known autophagy-modulating pathway consists of the mTOR complex1 (mTORC1), a downstream substrate of the phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT axis, which conveys extracellular and environmental stimuli to control cell growth, proliferation, protein synthesis and metabolism in response to bioenergetics and nutritional requests (70). Other well-known pathways that foster autophagy initiation consist of the activation of 5′ AMP-activated Protein Kinase (AMPK), and transcription factor EB (TFEB) or inhibition of glycogen synthase kinase 3 beta (GSK3-β) (58, 71).

A number of CNS disorders are characterized by dysregulated autophagy and related synaptic alterations, and/or oxidative and inflammatory processes connected with neuronal loss (51, 69). In line with this, autophagy provides neuroprotection in general, and for catecholamine neurons, which are mostly susceptible to oxidative-related alterations, in particular (49, 52, 53, 68). In fact, autophagy grants the survival of both DA- and NE-containing neurons during a variety of stressful conditions (49, 51, 52, 68, 70). Autophagy alterations are associated with the effects of abused substances (amphetamine, methamphetamine, cocaine, ethanol) on brain catecholamine neurons concerning their morphology, neuroplasticity, as well as neurotoxic and behavioral effects (51, 52, 56, 61). This is evident by a variety of behavioral effects produced by psychostimulants based on autophagy-dependent alterations (56, 61). Psychostimulants alter neuroplasticity of DA and NE neurons through receptor sensitization/desensitization while affecting their activity and metabolism (7, 49). In keeping with this, autophagy orchestrates the turnover and responsivity of various neurotransmitter receptors by intermingling with the proteasome system and intracellular trafficking and secretory pathways (66, 67). This is key to modulate neurotransmitter release while promoting either desensitization or recycling of neurotransmitter receptors to the plasma membrane. In this context it is worth noting that alterations in autophagy-dependent modulation of vesicular DA trafficking and amount of DA release contribute to maladaptive plastic changes underlying various behavioral disorders (51, 68). Conversely, autophagy induction *via* mTOR or GSK3β inhibition improves early psychomotor and cognitive alterations by rescuing neurotransmission defects in various DA-related disorders (50, 51, 59, 61, 68, 72–74).

This is not surprising since behavioral alterations are related to intracellular pathways being placed downstream of neurotransmitter receptors, which are bound to the autophagy machinery. For instance, D1/D2-like DRs, including D1DR and D5DR act as negative regulators of autophagy *via* mTOR activation (75). In detail, D1/5-DR silencing in cells lines increases LC3-II levels while attenuating mTOR activity as evident by the decrease in the levels of its downstream substrate phospho-p70-S6K, indicating activation of autophagy (75). Opposite results are obtained following D1/5-DR overexpression (75). Remarkably, when D1/5-DR silencing is combined with administration of the autophagy flux blockers bafilomycin/chloroquine, the latter are less effective in inhibiting autophagy compared with negative controls, as shown by higher

LC3-II and lower phospho-p70-S6K levels (75). This suggests that D1/D5-DRs may exert a powerful negative control on the autophagy machinery. Opposite results were obtained for D2-likeDRs, indicating that they act as autophagy stimulators through AMPK activation and mTOR inhibition (75). This was confirmed in several cells lines, including TH-positive primary midbrain neurons, where DRD2 and DRD3 activation by pramipexole and quinpirole promotes beclin 1-depedent autophagy activation (76). This is associated with neuroprotection and inhibition of alpha-synuclein/SNCA accumulation both in rotenone-treated catecholamine-containing cells that overexpress wild-type or mutant alpha-synuclein and in *SNCA* transgenic mice (76). More recently, D3DRs were shown to be specifically responsible for autophagy activation *via* AMPK stimulation and mTOR inhibition (77).

Despite such an evidence suggesting that D1-likeDRs may block while D2-likeDRs may promote the autophagy flux, further *in vivo* confirmatory studies are needed.

Autophagy is also variously altered by the signaling pathways cyclic AMP (cAMP)/protein kinase A (PKA)/protein kinase C (PKC) and TFEB/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG-α) which are triggered downstream of ARs (78–83). In particular, β2-ARs may induce autophagy, which is associated with NE-related protection (79, 82, 83). For instance, agonist-induced β_2-ARs activation prevents disruption of autophagy flux in skeletal muscle of mice with neurogenic myopathy, which is associated with improved skeletal muscle proteostasis and contractility properties (83). Autophagy blockade through either chloroquine or skeletal muscle-specific deletion of *Atg7* abolishes the beneficial effects of β_2-ARs activation. Similarly, administration of the β_2-agonist clenbuterol stimulates the autophagy flux in hepatic cells, while the β_2-antagonist propranolol produces opposite effects (79). These effects were confirmed by co-administering chloroquine and through both biochemical (LC3II and p62 quantification) and ultrastructural analyses (79). Nonetheless, the potentially beneficial effects of NE-induced autophagy remain to be confirmed.

STRESS AND BIDIRECTIONAL LC-VTA COMMUNICATION: POTENTIAL ROLE OF AUTOPHAGY

Abused psychoactive substances and stress engage shared DA and NE neural mechanisms, as shown for instance by numerous studies pointing to a reciprocal cross-sensitization (84, 85). Stress, similar to abused substances, strongly stimulates LC-NE and VTA-DA transmission, producing an activity overload that brings into play adaptation mechanisms based on feedback circuits between connected neural systems and molecular adjustments in the cells. When stress persists, these compensatory mechanisms fail to restore an ante-stress balance and foster neurodegeneration (6, 82, 86). In fact, during chronic/prolonged stress or drug abuse, a dysregulation of LC-VTA connectivity may occur, decreasing catecholamine release due to diminished LC and VTA activities (9, 86–92). While early being associated with apathy and depression, stress- or drug-induced alterations within LC and VTA may predispose to cognitive decline and neurodegeneration (86, 93). Here we consider available evidence to cast the hypotheses that (i) stress or drug-induced LC-NE overload may alter VTA-DA neurons activity, plasticity and metabolism; (ii) DA overload may in turn lead to a progressive reduction of LC activity that occurs during chronic stress; (iii) a reduction of LC activity may occlude the neurotrophic and neuroprotective effects of NE in LC projecting areas, including the VTA. These functional links between DA and NE brain systems suggest a crucial role for the catecholamine network in adaptive behavior and in stress demands to which the organism has to cope with. At the same time, some contradictory findings may be explained by the double-faceted effects of NE in the brain. While at physiological levels NE exerts neuro-protection, abnormally increased NE levels may induce apoptosis and neurodegeneration (94). In this frame, the autophagy machinery, as a major pathway that regulates both neuronal proteostasis and synaptic plasticity, may be involved in various steps of catecholamine systems' response to stress and drug intake/administration.

LC-NE and VTA-DA Transmission Overload

Brain DA and NE systems are connected through cortical-subcortical circuitry as well as through direct bidirectional LC–VTA pathways. The two-way communication between LC and VTA is key for drug-induced reward and reinforcement underlying maladaptive synaptic plasticity in striatal, limbic and cortical brain areas (7, 95). In detail, NE from the LC potently regulates drug-induced reward and reinforcement by stimulating DA release mostly within the ventral striatum (96). This may occur either directly or indirectly since NE-containing axons of the LC project to both VTA, and likely, the NAc shell (97). While DRD1-expressing medium spiny neurons of the NAc medial shell directly inhibit mesolimbic VTA-DA neurons, NAc lateral shell neurons mainly project to the VTA-GABA neurons to disinhibit VTA-DA neurons (98–100). These in turn, fire back to both the NAc lateral shell (98) and the LC (101). Selective stimulation of NAc lateral terminals in the VTA induces a potent reward phenotype, which is likely caused by a disinhibition of VTA-DA neurons (98). Acute exposure to stress, similar to drugs of abuse, alters inhibitory plasticity which may increase VTA excitability (42, 84, 100, 102). This occurs by blocking the induction of long-term potentiation at $GABA_A$ synapses while increasing GLUT release on VTA neurons (42, 43, 84, 100, 102).

In this scenario, autophagy is a key by acting at the level of both GABA and DA systems (103–106). Conditional deletion of *Atg7* in GABA inhibitory or excitatory neurons, similar to what observed in Unc-51 Like Autophagy Activating Kinase 2 heterozygous (*Ulk2*+/-) mice, leads to autistic-like behavioral abnormalities including social deficits, increased distress and anxiety along with cognitive alterations (103, 104). In detail,

autophagy deficiency within GABA neurons brings to hyper-excitability due to reduced membrane expression of $GABA_A$ receptors. In fact, these receptors are entrapped within SQSTM1/p62-positive aggregates (103, 104). Autophagy activation replaces $GABA_A$ receptors on the plasma membrane thus reducing abnormal hyperexcitability (104). At behavioral level this is evident by rescuing behavioral deficits in *Ulk2*+/- mice (104). Again, mice lacking *Atg7* specifically within DA neurons display increased evoked striatal DA secretion along with decreased DA re-uptake (105, 106). In line with this, activation of mTOR-dependent autophagy decreases evoked DA release in wild-type but not in transgenic mice (105). Thus, an impairment of mTOR-dependent autophagy at the synapse fosters unrestrained DA release (105). Consistently with this, abused substances activate mTOR signaling in the mesolimbic reward circuit while administration of the mTORC1 inhibitor and autophagy activator rapamycin reverses drug-induced relapse and reinforcement (61, 107–110). For instance, systemic treatment with rapamycin, similar to the infusion of lentivirus-expressing mTOR-shRNA into the NAc shell, suppresses the induction of methamphetamine-induced sensitization while rescuing morphological alterations in the NAc's dendritic spines (61). Again, selective deletion of *mTOR* within mouse VTA counteracts drug addiction by decreasing DA release in the NAc through potentiation of VTA-GABAergic neuron firing (110). Thus, mTOR-dependent autophagy regulates drug action by modulating both DA and GABA signaling within the VTA and subsequent DA release within target brain areas. This indicates that an autophagy impairment within the VTA may strengthen the feedback loop in which the VTA fires to the NAc, and back to the LC. In this way, the LC would then feedback into the VTA *via* α1-ARs to further evoke DA release in the NAc, potentially sustaining behavioral sensitization (7, 39).

Sustained LC-NE Transmission Predisposing to Oxidative-Related Neuronal Alterations Within VTA

When dealing with the multiple effects of α1-ARs, we may summarize that LC-NE transmission increases VTA-DA neurons activity both directly and indirectly by acting on α1-ARs within i) VTA-DA neurons, ii) GABA and GLUT terminals within the VTA, iii) DA and GLUT terminals in the NAc and mPFC (36–63). A crucial role of α1-ARs stimulation in the VTA by LC-NE is documented for the neurochemical and reward processes of abused substances, which leads to an increase in DA release through LC projections to VTA and NAc shell (7, 39, 47, 97). Overlapping with the effects of acute stress and drug exposure, activation of presynaptic α1-AR within the VTA depresses GABA while enhancing GLUT release and increasing AMPAR/NMDAR ratios within VTA-DA neurons (41, 42, 84, 111). Although different brain nuclei being targeted by LC-NE are known to serve as a source of GLUT to the VTA (including the prefrontal cortex and the bed nucleus of stria terminalis), α1-AR -induced GLUT inputs into the VTA seems to derive mostly from local GLUT neurons (41, 112, 113). While contributing to stress- and/or drug-induced behavioral alterations (41, 84, 111–113), an excess of α1-ARs-induced GLUT release onto VTA may increase its vulnerability to Ca2+-related excitotoxicity. In fact, stress- and drug-induced GLUT release onto VTA neurons is coupled to the calcium (Ca2+)-related PKC signaling pathway (41), which produces amphetamine-related oxidative damage going along with autophagy impairment (114, 115). In this context, autophagy is implicated in both GLUT-dependent synaptic plasticity and excitotoxicity. Transient exposure to low doses of NMDA induces autophagy through PI3K/AKT/mTOR pathway inhibition, which is key to promote AMPAR degradation in cultured rat hippocampal neurons and in rodent models of auditory fear reconsolidation (116, 117). On the other hand, neuroprotection against GLUT excitotoxicity is achieved by administering either mTOR-dependent or -independent autophagy inducers rapamycin or trehalose (118). This is in line with evidence showing that NMDAR antagonists may rescue autophagy flux and mitophagy to confer neuroprotection (119). These findings suggest that an autophagy failure being bound to either impaired degradation of plasma membrane AMPA receptors or NMDAR-mediated Ca2+ signaling, may be implicated in the responsivity of VTA neurons to α1-AR-induced GLUT release.

Again, the coupling of α1-AR signaling and the stress hormone corticotrophin-releasing factor (CRF) produces social stress enhancement of drug conditioning *via* NMDAR-mediated GLUT transmission within the VTA (8). Intriguingly, this is coupled to an amplification of IP_3-Ca^{2+}signaling, which is known to impinge on the autophagy pathway (57, 59). Within LC neurons, constitutive overexpression of CRF increases NE activity and redistributes beta-amyloid (Aβ) peptides from synapses to somato-dendritic processes, which occurs along with altered distribution and morphology of autophagy-related vacuoles (120). Again, CRF was recently shown to inhibit the autophagy pathway *in vitro* (121), suggesting that α1-AR and CRF stimulation following abnormal NE release may synergize to alter autophagy within VTA.

These findings are also in line with evidence on a deleterious role of high NE levels, which similar to stress/drug exposure, do occur in REM sleep deprivation (94, 122–124). In *ex vivo* and *in vivo* models of REM sleep deprivation, high NE levels lead to iron and calcium-related oxidative damage within neurons and glia *via* α1-ARs, which is accompanied by mitochondrial failure and altered levels of AKT (94, 122–124). This occurs in various brain regions including the LC itself, though the VTA remains to be examined. In this frame, it is likely that stress- and drug-induced catecholamine alterations may increase the susceptibility of LC and VTA to neuronal damage by increasing the formation of highly oxidative DA- and NE-derived metabolites, which are known to impair neuronal proteostasis (125, 126). This would explain why catecholamine-containing neurons are particularly susceptible to degeneration associated with an autophagy failure (127–129).

These findings support a correlation between early potentiation of NE-DA activity and autophagy-related alterations within the LC-VTA network following stressful stimuli or exposure to addictive substances (**Figure 1**).

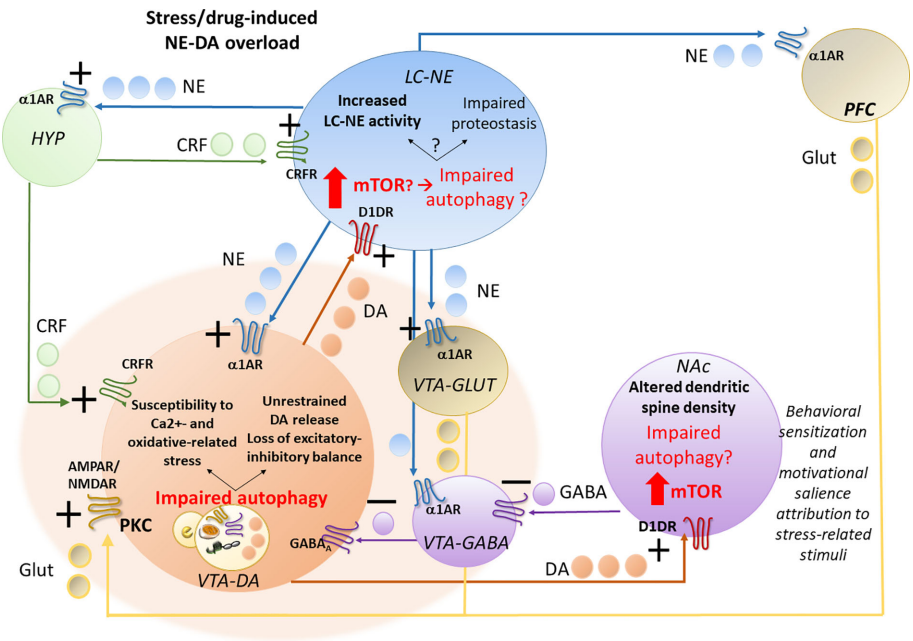

FIGURE 1 | Acute stress- or drug-induced potentiation of locus coeruleus–norepinephrine (LC-NE) and ventral tegmental area–dopamine (VTA-DA) activity and potential role of autophagy. Stress, similar to abused drugs, readily activates LC-NE and VTA-DA neurons, leading to a sustained NE transmission and increased NE release in the hypothalamic CRF-producing neurons, in the forebrain DA-targeted areas pre-frontal cortex (PFC) and nucleus accumbens (NAc) and also directly on VTA neurons. In fact, LC-NE excites VTA-DA neurons either directly through post-synaptic α1-ARs, or indirectly, through inhibition of VTA-GABA neurons and activation of VTA-GLUT neurons *via* stimulation of pre-synaptic α1-ARs. Again, NE in the PFC, through α1-ARs stimulation, might activate DA release on the NAc through GLUT cortical projections to VTA-DA cells. While altering the excitatory-inhibitory balance and increasing the metabolic rates within VTA-DA neurons, these NE-mediated events may predispose to oxidative stress, and glutamate-related alterations, which may in turn contribute to overwhelming autophagy. While increasing VTA neurons susceptibility to oxidative and Ca2+-related alterations, autophagy impairment within VTA neurons promotes an empowering of DA release to the NAc and likely, also back to the LC. In this way, through stimulation of post-synaptic D1DRs, which is known to promote mTOR activation, DA overload may in turn impair autophagy within the NAc, where it alters dendritic spine density while contributing to drug-induced behavioral sensitization and likely, altered motivational salience attribution to stress-related stimuli. VTA-DA overload, through stimulation of post-synaptic D1DRs, may also impair autophagy within the LC. This may lead to either potentiation of NE release or a progressive impairment of proteostasis impinging on LC neuronal integrity, which remains to be investigated.

CHRONIC STRESS AND DRUG EXPOSURE BRIDGING REDUCTION OF NE-LC AND VTA-DA ACTIVITY AND AUTOPHAGY-DEPENDENT NEUROPROTECTION

Reduction of NE-LC Activity May Occur Due to DA Overload

Dysregulations of NE activity during exposure to prolonged/chronic stress or abused drugs may include either an increase or a decrease of LC activity. In fact, cumulative cocaine self-administration in rats leads to functional reductions in the LC (92), and a decrease in baseline LC neuron activity and NE release occurs in rodent models of post-traumatic stress disorder - single prolonged stress (SPS), chronic unpredictable mild stress (CUMS), as well as chronic social defeat stress (CSDS) (9, 86, 92). The activation state of VTA-projecting LC-NE neurons, and the amount of NE released into the VTA are critical for determining the vulnerability to emotional stress (45). The loss of NE neurons projecting to the VTA leads to a potentiation of VTA-DA firing conferring an increased susceptibility to stress induced by social defeat compared with resilient mice (45). This is in line with evidence showing that selective lesion of LC increases the firing activity VTA-DA neurons (44). Since VTA-DA excites LC-NE neurons (101), it is expected that exaggerated firing of VTA neurons occurring during acute/repeated stress or abused substance intake/administration, may progressively impinge on LC thus altering this nucleus. In this context it would be worth investigating whether LC alterations are bound to VTA-DA-induced stimulation of D1DRs within the LC, which is essential for ethanol-triggered reinforcement behavior (48). This would be key in the light of evidence indicating that D1DRs that occur within LC neurons (48), may impair autophagy (75). Thus, similar to NE-dependent alterations affecting the VTA discussed in 3.2, autophagy may be implicated in DA-dependent alterations within the LC. In this scenario, a protective role for NE, which may act as an autophagy inducer (79, 82, 83), is substantiated by evidence showing that LC-NE dysfunction may predispose to degenerative phenomena involving various LC-NE-targeted brain areas (82, 86). Here we

hypothesize that these may include DA-containing neurons, where reduction of LC-NE release could contribute to undermining intracellular protection mechanisms.

Impairment of LC-NE to VTA-DA Neurons May Occlude NE-Induced Protective Autophagy

Neuroprotective effects of the LC-NE on DA neurons *in vivo* and *in vitro* have been documented emphasizing the effects of NE as a neurotrophic factor and its ability to stimulate the expression of other neurotrophic factors (28, 130–134). This is the case of brain-derived neurotrophic factor (BDNF), which is synthesized within glial cells or neurons mainly through β1/β2-ARs (133, 134). In fact, β2AR agonists reverse DA neurotoxicity *in vitro* and *in vivo* (130, 133). As shown in mice models, this occurs through the inhibition of microglial activation rather than exerting a direct effect on VTA-DA neurons, which in fact lack β2-ARs (133). NE-induced neuroprotection of DA neurons depends on the presence of β2AR complexed to β-arrestin (133), which intriguingly, mediates neuroprotection in experimental cerebral ischemia through coordination of BECN1-dependent autophagy (135).

In line with this, there is evidence indicating that NE induces protective autophagy mostly through β2-AR stimulation (79, 82, 83), and again, the protective effects of BDNF are bound to autophagy activation. In detail, BDNF works through inhibition of either mTOR (136) or GSK3β pathway (137), which are main upstream regulators of autophagy. *In vivo*, BDNF enhances autophagy flux and promotes mitophagy through the HIF-1α/BNIP3 pathway (138). BDNF and related autophagy ameliorate stress-induced behavioral and emotional alterations, suggesting that a direct association exists between autophagy impairment and BDNF deficiency (136, 138). This is not surprising since autophagy has been implicated in the regulation of neurogenesis, which is altered by psychological stress and represents a risk factor for the development of mood/neuropsychiatric disorders (63). In line with this, the administration of either antidepressant drugs or the naturally occurring autophagy inducer resveratrol alleviates depressive-like behavior in mice models of CUMS or post-partum depression by increasing BDNF and autophagy-associated proteins (136, 138). This goes along with reduced HPA axis hyperactivity, CRF and pro-inflammatory cytokines levels (136, 138). In line with this, blockade of autophagy by chloroquine abrogates whereas the autophagy inducer rapamycin protects against the pro-inflammatory effects of CRH in other mice tissues besides the brain (139). These findings suggest that VTA-DA neurons may benefit from glial β2-AR stimulation and BDNF/autophagy induction through reduction of CRF and microglial-mediated inflammation. Autophagy failure is expected to occlude the neurotrophic and anti-inflammatory effects of NE. In fact, microglia-specific *Atg5*-deficient mice show higher inflammation levels, reduced BDNF expression, and exacerbated depressive-like behavior compared with wild-type mice (138). However, the link between autophagy, NE levels and stress/drug-related behavior remains to be investigated.

VTA-DA Activity Decline in Chronic Stress and Drug Abuse: Potential Role of Autophagy and Future Issues to Address

As discussed above, an impairment of autophagy within VTA-DA neurons is expected to occlude neurotrophic and neuroprotective mechanisms depending in part on LC-NE activity. Remarkably, an early loss of VTA-DA neurons, which precedes LC neuronal degeneration, has been documented in models of Alzheimer's disease (93). The potential mechanisms underlying such a temporal dissociation between LC and VTA degeneration will not be dealt herewith. Here, we wish to point out that different patterns of stress exposure might induce hypo-DAergic states (88–90). In detail, acute stress readily activates whereas chronic stress exposure may lead to a compensatory downregulation of the DA system (88–90). In this context, also the timing of stress is critical since contrarily to adolescent stress, adult stress induces a depression-like hypo-DAergic state (140). Similarly, chronic drug intake/administration is associated with a decrease in DA release mostly within the striatum, which may explain the decreased sensitivity to natural rewards and the compulsive drug use as a means to temporarily compensate for this deficit (87). Such an effect is associated with reduced striatal levels of D2-/D3-DRs (87, 141, 142). In the light of an interdependency between autophagy, substance abuse, and D2-/D3-Rs activity discussed in section 3, a potential link deserves to be investigated in stress-related disorders. A reduction of DA activity may be the outcome of either abnormally increased LC-NE release predisposing VTA-DA cells to excitotoxicity, or LC neuronal loss occluding the protective effects of NE upon VTA-DA neurons, as discussed in sections 3.2 and 4.2, respectively. This may be explained by the double-faceted effects of NE in the brain, exerting neuroprotection at physiological levels, while inducing apoptosis at high concentrations (94, 122–124).

Chronic exposure to psychosocial stressors in adults seems to be associated with reduced striatal DA synthesis, mostly within the ventral striatum (90). Autophagy and stress-related VTA-DA alterations may be reminiscent of the molecular effects produced by psychostimulants, which alter autophagy vacuoles (50, 143). This hypothesis remains to be confirmed and may represent the starting point to explore the effect of stress at subcellular level within VTA-DA cells. Despite appearing in contrast with evidence indicating that autophagy blunts DA transmission, these findings suggest that autophagy regulation may be a finely-tuned and context-dependent process, depending on specific patterns of neuronal activity and metabolic demands. In fact, an autophagy impairment within different cell compartments may have different effects, for instance, driving neuropathological changes at the soma while producing striatal-driven behavioral changes by increasing the extracellular availability of DA at the synapse (106).

CONCLUSIONS

The evidence here discussed points to the remarkable action of autophagy in NE-LC and VTA-DA connections and its role in

NE-dependent neuroprotection, which is crucial for the organism adaptive response to stress and allostatic load. In fact, heavy and/or chronic stress is known to induce or foster neurodegeneration in brain catecholamine neurons involved in a number of psychiatric and neurodegenerative diseases. We proposed here the autophagy machinery as a relevant mechanism of regulation and dysregulation of catecholamine neurons. As for NE-containing neurons, autophagy is seminal for the survival of DA neurons and remarkably, it plays a central role in DA release. The role of autophagy in brainstem NE and DA neurons and their projections in response to psychostimulants indicates adaptive mechanisms that can be activated by stress following its impact on neurotransmission. LC and VTA are both crucial in the response of the organism to stressors and both orchestrate brain systems involved in the appraisal and in the management of psychological stress. Lessons from the effects of abused substances suggest that autophagy, with its role in catecholamine synapse and in neuronal protection, is crucial in modulating the effects of psychological stress on emotional and cognitive driven behavior to foster adaptive emotional outcomes aimed to wellbeing.

At cellular level, psychological stress may translate into intracellular stress-responsive events, such as ER- and oxidative-stress and inflammation (144), which are known to promptly recruit autophagy in the attempt to restore homeostasis. However, under conditions of chronic/persistent stress, when alterations in neurotransmitter activity translate into maladaptive neuronal changes, autophagy may be consistently affected, fostering progressive synaptic deterioration up to neurodegeneration. In the light of an interdependency between autophagy and the mechanisms governing neurotransmission and neuronal homeostasis, we strongly believe that the role of autophagy deserves to be further investigated in the context of catecholamine response to psychological stress specifically.

The possibility of relating stress-related emotional and cognitive experiences to functional aspects through molecular pathways, may facilitate the discovery of potential biomarkers identifying an early risk. Individual differences in the response to stress and autophagy activity, which can make some individuals more or less susceptible than others, should also be addressed by future studies.

AUTHOR CONTRIBUTIONS

SP-A, FL, and FF drafted and wrote the manuscript. FL, CB, and FB contributed to literature review, manuscript editing, and artwork. SP-A and FF are coordinators of the paper; they critically revised the article for important intellectual content.

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REVIEW
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Gender Differences in Depression: Evidence From Genetics

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Compared with men, female accounts for a larger proportion of patients with depression. Behavioral genetics researches find gender differences in genetic underpinnings of depression. We found that gender differences exist in heritability and the gene associated with depression after reviewing relevant research. Both genes and gene-environment interactions contribute to the risk of depression in a gender-specific manner. We detailed the relationships between serotonin transporter gene-linked promoter region (5-HTTLPR) and depression. However, the results of these studies are very different. We explored the reasons for the contradictory conclusions and provided some suggestions for future research on the gender differences in genetic underpinnings of depression.

Keywords: depression, gender difference, genetics, gene-environment interactions, heritability

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INTRODUCTION

Depression is a prevalent mental illness that seriously affects physical and mental health (Krishnan and Nestler, 2008; Ge et al., 2018; Ren et al., 2020). Women are more likely to suffer from depression (Young et al., 1990; Harkness et al., 2010; Wang et al., 2019). The susceptibility to depression is affected by diverse hereditary, epigenetic, environmental, and endocrine risk factors (Duman et al., 2016). With the rise of developmental behavioral genetics (using the research methods and techniques of psychology and behavioral genetics to examine the influence of genetics and environment on the development of human psychology and behavior), more and more researchers began to pay attention to the role of genetic factors in the occurrence of gender differences in depression. Behavioral genetics research methods include quantitative genetics (mainly through twins and adoption research to find evidence that genetics and the environment affect human psychology and behavior) and molecular genetics [identify susceptibility genes associated with specific psychology and behavior, including candidate gene association studies and genome-wide association studies (GWAS)]. Twin studies show differences in the heritability of depression between men and women, and molecular genetics studies show gender differences in depression caused by specific genes and their interaction with the environment. However, these findings are not consistent.

This manuscript reviews relevant studies on the genetic underpinnings of gender differences in depression. Besides, we explored the reasons for the contradictory conclusions and provided some suggestions for future research on the genetic underpinnings of gender differences in the depression. We hope this manuscript will help scientists better understand and study genetic underpinnings of gender differences in the depression.

EPIDEMIOLOGY

Many national and international studies display that sex ratio (women: men) of depressive disorders over 1.7 for lifetime prevalence and 1.4 for 12-month prevalence after the age of 18 (Kuehner, 2017). The gender difference in depression rates first emerge in adolescence and continues into old age (Angold and Worthman, 1993), although the gender gap of the adult is smaller than it is at younger ages (Patten et al., 2016; Kiely et al., 2019). Similar gender differences exist in different income countries, although significant cross-national variation exists (Van de Velde et al., 2010). But, gender differences do not exist across all race-ethnic groups (Kessler, 2003; Yancu, 2011). The female predominate in the incidence of depressive disorders; instead, there appears to be no gender difference in recurrence, remission, or chronicity of depression (Kessler, 2003; Otte et al., 2016). The symptom profile of men and women with depression is different. Women are more likely to show increased appetite, hypersomnia, somatic symptoms, etc. (Piepenburg et al., 2019). Especially, comorbidity of peripartum depression with anxiety disorders, obsessive-compulsive disorder, and post-traumatic stress disorder worth attention (Kuehner, 2017).

GENDER DIFFERENCES IN HERITABILITY

The family pedigree study finds depression is hereditary. According to reports, children of depressed parents have increased symptoms of depression and internalization (Rice et al., 2002). Later, the twin study divided the sources of phenotypic variation of depression into three aspects: genetic, shared environment, and non-shared environment, which provided the possibility of separating the role of genetic and environmental.

Most Scholars use the twin paradigm in quantitative genetics to investigate gender differences in the genetic basis of depressive symptoms. Research on gender differences in heritability of depressive symptoms mainly focuses on adolescents in European and American countries. Adolescence is a particularly good time when many people will experience the first onset (Eley et al., 2004). During adolescence, the prevalence rate of depression in men and women has begun to rise dramatically, especially in girls. Similarly, the heritability of depression increased from childhood to adolescents (Ksinan and Vazsonyi, 2019). Biological and pubertal changes, cognitive maturity occurs during adolescence, some genetic factors may be "switched on" to promote these changes, which in turn affect depressive symptoms (Lau and Eley, 2006). Jacobson and Rowe (1999) show that the heritability in depressed mood is higher in female adolescents than in male adolescents (self-rated depressive symptoms), however, Rice et al. (2002) shows the opposite result (self-rated depressive symptoms). McCaffery et al. (2008) reported that non-shared environment and the genetic factors contribute to the correlation of depressive symptoms in female adolescents and cigarette smoking; but In male adolescents, only non-shared environment. In an older twin study, the heritability of women was also higher than that of men, although no statistically significant (Jansson et al., 2004). Scourfield et al. (2003) show higher heritability for young girls (children) than young boys only from parent-rated depressive symptoms, not self-rated depressive symptoms. Some methodological differences exist in these surveys, including measurement methods, source of information (informant), the age range of the sample, number of samples, sibling-pairs sample, demographic characteristics (**Table 1**), which limits comparability between surveys. We are not sure whether the difference in the heritability of depressive symptoms exists between gender.

Several reasons can explain the divergence of the above conclusions. First, the genetic factors on depressive symptom vary according to the individual's developmental stage (such as childhood and adolescence) or age: Both self-reports and parent reports show that individuals with early adolescence have a higher heritability in depressed mood than individuals with mid-adolescence (Hou et al., 2012); genetic factors become more important from childhood to adolescence or less important (Rice et al., 2002; Scourfield et al., 2003). Most studies have analyzed adolescents at different developmental stages of adolescence and may have overlooked the change in genetic interpretation of depressive symptoms during adolescence. Like most complex behaviors, depression does not simply follow Mendel's single gene inheritance law but is affected by multiple genes, known as quantitative trait locus (QTL). Different genes are turned on in different time, the interaction between genes and the interaction between genes and the environment show different patterns at different stages of development, so the influence of genetics and environment on adolescents' depression is dynamically changing (Hou et al., 2012). Second, The inheritance rate varies according to the reporter and genetic influences may be less important for child-rated depression symptoms than for parent-rated symptoms (Rice et al., 2002): Proxy ratings can be influenced by the informant's symptoms of depression and anxiety; Self-reports and parental reports may have evaluated different aspects of depressive symptom or depressive symptom at different moments; in parents-report, parents need to rate two twins. In this process, two twins will be inevitably compared with each other, or the two children will be rated more similarly, or the rating will be less similar. In self-reporting, a child only needs to report themselves' emotional experience. Third, the small number of subjects may not be sufficient to produce convincing results. Modest heritability (30–40%) (Sullivan et al., 2000), clinical heterogeneity and complicated genetic architecture for major depression requires a larger sample size. In order to generate replicable and statistically significant findings, 75,000–100,000 major depressive disorder cases are needed in GWAS to identify gene loci involved major depressive disorder (Duman et al., 2016). Also, maximizing sample sizes is more informative to understand genetic heterogeneity of depression (Hall et al., 2018).

GENDER DIFFERENCES IN THE GENE ASSOCIATED WITH DEPRESSION

The twin studies found that the genetic factor affect depressive symptom of adolescents but gender difference in heritability of depressive symptom remains to be further studied. Molecular

TABLE 1 | Gender differences in heritability.

Sibling-Pairs Sample	Age Group	Methodology	Measurement instruments	Demographic Characteristics	Gender composition	Result	Source of information (informant)	References
2,302 pairs Sibling-Pairs	16 years (range = 11–20 years)	Cross-sectional Study	Depressed mood: Center for Epidemiological Studies-Depression (CES-D)	Caucasian, African American, other Ethnicity (A smaller percentage)	Female: 2285, Male: 2319	Heritability in depressed mood is higher in female adolescents than in male adolescents. Genetic factors were higher for female adolescents than male adolescent in correlations between family and school environment and adolescent depressed mood	Self-report	Jacobson and Rowe, 1999
959 twin pairs (123 female MZs, 90 male MZs, 207 same-sex female DZs, 109 same-sex male DZs, and 430 opposite-sex DZs)	50 years or older (mean age 72 years)	Cross-sectional Study	Depressive symptoms: Center for Epidemiological Studies-Depression (CES-D) and self-reported use of antidepressant medication.	Caucasians	Female: 1090, Male: 828	Higher heritability for women than men (no statistically significant).	Self-report	Jansson et al., 2004
287 MZ (143 male-male, 144 female-female pairs) and 441 DZ twin pairs (132 male-male, 113 female-female, and 196 male-female)	Mean age 16.1 years	Cross-sectional Study	Depressive symptoms. Center for Epidemiologic Studies–Depression Scale (CES-D)	Caucasian, African American, Hispanic/Latino other ethnicities (A smaller percentage)	Female: 710, Male: 746	In female, non-shared environment and genetic factors contribute to the correlation of depressive symptoms and cigarette smoking. In male, only non-shared environment.	Self-report	McCaffery et al., 2008
670 twin pairs (MZ and DZ)	5–17 years	Cross-sectional analyses longitudinal study	Depressive symptoms: Parent and self-report questionnaire data Mood and Feelings Questionnaire.	Wales	Female: 636, Male: 612	Only parent-report data show that girls show greater genetic effects than boys.	Self-report	Scourfield et al., 2003
1463 families	8–17 years	Cross-sectional analyses	Depressive symptoms: Mood and Feelings Questionnaire and Hospital Anxiety and Depression Scale	South Wales and Greater Manchester		For self-rated depressive symptoms, adolescents (11 years and over) show greater genetic effects than female	Self-rated parent-rated	Rice et al., 2002
508 MZ, 176 DZ	10–19 years	Longitudinal study	Depressive symptoms: Children's Depression Inventory, CDI	Chinese		No gender difference in the heritability of adolescent Depressive symptoms	Self-rated parent-rated	Hou et al., 2012

TABLE 2 | 5-HTTLPR alone and interaction with the environment contribute to the risk of depression.

Age	Gene	Measurement instruments	Environmental factor	Gender composition	Demographic Characteristics	Source of information (informant)	Study Design	Result	References
337 adolescents aged 10–20 years	5-HTTLPR (L/S), HTR2A, HTR2C, MAOA (monoamine oxidase type A) and tryptophan hydroxylase (TPH)	Short form of Mood and Feelings Questionnaire (SMFQ)	Family environmental risk: family social adversity; parental educational level; adverse life events	Female: 220, Male: 117		Self-report questionnaire	Cross-sectional Study	1. The main effect of 5HTTLPR "short" alleles was significant only in the female. an overall decrease in odds of depression for an increasing number of "short" alleles. 2. 5-HTTPLR "short-short" genotype interacts with high environmental risk increase depression risk only for female	Eley et al., 2004
16–19 years	5-HTTLPR (L/S)	[Depression Self-Rating Scale (DSRS)] of the DSM-IV	1. Type of residence 2. Separated families' 3. Traumatic conflicts within the family	Female: 11, Male: 81	Swede	Self-reports interview	Cross-sectional Study	1. Boys and girls carrying the short 5-HTTLPR allele react to different kinds of environmental factors. 2. Females rather than male carrying the short 5-HTTLPR allele tended to develop depressive symptoms with the environmental stress factor	Sjöberg et al., 2006
Study 1 288 participants mean age 58.3 Study 2 142 participants Mean age 34.0	5-HTTLPR (L/S)	Study 1 depressive symptomatology: Center for Epidemiologic Studies Depression Scale (CES-D) Study 2 depressive symptoms: The 40-item Obvious Depression scale (OBD)	Study 1 the stressor of caregiving status Study 2 childhood socioeconomic status (SES)	Study 1 215 females Study 2 64 females	Study 1 70.5% Caucasians Study 2 47.2% Caucasians	Study 1 home visit self-reports. Study 2 self-reports.	Cross-sectional Study	1. For females, the s allele, combined with caregiving stress (Study 1) or low childhood SES (Study 2), was associated with higher depression scores as compared to participants in the non-stressor group and those with the long (l) allele. 2. In males, the l allele, combined with a stressor, was associated with higher depression scores as compared to those in the non-stressor group and those with the s allele	Brummett et al., 2008
Between the ages of 22 and 26 (n = 4724)	5-HTTLPR (L/S)	Depression: using responses from two questions; depression symptoms (CES-D)	1. Stressful life events 2. Childhood maltreatment.	Male n = 2312, Female n = 2412	Non-Hispanic white	self-reported	Cross-sectional Study	1. 5-HTTLPR plays a role in moderating the impact of SLEs on depression status, a statistically significant only in males (for SS genotype). 2. For females carrying one or more of the S-alleles, the prevalence of suicide ideation increased with an increasing number of stressful life events. whereas, for males, the prevalence rates increased for carrying one or more L-alleles	Haberstick et al., 2016
Students 17–18 years	5HTTLPR (L/S)	Self-rating scale (DSRS) of the DSM-IV	Maltreatment	Male n = 765, Female n = 717	Scandinavian 1245 Non-Scandinavian 217	self-reported	Cross-sectional Study	A significant main effect and a G × E interaction effect of the SS allele was found only among girls.	Aslund et al., 2009

(Continued)

TABLE 2 | Continued

Age	Gene	Measurement instruments	Environmental factor	Gender composition	Demographic Characteristics	Source of information (informant)	Study Design	Result	References
346 youth mean age 23.7 years	5HTTLPR (L/S)	Depressive symptoms: Beck Depression Inventory—II	Negative acute life events Chronic family stress	132 males, 214 females	93% Caucasian	Interview measures	Longitudinal Study	A significant interaction between family discord and genotype only among females. The effect of family discord on BDI was stronger in SL and SS females compared to LL females	Hammen et al., 2010
In males: 12–19 years; In females: 12–20 years	5—HTTLPR (L/S)	Depressive symptom: Epidemiological Studies Depression Scale (CES—D)	Family structure Family—level socioeconomic status (SES) Social support County—level environment	Females (n = 560), Males (n = 524)	White (reference), African—American, Hispanic, Asian, and other race	In-home interview self-report	Longitudinal Study	1. Among females, the main effects models showed an association between the SL genotype and lowered risk of depressive symptoms. 2. Among males, interaction models showed an association between SL genotype and lowered risk of depressive symptoms in deprived counties only	Uddin et al., 2010
12–19 years, males; 12–20 years, females	5-HTTLPR (L/S)	Depressive Symptom: 17-item version of the Center for Epidemiological Studies Depression (CESD)	1. Respondent-level building conditions 2. Neighborhood-level building conditions	1. Male (n = 510) Female (n = 574) 2. Male (n = 377) and Female (n = 418)	White (reference), African American, Hispanic, Asian, and other races.	Self-reported	Cross-sectional Study	1. No gene-social environment interaction effects 2. Respondent-level building analyses provided some evidence for genetic influences on depressive symptom score in adolescent females 3. Neighborhood-level building analyses provided evidence for increased depressive symptom score among adolescent males only residing in neighborhoods with poorer building conditions, in both unadjusted and adjusted results.	Uddin et al., 2011
	5-HTTLPR (L/S) monoamine oxidase A-upstream variable number tandem repeat (MAOA-uVNTR)	Depressive Symptoms: Children's Depression Inventory (CDI)	Negative life events (NLE)	129 female, 180 male	89.3 % were White, 1.7 % African American, 1.7 % Hispanic, 1.2 % American Indian/Alaskan, and 6.15 % biracial or multiracial.	Self-reported	Longitudinal Study	1. Girls were most likely to exhibit elevated depressive symptoms when experiencing NLE if they possessed low-expression MAOA-uVNTR alleles and short 5-HTTLPR alleles 2. Low-expression MAOA-uVNTR alleles but long 5-HTTLPR alleles were implicated in boys at the age of 13	Priess-Groben and Hyde, 2013

(Continued)

TABLE 2 | Continued

Age	Gene	Measurement instruments	Environmental factor	Gender composition	Demographic Characteristics	Source of information (informant)	Study Design	Result	References
	Brain-derived neurotrophic factor (BDNF) val66met and the serotonin transporter region 5-HTTLPR (L/S),	depressive symptoms: Beck Depression Inventory-II (BDI-II)	Family environment quality	140 males, 223 females	92% White, 1.5% Asian, 6% biracial, and 0.5% other/not reported	Interview and self-report	Longitudinal Study	After age 15, the interaction of cumulative plasticity genotype (defined as presence of neither, either, or both 5-HTTLPR S and val66met Met alleles) and early family environment quality was only predictive of depression among females	Dalton et al., 2014
The average age 15.5 years	5-HTTLPR (L/S)	Depression symptoms: 17-item version of the Center for Epidemiologic Studies Depression Scale (CES-D;	Family support	56% of boys	Caucasian	Self-report in-home interview	Cross-sectional Study	In the presence of poor family support, boys with at least one short allele had more symptoms of depression. in the presence of high family support, boys with the short allele had the fewest depression symptoms	Li et al., 2013
Mean age 38.3 ± 10.3 years	A tri-allelic serotonin transporter promoter polymorphism (5-HTTLPR/rs25531) low-expressing tri-allelic analyses, S'(S, L_G) and high-expressing L'(L_A, XL_A) bi-allelic analyses (L/S)	Beck Depression Inventory (BDI) Maudsley Personality Inventory (MPI)		550 men, 589 women	Healthy Han Chinese	Self-report	Cross-sectional Study	1. Tri-allelic genotype-by-gender interaction: S′S′ homozygotic were associated with higher neuroticism and BDI scores in men. 2. Women showed a non-significant pattern across both the 5-HTTLPR classifications 3. In the bi-allelic analyses, there was only an association between SS genotype and MPI-neuroticism in men	Chang et al., 2017
Aged from 14 to 18	5-HTTLPR (L/S)	Depressive symptoms: Center for Epidemiological Studies Depression Scale (CES-D)	Negative life events	131 females and 121 males	Chinese healthy Han population	Self-report interview	Longitudinal Study	No main effect of 5-HTTLPR A significant 5-HTTLPR × stress interaction in females only. Females with at least one 5-HTTLPR S allele exhibited more depressive symptoms under stressful situations. No significant 5-HTTLPR × stress interaction was found in males	Ming et al., 2013

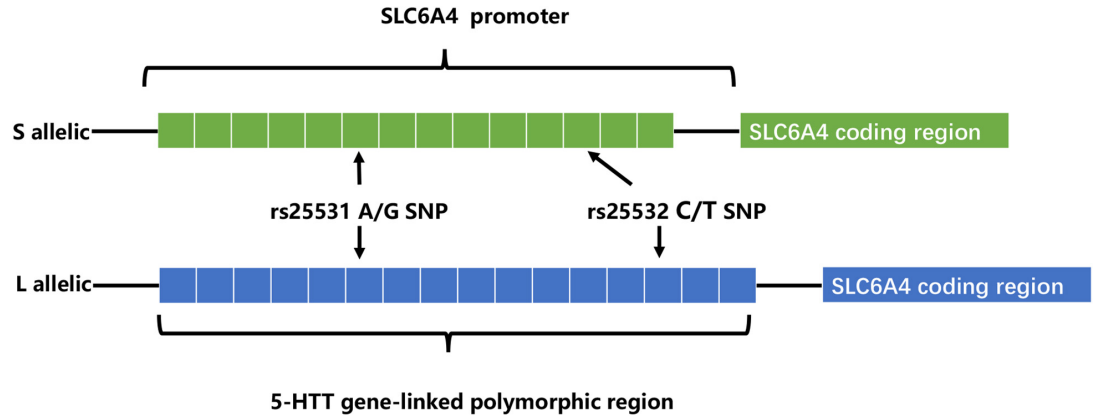

FIGURE 1 | SLC6A4 L and S allelic.

genetics attempts to locate the genes for gender differences in depression. At present, most candidate gene association studies have examined the relationship between serotonin system genes, dopamine system genes and depression (**Table 2**): loci implicated in the serotonin (5HT) system including serotonin (5-HT) transporter gene-linked promoter region (5-HTTLPR), 5HT receptor 2A (5HT2A), 5HT receptor 2C (5HT2C), monoamine oxidase type A (*MAOA*), tryptophan hydroxylase (*TPH1*). loci implicated in the dopamine system including catechol-*O*-methyltransferase (*COMT*), dopamine receptor genes *DRD1-DRD5*. Related candidate genes can regulate the level of neurotransmitters (serotonin or dopamine) in the synaptic space through degradation (e.g., *MAOA*, *COMT*) and transport (such as 5-HTTLPR), and can also change the number of receptors in the brain (5HT2A, *DRD2* gene) to regulate signal transmission, which in turn affects the level of individual depression.

Recently, extensive works of literature have investigated the relationships between 5-HTTLPR and depression, the serotonin transporter gene-linked promoter region (5-HTTLPR) is a variable number tandem repeats (VNTR) located in the promoter region of *SLC6A4* (the human 5-HTT-encoding gene) (Iurescia et al., 2016). In addition to most common alleles: the short (S, 14 repeats) and the long (L, 16 repeats), there are less common alleles: extra-long (XL, 17–24 repeats) and extra-short (XS, 11–13 repeats). The L allele possesses higher transcriptional activity and serotonin uptake rate than S allele positively affects serotonin reuptake rate. Also, two nearby single nucleotide polymorphisms (SNPs) rs25531 and 25532 (located in the 5-HTTLPR) contribute to the functional variations of *SLC6A4* expression (Iurescia et al., 2016; **Figure 1**). The 5-HT transporter (5-HTT), an integral membrane protein, moves 5-HT from synaptic space into presynaptic neurons (Damsbo et al., 2019; Möller et al., 2019). And then 5-HT was degraded by MAOA or recycled into synaptic vesicles. Duration and magnitude of 5-HT biological actions are closely related to 5HTT (Coleman et al., 2019). Also, effective drugs selective serotonin reuptake inhibitors (SSRIs), act on 5-HT transporter (Ananth et al., 2018; Kulikov et al., 2018). So, dysfunction in 5HTT leads to psychiatric disorders including depression.

Genes Directly Affect Depression in a Gender-Specific Manner

Different genes may directly affect depression in a gender-specific manner. 5HT2A, TPH may be a risk gene for depression in women, and COMT may have a greater impact on men. The relationship between 5-HTTLPR genotype and depression is highly controversial: although females carrying short alleles had a lower risk of depression than other genotypes (**Table 2**), these research results show inconsistent conclusions on specific genotypes (Eley et al., 2004; Aslund et al., 2009; Uddin et al., 2010). Animal studies have shown that individuals carrying short alleles, especially female animals, are more vulnerable to chronic stressors (Spinelli et al., 2012). But, Others showed no main effect of 5-HTTLPR on depression, which means 5-HTTLPR genotype cannot predict depression risk (Aslund et al., 2009; Risch et al., 2009; Ming et al., 2013). The study also indicated the main effects of 5HT2A, TPH on depression group exist in female subjects only (Eley et al., 2004). However, another study found direct effects of certain depression-related genes only exist in the male population. Individuals carrying the Met/Met of COMT genotype are less likely to suffer depression than those carrying the Val/Val genotype (Baekken et al., 2008).

In addition to candidate gene association studies, GWAS is another research strategy in the field of molecular genetics to find genes associated with individual psychological or behavioral phenotypes. Recent GWAS has identified 14 independent and replicated loci that were associated with MDD at the genome-wide level (Maul et al., 2020). Only a few scientists have reported gene loci related to gender differences in depression: SNP rs6602398, presented in interleukin receptor 2A gene (IL2RA), was significantly associated with males MDD (Powers et al., 2016); 2 SNPs rs619002 and rs644926, presented in the EH-domain containing 3 (EHD3) gene, were associated with female MDD (Wang et al., 2014). However, some scientists showed no evidence for genetic heterogeneity between the gender using GWAS summary statistics (Trzaskowski et al., 2019).

Candidate gene association studies and Genome-wide association studies (GWAS) are research methods in

developmental-behavioral genetic, aiming to find out whether genetics and environment affect human psychology and behavior development. Candidate gene association research is to directly select genes that may be related to individual psychological or behavioral phenotype variation based on existing genetic related information, biological related information, or empirical research results and then to determine whether a candidate gene is associated with this phenotype by case-control study or population-based association analysis. GWAS selects SNPs associated with individual psychological or behavioral phenotypes from sequence variations (single nucleotide polymorphism, SNP) throughout the human genome. The difference from candidate gene research strategies is that you do not need to know the function and characteristics of genes in advance. Also, there are no preset research assumptions. It offers opportunities for finding unknown susceptibility genes. Though more and more depression loci are identified, most GWAS has not yet made a replicable discovery of MDD (Hyde and Mezulis, 2020). Also, the GWAS study of depression has not achieved the same success as other mental illnesses; the complexity of the genetics and phenotype of depression may mean that a GWAS study will require a sample of thousands of participants (Howard et al., 2019). Compared with the huge cost of GWAS, candidate gene association studies are more economica and faster.

Gene-Environment Interaction Contribute to the Risk of Depression

Many studies suggest 5HTTLPR-negative environment interaction contributes to the risk of depression in the child, adolescent, and adult populations in a gender-specific manner (**Table 2**). Also, sex modulates 5-HTTLPR genotype-childhood adversity interaction on hippocampal volume [reducing hippocampal volume in depressed patients (Maller et al., 2018)] (Everaerd et al., 2012). But, results remain inconclusive. Some studies have shown females rather than males carrying the SS genotype of 5-HTTLPR tended to develop depressive symptoms under negative environment (Eley et al., 2004; Aslund et al., 2009; Hammen et al., 2010; Ming et al., 2013) or females carrying S allele are easier to develop depressive symptoms under negative environment (Sjöberg et al., 2006; Brummett et al., 2008; Hammen et al., 2010; Ming et al., 2013). However, many contradictions about 5HTTLPR-negative environment exist in males: the l allele-stressor interaction contributes to higher depression scores as compared to those control group and s allele (Brummett et al., 2008); Uddin et al. (2010) showed an interaction between SL genotype and deprived counties predicted lowered risk of depressive symptoms in males; Li et al. (2013) showed the interaction between poor family support and SL genotype predicted more symptoms of depression in males; Other studies showed SS genotype-negative environment interaction predicted higher risks of depression, a statistically significant only in males (Li et al., 2013; Haberstick et al., 2016; Chang et al., 2017). Basically consistent conclusion exist in the females but not males. Under negative environment, females carrying S alleles have higher depression levels. But, A Meta-Analysis of Interaction between 5-HTTLPR, stressful life events, and risk of depression, published in 2009, neither 5-HTTLPR genotypes alone or interaction with stressful life events predicted an increased risk of depression in females alone, males alone, or in both genders combined. The Meta-Analysis across 14 studies, subjects of most studies are adults (Risch et al., 2009).

Several reasons can explain the divergence of the above conclusions. First, Different countries and races have different distributions of alleles and genotypes of the 5-HTTLPR: e.g., different frequency of S/S and L/L genotype between older Taiwanese adults and western groups (Goldman et al., 2010); the higher frequency of S alleles in Asians than in Caucasians (Iurescia et al., 2016). Second, the dichotomous classification (S/L) of 5-HTTLPR genotypes may lead to influenced research results. Increased length of the 5HTTLPR may be associated with increased gene expression (S < L < XL) (Vijayendran et al., 2012). But, dichotomous classification of 5-HTTLPR genotypes exists in most studies (**Table 2**). Third, neglecting the two nearby SNPs rs25531 and 25532 may lead to a contradictory conclusion. SNP rs25531 contributes to different allelic subtypes S_A, S_G, L_A, and L_G. The different expression abilities exist in L_A/L_A genotype AND S/S, S/L_G, L_G/L_G, L_A/L_G. Fourth, gene-environment interaction may be more successful for studies that study a single gene with big environmental impact. For example, uninfected control group subjects, carrying 32 mutation in the ΔCCR5 chemokine receptor, were less infected with human immunodeficiency virus when they were highly exposed to the virus (Risch et al., 2009). However, The inheritance of depression does not follow a single-gene inheritance pattern like Huntington's disease but has a non-Mendelian, polygenic underpinning. As a complex psychological problem, depression is most likely the result of the synergistic effects of multiple genetic and environmental factors (Cao et al., 2018). In recent years, the studies of polygenic risk scores and gene-gene interaction studies have proved additive and interactive genetic effects of depression. Also, multi-genes affect the development of depression through interaction with environmental factors and gender differences exist in this complex interaction (Cao et al., 2016). e.g., Girls rather than boys possessed low-expression MAOA-uVNTR alleles and S 5-HTTLPR alleles, more likely to show increased depressive symptoms under stressful life events (Priess-Groben and Hyde, 2013). The interaction of both plasticity genotype (5-HTTLPR S and val66met Met allele)- early family environment quality predicted more depressive symptoms than either or neither plasticity genotype only in females (Dalton et al., 2014). Fifth, different study design, longitudinal study, and cross-sectional study have their advantages and disadvantages (**Table 3**). The cross-sectional study is a comparative study of people of different age groups at the same time (intergroup comparison), and the longitudinal study is a continuous study of the same population in various years (self-comparison). Sixth, different gene-environment results between objective measures (i.e., independent of the participants' report) and subjective measures (i.e., self-report) (Uddin et al., 2011), results of self-reported are more subjective (Sjöberg et al., 2006). Moreover, gene-environment interaction has a dynamic effect on depression. In a study of the influence of BDNF Val66met and 5-HTTLPR on depressive symptoms, Scientists report

TABLE 3 | Comparison of advantages and disadvantages between longitudinal research and cross-sectional research.

	Longitudinal Study	Cross-sectional Study
Advantages	1. No intergenerational effect 2. Systematically evaluate the behavioral changes of the subjects	1. Higher efficiency of data collection 2. Lower Research and control cost 3. Less affected by the loss of subjects
Disadvantages	1. Time-consuming 2. Higher Research and control cost 3. Loss of subjects brings confounding variables 4. Only observing the changes of one group, whether it has universal significance is still in doubt	1. More confusion variables 2. Can't systematically evaluate the behavioral changes of the subjects

that the gene-environment interaction conforms to differential susceptibility model when women are 15 years and that gene-environment interaction conforms to the diathesis-stress model after 15 years (**Figure 2**). Finally, measurement instruments, environmental factors, and source of information (informant) highly divergent across studies, so limiting the comparability and replication of the studies.

Besides, a SNP of the *HTR2C* gene, rs6318 (Ser23Cys), is Related to women's depressive symptoms with high stress levels and different cortisol release (Brummett et al., 2014). related genes of dopamine system (*DRD2*, *COMT*) (Vaske et al., 2009; Nyman et al., 2011), HPA axis system (*CRHR1*) (Roy et al., 2018), and immune system (IL-1β SNP) (McQuaid et al., 2019), can also interact with the environment to affect the occurrence of depression in a gender-specific manner (**Table 4**). Chinese scientists longitudinally studied the relationship of BDNF Val66Met (Fan et al., 2017), Preproghrelin Leu72Met (Su et al., 2017), oestrogen receptor alpha gene (*ESR1*) rs9340799 (Feng et al., 2017), adiponectin rs1501299 (Wang et al., 2015), tumor necrosis factor receptor-II (TNF-RII) rs1061622 (Memon et al., 2018) and insertion/deletion polymorphism at angiotensin-converting enzyme gene (ACE I/D) (Fan et al., 2018)with depression in adolescents after the 2008 Wenchuan earthquake. Results showed gene-environment interaction contributes to the risk of depression after the earthquake in a gender- and time-dependent manner. Dynamic genetic effects on depression across development were proved once again. However, the scientists also explained that their research is either different from previous research results or rarely reported. Therefore, we cannot yet draw a definitive conclusion on gender differences.

FUTURE DIRECTIONS

To date, few pieces of research have investigated gender differences in the polygenetic mechanisms of depression, and ignoring gender specificity may lead to inconsistent results. As a complex psychological problem, depression is most likely the result of the synergistic effects of multiple genetic and environmental factors (Cao et al., 2018). Therefore, future studies should further investigate the role of gender in the regulation of polygene genetic mechanisms (Cao et al., 2016). Second, gender differences in the genetic basis of depression may be caused by differences in the sensitivity of individuals to different types of environments (Cao et al., 2013). Future studies should examine the interaction between different types of the environment and genetic genes that affect gender differences in depression. The theoretical basis of the existing molecular genetics research on depression is mostly the "diathesis-stress

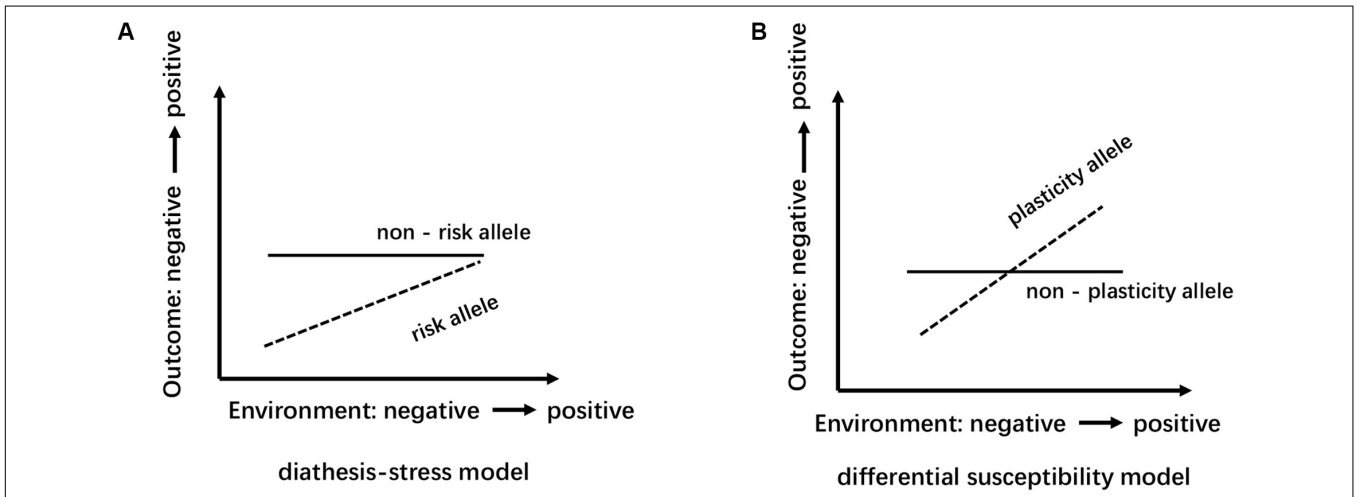

FIGURE 2 | Schematic diagram of the model of differential susceptibility and diathesis-stress: the abscissa represents the transition of environment factors from negative to positive; the ordinate indicates that the outcome variable range from negative to positive. **(A)** Diathesis-stress model points out that individuals with "risk" allele are only more likely to be negatively affected and to develop poorly than those with the "non-risk" allele. **(B)** Differential susceptibility model view, compared with the "non-plastic" allele carriers, "plastic" alleles individual has better sensitivity, more sensitive to both positive and negative environment accordingly develop better or worse.

TABLE 4 | Gene-environment interaction contribute to the risk of depression.

Age	Gene	Measurement instruments	Environmental stress factor	Gender composition	Demographic Characteristics	Source of information (informant)	Methodology	Result	References
Waves II and III (18–27) of the National Longitudinal Study of Adolescent Health (Add Health)	DRD2	Depressive symptoms: Center for Epidemiologic Studies-Depression Scale	Violent Victimization		African American, Caucasian	self-reported in-home interviews	Cross-sectional Study	1. Violent victimization has a strong independent effect on depressive symptoms for Caucasian females. violent victimization is associated with higher levels of depressive symptoms among African American females when they carry at least one A1 allele of DRD2. 2. DRD2 has a significant independent effect on depressive symptoms for males and African American females	Vaske et al., 2009
	Corticotrophin-releasing hormone receptor-1 gene (CRHR1) variant (rs17689918)	Depressive symptoms: Montgomery-Åsberg Depression Rating Scale; mood disorders: Mini-International Neuropsychiatric Interview	Stressful life events (SLE)	52.5% females	European origin	Self- and parent-reports	Longitudinal Study	1. A-allele males and GG females with higher SLEs reported greater depressiveness at age 18 2. Low SLE was associated with a lower risk for depression in males with the GG genotype at age 15	Roy et al., 2018
	SLC6A4, TPH2, COMT, MAOA, and the dopamine receptor genes DRD1–DRD5.	depressive symptoms: HSCL questionnaire	1. Early developmental risk 2. Social environment risk	2509 males, 2716 females	genetically isolated population-based Northern Finland Birth Cohort	Self-report	Longitudinal Study	1. No major genetic effects of the analyzed variants on depressiveness. Rs4274224 from DRD2 shows a significant association with depressiveness in males 2. Allelic variants of COMT interacted with high early developmental risk associated with depression in males	Nyman et al., 2011
Carleton University first-year students	IL-1β rs16944, IL-6 rs1800795 SNP, TNF-α rs1800629	Depressive Symptoms: 21-item Beck Depression Inventory (BDI)	Childhood Maltreatment	343 females and 132 males	various ethnic backgrounds	Self-report	Cross-sectional Study	Among females, higher childhood maltreatment was accompanied by elevated depressive symptoms irrespective of the IL-1β SNP, but among males, this relationship was particularly pronounced for those carrying the GG genotype of the IL-1β SNP.	McQuaid et al., 2019
	HTR2C gene, rs6318 (Ser23Cys)	Depressive symptoms: brief CES-D	Stressful life events	Men 2,366, Women 2,712	White	Self-report	Cross-sectional	Homozygous Ser23 C women who reported high levels of life stress had depressive symptom scores that were about 0.3 standard deviations higher than female Cys23 G carriers with similarly high stress levels.	Brummett et al., 2014

(Continued)

TABLE 4 | Continued

Age	Gene	Measurement instruments	Environmental stress factor	Gender composition	Demographic Characteristics	Source of information (informant)	Methodology	Result	References
High school (grades 11–12)	BDNF Val66Met	Depression severity: Beck Depression Inventory (BDI)	Wenchuan Earthquake	Males 306, Females 399	Chinese Han	Self-report	Longitudinal Study	1. Females constantly had higher depression prevalence than the males during the follow-up in the Met allele carriers 2. Compared to that at 6 months, the prevalence was lowered at 12 months in the male Met allele carriers, and at 18 months in all the females and the male Met allele carriers.	Fan et al., 2017
High school (grades 11–12)	Preproghrelin Leu72Met	Beck Depression Inventory (BDI)	Wenchuan Earthquake		Chinese Han	Self-report	Longitudinal Study	1. Females had a higher prevalence of depression than males at 6 months after the earthquake in 72Leu/Leu homozygotes 2. The prevalence was consecutively decreased in male 72Met allele carriers, but not in male 72Leu/Leu homozygotes, female 72Met allele carriers, or female 72Leu/Leu homozygotes during follow-up	Su et al., 2017
439 Chinese Han adolescents	Oestrogen receptor alpha gene (ESR1) rs9340799	Beck Depression Inventory (BDI)	Wenchuan Earthquake	Males 197, Females 242	Chinese Han	Self-report	Longitudinal Study	ESR1 rs9340799 maybe not associated with neither the prevalence nor the severity of depression in male individuals, but in female	Feng et al., 2017
Grade 11–12	Adiponectin rs1501299	Beck Depression Inventory (BDI)	Wenchuan earthquake.	Males 233, Females 304	Chinese Han	Self-report	Longitudinal Study	1. The decreases of the scores were found in the male subjects regardless of the genotypes in the time course of 6, 12, and 18 months after the earthquake. 2. The scores were decreased in the female T carriers, but not in the female GG homozygotes at 18 months when compared with those at 12 months after the earthquake.	Wang et al., 2015
High school students	Tumor necrosis factor receptor-II (TNF-RII) rs1061622	Beck Depression Inventory (BDI)	Wenchuan earthquake	Males 197, Females 242	Chinese Han	Self-report	Longitudinal Study	1. Female TT homozygotes had a higher depression prevalence than the male TT homozygotes at 6, 12, and 18 months. 2. The female G allele carriers had a higher depression prevalence than the male G allele carriers only at 6 and 12 months after the earthquake3. BDI scores declined in the male subjects with both genotypes and only in the female G allele carriers at 12 months when compared with those at 6 months	Memon et al., 2018

(Continued)

TABLE 4 | Continued

Age	Gene	Measurement instruments	Environmental stress factor	Gender composition	Demographic Characteristics	Source of information (informant)	Methodology	Result	References
	Insertion/deletion (I/D) polymorphism at angiotensin-converting enzyme gene (ACE)	Depressive symptoms: Beck Depression Inventory (BDI)	Wenchuan earthquake	Males 187, Females 244	Chinese Han	Self-report	Longitudinal Study	1. The D-allele carriers had lower depression prevalence than II homozygotes at 6, 12, and 18 months after the earthquake only in females, but not in males2. BDI scores were reduced in the female D-allele carriers when compared with those in the female II homozygotes at 6 and 12 months after the earthquake	Fan et al., 2018

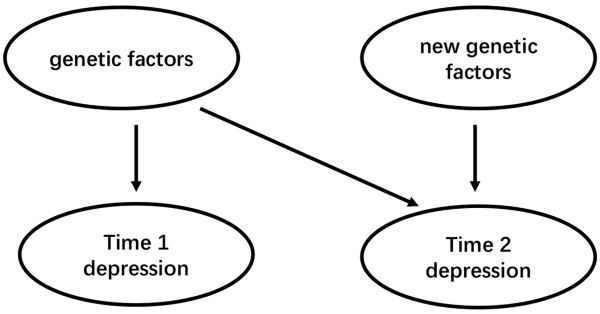

FIGURE 3 | The continuity and variability of genetic factors for depression.

model" because the many scientists believe that when individuals are under stress or high pressure, psychological and behavioral problems are prone to occur in individuals with a certain type of poor genetic quality, so studies based on this model mostly use the negative environment such as stressful life events as indicators to investigate the G × E effect of depression. However, the newly emerging theoretical model, the "differential susceptibility model," clearly puts forward and proves that individuals of certain genotypes are also more susceptible to the effects of positive growth environments and perform well or the opposite (**Figure 2**). Also, the existing research based on the "diathesis-stress model" fails to reveal multiple possible ways of G × E interaction. Whether there is a gender difference in the sensitivity of individuals with different genotypes to the positive environment is also needed for future research. Third, developmental behavioral genetics can investigate in depth whether genetics and the environment have an impact on human psychological and behavioral development and whether the effects were moderated by age. Compared to younger aged youth, older aged adolescents carrying SS/SL genotype has a higher risk of depressive episodes with greater chronic peer stress over the 3 years (Hankin et al., 2015). Besides, Depression is developmentally dynamic and may be affected by some new genetic factors across development (**Figure 3**). some new genetic factors emerge in depressive symptoms (Lau and Eley, 2006) or symptoms of anxiety and depression (Nivard et al., 2015) in adolescence. Compared with the 5-month-old baby, the negative emotionality of an 18-month-old baby was affected by persistent and new genetic factors (Schumann et al., 2017). So, it is necessary to use a longitudinal cohort design to investigate the gender differences in the genetic basis of depression at different ages and their developmental changes. Forth, Subjects suffering from mental disorders or various physical diseases may rate disease inaccurately (Chang et al., 2017). So, Selecting physically and mentally healthy, drug-free subjects to minimize these confounding factors and reveal the effect of the gene on depression more accurately. Finally, It is worth mentioning that to reduce the interference of confounding factors (e.g., ethnicity, gender, age, socioeconomic status), researchers should add the covariate × environment and covariate × gene interaction terms to the same model that tests the G × E interaction (Keller, 2014).

CONCLUSION

There is not enough evidence for genetic heterogeneity in men and women with major depression (Piccinelli and Wilkinson, 2000; Maciej et al., 2019). Genetic markers of major depression have not been successfully identified. Similarly, specific susceptibility genes on the X chromosome have not been successfully identified (Hyde and Mezulis, 2020). As a heterogeneous and multifactorial disease, the gender gap in depression may be caused by many biological, psychological, micro and macro environmental factors with varying interactions (Piccinelli and Wilkinson, 2000; Kuehner, 2017). Heredity may play a role in explaining gender differences. But, no sufficient evidence can explain the gender difference in depression from genetic underpinnings. In future research, scientists should pay attention to the influence of confounding factors on the results. such as different types of environments (positive or negative), demographic characteristics, measurement instruments, study design and so on.

AUTHOR CONTRIBUTIONS

LZ wrote the first draft and participated in the discussion of the manuscript. LZ, GH, YZ, YJ, TG, WY, RC, SX, and BL made major revisions to the logic of this manuscript and provided the critical revisions. All authors approved the final version of the manuscript for submission.

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Effects of Xiaoyaosan on Depressive-Like Behaviors in Rats With Chronic Unpredictable Mild Stress Through HPA Axis Induced Astrocytic Activities

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Abstract: Astrocytes in the hippocampus are immediately relevant to depressive-like behavior. By regulating their activities, Xiaoyaosan (XYS), a traditional Chinese medicine compound, works in the treatment of depression.

Objective: Chronic unpredictable mild stress (CUMS) rat model was established to observe the regulation of XYS. We investigated the behavioral changes of CUMS, the expression of corticosterone (CORT) of the hypothalamo–pituitary–adrenal (HPA) axis, the expression of Glu-NMDA receptor and astrocytes glial fibrillary acidic protein (GFAP) in the hippocampus. We also investigated whether these changes were linked to XYS.

Methods: 80 adult SD rats were randomly divided into four groups, control group, CUMS group, XYS group, and fluoxetine group. The rats in the control group and the CUMS group received 0.5 ml of deionized water once a day by intragastrically administration. Rats in the two treatment groups received XYS (2.224g/kg/d) and fluoxetine (2.0mg/kg/d) once a day, respectively. Rat hippocampus GFAP and Glu-NMDA receptor were respectively detected by real-time fluorescent quantitative PCR and western blot. The CORT of HPA axis was detected by Elisa. Body weight, food intake, and behavioral tests, such as open field tests, the sucrose preference test, and exhaustive swimming test, were used to assess depressive-like behavior in rats.

Results: In this work, significant behavioral changes and differences in expression of the CORT of HPA axis and hippocampal GFAP and Glu-NMDA receptor were presented in CUMS-exposed rats. Like fluoxetine, XYS improved CUMS-induced rat's body weight, food intake, and depressive-like behavior. The study also proved that XYS could reverse the CUMS-induced changes of the CORT of HPA axis and affect the astrocytic activities and down-regulate the NR2B subunit of NMDA receptor (NR2B) level in the hippocampus.

Conclusion: Changes in the hippocampus GFAP and Glu-NMDA receptor may be an essential mechanism of depression. Besides, XYS may be critical to the treatment of depression by intervention the HPA axis, GFAP and Glu-NMDA receptor.

Keywords: Xiaoyaosan, depression, astrocyte, glial fibrillary acidic protein, chronic unpredictable mild stress, hippocampus

INTRODUCTION

Due to the accelerated pace of life, depression is a common mental health problem in modern society (1). Under chronic stress, the neuroendocrine system of the body will produce pathophysiological changes (2), such as pyramidal cell atrophy, astrocyte dysfunction, and related signaling pathway changes that cause the hippocampus nerve regeneration disorders. It also causes memory loss, anxiety, and behavioral abnormalities, and also causes depressive symptoms or aggravating the emotional chaos of depressed patients (3, 4).

The causes of depression are very complicated, including social and psychological factors that affect the progress of the disease. Chronic stress is one of the highest risk factors for depression (5). Astrocytes are the most abundant and widely distributed cells in the central nervous system (6, 7). In recent years, we found that astrocyte plays an increasingly important role in the occurrence and development of neuropsychiatric diseases (8). At present, research on astrocyte has become a hot spot in the study of the pathophysiological mechanism of depression and even other neuropsychiatric disorders, such as epilepsy and Alzheimer's disease. The regulation of astrocytes is expected to become an essential target for the prevention and treatment of depression (9).

Besides, some studies have found that the number of astrocytes in the brain and the expression of its specific cytoskeleton protein Glial Fibrillary Acidic Protein (GFAP) are significantly decreased in some young patients with early-onset depression, and the expression level of GFAP can increase with age (10). GFAP is a biological marker protein of astrocytes (11). Numerous studies have shown that GFAP is involved in various physiological functions of astrocyte, such as maintaining the blood-brain barrier, synaptic plasticity, cell proliferation, and regulating the transport of vesicles and lysosomes in astrocyte (12). Decreased levels of GFAP protein in key brain regions such as the cortical, limbic system and hippocampus in patients with depression may be the critical cause of astrocyte and neuronal apoptosis, as well as specific brain changes in the brain. Furthermore, GFAP can enter the blood circulation from the brain through the blood-brain barrier, so the changes in protein levels of GFAP in the central nervous system and peripheral blood can reflect the degree of astrocyte damage in neurodegenerative diseases. At present, GFAP has become a new target for the occurrence, development, and treatment of depression (13, 14). But whether it affects the astrocyte and HPA axis is still unknown.

Xiaoyaosan (XYS) is a well-known traditional Chinese medicine formula composed of Bupleurum 30 g, Angelica 30 g, Radix Paeoniae Alba 30 g, Atractylodes 30 g, Poria 15 g, Zhigancao 15 g, Rhizoma Zingiberis Recens 10g, Mint 10 g. XYS was first described in the "Taiping Huimin Heji Jufang" and has been used to treat various diseases for hundreds of years and also extensively used in clinical and experimental research of depression (15). Previous studies have shown that XYS exerts an antidepressant-like effect by regulating brain regions such as the hippocampus, hypothalamus and locus coeruleus. My laboratory has been engaged in animal research on depression (3). We have established a CUMS depressive rat model and found that the classic compound XYS has a significant regulatory effect. It can works in a variety of neural circuits, with multiple targets, a multi-directed role (16). However, whether the GFAP and Glu-NMDA receptor is regulated, and the current mechanism is still unclear.

Some scholars have suggested that the Glu-NMDA receptor-NO pathway in the hippocampus may be damaged when the brain is stressed (17). The hippocampus limbic system has a clear role in declarative memory and spatial learning (18). Hippocampus is associated with the inhibition of the HPA axis and is susceptible to chronic stress, aging, stroke, and brain trauma (19). The hippocampus atrophy in patients with depression is associated with symptoms such as cognition and emotion (20). Chronic stress increases extracellular glutamate (Glu) concentration in the hippocampus may be a significant cause of neuronal atrophy and death (21). Glutamate is the most critical type of excitatory neurotransmitter in the brain. It is vital for learning, memory, and emotion (22). When the body is overwhelmed by stress, the HPA axis activates, resulting in the increase in glutamate release and secretion, which increases the excitability of the brain, increases the body's ability to adapt to stress and stimulation. It also leads to an increase in glucocorticoid, and strength of the body to overcome the stress. Increased sensitivity, this behavior is called "unsteady load", but it also causes neurotoxicity in the hippocampus. As the stimulation continues, it eventually causes necrosis of hippocampus neurons (23, 24).

Therefore, this paper puts forward the hypothesis: Chronic stress leads to hyperfunction of the HPA axis, followed by increased CORT content to a certain degree. Glu system is moderately activated, and the body's adaptability is enhanced. However, with the over-activation of the Glu system, the astrocyte (GFAP) content of hippocampal is reduced and neuron injury, eventually leading to the occurrence of depression. Furthermore, this study focused on the relationship between hippocampus neurons and astrocytes, and further observed the regulatory role of traditional Chinese medicine XYS.

MATERIALS AND METHODS

Animals

80 healthy male Sprague Dawley (SD) rats with the body of 180–200 g (12-week old; SCXK(JING)2016-0006 were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and then fed in a standard animal room (room temperature: 20°C–24°C; relative humidity: 30%–40% and light condition: 12-h dark/light cycle).

The experiment was approved by the Institutional Animal Care and Use Committee at Beijing University of Chinese Medicine and complied with the Animal Management Rules of the Chinese Ministry of Health and existing current animal welfare guidelines (NO. BUCM-4-2013101501-4001).

Preparation of Drugs

According to a previous publication (15), the traditional Chinese medicine compound stems from "Taiping Huimin Heji Jufang" in XYS (Bupleurum 30 g, Angelica 30 g, Radix Paeoniae Alba 30 g, Atractylodes 30 g, Poria 15 g, Zhigancao 15 g, Rhizoma Zingiberis Recens 10 g, Mint 10 g). All of the raw herbs were provided by Jiuzhitang Co., Ltd. and processed the herbs into a dry extract in Beijing University of Traditional Chinese Medicine under the Regulation on Processing of Traditional Chinese Medical Herbal Pieces of Beijing. Meanwhile, we have previously used an HPLC-LTQ-Orbitrap-MS eluted system to identify eight compounds from XYS samples (25), which matched the corresponding peaks in XYS. Fluoxetine hydrochloride capsule was employed in the experiment, Patheon France, packaged by Lilly Suzhou Pharmaceutical Co., Ltd. 20mg/granule. Product Lot Number 5545A.

CUMS Procedure and Drug Administration

Experimental Grouping

After seven days of adaptive feeding, the weight was labeled, and then 80 rats were randomly divided into four groups by Excel. There were 20 rats in each group: control group, model group, XYS group, and fluoxetine group.

Modeling

The model of rats with depression was replicated by CUMS. Selected items include (1) 45°C baking 5 min (Place the rat single cage under a 45°C electric heating lamp for 5 min), (2) 4°C swim 5 min (4°C ice water swimming for 5 min), (3) 85 dB noise 5h (85 db white noise stimulation for 5 h), (4) bondage stress 3h (Restrictive restraint for 3 h), (5) strange smell 24 h (Spray glacial acetic acid on the litter for 24 h), (6) strange objects 17 h (Put some plastic toys in the rat cage for 17 h) and (7) damp bedding 17 h (Put in damp litter for 17 h). Each model is selected every day, and each stress item is not displayed continuously. The CUMS random stress timetable is shown in **Table 1**. The experimental schedule is presented in **Figure 1**.

TABLE 1 | CUMS random stress timetable.

Date	45°C baking	4°C swimming	85 dB noise	Bondage stress	Strange smell	Strange objects	Damp bedding
Monday	√						
Tuesday		√					
Wednesday			√				
Thursday				√			
Friday					√		
Saturday						√	
Sunday							√

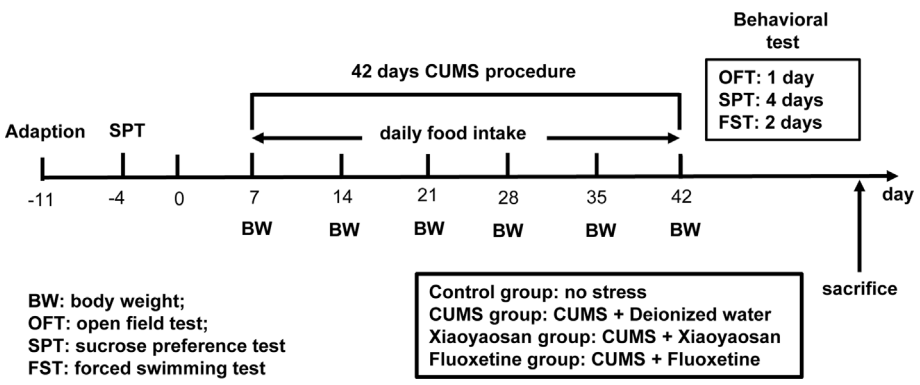

FIGURE 1 | The entire experimental plan. First, one week of adaptive feeding, sucrose preference test (SPT) was conducted as a baseline to determine the initial behavioral condition of the rats. During the 42 days chronic unpredictable mild stress (CUMS) procedure, rats in each group received the respective treatment. Open field test (OFT), SPT, and forced swimming test (FS)T were carried out to detect depressive-like behavior in rats after CUMS modeling. After completing all behavioral tests, animals were sacrificed, and tissue was collected.

Administration

The rats in the control group and the CUMS group received 0.5 ml of deionized water once a day by intragastrically administration. The rats in the two treatment groups received XYS (2.224 g/kg/d) and fluoxetine (2.0 mg/kg/d), respectively. From the first day of modeling, the rats in each group were intragastrically administered at 30-60 min after modeling and converted into equivalent drug doses of rats according to the adult body weight of 60 kg, the treatment of rats was 0.161/60 kg for adults and 2.224 g/kg/d for crude drugs. The fluoxetine group was administered with deionized water in an amount of 0.2 mg/100g (2.0 mg/kg/d) of body weight. The XYS powder was dissolved in deionized water. The CUMS group and control group were given the same amount of deionized water and were intragastrically administered at the end of the modeling. The deionized water or drugs were intragastrically administered for 42 days.

Sucrose Preference Test (SPT)

The SPT was performed at two steps, day 0 and day 42. Two days before the experiment, the rats were trained to adjust to drinking the sugar water. In the first step, the rats of each group were raised in single cages. Two bottles of 1% sucrose solution (sugar water) were placed in each cage, and the rats were allowed to drink freely for 24 h. Then, place a bottle of 1% sucrose solution and a bottle of purified water in each cage, and allow the rats to drink freely for 24 h. This period was the training period. The second step was to place 1 bottle of 1% sucrose solution and 1 bottle of purified water in each cage, allowing rats to drink freely for 1 h, and record the weight of the sucrose solution and purified water before and after the experiment. Sugar water consumption rate=sugar water consumption/total liquid consumption×100%. This experiment was used primarily to evaluate the degree of loss of pleasure in rats.

Forced Swimming Test (FST)

The forced swimming experiment was carried out in two steps: the top of a cylindrical transparent glass bucket (50 cm high and 20 cm diameter) was opened, and purified water (35 cm, 20–25°C) was added to the bucket. Mice were placed gently into the water and swimming behavior is recorded for 15 min, as a training period before the formal experiment, the formal experiment is conducted 24 h after the training is completed. The operator recorded the immobility time of each group of rats in the bucket within 5 min to evaluate the depressive behavior of each group of rats. The FST is the most extensive trial used to evaluate the effect of antidepressants.

Open Field Test (OFT)

Rats were subjected to OFT to evaluate the behavioral characteristics of spontaneous activity, exploration, anxiety. The OFT was performed respectively at day 0 and day 42. The rats were measured in a 40cm×40cm×15cm wooden box without ceiling, the floor was divided into 5×5 squares by white lines, each rat was placed right in the center and record the movement condition for 5 min with HD camera. Before the formal experiment, the rats in each group were moved to the operation room 30 min in advance. The operator gently placed the rats into the medium box of the field box and recorded the number of standing rats in each group within 5 min. At the same time, Etho Vision 3.0 software was used to analyze the behavioral parameters of the rats in each group, such as the total moving distance. At the end of each experiment, the cellar box was cleaned with 75% ethanol and water. After the odor was dispersed, the next test was performed, and the whole experiment was kept quiet. The total distance moved within 5 min, the number of crossings which at least three feet entering a square at the same time are counted as one time, and the number of groom times within 5 min were calculated.

Sample Collection and Preparation

After modeling and behavioral test, serum and the hippocampus tissues from five rats in each group were collected for protein analysis, and then collected the other five rats in each group with RNA preservation solution (Biotech, #2714) for Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) assay.

Enzyme-Linked Immunosorbent Assay (Elisa) Analysis

The serum CORT levels were determined with a commercial Elisa kit (CORT, Enzo ADI-900-097, New York, NY, USA). First of all, add 100 μl of each standard or sample to a 96-well plate depending on the manufacturer's instructions. Wrap with primary antibody and incubate at 37°C lasts for 1 h. Then wash holes with water 0.05% Tween 20 (PBST). After incubation and washing several times, 100 μl of 1:1000 horseradish peroxidase (HRP)-conjugated secondary antibody diluted in PBST was added to each well, and the plates were incubated at 37°C. for 1 h. Then, all wells were washed three times with PBST and produced with 100 μl of TMB (3,3',5,5'-tetramethylbenzidine solution) substrate per well until yellow appears. The reaction was stopped by the addition of 2 mol/L H_2SO_4. Read a sample absorbance at 450 nm using a Multiskan^{TM} GO (Thermo Fisher Scientific, Waltham, MA, USA) detector system. Hippocampus Glu was determined by using a commercial kit (Glu, SuoQiao, shanghai, SH, CH). About 0.2 g of hippocampus tissue was weighed, 2 ml of reagent one was added, and homogenization was carried out in an ice bath. Centrifuge at room temperature for 10 min, take the supernatant and place it on ice for testing. Then, the spectrophotometer was preheated for more than 30 min, the wavelength was adjusted to 570 nm, and the distilled water was zeroed. Add the following reagents to the covered EP tube:

The name of the reagent (μl)	Measuring tube	Control tube
Sample	1,000	
Reagent I		1,000
Reagent II	200	200

Mix well, 90°C water baths for 20 min (tighten to prevent water dispersion loss), water cooling, colorimetric at 570nm wavelength, △A=A determination tube - A control tube. The

regression equation was determined under standard conditions at y= 0.0074x - 0.5255; x is the glutamine acid content (μg/ml), and y is the absorbance.

RT-qPCR Analysis

Next, we continue to explore changes in GFAP and NR2B mRNAs in the hippocampus. The total RNA in the hippocampus of each rat was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of total RNA was determined by a spectrophotometer (Eppendorf, Germany), and the purity of RNA was measured by 1% agarose gel electrophoresis. The RNA from each sample was utilized to synthesize the first-strand cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a C1000 TouchTM Thermal Cycler (Bio-Rad, California, CA, USA). The sequences for primers showed in **Table 2** were designed based on published mRNA sequences in NCBI, and then synthesized by a specialized biotechnology company (Sangon Biotech Co., Ltd., Shanghai, China). The SYBR® Green PCR Master Mix (Thermo Fisher Scientific) was used to amplify the cDNA in the Multicolor Real-time PCR Detection System (Bio-Rad Laboratories Inc.), and the cycling parameters were as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 55°C (GFAP) or 58°C (NR2B) for 1 min, respectively, then followed by 65°C for 5 s and 95°C for 15 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the results.

Western Blot (WB) Analysis

Protein levels of the GFAP and NR2B in the hippocampus were identified by WB. Hippocampal tissues were used to prepare the total proteins with RIPA Lysis buffer (Biomiga, Santiago, CA, USA). According to the molecular weight of the target protein GFAP (50 kDa) and NR2B (166 kDa), 12% and 5% SDS-PAGE gels were selected, and then proteins were transferred onto polypropylene fluoride (PVDF) membranes. The 5% nonfat milk was used to block the membranes and dilute antibody, and the primary antibodies were anti-GFAP antibody (60190-1-lg, Mouse polyclonal to GFAP, diluted 1:5,000; Proteintech Group, Rosemont, IL, USA), anti-GRIN2B antibody (21920-1-AP, rabbit polyclonal to NR2B, diluted 1:500; Proteintech Group, Rosemont, IL, USA) and β-actin monoclonal antibody (66009-1lg, mouse monoclonal to β-actin, diluted 1:5,000; Proteintech Group, Rosemont, IL, USA). The enhanced chemiluminescence (ECL) detection reagent and Thermo Fisher Scientific was used to develop the membranes for 2 min, and then the Tanon-5200 system (Tanon, Shanghai, China) was used for exposure. The intensity of the protein band was read by Tanon Gis software (Tanon).

TABLE 2 | Primer sequences used in the RT-qPCR analysis.

Gene	Sequences
GFAP	
Forward	5′-GACCTGCGACCTTGAGTCCT-3′
Reverse	5′-AGCGAGTGCCTCCTGGTAAC-3′
NR2B	
Forward	5′-AGCTTCCGTCATGCTCAACA-3′
Reverse	5′-CGATGGTACTGCGGATCTTG-3′

Statistical Analysis

All dates were expressed as means ± standard errors of the means (SEM). And analyzed the date by SPSS 21.0, one-way analysis of variance (ANOVA), or non-parametric test was used for data processing based on normality test and homogeneity test for variance and least significant difference (LSD) method was adopted for the comparisons between groups. Furthermore, a multivariate analysis process of variance was also used to make comparisons. P-value < 0.05 was considered statistically significant difference.

RESULTS

XYS Improved Body Weight and Food Intake of CUMS-Exposed Rats

Figure 2A shows that: on the 7th day of modeling, the food intake of the model group was significantly lower than that of the control group ($P < 0.01$). On the 21st day of CUMS, the rats in the model group were significantly lower than the control group ($P < 0.01$), and there was no significant difference between XYS and fluoxetine group; on the 42nd day of modeling, the rats in the model group were significantly lower than the control group, XYS group and fluoxetine group ($P < 0.01$).

Figure 2B shows that: On day 0, there were no significant differences in the bodyweight of the four groups. On the 7th day of CUMS, the body weight of the model group began to be

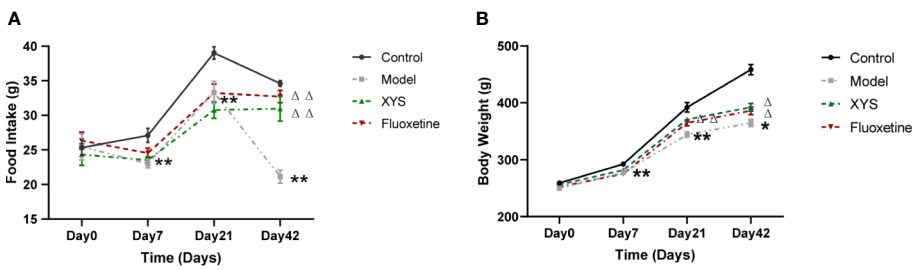

FIGURE 2 | 42 days of food intake and body weight were recorded once a week during the modeling period. **(A)** Changes in food intake. **(B)** Changes in body weight. Data were expressed as means ± SEM (n = 20), $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus the model group.

considerably lower than that of the control group ($P < 0.01$). On the 21st day of CUMS, the body weight of the model group was noticeably lower than that of the control group ($P < 0.01$). Compared with the model group, the body weight of the XYS group and the fluoxetine group were statistically different ($P < 0.01$). On the 42nd day of modeling, the body weight of the model group was considerably lower than that of the control group, and there was also a significant difference between the XYS group, the fluoxetine group and the model group ($P < 0.05$).

XYS Improved Depression-Like Behaviors of CUMS-Treated Rats

Continue to observe the behavior of depressed rats, some standard behavioral tests were carried out, including SPT, FST, OFT. From the results of SPT **Figures 3A, B**, it can be seen that the sucrose water consumption of the model group was significantly lower than that of the control group, and there was a significant statistical difference between the two groups ($P < 0.01$). The consumption of sucrose water in the XYS group was significantly higher than that in the model group, and the two were statistically different ($P < 0.05$). The FST is a standard behavioral despair test to evaluate the rat's depressive-like behaviors, where the immobility time of rats in the CUMS group was significantly longer versus the control group rats at day 42 ($P < 0.01$, **Figure 3C**). It also can be seen that the rats treated with XYS and fluoxetine were significantly lower than those of CUMS group rats (both $P < 0.01$, n = 20). The results show that both XYS and fluoxetine had antidepressant effects.

The OFT can reflect the independent and exploratory behaviors of rats intuitively. It includes the lattice number and groom time. At the beginning of the experiment, there were no significant differences in the results of the OFT at day 0 (both $P > 0.05$, **Figures 4A, C, E**). After CUMS modeling for 42 days, the lattice number, the groom times and the total distance travel of rats in the CUMS group were significantly less than those of control group rats (both $P < 0.01$, **Figures 4B, D, F**), while the XYS and fluoxetine treatment groups were significantly higher than that in the model group, and the two were statistically different ($P < 0.05$).

XYS Improved the Expression of Serum CORT and Hippocampus Glutamate of CUMS-Exposed Rats

Figure 5A shows that: Versus the control group, the serum CORT content in the model group was significantly higher ($P < 0.01$), and the serum CORT content in the XYS group and the fluoxetine group was considerably lower than that in the model group ($P < 0.05$, $P < 0.01$). **Figure 5B** shows that: Compared with the control group, the Glu content in the hippocampus of the model group was significantly higher ($P < 0.01$), and the content of Glu in the hippocampus of the XYS and fluoxetine groups was significantly lower than that of the model group ($P < 0.05$, $P < 0.01$).

XYS Improved the Expression of NR2B and GFAP in the Hippocampus of CUMS-Exposed Rats

To continue to detect changes at the molecular level. Detect whether XYS regulated the astrocyte (GFAP) and NMDA receptor system in the CUMS rat model, expressions of GFAP and NR2B were measured. First of all, the RT-qPCR and WB results revealed that the 42-day CUMS modeling could significant reduce the GFAP level in the hippocampus of CUMS rats (both $P < 0.01$, **Figures 6A, B**), and the rats in the two treatment groups showed a significant increase in GFAP level versus the CUMS group rats ($P < 0.05$). The 42-day CUMS modeling could also increase the NR2B level in the hippocampus of CUMS rats (both $P < 0.01$, **Figures 6C–F**), and the rats in the two treatment groups showed a significant decrease in NR2B level versus the CUMS group rats ($P < 0.05$, $P < 0.01$, respectively).

DISCUSSION

XYS has the effect of soothing the liver and relieving stagnation, nourishing blood, and strengthening the spleen (26). In the treatment of complex diseases caused by chronic stress such as depression, it shows superiority owing to the penetration of traditional Chinese medicine concepts such as overall regulation, syndrome differentiation, and individualized diagnosis and treatment (27). From the macroscopic point of view, the general condition of XYS group rats was significantly better than that of the model group rats. The food intake of XYS group rats was not significantly different from that of the model group on the 7th and 21st day of modeling, but it was considerably higher than that of the model group on the 42nd day ($P < 0.01$). The effect of XYS was also evident from the change in the body weight of rats. From the 7th day, the body weight of XYS group rats was significantly different from that in the model group. After the 21st day of modeling, the rats of the XYS group increased in body weight versus the model group ($P < 0.05$). From the results of body weight and food intake, it can be observed that XYS is a long-term process for regulating body weight and food intake. The effects of sugar

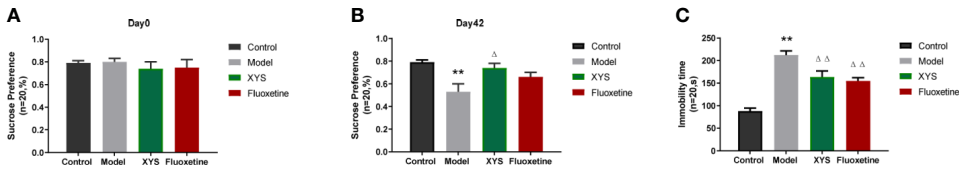

FIGURE 3 | **(A)** SPT of rats in each group at day 0 (baseline); **(B)** SPT of rats in each group at day 42; **(C)** FST of rats in each group at day 42. Data were expressed as means ± SEM (n = 20), $^{**}P < 0.01$ versus the control group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus the model group.

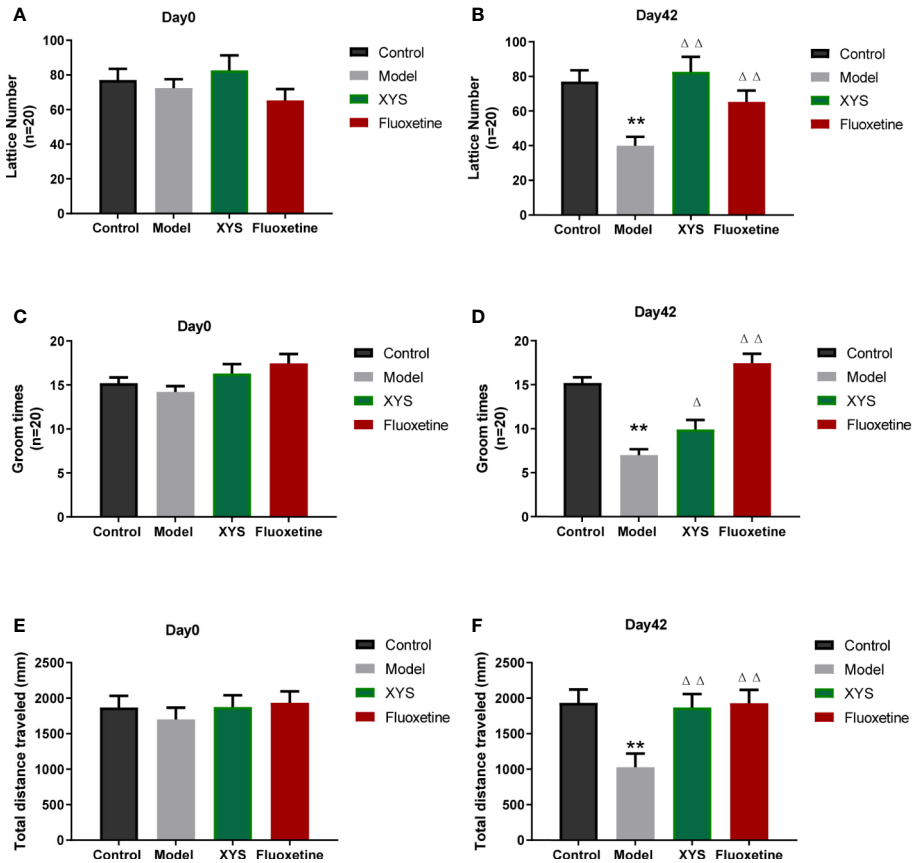

FIGURE 4 | **(A)** The results of lattice number in each group at day 0 (baseline); **(B)** The effects of lattice number in each group at day 42; **(C)** The results of groom times in each group at day 0 (baseline); **(D)** The effects of groom times in each group at day 42; **(E)** The results of total distance in each group at day 0 (baseline); **(F)** The results of total distance in each group at day 42. Data were expressed as means ± SEM (n = 20), $^{**}P < 0.01$ versus the control group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus the model group.

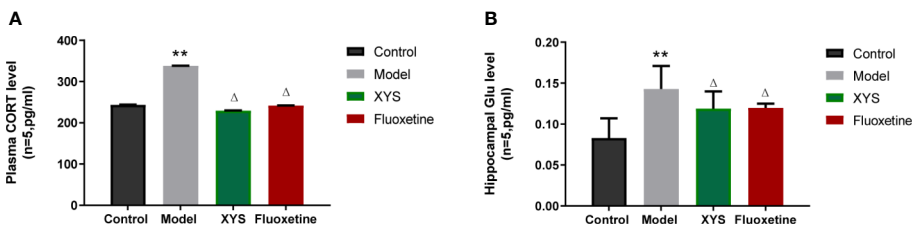

FIGURE 5 | Effects of Xiaoyaosan (XYS) on plasma corticosterone (CORT), hippocampus Glu levels in stress-induced rats. **(A)** Plasma CORT levels; **(B)** Hippocampus Glu levels. Data were expressed as means ± SEM (n = 5), $^{**}P < 0.01$ versus the control group; $^{\Delta}P < 0.05$ versus the model group.

water preference test and OFT also indicated that XYS prescript was restraint stress chronically. Fluoxetine was used as a positive control drug, it is widely used first-line antidepressant that selectively inhibits 5-HT transporters and blocks presynaptic membrane uptake (28). The results showed that the food intake, body weight, and sugar water preference of the fluoxetine group were better than the model group ($P < 0.05$).

The functional activities of the central nervous system are based on neurotransmitters and other information molecules (29). In the case of chronic stress, abnormal expression of molecules will cause damage to the brain (30). HPA axis activation is the most critical adaptive and protective response to stress in the body (31). However, in the chronic stress process, sustained HPA axis activation, and high secretion of glucocorticoid (GC) will produce

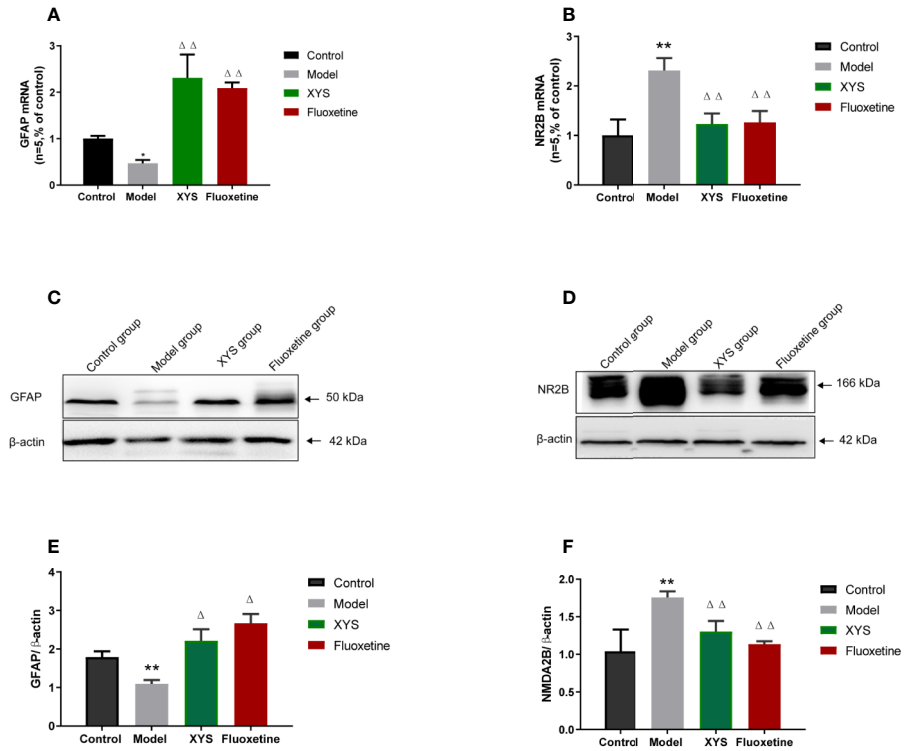

FIGURE 6 | Changes in the hippocampal of glial fibrillary acidic protein (GFAP) and NR2B in chronic unpredictable mild stress (CUMS) rats. **(A)** The mRNA results of GFAP (n = 5); **(B)** The mRNA results of NR2B (n = 5); **(C)** The protein results of GFAP (n = 5); **(D)** The protein results of NMDA2B (n = 5). **(E)** Relative GFAP/β-actin protein; **(F)** Relative NMDA2B/β-actin protein. Data were expressed as means ± SEM (n = 5), $^{**}P < 0.01$ versus the control group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus the model group.

many pathologies of the body (32). In this experiment, we detected a significant increase in the serum CORT content in the model group by Elisa, indicating that the HPA axis is continuously activated. The XYS group and the fluoxetine group reduced the serum CORT content. The HPA axis continues to activate damage to the hippocampus formation mainly through the following pathways: affecting glucose uptake, reducing the expression of glucose carriers, thereby interfering with cell metabolism, reducing cell viability; causing excessive release of excitatory amino acids and increasing extracellular accumulation of glutamate (33); increasing excitatory amino acid N-methyl-D-aspartate (NMDA) receptor-binding protein NR2A, NR2B subunit level, and receptor channel binding site number, thereby increasing hippocampus excitability (34). A large number of studies have shown that when the glutamate content at the synapse is too high. It can cause glutamate to exude synapses and bind to the extra-synaptic NMDA receptor (35). Recent studies have found that NMDA receptors located at the synapse can protect cells, and NMDA receptors outside the synapse stimulate cell cytotoxicity, promote cell necrosis and apoptosis, and NR2B subunits are mainly expressed in sites other than synapses such as cytoplasm and axonal membrane (36, 37). As a result, NR2B is also considered to be a necrosis factor of neurons. By RT-qPCR and WB detection, we can find that both XYS and fluoxetine can down-regulate NR2B at the level of genes and proteins. Thereby protecting neuronal damage.

In the nervous system of the brain, astrocyte is about ten times more quantitative than neurons (38). Astrocyte is located between neurons and capillaries. They are an essential part of the blood-brain barrier (39). Astrocyte regulates the transmission of neurotransmitters, regulates glucose metabolism, participates in glutamate metabolism and synaptic plasticity, participates in the regulation of immune mechanisms, and provides neurotrophic support (40). Under normal conditions, the Glu concentration of neuronal cytoplasm is 10 mM/L, the Glu concentration of AC cytoplasm is 50 μM/L to several hundred μM/L, the synaptic gap is 1 μM/L, and the synaptic terminal vesicles can reach 100 mM/L. The concentration of Glu inside and outside the cell varies greatly (41). During synaptic transmission, nerve impulses are transmitted to the synapses, and the nerve endings are depolarized. Synaptic vesicles are released from neurons by synaptic vesicles and plasma membrane fusion (42). Glu receptors acting on the postsynaptic membrane transmit nerve impulses and exert physiological functions. At the same time, they trigger a negative feedback regulation mechanism and are taken up by the glutamate transporter on the astrocyte cell membrane. The glial cells are very strong (43). The ingestion capacity of Glu and glutamine synthetase can convert Glu into

glutamine, transport it to the cytoplasm of presynaptic nerve endings, and deamination to Glu by glutaminase. Next, Glu enters the vesicle lumen through the glutamate transporter located on the vehicle and is stored in the vesicle. In resting neurons, Glu is stored in synaptic vesicles at the nerve endings in the form of small membrane-bound organelles (44). Thereby a "glutamate-glutamine cycle" between neurons and glial cells is formed, as showed in **Figure 7** below.

GFAP is mainly expressed in mature astrocyte, and it acts as a backbone protein to support and protect cells. GFAP is a commonly used marker protein of astrocyte and is recorded as a characteristic marker of astrocyte (45). In recent years, GFAP has been involved in many of the biological functions of astrocyte, including maintaining normal physiological functions of the blood-brain barrier, cell proliferation and division, autophagy, vesicles and lysosomes in astrocyte, maintenance of neurotransmitters in astrocytes and neuronal and glial cell balance (38). Besides, there is evidence that only by inhibiting the number of GFAP-positive glial cells is sufficient to induce depressive phase behavior in rats, classic anti-depression drugs such as fluoxetine can act as antidepressants to improve this pathological change and depressive-like behaviour (46).

In our past experiments, we have found that GFAP in the hippocampus of CUMS mice is down-regulated in the model group, and then XYS and fluoxetine can significantly up-regulate the expression of GFAP in the hippocampus (47). Other animal experiments have also found that different patterns of stimulation in the depression model, the expression in GFAP, and the number of positive cells of GFAP were found to be down-regulated in key brain regions such as the cortex-marginal system and hippocampus (48). We also found the same conclusion. Compared with the control group, the GFAP of the model group showed significant down-regulation of the gene and protein levels, while the XYS group and the fluoxetine group

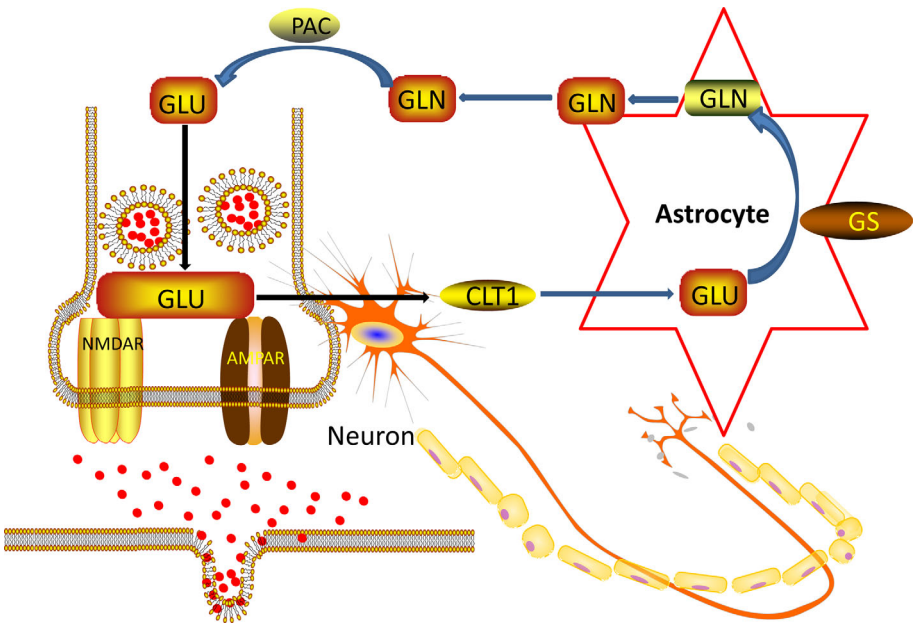

FIGURE 7 | Nerve cell and Ast Glu-Gln loop pattern.

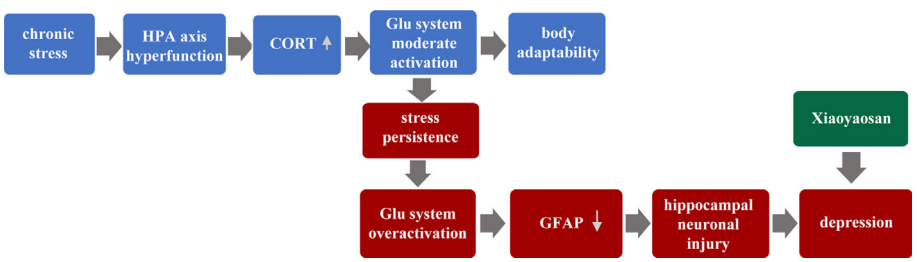

FIGURE 8 | Hypothetical pathogenesis of depression.

up-regulated the expression of GFAP. Astrocyte is considered to be an essential target in the treatment of depression. A large number of animal studies have shown that different types of antidepressants have different effects on astrocyte. Fluoxetine, a selective serotonin reuptake inhibitor, is effective in inhibiting the reduction in the amount of astrocyte caused by various stimulation. XYS also played a role similar to fluoxetine. So we speculate the pathogenesis of depression, which put forward at the beginning of the article. As shown in **Figure 8** below:

These studies are merely preliminary explorations of the above hypotheses, we preliminary explored the HPA axis, Glu system and astrocyte respectively in this study, but the correlation between the three aspects and the mechanism of causal relationship is not clear. We will continue to conduct further research around these three crucial components of depression. In recent years, the combinations of gas chromatography-quadrupole time of flight mass spectrometry (GC-Q-TOF/MS) and liquid chromatography-quadrupole time of flight mass spectrometry (LC-Q-TOF/MS) has been applied successfully in numerous metabolomics studies to achieve more sensitive and accurate metabolic profiling and screening of biomarkers. The use of GC-Q-TOF/MS is a possible area of future work. Functional electrophysiological research under disease models will help us further reveal the pathogenesis of the disease, we should use them to identify better the metabolite changes and pathways involved and support the proposed hypotheses (49).

CONCLUSIONS

In summary, XYS showed effects in improving the depression-behavior of CUMS rats. The study has proved that XYS could reverse the CUMS-induced changes of the CORT and affect the astrocytic activities and down-regulate the NR2B level in the hippocampus.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Beijing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MS, JC and JZ. Carried out the animal experiment: MS, XL, TW, and YL. Conducted molecular experiment: MS, TW and YZ. Analyzed the data: MS. All authors contributed to the article and approved the submitted version.

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Childhood Maltreatment and Depression in Adulthood in Chinese Female College Students: The Mediating Effect of Coping Style

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Depression is the most common psychological disorder of female, with high disability rate and remarkable mortality rate. There is a lack of knowledge about childhood experience, coping style, and adult depression. The aim of the present research was to enrich this knowledge by investigating the mediating effect of coping style between childhood maltreatment and depression in adulthood in Chinese female college students. Self-report questionnaires assessing childhood maltreatment, depression, and coping style were completed in 738 participants. The results illustrated that childhood maltreatment was positively related to depression in adulthood while coping style was negatively related to depression. In addition, childhood maltreatment could influence adult depression through the mediating role of coping style. These findings indicate that childhood maltreatment and negative coping style are associated with depression in adulthood. Psychological intervention strategies for coping style could provide effective treatment direction for depression caused by childhood maltreatment.

Keywords: childhood maltreatment, depression, coping style, mediating effect, psychological intervention

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INTRODUCTION

Depression is the leading cause of disability among people across the globe. It was estimated that the proportion of people suffering from depression in 2015 was 4.4%, and women (5.1%) were more common than men (3.6%) (1). Depression is common among the youth, and often indicates chronic and recurrent diseases in adulthood. A systematic review of medical students showed that the prevalence of depression or depressive symptoms was 27.2%, and the prevalence of suicidal ideation was 11.1% (2). Many psychosocial factors may affect depression, including cognitive impairment, stressor in life and circumstance, parental depression, interpersonal distress, and female gender (3). Childhood maltreatment, as one of the stressors in life, has been considered as a factor leading to depression (4). Therefore, the understanding of depression should not only consider its physiological susceptibility, but also focus on the individual's childhood experience and other psychosocial factors.

Childhood maltreatment (CM) includes many forms, involving emotional, physical and sexual abuse, as well as emotional and physical neglect (5). CM has been recognized as a crucial risk factor for depression. High levels of emotional and/or sexual abuse were associated with more significant severity of depression, and more markedly associated with depression than physical

abuse (6). In other words, although each type of CM was positively correlated with the severity of depression, emotional abuse and emotional neglect were the strongest (7). The probability of depression in adulthood with CM experience was 2.66–3.73 times higher than that of normal people. The early onset age was more likely to develop into chronic or refractory depression (8). Although CM increases the risk of depression, not all abused children become depressed. Some modifiable factors increase vulnerability to, or act as a buffer against, depression. Coping style had a significant mediating effect between stressor and psychological distress (9). Maladjusted coping was the main predictor of depression and reduction of maladjusted coping behavior might have the most positive effect on the relief of depression (10).

To sum up, depression is a high incidence of psychological disorders, seriously endangering the health of female youth, even life-threatening. There are many causes of depression and CM is a crucial one. Many empirical studies have revealed the link between CM and depression. It is less clear what coping strategies might be used to assist individuals in decreasing depression cause by CM. In this research, we hypothesize that CM is not only an important predictor of adult depression, but also might be used to directly predict the severity of depression. And through the mediating effect of coping style, the severity of depression is indirectly affected.

METHODS

Participants and Procedure

Seven hundred forty-five female college students were selected by cluster sampling. Seven hundred thirty-eight (99.1%) consented and took part in the current study, average age 19.4 years (SD = 1.1, range = 17–23). Two hundred eighty-four (38.5%) came from rural area and 454 (61.5%) from urban area. One hundred forty-six (19.8%) majored in science and engineering, 296 (40.1%) in medicine, 85 (11.5%) in art, and 211 (28.6%) in liberal arts.

The assessment was conducted in class under the supervision of research team members. The survey lasted 20 min. The study was approved by the Human Research Ethics Committee of NJUCM. Approval was also granted by each college. Research information was provided directly to participants and informed consent and consent were obtained.

Measures

The Hospital Anxiety and Depression Scale [HADS; (11)] is a self-evaluation scale which examines anxiety and depression symptoms, respectively. The total score of depression or anxiety can be regarded as the severity of symptoms. HADS had good reliability and validity (12). In the current study, internal consistency $\alpha = 0.81$.

The Simple Coping Style Questionnaire [SCSQ; (13)] contains 20 items assessing coping style which effectively reflect individual's coping style in the context of Chinese culture. Items 1–12 belong to positive coping and 13–20 belong to negative coping. The score of each item is from 0 (never) to 3 (always). If the average difference between positive coping and negative coping is >0, it is positive coping and <0 is negative coping. The internal consistency coefficient of the scale was 0.90 and the test-retest reliability was 0.89 (13). In the present study, total questionnaire internal consistency $\alpha = 0.75$.

Personal Report of Childhood Abuse [PRCA; (14)] is compiled in the context of Chinese culture. It contains 20 items and measures the frequency and severity of abuse in childhood. The scale includes four sub-scales of physical abuse (PA), emotional abuse (EA), sexual abuse (SA), and neglect. Higher score indicates more severe CM. The Cronbach's α coefficient of the total scale and the four sub-scales of PRCA ranged from 0.604 to 0.839 in college students (15). In the current study, internal consistency $\alpha = 0.88$.

Statistical Analysis

All analyses were performed using SPSS22.0. All statistical tests were two-sided and the significance level was set at $p < 0.05$. Partial correlation was used to examine correlations among depression, CM and coping style. Because the scores of PRCA and subscales did not conform to normal distribution, Kruskal-Wallis method was used for nonparametric test to compare the differences in different depression status. The bootstrap method of Preacher and Hayes (16) and Hayes (17) was used to test the mediating effect.

TABLE 1 | Partial correlation of various variables after controlling potential age effect.

	1	2	3	4	5	6	7
1. PRCA	1						
2. PA	0.816***	1					
3. EA	0.952***	0.723***	1				
4. SA	0.244***	0.224***	0.154***	1			
5. Neglect	0.751***	0.434***	0.588***	0.209***	1		
6. Coping style	−0.138***	−0.102***	−0.119***	−0.089*	−0.125***	1	
7. Adult depression	0.191***	0.119***	0.171***	0.106***	0.195***	−0.437***	1

PRCA, personal report of childhood abuse; PA, physical abuse; EA, emotional abuse; SA, sex sbuse.
**p < 0.05 (two-tailed), ***p < 0.001 (two-tailed).*

TABLE 2 | Nonparametric test of childhood maltreatment at different depression status.

Group	①	②	③	χ^2
PRCA	1.99 ± 4.23	3.79 ± 5.83	4.64 ± 5.79	22.06***
PA	0.43 ± 1.14	0.59 ± 1.22	0.86 ± 1.64	3.61
EA	1.04 ± 2.37	1.94 ± 3.39	2.60 ± 3.47	20.56***
SA	0.01 ± 0.12	0.06 ± 0.32	0.07 ± 0.34	8.37*
Neglect	0.49 ± 1.15	1.13 ± 1.87	1.02 ± 1.60	19.05***

*PRCA, personal report of childhood abuse; PA, physical abuse; EA, emotional abuse; SA, sex abuse; ①, asymptomatic; ②, suspicious depression; ③, symptomatic depression. *p < 0.05 (two-tailed), ***p < 0.001 (two-tailed).*

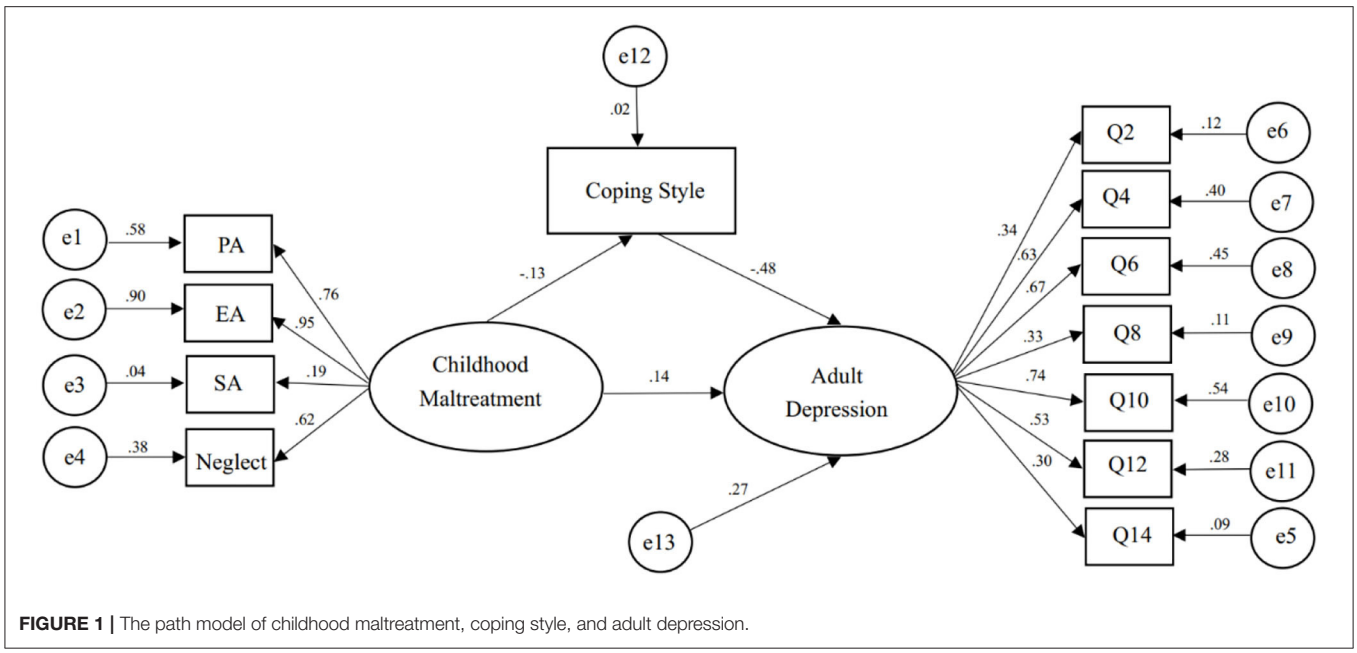

FIGURE 1 | The path model of childhood maltreatment, coping style, and adult depression.

TABLE 3 | Fitting index of intermediary model.

χ^2	df	χ^2/df	RMSEA	GFI	NFI	IFI	CFI
151.329	52	2.91	0.051	0.967	0.924	0.949	0.949

df, degree of freedom; RMSEA, root mean square error of approximation; GFI, goodness of fit index; NFI, normed fit index; IFI, incremental fit index; CFI, comparative fit index.

RESULTS

Correlations Among Childhood Maltreatment, Coping Style, and Adult Depression

The correlations among CM, adult depression and coping style are shown in **Table 1**. As revealed in the table, CM was positively related to adult depression, whereas coping style was negatively related to CM and adult depression.

The Relationship Between Childhood Maltreatment and Depression in Adulthood

According to the score of HADS, the participants were divided into three groups: asymptomatic (79.7%), suspicious depression (14.6%), and symptomatic depression (5.7%). The relationship between adult depression and CM is shown in **Table 2**. As revealed in the table, there were obvious differences in PRCA [$\chi^2_{(2,735)} = 22.06$, $p < 0.001$], as well as emotional abuse [$\chi^2_{(2,735)} = 20.56$, $p < 0.001$], sexual abuse [$\chi^2_{(2,735)} = 8.37$, $p < 0.05$], and neglect [$\chi^2_{(2,735)} = 19.05$, $p < 0.001$] among different depression status.

The Mediating Effect of Coping Style on the Relationship Between Childhood Maltreatment and Depression in Adulthood

Taking CM as latent variable, the path model between CM, coping style and adult depression was constructed to verify the mediating effect of coping style (**Figure 1**). The fitting index of

TABLE 4 | Analysis of total effect, direct effect and indirect effect.

	Effect value	Boot SE	Boot CI lower	Boot CI upper	Relative effect value
Total effect	0.207	0.054	0.109	0.321	
Direct effect	0.144	0.041	0.054	0.244	69.6%
Indirect effect	0.063	0.020	0.027	0.107	30.4%

Boot SE, Boot CI Lower, and Boot CI Upper refer to the standard error of indirect effect estimated by percentile bootstrap method corrected by deviation, the lower limit and upper limit of 95% confidence interval, respectively.

TABLE 5 | Regression equations of the mediating effect of coping style.

Variable	Path	Regression equation
PA, CS, AD	PA → CS	M = −0.10X
	PA, CS → AD	Y = 0.08X−0.43M
	PA → AD	Y = 0.12X
EA, CS, AD	EA → CS	M = −0.12X
	EA, CS → AD	M = −0.12X
	EA → AD	Y = 0.12X−0.42M
SA, CS, AD	SA → CS	M = −0.09X
	SA, CS → AD	Y = 0.07X−0.43M
	SA → AD	Y = 0.11X
Neglect, CS, AD	Neglect → CS	M = −0.13X
	Neglect, CS → AD	Y = 0.14X−0.42M
	Neglect → AD	Y = 0.20X

PA, physical abuse; EA, emotional abuse; SA, sex abuse; CS, coping style; AD, adult depression.

the model basically conformed to the standard of commonly used fitting statistics (**Table 3**). The standardized 95% CI of total effect of CM on depression was (0.109, 0.321), direct effect was (0.054, 0.244), indirect effects was (0.027, 0.107), excluding 0. Therefore, the overall effect, direct and indirect effects were significant. In the model, the overall effect of CM on adult depression was c = 0.207, a = −0.133, b = −0.476, c' = 0144, all of which reached a significant level. This presented that CM could not only directly predict adult depression, but also influence depression through the mediating effect of coping style. The direct effect (0.144) and mediating effect (0.063) accounted for 69.6 and 30.4% of the total effect (0.207), respectively (**Table 4**).

Furthermore, four subscales of PRCA were used as independent variables and adult depression as dependent variable to test the mediating effects of coping style. The results showed that physical abuse ($\beta = 0.12$, $t = 3.25$, $p < 0.01$), emotional abuse ($\beta = 0.17$, $t = 4.72$, $p < 0.01$), sexual abuse ($\beta = 0.11$, $t = 2.88$, $p < 0.01$) and neglect ($\beta = 0.20$, $t = 5.41$, $p < 0.001$) had significant positive effects on adult depression. After adding coping styles, physical abuse ($\beta = 0.08$, $t = 2.26$, $p < 0.01$), emotional abuse ($\beta = 0.12$, $t = 3.66$, $p < 0.001$), sexual abuse ($\beta = 0.07$, $t = 2.05$, $p < 0.05$), and neglect ($\beta = 0.14$, $t = 4.29$, $p < 0.001$) still had significant effects on adult depression. The regression equations of the mediating effect of coping style on the four subscales of PRCA and adult depression is shown in **Table 5**.

DISCUSSION

The consequences of CM can be short-term or even continue to adulthood, so it is a serious social and public health problem (18). CM is closely related to depression in adulthood. Therefore, it is important to understand the mechanism of their interaction for the provision of prevention and intervention services, which can help individuals with CM experience to better deal with emotional problems. CM is an important risk factor for health problems in adulthood, stress and coping strategies may affect this relationship (19). This study aims to explore the mediating role of coping style between CM and adult depression. In this study, we illustrate that compared with physical abuse, emotional abuse shows significant differences at different depression status, which is consistent with previous research (6). More importantly, the results indicate that coping style plays an exact mediating role between CM and adult depression. A study from Canada shows that positive coping strategies as a mediator can buffer the impact of childhood abuse on adult psychological distress, which is consistent with the results of this study, suggesting that the mediating role of coping styles between CM and adult depression may be cross-cultural (20). The findings of this study support the view that individuals who encounter CM will increase the severity of depression if they adopt negative coping style, while positive coping style can reduce the negative effects of CM to a certain extent. In addition, female youth with high degree of CM use more negative coping strategies and less adaptive coping skills, which affects their psychological health.

This study provides a valuable direction for the effective intervention of depression by investigating the mediating role of coping style on CM and adult depression. Coping is an individual's conscious, purposeful and flexible adjustment behavior to the change of real environment. Individuals can evaluate and regulate the physical and emotional responses related to stress events by coping. Although people may suffer different kinds of stress events, their coping styles are often stable and consistent. Some tend to adopt positive strategies, such as seeking support and changing the belief system, while others tend to adopt negative strategies, such as avoidance and emotional venting. Previous studies also found that inappropriate coping strategies such as self-blame, denial and abandonment were the main predictors of depression, anxiety, and stress (10). This study illustrated that coping style can be used as an intermediary variable to regulate the effect of CM on adult depression. For any person, childhood experience is an established fact that has happened and can't be changed. However,

coping style can be changed through purposeful training and intervention as to actively adjust the individual's mental and physical state.

In this study, we tested the hypothesis that CM not only positively predicted adult depression, but also indirectly affected adult depression through coping style. The contribution of this study is to verify the mediating effect of coping style on CM and adult depression, which can provide guidance for psychological intervention of depression. The limitation of this study is that how to adjust coping strategies to alleviate depression needs more empirical research. In addition, the subjects of this study are female college students, and the sampling scope will be further expanded in the future. Moreover, considering the differences of cultural factors in various countries on the specific manifestations of CM, we chose the questionnaire compiled by Chinese scholars. In future research, we might try to select tools with more items and details, such as Childhood Trauma Questionnaire (CTQ) to verify whether there would be more findings.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research Ethics Committee of NJUCM. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZZ is responsible for design and writing. WH and YZ are responsible for questionnaire test and data analysis. NZ is for revising. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.581564/full#supplementary-material

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ORIGINAL RESEARCH
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The Differential Role of Cytokines on Stress Responses in a Menopause Rat Model

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Menopause is a risk factor of anxiety and depression. Also, psychoneurological symptoms are shown in almost all women in the perimenopausal period. The present study investigated if repeated stress modulates behavioral changes or the balance of pro- and anti-inflammatory cytokines in ovariectomized (OVX) rats. Albino SD female rats were randomly divided into four groups: the naïve normal group (NOR), a surgically ovariectomized group (OVX), the only stressed group (ST), and the OVX and stressed groups (OVX + ST). We performed a battery of tests such as the forced swimming test (FST), the sucrose intake, and social exploration. In the same animals, corticosterone (CORT) was assessed in the serum, and also, two representative cytokines (IL-1β and IL-4) were examined in different brain regions after all the behavior sessions for all the experimental groups. The OVX + ST group showed more immobility time in FST than the OVX group or the ST group. Also, the OVX + ST group tended to have a decreased active social exploration and sucrose solution intake compared to the OVX group or ST group. The serum concentration of CORT of the OVX + ST group was higher than the OVX group or ST group and also the level of CORT in OVX + ST was markedly increased compared to the NOR group. In the brain, the number of IL-1β immunoreactive neurons of the OVX + ST group was increased compared to the NOR group. The OVX + ST group tended to have an increase in IL-1β-positive neurons compared to the OVX or ST group. However, the number of IL-4 immunoreactive neurons of the OVX + ST group was markedly decreased compared with the NOR group. Also, the IL-4-positive neurons in the OVX + ST group was significantly decreased when compared to the ST group. These results indicate that ovariectomy and stress combine to increase the depressive-like behaviors and neuroinflammatory responses. Together, these data show neuroinflammation as a potential contributor to depressive-like symptoms during menopausal transition.

Keywords: cytokines, depression, motor trigeminal nucleus (MTN), para ventricular nucleus (PVN), ovariectomy

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INTRODUCTION

Ovarian hormones play a pivotal role in regulating affective disorder (1). Depletion of ovarian hormone as menopausal implicated increased mood disorders and deficient learning and memory (2). Also, several studies proved that estrogen and testosterone regulate HPA axis responses (3, 4). The postmenopausal stage in women exacerbates mood disorder or insomnia (5). Some studies

implicated that mood disorders are related to change of neuroimmune systems (6–9). For example, chronic restraint stress or foot shock stress elicits depressive behaviors (learned helplessness, anhedonia, anxiety, etc.) and also chronic stress alter neuroimmune systems (10, 11). Other studies reported that psychological stress is associated with pro-inflammatory cytokine release or depressive behavior (12, 13). In a recent study, infectious challenge with IL-1β to IL-4 KO mice resulted in dysregulation of pro-inflammatory cytokines (14). IL-4 KO is capable of causing susceptibility to immune responses.

A recent study proved that the postmenopausal stage altered the neuroendocrine–immune system (15). Menopausal women experience difficulty in coping with stressful situations. Stress levels are perceived to vary with menopause (16). This is related to pro-inflammatory cytokine levels in the serum (16). Also, the ovarian hormone exerts potent immunomodulatory effects in neuroinflammation animal models (17–19). Although estrogen is associated with menopause-related neuronal inflammation changes, the mechanisms behind its effects are unclear.

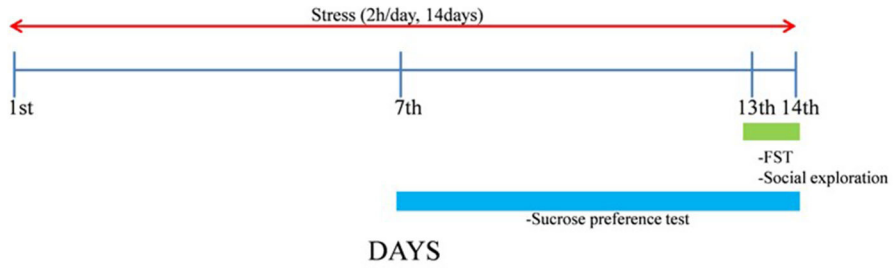

FIGURE 1 | Experimental schedule. The naïve normal group (NOR), a surgically ovariectomized group (OVX), the only stressed group (ST), and the OVX and stressed group (OVX + ST). Each group contained six rats ($n = 6$).

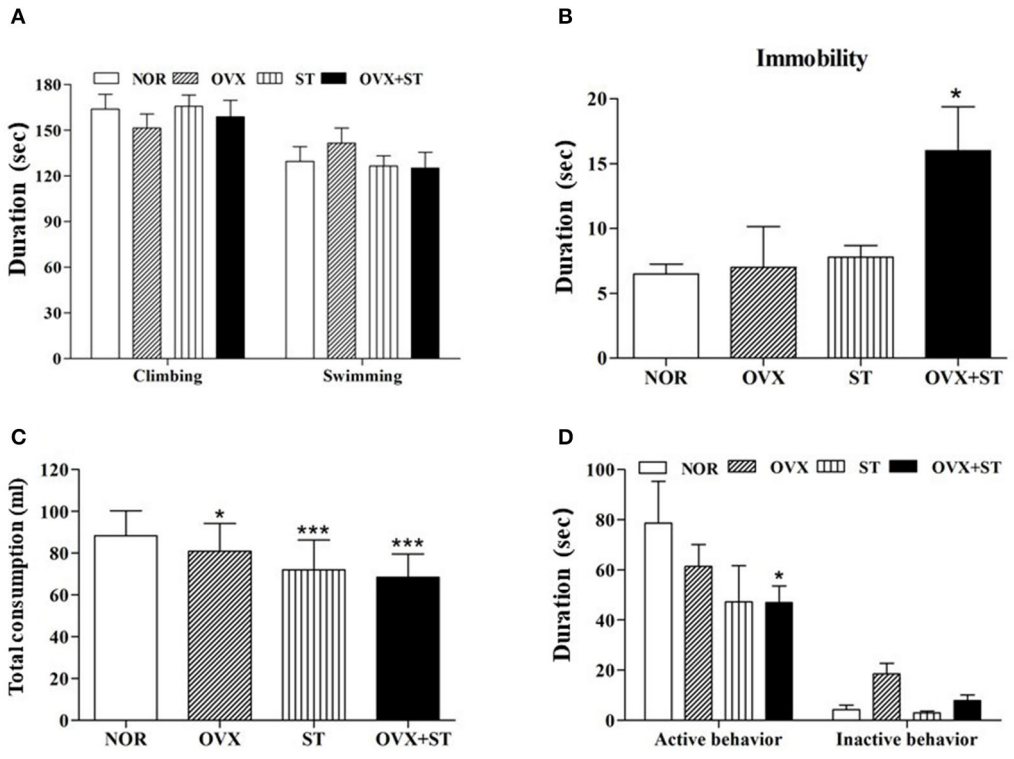

FIGURE 2 | The effects of repeated immobilization stress on the forced swimming test in the rats. The data represent the means ± SEM of the duration of climbing and swimming **(A)** and immobility **(B)** during the 5-min test session. Also, the effects of repeated immobilization stress on the sucrose intake **(C)** and social exploration **(D)**. The data represent the duration of active behavior during the 5-min test session. The naïve normal group (NOR), a surgically ovariectomized group (OVX), the only stressed group (ST), and the OVX and stressed group (OVX + ST). Each group contained six rats ($n = 6$). The results of behaviors were analyzed by performing separate one-way ANOVA among the groups. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to the normal group.

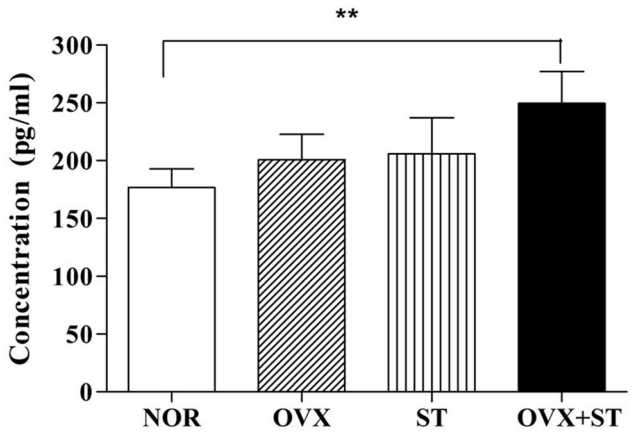

FIGURE 3 | The effects of the corticosterone level in the serum. The naïve normal group (NOR), a surgically ovariectomized group (OVX), the only stressed group (ST), and the OVX and stressed group (OVX + ST). Each group contained six rats ($n = 6$). The results of ELISA were analyzed by performing separate one-way ANOVA among the groups. Each value represents the mean ± S.E.M. $^{**}p < 0.01$ compared to the normal group.

In this study, we aimed to investigate whether repeated stress in the OVX rat model could change depressive-like behaviors and changes of pro- and anti-inflammatory cytokines in the brain regions. Depressive behavior was tested via a forced swimming test (FST), social exploration, and sucrose intake; moreover, we further assessed the changes of IL-1β- and IL-4-reactive neurons in the PVN and MTN using immunohistochemistry.

MATERIALS AND METHODS

Animals and Immobilization Stress

Albino SD female rats (over 3 months old, weighing 240–300 g) (Orient, Inc. Korea) underwent ovariectomy. All rats were housed in a plastic cage with stainless steel lips under controlled conditions (temperature: 22–24°C, 12-h light/dark cycle, and humidity 65 ± 5%). The rats had *ad libitum* access to food and water. All the experiments were approved by the Kyung Hee University Institutional Animal Care and Use Committee [approval no. KHUAP(SE)-13-041].

Albino SD female rats were randomly divided into four groups: the naïve normal group (NOR, $n = 6$), a surgically ovariectomized group (OVX, $n = 6$), the only stressed group (ST, $n = 6$), and the OVX and stressed group (OVX + ST, $n = 6$). Bilateral ovariectomy was performed under pentobarbital sodium (50 mg/kg, i.p.). The animals were monitored for 1 week post-surgery. Postoperative recuperation was monitored for 1 week.

After 1 week, ST groups were exposed to repeated immobilization stress using the disposable plastic cone (a disposable rodent restraint cone, Yusung, Korea) for 2 h (10:00–12:00 a.m.) for 14 days. The experimental schedule is shown in **Figure 1**.

Forced Swimming Test

Transparent Plexiglas cylinders (height: 50 cm × diameter: 20 cm) were used for FST. The room temperature water was filled to a 30-cm depth. Before test, all rats were taken pre-test for 15 min. After 24 h, rats were tested for 5 min. We used a video camera and analyzed. Total duration of immobility, climbing, and swimming were examined (20).

Social Exploration

On the first day, rats were introduced into their transparent home cage for 5 min. After 24 h, rats were recorded as doing "social exploration" for 5 min. We counted the behaviors (active behaviors: grooming of test rat, sniffing, and liking; inactive behaviors: boxing and kicking).

Sucrose Preference Test

All rats assessed adaptive training for 1 week. Training consisted of seven 12-h tests, and rats could freely choose between a bottle of water and a bottle containing a mild 1% [weight/volume (w/v)] sucrose solution. Two bottles were rotated daily. Each rat freely selected a pre-weighed tap water bottle or a 1% sucrose bottle. On the test day (13th day), the total consumption of each bottle was recorded after 12 h.

Corticosterone Measurement

The blood samples were collected and then centrifuged. The final supernatant was used for corticosterone analysis. Concentration of corticosterone was determined using ELISA kit (DuoSet ELISA development system, R&D Systems, Inc., Minneapolis, MN., USA) according to the manufacturer's protocols.

Immunohistochemistry

After all behavioral tests, rats were euthanized with an overdose of sodium pentobarbital (80 mg/kg, IP) and then they were transcardially perfused with phosphate buffered saline (PBS) into a protrusion of the left ventricle, with a steady flow of around 20 ml/min of saline solution. When all blood has been cleared from the body, we used a cold 4% paraformaldehyde solution at a flow rate of 7 ml/min (800 ml). The brains were removed and placed in a 4% paraformaldehyde solution for 24 h at 4°C. The brain was placed in O.C.T. compound at −20°C and cut into 30-μm serial coronal sections. Sections were rinsed with PBS containing Triton X-100. The primary antibody concentrations were at 1:100 and 1:1,000 for IL-1β (Santa Cruz Biotechnology, Delaware Avenue Santa Cruz, CA, USA) and IL-4 (Santa Cruz Biotechnology, Delaware Avenue Santa Cruz, CA, USA), respectively. The primary antibody was diluted with blocking serum in ABC kits (Vector Laboratories, Burlingame, CA) for 72 h at 4°C. Then, tissues were washed three times in PBS. For the secondary antibody use as the appropriate primary antibody (1:200) in PBST. Tissues were washed three times in PBS and stained using diaminobenzidine (DAB) chromogen with nickel intensification and coverslipped slides with mounting media. Light microscopy was used for acquiring photomicrographs. The stained cell was counted in paraventricular nucleus (PVN) and motor trigeminal nucleus (MTN) according to a brain atlas (21).

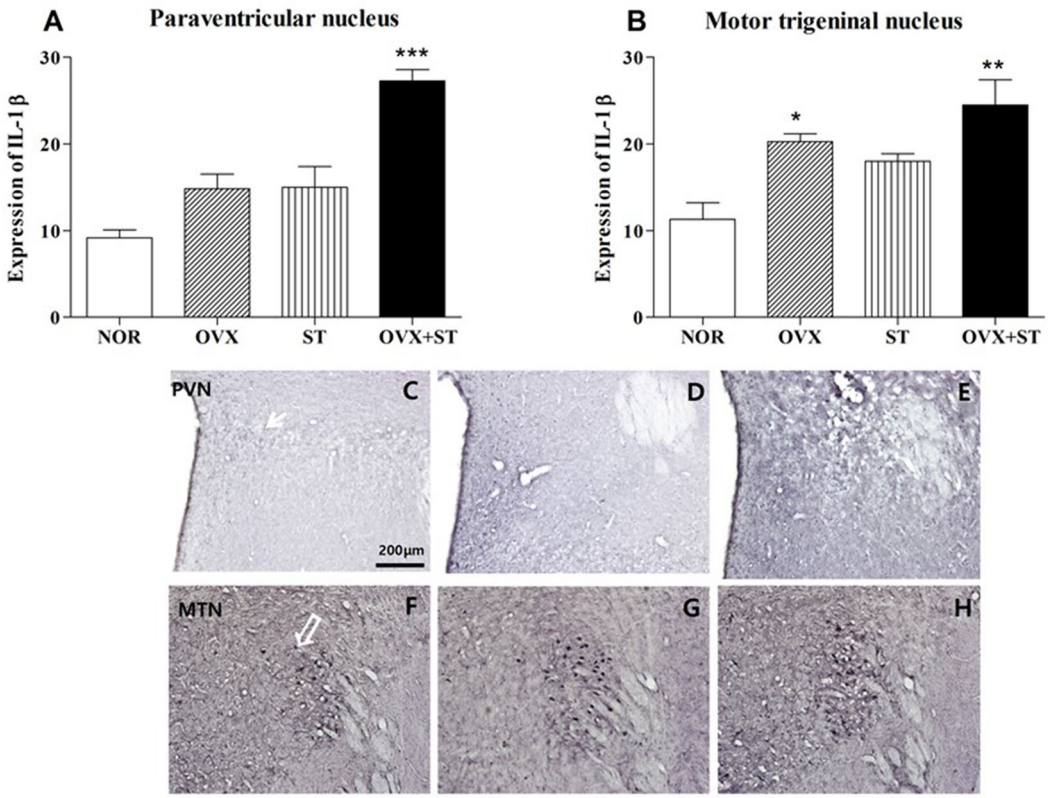

FIGURE 4 | The number of IL-1β immunostained nuclei in the paraventricular nucleus **(A)** and motor trigeminal nucleus regions **(B)**. The naïve normal group (NOR), a surgically ovariectomized group (OVX), the only stressed group (ST), and the OVX and stressed group (OVX + ST). Each group contained six rats ($n = 6$). The results of IL-1β-reactivity were analyzed by performing separate one-way ANOVA on the number of the IL-1β immunostained neurons among the groups. Each value represents the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the normal group. Photographs showing the distribution of IL-1β immunoreactive cells in the brain of NOR **(C,F)**, OVX **(D,G)**, and OVX + ST **(E,H)**. Sections were cut coronally at 30 μm and the scale bar represents 200 μm. PVN, paraventricular nucleus; MTN, motor trigeminal nucleus.

Statistical Analysis

SPSS 15.0 software (SPSS Inc., Chicago, IL) was used for statistical analysis. Differences among groups in the behavioral test and immunohistochemistry were analyzed using one-way ANOVA and LSD *post hoc* test. A $P < 0.05$ was considered statistically significant. GraphPad Prism 6.0 software was used for graph generation.

RESULTS

Behavioral Tests

As shown in **Figures 2A,B**, the immobility time of the OVX groups in the FST significantly increased compared to the NOR group [$F_{(3,20)} = 4.7$, $P < 0.05$]. When tested in the FST, the OVX and OVX + ST groups tended to show decreased active behavior (swimming and climbing) compared to the NOR group. **Figure 2C** shows that the sucrose preference test was significantly different when compared among the groups [$F(3, 20) = 22.9$, $P < 0.001$]. Also, the OVX + ST group showed more immobility time compared to the OVX group or the ST group. Furthermore, the OVX + ST group showed a decrease in consumption of sucrose solution by 30% compared with the NOR group ($P < 0.001$). Also, the OVX + ST group tended to decrease sucrose solution intake compared to the OVX group or the ST group. **Figure 2D** shows that the social exploration tests were markedly different among the groups [$F_{(3,20)} = 8.3$, $P < 0.01$]. The active behavior of the juvenile was decreased in the OVX + ST group compared with the NOR group ($P < 0.05$). Also, the OVX + ST group tended to have a decreased active social exploration when compared to the OVX group or the ST group.

Corticosterone

As shown in **Figure 3**, the serum concentration of CORT was significantly different among the groups [$F_{(3,20)} = 5.8$, $P < 0.01$]. Statistical results indicated the markedly increased serum concentration of CORT in the OVX + ST group compared with the NOR group ($P < 0.01$), and the level of CORT in OVX +ST was markedly increased compared to the NOR group.

IL-1β Immunohistochemistry

As shown in **Figures 4A–H**, the number of IL-1β-positive neurons in the PVN [$F_{(3,14)} = 23.7$, $P < 0.001$] and MTN ($F_{(3,14)} = 9.0$, $P < 0.01$) was significantly different among the groups. The

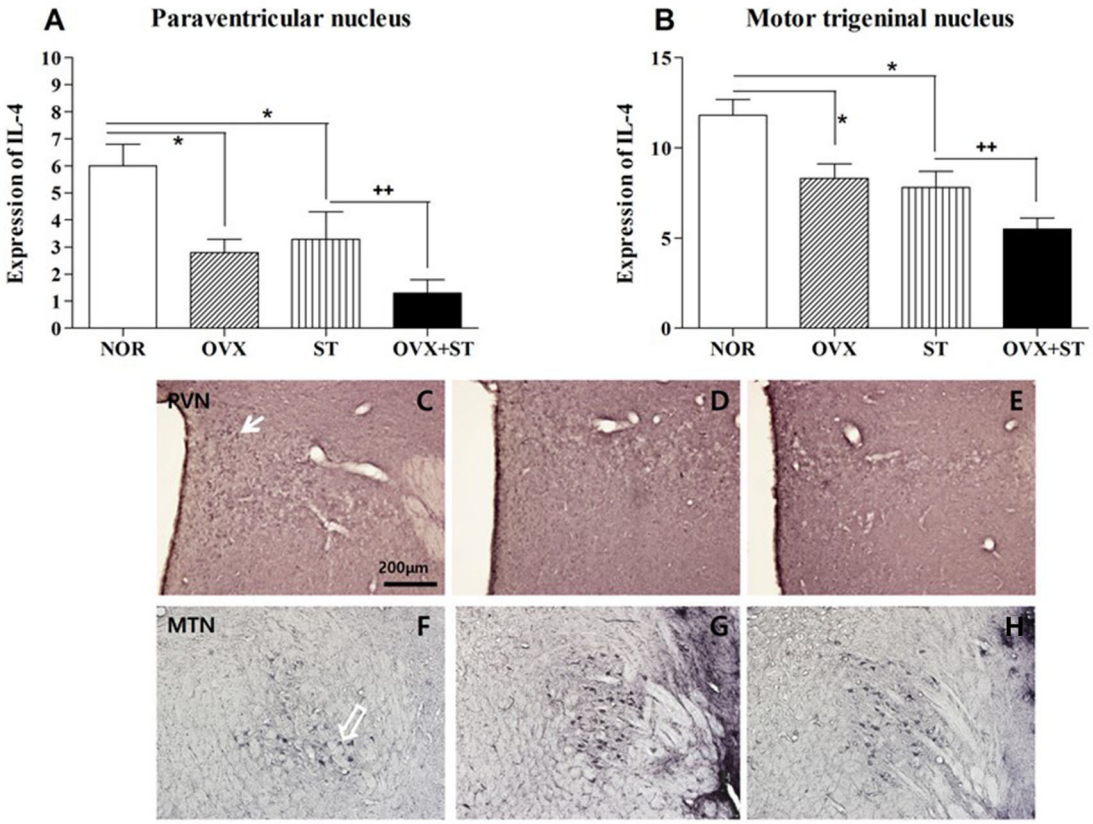

FIGURE 5 | The number of IL-1β immunostained nuclei in the paraventricular nucleus **(A)** and motor trigeminal nucleus regions **(B)**. The naïve normal group (NOR), a surgically ovariectomized group (OVX), the only stressed group (ST), and the OVX and stressed group (OVX + ST). Each group contained six rats ($n = 6$). The results of IL-4 reactivity were analyzed by performing separate one-way ANOVA on the number of the IL-4 immunostained neurons among the groups. Each value represents the mean ± S.E.M. $^{*}p < 0.05$ compared to the normal group. $^{++}p < 0.01$ compared to the ST group. Photographs showing the distribution of IL-4 immunoreactive cells in the brain of normal group **(C,F)**, OVX **(D,G)**, and OVX + ST **(E,H)**. Sections were cut coronally at 30 μm and the scale bar represents 200 μm. PVN, paraventricular nucleus; MTN, motor trigeminal nucleus.

expression of the IL-1β-positive neurons in the PVN of the OVX + ST group increased threefold compared with the NOR group ($P < 0.001$). Also, the expression of the IL-1β positive neurons in the MTN was significantly increased in the OVX + ST group compared with the NOR group ($P < 0.01$). The OVX + ST group tended to have an increase in IL-1β positive neurons compared to the OVX or ST group in the MTN and PVN.

IL-4 Immunohistochemistry

As shown in **Figures 5A–H**, the number of IL-1β-positive neurons in the PVN [$F_{(3,13)} = 7.2$, $p < 0.05$] and MTN [$F_{(3,15)} = 11.0$, $p < 0.001$] was significantly different among the groups. The number of IL-4 neurons in the PVN area of the OVX + ST group was decreased compared to that in the normal group. Also, the expression of the IL-4-positive neurons in the PVN was significantly decreased in the OVX + ST group compared with the NOR group ($P < 0.05$). In the MTN, the expression of the IL-4-positive neurons was markedly decreased in the OVX +ST group compared with the NOR group ($P < 0.05$). Also, the IL-4-positive neurons in the OVX + ST group was significantly decreased when compared to the ST group.

The Relationships Between IL-1β and IL-4 Expression

The Spearman's correlation test revealed significant relationships between IL-1β and IL-4. In the experiment groups, biochemical and neuro-immunological characteristics were changed. In particular, the mean levels of IL-1β increased significantly after OVX and repeated stress, whereas IL-4 decreased significantly. In the PVN ($r = 0.7$, $P < 0.05$) and MTN ($r = 0.7$, $P < 0.01$), the change in IL-1β and IL-4 correlates significantly after OVX and repeated stress.

DISCUSSION

The present results proved that repeated immobilization stress in the OVX rats poses an immune challenge that is capable of inducing depressive like behaviors, promoting exaggerated corticosterone responses and changing the cytokine expression in the brain. The present results showed that the interaction between the anti- and pro-inflammatory

mediators ultimately determines both the severity of the manifestation of depressive-like behavior and neuro-immune dysfunction.

Ovarian hormones have played a role in the regulation of cognitive and mood functions (22). They have been proposed as a contributor to depressive behavior and the synthesis, release, and reuptake of many receptors for neurotransmitters (23). This study also showed an increase of depression-like behaviors including reduction of preferences for sucrose solution, inhibition of locomotor activities, and induction of learned helplessness behavior. Also, the effects of female sexual hormones on both neuroinflammatory responses and depressive behaviors have also been well described by Azizi-Malekabadi et al. (22). 17β-estradiol (E2) replacement exhibits anti-inflammatory properties in the central nervous system (18). Overproduction of pro-inflammatory cytokine could be related to the inflammatory response system, which is upregulated during depression, and this is related to the shift of the pro-/anti-inflammatory cytokine balance. A previous study proved that psychological stress is associated with pro-inflammatory cytokines in the brain such as IL-6, interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) (24). You et al. proved that chronic mild stress induced inflammatory responses in the spleen and brain region, as well as increased depressive-like behaviors in rats (25). The present study also observed that the expression of IL-1β immunoreactive cells was increased in the PVN and MTN after OVX and repeated immobilization stress. These results indicated that life events and depressive symptoms are related to the rise of central pro-inflammatory cytokines such as IL-1β in major mood disorder patients (26–29) and stress-treated animals (30).

On the other hand, Myint et al. and Pavon et al. reported that depressed patients showed lower IL-4 levels in the serum (28, 31). Also, recent findings in laboratory studies have highlighted the role of interleukin-4, which is released from glia (32), in regulating the neuroinflammatory changes that occur in brain regions. We also observed that the IL-4 immunoreactivity was downregulated in the brain after repeated immobilization stress. The data in this current study indicate that there is an imbalance between anti-inflammatory and pro-inflammatory cytokines (IL-4 and IL-1β) in depressed female rats. The shift between anti-inflammatory and pro-inflammatory cytokines in different psychiatric disorders has been previously reported, and the dominance of macrophage cytokines in major depression has also been described (28). The present study showed that the increased IL-1β/IL-4 ratio may be associated with the activation of macrophage-induced inflammatory response. The present study also found that stimulated production of IL-1β and IL-4 displayed an opposite inflammatory pattern. These changes could be part of normal immune modulation. According to present results, we found an imbalance between anti-inflammatory and pro-inflammatory cytokines in depressed female rats that is associated with life events.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All the experiments were approved by the Kyung Hee University Institutional Animal Care (Approval No. KHUAP(SE)-13-041) and in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication number 80-23, revised 1996).

AUTHOR CONTRIBUTIONS

IS, HS, and HP designed the study. HP and HS acquired and analyzed the data. HP, HS, and IS wrote the article, which all other authors reviewed. All authors contributed to the article and approved the submitted version.

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Electroacupuncture Alleviates Cerebral Ischemia/Reperfusion Injury in Rats by Histone H4 Lysine 16 Acetylation-Mediated Autophagy

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Background: Electroacupuncture (EA) treatment in ischemic stroke has been highlighted recently; however, the specific mechanism is still elusive. Autophagy is considered a new target for cerebral ischemia/reperfusion (I/R), but whether it plays a role of protecting or causing rapid cell apoptosis remains unclear. Studies have reported that the reduction in lysine 16 of histone H4 acetylation coheres with autophagy induction. The primary purpose of the study was to explore whether EA could alleviate I/R via autophagy-mediated histone H4 lysine 16 acetylation in the middle cerebral artery occlusion (MCAO) rat model.

Methods: One hundred and twenty male Sprague-Dawley rats were divided into five groups: control group, MCAO group, MCAO+EA group, MCAO+EA+hMOF siRNA group, and MCAO+EA+Sirt1 inhibitor group. EA was applied to "Baihui" (Du20) and "Renzhong" (Du26) at 5 min after modeling and 16 h after the first EA intervention. The structure and molecular markers of the rat brain were evaluated.

Results: EA significantly alleviated I/R injury by upregulating the expressions of Sirt1, Beclin1, and LC3-II and downregulating the expressions of hMOF and H4K16ac. In contrast, the Sirt1 inhibitor lowered the increase in Sirt1, Beclin1, and LC3-II and enhanced the level of hMOF and H4K16ac expressions associated with EA treatment. Besides, ChIP assay revealed that the binding of H4K16ac in the Beclin1 promoter region of the autophagy target gene was significantly raised in the MCAO+EA group and MCAO+EA+hMOF siRNA group.

Conclusions: EA treatment inhibited the H4K16ac process, facilitated autophagy, and alleviated I/R injury. These findings suggested that regulating histone H4 lysine 16 acetylation-mediated autophagy may be a key mechanism of EA at Du20 and Du26 to treat I/R.

Keywords: HMOF, SIRT 1, autophagy, electroacupuncture, H4K16Ac

INTRODUCTION

Stroke is one of the leading causes of death and disability worldwide, leading to a heavy financial burden and mental stress. WHO data displayed that the stroke burden may rise from about 38 million disability-adjusted life years (DALYs) in 1990 to 61 million DALYs in 2020 globally (1). Ischemic stroke accounts for about 80–85% of all stroke cases that induce reperfusion and damage brain tissue (2, 3). Worse, it leads to physical defects and mental disorders. Meta-analysis shows that one-quarter of stroke survivors experienced poststroke anxiety and that one-third suffered from poststroke depression (4, 5). The neuropsychiatric sequelae of stroke may prevent the recovery process, affect quality of life, and cause caregiver's fatigue. Therefore, it is crippling. Unfortunately, the underlying mechanism of ischemic stroke is still unclear due to its complexity. Moreover, cerebral ischemia/reperfusion (CIR) injury exacerbates brain damage after ischemia. It refers to a pathological process in which neuronal apoptosis is further aggravated after blood perfusion is restored in a short period (6), including cell autophagy, inflammation, mitochondrial dysfunction, and oxidative stress (7, 8). However, few effective treatments could prevent CIR injury.

Acupuncture has a long history in ischemic stroke therapy, and "Baihui" (Du20) and "Renzhong" (Du26) are two classic acupoints for the treatment of the disease. Electroacupuncture (EA) is a new combination of acupuncture and modern electrotherapy. Previous studies have reported that EA is an apparent effective cure for cerebral ischemia tolerance (9, 10). However, the detailed mechanisms remain elusive. Some studies have manifested the affiliation between the effect of EA on cerebral ischemia and autophagy (11).

Active autophagy has been intensively observed in the ischemic penumbra (12), confirming that it has occurred in both cerebral ischemia physiological and pathological processes. Some studies have pointed out that autophagy plays a crucial role in providing additional energy and nutrients for ischemic cells (13, 14). Besides, researches have shown that autophagy induction of cerebral ischemia injury presents more functions on inhibiting neuronal damage and promoting neuronal survival than its blockade (15, 16). Several studies demonstrate that excessive autophagy could increase the risk of cell death (17, 18). Therefore, the role of autophagy in cerebral ischemia is not exact yet, and whether activation of autophagy will increase neuronal mortality or not remains controversial. By this dual mechanism of action, we believe autophagy has the potential to act on cerebral ischemia treatments. Here, we used autophagic markers LC3-II and Beclin1 to test the autophagic flux by western blot.

MOF is a member of the MYST family of histone acetyltransferases (HATs) (19), while hMOF is a 61-ortholog Drosophila MOF (20). It has been demonstrated that hMOF competes in DNA damage repair, cell cycle, and cell differentiation (21). Sirt1 is a member of the histone deacetylase (HDAC) protein family sirtuin and has been shown to regulate the multiple cellular stress responses (22). Histone acetylation is regulated by HATs and HDACs. It is one of the essential posttranslational modifications. Recent research has found that acetyltransferase (HATs) hMOF and histone deacetylase (HDACs) SIRT1 is a molecular histone switch on H4K16 acetylation, whose balancing effects regulate autophagy (23).

In summary, a correlation among EA, H4K16ac, and autophagy was found, while the mechanism is still unclear. Our purpose is to explore whether the effect of EA on autophagy is mediated by H4K16ac and to investigate its potential neuroprotective agent.

METHODS

Animal Preparation

Adult male Sprague-Dawley (SD) rats (6–8 weeks old, 260 $\pm$ 10 g) were provided by the Nanjing Traditional Chinese Medicine University experimental animal center. One hundred and twenty rats were randomly divided into the following five groups: control group, middle cerebral artery occlusion (MCAO) group, MCAO+EA group, MCAO+EA+hMOF siRNA group, and MCAO+EA+Sirt1 inhibitor group ($n = 24$ for each group). All the rats were placed in an environment with a temperature of $24 \pm 1^{\circ}$C, humidity of 50%, a cycle of light for 12 h/darkness for 12 h.

The study protocol was approved by the Animal Care and Use Committee of the First Affiliated Hospital of the Medical College at Nanjing Traditional Chinese Medicine University and strictly followed the guidelines of the Guide for the Care and Use of Laboratory Rats.

MCAO Model

According to the Longa thread embolization method, the right middle cerebral artery (MCA) was blocked in rats to establish a modified acute focal CIR model (MCAO) (24). SD rats were anesthetized with 10% chloral hydrate (0.36 ml/100 g) intraperitoneal injection, and a laser Doppler flowmeter probe was inserted into the rat skull to the surface of the rat cortex to monitor cerebral blood flow. In the rat's supine position, a midline neck incision was made to separate the extracranial branch of the internal carotid artery of the rat, the pterygopalatine artery, and it was closed with a micro-arterial clip. Then the common carotid artery and internal carotid artery were clamped with a micro-arterial clip, the external carotid artery was cut with microsurgical scissors, and a 4/0 nylon wire with a diameter of 0.18 mm was inserted into the internal carotid artery from the external carotid artery incision and sent into the skull (about 18–20 mm); there is a clear sense of resistance at this time, and the cerebral blood flow monitored by the laser Doppler cerebral blood flow instrument suddenly drops to <15% before the modeling, which was successful. After 1 h, the nylon thread was retracted to the external carotid artery, which was the beginning of reperfusion.

EA Stimulation

EA intervention was operated at 5 min and 16 h after the model was successfully established. Two acupuncture needles (0.25-mm outer diameter) were inserted into the Du20 acupoints (in the middle of the parietal bone) and Du26 (1 mm below the nasal tip, in the middle of the nasolabial fold) subcutaneously,

which were indwelled for 30 min. The G6805-II EA apparatus (Shanghai Medical Electronic Machine Co., Ltd., Shanghai, China) was connected above the two needles after 5 min and 16 h of ischemia, with sparse waves at 3.85 Hz for 1.28 s and dense waves at 6.25 Hz for 2.08 s. The strength was 0.8–1.0 mA for 30 min.

Lateral Ventricular Administration

After two EA treatments, the heads of the MCAO+EA+hMOF siRNA group and MCAO+EA+Sirt1 inhibitor group were fixed to the stereotype of the brain; the anterior fontanelle was marked. The puncture point was 1 mm to the right of the sagittal suture and 1.5 mm behind the coronal suture, and the depth of the injection was 1.5 mm. At high speed, the skull was drilled directly above the right ventricular injection site of the hMOF siRNA group and the Sirt1 inhibitor group, the microsyringe was inserted into the skull, and the configured hMOF siRNA and Entranster^{TM}-*in vivo* transfection reagent and the Sirt1 inhibitor nicotinamide were slowly injected with the configured hMOF siRNA. The injection rate was 0.5 L/min, and the injection volume was 20 L/n. The injection was completed, bone paraffin was applied, and the skin was sutured to complete the brain localization injection.

Evaluation of the Neurobehavioral Deficit Score

Neurological deficits were scored in a single-blind design at 24 h following reperfusion. There were eight grades (0–7 scores). Scoring criteria were as follows: a score of 0, no asymmetrical activities; a score of 1, left forepaw cannot completely extend when lifting the tail; a score of 2, left forepaw disability; a score of 3, left forepaw tightly closed to the chest wall; a score of 4, turning left when free-running; a score of 5, left forepaw makes an act of pushing back; a score of 6, the rotation was surrounding the original point; and a score of 7, the left limb cannot support the body (25).

Calculate Cerebral Infarction Volume

Twenty-four hours after reperfusion, the rat heads were quickly cut off, and the material was taken. After freezing, the brain was divided into 2-mm-thick brain slices and immersed in a 37/2% TT solution (TTC, Xinong Inc., Beijing, China) and incubated for 15 min. After TTC staining is completed, it was fixed, stored, photographed, and scanned, and the Ulead Photo Express 2.0 image analysis software was used to outline the cerebral infarction. Ischemic penumbra is measured with the medical injury measurement software, and the entire left hemisphere in each layer of brain slices is measured separately. Multiplying the area by 2 will get us the total tissue area; to get the infarct area, we multiply the total area of the left hemisphere by 2 and subtract the total area of the normal tissue; by multiplying infarct area with the thickness of the brain slice of 2 mm, we calculate the volume of each cerebral infarct tissue; and then by dividing the total volume of the left hemisphere by 2, we obtain the percentage of infarct volume.

Hematoxylin–Eosin Staining

Brain tissues were cleaned and fixed with PBS to prepare paraffin sections, which were dehydrated with conventional ethanol and xylene. The tissues were soaked in paraffin and embedded, sliced, and baked. The paraffin sections were dewaxed and rehydrated, stained with hematoxylin for 10 min, and washed with water. Eosin staining was re-dyed for 3 min, and the slices were sealed with neutral resin and observed under a microscope.

Western Blot

RIPA lysate was used to lyse the tissue and extract the supernatant by centrifugation; the protein concentration was determined by the BCA method; the same amount of protein sample (10 μl per well) was loaded, and the wavelength of the microplate reader was set at 562 nm for measurement. After electrophoresis, the target protein was transferred to PVDF membranes washed with TBST; and 5% skim milk was used to block at room temperature for 1 h. The primary antibody (Beclin1) was diluted 1:1,000, incubated for 1.5 h, and washed with TBST three times for 5 min each, and the secondary antibody was diluted 1:1,000, incubated at room temperature for 1.5 h, and washed with TBST four times for 5 min each. The ECL exposure solution was mixed according to a 1:1 (solution A:B) ratio, evenly covered the whole film, was reacted for 2 min, and was put into the exposure meter for exposure detection, and GAPDH was used as the internal reference for grayscale value comparison.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Fresh brain tissues were taken, RNA was extracted according to the instructions of the RNA extraction kit, reverse transcription to cDNA was carried out according to the instructions of the reverse transcription kit, and qPCR was performed using cDNA as the template. The reaction system is as follows: 2 × SYBR Mix 5 μl, 0.5 μl of each primer, 10 × cDNA 1 μl, and ddH2O 10 μl. The reaction parameters were set as follows: 50°C 2 min, 95°C 2 min, 95°C 15 s, and 60°C 1 min, for 40 cycles. The primer sequences are shown below. With GAPDH as the internal reference gene, the relative expression of Beclin1 mRNA was calculated according to the 2−ΔΔCt method. The primer sequences were as follows: hMOF, 5′-GAGCATGAGGCGATCACCA-3′ and 5′-CCCATAGTCCTCCGGGAAAG-3′; Sirt1, 5′-TGTCATAGGTTAGGTGGCGAGT-3′ and 5′-AGGTGTTGGTGGCAACTCTGAT-3′; Beclin1, 5′-CGAGTGTCAGAACTACAAACGCT-3′ and 5′-CTCCTCCTCCAAGGCCAACT-3′; and Rat-GAPDH, 5′-GGCAAGTTCAACGGCACAG-3′ and 5′-CGCCAGTAGACTCCACGACAT-3′.

ChIP-qPCR

Using 100–150 mg of rat brain tissue, follow the steps of the ChIP kit instructions. Briefly, first, use formaldehyde to perform DNA-protein cross-linking experiments; then perform nuclear preparation and chromatin fragmentation to obtain cross-linking chromatin preparation; the chromatin immunoprecipitation method was used; the chromatin is eluted from the antibody/Protein G Magnetic Beads and de-crosslinked. Finally, the spin column is purified, and DNA bound to the

antibody of interest is obtained. Quantify DNA by quantitative fluorescence PCR. The primer for the promoter region of Beclin1 gene is shown as follows: forward 5′-GGCGATGGGAACTCTG GA-3′ and reverse 5′-CCCCGACGCTCTTCACCT-3′; forward 5′-CGTCAAGGCGTCACTTCTGG-3′ and reverse 5′-ACCTC CAGAGTTCCCATCGC-3′; and forward 5′-CGGGCGATGGG AACTCTG-3′ and reverse 5′-CCCGACGCTCTTCACCTC-3′. Each value was normalized to the percentage of input DNA by using IP/INPUT = 2CtInput DNA – Ct IP DN (26).

Statistical Analysis

SPSS 22.0 was used for statistical analysis, data were expressed by $x \pm s$, t-test was used, comparison between multiple groups was done by one-way ANOVA, and $P < 0.05$ indicated a significant difference.

RESULTS

EA Improves Neurological Functions and Relieves Cerebral Infarction

As shown in **Figure 1**, the Longa test was used to evaluate the neurobehavioral function, and TTC staining was assessed to investigate cerebral infarction. Control group rats did not show any neurobehavioral impairment signs. In contrast, obvious white infarction areas were observed in the MCAO group (**Figure 2A**). As shown in **Figure 1**, the neurological scores of the MCAO+EA and MCAO+EA+hMOF siRNA groups were significantly decreased compared with those of the MCAO and MCAO+EA+Sirt1 inhibitor groups ($P < 0.01$). Simultaneously, when rats were treated with EA and hMOF siRNA, the infarct volume was obviously reduced compared to that in the MCAO group and MCAO+EA+Sirt1 inhibitor group (**Figures 2A,B**, $P < 0.05$). These results revealed that ischemia/reperfusion (I/R) and the Sirt1 inhibitor impaired neurologic function seriously, while the EA and hMOF inhibitor treatment alleviated it.

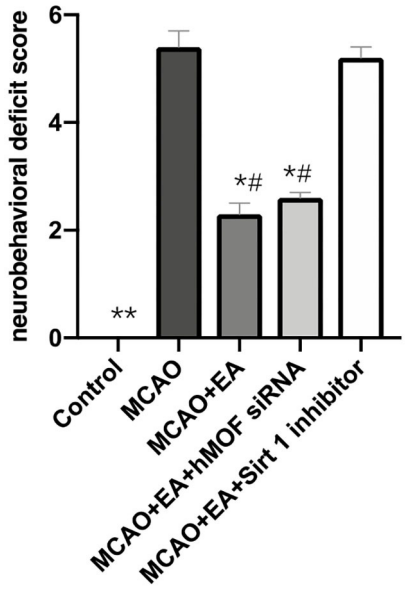

FIGURE 1 | Electroacupuncture improves neurological scores. Data are presented as means ± SEM; $n = 24$ rats per group, $^{*}P < 0.01$, $^{**}P < 0.001$, vs. the MCAO group; $^{\#}P < 0.01$, vs. the MCAO+EA+Sirt1 inhibitor group.

EA Reduces the Pathological Damage to Right Striatum Brain Tissues in MCAO Rats

Hematoxylin–eosin staining showed significant differences in histopathological changes of parietal cortex neurons in each group (**Figure 3**). In the control group, the right striatum neurons have a neat, tidy structure, abundant cytoplasm, clear nuclei, and seamless interstitial edema. The MCAO+EA group and MCAO+EA+hMOF siRNA group displayed a few incomplete cell structures, resulting in shrinkage. Compared with the MCAO+EA group and MCAO+EA+hMOF siRNA group, the MCAO group and MCAO+EA+Sirt1 inhibitor group exhibited neuronal cell disorder, cell shrinkage, nuclear fragmentation, disappearance of the nuclear area, and large vacuoles.

EA Alters hMOF, Sirt1, H4K16ac, LC3-II, and Beclin1 Expressions in the MCAO Rat Corpus Striatum

To test the involvement of autophagy in CIR injury, we investigated the protein expression of LC3-II and Beclin1, which are effective markers for detection of autophagy (**Figures 4E,F**). The results revealed that the administration of EA enhanced the I/R-induced increases in the level of LC3-II and Beclin1, confirming that EA resulted in increased autophagic flux (**Figures 4E,F**). Cotreatment with hMOF siRNA, which inhibited hMOF to decrease the acetylation status of H4K16, led to further increases in LC3-II and Beclin1 expressions. In contrast, remarkable decreases were detected upon EA and Sirt1 inhibitor treatments. To provide evidence that decreased apoptosis by autophagy induction was associated with acetylation of H4K16, the protein expressions of hMOF, Sirt1, and H4K16ac were detected by western blot (**Figures 4B–D**). Obviously, the observed upregulation of hMOF and H4K16ac combined with the downregulation of Sirt1 upon I/R was abrogated when rats were treated with EA. Besides, inhibition of hMOF repressed the acetylation of H4K16, while Sirt1 siRNA reversed it. These data revealed that treatment with EA decreased the acetylation status of H4K16. Collectively, the results showed that I/R is related to H4K16ac-mediated autophagy and that maybe the mechanism of EA alleviates CIR injury.

EA Effectively Regulates hMOF, Sirt1, and Beclin1 mRNA Expressions in the MCAO Rat Corpus Striatum

qRT-PCR was performed to analyze the mRNA expressions of hMOF, Sirt1, and Beclin1 in the rat corpus striatum. Expression analysis demonstrated that treatment with I/R significantly increased hMOF and Beclin1 mRNA expressions ($P < 0.05$, **Figures 5A,C**). EA intervention enhanced the increase in Sirt1 and Beclin1 mRNA expressions ($P < 0.05$). A combination with hMOF siRNA led to similar results. However, there was no

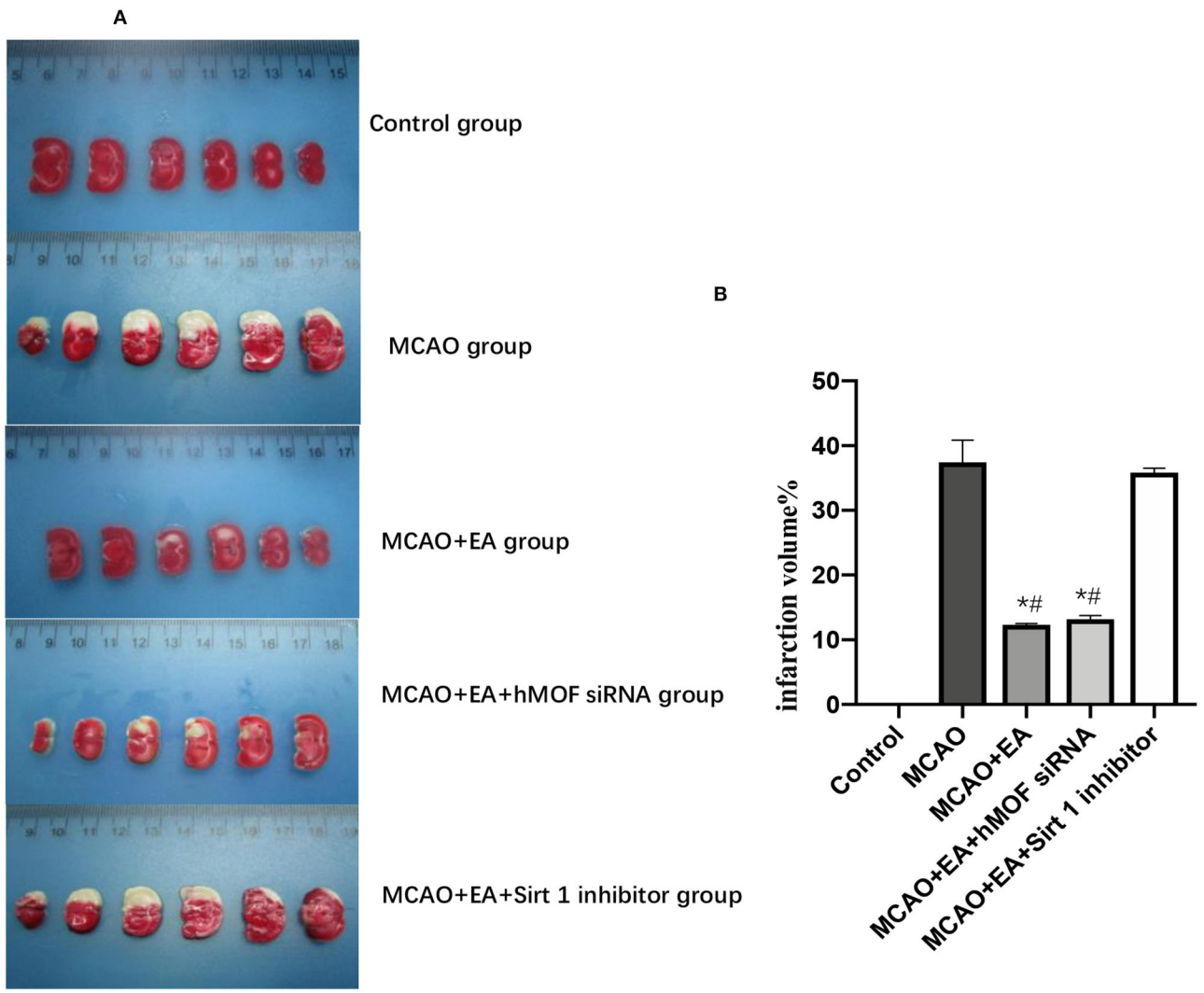

FIGURE 2 | Image showing changes of infarct volume. **(A)** Typical photo micrographs of ischemic penumbra identified with TTC staining. **(B)** Calculation results of infarct volume; $n = 6$ per group. $^{*}P < 0.05$, vs. the MCAO group; $^{\#}P < 0.05$, vs. the MCAO+EA+Sirt1 inhibitor group.

significant difference between the expression of hMOF mRNA in the MCAO+EA group compared with the MCAO group ($P > 0.05$). And EA cotreatment with the Sirt1 inhibitor inverted the tendency of the mRNAs of hMOF, Sirt1, and Beclin1, compared to EA treatment alone.

EA Treatment Prominently Augments H4K16ac Occupancy

To investigate whether EA regulated autophagy-induced transcription and epigenetic regulation, we detected the binding of H4K16ac on the three sites in the Beclin1 promoter. **Figure 6** shows the enrichment of H4K16ac chromatin on the three sites of the Beclin1 promoter. As shown, ChIP-qPCR assay increased the enrichment of the H4K16ac via EA treatment compared with the I/R condition ($P < 0.01$).

DISCUSSION

Ischemic stroke is one of the most ordinary and destructive stroke types, with high morbidity and mortality (27, 28). The occurrence and progress of ischemic stroke are caused by various factors (29). CIR injuries are often inevitable, while effective treatments for cerebral ischemia have not yet been discovered. Studies have confirmed that autophagy is involved in the regulation of various stages of cerebral ischemia (12–17). Autophagy refers to a catabolic process, which results in lysosomal degradation-dependent autophagy of large cytoplasmic contents and an excess of abnormal protein aggregates or damaged organelles. Autophagy is involved in various stages of cell growth, survival, and differentiation and has different assignments in anticancer, antiaging, antimicrobial defense, and neuroprotection. In summary, autophagy plays

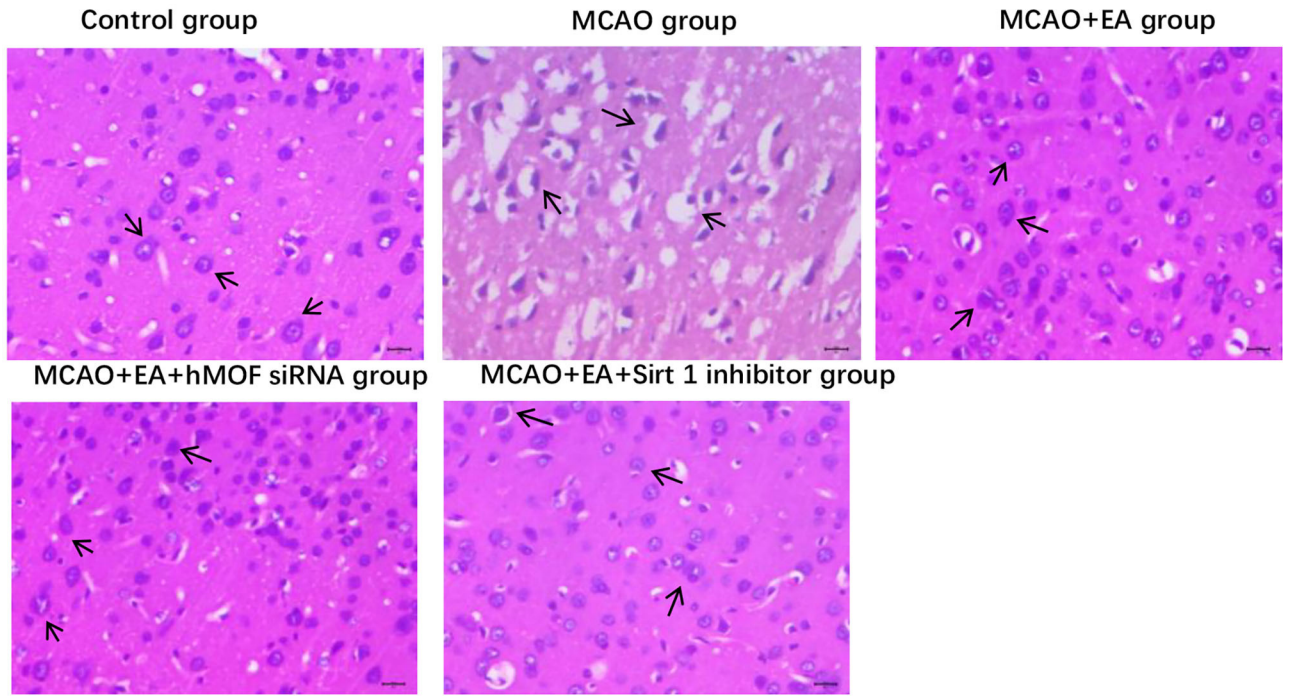

FIGURE 3 | The histopathological findings in the right striatum brain tissue, ×400.

multiple roles in cerebral ischemic progression, but whether it is beneficial or pernicious remains unclear.

More than 30 autophagy-associated (ATG) proteins have been identified in yeast, most of which are conserved in mammals. In mammalian cells, the ATG protein is necessary for the correct execution of autophagy procedures. Beclin1 plays a vital role in the initiation of autophagy, and it forms a trimer with PI3K and Atg14 to recruit autophagy-related proteins to mediate the initiation of autophagy (30). As an indispensable regulator of autophagy, Beclin1 dominates the process of autophagy by regulating the production of class III PI3K-dependent phosphatidylinositol 3-phosphate (PI3P) and subsequent supplementation of ATG protein that promotes the formation of autophagosomes (31–33). In the past few years, much insight has been gained into how the function of Beclin1 scaffolds is regulated. It is essential for making clear the ability of Beclin1-dependent autophagy or non-autophagy to manipulate processes.

LC3 formation is derived from the precursor LC3 through proteolysis or deionization of autophagy LC3-PE. Under the action of cysteine protease Atg4, the LC3 precursor was processed into soluble LC3-I, which was related to the formation of lipid-soluble LC3-II-PE under phosphatidylethanolamine (PE) action, which was associated with the extension of the cell membrane of the body until the appearance of autophagosome (34, 35). LC3-II is the only reliable marker protein associated with complete autophagosomes and is also localized to phagosomes. Therefore, LC3-II is currently the primary biochemical marker of autophagy (36). In this paper, we used the relative level of Beclin1 and LC3-II to indicate the autophagic flux. In the MCAO group, the expression of LC3-II and Beclin1 showed a rising tendency compared to the control group insignificantly ($P > 0.05$). EA treatment facilitated the level of these two proteins ulteriorly. These results indicated that EA increased autophagic flux to alleviate I/R.

Literature shows that I/R-induced autophagic activation is coupled with the acetylation of H4K16 (37–40). Researchers noted that a molecular histone switch exists, where the balancing effects of hMOF and SIRT1 on H4K16 acetylation regulate autophagy (23). Studies have shown that H4K16 acetylation is linked to the nuclear deacetylase Sirt1 accumulation, which antagonizes the enzymatic activity of hMOF. This leads to hMOF deacetylation and activation of the promoter region of related genes (40). That is, the acetylation of H4K16 depends on the counteraction of hMOF and Sirt1 (41, 42).

Acupuncture for ischemic stroke is recommended by the World Health Organization (43, 44). Accumulating reports confirm that EA could alleviate I/R damage (9, 10). An underlying interrelation between the protective mechanism of acupuncture on ischemic stroke and autophagy has been found (11). For instance, Liu et al. (45) suggested that EA activated the mTORC1-ULK complex–Beclin1 pathway to inhibit autophagosome formation to induce autophagic flux to alleviate I/R injury. Wang et al. (46) showed that EA triggered Pink1/Parkin-mediated mitophagy clearance to ameliorate CIR-induced neuronal injury. Our previous studies demonstrated that EA inhibited the level of autophagy-related protein LC3-II, suppressed CIR injury, and regulated the expressions of

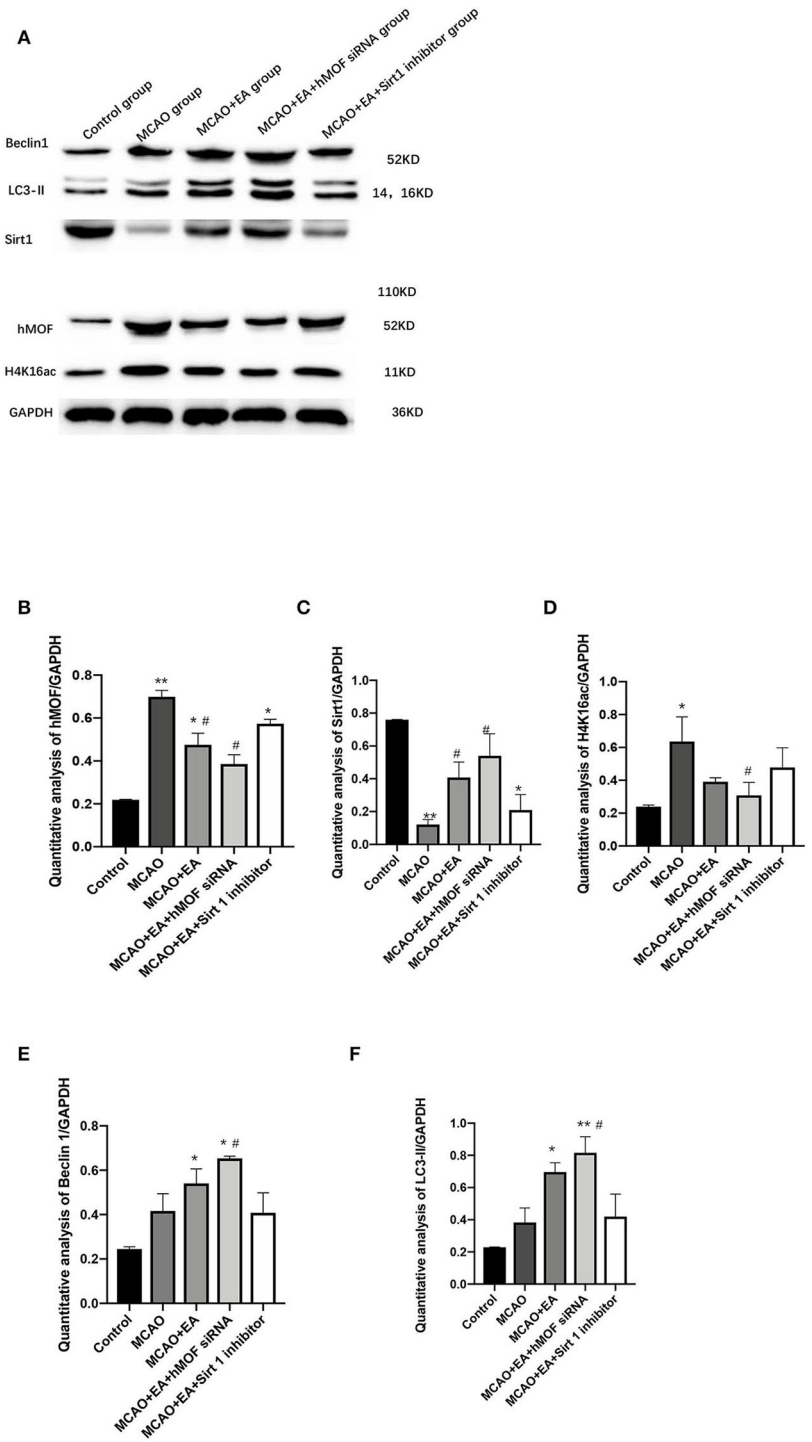

FIGURE 4 | Electroacupuncture alters hMOF, Sirt1, H4K16ac, LC3-II, and Beclin1 expressions in the MCAO rat corpus striatum. **(A)** Western blot results of Sirt1, Beclin1, LC3-II, hMOF, H4K16ac, and GAPDH. **(B)** Quantification analysis of hMOF. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. the control group; $^{\#}P < 0.05$, vs. the MCAO group. **(C)** Quantification analysis of Sirt1. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. the control group; $^{\#}P < 0.05$, vs. the MCAO group. **(D)** Quantification analysis of H4K16ac. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. the control group; $^{\#}P < 0.05$, vs. the MCAO group. **(E)** Quantification analysis of Beclin1. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, vs. the control group; $^{\#}P < 0.05$, vs. the MCAO group. **(F)** Quantification analysis of LC3-II values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. the MCAO group.

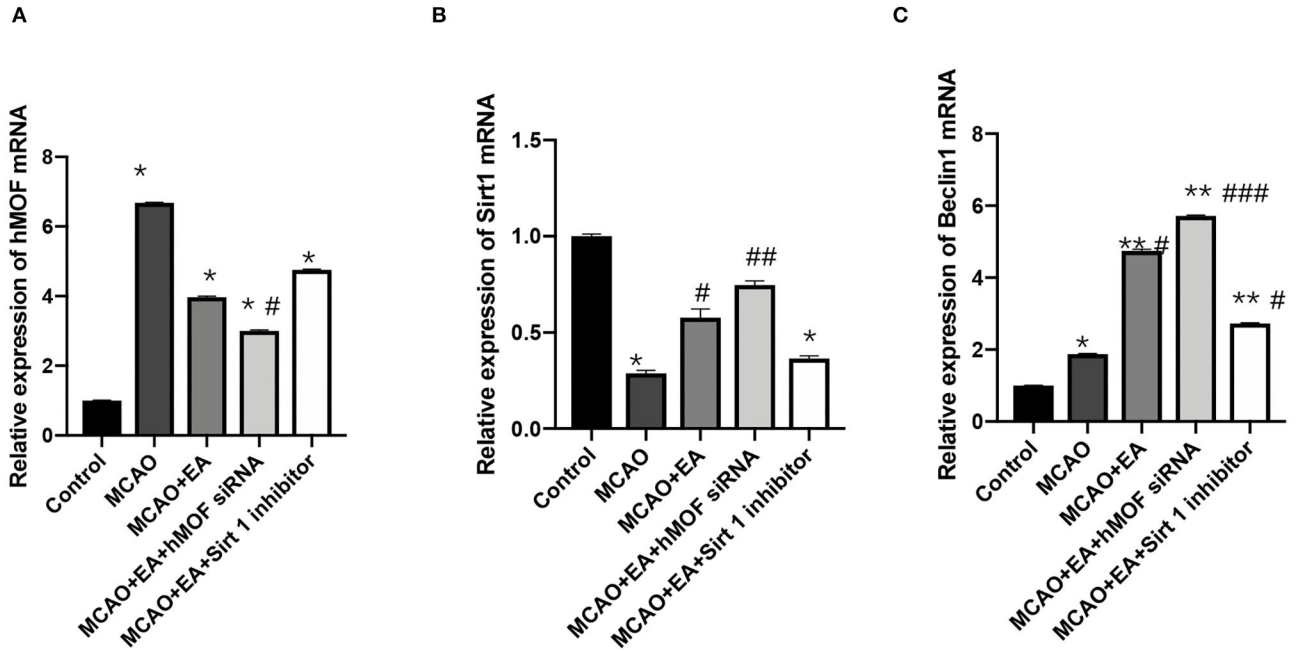

FIGURE 5 | Electroacupuncture effectively regulated hMOF, Sirt1, and Beclin1 mRNA expressions in the MCAO rat corpus striatum. **(A)** Relative expression of hMOF mRNA. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.001$, vs. the control group; $^{\#}P < 0.05$, vs. the MCAO group. **(B)** Relative expression of Sirt1 mRNA. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. the MCAO group. **(C)** Relative expression of Beclin1 mRNA. Values are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.01$, $^{**}P < 0.001$, vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, vs. the MCAO group.

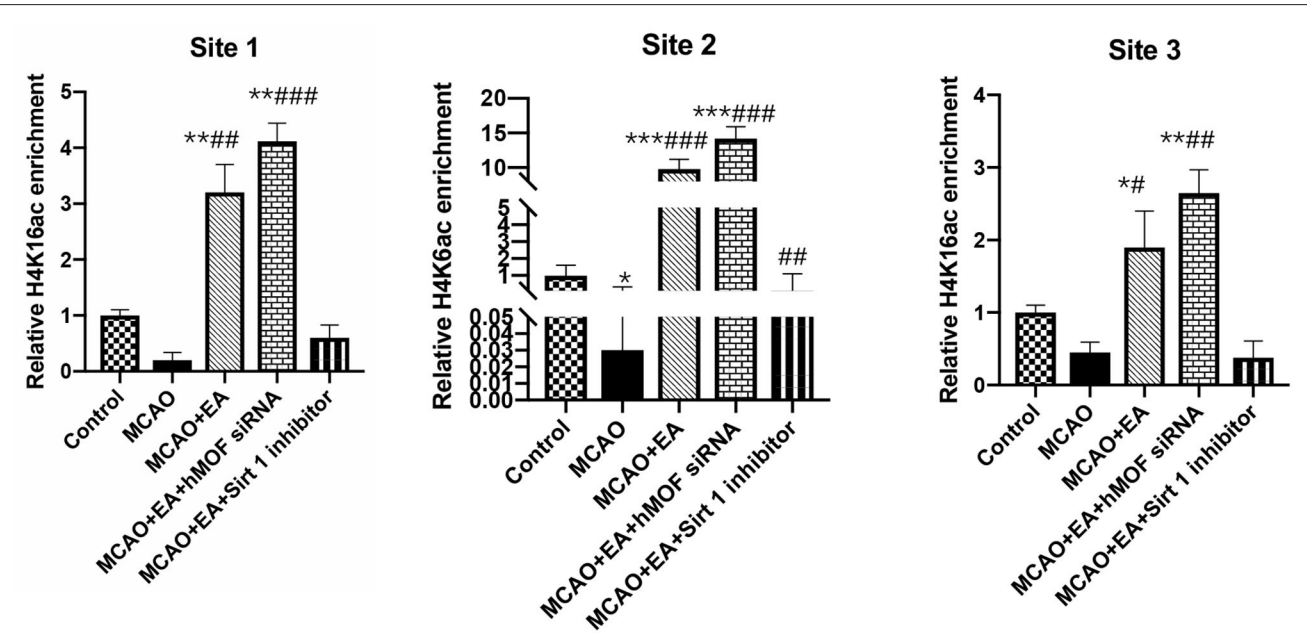

FIGURE 6 | ChIP analysis of the enrichment of acetylated H4K16 on the Beclin1 promoter. ChIP quantitative analysis of extracted chromatin from the brains of each group and precipitated with H4K16ac antibody. Quantitative PCR was used to amplify the Beclin1 promoter region. The following formula was used to normalize the input results and nonspecific IgG results: [2 – (ΔCt) specific antibody/2 – (ΔCt) nonspecific IgG], where ΔCp is the Cp (immunoprecipitated DNA) – Cp(input) and Cp is the cycle where the threshold is crossed. The position at the top of each figure shows three different transcription positions of the Beclin1 promoter region. Data were expressed as means ± SD; $n = 6$, $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$, vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the MCAO group.

histones H3K9 and H4K16 (47, 48). However, it remains elusive whether histone H4 lysine 16 acetylation plays a regulatory role in EA-induced suppression of I/R injury. Here, we showed that EA triggered the molecular histone switch of acetyltransferase (HATs) hMOF and histone deacetylase (HDACs) SIRT1 to reduce acetylation of H4K16 and facilitated MCAO-induced autophagy.

CONCLUSION

To sum up, we hypothesize that regulating histone H4 lysine 16 acetylation-mediated autophagy may be a key mechanism of EA at Du20 and Du26 to treat I/R damage. EA treatment inhibited the H4K16ac process to facilitate autophagy and alleviated I/R injury in the end. However, there are some defects in this study as the lateral ventricular injection injury has not been excluded because of the limited number of groups, and we did not demonstrate this mechanism *in vitro*. Besides, the results showed that MCAO induced a small increase of autophagic flux, while EA treatment enhanced autophagy progress. These data indicated that the role of autophagy might vary with its level. The most appropriate autophagy flux to promote neurological function recovery should be focused on in the future.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the First Affiliated Hospital of the Medical College at Nanjing traditional Chinese medicine University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

S-YX has made contribution to the conception and design of this work and taken part in the experiment studies. H-QL has done the experiment studies. W-QL has done data analysis and statistical analysis. HH has done the literature research. Y-JP and B-MZ have provided guidance throughout this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.576539/full#supplementary-material

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Wfs1 and Related Molecules as Key Candidate Genes in the Hippocampus of Depression

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Background: Depression is a prevalent mental disorder, which is difficult to diagnose and treat due to its unclear pathogenic mechanisms. The discovery of novel and effective therapeutic targets for depression is urgently needed. The hippocampus is a crucial region involved in depression and has been a therapeutic target for many antidepressants. Thus, it is beneficial for comprehensive research to be carried out on the molecular mechanisms of the hippocampus involved in the pathogenesis of depression. This study aims to investigate the differentially expressed genes (DEG) in the hippocampus in a chronic unpredictable mild stress (CUMS) mouse model.

Method: The study obtained GSE84183 from the GEO database. The R language screened the differential expression genes (DEG) in the hippocampus tissue of depressed mice, and the enrichment pathways of DEGs were analyzed. A protein-protein interaction (PPI) network was constructed in the STRING database and visualized in Cytoscape software. MicroRNAs for these DEGs were obtained from TarBase and mortar base databases, and transcription factors (TF) related to DEG were predicted from the ENCODE database. Both networks used the visual analysis platform NetworkAnalyst. Finally, the microRNA-TF network was integrated based on the above two networks and imported into Cytoscape for further analysis.

Results: This study screened 325 differentially expressed genes, containing 42 downregulated genes and 283 upregulated genes. Most of these genes are enriched in the cell cycle and the chemokine signaling pathway. Meanwhile, Wfs1, one of the top ten DEGs, was identified as the key regulator of the cell cycle and the participator in the highest number of modules screened out in PPI networks. Wfs1-related molecules, including UBTF, mmu-mir-17-5p, and mmu-mir-7b-5p, were therefore screened out. Furthermore, we confirmed the downregulation of Wfs1 and upregulation of UBTF/mmu-mir-17-5p/mmu-mir-7b-5p in the hippocampus of the CUMS mouse model. Our data indicate that Wfs1 and related molecules were predicted to be associated with the pathological process of depression. This research provided potential new molecular targets of stress-induced depression.

Keywords: depression, Hippcampus, Wfs1, CUMS, microRNAs

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INTRODUCTION

Depression is one of the most common and devastating neuropsychiatric diseases, and has become a severe health problem worldwide (Etkin et al., 2015). Studies have shown that about one-third of patients with depression are unresponsive to conventional antidepressant therapy (Rush et al., 2006). The development of new biomarkers for the treatment of depression is urgently required. A more in-depth exploration of the molecular mechanisms involved in the pathological process of depression may reveal new ways helps to combat this complex disease.

Depression is associated with smaller hippocampal volumes and changes in either the activation or connectivity of neural networks (Etkin et al., 2015; Ma et al., 2019). Depression also increases the level of inflammatory factors in the hippocampus (Fasick et al., 2015), downregulates BDNF (Krishnan and Nestler, 2008), and impairs synaptic plasticity (Otte et al., 2016), all of which contribute to the development and long-term pathophysiology of depression. These studies show that hippocampus dysfunction occurred in the neuropathology of depression.

Microarray technology can quickly screen out differential gene expression information related to depression. According to bioinformatics analysis, the functional pathway of differentially expressed genes (DEG) in the pathogenesis of depression can be analyzed. Herve et al. (2017) screened the gene expressions related to depressive behavior after chronic stress in the GSE84183 data set in 2017. It is worth noting that that study only paid attention to and studied the function of a select few of these genes. In order to obtain a more comprehensive understanding of the pathogenesis of depression, we used a variety of bioinformatics techniques to reanalyze some of the data in the GSE84183 data set. This study constructed a TF-DEG network and a microRNA-DEG network based on the DEG identified from CUMS samples and standard control samples. The regulatory association between TF and its target is obtained from the "Encyclopedia of DNA Elements" (ENCODE). The association between microRNA and its target is extracted from the TarBase and mortar base databases. These were then integrated into the network and the TF-microRNA core regulatory network was built, from which key microRNAs (mmu-mir-17-5p and mmu-mir-7b-5p) and TF (UBTF) in depression can be identified. The present study highlights and identifies Wfs1 and related molecules in the hippocampus as new potential targets for depression and improves our understanding of molecular mechanisms underlying depression pathophysiology.

MATERIALS AND METHODS

Microarray Data Data Set Collection and Identification of DEGs

The study obtained microarray expression data from the Gene Expression Omnibus[1]. Based on the Agilent GPL13912 platform (Agilent-028005 SurePrint G3 Mouse GE 8 × 60K Microarray), the exon expression profiling provided eight sham and eight CUMS hippocampus. The probes were converted to the corresponding gene symbols according to the annotation information of the raw data. To reduce multiple testing, each corresponded to a unique gene symbol. Only the probe set with the highest average expression was considered when multiple probe sets were associated with the same gene. The pre-treatment standardization on gene expression data for each experiment was performed using the R/Bioconductor Limma package. After the linear model fitting, the Bayesian linear model of the Limma package was estimated to identify DEGs. Statistically significant DEGs were defined with $P < 0.05$ and $|\log FC| > 1$ as a cut-off criterion in this study. Heatmap and volcano plot visualizations were performed using the R package "pheatmap" and "ggplot2," respectively.

Enrichment Analyses of DEGs

In this study, gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs was carried out using R. We also performed gene set enrichment analysis (GSEA) using the "clusterProfiler" package in R (Yu et al., 2012). All visualization was handled in R using the ggplot2 graphics package.

Metascape is a powerful enrichment analysis online tool[2], which can display the activation levels of biological pathways. Because the database is often updated, it has high credibility and accuracy, which helps bioinformatics analysis (Zhou et al., 2019).

Module Screening From the PPI Network

The comprehensive information of the proteins found by using STRING[3], a search tool for retrieving interacting genes/proteins, was used to evaluate protein-PPI information. Subsequently, a PPI network was constructed and visualized by Cytoscape software (version 3.7.1). Then, Molecular Complex Detection (MCODE) analysis, an APP of Cytoscape, was used to select the most significant PPI network modules. The criteria for selection were as follows: MCODE score > 3, degree cut-off = 2, node score cut-off = 0.2, and max depth = 100.

TF-DEG Network and MicroRNA-DEG Network Construction

NetworkAnalyst[4] is a comprehensive web-based tool for network-based visual analytics of comprehensive gene expression profiling and meta-analysis. All the identified DEGs were uploaded to NetworkAnalyst to obtain TF-gene and microRNA-gene interaction data. The generated list of datasets was exported to Cytoscape for further analysis.

TF-MicroRNA Synergistic Regulatory Network

The TF–miRNA regulatory network was built by integrating TF-target and miRNA-target, and the TF-target-miRNA network was

[1]https://www.ncbi.nlm.nih.gov/geo/

[2]http://metascape.org/gp/#/main/step1

[3]https://string-db.org/

[4]http://www.networkanalyst.ca/faces/home.xhtml

constructed using Cytoscape. The DERs that showed reciprocal changes in TF and microRNA were selected, and the microRNAs and TFs associated with them were extracted. Finally, the network was obtained and visualized in Cytoscape.

Animals and CUMS (Chronic Mild Unpredictable Stress, CMUS) Models

Adult male ICR mice (20–25 g) purchased from the Experimental Animal Center of Zhejiang Province (Hangzhou, China) were used in this study. The animals were housed in a temperature-controlled animal facility with a 12 h light-dark cycle. All procedures were approved by the Animal Care and Use Committee of Zhejiang University, following the guidelines for the Care and Use of Laboratory Animals by the National Institute of Health (NIH Publications No. 80-23).

Chronic unpredictable mild stress is a mouse model of depression in which animals are exposed to a random sequence of mild stressors and make a slight modification based on there already had studies (Jiang et al., 2019). Mice were subjected to 10 different stressors: tail pinching (3 min), electric shock to the sole (voltage 30 mV, 10 s/time, 1 min interval, 30 times in total), water deprivation (24 h), food deprivation (24 h), light-dark cycle reversal, hot environment (45°C, 5 min), swimming in cold water (4°C, 5 min), cage shake (15 min), wet bedding (24 h), cage tilt (24 h), and restraint (8 h). These stressors were performed every day in a random sequence.

Depression Behavioral Tests

Forced Swimming Test (FST)

The test was performed as previously described. Mice were individually placed into a glass cylinder (15 cm diameter, 30 cm height). The water depth was around 20 cm to prevent mice from touching the cylinder bottom with tails or limbs. The water temperature was maintained at 23–25°C. Black cardboard was placed between every two cylinders to minimize the interaction of mice. Each mouse was allowed to swim for 6 min. A digital camera videotaped all test sessions. The immobility time during the last 4 min was scored offline. Immobility time was defined as when the mice were motionless or passively floating on the water (only with movements necessary for keeping balance).

Sucrose Preference Test (SPT)

Mice were singly housed and habituated with one sucrose bottle (1%, w/v) and one bottle of water for 48 h (from day-2 to day-1). Bottle positions were switched after 24 h, and mice were then immediately water-deprived for 24 h. During the test session, mice were singly housed and exposed to one bottle of 1% sucrose and one bottle of water for 24 h. Bottle positions were switched after 12 h. Total consumption of each fluid was measured, and the sucrose preference was calculated according to the following ratio: sucrose preference (%) = [sucrose intake (g)/sucrose intake (g) + water intake (g)] × 100%.

Real-Time Polymerase Chain Reaction (RT-PCR)

The RT-PCR analysis was performed as previously described (Yang et al., 2019). Four weeks after CUMS, our control mice were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/kg i.p.) and sacrificed. Freshly isolated hippocampus tissues were collected on ice, immersed in TRIzol reagent (Invitrogen, Carlsbad, CA, United States), and immediately stored at −80°C until the time of RNA extraction. Complementary DNA (cDNA) synthesis was synthesized with the reverse transcription enzyme SuperScript II (Invitrogen, Carlsbad, CA, United States) together with the reverse transcription primer. The cDNA was then amplified using HiFiScript cDNA Synthesis Kit (CWBIO, Beijing, China) by ABI Q5 RT-PCR System (Applied Biosystems, Thermo Fisher, United States). All primers were synthesized by BGI Co., Ltd. (Shenzhen, China) (**Table 1**).

Data and Statistical Analysis

Data are presented as means ± SE. Analyses were performed using Microsoft Excel. Students' *t*-test tests were used for analyzing statistic differences, as indicated in Figure legends. $p < 0.05$ was considered as statistically significant.

TABLE 1 | Primer used in this study.

Gene	Primers	Sequence
GAPDH	Forward primers	AAGGTCGGTGTGAACGGATT
	Reverse primers	TGAACTTGCCGTGGGTAGAG
Wfs1	Forward primers	GGAAACTAACATGGCCCGGA
	Reverse primers	GTGATGGAGGGTCTTTGGGG
UBTF	Forward primers	ACAACCTCCCATCCAACGAC
	Reverse primers	TGTTGTGTTGACCCCTCCAC
U6	Forward primers	AGAGAAGATTAGCATGGCCCCTG
	Reverse primers	ATCCAGTGCAGGGTCCGAGG
mmu-mir-17-5p	Forward primers	ACACTCCAGCTGGGCAAAGTGCTTACAGTGCAG
	Reverse primers	CTCAACTGGTGTCGTGGA
mmu-mir-7b-5p	Forward primers	TCCAAGACATGTGATCCGTGTT
	Reverse primers	GGGTTACTCCCGTTTGGTTGT

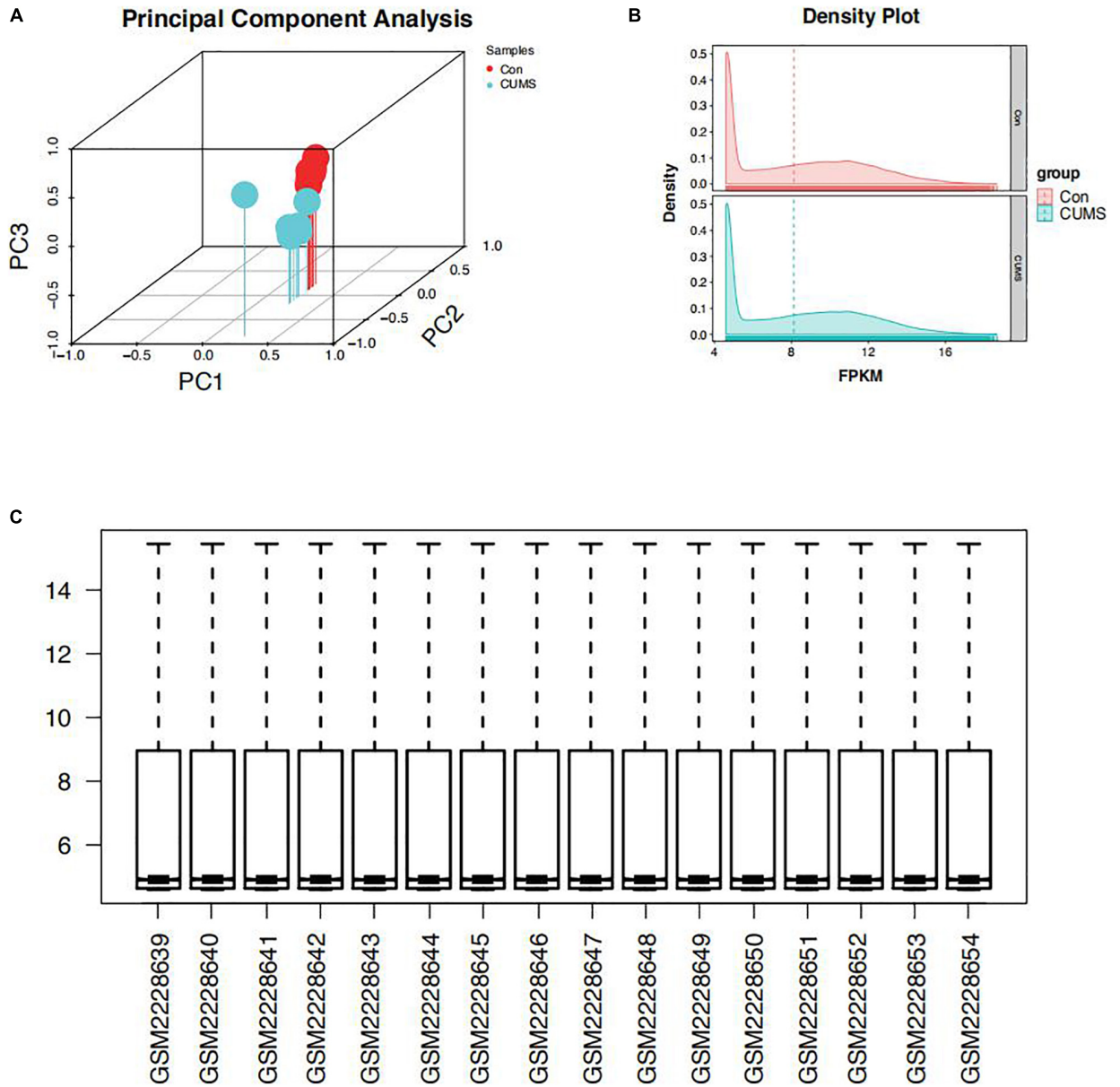

FIGURE 1 | Distribution features of the expression situation after normalization of all samples. **(A)** Principal component analysis (PCA) graph. **(B)** The density plots. **(C)** A box plot.

RESULTS

Data Normalization

The primary purpose of normalization is to eliminate technical and systematic variability from the data to compare different samples. After microarray data normalization, biological variability between different samples was assessed by plotting a principal component analysis (PCA) graph (**Figure 1A**). As can be noted, CUMS (n = 8) and Control (n = 8) overall had distinct, non-overlapping expression profiles. The density plots results demonstrated that the distributions of the samples' intensities were generally consistent and could be used for downstream analysis (**Figure 1B**). A box plot showed each sample's gene expression, and the black lines in the boxes were almost on the same straight line, indicating that the raw data were normalized successfully, which ensures the accuracy of the data (**Figure 1C**).

Identification of DEGs

Based on the Limma package in R language, $P < 0.01$ and $|\log FC| > 0.5$. A total of 325 DEGs were screened, including 283 upregulated and 42 downregulated DEGs. The volcano of DEGs is presented in **Figure 2A** and heatmaps plots in **Figure 2B**.

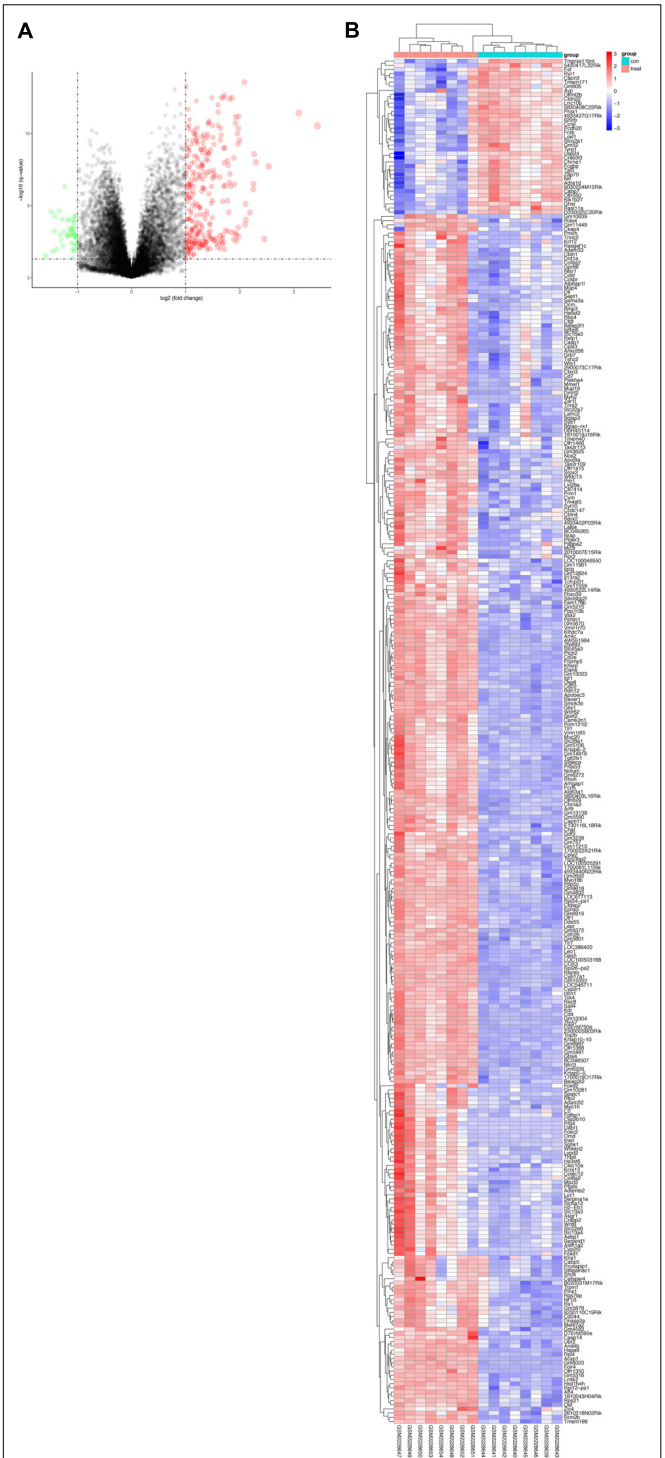

FIGURE 2 | The volcano plot **(A)** and clustering map **(B)** were generated using the R package "ggplot2." **(A)** Volcano Plot indicated upregulated and downregulated mRNAs of the hippocampus in CUMS model. Red represents the selected upregulated genes and green represents the selected downregulated genes. **(B)** RNA-seq comparing hippocampus from mice exposed to CUMS or control group. Eight biological replicates were used per condition for the hippocampus. Hierarchical cluster heatmaps of the hippocampus showing relative expression of genes across sham and CUMS samples.

Functional Enrichment Analysis of DEGs

To further investigate the function of DEGs, GO term, and KEGG pathway analyses were displayed in R. These DEGs were all divided into three major functional categories: biological processes (BP), molecular functions (MF), and cellular components (CC). The data collectively showed that DEGs were largely involved in circadian rhythm, cytokine biosynthesis processes, and prostaglandin biosynthesis processes (**Figure 3A** and **Table 2**). As for the molecular function (MF) group, DEGs were mainly enriched in endopeptidase activity (**Figure 3B**). In the CC analysis, the DEGs were predominantly enriched in the extracellular matrix receptor complex (**Figure 3C**). In these candidate DEGs, 14 signaling pathways were enriched in the KEGG database, such as Cell cycle, Oocyte meiosis, and Cellular senescence (**Figure 3D**).

Moreover, we used more than one kind of enrichment analysis to ensure the accuracy and reliability of the data analysis. Therefore, 325 differentially expressed genes were uploaded to the Metascape for standardized pathway and molecular function analysis. Biological processes (BP) and KEGG enrichment results show that DEGs were mainly enriched in rhythmic behavior, negative regulation of interleukin-1 beta production, and cell apoptotic pathways (**Supplementary Figure 1**). The enrichment results of meta scope are consistent with the R language. Also, we performed GSEA analysis for all genes on the microarray. The significantly enriched gene sets were set at a default cut-off as P-value < 0.05 and FDR < 0.25. Furthermore, the consequences of GSEA analysis suggested that the expression profiles of CUMS model were primarily enriched in "interferon-alpha response," "coagulation," "interferon-gamma response," and "epithelial-mesenchymal transition" (**Figure 3E**).

PPI Network and Module Analysis

To investigate the relationship between protein interaction by those DEGs, the PPI network was constructed in a STRING (V11.0), which consisted of 245 edges and 174 nodes. The STRING analysis showed that a total of 174 genes were filtered into the DEGs PPI network complex. The network was visualized using the software tool Cytoscape (**Figure 4A**). Moreover, eight significant models were screened out from the PPI network according to the module analysis by MCODE in Cytoscape (**Table 3**). They screened out the modules with the highest MCODE analysis scores, including Serpind1, Ckap4, Wfs1, Notum, Serpina1e, and Apol9a (**Figure 4A**). Furthermore, we found that Wfs1 was overlapping within the top 10 DEGs (Cplx2, COX3, Ptgds, Hspa8, Rgs7bp, Raver1, Gm4832, Rpl4, Mettl7a2, and Wfs1). We compared the normal hippocampus on the 4 weeks with the treated group to verify the screened genes in real-time PCR. This was the most obvious time point for depressive behavior (**Figure 4B**). Consistent with our bioinformatics results, the expression of Wfs1 decreased significantly after stress (**Figure 4B**). Due to differences in the laboratory environment, experimental details, and different strains of mice, the final sacrifice time was determined by

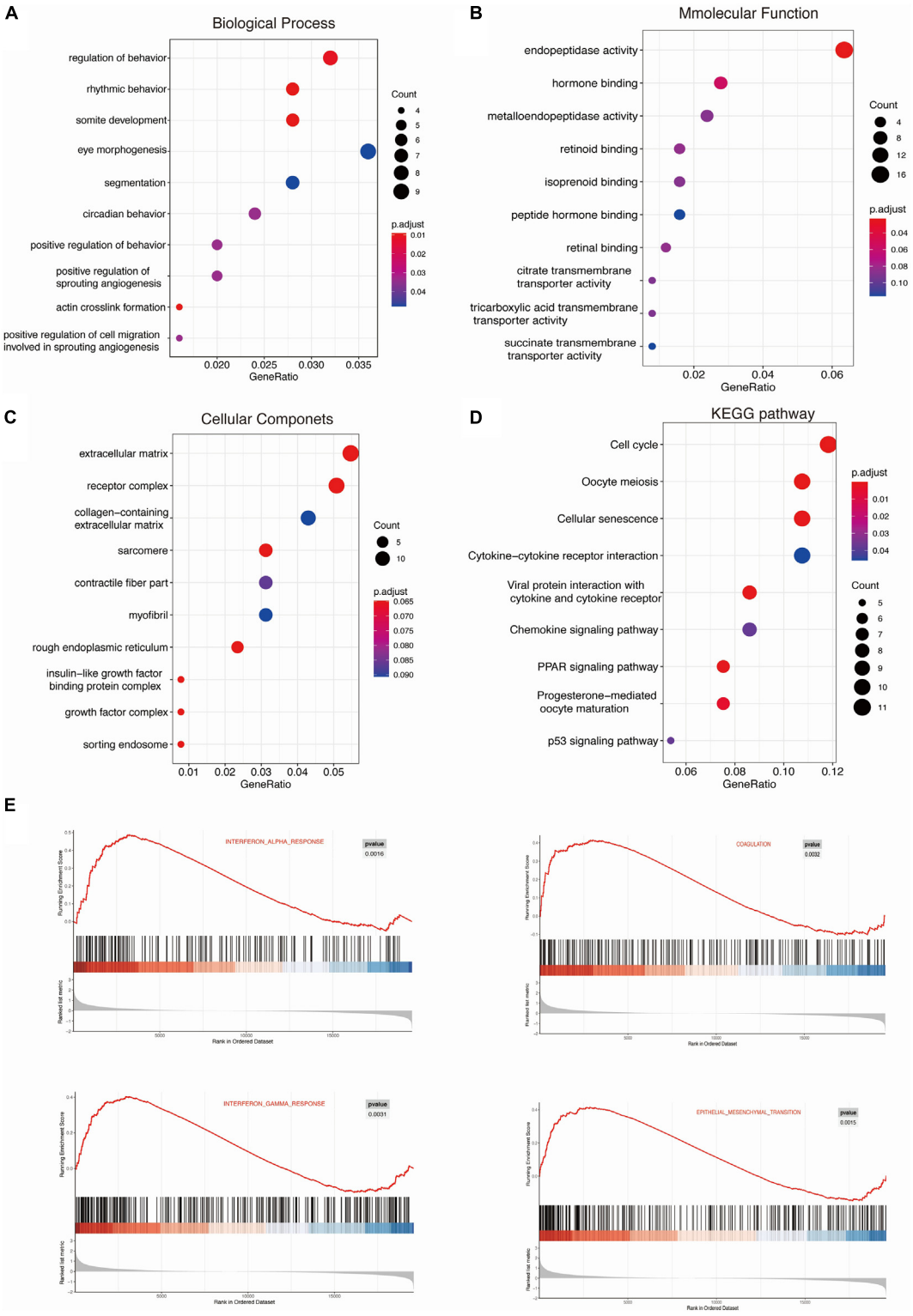

FIGURE 3 | KEGG and GSEA enrichment analysis of the DEGs were performed using ClusterProfiler. Gene ontology (GO) analysis for CUMS models DEGs: Biological Processes (BP), Molecular Function (MF), and Cellular Components (CC) **(A–C)**. **(D)** The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment of DEGs. **(E)** h.all.v 6.2.symbols.gmt [Hallmarks] gene set database was used to analyze the whole gene expression value of the PTB and HC samples. Significant gene sets were cut-off by FDR < 0.25 and P-value < 0.05.

TABLE 2 | Top 10 biological process of DEGs.

Term	Genes	*P* value
GO: 0050795 ~ circadian rhythm	Lepr/Ptger3/Mc3r/Ghsr/Pmch/Ptgds/Casp1/Cckbr	9.60E-06
GO: 0007622 ~ cytokine biosynthesis processes	Lepr/Chat/Ptger3/Mc3r/Pmch/Ptgds/Casp1	5.17E-06
GO: 0061053 ~ prostaglandin biosynthesis processes	Gdf3/Frzb/Ror2/Foxc2/Dmrt2/Cobl/Aldh1a2	7.71E-05
GO: 0048592 ~ synaptic vesicle fusion on the regulation of presynaptic active zone membrane	Kdr/Vax2/Hcn1/Trpm1/Rs1/Prox1/Rbp4/Nrl/Col8a2	0.000130269
GO:0030335~segmentation	Gdf3/Sema3a/Ror2/Foxc2/Dmrt2/Cobl/Aldh1a2	7.88E-13
GO: 0048512 ~circadian behavior	Lepr/Ptger3/Mc3r/Pmch/Ptgds/Casp1	5.02E-05
GO: 0048520 ~positive regulation of behavior	Ptger3/Ghsr/Pmch/Casp1/Cckbr	6.79E-05
GO:0045087~ positive regulation of sprouting angiogenesis	Kdr/Srpx2/Ghsr/Foxc2/Fgfbp1	2.01E-09
GO: 0051764 ~actin crosslink formation	Baiap2l2/Tnnt2/Baiap2l1/Cobl	4.11E-06
GO: 0090050 positive regulation of cell migration involved in sprouting angiogenesis	Kdr/Srpx2/Foxc2/Fgfbp1	6.85E-05

the depression phenotype of the mice. The asterisk indicates significant statistical difference ($P < 0.05$), four symbols $p < 0.0001$.

Construction of the TF-DEG- MicroRNA Network Analysis

To further investigate the functional roles of DEGs, the potential regulatory relationships between DEGs and TFs were screened according to TF binding site data, and genetic coordinate position information provided on ENCODE (**Supplementary Figure 2**).

Next, we identified microRNA-DEG pairs through network analysis of 325 DEGs using the TarBase and miRTarBase databases, and five large-pairing pictures were generated (**Supplementary Figure 3**). As a result, a total of 436 associations between 257 microRNAs and only 71 DEGs were found.

The co-regulated 20 DEGs modulated by microRNA and TFs were selected, and their related regulators were extracted, and then a TF-microRNA-interacted network in Cytoscape was constructed (**Figure 5A**). This network included 22 DEGs, 26 TFs, and 62 microRNAs, with 163 associations. We separately analyzed the degree of these 20 DEGs in the TF-DEG network and the microRNA-DEG network. Then, we found that Wfs1 regulated four TF (UBTF, ELF1, TBP, and MAZ) and directly regulated five miRNAs (mmu-mir-17-5p, mmu-mir-362-3p, mmu-mir-329-3p, mmu-mir-7b-5p, and mmu-mir-466i-3p) (**Table 4**).

Verification of Potential Experimental Target Expression by qRT-PCR

Four TF (UBTF, ELF1, TBP, and MAZ) and five miRNAs (mmu-mir-17-5p, mmu-mir-362-3p, mmu-mir-329-3p, mmu-mir-7b-5p, and mmu-mir-466i-3p) were verified, and mmu-mir-17-5p and mmu-mir-7b-5p were found to be highly reliable (cross-links $\geq$2) when targeting Wfs1. Then, using qRT-PCR analysis, selected molecules, including mmu-mir-17-5p, mmu-mir-7b-5p, and TF, were verified in the hippocampus (**Figure 5B**). Consistent with the prediction, the results showed that the expression levels of mmu-mir-17-5p, mmu-mir-7b-5p, and UBTF in the CUMS group were significantly higher than control mice. There was no significant difference in ELF1, TBP, and MAZ expression compared with the control group (Data not shown).

DISCUSSION

Depression is a complex neuropsychiatric disorder and alters multiple gene expression/signal cascades in the hippocampal region (Zandio et al., 2002). In this study, we screened 325 DEGs in the hippocampi of mice receiving chronic unpredictable stimulation. Based on a series of bioinformatics analyses, we identified Wfs1 and its related molecules as potential genetic targets for depression.

We screened out 325 differential genes and analyzed these DEGs through a variety of bioinformatics analysis methods. The results indicate that the GO terminology of 325 DEGs categorizes the depression-related biological processes as circadian rhythm, cytokine biosynthesis processes, and prostaglandin biosynthesis processes. It has been reported that the hippocampus regulates REM sleep disorders in depression. Chronic unpredictable stimulation will increase REM sleep time and downregulate the hippocampus and other brain regions (Lustberg and Reynolds, 2000; Zandio et al., 2002). A great amount of evidence supports the correlation between the upregulated expression of inflammatory cytokines in the hippocampus and depression (Makhija and Karunakaran, 2013; Song et al., 2018; Wang et al., 2018). KEGG pathway enrichment analysis shows that these DEGs are mainly related to the cell cycle, the interaction of cytokines and cytokine receptors, and the chemokine signaling pathways. The GSEA data is consistent with the above analysis results, and all genes are enriched in interferon-gamma alpha. Our findings suggest that the circadian rhythm, neuroinflammation, and cell cycle may play a vital role in the pathogenesis of depression.

Next, we constructed a PPI network to study DEG's interaction relationship and screened out the modules with the highest scores in MCODE analysis, including Serpind1, Ckap4, Wfs1, Notum, Serpina1e, and Apol9a. These genes are highly related to depression. Consistently, Serpind1 is considered a candidate gene for depression (Wang et al., 2019). We noticed that Wfs1 is the overlapping gene of the first ten differential genes. The qRT-PCR results also show that the expression of Wfs1 in the CUMS group is downregulated compared to the control group. The above results indicate the importance of this gene in depression.

Wfs1 is an ER membrane protein and expressed specifically in the adult mouse brain regions relevant to stress and depression,

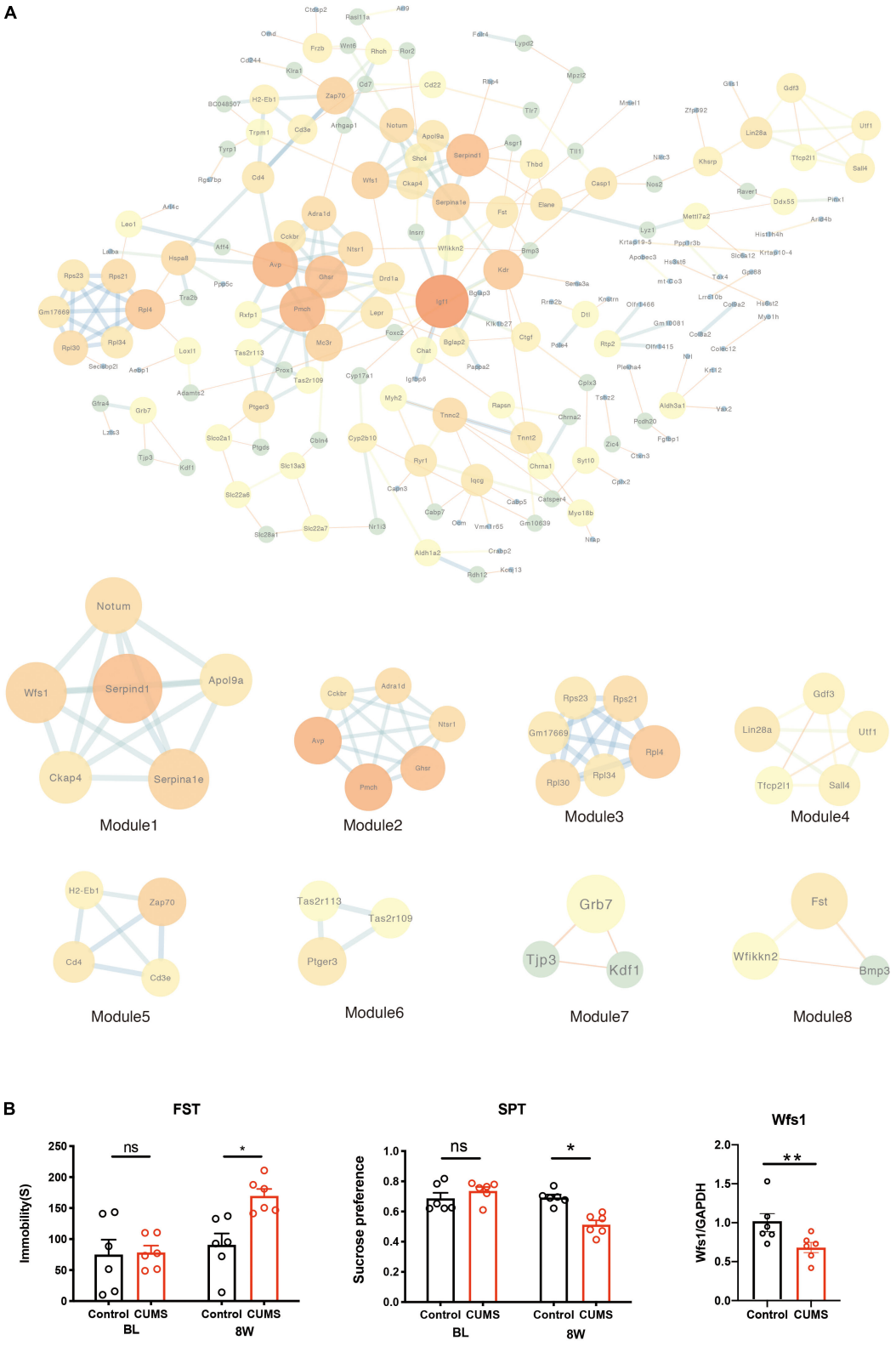

FIGURE 4 | PPI network of DEGs constructed by the STRING database. **(A)** The PPI network of DEGs was constructed using Cytoscape. Identification of a sub-network using MCODE in Cytoscape software. **(B)** Depression behavior after chronic mild unpredictable stress. CUMS increased the immobility time compared with control mice, and decreased the sucrose preference compared with control mice. Real-time PCR verified the downregulation of hippocampal Wfs1 gene expression after CUMS. one symbol $p < 0.05$, two symbols $p < 0.01$ ($n = 6$ mice/group).

TABLE 3 | Module analysis of DEGs using Cytoscape.

Cluster	Score	Nodes	Edges	Node IDs
1	6	6	15	Serpind1/Ckap4/Wfs1/Notum/Serpina1e/Apol9a
2	6	6	15	Ghsr/Ntsr1/Cckbr/Adra1d/Pmch/Avp
3	6	6	15	Gm17669/Rpl30/Rpl4/Rpl34/Rps21/Rps23
4	4.5	5	9	Gdf3/Lin28a/Sall4/Utf1/Tfcp2l1
5	4	4	6	H2-Eb1/Cd4/Cd3e/Zap70
6	3	3	3	Ptger3/Tas2r113/Tas2r109
7	3	3	3	Kdf1/Tjp3/Grb7
8	3	3	3	Bmp3/Wfikkn2/Fst
9	3	3	3	Rapsn/Chrna1/Chrna2

TABLE 4 | Co-DEGs regulated by TF and miRNAs.

TF	DEGs	Gene counts	miRNA	DEGs	Gene counts
UBTF	Arid4b, Aff4, Raver1, Wfs1, Dtl, Camk2n1	6	mmu-mir-17-5p	Wfs1, Camk2n1, Aff4	3
ELF1	Aff4, Utf1, Raver1, Wfs1, Ckap4	5	mmu-let-7b-5p	Ptgds, Khsrp, Arhgap1	3
CHD2	Arhgap1, Khsrp, Dtl, Lin28a, Sall4	5	mmu-mir-362-3p	Lin28a, Wfs1, Igf1	3
HCFC1	Prox1, Raver1, Aff4, Ror2, Cbln1	5	mmu-mir-329-3p	Lin28a, Wfs1, Igf1	3
TBP	Aff4, Raver1, Wfs1, Dtl	4	mmu-mir-3473c	Camk2n1, Ror2, Shc4	3
MXI1	Utf1, Raver1, Ckap4, Khsrp	4	mmu-mir-7b-5p	Camk2n1, Raver1, Ubr2	3
MAZ	Raver1, Wfs1, Ckap4	3	mmu-mir-19b-3p	Arid4b, Camk2n1, Aff4	3
E2F4	Wfs1, Dtl, Khsrp	3	mmu-mir-340-5p	Arid4b, Camk2n1, Aff4	3
BHLHE40	Ckap4, Utf1, Shc4	3	mmu-mir-30e-5p	Arid4b, Camk2n1, Aff4	3
ETS1	Igf1, Dtl, Raver1	3	mmu-mir-466f-3p	Wfs1, Arid4b	2
USF1	Khsrp, Ptgds, Nrl	3	mmu-mir-466i-3p	Wfs1, Igf1	2
SIN3A	E2F4, Igf1, Dtl	3	mmu-let-7c-5p	Sall4, Lin28a	2
TCF12	Aff4, TCF12	2	mmu-mir-124-3p	Cbln1, Prox1	2
MYC	Utf1, Raver1	2	mmu-mir-26a-5p	Shc4, Ckap4	2
MYB	Utf1, Raver1	2	mmu-mir-9-5p	Ptgds, Ror2	2
ZMIZ1	Utf1, Raver1	2	mmu-mir-181a-5p	Aff4, Prox1	2
MEF2A	Ubr2, Shc4	2	mmu-mir-758-3p	Khsrp, Arid4b	2
KAT2A	Dtl, Raver1	2	mmu-mir-15a-5p	Igf1, Cbln1	2
GATA1	Raver1, Aff4	2	mmu-mir-149-5p	Cbln1, Arid4b	2
RCOR1	Raver1, Dtl	2	mmu-mir-301b-3p	Camk2n1, Aff4	2

including superficial layers of the cerebral cortex, the central extended area of the amygdala, and the hippocampus (Shrestha et al., 2015). Under external stimuli, Wfs1 knockout mice show stress responses. Locomotor activity of Wfs1-deficient mice was significantly lower only in a brightly lit environment. Short-term isolation had a significant anxiogenic-like effect on the behavior of Wfs1-deficient mice in a dark/light exploration test (Kato et al., 2008). To assess the regional specificity of Wfs1 loss in the mice, Shrestha et al. (2015) studied conditional knockout Wfs1 from forebrain neurons. Exposure of Wfs1 CKO (mPFC) mice to ARS (acute restraint stress) resulted specifically in enhanced responses to stress, including increased immobility in the FST and a suppressed preference for sucrose, and was accompanied by hyperactivation of the HPA axis and elevation of serum corticosterone (Shrestha et al., 2015). It should be noted that the depression model used in this study was ARS, an acute stress-induced depressive state. Here, we have reported the change of the Wfs1 mRNA level in the CUMS model, suggesting that Wfs1 is involved in the early state and the long-term development of depression. The present analysis supports a possible contribution of hippocampal Wfs1 to depression in the hippocampus is in its beginning stages. However, the detailed mechanism of Wfs1 in depression requires further investigations.

Transcription factors and microRNA have been proven to be important regulators in developing depression (Carlezon et al., 2005; Gururajan et al., 2016). Therefore, we constructed a TF-microRNA interaction regulatory network to predict the potential interaction of TF, microRNA, and DEGs in depression. UBTF is connected to the six target genes of the TF node (Arid4b, Aff4, Raver1, Wfs1, Dtl, and Camk2n1). Screening by qRT-PCR found that the expression of UBTF in the CUMS group was upregulated compared to the control group, suggesting a correlation in the pathogenesis of depression. UBTF can activate ribosomal RNA (rRNA) transcription in nucleoli (Sanij and Hannan, 2009; Pederson, 2011). Induction of rRNA and other nucleolar activities play a role in normal neural processes, such as neurite outgrowth and memory consolidation during spatial

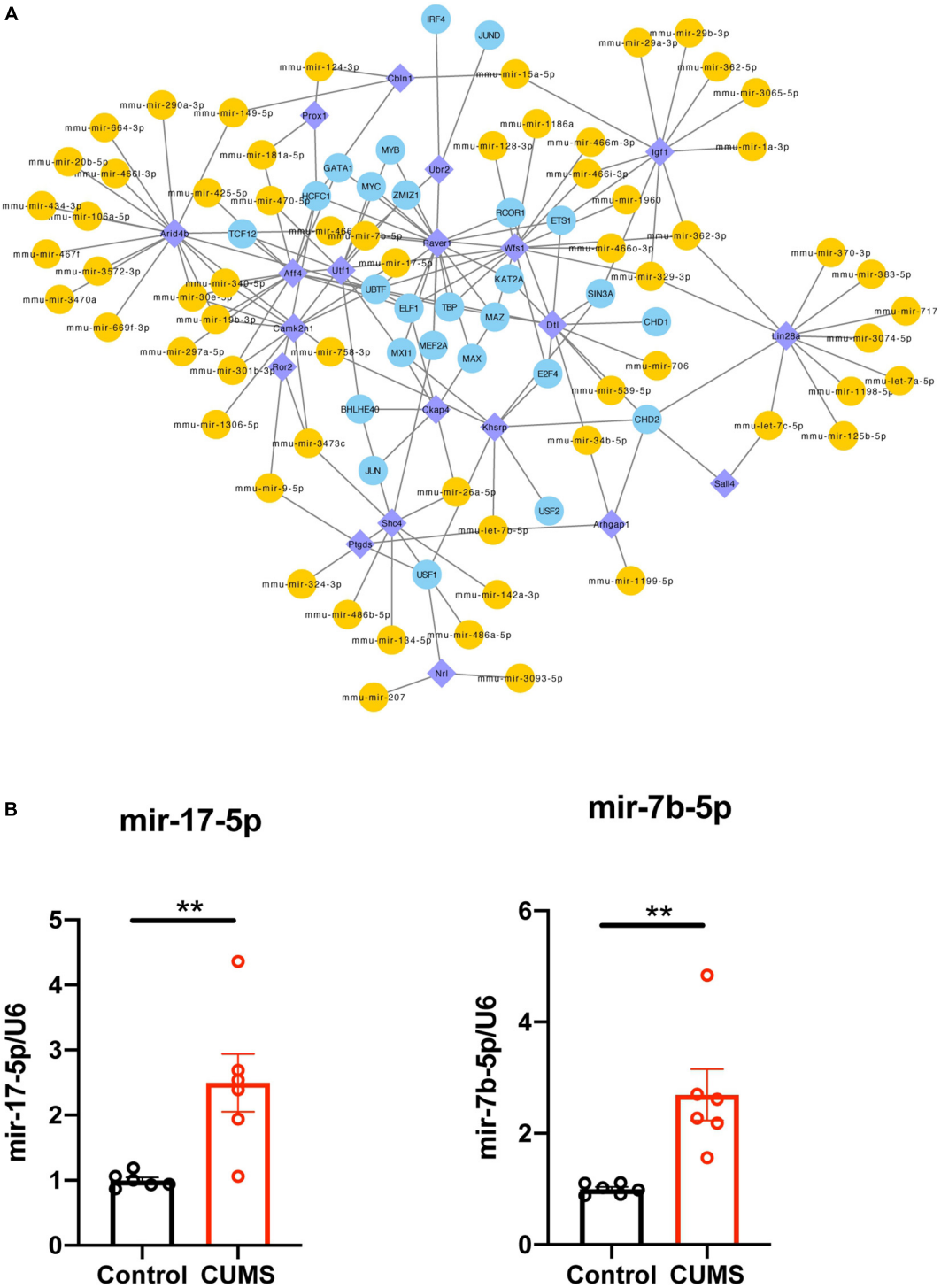

FIGURE 5 | (A) Integrative regulatory network of TF-DEG-miRNA, DEG, differentially expressed gene; miRNA, microRNA; TF, transcription factor. **(B)** mRNA expression level of UBTF, mmu-mir-17-5p and mmu-mir-7b-5p were upregulated in hippocampus after CUMS group compared with control group. one symbol $p < 0.05$, two symbols $p < 0.01$ ($n = 6$ mice/group).

training (Capitano et al., 2016). As shown in the GO terminology, the target genes of UBTF are mainly enriched in this terminology (GO: 0070997 ∼ neuron death; GO: 1901990 ∼ regulation of mitotic cell cycle phase transition), including Wfs1 and Dtl. Wfs1 is required for normal endoplasmic reticulum function, while Wfs1-deficiency increases endoplasmic reticulum stress,

impairs cell cycle progression, and triggers the apoptotic pathway, specifically in pancreatic beta-cells (Yamada et al., 2006). In response to ARS in Wfs1 CKO animals, downregulation of WNT7A in mPFC was observed (Yamada et al., 2006). WNT7A is a secreted signal transduction factor involved in axonal remodeling and synaptic differentiation (Ciani et al., 2011). These results suggest that Wfs1 may alleviate stress-induced depressive behaviors by regulating neuronal synaptic plasticity. Wfs1 and UBTF can participate in similar biological processes. Our results suggest that Wfs1 and UBTF may have the potential to be an anti-depressive drug target.

The miRNA is an endogenous non-coding RNA whose function is to degrade or inhibit the target gene's translation process, thereby regulating gene expression at the post-transcriptional level. In this study, we observed nine miRNAs, that targeted three DEGs. It is expected that Wfs1 can adjust mmu-mir-17-5p, mmu-mir-466f-3p, mmu-mir-466m-3p, mmu-mir-466o-3p, mmu-mir-466i-3p, mmu-mir-362-3p, mmu-mir-329-3p, mmu-mir-1960, mmu-mir-128-3p, and mmu-mir-1186a, and Dtl can adjust mmu-mir-539-5p, mmu-mir-34b-5p, and mmu-mir-706. Screening by qRT-PCR revealed that mmu-mir-17-5p and mmu-mir-7b-5p were upregulated. Although there is no current evidence to prove their direct role in depression, the target gene Wfs1 of mmu-mir-17-5p is considered a candidate genetic marker related to the relief of depression (Ferrua et al., 2019). Therefore, Wfs1/UBTF/mmu-mir-17-5 may play an important role in the pathogenesis of depression caused by long-term stress. The molecular mechanism of Wfs1 and its related molecules participating in depression need to be further investigated. Further in-depth studies of their mechanisms would contribute to a more precise diagnosis and efficient treatment of depressive disorder.

CONCLUSION

Our current study found that the cell cycle and the chemokine signaling pathway are important in stress-induced depression. Then, we identified Wfs1, which has significant relevance with the cell cycle biological processes, and screened out its related TF and miRNAs, including UBTF, mmu-mir-17-5p, and mmu-mir-7b-5p as potential experimental targets in the hippocampi of depressed mice. Further qRT-PCR results show that Wfs1 was down-regulated and UBTF, mmu-mir-17-5p, and mmu-mir-7b-5p were up-regulated in the CUMS group with healthy controls. In conclusion, Wfs1 and related molecules are key genes involved in depression. Further experiments are required to probe the functions of these genes in the pathogenesis of depression.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Zhejiang University.

AUTHOR CONTRIBUTIONS

JY and CC designed the experiments. LL performed the experiments. JY and XJ wrote the manuscript, analyzed the data, and collected the samples and delivered them. English has been professionally checked. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.589370/full#supplementary-material

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ORIGINAL RESEARCH
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Early-Life Neglect Alters Emotional and Cognitive Behavior in a Sex-Dependent Manner and Reduces Glutamatergic Neuronal Excitability in the Prefrontal Cortex

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Early-life neglect in critical developmental periods has been associated with emotional and cognitive consequences. Maternal separation (MS) has been commonly used as a rodent model to identify the developmental effects of child neglect. However, reports have shown considerable variability in behavioral results from MS studies in both mice and rats. Difficulties in developing reliable child neglect models have impeded advances in identifying the effects of early-life stress. Accumulating evidence shows that neuronal intrinsic excitability plays an important role in information processing and storage in the brain. The prefrontal cortex (PFC) integrates information from many cortical and subcortical structures. No studies to date have examined the impact of early-life stress on glutamatergic neuronal excitability in the PFC. This study aimed to develop a reliable child neglect rat model and observe glutamatergic neuronal excitability in the PFC. An MS with early weaning (MSEW) rat model was developed. Rats were separated from the dam for 4 h per day on postnatal days (PNDs) 2–5 and for 8 h per day on PNDs 6–16 and then weaned on PND 17. A battery of behavioral tests was used to assess anxiety-like behavior, coping behavior, working memory, spatial reference memory, and fear memory. The action potentials (APs) of glutamatergic neuronal membranes were recorded. MSEW resulted in anxiety-like behavior, a passive coping strategy and increased fear memory in male rats and decreased locomotor activity in both sexes. MSEW slightly impaired working memory during non-stressful situations in female rats but did not change spatial reference memory or associative learning under stressful circumstances in either sex. MSEW reduced the number of glutamatergic neuron APs in male rats. Our findings showed that MS with early weaning induced anxiety-like behavior in male rats. The reduced glutamatergic neuronal excitability may be associated with the emotional alteration induced by MSEW in male rats. In addition, MSEW induced adaptive modification, which depended on a non-stressful context.

Keywords: child neglect, maternal separation, learning and memory, anxiety like behavior, neuronal excitability, rats

INTRODUCTION

Child neglect is the most common form of early-life stress in both Western and Eastern countries (1–3). The number of left-behind children has been increasing dramatically in recent decades in China (4). The developing brain is particularly sensitive to early-life neglect. It is well-known that child neglect increases the risk for the development of many psychiatric disorders, including anxiety disorder, post-traumatic stress disorder (PTSD), and depression (5, 6). Considering the importance of mother–infant attachment in the early-life period, maternal separation (MS) is one of the most widely used models to elucidate the effects and neurobiological mechanisms of child neglect (7). However, due to the different MS paradigms and strains, the findings of the animal behavioral changes in both mice and rats are inconsistent. Some studies have reported that MS induces impaired memory (8), anxiety, and depressive behavior in rodents (9, 10). However, other studies observed paradoxical behavioral effects (11). Difficulties in developing reliable child neglect models have impeded advances in identifying early-life stress effects. The two- or three-hit stress model, which often induces a consistent behavioral phenotype, has been increasingly studied in recent years (12). Early weaning is another early-life neglect model used to replicate early-life adversities (13, 14). George et al. (15) developed a "two-hit" model that combined MS with early weaning (MSEW), which also elicited inconsistent behavioral outcomes in mice (15–17). The brain development of rats is different from that of mice, and the MS rat model seems to elicit more consistent stress-related alterations than that of mice (18). For example, MS causes a significant increase in defensive-exploratory behavior, a relatively conserved circuit between humans and rodents, in rats but not in mice (19). In the current study, we developed an MSEW rat model to observe long-lasting behavioral changes and provided a reliable animal model of child neglect.

Accumulating evidence shows that neuronal intrinsic excitability plays an important role in information processing and storage in the brain (20). A previous study reported the effect of stress on neural intrinsic excitability in the amygdala, which is involved in governing stress responsivity (21, 22). The prefrontal cortex (PFC) integrates information from many cortical and subcortical structures, including the ventral hippocampus, amygdala, and mediodorsal thalamus. There is growing evidence that the PFC is involved in the generation and regulation of complex cognitive functions such as emotional regulation, working memory, decision-making, planning, and reasoning (23). It is important to understand how early-life stress can influence neural excitability. Until now, the effect of early-life stress on neuronal excitability in the PFC has remained unclear. Here, we explored the possible influence of MSEW on glutamatergic neuronal excitability in the PFC in rats.

Abbreviations: MSEW, maternal separation with early weaning; MS, maternal separation; AP, action potential.

MATERIALS AND METHODS

Animals

Adult male and female Sprague–Dawley (SD) rats were purchased from Beijing HFK Bioscience Co., Ltd., and habituated to the animal facilities for 2 weeks. For breeding, a single male rat and two female rats were group-housed in cages for 10 days. Females were housed individually in the last week of pregnancy and observed for births, denoted as postnatal day (PND) 0. All animals were maintained under standard conditions (22–23°C with a 12/12-h light/dark cycle) and received standard food and tap water *ad-libitum*.

Maternal Separation With Early Weaning Protocol

The protocal was performed that described by George et al. (15). Entire litters were randomized to the control group (male, eight per group; female, 13 per group) or the MSEW group (male, nine per group; female, 13 per group) on PND 0. Control animals were left undisturbed and weaned at PND 21. The MSEW procedure was conducted as described previously (16). MSEW animals were separated from the dam for 4 h per day on PNDs 2–5 and for 8 h per day on PNDs 6–16 and then weaned on PND 17. Litters were placed individually in a separate room on a heating pad (32°C). After weaning, animals were group-housed with littermates of the same sex.

Behavioral Experiments

At the age of 3 months, the male and female offspring underwent a battery of behavioral tests. All experiments were conducted during the light phase of the cycle and in a temperature-controlled room (22–23°C, humidity was between 40 and 70%) with low-light intensity (10 lux). On the day of the experiment, rats were moved to the testing room, where they remained in their home cage for a 60-min acclimation period. The tests were completed in the order of the least to the most stressful. The open field test, novel object recognition (NOR) test, elevated plus maze test, Y maze, and Barnes maze were conducted using EthoVision video tracking software. Tests were conducted in the following order: (1) open field; (2) NOR test; (3) elevated plus maze; (4) Y maze; (5) Barnes maze; (6) forced swim test; and (7) fear conditioning. Testing areas were thoroughly cleaned with 75% alcohol solution between trials to remove any olfactory traces.

Open Field Test

The open field test was used to study spontaneous locomotor activity and anxiety behavior. The experimenters were blinded to the treatment of the rats. The open field (80 × 80 × 50 cm) was divided into a central zone and the surrounding border zone (15 cm from the wall). The rats were gently placed in the open field for 5 min of testing. The total distance moved, the distance moved in the border area, time in the center area, velocity, and number of fecal droppings were recorded and analyzed to evaluate locomotor activity and anxiety-related behavior. Thigmotaxis was assessed by the ratio of the distance moved in the border area to the total distance moved, expressed as a percentage.

Elevated Plus Maze Test

The elevated plus maze test was used to assess animal anxiety and exploratory behavior. The maze comprised two open arms (50 × 10 cm) and two closed arms (50 × 10 × 30 cm) with a central platform (10 × 10 cm). The maze was elevated 70 cm above the floor. The rat was placed in the central platform with its head facing an open arm for a 5-min test. The time spent on the open and closed arms, center area, and the distance moved were recorded. The head-dipping frequency was scored from the video by a trained observer who was blinded to the treatment of the rats. The percentage of time spent on the open arms (%OT = 100 × time spent on open arms/(time spent on open arms + time spent on closed arms) was calculated.

Novel Object Recognition Test

The NOR test was used here to study non-spatial memory. The test was performed in the same arena employed for the open field test. For habituation, the rat was placed into the open field without stimuli for 10 min for 2 days. For training, two identical objects were placed near the two corners of the open field (15 cm from each adjacent wall). The rat was placed into the center of the open field facing the opposite wall and was allowed 5 min for free exploration of the arena. The test began after a delay of 2 h, with the same object that was used in the training phase (familiar object) and a novel object set into the arena. The rat was placed into the arena and allowed to explore the objects for 3 min. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. The time that each rat interacted with the familiar object and the novel object was recorded. The total exploration time (e1) was calculated during the training session for two identical objects. The discrimination index (d1) was calculated as the time spent exploring the novel object minus the time spent exploring the familiar object. The discrimination ratio was calculated as the time spent exploring the novel object minus the time spent exploring the familiar object divided by the total exploration time.

Y Maze Novel Preference Arm Test

The Y maze novel preference arm test was used to study spatial working memory.

The Y maze consisted of three identical arms (50 × 10 × 30 cm) diverging at a 120° angle from one another. The rat was placed inside the start arm of the Y maze while the novel arm was closed. Then, the rat was allowed to explore the start and other arms but not the novel arm during the 5-min trial. After a 15-min delay, the rat was placed in the start arm and was allowed to explore all three arms for 5 min. The number of entries made into each arm was counted to determine spatial working memory. The percentage of entries into the novel or other arm was calculated as the number of entries into the novel or other arm divided by total entries into the three arms.

Barnes Maze Test

The Barnes maze test was used to assess hippocampus-dependent spatial learning and memory. The maze was a circular open platform (diameter: 120 cm, height: 65 cm) with 12 equally spaced holes (diameter: 10 cm) around the edges. The escape box (l × w × h: 25 × 6 × 6 cm) was under one of the holes. On the habituation day, the rat was placed under a start box in the center of the circular platform for 10 s. Then, the start box was removed, the buzzer was turned on, and the rat was motivated to escape and gently guided to the hole connected to the escape box (target hole). Immediately after the rat entered the escape box, the buzzer was turned off. Each rat was allowed to remain in the escape box for 1 min, removed, and then returned to the home cage. In the training phase, the rat was placed in the center of the platform and allowed to explore the maze for 3 min. The training tests consisted of three trials for 3 days. In the probe test, 24 h after the last training test, the escape box was removed. Each rat was given 3 min to explore the maze. Escape latency, distance, and velocity were recorded for later analysis.

Forced Swim Test

Immobility in the forced swim test is interpreted as an inability to actively cope with an aversive situation, and high immobility is believed to reflect increased depressive behavior. On the 1st day, rats were individually placed in a cylinder (diameter: 22 cm; height: 45 cm) filled with water (25°C, depth: 35 cm) for 15 min. Twenty-four hours later, rats were placed in the same cylinder again for 5 min. The climbing time and immobility time were recorded by a trained observer who was blinded to the treatment of the rats. Rats were dried and returned to their home cages after the test. Immobility was defined as the animal floating and making minimal movements necessary to keep its head above water. Climbing behavior was defined as upward-directed movement of the forepaws.

Contextual Fear Conditioning Test

Contextual fear conditioning was performed to assess hippocampus-dependent associative learning. For habituation, the rat was placed into the conditioning chamber without stimuli for 5 min. In the training phase, the rat was placed into the conditioning chamber (Med Associates, USA) for 3 min and then received five shock and tone pairs (30-s tone; 5 kHz; 70 dB; 1-s foot shock; 0.65 mA DC current) at an interval of 30 s. Contextual fear conditioning was measured 24 h after the training phase in the same chambers. The rat was placed into the same chamber, and no shock or tone was delivered. Freezing behavior was recorded for 5 min with specialized software (Video Freeze, Med Associates, USA).

Acute Slice Preparation and Electrophysiological Recording

Slice Preparation

At 3 days following behavioral testing, rats (n = 3–5 per group) were sacrificed under deep pentobarbital sodium anesthesia [50 mg/kg body weight, intraperitoneally (i.p.)], and their whole brains were rapidly dissected and submerged in ice-cold, oxygenated (95% O_2, 5% CO_2) cutting solution containing (in mM) 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 10 $MgSO_4$, 26 $NaHCO_3$, 10 glucose, and 230 sucrose, pH 7.4, 300–310 mOsm. Coronal slices (250 μm) containing the medial PFC were cut with a Leica VT1000S vibrating microtome (Leica Instruments,

Germany) and transferred to an incubation chamber with oxygenated, warm (32°C) regular artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 10 glucose, pH 7.4, 290–300 mOsm. Slices were then allowed to equilibrate for ∼1 h at room temperature.

Whole-Cell Recording

The excitability of glutamatergic neurons in the PFC was assessed following MSEW stress by recording the action potentials (APs) of glutamatergic neuronal membranes. After the recovery period, individual slices were placed in the submerged recording chamber, and the tissue was continuously perfused (2 ml/min) with ACSF. The recording chamber was placed on the fixed stage of an Olympus BX51 microscope (Olympus, Germany) equipped with video-enhanced infrared differential interference contrast. Whole-cell recordings were obtained from cortical neurons of medial PFC layer II/III. The patch pipettes were pulled from borosilicate glass capillary tubes (Sutter 150-86-10, USA) using a PC-10 pipette puller (Narishige, Japan). The resistance of pipettes varied between 5 and 8 MΩ when filled with a K^+ Met sulfonate intracellular solution containing (in mM) 140.5 K+ Met sulfonate, 7.5 NaCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) hemisodium salt, 2 Mg_ATP, and 0.2 Na_GTP, pH 7.33, 300–310 mOsm. Data were recorded using a Multiclamp 700B amplifier and a Digidata 1440A interface controlled by Clampex 10.6 (Molecular Devices, USA). Signals were digitized at 20 kHz and low-pass filtered at 10 kHz. Series resistance was on the order of 10–30 MΩ and was approximately 60%−80% compensated. Data were discarded when the series resistance increased or decreased by more than 20% during the course of the recordings. Input resistance resulted from ΔV divided by the injection of unit current (i.e., 100pA). Afterhyperpolarization (AHP) was measured as previously described (24).

In the PFC, the threshold, amplitude, and half spike time of the glutamatergic neuron single APs were recorded. The number of APs obtained in response to a series of 600-ms current steps from 0 to +500 pA with increments of 100 pA at the fixed potential of −80 mV was obtained. Glutamatergic neurons were distinguished as described previously (25).

Statistical Analysis

SPSS 20 was used for statistical analysis. All results are presented as the mean ± standard error of the mean (SEM). Data were analyzed using two-way analysis of variance (ANOVA) (sex × MSEW). A repeated-measure ANOVA was performed to assess Barnes maze test training. Student's *t*-test was used when comparing only two groups on one behavioral measure. The normality and homogeneity of variances were tested using the one-sample Kolmogorov–Smirnov test ($P > 0.05$) test and Levene's test ($P > 0.05$). When the assumption of the normality and homogeneity of variances was not met, non-parametric tests (Kruskal–Wallis and Mann–Whitney U) were used to detect significant differences. In the Y maze, paired Student's *t*-test was used to determine whether the percentage of entries into the novel arm differed from the percentage of entries into the other arm. $P < 0.05$ was considered statistically significant for all data. Graphs were prepared using GraphPad Prism 5 (GraphPad Software, United States).

RESULTS

Effect of Maternal Separation With Early Weaning on Anxiety-Like Behavior and Locomotor Activity

Open Field Test

There was no interaction of sex and MSEW in the distance moved [$F_{(3.33)} = 0.106$, $P = 0.747$], velocity [$F_{(3.33)} = 0.043$, $P = 0.838$], or the index of thigmotaxis [$F_{(3.33)} = 0.931$, $P = 0.342$]. Significant main effects of MSEW and sex were found on the distance moved [$F_{(3.33)} = 11.610$, $P = 0.002$; $F_{(3.33)} = 43.029$, $P = 0.000$] and velocity [$F_{(3.33)} = 12.347$, $P = 0.001$; $F_{(3.33)} = 44.293$, $P = 0.000$]. MSEW significantly reduced the distance moved and velocity in both sexes (**Figures 1A,B**). Control female rats were more active in the distance moved and velocity than control male rats. MSEW increased the index of thigmotaxis in male rats (**Figure 1C**). There was no interaction of sex and MSEW in the center time in the open field [$F_{(3.33)} = 2.112$, $P = 0.156$]. A significant main effect of sex was found on the center time [$F_{(3.33)} = 8.859$, $P = 0.005$]. MSEW reduced the center time in males (**Figure 1D**). There was no interaction of sex and MSEW in the number of feces [$F_{(3.33)} = 0.054$, $P = 0.817$]. A significant main effect of MSEW was found in the number of feces [$F_{(3.33)} = 14.062$, $P = 0.001$]. The assumption of normality for the number of feces was not met ($P = 0.042$), and a Mann–Whitney test was conducted. MSEW increased the number of feces in both sexes (**Figure 1E**).

Elevated Plus Maze Test

There was no interaction of sex and MSEW in the percentage of time spent on the open arms (%OT) [$F_{(3.33)} = 0.05$, $P = 0.824$], head-dipping frequency [$F_{(3.33)} = 1.397$, $P = 0.246$], distance moved [$F_{(3.33)} = 1.106$, $P = 0.301$], time in the closed time [$F_{(3.33)} = 1.593$, $P = 0.216$], or total entries [$F_{(3.33)} = 0.209$, $P = 0.989$]. Significant main effects of MSEW and sex were found on head-dipping frequency [$F_{(3.33)} = 3.916$, $P = 0.050$; $F_{(3.33)} = 16.603$, $P = 0.000$] and distance moved [$F_{(3.33)} = 23.117$, $P = 0.000$; $F_{(3.33)} = 11.654$, $P = 0.002$]. The assumption of normality for %OT was not met ($P = 0.023$), and a Mann–Whitney test was conducted. MSEW reduced %OT, head-dipping frequency, distance moved, and total entries in male rats (**Figures 2A–D**). A sex × MSEW interaction for the time in the center was observed [$F_{(3.33)} = 6.306$, $P = 0.017$]. Significant main effects of MSEW and sex were found on the time in the center [$F_{(3.33)} = 5.170$, $P = 0.030$; $F_{(3.33)} = 6.171$, $P = 0.018$] and the time in the closed arms [$F_{(3.33)} = 4.051$, $P = 0.052$; $F_{(3.33)} = 6.306$, $P = 0.020$]. MSEW reduced the time in the center area and increased the time in the closed arms in male rats (**Figures 2E,F**).

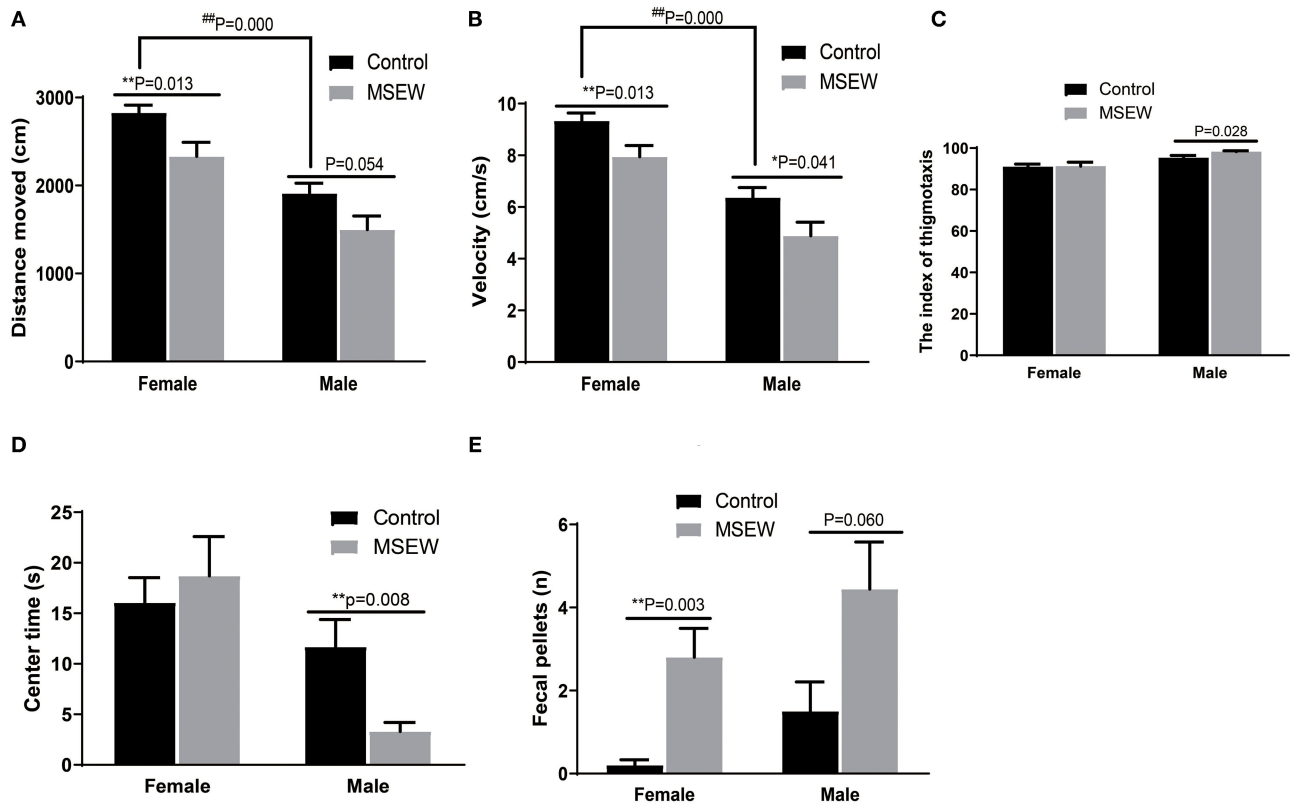

FIGURE 1 | Maternal separation with early weaning (MSEW) induced anxiety-like behavior in the open field test in male rats and decreased locomotor activity in both males and females. MSEW significantly reduced the distance moved and velocity in the open field in both female and male rats **(A,B)**. MSEW increased the index of thigmotaxis in male rats and reduced the center time only in males **(C,D)**. Female and male MSEW rats had increased numbers of feces. **(E)** All results are presented as the mean ± standard error of the mean (SEM) of $n = 8$–10 rats per group. $^{**}P < 0.01$, control vs. MSEW of the same sex; $^{\#\#}P < 0.01$, male vs. female in controls.

Effect of Maternal Separation With Early Weaning on Memory

Novel Object Recognition Test

During the training session, there was no interaction of sex and MSEW in the total exploration time (e1) [$F_{(3.33)} = 0.01$, $P = 0.978$]. A significant main effect of sex was found [$F_{(3.33)} = 14.638$, $P = 0.01$]. The total exploration time did not differ between the control and MSEW groups of the same sex (**Figure 3A**). Exploration time was longer in control female rats than in control male rats (**Figure 3A**). The total exploration time of control male rats did not reach 20 s. During the test session (2 h later), MSEW female rats had a significantly reduced d1, but their discrimination ratio did not change compared to that of the control females (**Figures 3B,C**). MSEW male rats showed a very low exploration level of both the novel object and the familiar object (**Figure 3D**).

Y Maze Novel Preference Arm Test

The 1st min that contained the greatest locomotor activity was investigated. There was no interaction of sex and MSEW in the discrimination preference in the 1st min [$F_{(3.33)} = 1.943$, $P = 0.173$]. Significant main effects of MSEW and sex were also not found [$F_{(3.33)} = 1.230$, $P = 0.275$; $F_{(3.33)} = 2.565$, $P = 0.119$]. Control female rats entered the novel arm more than the other arm (**Figure 4A**). MSEW female rats showed a preference for the novel arm (**Figure 4A**). Both control male rats and MSEW male rats entered the novel arm more than the other arm (**Figure 4B**).

Effect of Maternal Separation With Early Weaning on Spatial Reference Memory

Barnes Maze Test

In the training phase, for the latency to find the target hole over the 3 days of training, there was no effect of a treatment × days interaction [$F_{(3.33)} = 0.781$, $P = 0.513$]. There was an effect of days [$F_{(3.33)} = 49.561$, $P = 0.000$] but no effect of treatment [$F_{(3.33)} = 0.452$, $P = 0.711$]. Three-way ANOVA analysis showed that significant main effect of sex was not found [$F_{(3.33)} = 0.439$, $P = 0.512$]. Compared to control female rats, MSEW female rats had a tendency to spend more time finding the platform on the 1st and 2nd days but similar time on the 3rd day (**Figure 5A**). MSEW and control male rats spent similar time finding the platform (**Figure 5B**). For the probe test, there was no interaction of sex and MSEW in the latency to find the escape box [$F_{(3.33)} = 1.722$, $P = 0.199$]. No significant differences between MSEW rats and their respective controls were found for latency to the target

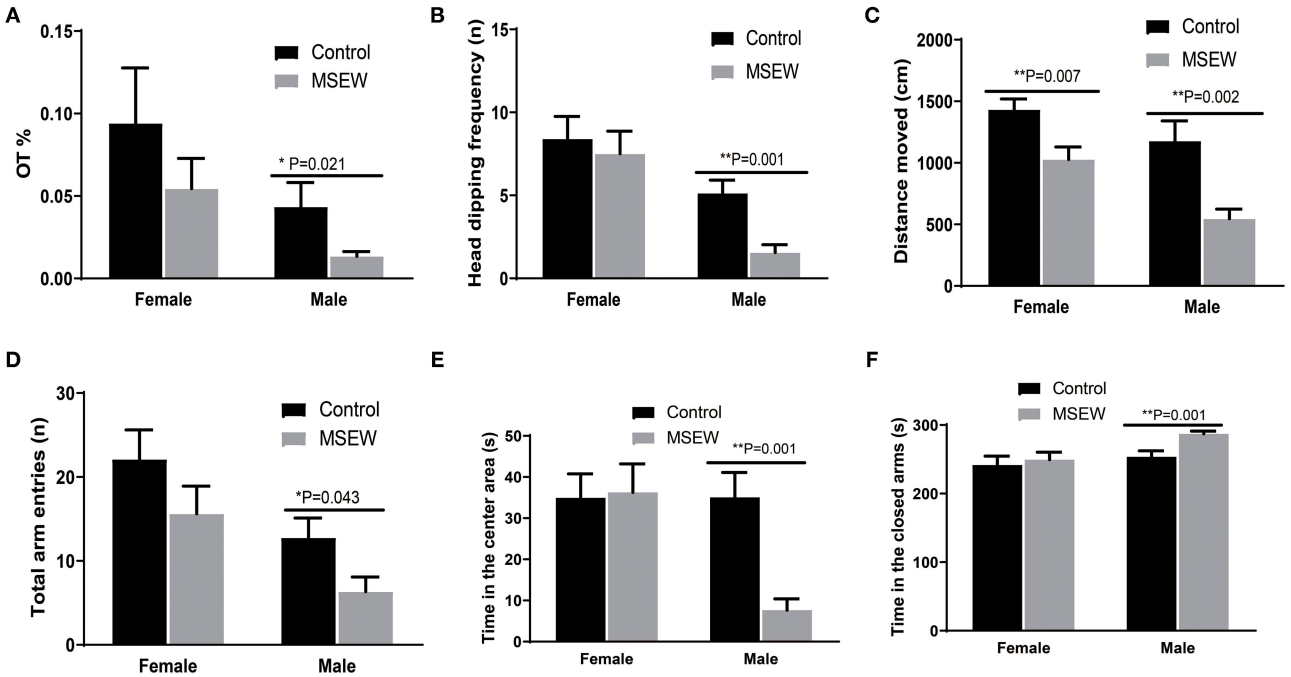

FIGURE 2 | Maternal separation with early weaning (MSEW) induced anxiety-like behavior in the elevated plus maze test in male rats and decreased locomotor activity in both sexes. Male MSEW rats exhibited reduced percentage of time spent on the open arms (%OT), head-dipping frequency, distance moved, and total entries **(A–D)**. MSEW reduced the time in the center area and increased the time in the closed arms in male rats **(E,F)**. All results are presented as the mean ± standard error of the mean (SEM) of $n = 8$–10 rats per group. $^{**}P < 0.01$, control vs. MSEW of the same sex; $^{*}P < 0.05$, $^{**}P < 0.01$, control vs. MSEW of the same sex.

hole, time in the target hole, velocity, or distance moved in either sex (**Figures 5C–F**).

Effect of Maternal Separation With Early Weaning on Coping Behavior

Forced Swim Test

There was no interaction of sex and MSEW in the immobility time [$F_{(3.33)} = 1.628$, $P = 0.211$] or climbing time [$F_{(3.33)} = 0.923$, $P = 0.344$]. A trend-level effect of sex was found [$F_{(3.33)} = 0.295$, $P = 0.057$]. MSEW increased the immobility time in males (**Figure 6A**). MSEW did not change the climbing time in either sex (**Figure 6B**).

Effect of Maternal Separation With Early Weaning on Fear Memory

Fear Conditioning Training

In the first 3-min habituation, there was an interaction of sex and MSEW in the freezing time [$F_{(3.33)} = 6.359$, $P = 0.017$]. Significant main effects of MSEW and sex were found on the baseline freezing time [$F_{(3.33)} = 11.218$, $P = 0.002$; $F_{(3.33)} = 6.146$, $P = 0.018$]. The MSEW male rats showed more freezing behavior than the control male rats (**Figure 7A**). In the fear conditioning training phase, there was no interaction effect of sex and MSEW in the freezing time [$F_{(3.33)} = 1.467$, $P = 0.234$]. Significant main effects of MSEW and sex were also not found [$F_{(3.33)} = 0.345$, $P = 0.561$; $F_{(3.33)} = 0.089$, $P = 0.767$]. Female and male MSEW rats did not demonstrate impaired associative learning (**Figure 7B**).

Contextual Fear Conditioning

There was no interaction of sex and MSEW in the freezing time in the contextual fear conditioning test [$F_{(3.33)} = 1.460$, $P = 0.235$]. A significant sex effect was found [$F_{(3.33)} = 0295$, $P = 0.000$]. No significant main effects of MSEW were found [$F_{(3.33)} = 1.985$, $P = 0.168$]. MSEW increased freezing time in male rats (**Figure 7C**).

Effect of Maternal Separation With Early Weaning on Neuronal Excitability

MSEW did not change the threshold or amplitude of single APs in the PFC in either sex (**Figures 8A,B**). MSEW decreased the half-spike time in female rats (**Figure 8C**). AHP for each group was presented and input (**Figure 8D**). Representative traces of glutamatergic neurons were presented (**Figure 8E**). With 400 and 500 pA current injections, MSEW male rats exhibited significantly decreased numbers of glutamatergic neuron APs compared to control males (**Figure 8F**). Representative traces obtained in the prefrontal cortex neurons from control and MSEW male rats were presented (**Figures 8G,H**). Input resistance for each group was presented (**Figure 8I**). MSEW female rats showed a trend for decreased numbers of glutamatergic neuron APs (**Figure 8J**). Representative traces obtained in the prefrontal cortex neurons from control and MSEW female rats were presented (**Figures 8K,L**).

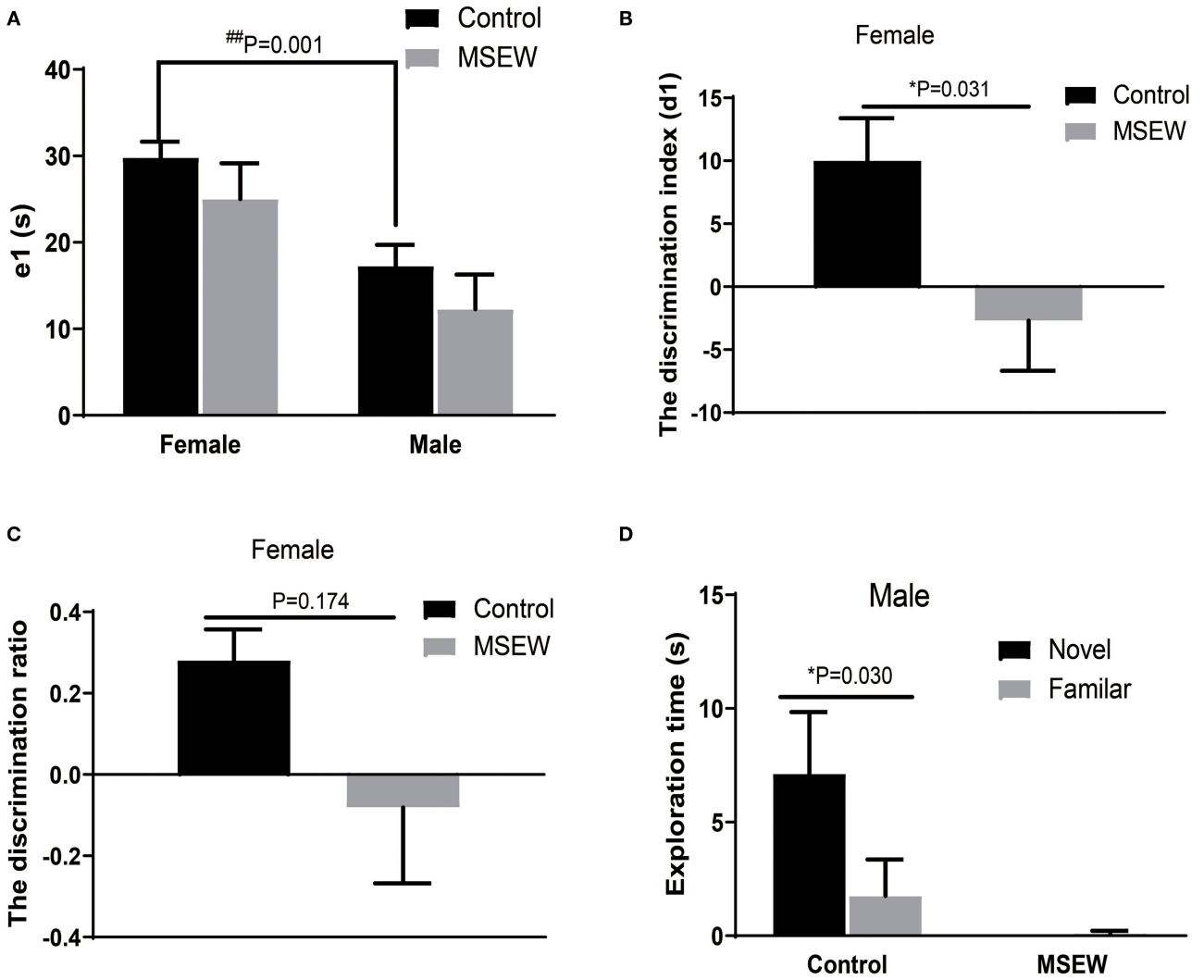

FIGURE 3 | Maternal separation with early weaning (MSEW) slightly impaired non-spatial working memory in the novel object recognition test in female rats. The total exploration time did not differ between the control and MSEW groups of the same sex, and female control rats showed a longer exploration time than male control rats **(A)**. During the test session (2 h later), female MSEW rats showed significantly reduced discrimination index (d1) but no change in the d1 compared to the control females **(B,C)**. Male MSEW rats showed very low exploration levels of both the novel object and the familiar object **(D)**. All results are presented as the mean ± standard error of the mean (SEM) of $n = 8$–10 rats per group. $^{*}P < 0.05$, control vs. MSEW of the same sex; $^{\#\#}P < 0.01$, male vs. female in controls.

DISCUSSION

Despite an extensive body of literature, there is no consensus on the behavioral effects induced by MS and the extent of these effects. A longer MS period was used to reduce any potential for compensatory maternal care after MS. In addition, a previous study indicated that the early-life stress phenotype was strongest when early-life stress was combined with other negative experiences. In our study, we observed a longer MS period with early weaning-induced long-lasting behavioral changes and electrophysiological alterations in the PFC that persisted into adulthood.

Maternal Separation With Early Weaning Induced Anxiety-Like Behavior and Passive Coping Strategy in Male Rats

In preclinical research, the open field test and elevated plus maze test are widely used to assess anxiety-like behavior in rodent animals (26, 27). In our study, only male MSEW rats showed an anxiogenic phenotype with decreased central activity in the open field test, as well as decreased time in the open arms and head-dipping frequency in the elevated plus maze test, which is in agreement with previous work showing strong sex-dependent bias on anxiety behavior with males (28–31). Few studies have reported that MS induces anxiety behavior in female animals

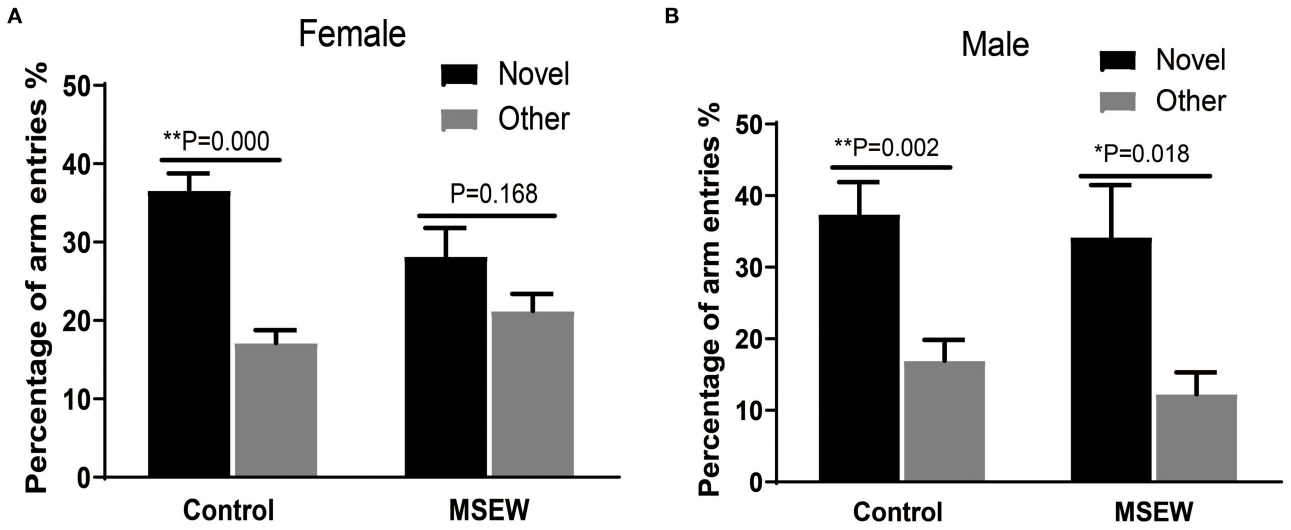

FIGURE 4 | Maternal separation with early weaning (MSEW) slightly impaired spatial working memory in the Y maze in female rats. Female control rats entered the novel arm more than the other arm **(A)**. Female MSEW rats also showed a preference for the novel arm, but the difference was not significant **(A)**. Both control and MSEW male rats entered the novel arm more than the other arm **(B)**. All results are presented as the mean ± standard error of the mean (SEM) of $n = 8$–10 rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, control vs. MSEW of the same sex.

(32). Notably, in our subsequent study, we found that MSEW also induced anxiety-like behavior in adolescent SD rats (data not shown). Taken together, our findings suggested that MSEW induced a sustained anxiety phenotype in male rats. Conversely, in the clinic, women are twice as likely as men to exhibit anxiety disorders and are believed to possess an innate vulnerability that makes them susceptible to anxiety disorders (33). The difference in developmental timing between rodents and humans may be relevant to the discrepancy. Another possibility is that the method of what is detected in rodents does not accurately reflect the human condition (34).

Similar to the anxiety phenotype, inconsistent behavior results for depressive-like behavior were reported for MS in rats (35–37). Our results showed that MSEW induced a passive coping strategy with increased immobility time in the forced swim test in male rats. More behavioral tests, such as anhedonic assessments, are needed to identify the depressive-like behavior induced by MSEW.

Maternal Separation With Early Weaning Induced Decreased Locomotor Activity in Both Sexes

In the present study, MSEW induced decreased locomotor activity in the open field test, elevated plus maze test, and Y maze test in both sexes. MS induced paradoxical behavior results for the locomotor activity effect. MS on PNDs 3–14 and on PNDs 3–21 did not affect locomotor activity in Long-Evans rats (31). A single 24-h period of maternal deprivation on PND 9 resulted in a decrease in locomotion in Wistar rats (38). MSEW caused hyperactivity in the open field test in mice (16). Different methodologies of MS may lead to these inconsistent results. Interestingly, our findings showed that MSEW did not affect locomotor activity in either sex in the Barnes maze test. Notably, the open field test, elevated plus maze test, and Y maze test are based on the spontaneous exploratory behavior of rodents, while the Barnes maze test is based on negative reinforcement of behavior (bright lights and loud buzzing). A previous study reported that MS may improve stress coping in adult rats, reflected by increased offensive-like behavior during juvenile play-fighting and aggression resident–intruder tests (39, 40). As such, our results suggested that negative reinforcement in the Barnes maze test increased the locomotor activity level of MSEW rats and, to some extent, improved their stress coping in a stressful circumstance. In addition, our study indicated that female rats showed higher levels of exploration and locomotion than male rats, as measured by a significant increase in distance moved and velocity in the open field test and open arm entries in the elevated plus maze, as well as total arm entries in the Y maze and exploration time of the familiar object in the NOR test. These findings supported previous work that reported that female SD rats exhibited more curiosity and showed greater locomotion than males in social and non-social behavioral tests (41).

Maternal Separation With Early Weaning Slightly Impaired Working Memory in Female Rats but Did Not Impair Spatial Reference Memory in Either Sex

Working memory in humans is considered a distinct short-term memory process. Working memory reflects the capacity to temporarily maintain and manipulate recently acquired information actively for further goal-directed actions (42). Working memory is impaired in some neurodegenerative diseases (43) and most psychiatric and developmental disorders,

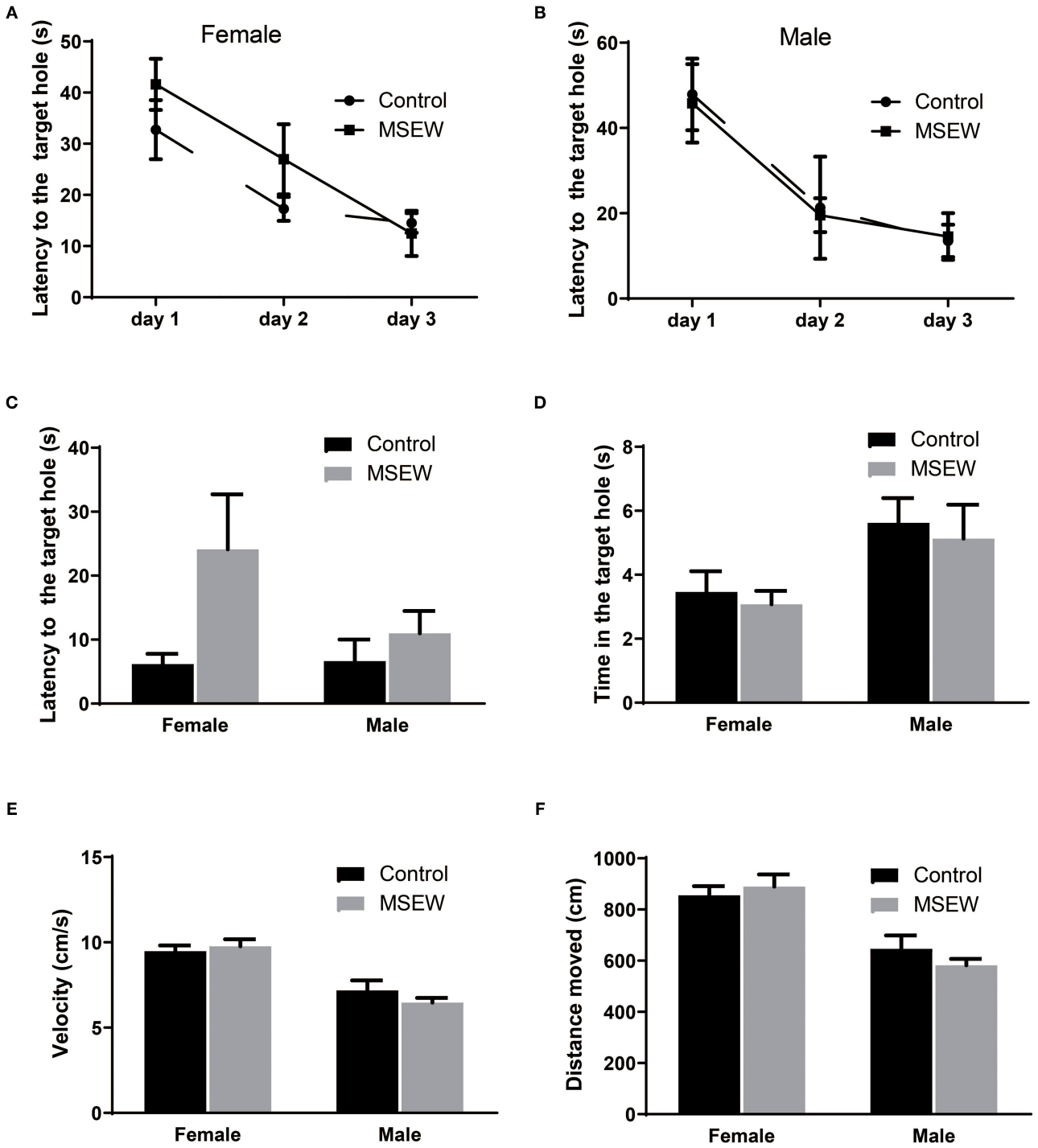

FIGURE 5 | Maternal separation with early weaning (MSEW) did not impair spatial reference memory in either sex. Compared with control females, MSEW female rats had a tendency to spend more time finding the platform (latency) on the 1st and 2nd days and spent similar time on the 3rd day **(A)**. MSEW and control male rats spent similar amounts of time finding the platform **(B)**. For the probe test, MSEW rats showed no significant difference compared to their respective controls in latency to the target hole, time to the target hole, velocity, or distance moved for either sex **(C–F)**. All results are presented as the mean ± standard error of the mean (SEM) of $n = 8–10$ rats per group.

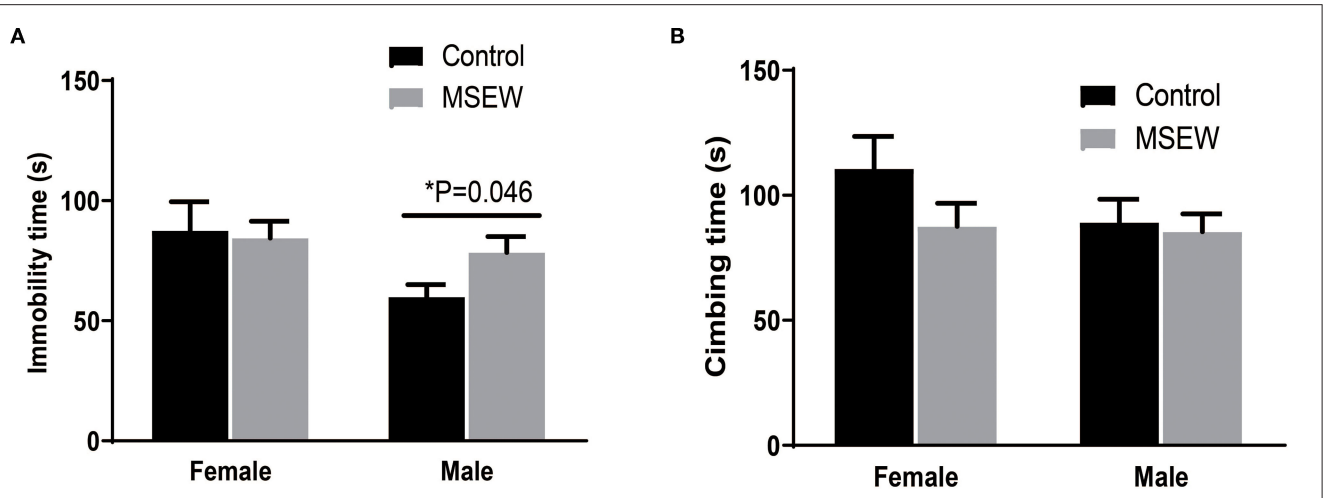

FIGURE 6 | Maternal separation with early weaning (MSEW) induced a passive coping strategy in male rats. MSEW increased the immobility time in males. **(A)** MSEW did not change the climbing time in either sex. **(B)** All results are presented as the mean ± the standard error of the mean (SEM) of $n = 8$–10 rats per group. $^*P < 0.05$, control vs. MSEW of the same sex.

such as schizophrenia and attention-deficit hyperactivity disorder (44). Clinical practice has reported that early-life stress is related to working memory deficits (45). Spatial spontaneous alternation behavior in the Y maze has been viewed as a test of spatial working memory in rodents that requires active maintenance and the manipulation of information in a limited time (46). We used the Y maze delay spontaneous alternation protocol to determine the effects of MSEW on spatial working memory. Our results showed that MSEW female rats, but not MSEW male rats, displayed a lower preference for the novel arm than control females in the 1st min, which indicated that MSEW led to deficits in spatial working memory in female rats. The sex-dependent bias on working memory deficits in females in our study differs from those of some studies that reported that working memory was also impaired in males (47, 48). We did not find MSEW-induced deficits in recognition memory in the Y maze novel preference arm test in male rats.

The NOR test is a simple and sensitive behavioral assay for the evaluation of non-spatial memory. In the present study, after a 2-h retention delay, our results showed that MSEW decreased the discrimination index in female rats, which indicated that MSEW impaired non-spatial memory in female rats. Statistically significant differences were not found between control and MSEW female rats in the discrimination ratio. We could not estimate whether MSEW impaired the recognition ability in male rats due to their low level of exploration.

In the present study, the Barnes maze test was used to detect the effect of MSEW on spatial reference memory. Our results showed that MSEW did not impair learning in the training phase or spatial memory in the probe test in either sex, although MSEW had a tendency to impair learning on the 1st and 2nd days in the training phase in females. MS resulted in inconsistent behavior in the spatial reference test. This result is in line with previous studies that have reported that MS (10 and 21 days) did not alter spatial long-term memory in the Morris water maze test (a similar paradigm to the Barnes maze test for evaluating spatial reference memory) in female or male Wistar rats (49, 50).

Maternal Separation With Early Weaning Did Not Impair Associative Learning in Either Sex but Increase Fear Memory in Male Rats

In the habituation phase of the fear conditioning test, MSEW male but not female rats showed more anxious behavior, measured by greater freezing behavior, than control male rats. This result is consistent with our findings in the open field test and elevated plus maze test, which also showed that MSEW induced anxiety-like behavior in male rats. In the fear conditioning training phase, MSEW did not impair fear learning in either sex. In the contextual fear conditioning test, MSEW male rats exhibited increased fear memory. No effect was observed in females. Our current finding concurred with previous work that suggested that anxious rats exhibited greater fear memory (51). Contextual fear conditioning is hippocampus-dependent. Further research is needed to investigate the role of the hippocampus in the effect of early-life stress on anxiety-like behavior and fear memory.

Notably, the NOR test and Y maze test are based on the spontaneous exploratory behavior of rodents and are non-stressful, while the Barnes maze test and fear conditioning test are based on stressful situations. Bonapersona et al. (30) used a large-scale three-level meta-analysis of all peer-reviewed preclinical literature and provided extensive evidence that early-life stress impaired non-stressful learning and enhanced memory formation during stressful learning. In the current study, MSEW increased locomotor activity and improved stress coping in stressful situations, which suggested that MSEW induced adaptive modification in stressful situations. MSEW female rats showed slight working memory deficits in the novel recognition

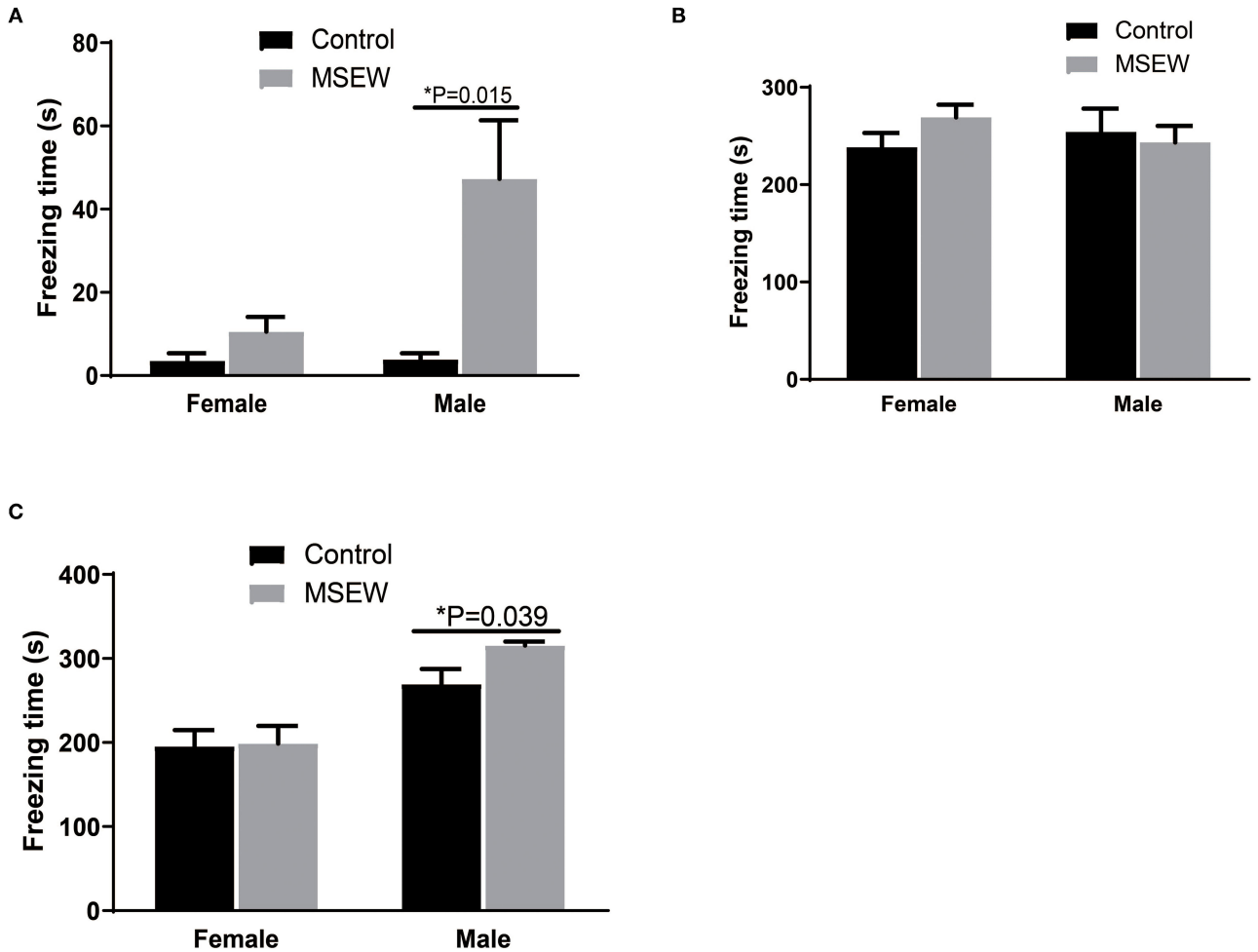

FIGURE 7 | Maternal separation with early weaning (MSEW) did not impair associative learning in either sex or increase fear memory in male rats. MSEW male rats showed more freezing behavior than control male rats **(A)**. Female and male MSEW rats did not show impaired associative learning **(B)**. MSEW male rats exhibited increased freezing time compared to the control male rats **(C)**. All results are presented as the mean ± the standard error of the mean (SEM) of $n = 8$–10 rats per group. $^*P < 0.05$, control vs. MSEW of the same sex.

test and Y maze test and a trend-level effect on spatial learning impairment in the Barnes maze, which supports theories in which there is a close link between working memory mechanisms and long-term memory mechanisms (52).

Maternal Separation With Early Weaning Reduced Glutamatergic Neuronal Excitability in the Prefrontal Cortex in Male Rats

Neural intrinsic excitability determines the net output of neurons by integrating synaptic inputs and consecutively translating them into AP firing (20). Our study demonstrated that MSEW reduced glutamatergic neuronal excitability significantly in the PFC in male rats, as shown by decreased numbers of glutamatergic neuron APs upon current injection, although the parameters of single APs did not change. Neuronal intrinsic excitability reflects global changes (53). Many voltage-gated and ion channels are implicated in shaping the spiking output (20). Further research is needed to identify the mechanism by which MSEW affects glutamatergic neuronal excitability. The reduced glutamatergic neuronal excitability may be associated with the emotional alteration induced by MSEW in male rats (54, 55). Although MSEW reduced the half-spike time, we could not infer the effect of MSEW on glutamatergic neuron single APs in female rats. The effect of MSEW on the glutamatergic neuronal excitability and its relationship with the damage of working memory in female rats need further study.

There is a limitation to this study. Growing evidence has shown that sex hormone status plays an important role in modulating rodent behavior (56). In the current study, the estrous cycle stage was not recorded, and the effect of sex hormones on the behavior of female rats was ignored. In addition, previous reports have shown that the female estrous cycle also

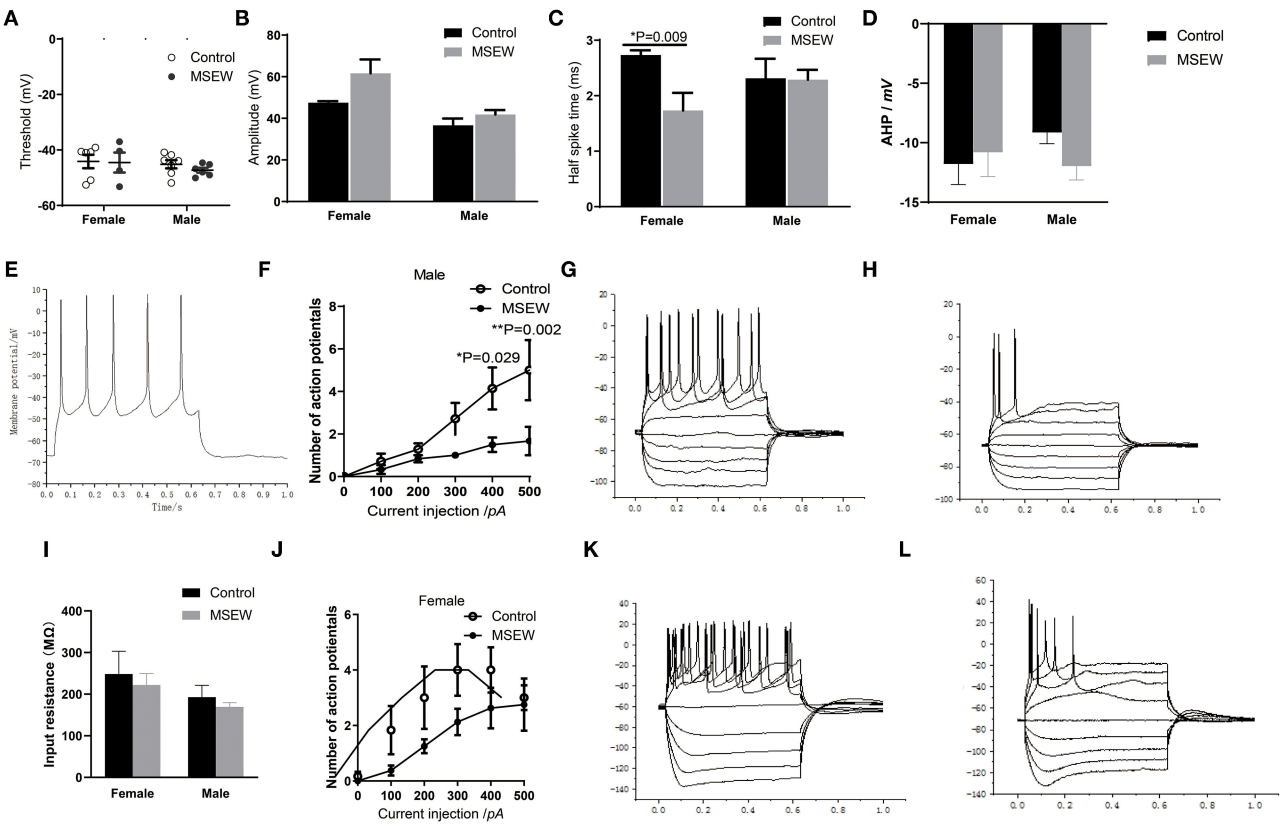

FIGURE 8 | Maternal separation with early weaning (MSEW) reduced glutamatergic neuronal excitability in the prefrontal cortex in male rats. MSEW did not change the threshold or amplitude of single action potentials in the prefrontal cortex in either male or female rats **(A,B)**. MSEW decreased the half-spike time in female rats **(C)**. Afterhyperpolarization (AHP) in the four groups **(D)**. Representative traces of glutamatergic neurons (150 pA, 600 ms) **(E)**. MSEW male rats had decreased numbers of glutamatergic neuron action potentials (APs) with current injections of 400 and 500 pA **(F)**. Representative traces obtained in the prefrontal cortex neurons from control and MSEW male rats **(G,H)**. Input resistance in the four groups **(I)**. MSEW female rats showed a trend for decreased numbers of glutamatergic neuron APs **(J)**. Representative traces obtained in the prefrontal cortex neurons from control and MSEW female rats **(K,L)**. All results are presented as the mean ± standard error of the mean (SEM) of $n = 3$–5 rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, control vs. MSEW of the same sex.

induced I sex differences in neuron excitability and behavior (57, 58). Hippocampal excitability is more variable at proestrous (59). Our results showed a high level of variability in the number of APs in female rats across a range of current steps, which may be associated with female estrous cycle effects. MSEW affects different aspects of glutamate neuron properties in males and females, which may be partly linked to the estrogen cycle (60). Collectively, these data provide the first evidence that early-life stress modulates the intrinsic excitability of glutamatergic neurons in the PFC.

CONCLUSION

In summary, we combined MSEW to create an early-life neglect rat model, inducing long-lasting anxiety-like behavior in male rats, to unravel possible influences of early-life stress on adult anxiety disorder and screen for potential therapies. The reduced glutamatergic neuronal excitability may be associated with the emotional alteration induced by MSEW in male rats. MSEW affected memory formation in a sex-dependent manner. In addition, MSEW slightly impaired memory during non-stressful situations and did not change learning or memory under stressful circumstances. This finding suggested that child neglect induced an adaptive modification only in a stressful context.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethical committee for the use of experimental animals of the Institute of Laboratory Animal Sciences.

AUTHOR CONTRIBUTIONS

CQ conceived, designed the experiments, and acquired the funding. XS designed, performed the behavioral

tests, and wrote the manuscript. YZ performed the electrophysiological tests. XLi performed the behavioral tests. XLiu conceived the experiments and acquired the funding. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Locus Coeruleus-Norepinephrine System in Stress and Arousal: Unraveling Historical, Current, and Future Perspectives

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Arousal may be understood on a spectrum, with excessive sleepiness, cognitive dysfunction, and inattention on one side, a wakeful state in the middle, and hypervigilance, panic, and psychosis on the other side. However, historically, the concepts of arousal and stress have been challenging to define as measurable experimental variables. Divergent efforts to study these subjects have given rise to several disciplines, including neurobiology, neuroendocrinology, and cognitive neuroscience. We discuss technological advancements that chronologically led to our current understanding of the arousal system, focusing on the multifaceted nucleus locus coeruleus. We share our contemporary perspective and the hypotheses of others in the context of our current technological capabilities and future developments that will be required to move forward in this area of research.

Keywords: arousal, norepinephrine, locus coeruleus, electroencephalography, transmission electron microscopy

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INTRODUCTION

The concept of arousal is one that is best defined on a spectrum. Through a clinical lens, behaviors relating to inattention or cognitive dysfunction associated with under-stimulation may reflect mild to moderate levels of an arousal deficiency, with excessive daytime sleepiness and sedation being symptoms of the most extreme cases. On the other hand, behavior associated with hypervigilance, insomnia, overstimulation, and fear or panic may indicate a mild or moderate level of excessive arousal, with psychosis and hallucinations being symptoms in the most extreme cases. In the center of the spectrum, arousal is a state of wakefulness in which an individual is awake, alert, able to create and problem solve (1). However, as a subject of investigation, arousal can be difficult to define, particularly when considering the dynamic neural processes that underlie this spectrum of brain states. For example, the brain state associated with overstimulation of arousal systems may describe a stressed brain state. Continued efforts to define stress and its relationship to arousal reflect a longstanding issue that has been described by others (2). Both have been characterized across scientific disciplines using a broad range of descriptors, many of which are nearly synonymous, making it challenging to converse across neuro-behavioral study fields.

Cognitive psychology, neurobiology, neuroendocrinology, and cognitive neuroscience have shared technological advancements expanding our understanding of the behavioral, network-, circuit- and synaptic-level responses to stimuli. However, silos of advancements within each discipline persist, continuing to hinder the potential for interdisciplinary synergism. In perspective

shared by Pessoa, Pessoa describes a significant modern challenge in brain-behavior research embodied by a granular focus on "causal explanations" at the expense of theoretical and conceptual descriptions that may be shared and provide deeper understanding across disciplines (3).

In the sections that follow, we discuss the Locus Coeruleus (LC)- Norepinephrine (NE) system in the context of this quandary as it illustrates a system with interdisciplinary interest that has made significant strides in recent years and yet lacks a common vernacular to facilitate cross-disciplinary communication. The LC-NE system is involved in various neurobiological processes, including the modulation of sleep-wake cycles, facilitation of attention, responses to stress, initiation memory formation, and retrieval. It modulates blood flow, metabolism, and the distribution of oxygen and glucose throughout the brain. Thus, it is evident that the LC is a brain region that directly or indirectly has been investigated or described across several scientific disciplines. Though scarce, we discuss works in the literature that integrate the findings of these efforts across multiple levels of neurobiological function (4–8).

We start by providing historical context, first by the recollection of the neuroanatomical and physiological studies that led to our current understanding of the LC-NE system and subsequently discussing the evolution of Arousal Theory. Because the broader fields of cognitive psychology and neurobiology have historically advanced our understanding of arousal, primarily independently of each other, we first discuss them separately. We then transition to a synthesis of recent literature that brings to light a critical intersection of these fields by discussing current theories that directly or indirectly implicate the LC-NE system in large-scale network dynamics under various arousal states. Finally, we discuss pathology related to the LC-NE system dysfunction, emphasizing hyper-arousal in stress-related disorders. Along the way, we discuss technological advancements that preceded each milestone of conceptual evolution and the past and current day challenges that have emerged over time.

Abbreviations: ACTH, adrenocorticotropin releasing hormone; aINS, anterior insula; AR, adrenergic receptor; BOLD, Blood Oxygen Level Dependent; CEN, Central Executive Network; CRF, Corticotropin-Releasing Factor; CRF-OE, overexpressed CRF; CRFR1, CRF receptor 1; DAB, 3,3a-diaminobenzidine tetrahydrochloride; dACC, dorsal anterior cingulate cortex; dlPFC, dorsolateral prefrontal cortex; DMN, Default Mode Network; dMPFC, dorsal medial prefrontal cortex; DOX, doxycycline; dPFC, dorsal frontal cortex; dPPL, dorsal posterior parietal lobe; DREADDs, designer receptors exclusively activated by designer drugs; DβH, dopamine-beta-hydroxylase; FEF, Frontal Eye Field; GANE, Glutamate Amplifies Noradrenergic Effects; HRP, Horseradish Peroxidase; ICA, independent component analysis; ICNs, Intrinsic Connectivity Networks; IPL, inferior parietal lobe; LC, Locus Coeruleus; LDCV, Large Dense-Core Vesicle; lPPL, lateral posterior parietal lobe; LTC, lateral temporal cortex; NE, Norepinephrine; PACE, Probability Associated Community Estimation; PCC, posterior cingulate cortex; pCu, precuneus; PET, Positron Emission Topography; PLACE, Path Length Associated Community Estimation; RAS, reticular activating system; REM, rapid eye movement; SN, Salience Network; SSV, Small Synaptic Vesicle; SWS, slow-wave sleep; TEM, Transmission Electron Microscope; TH, Tyrosine Hydroxylase; TPL, temporoparietal lobe; VAN, Ventral Attention Network; vMPFC, ventral medial prefrontal cortex; vPFC, ventral frontal cortex.

THE STUDY OF STRESS: A HISTORICAL PERSPECTIVE

The Origin of "Stress" and the Divergence of Its Scientific Study

Early scientific observations on how the body adapts to a life-threatening situation, aging, or disease, are the foundations of stress-related research as we know it today (9). The earliest scientific report of such an adaptation was published in 1914 by Walter Cannon and described the adrenal medullary response to pain, asphyxiation, and emotions such as fear and rage as an "emergency response." He notes that the release of catecholamines norepinephrine and epinephrine produces 'striking bodily alterations' in which blood flow shifts away from the abdomen and toward the lungs, heart, limbs, and central nervous system (10). Cannon later conceptualized the sympathoadrenal system as a "mobilizer of bodily forces for struggle" that connected the adrenal medulla's endocrine functions with the sympathetic nervous system to coordinate physiological responses to crises. Thus, Cannon's observations founded the physiological basis for the 'fight or flight' response. Throughout the 1920s, Cannon published highly impactful work on regulating internal states and coined the term "homeostasis." Homeostasis describes the intricate coordination of mechanisms between the autonomic nervous and endocrine systems to regulate physiological parameters first observed by Bernard (11). Bernard and Cannon's observations fundamentally contributed to the body's conceptualization as a self-regulating system that required a balance of physical and chemical states with the external environment. Importantly, Cannon brought to light the clinical impact of emotional distress, which he asserted could dysregulate these homeostatic mechanisms and generate disease.

In the decades that followed the initial conception of "stress," scientists across disciplines debated how to define the stress response and measure it under experimental conditions (2). Some took a reductionist approach, derived from the concept of a body economy (12), that defined the body as an energy system from which elements of the environment would mobilize resources to respond physiologically or behaviorally (13, 14). Others emphasized the emotional aspects of stress (15, 16) in which emotions manage both motivational resources and regulate behavioral and cognitive activation as a preparatory step to formulating action (17, 18).

Meanwhile, other investigators refuted the idea of a unified explanation of such responses. They emphasized the diversity of stress effects (19), noting that sleep deprivation caused specific detriments while other stimuli such as noise or heat compromised other factors (2). Creating a single definition across disciplines proved an insurmountable task that left each field to define "stress" on its terms. One could certainly argue that divergence from the singular concept of "stress" in the scientific study was necessary to expand our understanding of a complex system. However, beneficial the departure was, it also created hindrances as silos of neurobiology advancements,

neuroendocrinology, and cognitive neuroscience occurred in parallel, without synergism.

The Discovery of the Hypothalamic-Pituitary-Adrenal Axis

Technological advancements facilitated continued growth in neurobiology and neuroendocrinology, particularly the monumental discovery of microelectrodes (20). Although primitive in their design, the creation of such electrodes by the "founding fathers of neurobiology" allowed for recording electrical impulses for the first time (21, 22). Techniques of this kind sparked the interest of Geoffrey Harris, credited as the founding father of neuroendocrinology. Harris found that stimulation of specific regions of the hypothalamus and anterior pituitary caused ovulation, from which he hypothesized that nerve fibers from the hypothalamus mediated the response to the hypophysis. Harris went on to invent a 2-electrode system capable of stimulating the hypothalamic brain region over several days and weeks remotely in conscious animals. His seminal invention used a skull-fixed insulated coil of enameled copper wire connected to an insulated platinum wire stimulating electrode that descended into the brain. A silver plate placed beneath the scalp and over the frontal bones served as a second electrode (23). The animal with the implanted coil was placed in a strongly fluctuating magnetic field to achieve electrode stimulation. Following years of technological refinement of the system and numerous collaborations, Harris elucidated the adenohypophysis's innervation and blood supply. Harris' work culminated in providing definitive evidence that a neurohormonal factor, traveling through the adenohypophysis portal system, was essential for controlling hormone production in the pituitary (24).

Emanating from Cannon's work, and during Harris's time, the endocrinologist Selye became known for conceptualizing the first stress model, known as General Adaptation Syndrome. Selye published widely on General Adaptation Syndrome, using his work and his peers (9) to support his proposal of a 'syndrome of diverse nocuous agents' (25). Selye describes three stages of responses to acute, non-specific damage he called "stressors" (26, 27), that ranged from excessive muscle use to spinal shock (25). The first phase, known as the alarm response, is what we would identify today as the "fight or flight" response coined by Cannon (28). The second phase is the stage of resistance, defined as a continued state of arousal, and the third phase is exhaustion. Selye eloquently articulated stress as "passive non-specific damage intricately mixed with those of active defense," which gradually eroded health over time as the body's neurological and endocrinological defense systems continually responded to and became exhausted by stressors.

The foundation of neuroendocrinology provided by Harris' work was significantly advanced when a trainee of Selye, Guillemin, and Schally independently demonstrated that a hypothalamic factor whose chemical structure was not yet defined stimulated the release of adrenocorticotropin releasing hormone (ACTH) from the pituitary. Thus, emerged the first description of the corticotropin-releasing factor (CRF). The scientific lineage of Selye's work, from his trainee, Guillemin, and later, Guillemin's trainee, Wylie Vale, would define the adrenal-hypothalamic-pituitary response to stress as we know it today (29).

LC-NE Anatomy and Physiology of the Stress Response

The initial conception in 1931 by Ernst Ruska, and the later application of the transmission electron microscope (TEM) to biological samples in the 1940s, enabled scientists to observe the subcellular structure of neurons for the first time (30). The use of thin tissue sectioning combined with plastic embedding of samples made it possible to keep organelles in their natural subcellular compartments (31, 32). These early ultrastructural studies provided unequivocal support for the neuron doctrine by capturing micrographs of individual neural cells (33). TEM studies identified synaptic contacts' morphological features and confirmed dendritic spines' existence; a feature previously observed using the Golgi Method (34). High-resolution TEM analysis of axon terminals led to the identification of two types of neurotransmitter -containing vesicles; small synaptic vesicles (SSV) later shown to store and release fast-acting transmitters such as glutamate and GABA, and large dense-core vesicle (LDCV), the primary site for neuropeptide storage and release (35). Monoamine transmitters, such as norepinephrine, can be stored in either SSV or LDCV (36). TEM studies also provided evidence for putative extrasynaptic release of LDCV (36, 37) and non-synaptic transmission or volume transmission (38). The theory of volume transmission was supported by anatomical studies of the time that indicated neuropeptide receptors were not frequently found at the synaptic cleft, thus raising the possibility of asynaptic peptide release, distant receptor binding, and activation of neurons or glial cells in the microenvironment (39). Additionally, the low synaptic incidence of monoaminergic terminals and their receptors' extrasynaptic localization (40–42) suggest that these transmitters may be preferentially released in a manner consistent with volume transmission.

The work of Coons et al., who first used fluorescein-labeled antibodies to localize antigens at the light microscopic level, inspired increasingly advanced antigen-detection methods (43, 44). The introduction of the radio immuno-assay for direct immuno-labeling of cellular components by Yalow and Bernson and the subsequent development of the immunocytochemistry indirect labeling techniques provided the tools necessary for cell-type-specific labeling of neurons by antibodies (45–47). Further, Singer's development of an antigen visualization technique that employed electron-dense substances such as colloidal gold conjugated to the antibody to improve its detection under the electron microscope significantly increased TEM studies' success (48). Under the electron microscope, the electron-dense colloidal gold particles are easily observed as dark puncta and quantified using the TEM (49). Another significant advancement in this regard was the discovery and subsequent widespread use of Horseradish peroxidase (HRP) as an enzymatic label for the detection of biomolecules in immunohistochemistry (50). The common use of HRP has been attributed to its high enzymatic

activity, that when combined with electron-dense chromogen substrates such as 3,3α-diaminobenzidine tetrahydrochloride, created a substantial reaction product that could be easily visualized under a light or electron microscope (51).

A line of neuroanatomical studies employed indirect labeling techniques to visualize Tyrosine Hydroxylase (TH), the first and rate-limiting enzyme of catecholamine biosynthesis (52). These seminal studies approximated dopaminergic and noradrenergic neuronal cell bodies (53, 54). The LC is localized in the fourth ventricle base and is known for its vast and divergent efferent system, whose noradrenergic fibers reach nearly the entire neuraxis (53). NE is stored in both SSVs and LDCVs that can be localized throughout the somatodendritic processes LC neurons (55–57). These findings were preceded by the stereotaxic mapping of the ascending monoaminergic neurons (58) and followed with advanced immunohistochemical studies that employed high-resolution immunoelectron microscopy to visualize the cellular and subcellular distribution of TH within LC neurons (59, 60). Later studies compared the immunohistochemical labeling of TH and dopamine-β-hydroxylase (DβH), the final enzyme in NE biosynthesis, to classify catecholaminergic neurons into distinct populations of noradrenergic and dopaminergic neurons (61). Subsequent high-resolution electron microscopy analysis of the LC revealed CRF-immunoreactive axon terminals that formed synaptic specializations with rostral LC dendrites (62), and were later found to be primarily co-localized with excitatory amino acids (63), suggesting that CRF afferents to this region directly control LC neuronal excitability and activity (64).

Wylie Vale et al. seminal work characterized a 41-residue peptide found to induce corticotropin-like and endorphin-like immunoreactivity in the anterior pituitary cells. Subsequent studies by this group characterized CRF-immunoreactive nerve fibers in the hypothalamus (65), its effects on the sympathetic nervous system and metabolism (66), and its role in stimulating the release of ACTH during stress (67). Further, Vale's group identified the CRF receptor 1 (CRFR1) and three urocortin receptors (68). Vale's continued study of the system led to the discovery that human patients with major depression had elevated CRF (69) and that stress could inhibit reproductive function in the rat (70). Other fascinating studies found that CRF could stimulate the secretion of the chemokine IL-1 involved in the immune response under conditions of stress (71). In many ways, Vale et al. findings echo and further validate Cannon and Selye's observations of the profound effects of stress on the body.

At this time, scientists believed CRF could mediate stress-induced LC activation based on anatomical evidence that demonstrated dense CRF-immunoreactive fibers within the dendritic pericoerulear regions surround the LC core (72). The refinement of axonal tract-tracer technology enabled a complete understanding of this region and others, as it allowed for examining connectivity between brain regions. Tract-tracing experiments can employ two types of tract tracers. The first, anterograde tracers, are taken up into the cell via endocytosis in the somatodendritic processes of neurons and then transported to their axon terminals where they may be detected using various immunohistochemical techniques including TEM. Alternatively, the second type of tract tracer travels retrogradely, as axon terminals endocytose the tracer and transport it back to the soma. The first anterograde tract tracer (73) was derived from the kidney bean lectin Phaseolus vulgaris leucoagglutinin (74) in 1978. Retrograde tracers, including fluorogold and wheat germ agglutinin, are commonly used retrograde transporters that have been used in combination with TEM (75–80). More recent approaches to mapping neural circuitry also include genetic tracers embedded within recombinant viruses that can travel across synapses to define multiple synaptic connections (81–83). Studies that employed tract-tracing techniques with immunocytochemistry revealed that dense bundles of neuronal fibers extend from diverse brain regions (84) and are composed of distinct neurochemical profiles that vary by brain region (85, 86).

Later physiological studies supported earlier neuroanatomical studies by confirming that CRF engages the LC during acute and chronic cognitive and physical stressors (64, 87, 88). These methods helped establish that the axon terminals of several brain structures release CRF onto the LC (72, 89). When presented with a stressful stimulus, afferents from the paraventricular nucleus of the hypothalamus release CRF onto the anterior pituitary (90) and onto LC neuronal cell bodies and dendritic zones (91), regions of the LC densely populated with CRFR1 (92). The activation of this pathway during the stress response came to be understood as a parallel but intricately connected process that occurs alongside the HPA axis-mediated peripheral stress response. Depolarization of LC neurons results in the production and release of NE from axon terminals throughout the neuraxis (93, 94). This has been demonstrated in microdialysis studies investigating the effects of restraint, tail shock, auditory, and hypotensive stressors on extracellular levels of NE in the terminal areas of the LC (95, 96). Of particular interest are the afferents expressing CRF from the central nucleus of the amygdala, which are thought to activate the LC to engage cognitive processes in response to environmental stressors (72, 91) thus, has been conceptualized as the cognitive limb of the stress response (64).

LC-NE Anatomy, Physiology, and Function in Arousal

Initially described in 1929, Berger described an instrument capable of measuring electrical activity waves that send pulses across the brain (97). Today, those electrical pulses are known as neuronal oscillations, and they are measured as a frequency in Hertz (Hz). Because the EEG directly measures neural activity with very high time resolution, it is considered an outstanding tool for studying the broad range of neurocognitive processes that dictate human behavior (98). When the full potential of the EEG came to fruition, it brought revolutionary advancements to the scientific research community as investigators across disciplines characterized the stages of the sleep-wake cycle, epileptic seizures, and for the first time, glimpsed global brain dynamics.

The EEG provides information about the speed of an oscillation (frequency), the amount of energy in a frequency band (Power, expressed as amplitude^2), and the amount of synchronization across neurons (Phase; measured in radians). These are the essential elements of modern neural dynamics and

set the foundation for the rapidly growing field of Neural Field Theory. Five EEG frequency bands are defined by the frequency ranges in which they occur and are associated with diverse cognitive processes. The delta band (1–4 Hz) is the slowest but has the highest amplitude and is related to non-REM slow-wave sleep (SWS) (99). The theta band (4–8 Hz) is associated with mental tasks that require a high degree of focus, such as learning or recalling information (100). Meanwhile, the alpha-band (8–12 Hz) that was first described in Hans Berger's initial 1929 publication is now associated with sensory, motor, and memory functions and is known to be active during states of mental or physical relaxation while the eyes are closed (100). In contrast, with open-eyes and in a task-focused state, alpha frequency waves are suppressed. The higher frequency Beta Band (12–25 Hz) is associated with anxious thinking or active concentration (101), and while less is known about the highest frequency gamma-band (>25 Hz) (99), abnormalities in this frequency are common in schizophrenia and likely reflect large scale network dysfunction (102).

The use of the EEG in research observing brain states provided the first evidence for the involvement of the LC and other brainstem structures in arousal. The pivotal work of Moruzzi and Magoun revealed that stimulation of the reticular formation elicited an excitation that ascended from the lower bulbar region of the brainstem, through the pons, midbrain tegmentum, and into the caudal diencephalon (103). Moreover, the activation of these brain structures resulted in the switching of EEG high-voltage slow-wave activity to low voltage fast activity. Importantly, the resulting ascending activation resulted in the de-synchronization of cortical structures. As clinician-scientists, Moruzzi and Magoun were struck by the similarity of this response to the subtle but significant alpha-wave blockade of EEG activity that was commonly observed when patients turned their attention to a visual stimulus and characterized the state transition from sleep to wakefulness (103). The thalamic and reticular regions involved in this activation response collectively became known as the reticular activating system (RAS). The degree of RAS activation could induce three distinct arousal states: waking, SWS, and rapid eye movement (REM) sleep (104).

Subsequent lesion studies implicated a mid-pontine, pre-trigeminal area of the brainstem in behavioral state-dependent EEG activity that was varied when the animal was awake and alert but synchronized during sleep (105). Subsequent pharmacological studies indicated that arousal EEG patterns induced by injection of NE had an activation site at the midbrain level (106), showing a close relationship between thalamic and reticular sites during sleep-wake cycles (107). Other physiological properties of LC neurons came to be understood by electrophysiological studies enabled by the development of tungsten electrodes. Hubel's advancement to probes composed of sharpened tungsten enabled physiologists to record much smaller neurons and axons (20, 108). Now armed with pharmacological agents, EEG, and electrophysiology, neurobiologists had the unprecedented insight into the brain's neural underpinnings, which allowed for a closer, more detailed understanding of small groups of neurons such as the LC. In a seminal study of this nature, Chu and Bloom used microelectrodes combined with EEG to correlate LC discharge activity with global brain wave activity in parallel with behavioral observation and reported sleep-stage dependent changes in discharge patterns (109). Later studies by Aston-Jones and Bloom not only confirmed but also more accurately described the involvement of LC-NE activity in RAS. Their findings demonstrated that LC tonic discharge rates were highest in the waking state, slower in SWS, and absent in REM sleep (110), thus firmly establishing a foundation for LC-NE involvement in the RAS that controls sleep-wake cycles.

It is important to note that the spectrum of arousal states is the result of a distinct balance of multiple transmitters derived from several brain structures. The discovery of Orexin peptides in the lateral hypothalamus (111) and the subsequent immunohistochemical characterization of their distribution throughout the CNS were the first to suggest a complex multi-transmitter integrative system controlled arousal (112). Dense orexin-immunoreactive fibers in the LC were found to have a significant excitatory effect *in vitro* and were demonstrated to have an essential role in mediating transitions of sleep to wake *in vivo* (112–114). Orexin also innervates the basal forebrain, a primarily of cholinergic and GABA-ergic nucleus that is also sufficient for cortical activation and transition from NREM sleep arousal. Although orexin has different effects on cholinergic and non-cholinergic cells of the basal forebrain, both stimulate the transmission of acetylcholine in the cortex, resulting in stimulating wakefulness [reviewed in (115)]. Interestingly, while orexin clearly plays a pivotal role in wakefulness and arousal, it is considered a stabilizer of wakefulness rather than a sole determinant. This stems from selective optogenetic studies that demonstrate an inability of orexin to stimulate sleep to wake transitions under conditions of selective LC silencing. Thus, it has been proposed that a common theme of orexinergic actions is the integration of homeostatic or external goal-oriented signals of danger or reward to promote motivation (116).

Early studies on LC stimulus-evoked neuronal responses in unanesthetized monkeys indicated that iontophoretically applied NE was more effective in reducing spontaneous cortical activity "noise," to a greater extent than it could reduce the activity evoked in response to the stimulus "signal" (117). Moreover, during excitatory responses, NE reduced a more significant proportion of low-discharge rates than high-discharge rates in cortical regions (117). This was a critical discovery, as it suggested that NE could silence some signals while enhancing others. Further physiological studies in the awake rat demonstrated that LC-NE neuron activity varied as a function of sensory stimulation and arousal. These studies were the first to show that LC neurons responded vigorously to mild, non-noxious, physiologically relevant stimuli, and the previously established responses to noxious stimuli under anesthesia (118). Notably, electrical stimulation increased neuronal responses to strong or preferred stimuli while decreasing responses to weak inputs, thereby enhancing "signal-to-noise" ratios in target cell impulse activity (110). This concept pervades the LC-NE literature even today, as interpretations evolve with the field (6, 119).

A critical line of inquiry still under investigation is in determining how salience is encoded and how a system the system involved in such encoding filters and initiates

memory processing in the hippocampus. Previous studies had demonstrated that phasic activation of the LC produced a protein synthesis-dependent long-term potentiation 24 h after stimulation, suggesting that phasic firing of the LC could encode the initiation of long-term memory processes (120). Subsequent studies discovered a mechanism by which the LC may facilitate such an effect by identifying two sub-populations of functionally distinct inhibitory interneurons in the dentate gyrus that respond to NE in an opposing manner (121). It was demonstrated that NE could increase the excitability of one population of interneurons while decreasing the excitability of the second population of interneurons (121).

The Evolution of Arousal Theory

Cognitive psychologists focused on measures of attention and arousal in their study of "stress," resulting in the theoretical emergence of a two-stage process of stimulus-related information processing required for a stress response to occur. The first stage, termed the "evaluative reflex" (122), was believed to be an automated, non-specific response to environmental stimuli followed by the second stage of higher-order cognitive information processing. Here, many studies utilized experimental paradigms that assessed the impact of various levels of increasing task difficulty or mental load during tests of visual attention. Early studies suggested that an optimal level of stress, embodied by the inverted-U shape curve, could be achieved to maximize performance on cognitive and physical tasks (123). While this original study has been heavily scrutinized in more recent years (2, 123), ultimately, multiple lines of evidence converged on the idea that moderation was key. Easterbrook's studies of human performance in the context of stress supported the notion in his observations of his personal experiences that suggested, while long-term (chronic) stress was detrimental, a certain amount of short-term (acute) stress could be beneficial in performing physical and cognitive tasks (124). Stress neurobiologists of today have generally come to accept a conceptual paradigm in which stress and workload can elicit a reduction in an individual's perceptive field or ability to scan the environment for cues, thus creating a "tunnel" of attention.

One of the most significant concepts of this time, the Tunnel Hypothesis, was guided by Easterbrook's observations and others in the study of attention and information processing under conditions of stress. In line with this rationale, the continued study of arousal and the processing of auditory stimuli brought to light an understanding that noise could increase arousal and result in a narrowed attention span (125). In other words, in the aroused state, information processing becomes limited to stimuli related to environmental cues, particularly those that may signal threat, such as noise. This led to the first assertion that arousal may moderate information processing during stress (2). The work of Beatty supported this notion, demonstrating correlated changes in pupil diameter with increases in task difficulty, finding that pupil dilations had a significant relationship to information processing (126). From this direct relationship, these investigators inferred that pupil dilation could be an indicator of resource mobilization. To further understand how stress may hinder performance, Rachman et al. studied performance on cognitive tasks under conditions of bomb disposal. Subjects were described as experiencing significant physiological changes consistent with anxiety and fear, including increased heartbeat, labored breathing, and trembling (127). The subsequent observation that once a stress or anxiety response was provoked, mental resource allocation could be diverted away from the task at hand and toward task-irrelevant stimuli, resulting in performance deficits (128). This idea is still relevant today as cognitive psychologists continue to discuss theoretical mechanisms for selective attention and the ability of stress to both improve or weaken performance in a state-specific manner.

In his theory of "stress," Lazarus influenced the field by suggesting that psychological stress occurs when a situation is perceived as threatening (129). This concept gained importance over time, as Kahneman put forth the notion that cognitive processing required mental resources that are limited in quantity (130), and was coined by Norman and Bobrow as the limited-capacity resource model (131). Thus, Kahneman conceptualized cognitive tasks as processes that compete for resources that are allocated based on the energetic requirements for the neuronal populations involved (metabolism of glycoproteins or blood flow/oxygenation) (132, 133). Ultimately, this came to be understood from an evolutionary perspective that echoed Lazarus's focus on "threatening" stimuli. It was conceived that cognitive tasks required for- or related to- survival would be prioritized in terms of importance and resource allocation. This ideology likely influenced the concept of "salience," which describes the prioritization of a stimulus or mental representation based on its importance in achieving a goal. In the case of a threatening stimulus, survival-related actions or stimuli would be designated as a high priority. The related "neural representations" would be conveyed as salient information, and therefore would be granted more resources to survive. Wickens et al. asserted that the performance of tasks was fundamentally dependent on a pool of limited resources, using capacity, attention, and effort as examples (134). Wickens went on to create a formula for optimal performance, in which he proposed that optimal performance would be equal to the resources available divided by the task difficulty (135).

The late 90's into the early 2000s brought some clarity in the conceptual delineation of arousal from stress. Razmjou provided a framework for the concept of arousal (136), asserting that, "arousal is a hypothetical construct that represents the level of central nervous system activity along a behavioral continuum ranging from sleep to alertness" (p. 530). This concept was further developed with a comprehensive definition of stress, in which Wofford and Daly proposed that there were three domains of a stress response. The first domain, physiological arousal, included physiological indices such as heart rate, blood pressure, and temperature. The second domain composed psychological responses such as dissatisfaction, anxiety, sleep problems, depression, or irritation (137). Finally, the third domain was that of behavioral responses, for example, job performance, drug abuse, eating disorders, aggression, or poor relationships (2, 137). Moreover, Gaillard et al. used the concept of energy mobilization to distinguish the arousal further- related mental load from stress, stating that mental load represents the energy mobilization

required to adapt to an environment in a healthy manner, while stress induces a heightened state of activation that, presumably requires increased energy mobilization, and fails to improve performance (138). Thus, while the field of Cognitive Psychology did not determine a unitary model of stress, the work of many researchers converged on the multifaceted nature of arousal, that, when paired with a perceived inability or insufficient resources, could manifest as a stress response that was composed of physical, cognitive, behavioral or performance characteristics.

Later, a growing interest in the effects of stress on attention led to the concept of active and passive attentional states that were driven by top-down and bottom-up information processes, respectively. Ohman et al. asserted that top-down processing implies a voluntary action in which the organism directs attention, while bottom-up processing was a stimulus-driven process that involved environmental cues to draw-in attention (139). The renewed interest called back to earlier models of attention that focused on a reflexive evaluation followed by an appraisal by higher-order cognitive processing. Rohrbaugh viewed the purpose of the orienting reflex as preparation for stimulus perception (140), and it likely occurs before higher-order cognitive assessment (141). Additionally, Sokolov and Vinogradova suggested that once a given stimulus is detected, it enters into a pattern recognition system through which it is compared to a pre-existing "library of internal representations (neuronal models) of previous stimulations" [(133), p. 324]. Further developments from Crawford and Cacioppo examined the asymmetrical and negative bias that humans have toward the automatic processing of information, concluding that humans are wired to evaluate the environment and that this evaluation likely takes place subcortically, before any conscious awareness of emotion or higher-order cognition occurring (122). The importance of these developments will be clarified in future sections that describe the role of the LC-NE system in information processing.

The LC-NE System and Allostasis

The LC-NE system is uniquely positioned to influence global brain states, evidenced, at least in part, by the finding that NE can be released from synaptic boutons to effectively diffuse beyond the bounds of a typical synapse to interact with adrenergic receptors (AR) on surrounding neurons and glial cells (142, 143). This becomes particularly important when considering the protective role of NE in modulating central inflammatory responses (144). Thus, the design of LC neural architecture reflects its broad functionality, as it is critical for promoting attention, wakefulness, and cognition (145), processes that require the coordination of multiple brain regions across large scale networks (146, 147). Several lines of evidence indicate that LC activation is sufficient to initiate and maintain states of arousal, and LC activity is positively correlated with vigilance (148) and attentiveness, supporting the idea that the LC mediates scanning of the environment for potentially threatening stimuli (64, 145). Further, LC discharge frequency indicates state changes in arousal and attention processing. For example, low rates of tonic activity suggest a state of potential hypo-arousal and is associated with disengagement from the environment (145). In contrast, optimal tonic activity levels reflect an arousal state associated with high responsivity to sensory stimuli in the environment. Optimal tonic activity in this state enables phasic burst firing, in which electrotonically coupled LC neurons fire together in synchrony (149, 150). This level of arousal is associated with focused attention on task-related, novel, or unpredictable stimuli (145, 146, 151).

Amidst the progress of charting the involvement of CRF in stimulating the LC-NE system in the physiological response to stress, Sterling and Schulkin and Sterling introduced the concept of allostasis as a process of reestablishing stability in response to a challenge (152, 153). Shortly after, Bruce McEwen built upon this concept by introducing the concept of "allostatic load" and "allostatic overload" (154). McEwen argued for the reinterpretation of Selye's General Adaptation Syndrome based on the understanding that stress could have protective and deleterious effects on the body. McEwen reinterprets Selye's alarm response as "the process leading to adaptation, or allostasis, in which glucocorticoids and epinephrine, as well as other mediators, promote adaptation to the stressor." Perhaps the most significant reinterpretation arises in McEwen's view of the third stage, in which he asserts that exhaustion of defense mechanisms was insufficient to account for the alterations in the body. Instead he proposed that at a certain point, the stress mediators themselves no longer served a protective role, but rather became counterproductive, exacerbating damage to the body. Thus, noting that the word "stress" is overused and imprecise, he introduced the term "allostatic overload," defined as circumstances under which the hormones of the HPA axis, catecholamines, cytokines, and other physiologic mediators are over-worked, resulting in active contributions to the cumulative effects of daily life. Importantly, McEwen's framework emphasized the wear and tear of the regulatory systems caused by these mediators in the brain and body (155). McEwen states further that allostatic overload is a state in which physiological mediators are no longer purposeful, and these circumstances predispose individuals to disease (156, 157).

MODERN INTEGRATED PERSPECTIVES OF AROUSAL AND THE LC-NE SYSTEM

The Growing Field of Large-Scale Network Dynamics

The emergence of brain imaging technologies, first Positron Emission Topography (PET) in 1974, and subsequently Blood Oxygen Level Dependent (BOLD) functional magnetic resonance imaging (fMRI) in 1990, enabled scientists across the spectrum of neurobehavioral research to collect and analyze precise spatial information about the brain regions that gave rise to cognitive processes [(158); reviewed in (159)]. From these developments, emerged a number of new strategies to process and interpret fMRI data (160). Seed-based correlation analyses deliver information about the activity of a pre-selected region of interest in relation to the rest of the brain. Principal Component Analysis is another common univariate approach that focuses on the high-resolution spatial component of the data rather than

the limited temporal information available (161). These analyses were sufficient to enable researchers to couple a specific observed cognitive task or deficit to a localized brain region. However, because BOLD is a fundamentally metabolic measure of neural activity, the temporal specificity of these scientific observations is limited by hemodynamic response time. This translates to the identification of broad stimulus-response areas of the brain without an understanding of the functional specificity of each brain region that could only be parsed out with high temporal resolution.

A pivotal advancement came with the discovery that fMRI and EEG could be used in combination to derive high-resolution spatio-temporal information about neuronal events at the timescale of tens of milliseconds (162). This advancement necessitated the development of more complex analytical systems that are capable of collecting, processing and integrating large data sets. The advancement of computational neuroscience has addressed this need, as model-based (163) and data-driven EEG-fMRI fusion techniques, including independent component analysis (ICA) and canonical correlation analysis, have been described (164, 165). Structure-function connectome analyses of EEG-fMRI data using graph theory translated to maps of nodes (brain regions) connected by edges (network connections). The strength of within-network connections (between nodes in the same network) could be represented by assigning a weight based on the correlation coefficient between the two brain regions (160). This enabled researchers to hypothesize about the time-dependent functional roles of specialized regions within a system that can dictate complex behavioral responses when brought together at a network level. This conceptual line of inquiry, combined with continued methodological advancements, shifted our understanding of the neural basis for cognition. The advent of ICA moved the field away from univariate models that coupled specific deficits to individual brain regions and toward multivariate approaches. ICA uses global EEG-fMRI temporal and spatial data to identify Intrinsic Connectivity Networks (ICNs), sub-components of the data composed of highly connected large-scale brain regions whose activity could be reliably observed during a specific set of cognitive responses (166).

Identification and Characterization of Intrinsic Connectivity Networks

In their synthesis of Arousal Theory with PET and BOLD fMRI data available at the time, Corbetta and Shulman build on the concept of top-down and bottom-up processing of information during the orienting reflex, suggesting that attention is controlled by two neuroanatomically defined systems (167). The Dorsal Attention Network (DAN) relies on top-down cognitive information processing to build task-related stimulus-response maps that pair cognitive cues with associated motor responses. In contrast, the Ventral Attention Network (VAN) is concerned with the salience or novelty of a stimulus in the environment (168). In this system, bottom-up sensory cues in the environment can interrupt an ongoing task-related cognitive activity to quickly reorient attention (within 50 ms) to novel or infrequent events. In broad strokes, Corbetta and Shulman argue that DAN is primarily an "endogenous orienting system" while VAN is an "exogenous orienting system" (**Figure 1**). Considering the trajectory of this line of research, the authors provide a functional interpretation of data beyond their time. This is relevant in light of the findings of Schupp et al., who affirmed the earlier ideas of cognitive psychologists that conceptualized a two-stage process of stimulus-related information processing, including an automatic "evaluative reflex," followed by a stage of higher-order cognitive information processing (169). While investigating electrocortical activity during the processing of emotional images using event-related potentials and fMRI, these investigators found that the processing of emotional images is related to the degree to which the stimuli emotionally engage individuals. Those with strong affective cues were processed more for both pleasant and unpleasant images. It was determined that selective attention to the stimulus's location occurred within 100 ms and that attention to its features such as color, orientation, and shape occurred between 150 and 200 ms (169).

The continued investigation of cognitive processes using combined fMRI and EEG led to the discovery of the first resting-state large-scale brain network. Studies in non-human primates and humans converged to identify the Default Mode Network (DMN), defined by core regions that were reliably activated during internally focused tasks such as forming perceptions of others or retrieving memories (170–172). The discovery of the DMN brought about a new line of investigation concerning the maintenance and switching between brain states, particularly transitions from DMN-mediated internally focused activities to those that involved the external environment (173). In this regard, Corbetta and Shulman's DAN could account for attentional control involved in the preparation, coordination, and execution of motor responses to task-related stimuli, while the VAN could facilitate task switching upon exposure to a novel or infrequent sensory stimulus in the environment (167). During this time, an ICN engaged selectively during working memory, problem-solving, and decision making, was discovered and called the Central Executive Network (CEN) (174).

Regions of the VAN are often combined with several limbic structures to collectively form the Salience Network (SN), which detects, integrates, and filters incoming sensory, autonomic, and emotional information to determine the relative importance of a stimulus (4, 8). These discoveries shifted our understanding of the neural basis for cognition to a paradigm focused on a balance between three core ICNs: the DMN, CEN, and SN (8). In this triple network model, each ICN serves a specialized function, that when optimally coordinated, results in the emergence of cognition, goal-directed, and stimulus-directed behavior [(8, 175); **Figure 2**].

The Hierarchy of Large-Scale Networks Dictates their Functional Balance

In terms of network analysis, the strength and direction of a relationship between large scale networks are represented as edges weighted by between-network correlation coefficients. Importantly, in addition to positive correlations (excitatory/engaged), there can also be negative or anti-correlation (inhibitory/disengaged) between edges (177). Anti-correlations were once thought to be an artifact; thus,

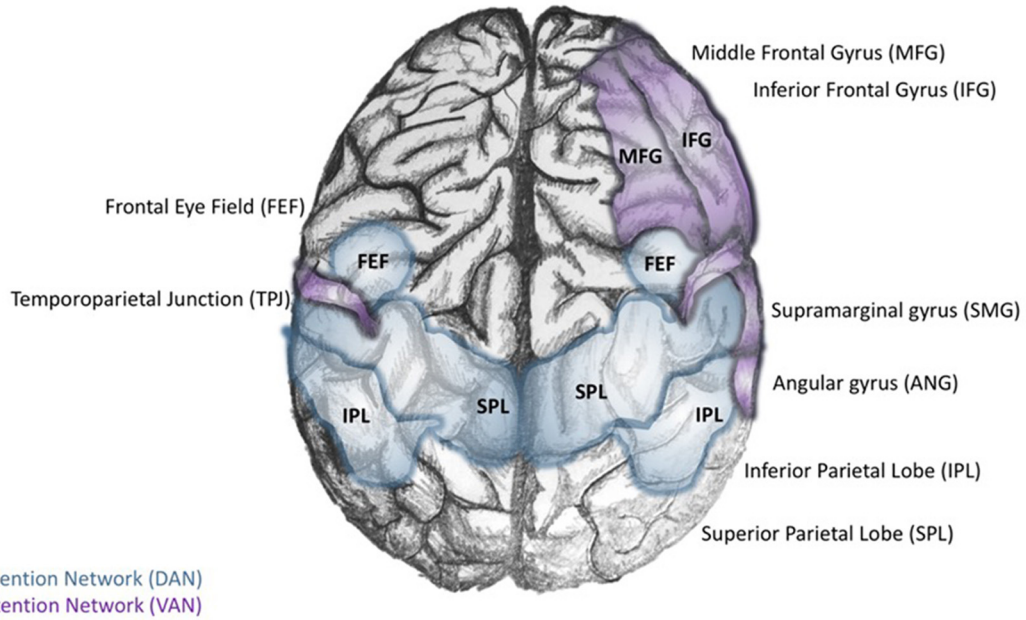

FIGURE 1 | Core regions of the dorsal and ventral attention networks. The Dorsal Attention Network (DAN; blue) is defined by the interconnectivity of the dorsal posterior parietal lobe (dPPL) and dorsal frontal cortex [dPFC-Frontal Eye Field (FEF)]. The activity of the left dPPL may be a preparatory step for stimulus-response tasks; selective attention to task-relevant stimuli results in the development of cognitive cues. Meanwhile, activity in the (dPFC-FEF) coordinates motor responses to the task-relevant stimulus. In contrast, the Ventral Attention Network (VAN) is anchored in the right temporoparietal lobe (TPL), and ventral frontal cortex (vPFC; composed of the inferior and middle frontal gyri and the frontal operculum).

many forms of data "optimization" methods only took positive values into account by pre-processing the data using binary thresholds in which values below the threshold became zero and above the threshold became one (178, 179). However, several recently developed approaches, such as Path Length Associated Community Estimation [PLACE; (180, 181)], and Probability Associated Community Estimation [PACE; (177)], that do not use thresholds or signal-magnitude for correlations overcome these challenges. In particular, PACE is designed to interpret higher probabilities of an edge being correlated or anti-correlated as optimal relationships to capture because regardless of their direction, those relationships are more likely to reflect connectivity between different regions or different networks (177).

These factors become important when considering how large-scale networks interact with each other. Based on their specialized functions, it is advantageous for large-scale networks to operate independently, in synchrony, or in opposition, under various circumstances. The DMN, known to be engaged during stimulus-independent tasks or those related to internal thought, is usually suppressed during CEN activation (182–184). While mechanisms of large-scale network suppression are not well-understood, it is well-established that the suppression of opposing networks such as the DMN and CEN is crucial for the function of specific cognitive processes, including focused attention, working memory, and other executive functions (185). In line with this, a reduced ability to suppress DMN activity or effectively switch between DMN and CEN states has been linked to a wide range of cognitive and psychiatric disorders (8, 170).

A recent study employed hierarchal cluster analysis and spectral dynamic causal modeling to demonstrate that core regions of the SN and DAN that terminate in core regions of the DMN were negative (inhibitory), while connections arising in the core of DMN that terminate in SN or DAN regions were weakly positive (excitatory) (186). Moreover, there were positive (excitatory) bidirectional connections between SN and DAN. Further analysis of the effective connectivity matrix confirmed that the SN was highest in the hierarchy, suggesting that it may play a role in switching between anti-correlated networks (186). It is important to note, however, that these authors included the left and right anterior PFC, including the dlPFC (BA9), which is typically considered a core hub of the DMN. Indeed, the large-scale network literature has inconsistencies in nomenclature, as well as diverse analytic methods, that make direct comparisons between studies difficult (187).

Is the LC-NE System a Master Switch?

In discussing their physiological study on LC responses under different stages of arousal in 1981, Aston-Jones and Bloom proposed that the LC-NE "system may serve to facilitate transitions between global behavioral states" (110). At the time, these investigators reported the spontaneous discharge of LC-NE neurons across the sleep-wake cycle. However, nearly 40 years later, the notion that the LC plays a role in transitioning between global behavioral states is a continued investigation.

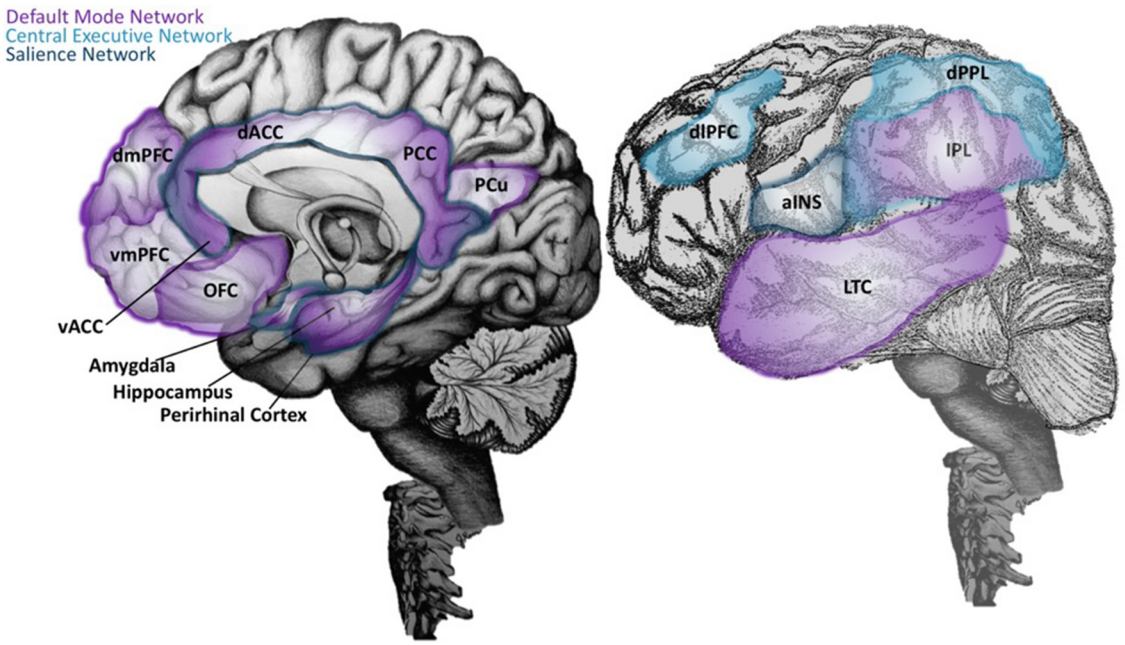

FIGURE 2 | Neuroanatomical structures of the Default Mode, Central Executive, and Salience Networks. On the right, an illustration of the brain with mid-sagittal view reveals key neuroanatomical structures of the Default Mode Network (DMN) (purple), which includes the ventral medial prefrontal cortex (vMPFC; or infralimbic region), posterior cingulate cortex (PCC), precuneus (pCu), inferior parietal lobe (IPL), lateral temporal cortex (LTC), the dorsal medial prefrontal cortex (dMPFC), and the hippocampal formation (170, 171). The most recently evolved dorsolateral prefrontal cortex (dlPFC) and the lateral posterior parietal lobe (PPL) are key regions of the Central Executive Network (CEN; light blue) (174), and are depicted in the lateral view of the brain in the left panel. In the same panel, the anterior insula (aINS) is labeled, along with other regions of the Salience Network (SN; dark blue) that are visualized on the mid-sagittal illustration in the right panel. This includes the dorsal anterior cingulate cortex (dACC), amygdala, and ventral tegmental areas. The SN detects, integrates, and filters incoming sensory, autonomic, and emotional information to determine the relative importance of a stimulus (4, 176). Regions labeled with two or more colors are overlapping network areas that are believed to be important for between-network interactions.

Dalley et al. proposed that an influx of NE to the PFC for novel stimuli may signal a mismatch between action and reward to promote behavioral modification (188). An action of this sort would require a switch from the current activity to a new behavioral response, an observation that Corbetta and Shulman likened to the "circuit-breaker" function of VAN when unexpected or novel stimuli are detected [reviewed in (167)]. Further inspired by the work of Corbetta and Shulman, Aston-Jones et al., Pardo et al., and Morrison and Foote, suggest that the activity of the right-lateralized VAN depends on LC-derived cortical NE, which is more densely concentrated in the right hemisphere, and implicated in arousal, vigilance, and selective attention (167, 189–191).

In a severe departure from the heightening complexity of LC-NE functional theories at the time, Bouret and Sara put forth the elegant notion that the evolved LC-NE system operates analogously to a small number of synchronized neuromodulatory cells observed in crustacean (146). The crustacean neuromodulatory system can abruptly re-orient widespread neural networks to adapt to environmental conditions, a fascinating parallel to the widespread environmentally engaged activity of the LC system. Further, the two systems share global reaching targets capable of inducing abrupt, widespread changes in activity on a global scale (146). Together with an understanding of LC-NE system dynamics, investigators in several groups suggested that the LC initiates brain state changes in response to the environment to facilitate behavioral responses tailored to the most critical information. Thus, it has been proposed that the activation of the LC and release of NE terminates the resting state and begins a brain-state adjustment that involves cortical, subcortical, and autonomic activity, to facilitate focused attention (171, 173, 184, 192) and allows for behavioral output toward a task-related stimulus (119). Bouret and Sara propose a model in which the NE signal has a general reset function that mediates changes in widespread forebrain networks that are mediating specific cognitive functions. These investigators go on to present evidence using a multi-electrode dual recording of LC and cortical neurons to demonstrate that both within-trial and between trials, LC neuron depolarization occurs before forebrain neuronal activity and is closely related to shifts in cognition. Furthermore, the investigators demonstrate that LC-mediated stimulus-induced cognitive shifts do not occur in the absence of an external cue or when the presentation of a stimulus is predictable, thus delineating LC-NE induced changes are not a reflection of decision making or reward anticipation, but rather to facilitate the dynamic reorganization of neural networks to adapt to a changing environment quickly (146).

As previously discussed, during task states that do not require focused attention, the LC-NE system is characterized by a high tonic baseline, and investigators have proposed that this state is linked with the generation of spontaneous thoughts (193). In contrast, moderate LC tonic activity levels may promote optimal engagement in the environment to enhance performance in task- goal- or survival-oriented behaviors (119). Thus, it has been suggested that the deactivation of certain regions overlapping with DMN may be caused, at least in part, by high tonic activity associated with the LC-NE system, reflected by theta oscillations (194). A recent study utilizing a chemo-connectomics approach that employed Chemogenetic designer receptors exclusively activated by designer drugs (DREADDs), paired with resting-state fMRI, supports this notion (195). The investigators demonstrate that selective LC activation rapidly increases brain-wide functional connectivity in a manner consistent with AR distribution and the processing of salient information (195).

The opposing view does not disqualify LC-NE involvement in the switch *per se* but instead asserts that the anterior insula, a critical component of the SN, initiates the switch in behavioral states through large-scale network dynamics. This side argues that effective switching between brain states is at least partially dependent on the function of the SN, consisting of anterior insular and dorsal anterior cingulate regions (196, 197). In this light, a recent study examining the directional influences exerted by specific nodes of the SN on other brain regions concluded that the anterior insula plays a causal role in switching between the CEN and DMN, two networks that undergo competitive interactions across task paradigms and stimulus modalities and are thought to mediate attention to the external and internal worlds, respectively (196). Specifically, it was found that a touch stimulus resulted in activation of the mid-to-posterior insula, whereas anticipation of the touch stimulus activated the anterior insula and is correlated with the amount of activation in the caudate and posterior insula during information processing of the stimulus (198).

Current Theories on Attention and Arousal

Few authors have attempted to explain the range of network, circuit, cellular, and molecular mechanisms involved in Emotion-Cognition human behavior theories. The two groups that made progress in this ambitious endeavor start by looking at human studies of the visual attention system and go on to describe parallel mechanisms for the selection of high priority stimuli. Both groups refer to a vast literature that, in addition to behavior, covers large-scale networks, local circuitry, neuronal populations, and single neuron responses to stimuli. Interestingly, the conclusions of both studies echo the findings of Aston-Jones and Cohen, who have long described "increased signal to noise" and "gain" as a characteristic of the LC-NE system (119).

The first study is primarily shaped around the concept of attention, which authors Buschman and Kastner define as the "selective prioritization of the neural representations that are most relevant to one's current behavioral goal." These authors propose that this cognitive process arises from an "attentional modulation" system that interacts with pyramidal neurons and inhibitory interneurons to suppress the sensitivity of some stimuli while also increasing high-frequency synchronous oscillations associated with other stimuli. The authors further suggest that this mechanism primarily relies on lateral inhibition (5). While NE is not discussed in the review, the net effect of the proposed mechanism "results in increased sensitivity and decreased noise correlations." The authors conclude that the cognitive process of attention is a result of increasing the inhibitory gain of the activated network (5).

The second study, primarily based around the concept of arousal, is defined by Mather et al. as, a state "evoked by emotional events" that "enhances some aspects of perception and memory but impairs others" (6). The authors put forth the Glutamate Amplifies Noradrenergic Effects (GANE) theory (2016) to explain the ability of arousal to both enhance and suppress cognitive processes based on the priority of a stimulus. The GANE model proposes that arousal-induced NE release from the LC biases perception and memory to magnify the signal of salient, high priority neuronal ensembles while suppressing the signal of lower priority ensembles. In order to do so, the model proposes that the phasic activity of the LC, together with elevated levels of glutamate at the site of prioritized representations, increases NE release, creating "NE Hot Spots" (**Figure 3**). These regions are characterized by glutamate and NE co-release that advance the transmission of high priority neuronal ensembles (6). This excitatory effect is intensified by widespread NE-mediated suppression of weaker, low-priority neural responses via lateral and auto-inhibitory processes (6). This tenor is reminiscent of the 2015 report asserting that "attention" affects neuronal dynamics by altering the responsivity associated with a particular stimulus (5, 199), an effect thought to be mediated by interneuron-mediated lateral inhibition (**Figure 4**). Moreover, this mechanism is thought to normalize activity and suppress competing stimuli, thus resulting in an increased signal to noise ratio (5). Further, the authors suggest that these signals interact within the local cortical circuit to produce oscillatory synchrony so that "relevant representations" are selected and routed through the brain (5). Thus, a critical convergence in cognitive psychology, neuroscience, and neuroendocrinology bring together the concept that LC-NE mediated selective attention highlights salient stimuli, likely signaled by glutamate hotspots (6) and concurrently silencing irrelevant stimuli via lateral inhibitory mechanisms mediated by inhibitory interneurons (5, 6).

Modules of Task-Specific Neuronal Sub-populations: Redefining LC-NE Function

Several recent methodological advancements in genetics and molecular biology have provided neuroscientists with tools to test direct causal hypotheses on neuroanatomical structure and function with exquisite temporal and spatial resolution. The application of intersectional genetics allows one to precisely target a specific cell type using a combination of enhancers and promoters unique to that cell type. The addition of an element for temporal control of the experimental manipulation,

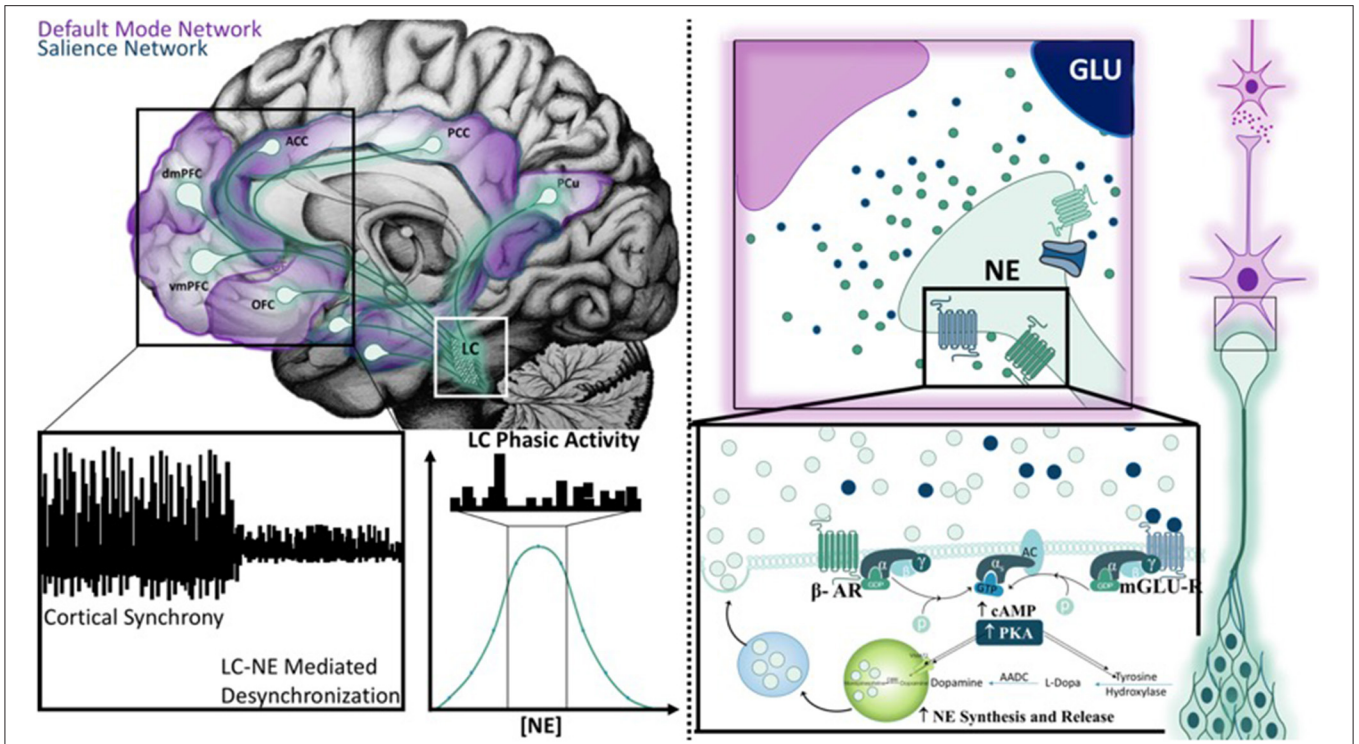

FIGURE 3 | Mechanisms of the Glutamate Amplifies Noradrenergic Effects Theory Hot Spot. The mid-sagittal illustration in the top left corner is overlaid with relevant projection sites of the locus coeruleus system in the context of the default mode and salience networks. Novel stimuli or unexpected events results in a brief cortical desynchronization (as illustrated on the left bottom panel), and is thought to arise from phasic burst firing of the LC (middle bottom panel). The Glutamate Amplifies Noradrenergic Effects Theory asserts that glutamate and norepinephrine work together at "hot spots" to expedite signals from neuronal ensembles that represent salient information. Hot spots develop only when multiple action potentials release high levels of norepinephrine and glutamate is released from nearby terminals. Both signals are mutually enhanced through the activation of β-adrenergic receptors (β-AR), metabotropic glutamate receptors (mGLU-R), and ionic NMDA receptors. As a result, additional norepinephrine and glutamate is synthesized and released to create a hot spot.

such as doxycycline-dependent t/TA or r/TA, allows for an even more powerful approach (200). Stemming from these technologies, genetic coding of microbial opsins that can be directed toward specific cell types with an enhancer-promoter approach enables neuroscientists to activate or inhibit neuronal activity with exposure to light (201). Such unprecedented advances have accelerated the study of neuroanatomy and physiology tremendously in recent years. Researchers using a novel optogenetic approach to study tonic and phasic activity in the LC, confirmed that the LC could fine-tune levels of arousal based on the frequency of its neuronal firing activity (202).

The concept of LC-NE mediated alterations in local cortical network interactions that ultimately dictate global brain states is supported by a study that demonstrates significant arousal induced, NE-dependent influence on cortical dynamics (203). The authors emphasize the potential role of ARs in mediating these effects, noting that low levels of NE during SWS and anesthesia preferentially recruit the high-affinity α-2 receptors coupled to inhibitory G proteins. In contrast, high NE levels during wakefulness may recruit low-affinity α-1 and β-ARs that couple to Gq or Gs proteins (203). More recent studies have examined the relationship between local desynchronization states and pupil-linked arousal in healthy human participants, providing intriguing evidence of LC-NE involvement in global network dynamic balance (204). The investigators used EEG recordings and pupillometry, while participants performed a challenging auditory discrimination task. EEG data from the auditory cortex were analyzed by a weighted permutation entropy algorithm that allowed investigators to capture not only features of desynchronization such as oscillatory power but also central underlying mechanisms detected as fluctuations in excitatory/inhibitory balance (204). This powerful approach elucidated two independent but synergistic processes that contribute to neural gain. First, local cortical desynchronization (EEG entropy) is dictated by regions of the sensory cortex concerned with task-related stimuli and is dependent on selective attention. Second, global brain states arise from processes related to pupil-linked arousal, a mechanism putatively dependent on LC-NE afferent projections. Together, the additive selective-attention mediated (local) gain and propagative arousal-mediated (global) gain optimize sensory perception (204).

An ongoing challenge emerges as we aspire to relate information about large-scale network dynamics on the scale of seconds to hours to the vast electrophysiological and neuroanatomical data measured on fast (sub-second) timescales (205, 206). A recent study approached this herculean task by

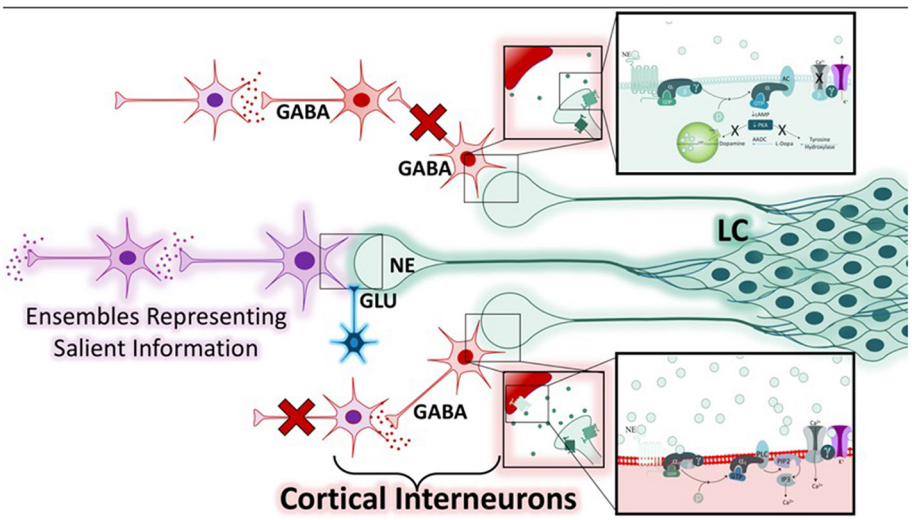

FIGURE 4 | Mechanisms of norepinephrine-mediated lateral inhibition within the Glutamate Amplifies Noradrenergic Effects Framework. While neuronal ensembles transmitting prioritized information are expedited at noradrenergic hot spots (purple), regions containing low levels of norepinephrine and with low or absent glutamatergic input are suppressed. There are two mechanisms by which the noradrenergic system can accomplish this, both involve modulation of GABA-ergic interneurons (red). Depending on the adrenergic receptors expressed, and the concentration of norepinephrine at the synapse, various cellular responses may occur. Two examples are illustrated. The bottom neuronal path shows a cortical interneuron that expresses the α-1-adrenergic receptor (α1-AR) and is activated by moderate levels of norepinephrine. This results in the activation of the $G\alpha_q$ in the post-synaptic cell, resulting in the depolarization of the GABAergic interneuron and inhibition of the neuronal path. The top neuronal path shows two GABA-ergic interneurons with feed-forward connections that are suppressed when pre-synaptic α2-adrenergic receptors are activated by low levels of norepinephrine, resulting in hyperpolarization and inhibition of neuronal firing. Thus, the varied affinity of the adrenergic receptors for norepinephrine, combined with the presence or absence of glutamate, is the cellular basis for this lateral-inhibition response, and ultimately serves to heighten the response of other neurons to salient information.

measuring the activity of mPFC neuronal populations across 0.01–100 Hz frequencies using multi-site silicon probes (206). While slow timescale (global) dynamics were approximated by measuring a neuron's power spectrum on a global scale, fast timescale (local) dynamics that reflect the strength of a neuron's coupling within a population are approximated by frequency. The data, analyzed using frequency domain analysis, revealed no relationship in population neuronal coupling between time scales (i.e., neurons strongly coupled to a population on fast timescales could be weakly correlated to the same population on slow timescales). However, the results demonstrate a significant positive correlation between pupil-linked arousal and neuronal population dynamics at infraslow frequencies of 0.01–1 Hz (206). These findings are consistent with those of Waschke et al., as global, slow-timescale brain states were associated with LC-NE mediated pupil-linked arousal, while fast timescale, frequency-dependent local cortical dynamics, such as those involved in selective-attention within the sensory cortex, were not directly related. Thus, these analyses illustrate the breadth of responses of neuronal ensembles across fast and slow time scales (204, 206).

An excellent, recent review (207) highlights the LC-NE system as a prime candidate for such analyses. LC activation during attention orientation by salient somatosensory stimuli triggers rapid phasic LC responses offset by ∼20 ms (110), while novel visual stimuli elicit a phasic LC response occurs over a timescale of 50–100 ms (118, 208, 209). Further, LC activity may also vary over a few seconds in working memory tasks, as well as over tens of seconds to minutes during cue-induced adjustments in behavioral task-related strategies (188, 210–218). These time-dependent processes have been studied using a combination of pupillometry, microdialysis, and single-unit recordings under conditions of pharmacological manipulation (207). The author also cites instances of behavioral states during learning (219), vigilance, and arousal that may vary over many minutes to hours (110, 118, 220, 221). Importantly, a recent study employing an optogenetic rodent model of LC activation was able to reproduce the temporal dynamics of stimulus-induced phasic LC activity for the first time (151). The study revealed that LC activation modulates cortical encoding of salience in a temporally and cell-type-specific manner in the somatosensory cortex. Two populations of LC-responsive cortical neurons could be distinguished; the first population was directly responsive to sensory detection in time with firing of the LC, while the second population was characterized by a lower basal firing rate and a "gating" long latency signal that only occurred after LC activation. Further, because the population of LC modulated cells could recruit the population of LC gated cells under phasic firing conditions, the authors hypothesize that these cortical cells were specialized to express highly salient information. Because the study exclusively tested photoactivation frequencies that do not induce arousal, these investigators delineated an arousal-independent role for LC in influencing cortical sensory processing, concluding that LC-induced attentional processing does not depend upon LC-NE–induced changes in arousal (151).

Emerging information on the LC-NE system indicates a growing need for computational tools. Until recently, the

LC-NE system had been considered a homogenous nucleus of noradrenergic cells that fire synchronously and serve as a reactionary system that both centrally alerts and peripherally prepares for a behavioral response in the face of environmental threats to survival. However, accumulating data suggest that the LC may operate in a modular fashion, with specialized sub-regions that facilitate specific aspects of behavioral responses and cognition. The data are convincing, presenting anatomical, molecular, and electrophysiological evidence that at least two distinct populations of LC neurons exist that project to the prefrontal vs. motor cortex and are electrophysiologically and biochemically distinct (222). In support of this notion, a recent developmental genetic analysis study identified two subpopulations of LC neurons, a majority of which were derived from the alar plate, and a smaller portion, previously undescribed, is negative for a typical marker of alar-plate derived LC neurons (223).

A Theory of Stress-Induced LC-NE Mediated Network Desynchronization

Compelling pre-clinical (224, 225) and clinical evidence (226, 227) supports the involvement of the LC-NE system in Alzheimer's Disease (AD) via the influence of Ars (**Figure 5**) on the processing of amyloid precursor protein (APP) (228–230), dysregulation of the stress-signaling axis (231), and exacerbation of neuroinflammation (144). Throughout the lifetime, APP can undergo sequential β- and γ-secretase proteolytic processing to release a 42-amino acid length amyloid beta-peptide [$A\beta_{42}$; (232)]. While $A\beta_{42}$ is best known for its role as a self-nucleating peptide that readily forms concentration-dependent neurotoxic aggregates called plaques (233), it is now widely accepted that endogenous picomolar levels of $A\beta_{42}$ are produced and efficiently broken down throughout the lifetime (234). However, the efficiency of $A\beta_{42}$ metabolism is decreased with age [reviewed in (235)], causing an imbalance in the ratio of $A\beta_{42}/A\beta_{40}$, (236) resulting in the formation of amyloid plaques and initiating factors in a cascade of events that lead to neurodegeneration (237).

The work of Muresan and Muresan lay a critical, and potentially underappreciated, foundation for cellular mechanisms of APP processing and transport in brainstem neurons under normal and degenerative conditions *in vitro* (238, 239). Most pertinent to this review, a series of early studies that employ cultured LC-derived CAD cells suggests that the LC may be a region for $A\beta_{42}$ plaque seeding, from which $A\beta_{42}$ oligomers are released over the widespread terminal regions of the LC (240). Further investigation of a subpopulation of CAD cells that were prone to accumulatio of large amounts of intracellular $A\beta_{42}$ at the terminals of their processes revealed significantly increased β-secretase immunoreactivity in noradrenergic axon terminals, that colocalized with $A\beta_{40,42}$ endosomal and autophagic marker immunoreactivity. The authors conclude that the initial seeds of aggregated $A\beta_{42}$ are produced at the neurite terminals of LC neurons that project into the brain regions prone to AD pathology and these seeds then trigger further aggregation of soluble, extracellular $A\beta_{42}$ into plaques, which are eventually exocytosed upon LC neuronal degeneration (240). In line with these findings, recent studies employing an unbiased, semi-quantitative stereotaxic neuropathology approach demonstrate significant changes in LC volume in AD post-mortem brains during various stages of disease progression (241), and suggests LC imaging may have biomarker potential for NE dysfunction in aging diseases (242).

From a large-scale circuit perspective, the Van Bockstaele lab put forth the similar hypothesis that chronic stress, or hyper-arousal-induced hyperactivation of the LC-NE system, would result in the aberrant production of endogenous $A\beta_{42}$ over the course of decades; a dysfunction that would be relatively silent in the early stages of life but would contribute to cognitive dysfunction of dementia later in life [(243); **Figure 5**]. Fundamentally, our hypothesis was inspired by the work of Palop and Mucke, that emphasize a balance between inhibitory and excitatory networks for regular network synchronization and, therefore, cognitive functioning (244, 245). Palop and Mucke demonstrate that network desynchronization resulting from an imbalance in inhibitory and excitatory neurotransmission is a mechanism of cognitive disturbance in several mouse models of AD (245). Our group expanded on this notion by applying this circuit-perspective to the LC-NE system (243). In our review, we present evidence from the literature that the LC-NE system has a profound influence over glutamatergic (246) and GABAergic systems (247). Moreover, we suggest that a disturbance in the LC-NE system, caused by the deleterious effects of chronic stress and exacerbated by the presence of $A\beta_{42}$, would be sufficient to induce the inhibitory/excitatory imbalance resulting in global network desynchronization (243). We emphasize the global architecture of the LC-NE system, its role in modulating the stress response, learning and memory, and inflammatory processes as unique features of the system that are also key disturbances in AD patient populations and post-mortem AD brain tissue (243).

Our laboratory is the first to have localized endogenous $A\beta_{42}$ peptides to LC neurons of the naïve rat *in vivo* using fluorescence microscopy and immunoelectron microscopy (248). Using highly sensitive ELISA for $A\beta_{42}$, we demonstrated that NE and $A\beta_{42}$ levels positively correlate in the naïve rat. In mice null for the DBH gene and rats treated with the LC-selective neurotoxin DSP-4, we found decreased endogenous $A\beta_{42}$ levels. Taken together with studies demonstrating β- and α- AR-mediated production of $A\beta_{42}$, we believe that NE may play a role in modulating $A\beta_{42}$ levels in the LC (248). This notion is supported by earlier pharmacological studies that indicate the activation of β-AR or α-AR can influence $A\beta_{42}$ production, and that stimulation of β-AR on microglial cells can upregulate insulin-degrading enzyme, an enzyme responsible for the break-down of $A\beta_{42}$ peptides (249). Thus, well-established mechanisms of NE-mediated $A\beta_{42}$ production and degradation described in the literature would support our observations.

Subsequent studies in our lab examined the relationship between $A\beta_{42}$ and the LC-NE system using a genetic model of chronic stress (250). The mice employed in these experiments conditionally overexpressed CRF (CRF-OE) in the forebrain upon administration of doxycycline (DOX) in their chow. Male

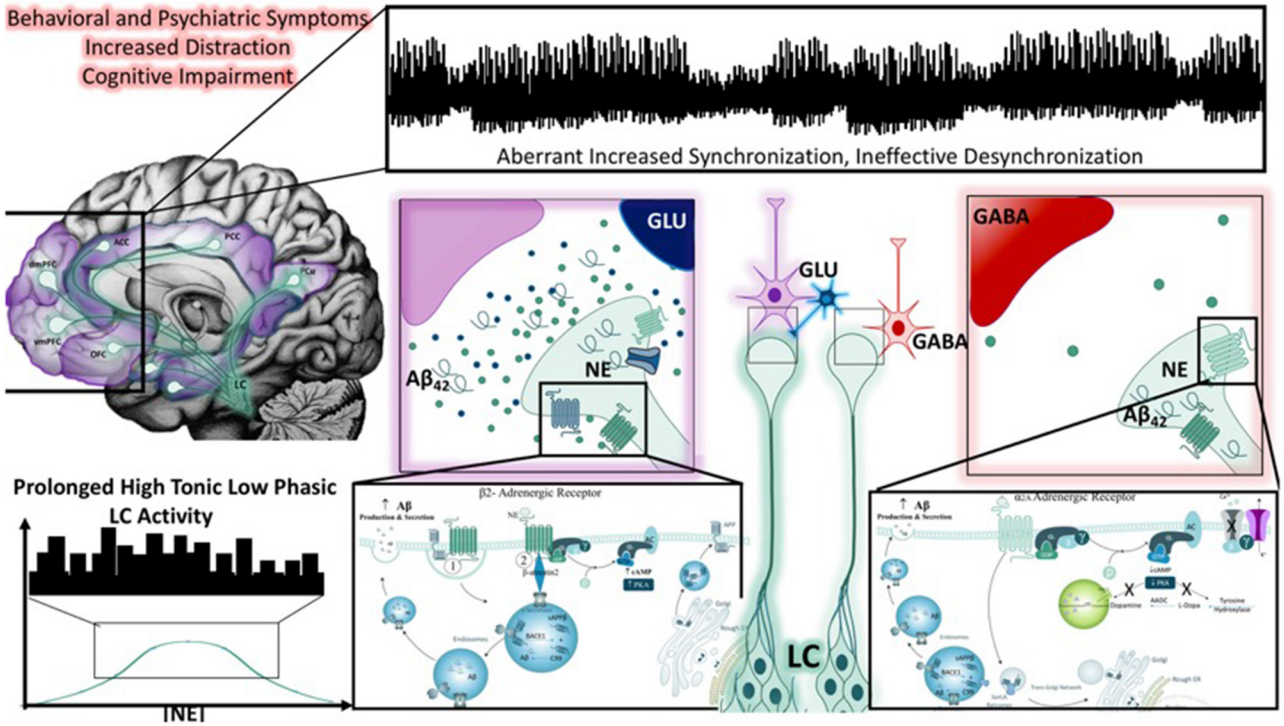

FIGURE 5 | Noradrenergic modulation of $A\beta_{42}$ peptide production and the impact of LC neuronal dysfunction. Behavioral and psychiatric symptoms resulting from global brain impairments have been associated with a number of stress-related disorders, including the dementia resulting from Alzheimer's disease. Throughout the literature, studies have demonstrated that cognitive impairment and epileptiform activity associated with dementia are associated with aberrant increased cortical synchronization, and reductions in desynchronization (illustrated in top panel). This may also reflect the inability of large-scale network switching between the default mode and salience networks, an activity thought to be heavily influenced by LC-NE activity (top left panel). One theory posits that chronic stress, marked by high tonic, low phasic LC activity (as depicted in the bottom left corner), promotes the accumulation of $A\beta_{42}$ peptides LC neurons and results in neuronal injury. As depicted in the right panel, the adrenergic receptors that transmit or suppress information in a concentration-dependent, receptor-dependent fashion, are also known to influence the production of $A\beta_{42}$ peptides. Prolonged periods of such activity, combined with aging could initiate LC neuronal degeneration. Importantly, in the presence or absence of LC degeneration, the impairment or low frequency of phasic firing may impact the transmission of salient information based on a flattening of the inverted U-curve on which the norepinephrine system operates. Salient information would be expected to be less quickly conveyed, and less important information may take longer to suppress.

and female DOX-treated mice were compared to their saline-treated littermates as the control. These studies revealed that CRF OE was sufficient to elicit a redistribution of $A\beta_{42}$ peptides in LC somatodendritic processes in male and female mice without altering $A\beta_{42}$ total protein levels. Under the electron microscope, lipofuscin, and abnormal morphology of lysosomal compartments were apparent, indicating that the compartments that usually clear $A\beta_{42}$ peptides could have been worn down or become dysfunctional with CRF OE (250). We also observed swollen microvessels with lipid-laden vacuoles, a sign of blood-brain barrier dysfunction. Other potential injury signs were evident as CRF OE mice exhibited high glial astrocytic protein immunoreactivity (250).

While considering that hyperactivity of the LC, driven by chronic stress, could be deleterious to the long-term integrity of the LC-NE system, we sought to better understand mechanisms of LC regulation. There is evidence for LC auto-regulation by the release of NE from somatodendritic processes, but only when the firing rate is high (15–20 Hz), likely under phasic conditions (145, 251, 252). The high-frequency action potential most likely elevates residual calcium level in LC somata, which leads to NE release, as shown in chromaffin cells (253). NE is stored in central neurons within SSVs and LDCVs that are present in both cell somata and dendrites of LC neurons (55–57). Thus, we argue that NE somatodendritic release from LDCVs plays a protective role in maintaining LC-NE system integrity by preferentially activating α-2ARs coupled to inhibitory G proteins, therefore, decreasing the excitability of LC neuronal cell bodies.

In line with our hypothesis, a recently published theory presents a compelling argument for the impaired phasic discharge of LC neurons in neurodegenerative disease. Janitzky proposes that the presence of persistent high tonic discharge may impair the function and protective actions of phasic discharge (254). First, the conditions described by Janitzky are consistent with our hypothesis of stress-induced LC-NE dysregulation, as we would predict that LC-NE firing would be altered to reflect a "hyper-aroused" state, defined by high tonic and low phasic activity. Second, the idea that an impairment in phasic discharge could exacerbate the hyper-activity of the LC by preventing auto-regulatory mechanisms derived from

LDCV somatodendritic release of NE and other neuropeptides is consistent with our hypothesized protective role of LDCV NE release. Further, the dysregulation of such mechanisms in stress-related disorders such as AD could contribute to LC neuronal degeneration (254). We continue to expand this hypothesis with a further inquiry into the behavioral and psychological symptoms of dementia, drawing lines of parallel between depression and AD. We put forth the notion that chronic stress is a factor that connects disparate aspects of both disorders and which profoundly alters LC-NE system integrity (255).

In the context of Mather and Harley's GANE hypothesis, the role of GABAergic interneurons is of particular interest. In this regard, the LC-NE system utilizes interneurons as lateral agents of inhibition to heighten the signal of salient information while minimizing the signal of irrelevant information. A study by the Mucke and Palop group (256) suggests that a voltage-gated sodium channel subunit Nav1.1, that is predominantly localized to parvocellular interneurons are responsible for the interneuron dysfunction at the root of pathological oscillatory rhythms and network synchrony in mouse models of AD (256). The study was conducted in cortical neurons via EEG, with a gamma frequency band reflecting the activity and function of such GABAergic interneurons. Interestingly, there is evidence of NE-regulation of parvocellular interneurons of the paraventricular nucleus of the hypothalamus (257, 258), as well as pyramidal interneurons of the cortex (259), although it is not specified if the interneurons are of the parvocellular subtype. The fascinating work of Dr. Tsai has elucidated a non-invasive method of induced 40 Hz gamma-band stimulation for the improvement of memory impairment and neuronal loss in AD by improving the clearance of $A\beta_{42}$ plaques and hyperphosphorylated tau pathology in several mouse models of AD (260, 261). Thus, while it remains unclear if the LC-NE system is involved in this gamma oscillation related mechanism, there appears to be a promising rationale for further investigation.

Aside from accelerating the advancement of our understanding of the physiological properties of the brain, computational neuroscience is also a leading edge of modern neuroanatomy. The emergence of 3D EM reconstruction has allowed for the in-depth spatial, and morphological analysis of the brain microenvironment of healthy and pathological disease states that are complex and varied, such as in AD. One facet of AD pathology is an inflammatory state in which microglia and astrocytes surrounding affected neurons and $A\beta_{42}$ plaques are activated—an event thought to exacerbate cognitive deficits in AD patients. Nuntagij et al. utilized 3D reconstruction EM in both 3 × Tg mice and naturally aged dogs to extensively describe the close spatial relationship between $A\beta_{42}$ deposits and the neutrophil, revealing entangled and branched plaques engulf soma, and apical dendrites (262). The ability of 3D EM reconstruction to portray the complex, disruptive forces of Aβ plaques and neurofibrillary tangles are evident in Fiala et al. work, which describes swollen, dystrophic neurites within late-stage plaques that form pouches impaled with microtubules, forming loops that trap mitochondria and other organelles, rendering them non-functional (263). Thus, 3D EM analysis has been instrumental in establishing that intraneuronal $A\beta_{42}$ aggregation may disrupt intracellular transport, leading to the dysfunction of mitochondria, potentially resulting in autophagic degeneration (264).

DISCUSSION

Implications for Health and Disease

A recent study used structural equation modeling on an open-access dataset of magnetization transfer images from the Cambridge Center for Aging and Neuroscience cohort to test the hypothesis that LC signal intensity values would be more closely related to NE-dependent functions in older adults compared to younger adults. The investigators concluded that age-related reduction of LC structural integrity is associated with cognitive and behavioral impairments (265). In line with this, a study examining functional connectivity between the LC and SN in healthy young and older adults used regression and functional connectivity analyses on resting-state fMRI data over a time course of LC activity. The study provides evidence that older adults had reduced functional connectivity between the LC and SN compared with younger adults, evidenced by increased coupling of the CEN network to the SN than the DMN. These authors conclude that the reduced interactions between the LC and SN impair the ability to prioritize the importance of incoming events. In turn, the SN fails to initiate network switching (4, 176), which would promote further attentional processing (266). The continued study of open access data sets using computational tools such as multivariate analysis will increase the amount of information decoded from brain activity (267). In particular, the LC will be a fascinating subject of study over varied timescales and sub-regions, as it likely mediates the vast array of behavioral and physiological outputs by coding responses differentially over short and long timescales. A number of disorders arise from hyper-activation of the SN that directly or indirectly relates to dysfunction of the LC-NE system. Among them, a convincing case can be made for post-traumatic stress disorder and opiate use disorder, in which the aberrant assignment of high salience to trauma- or drug- experience related stimuli hinder patient progress to recovery (268).

Future Directions

In light of the computational advancements discussed throughout this review, the expansive field of single-cell transcriptomics warrants mention here. Recent studies from the Allen Institute and others unveil a comprehensive view of genomic cortical neuron heterogeneity that cannot be overstated. The results predict the existence of at least 37 distinct peptidergic neuron types that are characterized by transcript abundance and taxonomic profiling, and compose cortical neuromodulatory networks (269). As we progress further into the age of transcriptomics, proteomics, and connectomics, a trend of increasing complexity in the form of neuronal heterogeneity emerges. While investigators continue to gather evidence of this diversity in evolutionarily conserved brain regions such as the LC, it is most apparent in brain regions that rapidly expanded in human evolution, such as the dl PFC. As pointed out by Arnsten et al. in response to Mather and Harley's 2016 GANE Theory (270), the dlPFC is a region associated with higher-order cognitive processing that is modulated in a

specialized, often opposing, manner compared to the classic synapses of the sensory cortex, amygdala, and hippocampus (271). Thus, a critical future direction for LC-NE research will be in testing the GANE hypothesis in both classical and more newly evolved synapse types as they relate to neuronal health and disease. In this regard, leading studies have begun to examine transcriptomic profiles of the human dlPFC with the 10x genomics platform to uncover cortical layer-specific expression profiles (signatures), and promises to continually advance the notion of Psychiatric Genomics (272).

Concluding Remarks

As we have moved forward, technological advancements in the evolving field of computational neuroscience have afforded scientists unprecedented resolution in the study of neural dynamics across spatial and temporal scales. In reference to the LC-NE system, this evolution has occurred across disciplines for decades. Today, with instruments of ever-increasing spatial and temporal resolution and data-driven analytics, the decades to come promise to reveal LC-NE system mechanics as they progressively unfold in space over time. The continued study of this multifaceted system in the context of stress- or arousal-related psychiatric disorders, including neurodegeneration, may facilitate a deeper understanding of within-network and between-network LC-NE dynamics. In time, undoubtedly, this will directly or indirectly lead to the discovery of novel therapeutics to treat the underlying systemic brain imbalances that drive the symptoms and progression of a wide array of such disorders.

AUTHOR CONTRIBUTIONS

EV and JR conceived the idea. JR wrote the manuscript. EV checked the manuscript for accuracy. Both authors contributed to the article and approved the submitted version.

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The Effect of Early Maternal Separation Combined With Adolescent Chronic Unpredictable Mild Stress on Behavior and Synaptic Plasticity in Adult Female Rats

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Our aims were to evaluate the depression model of early maternal separation (MS) combined with adolescent chronic unpredictable mild stress (CUMS) in female adult SD rats to observe the behavior and the expressions of synaptic proteins in rats and to provide a reference for the screening of antidepressant drug activity. In our study, MS and CUMS were conducted to establish a dual stress model on female rats. Behavioral tests, including the sucrose preference test, open field test, and zero maze test, were used to detect depression-like and anxiety-like behavior of animals. Nissl staining was used to detect the number of neuronal cells in the hippocampus CA1 and DG regions of rats from each group. Synaptophysin (SYN), postsynaptic density-95 (PSD-95), and growth-associated protein-43 (GAP-43) expressions in the hippocampus were detected by western blot. Expression of the hippocampus SYN protein was further detected by immunohistochemistry. Rats in the MS+CUMS group presented more serious depression-like and anxiety-like behavior than in the MS group. Also, few Nissl bodies in the hippocampus CA1 and DG regions, less percentage of SYN-positive cells, and downregulated expressions of SYN, PSD-95, and GAP43 were found in the hippocampus of rats in MS+CUMS group. In conclusion, adult female rats that underwent MS and CUMS performed more critical depression-like and anxiety-like behaviors, and this process may be resulted from synaptic plasticity impairment.

Keywords: maternal separation, depression, anxiety, synaptic plasticity, early stress

INTRODUCTION

Depression is a common psychiatric disorder with a high incidence, and its pathogenesis is still not clear. The World Health Organization points out that depression would be one of the three major diseases in 2030 (1). However, due to the complexity of the etiology of depression, how to establish an effective model of depression has become one of the key issues when researching depression. Additionally, although the etiology of depression has not been confirmed, most scholars believe that the occurrence of depression is influenced by environment and heredity (2). Berton et al. found

that genetic factors account for 40–50% of all factors leading to depression, and the remaining 50–60% are closely related to life stress in early childhood (3). Another study has demonstrated that the one who has adverse experience at a young age may have a risk of depression 4-fold higher than normal persons (4).

Contemporary studies on depression are mostly in male animals, while female animals are often excluded because of interference of estrogen (5–7). In fact, there are reports that women are twice as likely as men to experience depression and anxiety disorders (8, 9). So, it is more valuable to study depression in female rats. In addition, adolescence is the most critical period of physiological and psychological changes, and it plays an indispensable role in neurodevelopment and mental diseases. Maternal separation (MS) is a classical model of early stress study. Moreover, it is known that chronic unpredictable mild stress (CUMS) overcomes the stress habit and is commonly used to simulate depressive behavior (10). We chose female rats with MS combined with CUMS as experimental subjects of early stress. On the one hand, the complexity of the etiology of depression was in our consideration. On the other hand, it could also provide a novel model design for the construction of a new model to explore the impact of early stress on adult depression. As anxiety and depression often occur together (11), we would explore both depression-like and anxiety-like behaviors in our study.

Clinical studies have found that the hippocampus of depressed patients is reduced in volume (12, 13). Animal studies have found that neurons and glial atrophy are lost in the hippocampus of depression-like model animals (14). The reason may be that stress not only reduced dendritic spine density and number of branches but also thinned postsynaptic density (15). The hippocampus, a brain structure regulating stress and related to depression (16), is more vulnerable to stimulation damage (17), which may lead to neuron reduction (18, 19). Previous studies have shown that depression is closely related to the hippocampus synaptic plasticity (20, 21). Menard et al. have shown that the number of synapses in depression animals is significantly reduced, and the residual synaptic structure and function have different degrees of damage (22). Increasing evidence had found that dysregulation of synaptic plasticity is related to depression (21). However, there are so far only a few papers on synaptic plasticity proteins (2, 23). Thus, changes in synaptic plasticity proteins in the depression model are the focus of this study. This study provides a basis for research on synaptic plasticity protein-related depression and suggests that drugs may be able to improve depression by changing synaptic plasticity.

Based on the researches above, the stress model of MS, CUMS, and MS combined with CUMS were used in this study. We tried to observe the effects of different stress on the depression-like and anxiety-like behaviors of adult female rats and synaptic plasticity of the hippocampus.

MATERIALS AND METHODS

Animals and Groups

A total of 10 Sprague Dawley (SD) pregnant rats were purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine (China). Animals were housed in a constant animal facility at a temperature of 20–25°C, relative humidity of 50–60%, food and water *ad-libitum*, and a 12 h light/dark cycle. On the day the pups were delivered (PND0), only female pups were selected and then were divided into four groups ($n = 8$ animals/group) randomly: the non-maternal separation (CON group), maternal separation only group (MS group), chronic unpredictable mild stress only group (CUMS group), and maternal separation group plus chronic unpredictable mild stress (MS + CUMS group). The body weight was detected once a week. The study design flowchart is illustrated in **Figure 1**. After behavioral tests, on PND62, animals were anesthesia with 10% chloral hydrate, and the brain tissues were removed. All experimental protocols were approved by the Animal Experimental Committee of Guangzhou University of Chinese Medicine (Ethical approval number: 20190605015).

Maternal Separation (MS)

The protocol of MS we used was established in our previous study (24). In brief, from PND1 to PND21, the pups in the MS group and MS+CUMS group were separated from their mother for a total of 360 min each day (8:00–11:00 a.m. and 14:00–17:00 p.m.). During separation time, these pups were placed into cages filled with cotton for the purpose of maintaining the temperature. Rats in the CON group and CUMS group remained with their mother and did not undergo any interference during the MS period. Since PND21, rats were put into cages without their mother with four pups per cage.

Chronic Unpredictable Mild Stress (CUMS)

Similar to the previous studies (25, 26), from PND28, pups received one of nine trials (**Table 1**) every day until the end of the whole study (PND61). Each trial was randomly taken every day, and the same trial was not used twice in a row.

Sucrose Preference Test (SPT)

On PND56, rats were given two bottles of 1% sucrose solution for 24 h; one bottle was replaced with pure water on PND57, and the position of two bottles was exchanged after 12 h; on PND58, food and water were deprived from rats for 24 h; on PND59, the rats were given a bottle of pure water and a bottle of 1% sucrose solution. After 2 h, the lost weight of each bottle was recorded to determine the intake of the rats. Sucrose Preference rate (%) = Sucrose consumption/(Sucrose consumption + water consumption) * 100%.

Open Field Test (OFT)

OFT was conducted on PND56. The rats were placed in the center of a 100 × 100 × 60 cm black box, and the movement of each rat in the box was automatically recorded by the video-tracking analysis system for 3 min. The time in the central area and the total distance would be used as indicators.

Zero Maze Test (ZMT)

ZMT was conducted on PND60. The maze (outer diameter: 100 × 100 cm; inner diameter: 80 × 80 cm) was divided into four quadrants (two are opposing open quadrants and two are opposing closed quadrants). Rats were placed in an open quadrant, and the tracking system was triggered (Shanghai

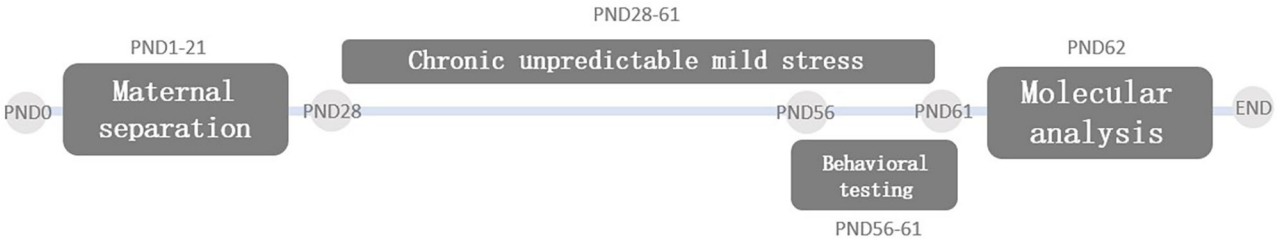

FIGURE 1 | Study design flowchart. PND, postnatal day.

TABLE 1 | Method for CUMS comprises nine trials.

Trials	
1	Food deprivation for 24 h
2	Water deprivation for 24 h
3	Thermal water stimulation (45°C) for 5 min
4	Day and night reversal for 24 h
5	Ice water stimulation (4°C) for 5 min
6	Cage tilting 45° for 24 h
7	Wet padding for 24 h
8	Crowded squirrel cage for 24 h
9	Empty water bottle for 2 h

Xinsoft Information Technology Co., Ltd., China). Each rat was tested for 5 min, and the time rat spent in open quadrants would be used as an index.

Nissl Staining

On PND62, brain tissues were removed and then embedded and cut at 5 μm thickness. The staining was carried out with Nissl staining solution (Shanghai Biyuntian Biotechnology Co., Ltd., China) for 40 min at 37°C after the sections dewaxing and rehydration. The number of Nissl bodies in the CA1 and DG region was observed under a field of view of a microscope (Leica Microsystems Wetzlar GmbH) at 400× magnification. Select cells that clearly observe the nucleus and nucleolus for statistics.

Immunohistochemistry

The embedded brain tissues were cut into 5 μm sections. Firstly, after dewaxing, the sections were immersed in 0.01 M sodium citrate buffer solution at 95–100°C for 15 min and then incubated with 3% hydrogen peroxide treatment for 10 min. Secondly, after being incubated with goat serum working solution for 15 min, the sections were incubated with anti-rabbit SYN antibody (Affinity, USA) at 4°C overnight. Thirdly, on the following day, the sections were incubated with biotinylated IgG, streptavidin, and DAB following the DAB detection kit (ZSGB-BIO, China). The sections were digitized and analyzed by a Leica microscope (Leica Microsystems Wetzlar GmbH). Each section was imaged at 400× magnification and measured the percentage of SYN-positive area in each field of view by quantitative image analysis with ImageJ 1.45 software.

Western Blot

Total protein of the hippocampus was lysed by using RIPA lysis buffer, and protein concentrations were quantified by using a BCA kit (Beijing Dingguo Changsheng Biotechnology co., Ltd., China). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to PVDF membranes and blocked with 5% skim milk powder. Antibodies against SYN (Affinity (USA), AF0257-50; 1:1000), PSD-95 (Affinity (USA), AF5283-50; 1:1000), GAP-43 (Affinity (USA), DF7766-50; 1:1000), and Tubulin (Affinity (USA), AF7018-50; 1: 5000) were incubated with membranes overnight at 4°C. Finally, the IgG-HRP antibody (CST (USA), BST11L22C51, 1:5000) was incubated with membranes for 1 h at room temperature. After the above steps were completed, membranes were combined with ECL Plus reagent (Beijing Lanjieke Technology Co., Ltd.) for a color reaction. Besides, the gray value was quantified by using Image Lab software.

Statistical Analysis

SPSS22 software was used to perform analysis and all data are expressed as mean ± standard error (SEM). One-way analysis of variance (ANOVA) was used for statistical analysis. When the variances were homogeneous, we used an LSD *post-hoc* test; otherwise, we used Dunnett's T3 test. The results were performed using GraphPad Prism 6.0. $p < 0.05$ was considered statistically significant.

RESULTS

The Effect of MS and/or CUMS on Body Weight

We observed the effect of MS and CUMS on body weight in this study, including PND28 and PND60 time points (**Figure 2**). As shown in **Figure 2A**, after MS for 28 days, rats in the MS group showed no significant influence on body weight compared with the non-MS group. After CUMS, rats in CUMS group [$F_{(3, 31)} = 34.76$, $p < 0.01$] and MS+CUMS group [$F_{(3, 31)} = 34.76$, $p < 0.01$] showed lower body weight than rats in the control group, while rats in the MS group showed no significant difference (**Figure 2B**). Moreover, there was no significant difference between CUMS and MS+CUMS groups on PND60.

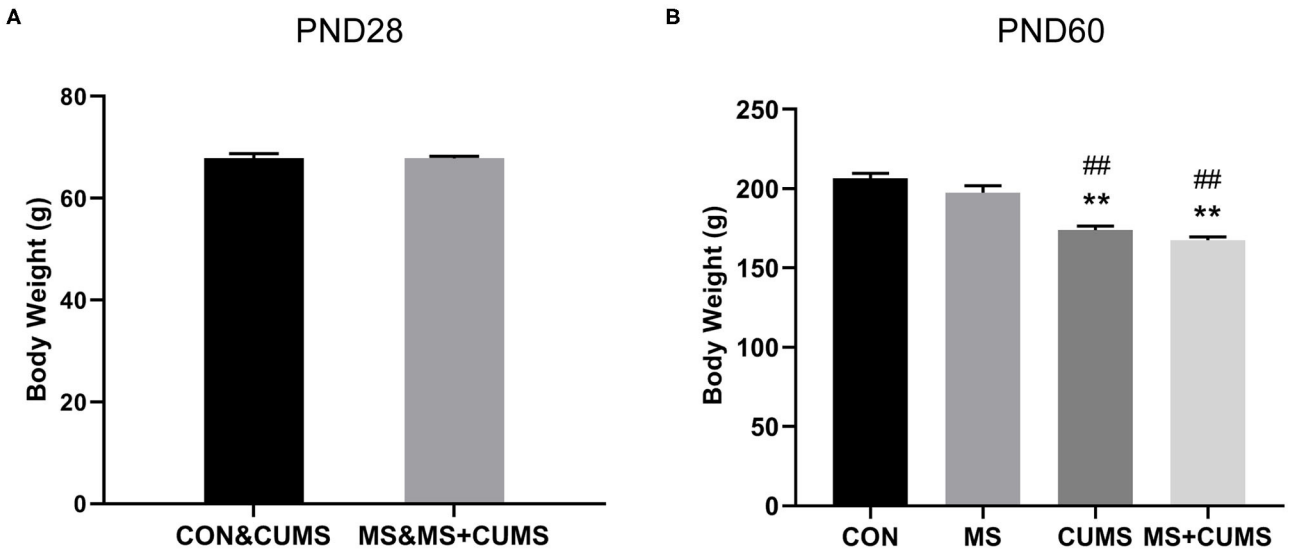

FIGURE 2 | Body weight record of rats on PND28 **(A)** ($n = 16$) and PND60 **(B)** ($n = 8$). The values represent the mean ± SEM. $^{**}p < 0.01$ vs. CON; $^{\#\#}p < 0.01$ vs. MS. CON, control; MS, maternal separation; CUMS, chronic unpredictable mild stress.

The Effect of MS and/or CUMS on Behavioral Tests

In SPT (**Figure 3A**), MS+CUMS group presented the lowest sucrose preference rate. MS showed a slight reduction in the sucrose preference rate, differing from the control group [$F_{(3,28)} = 14.64$, $p < 0.01$]. The CUMS group and MS+CUMS group presented a more noticeable decline in sucrose preference rate compare with the control group [$F_{(3,28)} = 14.64$, $p < 0.01$ and $p < 0.01$). Importantly, the sucrose preference rate reduction was the most significant in rats that underwent MS as well as CUMS, surpassing the effect of MS only [$F_{(3,28)} = 14.64$, $p < 0.05$]. By the way, there was no difference between CUMS group and MS+CUMS group.

In OFT (**Figure 3B**), although the MS group, CUMS group, and MS+CUMS group showed a noticeable reduction in central region time, the MS group showed no statistical difference compared to the control group, while the CUMS group [$F_{(3,28)} = 6.51$, $p < 0.05$] and MS+CUMS group [$F_{(3,28)} = 6.51$, $p < 0.05$] presented a significant reduction. Double stress of MS and CUMS led to the shortest total distance among four group (**Figure 3C**). The MS group showed no obvious decline in total distance, and the CUMS group presented a reduction with no statistical difference. However, the total distance revealed a significant difference in MS+CUMS group compared with the control group [$F_{(3,28)} = 5.11$, $p < 0.05$] and also with the MS group [$F_{(3,28)} = 5.11$, $p < 0.05$].

In ZMT, MS+CUMS group showed the least time spent in open areas (**Figure 3D**). The CUMS group [$F_{(3,28)} = 13.18$, $p < 0.01$] as well as MS+CUMS group [$F_{(3,28)} = 13.18$, $p < 0.01$] presented decreased time spent in open areas compared with the control group. Notably, double stress caused the most significant reduction in time spent in open areas, even less than MS only [$F_{(3,28)} = 13.18$, $p < 0.01$] and CUMS only [$F_{(3,28)} = 13.18$, $p < 0.05$, not shown]. Incidentally, the CUMS group showed less time spent in open areas than the MS group [$F_{(3,28)} = 13.18$, $p < 0.05$].

The Effect of MS and/or CUMS on Nissl Bodies

According to Nissl staining, the neurons of the hippocampus in control group rats were in a large quantity and in a compact arrangement, while neurons in MS, CUMS, and MS+CUMS group rats were sparsely arranged and low in number, with neurons in the MS+CUMs group being the sparsest and the least in number (**Figure 4C**). Both in CA1 and DG of hippocampus, fewer Nissl positive cells were found in the MS group [CA1: $F_{(3,8)} = 21.50$, $p < 0.01$; DG: $F_{(3,8)} = 21.80$, $p < 0.01$] and CUMS group [CA1: $F_{(3,8)} = 21.50$, $p < 0.01$; DG: $F_{(3,8)} = 21.80$, $p < 0.01$) compared to the control group (**Figures 4A,B**). Also, both in the CA1 and DG of the hippocampus, double stress of MS and CUMS exhibited fewer Nissl-positive cells than the control group [CA1: $F_{(3,8)} = 21.50$, $p < 0.01$; DG: $F_{(3,8)} = 21.80$, $p < 0.01$], which is even fewer than the MS only [CA1: $F_{(3,8)} = 21.50$, $p < 0.01$; DG: $F_{(3,8)} = 21.80$, $p < 0.01$] and CUMS only groups [CA1: $F_{(3,8)} = 21.50$, $p < 0.05$; DG: $F_{(3,8)} = 21.80$, $p < 0.01$, not shown].

The Effect of MS and/or CUMS on Expressions of Synaptic Plasticity Proteins in Hippocampus

Synaptic plasticity proteins were detected by western blot, **Figure 5** shows that the expressions of PSD-95, GAP-43 and SYN in CUMS group [PSD-95: $F_{(3,8)} = 5.54$, $p < 0.05$; GAP-43: $F_{(3,8)} = 11.84$, $p < 0.01$; SYN: $F_{(3,8)} = 8.60$, $p < 0.01$] and MS+CUMS group [PSD-95: $F_{(3,8)} = 5.54$, $p < 0.01$; GAP-43: $F_{(3,8)} = 11.84$, $p < 0.01$; SYN: $F_{(3,8)} = 8.60$, $p < 0.01$] were downregulated. The expressions of those proteins were significantly lower in MS+CUMS group than MS group [PSD-95: $F_{(3,8)} = 5.54$, $p <$

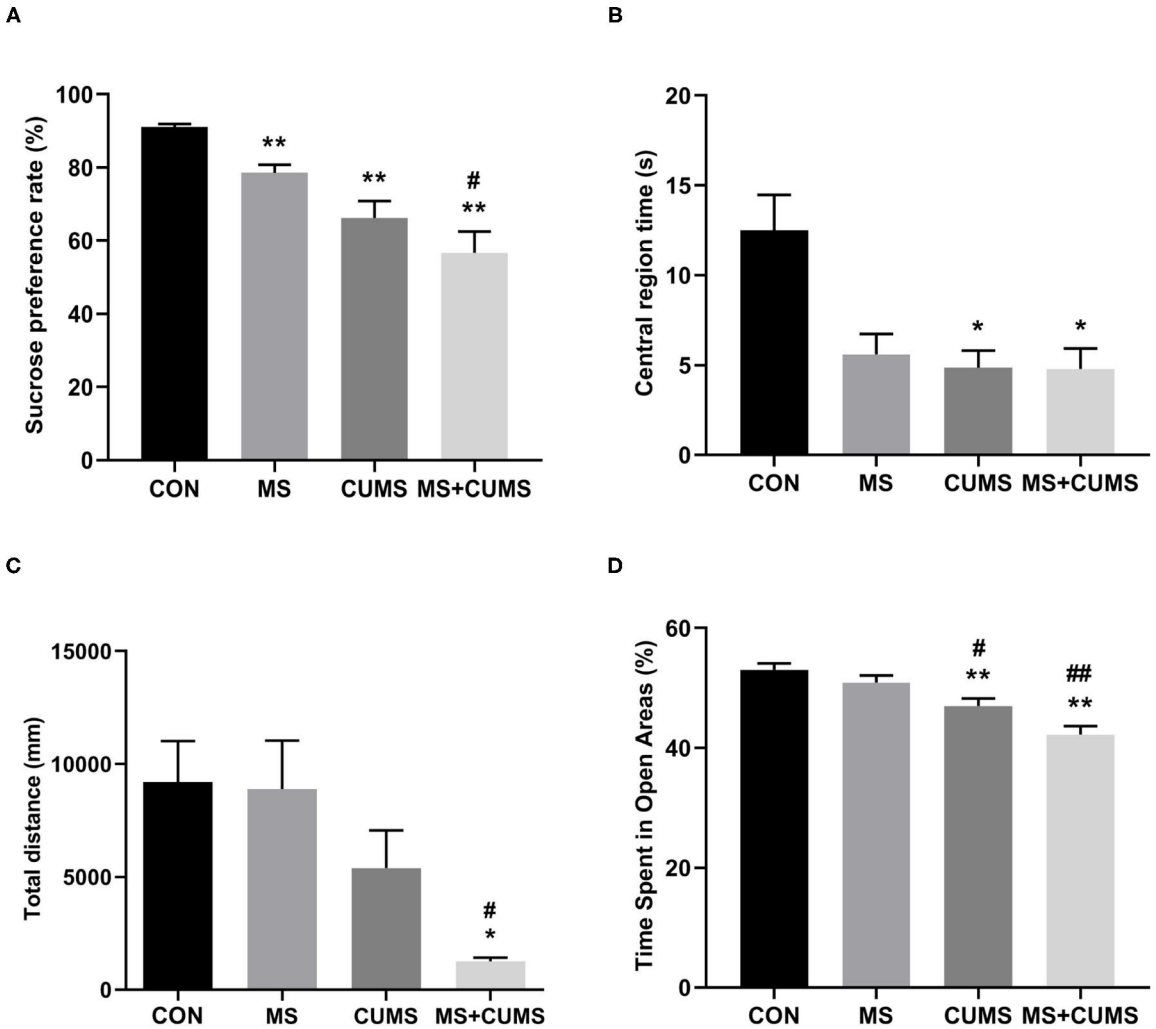

FIGURE 3 | The effects of MS and CUMS on behavior in female rats. After undergoing MS and/or CUMS, all groups of rats performed behavior tests. In the sucrose preference test, the sucrose preference rate was analyzed as factors **(A)**; in the open field test, central region time **(B)** and total distance **(C)** were analyzed as factors; in the zero maze test, time spent in open areas was analyzed as factors **(D)**. The values represent the mean ± SEM, $n = 8$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. CON, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MS. CON, control; MS, maternal separation; CUMS, chronic unpredictable mild stress.

0.05; GAP-43: $F_{(3,8)} = 11.84$, $p < 0.01$; SYN: $F_{(3,8)} = 8.60$, $p < 0.05$]. Here, the double stress of MS and CUMS led to the lowest expressions of those proteins among all groups (**Figure 5A**).

In order to further determined the expression of SYN, immunohistochemistry was detected (**Figure 6**). Both in CA1 (**Figure 6A**) and DG (**Figure 6B**) of hippocampus, SYN expression was radically diminished in MS group [CA1: $F_{(3,8)} = 45.45$, $p < 0.05$; DG: $F_{(3,8)} = 14.89$, $p < 0.01$] and CUMS group [CA1: $F_{(3,8)} = 45.45$, $p < 0.01$; DG: $F_{(3,8)} = 14.89$, $p < 0.01$] as well as MS+CUMS group [CA1: $F_{(3,8)} = 45.45$, $p < 0.01$; DG: $F_{(3,8)} = 14.89$, $p < 0.01$] compared with control group. MS+CUMS group presented the least percentage of the SYN-positive area both in CA1 and DG (**Figure 6C**). Both in CA1 and DG of the hippocampus, double stress of MS and CUMS led to lower SYN expression than the MS group [CA1: $F_{(3,8)} = 45.45$, $p < 0.05$; DG: $F_{(3,8)} = 14.89$, $p < 0.01$].

DISCUSSION

In the present study, we examined the effects of MS, CUMS, and MS combined with CUMS on body weight, depression-like and anxiety-like behaviors, and expression of synapse-associated proteins. We found that with the experience of MS at an early age and CUMS in adolescence, adult female rats showed very intense depression-like and anxiety-like behaviors, which were more severe than those who underwent only MS or only CUMS. Moreover, depression-like and anxiety-like behaviors were related to the abnormal expressions of synapse-associated proteins, including PSD-95, GAP-43, and SYN. This study demonstrated for the first time that MS combined with CUMS induced more critical depression-like and anxiety-like behaviors than MS only or CUMS only in female rats, and the

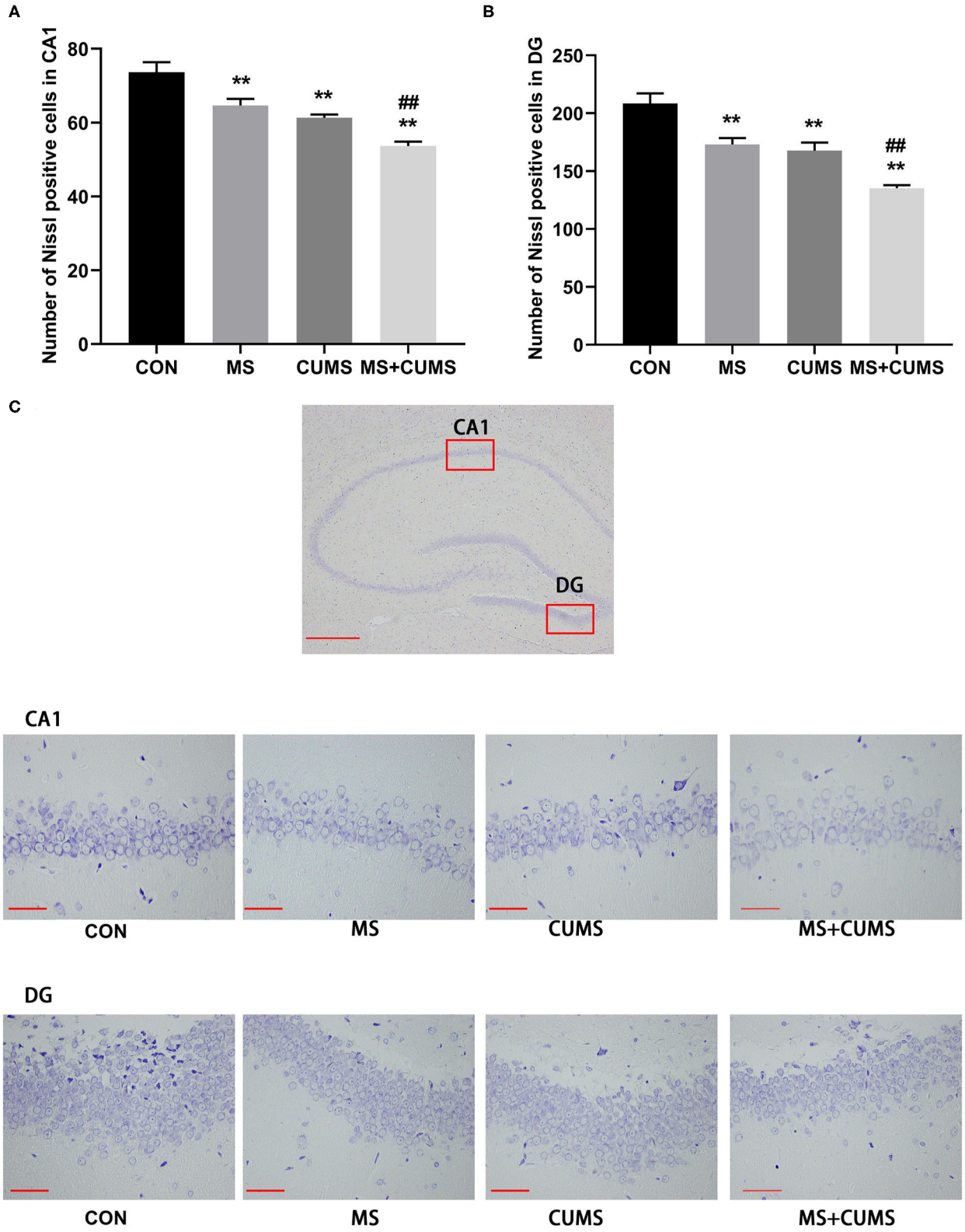

FIGURE 4 | Nissl staining in the hippocampus. **(A)** Number of Nissl positive cells in CA1; **(B)** Number of Nissl positive cells in DG; **(C)** Representative photomicrograph of Nissl staining. All scale bars were 50 μm. The values represent the mean ± SEM, $n = 3$. $^{**}p < 0.01$ vs. CON, $^{\#\#}p < 0.01$ vs. MS. CON, control; MS, maternal separation; CUMS, chronic unpredictable mild stress; CA1, cornu ammonis 1; DG, dentate gyrus.

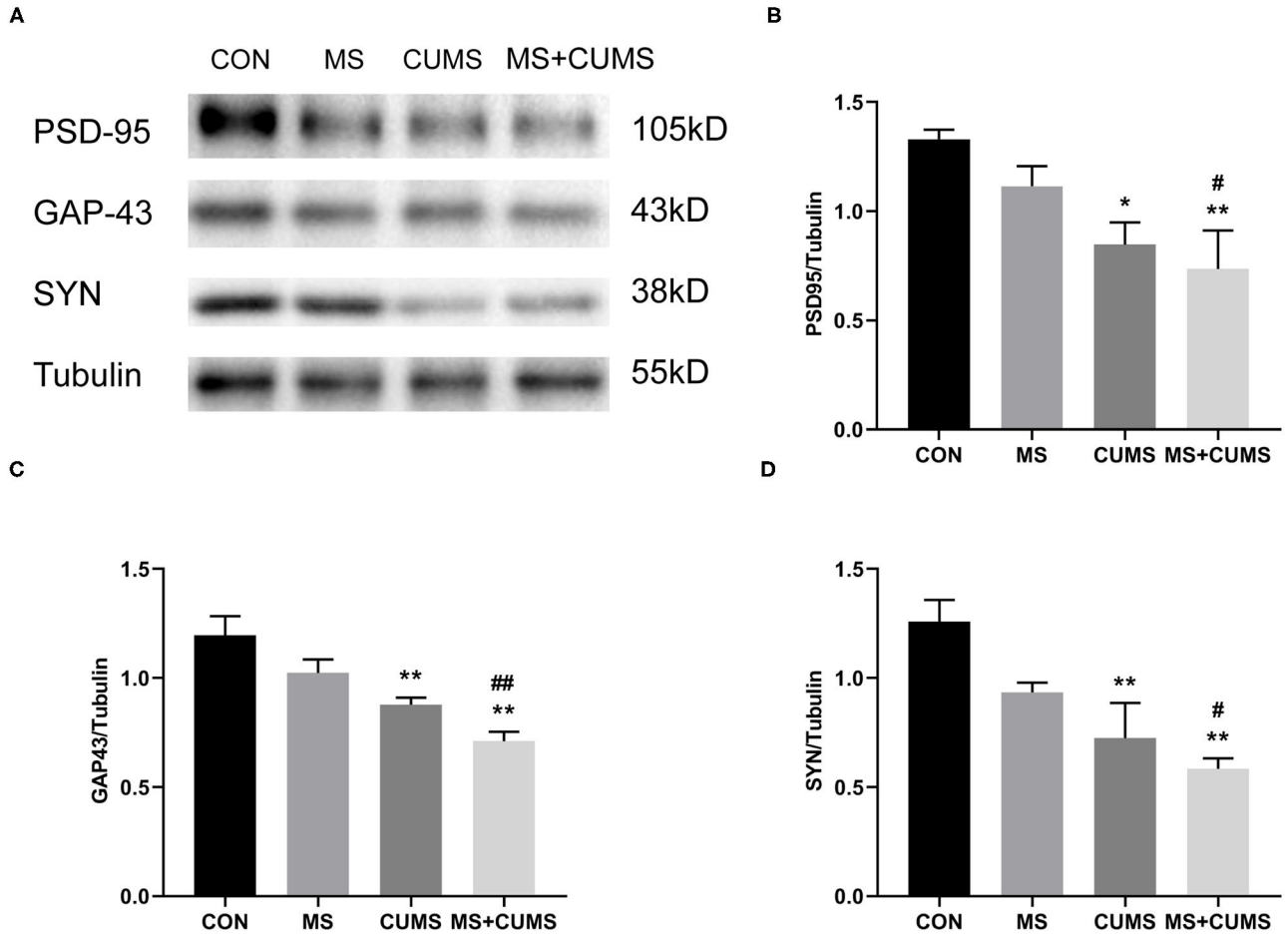

FIGURE 5 | Western blot analysis. The expressions of synaptic plasticity proteins were determined by western blot **(A)**, including PSD-95 **(B)**, GAP-43 **(C)**, and SYN **(D)**. The values represent the mean ± SEM, $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. CON, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MS. CON, control; MS, maternal separation; CUMS, chronic unpredictable mild stress.

impairment of hippocampal synaptic plasticity may be the potential mechanism.

In this study, we observed the effects of MS, CUMS, and MS combined CUMS on body weight and behaviors of rats. Knowing that losing weight could be one of the symptoms of depression (27), the treatment of CUMS and MS+CUMS led to an obvious loss in weight in our study, which indicated that animals were in a progression of depression-like behavior, and this was consistent with previous studies (28, 29). Gracia-Rubio et al. reported that maternal separation caused emotional alterations but had no impact on body weight (30). Consisted with this report, our study showed that MS had no effect on body weight, while CUMS had.

Here, we revealed that rats that underwent MS showed significantly increased depression-like and anxiety-like behaviors after exposure to a CUMS environment. These findings are in agreement with previous studies showing that stress at an early stage is an important factor in the development of depression-like behaviors when exposed to adult stress, and this resulted in neurodevelopmental abnormalities through stress (31). This is consistent with studies showing that early life experience is associated with susceptibility to depression (32–35). In our study, only MS treatment of rats did not lead to anxiety-like behavior. Similarly, Chen's study showed anxiety-like behavior did not occur in MS rats (36). However, on the contrary, MS could present anxiety-like behavior in some studies (37, 38), we cannot definitely explain the different behavioral performances owing to different MS protocol and animals. So far, the opinion that a longer period of MS could induce anxiety-like behavior was confirmed in Arias' study (39). Interestingly, from the result of Susana Roque, only rats in MS_{2-15} group (MS from PND2-15) displayed depressive and anxiety-like behaviors, while rats in MS_{7-20} group (MS from PND7-20) did not display these (40). These questions need more experiment to verify.

Nissl staining is often used to detect the activity of neurons and impairment of neurons would lead to the reduction of Nissl bodies (41, 42). A small amount of Nissl positive cells was found in the hippocampus of rats underwent MS as well as CUMS. In

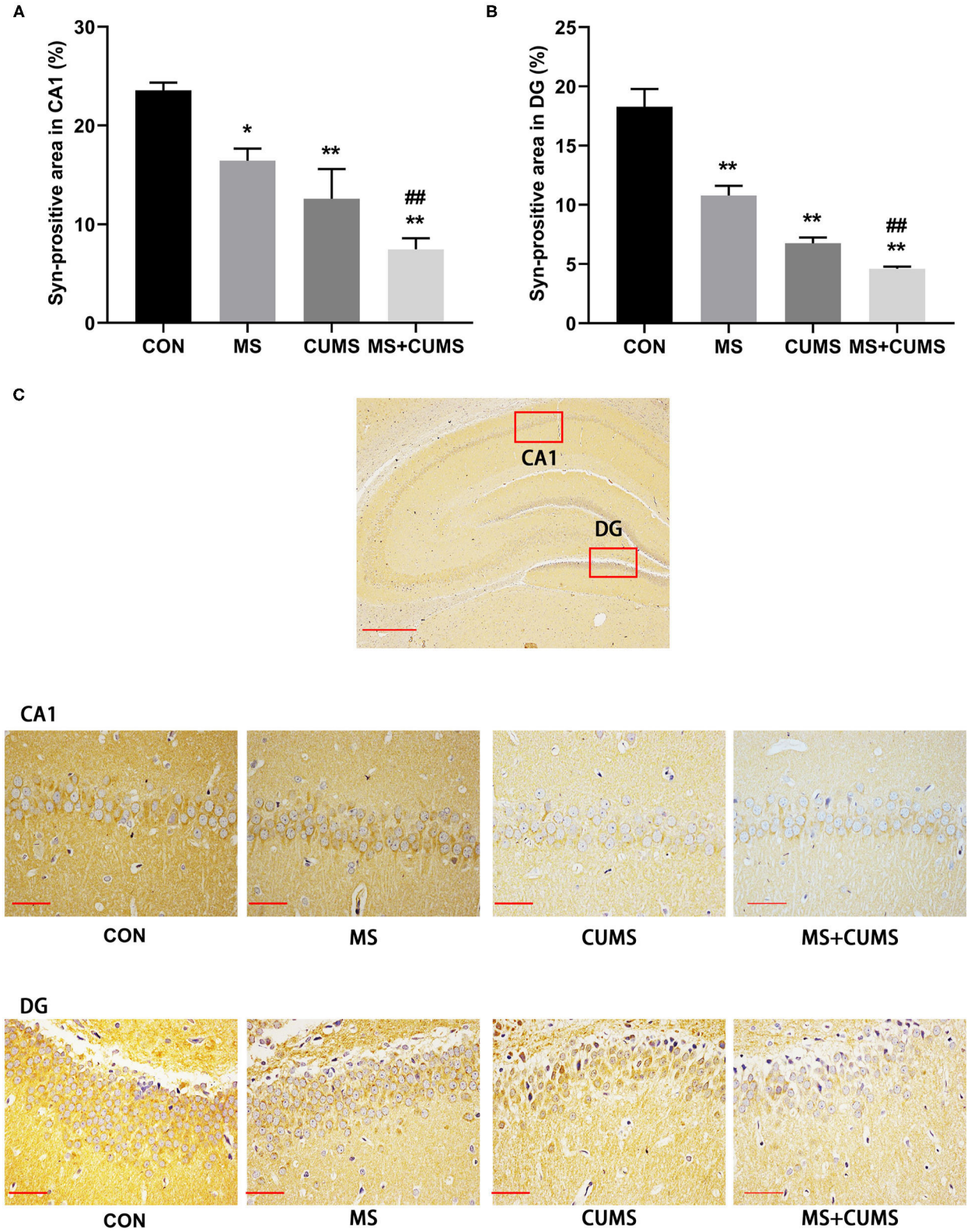

FIGURE 6 | Anti-SYN immunohistochemistry analysis in the hippocampus. **(A)** Syn-positive area in CA1; **(B)** Syn-positive area in DG; **(C)** representative photomicrograph of immunohistochemistry. All scale bars were 50 μm. The values represent the mean ± SEM, $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. CON, $^{\#\#}p < 0.01$ vs. MS. CON, control; MS, maternal separation; CUMS, chronic unpredictable mild stress, CA1, cornu ammonis 1; DG, dentate gyrus.

other words, those stress may affect the activity of neurons. This conjecture is further confirmed by the following experiments.

Clinical studies have found the reduction of synaptic signaling proteins expressed in the brain regions (such as the hippocampus) with major depressive disorder patients (43, 44). SYN, PSD-95, and GAP-43 are considered to be key proteins affecting synaptic plasticity (45, 46) as well as normal signaling between neurons in the central nervous system (47). SYN is a vesicle protein of presynaptic membrane, which is closely linked to the regulation of synaptic structure and functional plasticity, which widely exists in the synaptic membrane of neurons (48) as well as rapidly recruits to the presynaptic end in response to presynaptic neuronal activity (49). In our study, low expression of SYN was detected in the hippocampus of rats that underwent MS and CUMS. Studies have shown that abnormal SYN expression is associated with depression (50). The atrophy of dendrites in the vertebral hippocampal neurons and the downregulation of SYN expression existed in chronic stress and depression (51).

PSD-95 is one of the postsynaptic densities (PSDs), which plays a key role in synaptic plasticity signal transduction (52, 53). Studies have found that most neurological diseases, such as depression, show abnormal expression of PSD-95 (54, 55). GAP-43 is located on the growth cone of the axon and participates in the synaptic plasticity of the nervous system (56, 57). In addition, GAP-43 is essential for synaptic plasticity, axon elongation, and nerve germination during the development and maturation of neurons in adult rats (58, 59). Our results are similar to Li and Yang et al. in that the expression of GAP43 in rats with depression-like and anxiety-like behaviors is significantly downregulated (60). The results showed that the expression of PSD-95 had the same trend as GAP-43. This evidence further suggested that MS combined CUMS had an effect on the impairment of synaptic plasticity. Moreover, when fewer synaptic plasticity proteins were expressed, the rats displayed more serious depression-like and anxiety-like behavior.

As the model of early MS combined with adolescence, CUMS produced critical depression-like and anxiety-like behavior as well as synaptic plasticity impairment, our finding may help to provide a novel convincing depression model. However, what is the specific mechanism? Further study would verify whether synaptic plasticity proteins be the important targets for antidepressant interventions.

CONCLUSION

In conclusion, we demonstrated that early MS combined with adolescent CUMS in female rats could induce more critical depression-like and anxiety-like behaviors. The expressions of synaptic-related proteins and the impairment of synaptic plasticity may be the potential mechanisms.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experimentation Committee at Guangzhou University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

JH wrote the manuscript and conducted animal experiment. CS and RY conducted animal and analysis experiment. YS analyzed data and corrected the manuscript. WL controlled all work and revised manuscript. All authors contributed to the article and approved the submitted version.

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Perceived Stress and Life Satisfaction Among Chinese Clinical Nursing Teachers: A Moderated Mediation Model of Burnout and Emotion Regulation

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Our previous study indicated that clinical teaching nurses in China suffered high levels of perceived stress and burnout, mainly because they were taking double responsibilities of nursing and teaching at the same time. The study aimed to investigate the underlying mechanisms of how and when perceived stress increased the risk of burnout and decreased life satisfaction among clinical teaching nurses. Questionnaires about perceived stress, burnout, emotion regulation, and life satisfaction were self-administered to 1,372 teaching nurses from eight tertiary military hospitals in China. Correlation and hierarchical multiple regressions were employed for data analysis. The results revealed that perceived stress had direct and indirect impacts on life satisfaction, with the principal element of burnout—emotional exhaustion—acting as a mediator. Moreover, the association between perceived stress and emotional exhaustion was moderated by emotion suppression—a key emotion regulation strategy. The negative impact of perceived stress on burnout was stronger among teaching nurses with high emotion suppression than among those with low emotion suppression. The present study contributed to a deeper understanding of the relationship between perceived stress and life satisfaction and also suggested further research into emotion regulation interventions to alleviate or eliminate the impact of perceived stress on burnout and eventually improve the life satisfaction for Chinese clinical nursing teachers.

Keywords: perceived stress, burnout syndrome, emotion regulation, life satisfaction, clinical nursing teachers

INTRODUCTION

Clinical teaching is a requisite part of nurse education. Through clinical training, nursing students are expected to successfully transit from students in classrooms into nurses in clinical settings and to develop abilities and skills essential for providing the best possible care to their patients. In other words, good quality of clinical education guarantees the quality of the nursing care and eventually impacts patients' health outcomes. Undoubtedly, clinical nursing teachers play a core role in clinical

teaching. Their clinical expertise and teaching strategies greatly impact the quality and outcomes of clinical education. Therefore, most current studies have been focused on role transitions (1, 2) and preparation of clinical nursing teachers to promote competence and confidence in clinical teaching (3).

Nursing is considered a strenuous job with complicated demands, which leads to occupational stress. In a study of investigating stress and associated risk factors among 2,895 Iran nurses, 78.4% of the respondents reported a high level of occupational stress (3). The risk factors for high stress included increased work hours and working in emergency, intensive care wards, and teaching hospitals. Clinical nurses working in teaching hospitals were regarded as particularly vulnerable compared to those working in non-teaching hospitals; they reported greater job stress and dissatisfaction and higher intention to leave (3). Clinical nursing teachers frequently complained that clinical expert knowledge and skills could not always translate into clinical teaching expertise (4). Their stresses got doubled during the preparation of being "double-certified" and expertise in two different professions: nursing and education (4). Clinical nurses have already struggled with high patient care demands, lack of resources, long work hours, heavy workloads, and even workplace violence (5, 6); they have also been overwhelmed by responsibilities associated with clinical teaching. Other sources of stress for nurse educators included insufficient time for teaching preparation (7), role conflicts with peers and supervisors, strict appraisal standards for teaching abilities, pressure of conducting scientific researches, worries about accidents at work, and so on.

Work-related stress has been implicated as a major factor for mental health difficulties such as compassion fatigue, burnout syndromes, anxiety, and reduced job satisfaction, which negatively affected life satisfaction (8) and quality of life (QOL) (9–11). These stress-related problems reduced work productivity, increased incidence of practice errors, and were unfavorably associated with quality of health service (12). Moreover, occupational stress has been recognized as a risk factor for psychiatric sickness absence (13), intention to leave (14), and eventually nurse shortage (15). On the other hand, stress and its poor outcomes among clinical nursing teachers could result in negative impacts on role preparedness and confidence in clinical teaching and eventually reduced the effectiveness of clinical teaching and quality of education outcomes (16).

Burnout Symptom as a Mediator

Occupational stress can not only directly damage life satisfaction but also indirectly reduce life satisfaction through mediating variables, such as burnout. Burnout was one of the most common psychological disorders among healthcare professionals, in particular among nursing workers. Described as a state of emotional fatigue and exhaustion, burnout was caused by repeated and prolonged exposure to stressful working environments and situations. A long-term status of burnout resulted in a decrease or loss of motivation for work that can elicit exhaustion and a sense of failure (17, 18). According to Maslach's burnout model, three core characteristics were illustrated: depersonalization (DP), prolonged emotional exhaustion (EE), and reduced personal accomplishment (PA) at work (19, 20). There was a high prevalence of burnout among healthcare workers, in particular among nurses who cared for critically ill patients. Professor Li and Dr. Lu investigated on intensive care unit (ICU) nurses and found out that 84% of the participants have at least one aspect of burnout symptoms: 23% were positive for EE, 27% were positive for DP, and 77.8% were positive for lack of PA (19, 20).

Higher perceived stress was closely connected to higher levels of burnout. A study among 366 female nurses revealed that 85.5% of the participants experienced psychological distress and that burnout was positively associated with psychological distress (21). A high positive correlation between job stress and burnout has been found among 79 head nurses ($r = 0.426$, $p < 0.01$) and 145 senior nurses ($r = 0.554$, $p < 0.01$) (22). Similar results were found in nurse teachers. Our previous study has investigated on perceived stress and burnout among 835 nurse teachers in military hospitals. The results have revealed high rates in three aspects of burnout: loss of passion for work (18.8%), DP (12.5%), and reduced PA (28.1%). These aspects of burnout have a significant positive correlation with perceived stress, with correlation coefficients ranging from 0.28 to 0.48. Moreover, perceived stress has been demonstrated as a predictor of burnout through a multiple stepwise regression analysis (22).

Emerging evidence has suggested that associations may exist between occupational burnout and decreased life satisfaction (10). As an important component of subjective wellbeing (SWB), life satisfaction referred to a comprehensive evaluation of satisfaction and happiness about people's own overall life (23). Compared to other primary healthcare professionals, nurses demonstrated higher perceived stress, lower positive affect, and lower levels of mindfulness (24). A national study across China has been conducted to examine the association of job burnout with life satisfaction among 7,289 employees. One-third of the participants reported decreased life satisfaction. The logistic regression analysis showed that EE, DP, and reduced sense of PA were risk factors for low life satisfaction ($p < 0.001$) (25). In another study, registered nurses working in critical care units reported moderate to high levels of burnout and low levels of job satisfaction; and burnout acted as a predictor of the decreased job satisfaction (26).

Emotion Regulation as a Moderator

Emotion regulation (ER) was a process to evaluate and control expression of emotions. It reflected an individual's psychosocial adaptation, emotional flexibility, and coping capability to a stressful situation. Gross's process model of ER pointed out that two of the most widely used strategies were cognitive reappraisal (CR) and expressive suppression (ES). CR concentrates on the emotion generative process, referring to a reinterpretation to the situation so as to alter its emotional response. ES addresses the response tendency, referring to individuals' tendency of inhibiting or controlling emotional expressions and behavioral reactions. According to the transactional model of stress (27, 28), individual's perceived stress depended on the process of cognitive appraisal and ER, rather than the event itself. This process determined emotional responses and eventually affected

life satisfaction and SWB. Therefore, the relationship of stress, burnout, and life satisfaction was assumed to be moderated by ER strategies.

Emotion regulation can modify the effects of stressful events. Lack of ER capacity was associated with mental health disorders and may result in decreased life satisfaction and QOL among healthcare workers. Individuals with negative ER skills were prone to experience negative emotions such as anxiety, EE, and insufficient feeling of job accomplishment. What remained unclear was the role of ER in the association between perceived stress and burnout among clinical nursing teachers.

Aim of the Present Study

Although it is widely acknowledged that nurses have been subjected to stress, burnout, and decreased life satisfaction (29, 30), few literature have investigated the perceived stress and burnout among teaching nurses and the mechanisms of how perceived stress influences burnout and life satisfaction. Such an investigation is imperative for clinical nurse teachers in order to develop strategies to manage stress, prevent burnout syndromes, improve mental health outcomes, and promote overall QOL. Therefore, this study aimed to (1) explore the relationship of perceived stress with burnout and life satisfaction among Chinese clinical nursing teachers and (2) examine a moderated mediation model of this relationship. Firstly, we investigated the mediating effect of burnout syndromes on the relationship between perceived stress and life satisfaction. Secondly, we explored the moderating effect of ER on the relationship between perceived stress and life satisfaction via the mediation of burnout syndromes.

METHOD

Participants

This cross-sectional survey was conducted between June 2018 and May 2019. A convenience sampling procedure was used to recruit clinical nurse educators from eight military tertiary hospitals. These hospitals were teaching hospitals affiliated to the only three military medical universities across China, which were an army medical university, navy medical university, and air force medical university. Our researchers contacted nurse managers to help us release adverts to clinical nurses and invited them to participate if they met the following inclusion criteria: (1) working as a nurse in clinics and hospitals; (2) getting trained and certified on teaching; (3) undertaking clinical teaching in at least one nursing course; and (4) giving consent to participate. Participants were excluded if they (1) failed to reach a total teaching hour of 160 annually and (2) were not involved in clinical teaching for more than 1 year.

Measures

Perceived Stress

The Chinese Perceived Stress Scale (CPSS) revised by Yang and Huang (31) was adopted to evaluate perceived stress in one's life over the past month. This scale included 14 items in two dimensions: sense of control (seven items) and sense of tension (seven items), rating on a 5-point Likert scale (0 = "not at all"; 6 = "always"). The total score ranged from 0 to 56, with higher scores indicating a higher level of perceived stress. The internal consistency coefficient was 0.82, 0.86, and 0.77 for the total scale and the subscales of "sense of control" and the "sense of tension," respectively (32). A CPSS $\geq$ 26 could be judged as having health risk pressure (33). In our subjects, the internal consistency value of the α coefficient was 0.67.

Burnout

The Maslach Burnout Inventory-Human Service Survey (MBI-HSS) was the most commonly used scale to evaluate burnout syndromes among healthcare professionals. The Chinese version of MBI was translated and validated by Professor Peng Mei-ci from the Hong Kong Polytechnic University and was published in the book of Shang et al. (34). The 22-item MBI-HSS scale included three subscales: a nine-item subscale of EE, a five-item subscale of DP, and an eight-item subscale of PA. Each item evaluated the degree of burnout experience and feeling on a 7-point Likert scale (0 = "never"; 6 = "every day"). The total scores on the EE and DP subscales were positively related to burnout, while the scores on PA were inversely related to burnout. According to Maslach et al., this study defined "low burnout" by scores on EE of $\leq$16, on DP of $\leq$6, and on PA of $\geq$39; "moderate burnout" by scores on EE ranging from 17 to 26, on DP ranging from 7 to 12, and on PA ranging from 38 to 32; and "high burnout" by scores on EE of $\geq$27, on DP of $\geq$13, on and PA of $\leq$31. In our participants, Cronbach's α score of each domain ranged from 0.70 to 0.85, with Cronbach's α coefficient for the overall scale of 0.74.

Emotion Regulation

The Emotion Regulation Questionnaire (ERQ) developed by Gross (35) was used to evaluate participants' ER strategies. A Chinese version translated and validated by Wang et al. (36) was adopted in this study. This questionnaire was composed of a six-item CR subscale and a four-item "expression suppression" (ES) subscale. A 7-point Likert rating method was adopted, with 1 to 7 representing "strongly disagree" to "strongly agree." The alpha reliability was 0.79 for "cognitive reappraisal" and 0.73 for ES, respectively. Test–retest reliability across 3 months was 0.69 for both subscales. Cronbach's α value was 0.75 in our sample.

Life Satisfaction

The Satisfaction with Life Scale (SWLS) developed by Diener et al. (37, 38) offered a global measure of satisfaction with life and SWB. It comprised five items answered with a 7-point Likert scale ranging from "1 = totally disagree" to "7 = totally agree." Higher scores represented greater perceived life satisfaction. The Chinese version of SWLS indicated good validity and high internal consistency reliability (Cronbach's alpha = 0.84) (39). Total scores can be categorized as follows: very high scores/highly satisfied (30–35 points), high scores (25–29 points), average scores (20–24 points), slightly below average in LS (15–19 points), dissatisfied (10–14 points), and extremely dissatisfied (5–9 points) (40). Cronbach's α value was 0.89 in this study.

TABLE 1 | Descriptive statistics and bivariate correlations of the major study variables (n = 1,372).

Variables	$M \pm SD$	1	2	3	4	5	6	7
1. Perceived stress (PSS)	39.32 ± 6.52	1						
2. Emotional exhaustion (MBI_EE)	18.50 ± 10.48	0.483**	1					
3. Depersonalization (MBI_DP)	5.15 ± 4.82	0.307**	0.552**	1				
4. Personal achievement (MBI_PA)	31.12 ± 9.37	−0.395**	−0.170**	−0.234**	1			
5. Cognitive reappraisal (ERQ_CR)	30.22 ± 6.18	−0.322**	−0.153**	−0.206**	0.378**	1		
6. Expression suppression (ERQ_ES)	14.35 ± 4.53	0.118**	0.114**	0.117**	0.001	0.110**	1	
7. Life satisfaction (SWLS)	21.03 ± 6.39	−0.498**	−0.339**	−0.231**	0.330**	0.322**	0.010	1

*PSS, Perceived Stress Scale; MBI, Maslach Burnout Inventory–Human Service; ERQ, Emotion Regulation Questionnaire; SWLS, Satisfaction with Life Scale. **$p < 0.01$.*

Procedure

The participants filled out a self-administered questionnaire in an anonymous way after reading written instructions and giving informed consent through an online platform. A total of 1,395 questionnaires were collected, in which 1,372 were valid for further analysis. This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the human ethics committee for Scientific Research at the first researcher's university.

Data Analysis

The software SPSS (version 21.0) and the Hayes SPSS macro program PROCESS (version 3.2) were employed to organize and analyze the data, including the nature of the moderated indirect effects. Descriptive statistics and bivariate correlation analysis were first conducted. All regression coefficients were tested by the bias-corrected percentile bootstrap method. This method produced 95% confidence interval (CI) for these effects from 5,000 bootstrap samples; 95% CIs that do not include 0 indicated a significant effect. In the current study, we selected the mediation model (Model 4) to analyze mediation effects.

RESULTS

Sample Characteristics

The participants consisted of 1,348 females and 24 males. Regarding professional titles, 63.8% (876/1,372) held a primary level, 31.7% (435/1372) held a mediate level, and only 3.9% (53/1,372) held an advanced level. Among all of the participants, 12.8% (176/1372) have been working in the hospital for less than 5 years; 44.5% (611/1,372) for 5–10 years; 22.6% (310/1,372) for 11 to 15 years; 13.2% (181/1,372) for 16–20 years; and 6.9% (94/1,372) for more than 20 years. The average length of clinical teaching experience was 3.06 ± 1.38 years.

Descriptive Analysis

There is a prevalence of burnout syndrome according to Maslach's cutoff of burnout and its dimensions illustrated in the Method section. Almost 80% (1,091/1,372) of participants showed a moderate degree of burnout, and 5% (65/1,372) indicated a high degree of burnout. Regarding to each dimension of burnout, 52% (714/1,372) of the clinical nursing teachers were positive for moderate to high levels of EE, 28.9% (396/1,372) were positive for moderate to high levels of DP, and 74.5% (1,036/1,372) were positive for moderate to high levels of insufficient PA. The mean scores of perceived stress were 39.32, higher than the cutoff score of 26, indicating significant health risk pressure; the mean scores of SWB was 21.03, a little higher than the midpoint of 20 (see **Table 1**).

Correlation Analysis

Table 1 also presented the results of bivariate correlations between different main variables. The perceived stress was significantly positively correlated with EE ($r = 0.483$), DP ($r = 0.307$), and the ER strategy of ES ($r = 0.118$) and negatively associated with PA ($r = -0.395$), the ER strategy of CR ($r = -0.322$), and SWB ($r = -0.498$) (all $p < 0.01$). SWB was significantly positively associated with PA ($r = 0.330$) and CR ($r = 0.322$) and negatively related to EE ($r = -0.339$) and DP ($r = -0.231$; all $p < 0.01$).

Mediation Effect Analysis

In the SPSS macro PROCESS software, the analysis of mediating effect was conducted by Model 4 to investigate EE on the relationship between perceived stress and SWB. Model 1 in **Table 2** indicated that the perceived stress was a significantly negative predictor on SWB ($B = 0.487$, $t = -21.249$, and $p < 0.001$). It was suggested in Model 2 that perceived stress was a significantly positive predictor of EE ($B = 0.776$, $t = 20.396$, and $p < 0.001$), while EE had a significantly negatively predicted effect on SWB ($B = -0.078$, $t = -4.843$, and $p < 0.001$). Moreover, as shown in Model 3, the direct effect of perceived stress on SWB was still significant ($B = -0.427$, $t = -16.425$, and $p < 0.001$) when mediating variables were added. In addition, the upper and lower bounds of the bootstrap 95% CI for the direct effect of perceived stress on SWB and the mediating effect of EE did not include 0, indicating that the mediating effect was significant. The mediation effect accounted for 26.1% of the total effect, which confirmed that EE played a partial mediating role in the relationship between perceived stress and SWB.

Moderated Mediation Effect Analysis

As shown in **Table 3**, after the demographic variables of age and marital status were controlled for, the entry of perceived stress in step 2 indicated that perceived stress was positively associated with EE. After the entry of ES in step 3, the result revealed a

TABLE 2 | Mediation analysis ($n = 1,372$).

Variables	Model 1 (SWLS)				Model 2 (MBI_EE)				Model 3 (SWLS)			
	β	*t*	Bootstrap 95%CI		*β*	*t*	Bootstrap 95%CI		*β*	*t*	Bootstrap 95%CI	
			LLCI	ULCI			LLCI	ULCI			LLCI	ULCI
PSS	−0.487	−21.249***	−0.532	−0.442	0.776	20.396***	0.701	0.850	−0.427	−16.425***	−0.478	−0.376
MBI_EE									−0.078	−4.843***	−0.110	−0.047
R^2	0.248				0.233				0.261			
F	451.502**				415.995***				241.176***			

*PSS, Perceived Stress Scale; EE, Emotional Exhaustion; SWLS, Satisfaction with Life Scale. **p < 0.01, ***p < 0.001.*

TABLE 3 | Hierarchical multiple regression analyses of perceived stress and expression suppression on emotional exhaustion ($n = 1,372$).

Steps and independent variables	Emotional exhaustion		
	β	Total R^2	ΔR^2
Step 1			
Age	−0.74		
Marital status	−0.43	0.010	0.008
Step 2			
Perceived stress	0.478	0.234	0.233
Step 3			
Expression suppression	0.090	0.242	0.240
Step 4			
Perceived stress × expression suppression	0.062	0.246	0.243

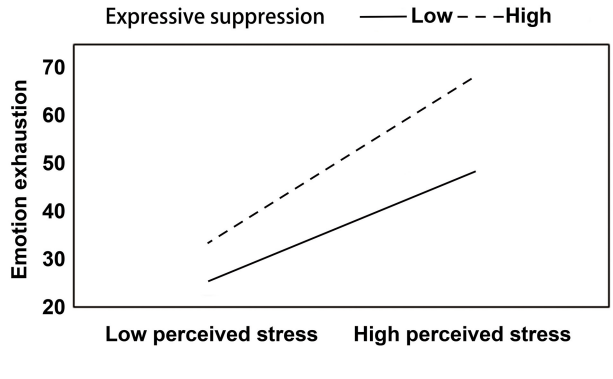

FIGURE 1 | The moderating effect of expression suppression on the relation between perceived stress and emotional exhaustion.

unique variance in EE while controlling for perceived stress, age, and marital status.

The entry of the interaction term perceived stress × ES in step 4 results in a significant result related to EE. This indicated that ES strengthened the effect of perceived stress on EE. A simple slope analysis showed that perceived stress predicted EE at both low ($B = 0.477$, $t = 5.131$, and $p < 0.001$) and high ($B = 0.732$, $t = 8.187$, and $p < 0.001$) levels of ES, but the association between perceived stress and EE was stronger when the ES level was high (see **Figure 1**).

DISCUSSION

During the past decades, an increasing number of research has been published in terms of burnout and SWB among clinical nurses. The mental health and overall QOL of clinical nursing teachers, as a special group of nurses, required increasing concerns. This study investigated the relationship of perceived stress with burnout and life satisfaction. Consistent with previous research, our research added evidence to confirm direct and indirect impacts of perceived stress on life satisfaction among Chinese nursing teachers (9). Moreover, to our best knowledge, this study was the first to propose a moderated mediation model to analyze the mechanism underlying the association between perceived stress, job burnout, ER, and life satisfaction. According to previous empirical studies and theories, we proposed a model that incorporated the mediating variable of burnout and the moderating variable of ER ability in the relationship between perceived stress and life satisfaction (**Figure 2**).

In particular, our findings indicated that the mediating role of burnout on the indirect relationship between perceived stress and life satisfaction was significant in the dimension of EE. The perceived stress was a significantly positive predictor of EE. EE, as the most core element in the Maslach burnout model, was regarded as feelings of being emotionally drained and depleted of one's emotional resources (41). Just as Maslach et al. (42) stated, "when people describe themselves as experiencing burnout, they are most often referring to the experience of emotional exhaustion". In addition, it has been shown that EE could result in other characteristics of burnout: DP and cynicism (42). The significance of EE could be predicted and explained according to Hans Selye's stress model of General Adaptation Syndrome (GAS). Hans Selye's stress model was a three-stage response that occurred when people encountered stress: alarm, resistance, and exhaustion. EE was an inevitable outcome when the stress response proceeded to the end stage. EE occurred if long-term stress continued beyond coping capacities and one's adaptation, which further increased the likelihood of suffering from a decreased level of life satisfaction. Khoo et al. illustrated

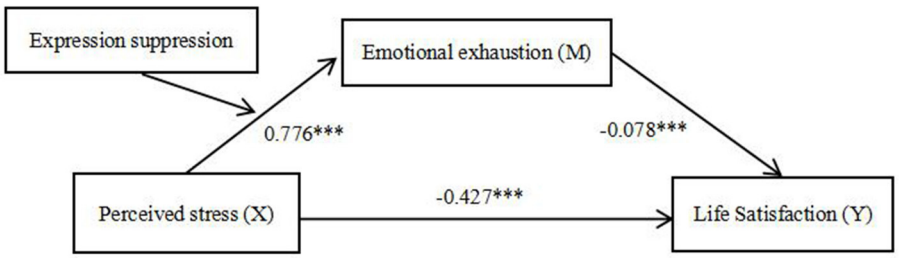

FIGURE 2 | The finalized moderated mediation model ($n = 1,372$). ***$p < 0.001$.

in their multicenter survey that stress in the workplace was the most significant factor for EE through multivariate analysis. About three-quarters of 38 sources of job stress were significantly associated with EE; and the most common source of stress was dealing with difficult parents (80.2%) (43).

Another finding worthy of note was that the perceived stress not only had a direct effect on EE but also had an indirect influence on EE, via mediation by the ER strategy of ES. The effect of perceived stress on EE could be strengthened by ES. For nursing teachers with high-level ES, the impact of perceived stress on EE was stronger than those with low-level ES. In other words, the more likely that clinical nursing teachers adopted an improper strategy of ER such as ES, the more they were vulnerable to suffer from EE. ES was a response-focused strategy, referring to individuals' efforts to suppress the expression or experience of emotion, especially in an attempt to control the behavioral component of the emotional response. For example, individuals with a high level of ES tended to "try to behave in such a way that a person watching you would not know you were feeling anything" (44). The association of ES with negative indicators of well-being was evident in previous studies. For individuals with cancer, those who tended to use more ES had reported lower QOL across all aspects (45).

The following reasons may explain the significance of ES among nursing teachers. The first reason might be the stressful situations that they have confronted. They were fully occupied with various sources of demands and problems, from both patients and intern students so that they have limited time and opportunities to consider and express their own feelings, emotions, and thoughts. Secondly, the implicit communication style in Chinese culture may account for the emotion expression among Chinese clinical nursing teachers. Influenced by Confucian philosophy, the Chinese are socialized to control the expression of their emotions and repress overt feelings. Only in this way could nursing teachers maintain professional authority among students. The third reason was the preference and role requirements of adopting emotional labor strategies among most nurses (46). Based on Hochschild's work (47), emotional labor strategies mainly comprised surface acting and deep acting. Surface acting stands for suppression of one's felt emotions, while deep acting represents an attempt to change one's felt emotions to meet the role demands (48). Suppression and control of emotion expression were thought to be a desired emotional state for nurses. They worried that too much emotional engagement with patients and students may disrupt medical equity and lead to a preference during decision making and practice. However, stress came up when nurses felt a mismatch between their actual feelings and displayed emotions during their interactions with patients and students, which was confirmed to result in burnout and negatively affected life satisfaction and wellbeing (48).

The study has significant theoretical and practical implications. Firstly, the results may help explain the underlying mechanisms of relationships among perceived stress, burnout, ER, and life satisfaction. Secondly, given the mediation role of ES, this study may inform initiatives to carry out ER training (ERT) to strengthen stress management. Generally, ERT incorporates techniques including but not limited to mindfulness (49), cognitive-behavioral therapy (50), and emotion-focused mediation (51) in order to improve ER skills to deal with stressful situations. ERT is expected to reduce or eliminate burnout syndromes and improve life satisfaction among workers with highly occupational stress such as clinical teaching nurses. Previous studies reveal that mental health (52) and professional QOL (53) can be increased through ERT among intensive and critical care nurses. Therefore, further research should be designed and conducted to examine the effectiveness of ERT that aims to alleviate or eliminate the impact of perceived stress on burnout and eventually improve life satisfaction for Chinese clinical nursing teachers.

In addition to its strengths, several limitations should be pointed out. Firstly, our sample was nurses all recruited from military hospitals. Considering that they were also taking responsibilities of military missions and disaster rescues, this group of nurses was regarded as having a higher level of perceived stress and burnout compared to nurses working in civilian hospitals. Besides, it was hard to determine that their stress was only from duties as clinical teachers and not from their duties as military nurses. Further research was required to explore the exact factors contributing to their perceived stress, in order to develop targeted interventions of stress management in this group of nurses. Secondly, this study failed to demonstrate the cause and effect relationships among the variables as a cross-sectional design was employed. For this reason, future studies might wish to apply a longitudinal or an experimental research design. Thirdly, it should be noted that the bias might occur when

we used a cross-sectional study design to explore the mediational processes, although most of the previous studies on mediation analysis did not consider the role of time. However, as early as the year of 1981, Dr. Judd and Kenny have declared the potential importance of studying meditation from a longitudinal design. They emphasized that failing to include prior assessments of the mediator and the outcome could lead to bias. In this respect, a longitudinal study was required in the future to give a more accurate examination of the mediation model (54).

CONCLUSION

This study investigated the mechanisms underlying associations between perceived stresses, job burnout, and life satisfaction among Chinese clinical teaching nurses using the moderated mediation model. The results indicated the direct and indirect relationships of perceived stress with burnout symptoms and life satisfaction. In particular, the findings demonstrated the mediator role of the principal element of burnout—EE—in the association between stress and life satisfaction. And ES partially moderated the strength of the relationship between perceived stress and job burnout. As a result, ERT was implied as a useful intervention to alleviate or eliminate the impact of perceived stress on burnout and eventually improve the life satisfaction for Chinese clinical nursing teachers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was obtained from the Ethics Committee of Daping Hospital affiliated to Army Medical University (number: 181).

AUTHOR CONTRIBUTIONS

XX, ZW, and FL: study design. XX, LC, YY, MX, and XT: data collection. ZW, LC, and FL: data analysis. ZW and FL: manuscript writing. All authors contributed to the article and approved the submitted version.

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