Antidepressant-like effects of Z-ligustilide on chronic unpredictable mild stress-induced depression in rats

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Abstract. Depression is a significant public health issue and its neuropathogenesis is associated with the dysfunction of progesterone and allopregnanolone biosynthesis. Z-ligustilide (LIG), one of the main components of the herb *Angelica sinensis (Oliv.) Diels* (AS), is reported to have antidepressant activities. The present study aimed to evaluate the antidepressant-like effects of LIG via behavioral tests and to measure the levels of progesterone and allopregnanolone in the prefrontal cortex and hippocampus. The results demonstrated that LIG (20 and 40 mg/kg) exerted antidepressant-like effects, confirmed by increased mobility, locomotion, rearing frequency and preference to sucrose. Furthermore, the levels of progesterone and allopregnanolone in the prefrontal cortex and hippocampus were markedly increased following treatment with LIG (20 and 40 mg/kg), indicating that both neurosteroids could serve a significant role in the antidepressant-like effects of LIG.

Introduction

Depression is a mental health condition with a high clinical incidence. The prevalence of depression in younger patients (age, 12-17 years; incidence rate >12% in the USA in 2015) is higher compared with elderly ones (age, ≥18 years; incidence rate <10% in the USA in 2015) (1,2). Globally, depression is considered to be one of the single largest contributors to non-fatal health losses, accounting for 7.5% of all years lived with disability (YLD). It has been reported that ~80% of depression cases occur in low to middle-income countries (3). There are several typical antidepressant drugs in common usage, including selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), serotonin-noradrenergic reuptake inhibitors (SNRIs) and monoamine oxidase inhibitors (MAOIs) (4). However, ~30-40% of patients do not respond to these drugs during the first 4-6 weeks of treatment. In addition, the efficacy of these drugs is often controversial, while a number of them are accompanied by numerous side effects, including apathy, sedation and cognitive and sleep disorders (5,6). Therefore, more effective and better tolerated antidepressants are urgently needed to treat depression.

Angelica sinensis (Oliv.) Diels (AS) is a well-known traditional Chinese medicine, which has been applied as a treatment for gynecological diseases. The antidepressant-like effects of AS extracts have also been reported (7,8).

Z-ligustilide (LIG; Fig. 1) is the main component of AS, accounting for 2-3.5% of all composite compounds (9-13). Studies have indicated that LIG may significantly improve blood circulation, protect against nerve damage and attenuate painful behavior (11,14-17). The neuroprotective effects of LIG have also been reported in several animal models of cerebral ischemia, indicating that LIG may reduce the infarct size in the ischemic area, decrease edema in the brain and improve neurobehavioral defects (18). Furthermore, LIG is thought to protect against hypoperfusion-induced nerve damage in the cerebral cortex, inhibit cortical neuron apoptosis and maintain the integrity of the neuronal structure (19).

The active antidepressant constituents of AS have been identified and LIG is thought to be one of the most important (20,21). A previous study demonstrated that LIG easily penetrates the blood-brain barrier and quickly enters the brain (22). By applying pharmaceutical analysis and pharmacokinetics, LIG is expected to be a major constituent of antidepressants (23,24).

Progesterone and allopregnanolone are neurosteroids, mediating both the transport of cholesterol from the outer to the inner mitochondrial membrane and the activation of a series of enzymatic reactions to regulate the biosynthesis of neurosteroids (25,26). Studies indicate that reduced levels of progesterone and allopregnanolone are associated with the development of various mental disorders, including anxiety and depression (27,28). Conversely, exogenous administration of progesterone and allopregnanolone may significantly improve depression (29-32).

The present study aimed to evaluate the antidepressant-like effects of LIG and assess its association with the secreted

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levels of neurosteroids (progesterone and allopregnanolone) in the brain using a rat model.

Materials and methods

Animals. A total of 80 male Sprague-Dawley (SD) rats (weight, 160-200 g; age, 7 weeks) were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). Prior to experimental procedures, all animals were maintained under controlled environmental conditions for ≥7 days (temperature, 22±2˚C; humidity, 45-65%; 12-h light/dark cycle) with free access to food and water. All animal experiments were conducted according to the Guidelines for the Management and Use of Experimental Animals (33). The experimental procedures were approved by the Animal Care Research Council of Guangdong Pharmaceutical University and complied with the principles of Laboratory Animal Care (34) to minimize the suffering of animals.

Drug preparation and administration. LIG (purity, ≥98%) was obtained from Nanjing Dasf Biotech Ltd. Sertraline (Ser) was purchased from Sigma-Aldrich (Merck KGaA). Mifepristone (Mp) and finasteride (Fin) were acquired from Shanghai Yien Chemical Technology Co., Ltd, and chloral hydrate (CH) from Qingdao Yulong Algae Co., Ltd. Rats were randomized into eight groups. Rats in the normal [not exposed to chronic unpredictable mild stress (CUMS)] and vehicle-treated groups (exposed to CUMS) were treated with 0.9% physiological saline. For the behavioral tests, rats in the Ser positive control group were treated with 15 mg/kg Ser (35,36).

Each behavioral trial consisted of two parts: The pharmacodynamic evaluation of LIG and the evaluation of the effect of Mp and Fin antagonists on LIG. For the pharmacodynamic evaluation of LIG, rats were divided into three groups, where rats were intraperitoneally (i.p.) injected with 10, 20 or 40 mg/kg LIG, as previously described (21,37). For the evaluation of the effect of Mp and Fin antagonists on LIG, rats were divided into the following two groups: i) LIG (20 mg/kg) + Mp (30 mg/kg) (38,39); and ii) LIG (20 mg/kg) + Fin (50 mg/kg) (40,41). LIG, Ser, Mp and Fin were diluted in 0.9% physiological saline. LIG and Ser were administered once daily, while Mp and Fin were administered 1 h prior to the behavioral tests.

CUMS. For CUMS, all rats were treated as previously described (21,42). Briefly, single housed rats were exposed to one stress stimuli per day, which could not be predicted by the animal (Table I). The full experimental procedure lasted for 41 days. Stress stimuli induction occurred from day 1-35 followed by drug administration from day 36-48. The forced swimming test (FST), sucrose preference test (SPT) and open field test (OFT) were performed 1 h following drug administration on day 43-48 (Fig. 2).

FST (day 43). FST is a behavioral test used to evaluate the effect of antidepressants. This test was carried out as previously described (21,43,44). Briefly, all rats were placed in separated glass cylinders filled with 20 cm of freshwater at 24±2˚C and forced to swim. Each animal was forced to swim for 6 min and the duration of immobility in the last 4 min was recorded. A rat was considered immobile when stationary or when making only the necessary moves to keep its head above the water.

SPT (day 46). Low SPT is utilized to evaluate the state of depression (21,43,45). Prior to SPT, two bottles (volume, 200 ml) with 1% sucrose solution (w/v) were provided to each rat for 24 h. The following day, the sucrose solution in one bottle was replaced with pure water. During the test, rats were allowed to drink 1% sucrose solution or pure water for 3 h, and the consumed volume was recorded. Sucrose preference was calculated according to the following equation: Sucrose preference (%)=sucrose intake (g)/[sucrose intake (g) + water intake (g)] x100.

OFT (day 48). OFTis a behavioral test for evaluating locomotor activity. Due to chronic stress, rats show an unavoidable tendency to reduce their locomotor activity in the open field (21,43,46). The apparatus was placed in a plastic enclosure (dimensions, 100x100x60 cm), with floor and walls painted black. The floor was divided into 25 equal squares, and a 60-W light bulb was hung 40 cm above the center. During the experiment, incidences of crossings (each 25 square crossed with all four paws) and rearings (vertical activity with rats standing on hind legs) were recorded for 3 min.

Determination of progesterone and allopregnanolone levels. Emerging evidence has suggested that the pathogenic mechanisms of depression areassociated withdysfunction ofprogesterone and allopregnanolone biosynthesis (31,32). Following the behavioral tests, rats were anesthetized with CH (400 mg/kg; i.p.) (47) prior to decapitation. Subsequently, the brain regions of interest were removed and dissected on ice. The prefrontal cortex and hippocampus were extracted using 1 ml extraction buffer[containing 50 mM Tris-HCl (pH, 7.4), 150 mM NaCl, 1% NP-40]/100 mg of tissue. To collect the supernatants, all brain samples were homogenized in a tissue homogenizer 20 times with ice-cold lysis buffer [containing 137 mM NaCl, 20 mM Tris-HCl (pH, 8.0), 1% NP40, 10% glycerol, 1 mM PMSF 10 μg/ml aprotinin, 1 μg/mlleupetin and 0.5 mM sodium vanadate]. The homogenate supernatants were centrifuged for 25 min at 4,360 x g and 4˚C. The levels of progesterone (cat. no. ADI-900-011; Enzo Life Sciences, Inc.) and allopregnanolone (cat. no. E1963Ge; EIAab) were determined in the supernatants using ELISA. ELISA was performed in accordance with the manufacturers' instructions and optical density (OD) was measured at a wavelength of 450 nm.

Statistical analysis. All data are presented as the mean ± SEM. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests using GraphPad prism 5.0 software (GraphPad Software Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of LIG on FST in rats. As shown in Fig. 3, following CUMS, the immobility duration of rats was significantly increased.

Table I. Schedule of chronic unpredictable mild stressors applied.

Treatment week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1st	3	1	4	5	2	7	8
2nd	1	8	2	6	4	5	7
3rd	2	7	3	1	8	6	4
4th	4	5	1	8	7	3	2
5th	5	2	6	7	1	4	8

1, food deprivation (24 h); 2, water deprivation (24 h); 3, forced swimming (6 min); 4, lighting (24 h); 5, cage tilting (24 h); 6, wet bedding (24 h); 7, rocking bed (200 Hz; 5 min); 8, tail suspending (5 min).

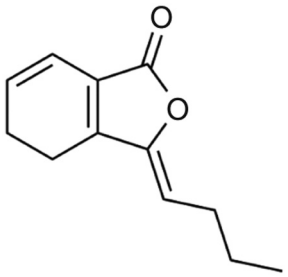

Figure 1. Molecular structure of Z-ligustilide.

Consistent with Ser treatment (15 mg/kg; i.p.), LIG administration (20 and 40 mg/kg; i.p.) exhibited antidepressant-like effects on rats, as demonstrated by the reduced immobility time (P<0.0001 for both 20 and 40 mg/kg). Furthermore, treatment with Mp (P=0.0270) and Fin (P=0.0246) significantly reversed the LIG-mediated reduction in immobility time. These results suggested that LIG may produce antidepressant-like effects.

Effect of LIG on SPT in rats. The results of SPT are presented in Fig. 4. Treatment of CUMS rats with Ser (15 mg/kg; i.p.) and LIG (20 and 40 mg/kg; i.p.) notably increased sucrose preference (P=0.0284, 20 mg/kg; P=0.0061, 40 mg/kg). However, Mp (P=0.0209) and Fin (P=0.0238) could significantly reduce sucrose preference in comparison with LIG alone. These findings further confirmed the antidepressant-like effects of LIG.

Effect of LIG on OFT in rats. The antidepressant-like effects of LIG confirmed by OFT are shown in Fig. 5. Following CUMS, rats in the vehicle-treated group showed markedly reduced crossings and rearing time compared with the normal group (P=<0.0001). Similarly to the effects of Ser (15 mg/kg; i.p.), treatment with LIG (20 and 40 mg/kg; i.p.) reversed the number of crossings (20 mg/kg, P=0.0068; 40 mg/kg; P=0.0005; Fig. 5A) and the rearing time (20 mg/kg, P=0.0036; 40 mg/kg, P=0.0013; Fig. 5B) of CUMS rats. In addition the increased number of crossings and rearing time mediated by LIG (20 mg/kg; i.p.) was inhibited by Mp (crossings, P=0.0467; rearing, P=0.0158) and Fin (crossings, P=0.0447; rearing, P=0.0058). The aforementioned results supported the hypothesis that LIG had antidepressant-like effects.

Role of progesterone and allopregnanolone on the antidepressant effects of LIG. The levels of progesterone and allopregnanolone were evaluated at the end of each behavioral test (Fig. 6). The levels of progesterone and allopregnanolone in the prefrontal cortex and hippocampus were significantly decreased in the vehicle-treated group compared with the normal group. However, treatment with Ser (15 mg/kg; i.p.) or LIG, restored the levels of progesterone in the prefrontal cortex (20 mg/kg, P=0.0440; 40 mg/kg, P=0.0024; Fig. 6A) and hippocampus (20 mg/kg, P=0.0020; 40 mg/kg, P=0.0033; Fig. 6B). Similar results were observed in the levels of allopregnanolone in the prefrontal cortex (20 mg/kg, P=0.0033; 40 mg/kg, P<0.0001; Fig. 6C) and hippocampus (20 mg/kg, P=0.0166; 40 mg/kg, P<0.0001; Fig. 6D). Following treatment with Mp, levels of progesterone were decreased in the prefrontal cortex (P=0.0088) and hippocampus (P=0.0017), while Fin notably attenuated the increased levels of allopregnanolone in the prefrontal cortex (P=0.0434) and hippocampus (P=0.0224) compared with LIG alone. Overall, the aforementioned findings indicated that the antidepressant-like effects of LIG were associated with the biosynthesis of progesterone and allopregnanolone in the prefrontal cortex and hippocampus.

Discussion

Studies have indicated that stressful life events, including chronic, low-intensity and long-term daily stressors are involved in the development of depression (48-50). CUMS is a widely used animal model, which is commonly established to simulate the stressors of depression and investigate its pathogenesis (51,52). Therefore, to evaluate the antidepressant effects of LIG, a CUMS rat model was established. FST, SPT and OFT are common behavioral tests applied to evaluate depression in animals (43-46). In the present study, a series of behavioral manifestations of CUMS on FST, SPT and OFT in rats indicated that the depression model was successfully established.

Long-term treatment with antidepressants may reverse CUMS-induced behavioral anomalies. Ser, an SSRI, is used to treat depression-associated symptoms, including depression with or without history of mania (53,54). Ser was selected as a positive control drug. In addition, the effective doses of LIG (20 and 40 mg/kg) and Ser were selected based on a series of experiments. The results suggested that treatment of rats with LIG significantly reduced their immobility and increased their frequency of crossings, rearing time and sucrose preference in comparison with a vehicle. Therefore, the findings

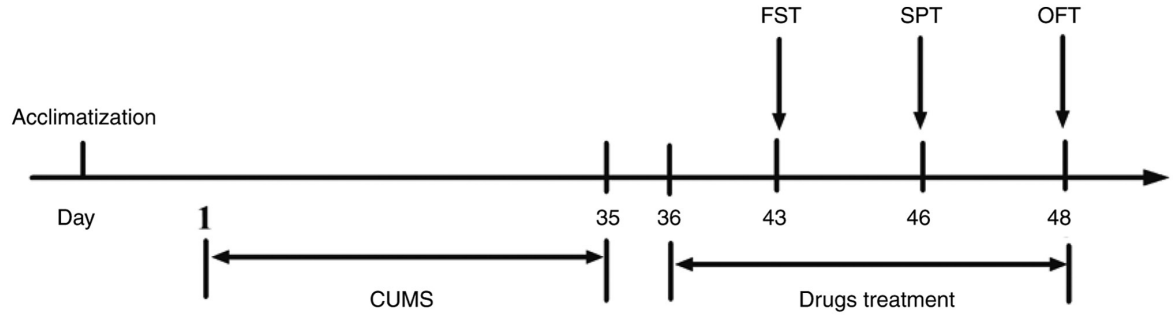

Figure 2. Schematic depiction of the application of CUMS and behavioral tests. Following CUMS and drug administration, the animals underwent FST (day 43), SPT (day 46) and OFT (day 48). Ser (15 mg/kg; i.p.) and LIG (10, 20, 40 mg/kg; i.p.) were administered daily from day 36 to 48. CUMS, chronic unpredictable mild stress; FST, forced swimming test; SPT, sucrose preference test; OFT, open field test; Ser, sertraline; LIG, Z-ligustilide.

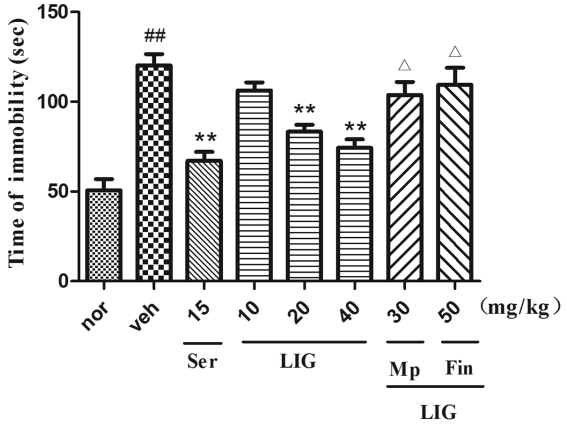

Figure 3. Antidepressant-like effects of LIG on FST in rats. Treatment with LIG (20 and 40 mg/kg; i.p.) significantly reduced immobility time. Mp and Fin reversed the LIG-mediated (20 mg/kg) reduced immobility time. $^{\#\#}$P<0.01 vs. normal group; **P<0.01 vs. vehicle-treated group; $^{\Delta}$P<0.05, vs. LIG (20 mg/kg) group (n=10). LIG, Z-ligustilide; FST, forced swimming test; Mp, mifepristone; Fin, finasteride; nor, normal; veh, vehicle.

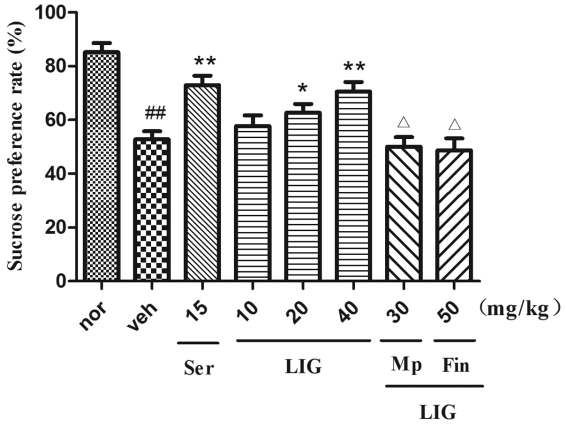

Figure 4. Effect of LIG on SPT in rats. Treatment with LIG (20 and 40 mg/kg) significantly increased sucrose preference. Treatment with Mp or Fin decreased the LIG-mediated (20 mg/kg) sucrose preference. $^{\#\#}$P<0.01 vs. normal group; *P<0.05, **P<0.01 vs. vehicle-treated group; $^{\Delta}$P<0.05, vs. LIG (20 mg/kg) group (n=10). LIG, Z-ligustilide; SPT, sucrose preference test; Mp, mifepristone; Fin, finasteride; nor, normal; veh, vehicle.

of the present study further supported the hypothesis for the antidepressant-like effects of LIG.

Studies showed that long-term treatment with Ser exerted antidepressant-like effects, when administrated 1 h prior to behavioral tests (55,56). Therefore, in the present study the same treatment scheme with Ser was adopted for LIG, and the results confirmed that this treatment approach was effective. Behavioral tests were performed one week following drug administration, suggesting that, similar to SSRI, the antidepressant-like effects of LIG were time-dependent (57,58).

Mp is an anti-progesterone drug, which blocks progesterone through binding to the progesterone receptor. The receptor itself has no progesterone or estrogen activity (38,39). Fin, a steroidal molecule, selectively inhibits type II 5α-reductase, a rate-limiting enzyme, which affects the antidepressant and anti-anxiety activities of several neurosterols, such as allopregnanolone. Treatment with Fin has been associated with several neuropsychiatric side effects, including emotional sensitivity, depression and anxiety (40,41). In the present study the levels of progesterone and allopregnanolone were reduced in the Mp- and Fin-treated groups, respectively. In addition, Mp and Fin antagonized the antidepressant-like effects of LIG, suggesting that Mp and Fin were antagonized the LIG receptor.

Several functional abnormalities have been found in the brain regions implicated in depression, including the prefrontal cortex and hippocampus. These brain regions are comprised of multiple neuron networks, which serve an important role in several processes, including emotional regulation, self-reference processing, memory and internal psychological activities (59-62). Therefore, in the present study, the effect of LIG on the prefrontal cortex and hippocampus was investigated. The results indicated that treatment with LIG significantly increased the levels of progesterone and allopregnanolone in both of these brain areas.

Allopregnanolone is a well-known positive allosteric modulator of γ-aminobutyric acid (GABA) A receptors and is considered to be a selective endogenous modulator of the effects of GABA A on GABA A receptors (63,64). Progesterone upregulates the expression of the GABA A receptor α2, α3, α4 and δ subunits, which are associated with antidepressant-like activities. It has been suggested that the effects of GABA A receptors may be mediated by their conversion to allopregnanolone (65).

Several studies have confirmed an association between reduced levels of neurosteroids (progesterone and allopregnanolone) and depression (66,67). Clinical studies have demonstrated that the levels of progesterone and

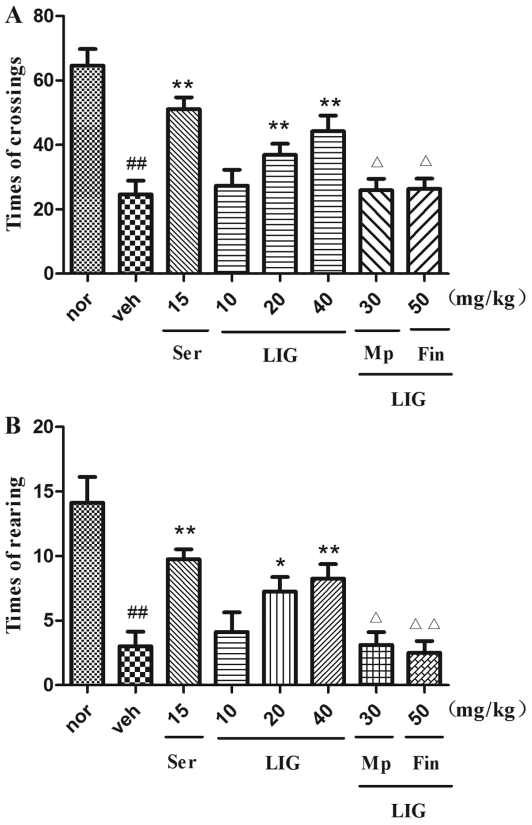

Figure 5. Antidepressant-like effects of LIG on OFT in rats. Treatment with LIG (20 and 40 mg/kg) significantly reversed (A) the crossings and (B) rearing time of CUMS rats. Treatment with Mp or Fin reduced the LIG-mediated (20 mg/kg) increase in the number of crossings and rearing time. ##P<0.01 vs. normal group; *P<0.05, **P<0.01 vs. vehicle-treated group; ΔP<0.05, ΔΔP<0.01 vs. LIG (20 mg/kg) group (n=10). LIG, Z-ligustilide; OFT, open field test; CUMS, chronic unpredictable mild stress; Mp, mifepristone; Fin, finasteride; nor, normal; veh, vehicle.

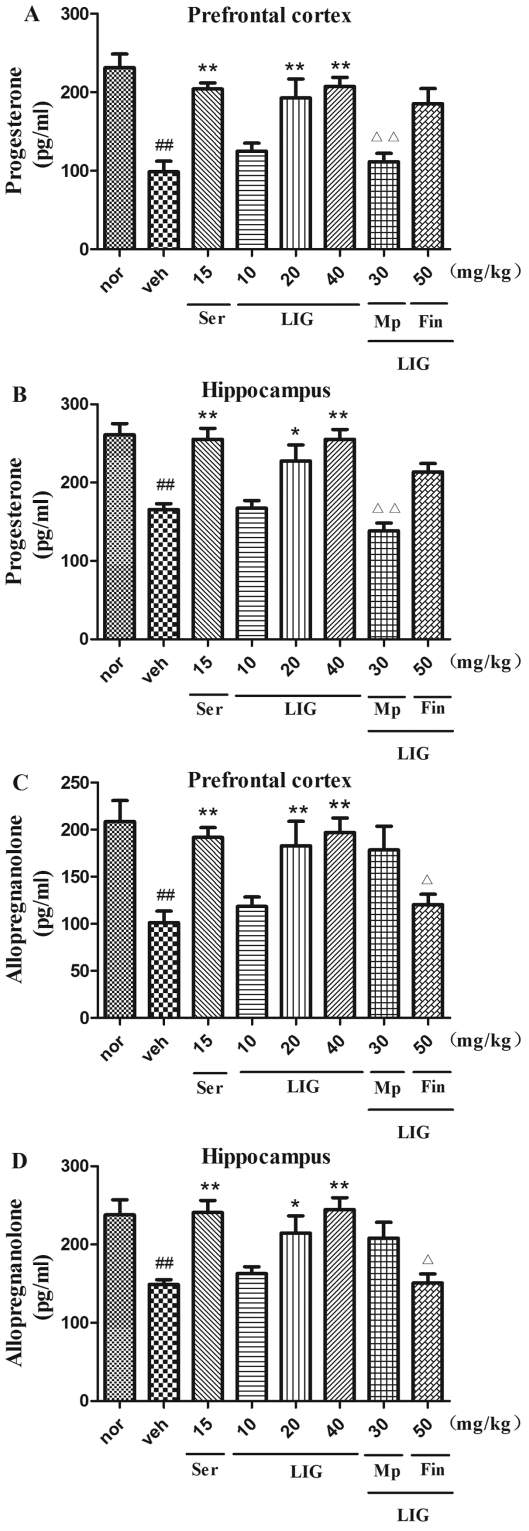

Figure 6. Role of progesterone and allopregnanolone in the antidepressant-like effects of LIG. The levels of (A) progesterone and (B) allopregnanolone in the prefrontal cortex and the levels of (C) progesterone and (D) allopregnanolone in the hippocampus were markedly reversed following treatment with LIG. Mp and Fin attenuated the increased levels of progesterone and allopregnanolone, respectively. ##P<0.01 vs. normal group; *P<0.05 **P<0.01 vs. vehicle-treated group; ΔP<0.05, ΔΔP<0.01 vs. LIG (20 mg/kg) group (n=10). LIG, Z-ligustilide; Mp, mifepristone; Fin, finasteride; nor, normal; veh, vehicle.

allopregnanolone are significantly decreased in patients with depression. Therefore, it is considered that the reduced biosynthesis of progesterone and allopregnanolone may be involved in the pathogenesis of depression (30-32). A limited number of studies have focused on the role of progesterone and allopregnanolone in the antidepressant effects of LIG. In the present study levels of progesterone and allopregnanolone were increased following treatment of CUMS rats with LIG, suggesting that the biosynthesis of both neurosteroids could play an important role in the antidepressant effects of LIG. However, more experiments are needed to further elucidate this mechanism. This study could provide novel perspectives on the antidepressant-like effects of LIG and its possible underlying mechanisms.

In the present study the levels of progesterone and allopregnanolone were determined using ELISAs and certain chemicals in the lysis buffer may affect the results of ELISA experiments . Therefore, more accurate methods, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), should be applied in future studies to determine the levels of neurosteroids in the brain.

The present study evaluated the antidepressant-like effects of LIG on rats by measuring immobility time by FST, sucrose preference by SPT as well as locomotion and rearing time by OFT. Furthermore, the levels of progesterone and allopregnanolone in the prefrontal cortex and hippocampus were evaluated. The results demonstrated that the antidepressant-like effects of LIG could be promoted by the

biosynthesis of progesterone and allopregnanolone in the brain. The current study preliminarily explored the antidepressant effects and possible mechanisms of LIG, therefore, further studies should be performed in the future to evaluate the antidepressant-like effects of LIG using more animal models, in order to provide a safe and effective treatment for depression.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JCM and ZKQ conceptualized the study, designed the experiments and wrote the manuscript; HPH, ZLM and SFC performed the experiments. JSC and HLZ analyzed the data. JCM and KSC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experimental procedures were approved by the Animal Care Research Council of Guangdong Pharmaceutical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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