

REGULAR RESEARCH ARTICLE

Leonurine Exerts Antidepressant-Like Effects in the Chronic Mild Stress-Induced Depression Model in Mice by Inhibiting Neuroinflammation

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Abstract

Background: Leonurine is an active alkaloid that is extracted from Traditional Chinese Medicine *Herba leonuri*. Emerging evidence indicates that leonurine produces neuroprotective effects in ischemic stroke, Parkinson's disease, and Alzheimer's disease. However, the effect of leonurine in neuropsychiatric disorders, especially in major depression, remains unknown.

Methods: We used the chronic mild stress mouse model to explore the antidepressant effects of leonurine and the potential mechanisms. Behavioral tests including sucrose preference test, forced swimming test, and tail suspension test were taken to evaluate depression symptoms. Moreover, the contents of monoamine neurotransmitters in hippocampus and prefrontal cortex were measured by high-performance liquid chromatography. Neuronal morphology was detected by transmission electron microscopy.

Results: Administration of leonurine (60 mg/kg) for 4 weeks significantly alleviated depression-like behaviors of chronic mild stress mice, including increased sucrose preference and reduced immobility time in forced swimming test and tail suspension test. We further found that leonurine (60 mg/kg) effectively restored the levels of 5-hydroxytryptamine, noradrenaline, and dopamine in the hippocampus and prefrontal cortex of chronic mild stress mice, accompanied by amelioration of hippocampal neuronal damage. Furthermore, leonurine (60 mg/kg) significantly inhibited the production of proinflammatory cytokine interleukin-1 β , interleukin-6 and TNF- α , and suppressed the nuclear factor kappa B signaling pathway.

Conclusions: These findings demonstrate that leonurine exerts antidepressant-like effects, which may be mediated, at least in part, by improving monoamine neurotransmitters and inhibiting neuroinflammation. Our study provides insight into the potential of leonurine in depression therapy.

Keywords: leonurine, depression, chronic mild stress, monoamine neurotransmitters, neuroinflammation

Introduction

Depression is a prevalent and serious psychiatric disorder with high mortality and morbidity, characterized by hopelessness, anhedonia, cognitive deficits, and recurrent thoughts of death

or suicide (Milanovic et al., 2015). It is predicted that depression will be the leading public health problem and the most prevalent cause of disease burden globally by 2030 (Abou-Saleh et al.,

Received: April 10, 2017; Revised: July 17, 2017; Accepted: July 20, 2017

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Significance Statement

Depression is a prevalent and serious psychiatric disorder worldwide. However, the current available treatments and their clinical efficacy are still unsatisfactory. Identifying new drugs and thereby developing more effective treatments are primary goals in that research field. Studies have found that leonurine is an alkaloid isolated from *Herba leonuri*, which is used to calm pregnant women suffering from anxiety. And leonurine exerts considerable neuroprotective effects in animal models of ischemic stroke, Parkinson's disease (PD), and Alzheimer's disease (AD). It remains unknown whether leonurine exerts therapeutic effects on depression. In the present study, we provide evidence to show that improvement of monoamine neurotransmitters and inhibition of inflammation may contribute to the antidepressant effects of leonurine. Our study provides insight into the potential therapeutic value of leonurine for depression treatment.

2017). Abnormal serotonergic, noradrenergic, or dopaminergic systems are widely found in the pathophysiology of depression (Coppen, 1967; Carneiro et al., 2016). Over the past few decades, the monoaminergic hypothesis had received more attention in neurobiological studies of depression and acted as the basis for antidepressant drugs (Schildkraut, 1965). Currently, clinical antidepressants mainly increase the levels of 5-hydroxytryptamine (5-HT), dopamine (DA), and norepinephrine (NE) neurotransmitters in synaptic cleft to mitigate the symptoms of depression (Chandrasekhar et al., 2017). However, there was no effect on approximately one-third of depression patients in clinic therapy, suggesting the complexity of the pathogenesis underlying depression (Murphy and Byrne, 2012). Therefore, it is urgent to develop more effective treatment for depression.

Increasing evidence has shown that inflammatory processes take part in the development of depression (Miller and Raison, 2016). Psychosocial stressors could increase the levels of pro-inflammatory cytokines, such as interleukin (IL)-1 β and IL-6 in both brain and peripheral organizations, leading to sickness behavior syndrome (Wager-Smith and Markou, 2011). The central immune response is mainly modulated by microglia, which exerts an inflammatory role in response to cell injury signals. An increase in microglial activation was found in postmortem individuals suffering from MDD (Steiner et al., 2008). Various antidepressants may have a direct inhibitory effect on microglia (Tynan et al., 2012). NF- κ B, a transcription factor regulating many immune and inflammatory genes, has been recognized to play an important role in depression (Caviedes et al., 2017). These findings illustrate that inhibition of neuroinflammation contributes to mitigate depression. Some Traditional Chinese Medicine herbs exhibit effective anti-inflammatory activity, suggesting the clinical potential of these herbs in depression treatment.

Leonurine, also named SCM-198 (4-guanidino-n-butyl syringate), is a chemically synthesized compound based on a bioactive alkaloid extracted from *Herba leonuri* (Liu et al., 2010). Previous studies reported that leonurine exerted several biological effects, such as antidiabetic, cardiovascular, and bovine mastitis protection (Xin et al., 2009; Qi et al., 2010; Huang et al., 2012; Zhang et al., 2012). Shikov et al. (2011) reported that *Herba leonuri* was used to calm pregnant women suffering from anxiety. In recent years, it is found that leonurine possess neuroprotective effects in animal models of ischemic stroke, PD, and AD (Liu et al., 2010; Shi et al., 2011; Hong et al., 2015). Leonurine treatment significantly rescued behavioral deficit of animals, promoted neuronal survival, and inhibited inflammation. However, it remains unknown whether leonurine exerts therapeutic effects on depression. To verify this speculation, a chronic mild stress (CMS) model was used in this study to investigate the antidepressant-like effects of leonurine and its underlying mechanisms. Our study showed that leonurine alleviated depression-like behaviors, recovered the levels of monoamine

neurotransmitters, and ameliorated hippocampus pathological damage of CMS mice. Furthermore, leonurine inhibited inflammation via suppressing the activation of NF- κ B signaling pathways. These findings imply that leonurine may be a promising drug for depression therapy.

Materials and Methods

Animals and Reagents

Male C57BL/6 (8–10 weeks) mice with a body weight of 18 to 22 g were purchased from the Comparative Medicine Center of Yangzhou University (Yangzhou, China) (Animal Production license no. SCXR20100004). Animals were housed in a standardized environment (22 \pm 2°C, 55 \pm 5% humidity, and 12-hour-light/-dark cycle) and given ad libitum access to food and water. All experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all animals were treated in strict accordance with protocols approved by Institutional Animal Care and Use Committee of Nanjing Medical University.

The following reagents were used: leonurine (purity >99%) was kindly provided by Professor Yizhun Zhu (Macau University of Science and Technology). Fluoxetine, 5-HT, NE, and DA standards were purchased from Sigma-Aldrich. The following different primary antibodies were used: rabbit anti-phosphor-IKK β (Ser177/181) (16A6) (1:1000, Cell Signaling, 2697), rabbit anti-IKK β (D30C6) (1:1000, Cell Signaling, 8943), rabbit anti-phosphor-NF- κ B p65 (1:1000, Cell Signaling, 2697) (1:1000, Cell Signaling, 8242S), rabbit anti-NF- κ B p65 (D14E12) (1:1000, Cell Signaling, 8242S), rabbit anti-TNF- α (1:1000, Abcam, ab9739), rabbit anti-IL-1 β (1:1000, sigma, 13767), rabbit anti-IL-6 (1:1000, Abcam, ab83339), rabbit anti-Iba1 (1:1000, Wako, 019-19741) and mouse anti- β -actin (1:1000, Sigma, A5441).

Experimental Design

Animals were housed in the laboratory for 1 week to adapt to the environment before initiation of the study. Mice were randomly divided into 5 groups (n = 15/group) as follows: normal control group, CMS group, CMS + leonurine (30 mg/kg/d) group, CMS + leonurine (60 mg/kg/d) group, and CMS + fluoxetine (20 mg/kg/d) group. For intragastric administration, leonurine was dissolved in 0.9 % saline containing 1% (w/v) sodium carboxymethyl cellulose (CMC-Na), according to the previous study (Hong et al., 2014). Fluoxetine was dissolved in 0.9% saline containing 1% (w/v) CMC-Na. Non-drug groups were intragastrically administered with 0.9% saline containing 1% (w/v) CMC-Na. All drugs were administered by oral gavage for 4 weeks after the establishment of CMS model.

CMS Model

The CMS model, a widely used depression model (Willner et al., 1992), was carried out as previously described (Lu et al., 2014). A series of procedures was included, for example, clipping (10 minutes), reversed light/dark cycle (24 hours), cage tilt (45°, 12 hours), restraints (12 hours), wet cage (12 hours), food and water deprivation (12 hours), pairing (2 hours), crowded a mice into an empty bottle (6 hours), continuous light (12 hours), etc. Two or three stressors were given daily at random that could not be repeated for 3 consecutive days. The sucrose preference was tested once a week during the CMS. The CMS procedure was continued during the entire drug treatment period.

Behavioral Evaluations

Sucrose Preference Test

Sucrose preference test (SPT) was performed as described previously (Du RH et al., 2016). At 72 hours before the test, all mice were habituated to 1% sucrose solution: 2 bottles of 1% sucrose solution (w/v) were placed in each cage. Then 24 hours later, mice were deprived of water and food for 24 hours followed by the SPT; each mouse was given 2 bottles that were placed on the cage containing 1% sucrose solution (w/v) and tap water to drink freely for 12 hours. To avoid location effects, 6 hours later, 2 bottles were exchanged. Twelve hours later, the consumption of tap water and sucrose solution was recorded. The sucrose preference was calculated as sucrose preference (%) = (sucrose solution consumption) / (sucrose solution consumption + tap water consumption) × 100%.

Forced Swimming Test

Forced swimming test (FST) was carried out and the total immobility was estimated according to described as our previous report (Du RH et al., 2016).

Tail Suspension Test

Tail suspension test (TST) was carried out based on the previous reported methodology (Du RH et al., 2016).

Tissue Preparation

At the end of the behavioral test, all animals were anesthetized with 4% chloral hydrate. To prepare tissues for electron microscopy, mice were perfused transcardially with saline followed by 2.5% glutaraldehyde phosphate buffer in 0.1 M phosphate buffer (pH 7.4). To prepare tissues for high performance liquid chromatography and western blot, mice were quickly decapitated. Hippocampus and prefrontal cortex were dissected, quickly frozen in liquid nitrogen, and stored at -80°C until use. To prepare tissues for immunohistochemistry, mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Whole brains were quickly removed from the skull. To prevent pressure artefacts, brains were additionally postfixed in the same fixative overnight at 4°C, dehydrated with increasing gradients of sucrose (20% and 30%) in phosphate buffered saline, and frozen at 4°C. Frozen brains were embedded in OCT and sliced serially at 30 µm using a cryostat (Leica).

High Performance Liquid Chromatography Analysis

5-HT, NE, and DA in the hippocampus and prefrontal cortex were measured by high-performance liquid chromatography with electrochemical detector (Ultimate 3000 Autosampler) as described previously (Lu et al., 2014). Hippocampus and prefrontal cortex tissues were homogenized in extract solution, which

consisted of 0.1 M HClO₄ and 0.1 mM EDTA buffer, and the mixtures were centrifuged at a speed of 20000 rpm for 30 minutes at 4°C. Then, 50 µL of the resultant supernatant was injected into the liquid chromatography system equipped with a reversed-phase C 18 column (2.2 µm, 120 Å, 2.1 × 100 mm, DIONEX) and was detected by ESA Coulochem III Electrochemical Detector. The detector was set at 350 mV. The mobile phase consisted of 90 mM NaH₂PO₄, 1.7 mM 1-octanesulfonic acid, 50 mM citrate, 50 µM EDTA - 2 Na, and acetonitrile (0.05 w/v) (pH 2.6). The identification and purity were evaluated by the chromatographic peaks as well as their quantitative evaluation by comparing their retention times and peak areas with those of standard solutions.

Transmission Electron Microscopy

Whole brains were quickly removed from the skull, and pieces of approximately 1-mm³ slabs containing the whole hippocampus were cut. The hippocampal tissues were immersed in the same fixative for 24 hours at 4°C. Tissue blocks were then post-fixed in 1% osmium tetroxide for 2 hours and dehydrated in a series of graded ethanol, and tissues were embedded in propylene oxide. After complete dehydration in ascending ethanol-series, tissue blocks were penetrated in acetone and resin (1:1) for 2 hours and flat-embedded in durcupan resin overnight. The tissue blocks were polymerized at 60°C for 72 hours. Serial ultrathin sections (70–80 nm) were collected on formvarcoated copper slot grids and stained by means of uranyl acetate and lead citrate. Sections were viewed under a light microscope, and hippocampi were chosen for reembedding and electron microscopic sectioning. Sections were examined with a transmission electron microscope (JEM-1010, Tokyo, Japan). Whole images were level adjusted, sharpened, and cropped in Photoshop without changing any specific feature within, and quantitative analysis of myelinated axonal transversal areas was performed using Image Pro Plus. A minimum of 100 myelinated axons was measured per mouse.

Western Blotting

Hippocampi were homogenized on ice in RIPA buffer (consisting of cocktail, NaF, and Na₃VO₄), and protein concentration was quantified by BCA assay kit (Beyotime Biotech Inc.). Protein samples were separated by 10% to 15% Tris-HCl polyacrylamide gels (Bio-Rad) and then electrotransferred to polyvinylidene difluoride membrane (Millipore). The blots were blocked with 5% skim milk in Tris-buffered saline containing Tween-20 for 1 hour at room temperature. Primary antibody incubations were performed overnight at 4°C followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Immunoreactivity bands were detected by the enhanced chemiluminescence plus detection reagent (Pierce) (Thermo) and quantified with Image J software.

Immunohistochemistry

The immunostaining method was described in a previous publication (Hu et al., 2010). The hippocampal slices were selected according to the mouse brain map. Primary antibody rabbit anti-Iba1 (1:1000, Wako, 019-19741) and biotinylated secondary antibody (1:1000, invitrogen) were used in the present study. Serial images of the dentate gyrus (DG) of each section were captured as tiffs and then exported to Image J software to count number of positive cells.

Statistical Analysis

All analyses were performed using SPSS Version 20.0 (SPSS Inc., USA). Differences between mean values were evaluated using

1-way ANOVA followed by the posthoc LSD test. All data are expressed as mean \pm SEM. Differences were considered significant at $P < .05$.

Results

Leonurine Ameliorated Depressive-Like Behaviors of CMS Mice

In this study, we evaluated the antidepressant effects of leonurine in the chronic mild stress model. The SPT, TST, and FST are extensively used for the evaluation of antidepressant activity (Cryan and Holmes, 2005; Stepanichev et al., 2016). The whole experimental process is shown in Figure 1A. At the beginning of the experiment, there was no significant difference in the percent of sucrose preference among the groups (Figure 1B). After 6 weeks of CMS modeling, the percent of sucrose

preference significantly decreased compared with that in the control group ($P < .01$) (Figure 1B-C). After leonurine (60 mg/kg) administration for 4 weeks, the sucrose consumption markedly increased compared with the CMS group (Figure 1B-C; $F_{4,50} = 13.552$, $P < .05$).

We also performed the TST and FST to assess the antidepressant effects of leonurine (Figure 1D-E). The immobility time of mice that were exposed to CMS significantly increased in FST ($P < .001$, Figure 1D) and TST ($P < .01$, Figure 1E) compared with that in the control group. Leonurine (60 mg/kg) treatment for 4 weeks induced a remarkable decrease of immobility time in FST (Figure 1D, $F_{4,50} = 6.755$, $P < .01$) and TST (Figure 1E, $F_{4,50} = 7.263$, $P < .05$). Similar effects were obtained after fluoxetine (20 mg/kg) treatment ($P < .01$, Figure 1B-E), but leonurine (30 mg/kg) treatment for 4 weeks failed to ameliorate behavioral symptoms in the CMS group. These data indicated that leonurine (60 mg/kg) exerted antidepressant effects in CMS-treated mice.

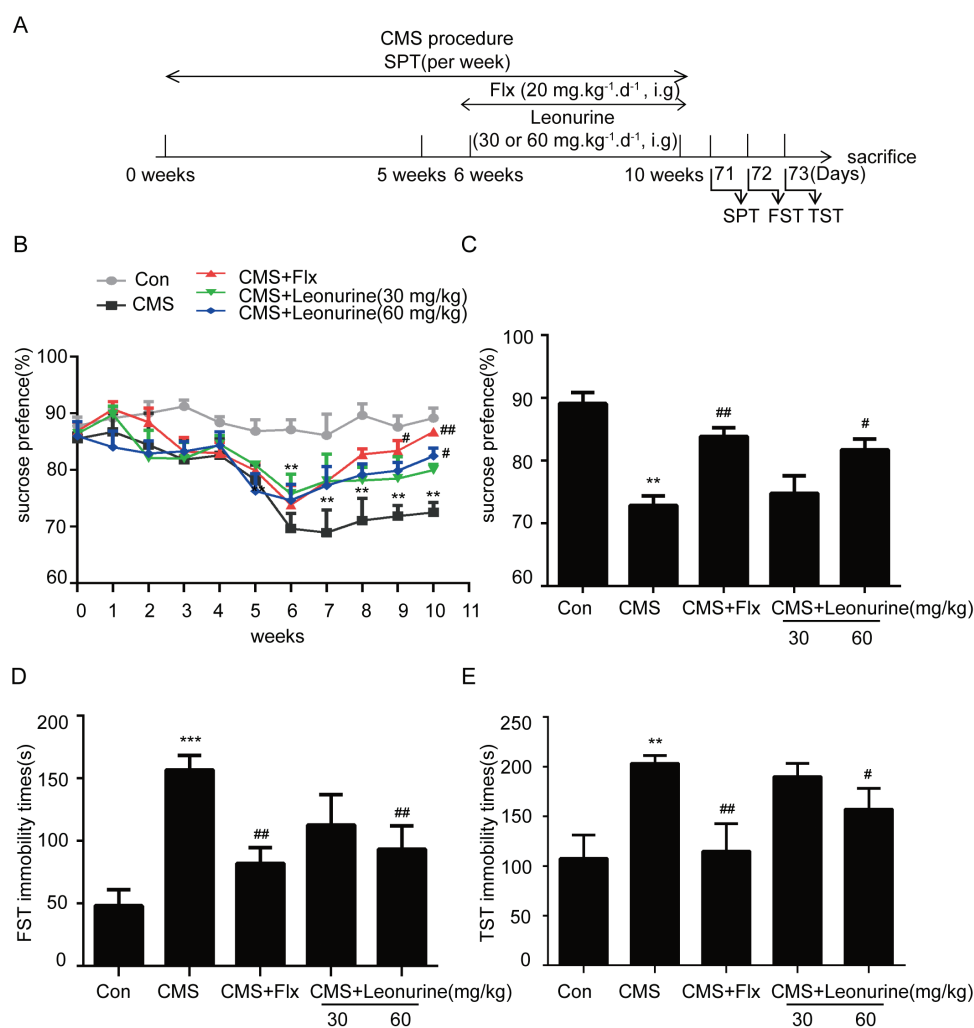


Figure 1. Leonurine ameliorated depressive-like behaviors in chronic mild stress (CMS) mice. (A) The schedule of CMS, drug administration, and behavioral tests. CMS was performed for 6 weeks. C57BL/6 mice were intragastric administered (i.g.) with a single dose of vehicle (control, 0.9% saline containing 1% carboxymethyl cellulose), fluoxetine (20 mg/kg), or leonurine (30 or 60 mg/kg) once daily for another 4 consecutive weeks. Sucrose preference test (SPT), forced swimming test (FST), and tail suspension test (TST) were then conducted. The CMS procedure was continued during the entire drugs treatment period. SPT was performed every week during the CMS procedure. (B) Changes in sucrose preference percentage of mice over 10 weeks. The percentage of sucrose preference (C), immobility times in the FST (D) and TST (E) were conducted half an hour after the last administration. Data are expressed as mean \pm SEM. The behavioral tests were performed in the control ($n = 9$), CMS ($n = 11$), CMS+Flx ($n = 12$), CMS+leonurine (30 mg/kg) ($n = 11$), and CMS+leonurine (60 mg/kg) ($n = 12$) mice. * $P < .01$, ** $P < .001$ vs the control group; # $P < .05$, ## $P < .01$ vs the CMS group. Con, control; Flx, fluoxetine.

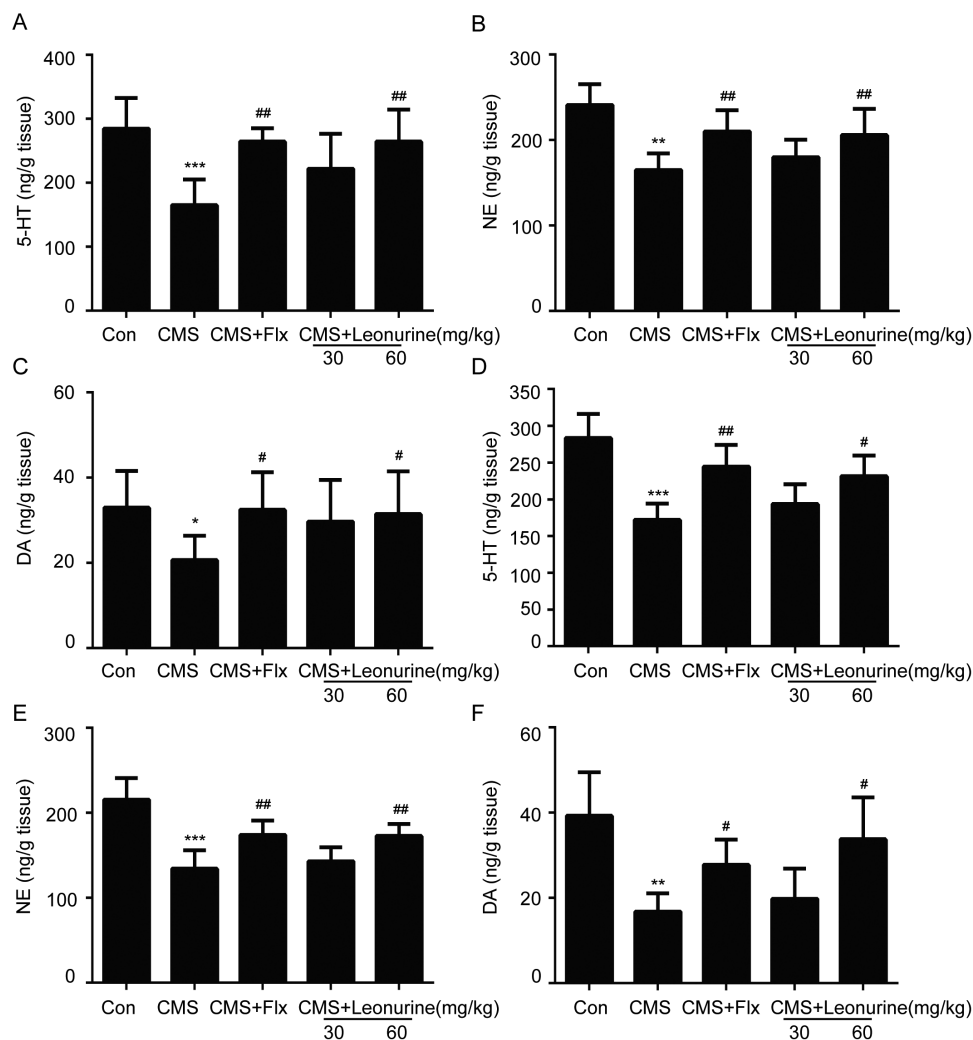


Figure 2. Leonurine increased the levels of 5-hydroxytryptamine (5-HT), dopamine (DA), and norepinephrine (NE) in the hippocampus and prefrontal cortex of chronic mild stress (CMS) mice. 5-HT, NE, and DA in hippocampus (A-C) and prefrontal cortex (D-F) were analyzed by high performance liquid chromatography. Data are expressed as mean \pm SEM. The high performance liquid chromatography experiment was performed in the control (n = 6), CMS group (n = 8), CMS + Flx (n = 9), CMS + leonurine (30 mg/kg) (n = 8), and CMS + leonurine (60 mg/kg) (n = 9) mice. * $P < .05$, ** $P < .01$, *** $P < .001$ vs the control group; # $P < .05$, ## $P < .01$ vs the CMS group. Con, control; Flx, fluoxetine.

Leonurine Increased the Levels of 5-HT, NE, and DA in the Hippocampus and Prefrontal Cortex of CMS Mice

We examined whether leonurine improved monoamine neurotransmitter deficits by high performance liquid chromatography. As shown in **Figure 2**, the levels of 5-HT, NE, and DA were significantly decreased in the hippocampus (**Figure 2A-C**) and prefrontal cortex of CMS mice (**Figure 2D-F**) compared with those in the control group. However, treatment of CMS mice with leonurine at 60 mg/kg, but not 30 mg/kg, for 4 weeks significantly increased the levels of 5-HT (**Figure 2A**, $F_{4,35} = 7.47$, $P < .01$), NE (**Figure 2B**, $F_{4,35} = 8.725$, $P < .01$), and DA (**Figure 2C**, $F_{4,35} = 2.403$, $P < .05$) in the hippocampus. The same effects were seen in the prefrontal cortex; leonurine at 60 mg/kg caused a noticeable increase in the levels of 5-HT (**Figure 2D**, $F_{4,35} = 16.547$, $P < .05$), NE (**Figure 2E**, $F_{4,35} = 21.948$, $P < .01$), and DA (**Figure 2F**, $F_{4,35} = 9.335$, $P < .05$). In addition, similar effects were obtained after fluoxetine (20 mg/kg) treatment ($P < .05$, $P < .01$, respectively) (**Figure 2**). These results indicated that leonurine ameliorated the monoamine neurotransmitter abnormalities.

Leonurine Attenuated Neuronal Damage in the Hippocampus of CMS Mice

In addition, we observed the ultrastructural changes of hippocampal neurons. Transmission electron microscopy showed that the ultrastructural features of neuron morphology was normal, manifested as clear membrane structure and homogeneous nucleus chromatin in the control group (**Figure 3A**). With the establishment of the CMS model, neurons showed abnormalities, represented as swollen and vacuolated mitochondria and increased chromatin (**Figure 3A**). Treatment of leonurine (60 mg/kg) for 4 weeks improved hippocampal neuronal abnormalities (**Figure 3A**).

Abnormal neuronal morphology is associated with axonal injury and may be involved in neuropsychiatric disorders (Licznarski and Duman, 2013). Myelination is a unique cellular process that has a significant effect on the structure and physiology of axons and its surrounding tissues (Chomiak and Hu, 2009). Myelinated fibers contribute to the transmission of nerve impulses between the hippocampus and other brain regions and are considered the structural basis of the hippocampal function. Furthermore, it has been reported that hippocampal myelination

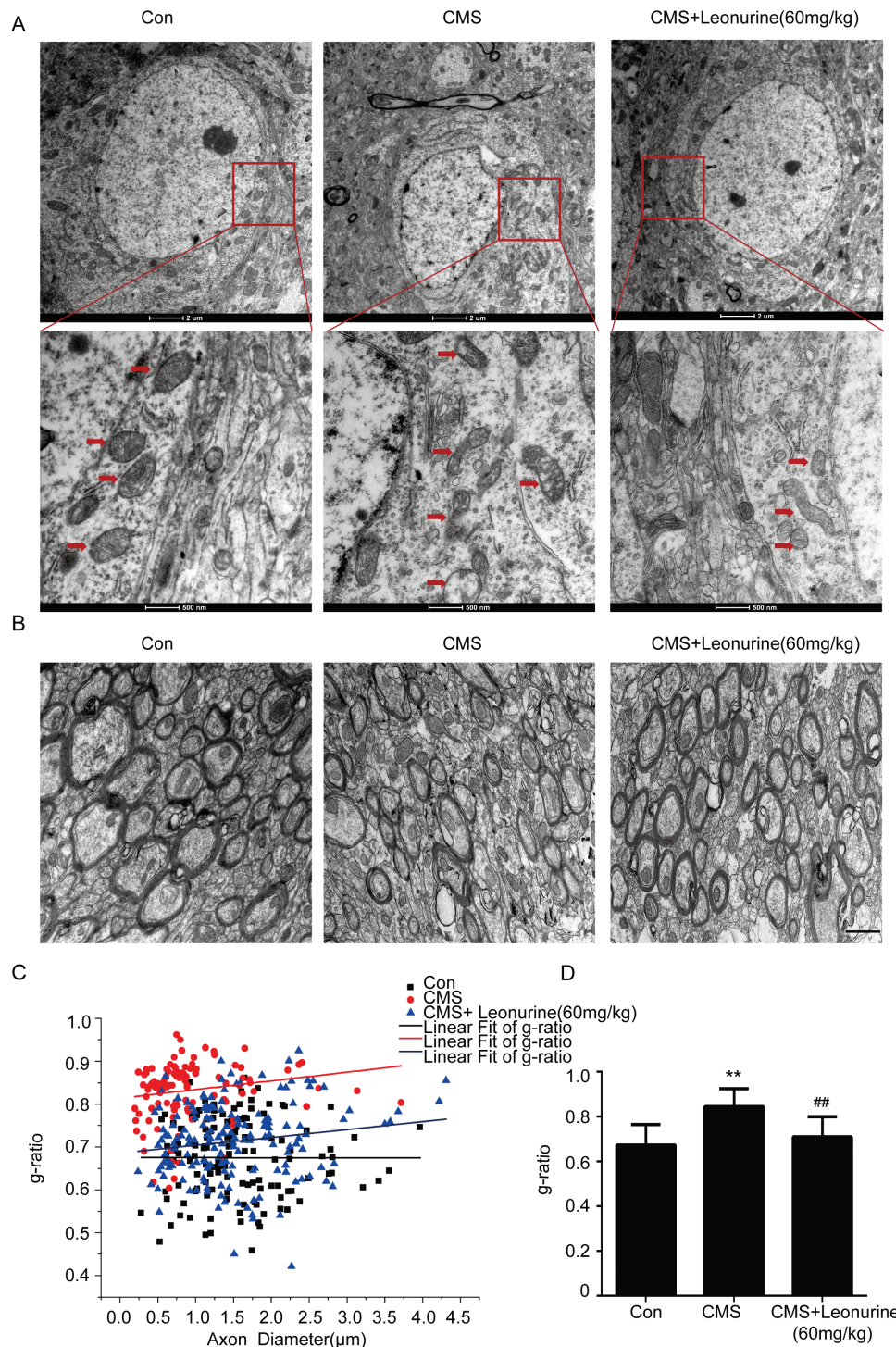


Figure 3. Leonurine attenuated neuronal damage in the hippocampus of chronic mild stress (CMS) mice. (A) Transmission electron microscopy analysis of hippocampal neuronal ultrastructural features at a magnification of $\times 2900$ (upper) and a higher magnification of $\times 11000$ (below). Mitochondria (arrows). The transmission electron microscopy experiment was performed in the control ($n = 3$), CMS group ($n = 3$), and CMS + leonurine (60 mg/kg) ($n = 3$) mice. (B) Ultrastructural images of hippocampal myelinated axons captured with transmission electron microscopy. Scale bar = 2 μm . (C-D) Scatter plot and quantitative analysis of g-ratio (myelin inner diameter/outer diameter) values in the hippocampus in the control group ($n = 167$ axons), CMS group ($n = 162$ axons), and CMS + leonurine (60 mg/kg) group ($n = 178$ axons). * $P < .01$ vs the control group; ** $P < .01$ vs the CMS group. Con, control; SCM-198, leonurine.

affects spatial memory and synaptic structure. Myelination injury may indirectly result in neuronal loss or death (Suzuki et al., 2016). Transmission electron microscopy showed that myelinated axons in the CMS group displayed thicker myelin sheaths compared with the control group, indicated by the significant

increase of g-ratio (myelin inner diameter/outer diameter) ($P < .01$) (Figure 3B-D). Leonurine (60 mg/kg) administration for 4 weeks significantly improved the injury of myelin (Figure 3B) and remarkably decreased the g-ratio compared with the CMS group (Figure 3C-D, $F_{2,504} = 119.888$, $P < .01$).

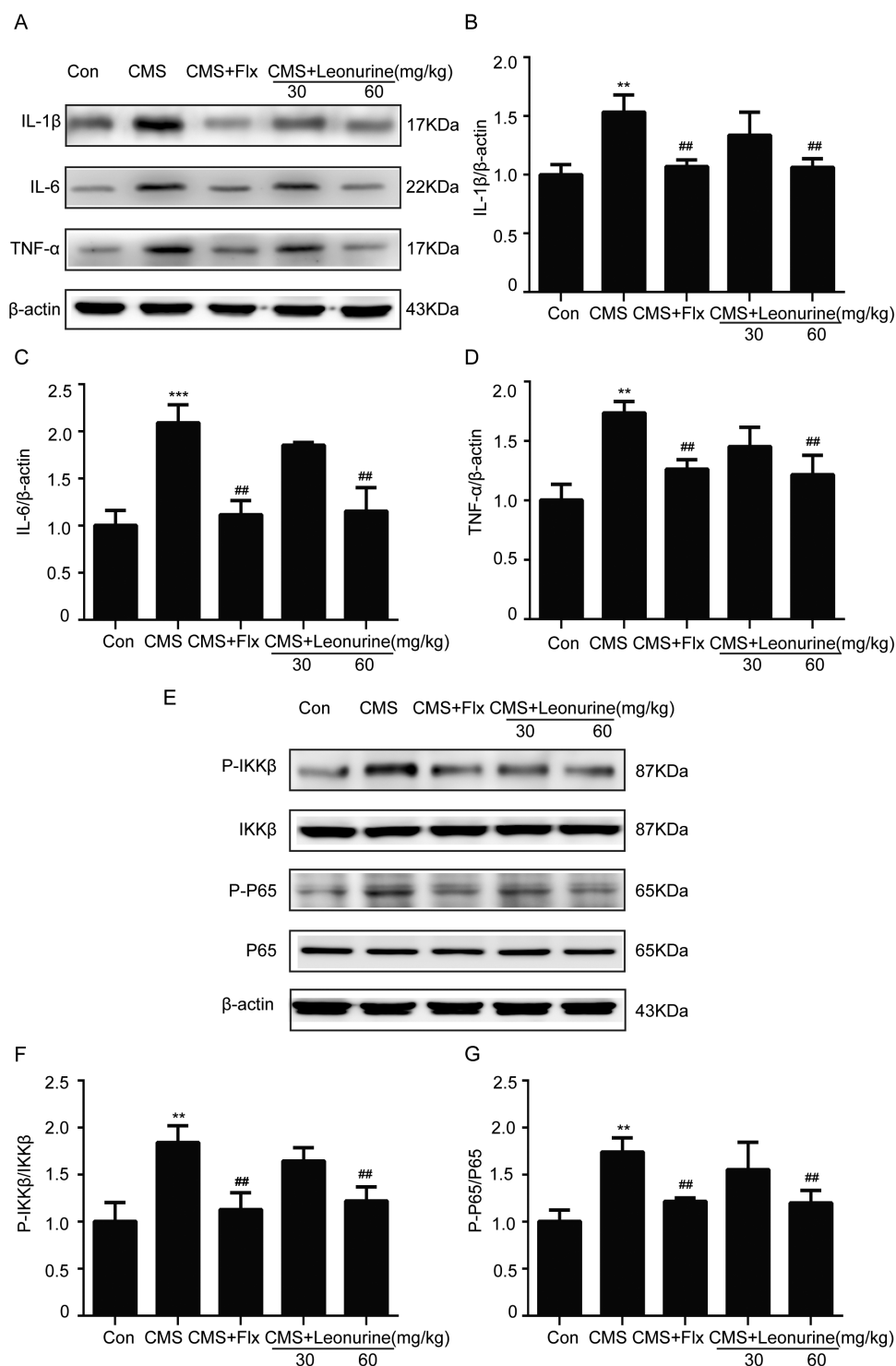


Figure 4. Leonurine suppressed inflammation in the hippocampus of chronic mild stress (CMS) mice. (A) Western blotting analysis of proinflammatory cytokines IL-1 β , IL-6, and TNF- α protein levels in hippocampus. Quantitative analysis of proinflammatory cytokines IL-1 β (B), IL-6 (C), and TNF- α (D). (E) Western blotting analysis of phosphorylated and total IKK β , and p65 in hippocampus. (F-G) Quantitative analysis of phosphorylated and total IKK β , phosphorylated and total p65. Data are expressed as mean \pm SEM. The western blot experiment was performed in the control (n = 3), CMS group (n = 4), CMS + Flx (n = 5), CMS + leonurine (30 mg/kg) (n = 4), and CMS + leonurine (60 mg/kg) (n = 4) mice. * $P < .01$, ** $P < .001$ vs the control group; ## $P < .01$ vs the CMS group. Con, control; Flx, fluoxetine.

Leonurine Suppressed Inflammation in the Hippocampus of CMS Mice

Inflammatory processes participate extensively in the etiology of major depression (Miller and Raison, 2016). We next

investigated whether leonurine played an antiinflammatory role in CMS mice. Western blotting analysis of proinflammatory cytokines showed that the levels of proinflammatory cytokines like IL-1 β , IL-6, and TNF- α markedly increased in the CMS group ($P < .01$, $P < .001$, and $P < .01$, respectively; Figure 4A-D) compared

with the control group. Leonurine (60 mg/kg) administration for 4 weeks dramatically downregulated the levels of proinflammatory cytokines (Figure 4A-D, $F_{4,15} = 12.935$, $P < .01$). No significant antiinflammatory effect was detected in the leonurine (30 mg/kg) group.

Nuclear factor kappa B (NF- κ B), an inflammatory signaling molecule, plays a crucial role in depression (Caviedes et al., 2017). To further study the potential mechanism of antiinflammatory effects of leonurine in CMS mice, NF- κ B signaling pathway was detected. Western blotting analysis showed that expression of phosphorylated IKK β and p65 in hippocampus significantly increased after CMS exposure ($P < .01$, $P < .01$, Figure 4E-G), suggesting that the NF- κ B signaling pathway was activated. Leonurine (60 mg/kg) administration inhibited NF- κ B signaling pathway activation (Figure 4E-G, $F_{4,15} = 9.607$, $P < .01$), but no significant difference was measured between the CMS + leonurine (30 mg/kg) group and the CMS group.

Leonurine Alleviated Microglial Activation in the Hippocampus of CMS Mice

Inflammation in the context of the nervous system, termed neuroinflammation, is typically associated with microglial activation (Najjar et al., 2013). We further verified antiinflammatory effect of leonurine on microglia activation. Immunostains of brain slices against Iba-1 showed an increased number of Iba1-positive microglia in the hippocampus DG of CMS mice (Figure S1, $P < .01$). Leonurine (60 mg/kg) administration for 4 weeks significantly decreased the number of Iba1-positive microglia ($F_{2,12} = 12.74$, $P < .05$).

Discussion

Previous studies showed that leonurine, an alkaloid isolated from *Herba leonuri*, exerts the potential neuroprotective effects in animal models of ischemic stroke, PD, and AD (Qi et al., 2010; Shi et al., 2011; Hong et al., 2015). However, the effect of leonurine on depression remains unclear. The current work is a further exploration based on previous reports and shows that leonurine has antidepressant-like effects. We found that administration of leonurine (60 mg/kg) significantly increased sucrose preference percentage in SPT and reduced immobility time in both the FST and TST. Our results further showed that leonurine (60 mg/kg) treatment alleviated CMS-induced neuronal injury and improved the levels of monoamine neurotransmitters in vivo. Furthermore, leonurine (60 mg/kg) inhibited CMS-induced neuroinflammation. Together, our present study demonstrates that leonurine may be a promising drug for treatment of depression, acting with multiple mechanisms.

Depression is considered as an immune activation-related disease (Kaufmann et al., 2017). Part of patients with depression exhibit increased levels of proinflammatory cytokines that return to normal levels after treatment with antidepressants (Miller et al., 2009). In the present study, we found that leonurine exerted an antiinflammatory influence, with a corresponding decrease in NF- κ B activity, this is consistent with previous reports (Xu et al., 2014). Central immune and inflammatory response is mainly modulated by microglia, which exerts a pro- or antiinflammatory role in response to cell injury signals. An increase in microglial activation was found in postmortem individuals suffering from MDD (Steiner et al., 2008). Various antidepressants may have a direct inhibitory effect on microglia (Tynan et al., 2012). In the present study, microglia was significantly activated in CMS mice, and leonurine (60 mg/kg) treatment effectively inhibited

microglia activation. These results indicate that antiinflammatory effects of leonurine may be mediated by inhibiting microglia overactivation in CMS model.

The classical monoamine hypothesis of depression (Schildkraut, 1965) still is one of the proposed theories regarding the etiology of depression (Chandrasekhar et al., 2017). Deficiency of 5-HT, NE, and DA in the brain are commonly observed both in animals and patients experiencing stress and depression. Fluoxetine, a classic antidepressant, plays an antidepressant effect by effectively raised the level of 5-HT and marginally improved serotonergic transmission. In addition, fluoxetine uniquely increases extracellular levels of DA and NE as well as 5-HT (Pozzi et al. 1999; Bymaster et al. 2002). It was also shown that fluoxetine had moderate affinity for animal 5-HT_{2C} receptors and acted as an antagonist at 5-HT_{2C} receptors (Ni and Miledi, 1997). Furthermore, blockade of the 5-HT_{2C} receptor increases extracellular DA and NE in the prefrontal cortex of the rat brain (Millan et al. 1998; Gobert and Millan 1999). It has been reported that inflammation has effects on neurotransmitter changes in major depression (Moller et al., 2015). The proinflammatory cytokines, acting as immunotransmitters, then initiate changes in the neurotransmitter networks, thereby precipitating depression (Leonard, 2007). Leonard et al. (Leonard et al., 2014) reported that proinflammatory cytokines can regulate the function of SERT to influenced serotonin signaling. Clinical nonsteroidal antiinflammatory drugs, such as the cyclooxygenase-2 inhibitor celecoxib, have antidepressant effects by enhancing the cortical release of dopamine and noradrenaline (Muller, 2010). Since 5-HT, NE, and DA are considered to be extensively involved in the regulation of mood and cognitive functions in depression (Mao et al., 2011), in the present study monoamine levels in hippocampus and prefrontal cortex were analyzed. We found that the levels of 5-HT, NE, and DA were significantly decreased in the hippocampus as well as prefrontal cortex of CMS mice, which was consistent with previous study (Liang et al., 2015). Leonurine (60 mg/kg) treatment restored to control values the 5-HT, NE, and DA levels decreased by CMS exposure, and this effect may be mediated by suppressing inflammatory response.

In addition, inflammation also influences neuronal functions (Berk et al., 2013). Alterations of the myelin sheath are involved in spatial memory and the synaptic structure, leading to neurobehavioral deficits (Fan et al., 2008). Previous study showed that LPS exposure-induced inflammation caused impaired myelination as indicated by the disintegrated myelin sheaths in the juvenile rat brain (Fan et al., 2008). This finding supports that inflammation is linked to myelin morphological change. G-ratio (the ratio of the inner axonal diameter to the total outer diameter) is a highly reliable ratio for assessing axonal myelination. Increased g-ratio means that the myelin is thinner, the nerve conduction velocity is reduced, and hippocampus structure is abnormal (Chomiak and Hu, 2009). In the present study, we found that leonurine (60 mg/kg) administration significantly decreased g-ratio and rescued CMS-induced hippocampal myelin degeneration. These findings indicate that inhibition of neuroinflammation may be involved in alleviating neurological damage by leonurine, which in turn exerts antidepressant effects in behavioral tests.

In conclusion, our results provide evidence to show that improvement of monoamine neurotransmitters and inhibition of inflammation may contribute to the antidepressant effects of leonurine. Our study provides an insight into the potential therapeutic value of leonurine for depression treatment. However,

the precise mechanisms underlying the antiinflammatory effect of leonurine in CMS mice is in the need of further exploration.

Acknowledgments

Thanks to Professor Yizhun Zhu for providing leonurine.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (nos. 81630099, 81473196, and 81573403) and the key project of Natural Science Foundation of the Higher Education Institutions of Jiangsu Province (no. 15KJA310002).

Statement of Interest

None.

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