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ORIGINAL PAPER

Licorisoflavan A Exerts Antidepressant-Like Effect in Mice: Involvement of BDNF-TrkB Pathway and AMPA Receptors

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Abstract

Depression is a highly debilitating and life-threatening psychiatric disorder. The classical antidepressants are still not adequate due to undesirable side effects. Therefore, the development of new drugs for depression treatment is an urgent strategic to achieving clinical needs. Licorisoflavan A is a bioactive ingredient isolated from Glycyrrhizae Radix and has been recently reported for neuroprotective effects. In this study, the antidepressant-like effect and neural mechanism of licorisoflavan A were explored. In the mice behavioral despair test, we observed that licorisoflavan A exhibited powerful antidepressant-like effect in forced swimming test (FST), tail suspension test (TST), without affecting locomotor activity in open field test (OFT). Additionally, licorisoflavan A administration significantly restored Chronic mild stress (CMS)-induced changes in sucrose preference test (SPT), FST, and TST, without altering the locomotion in OFT. In chronical-stimulated mice, the licorisoflavan A treatment effectively attenuated the expressions of Brain-derived neurotrophic factor (BDNF), tyrosine kinase B (TrkB), the phosphorylations of cAMP response element binding protein (CREB), extracellular signal-regulated kinase (ERK)-1/2, eukaryotic elongation factor 2 (eEF2), mammalian target of rapamycin (mTOR), initiation factor 4E-binding protein 1 (4E-BP-1), and p70 ribosomal protein S6 kinase (p70S6K) in hippocampus of CMS-induced mice. Additionally, licorisoflavan A could reverse the decreases in synaptic proteins post-synaptic density protein 95 (PSD-95) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor subunit glutamate receptor 1 (GluR1) caused by CMS, and its antidepressant-like effect was blocked by the AMPA receptor antagonist NBQX. These findings served as preclinical evidence that licorisoflavan A exerted potent antidepressant-like effects involving BDNF-TrkB pathway and AMPA receptors. Licorisoflavan A might be used as a potential medicine against depression-like disorder.

Keywords Licorisoflavan A · Antidepressant-like effect · Chronic mild stress · BDNF-TrkB signaling pathway · AMPA

Abbreviations

FST	Forced swimming test
TST	Tail suspension test
SPT	Sucrose preference test
OFT	Open field test
CMS	Chronic mild stress
BDNF	Brain-derived neurotrophic factor
TrkB	Tyrosine kinase B
CREB	cAMP response element binding protein
ERK	Extracellular signal-regulated kinase
eEF2	Eukaryotic elongation factor 2
mTOR	Mammalian target of rapamycin
4E-BP-1	4E-binding protein 1
p70S6K	p70 ribosomal protein S6 kinase
PSD-95	Post-synaptic density protein 95
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
GluR1	Glutamate receptor 1
NBQX	2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F) quinoxaline

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Introduction

Depression is a prevalent mental disorder affecting up to 20% of the world population [1]. Depression can diminish life quality, increase suicidality, and become an important global public health issue [2]. According to the estimation from the World Health Organization, depression will become the second leading cause of global disease problems by 2020, only behind ischemic heart disease [3].

Although significant progress has been made for revealing depression pathogenesis and the mechanism of antidepressants, the common molecular mechanisms provoking depression have not been entirely explained. Many factors, including genetic predisposition, obesity, sedentary lifestyle, eating habits, traumatic events, stress, anatomic and neurochemical alterations have contributed to the development of depressive disorder [4]. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family involved in synaptic plasticity and neuronal survival, has an important function in the pathophysiology of depression [5]. Chronic stress, a risk factor for depression, was also demonstrated to reduce the expressions of BDNF and its receptor tyrosine kinase B(TrkB) in the hippocampus, prefrontal cortex and locus coeruleus in animal experiments [6]. The upregulation of BDNF stimulates several downstream signaling targets, and consequently activates mammalian target of rapamycin (mTOR), which plays an important role in rapid antidepressant responses associated with critical synaptic proteins such as post-synaptic density protein 95 (PSD-95), glutamate A1 (GluA1) and synapsin I [7–9]. In addition, the glutamatergic system, which drives the synaptic plasticity and memory, is also regarded as an important relevant target for antidepressants [10].

Up to date, there are several antidepressant drugs available for depression, most of them affecting the monoaminergic system directly or indirectly [11]. However, no antidepressant agents are adequate for the majority of depressed patients because of their limited efficacy and slow response on set. Furthermore, most of these antidepressants produced side effects like sedation, blurred vision, constipation, seizures, sexual dysfunction, and weight gain [12]. Therefore, it is desirable to develop more effective antidepressants with fewer adverse effects to address the clinical needs. Glycyrrhizae Radix (also known as Gan Cao), a perennial plant extensively used in traditional oriental medicine to strengthen body functions, is applied for the treatments of various injury and detoxification. Generally, modern pharmacological studies on G. Radix have focused on its anti-inflammatory and antioxidative actions. Whereas recently, G. Radix has been reported to show significant antidepressant-like effect in murine immobility test [13–15]. However, the active ingredients of G. Radix responsible for its antidepressant-like activity are currently unknown. We have isolated and purified licorisoflavan A, an active isoflavane component from G. Radix. Our previous investigation demonstrated that the main component isolated from Glycyrrhizae Radix, such as liquiritigenin, isoliquiritin, and licorisoflavan A [16]. In addition, we reported the anti-depressant effect of liquiritigenin [17]. Isoliquiritin was also elicited to exerted protective effect on CUMS and the corticosterone-induced PC12 cells which served as the classical in vitro model for depression [18–20]. Thus, it was hypothesized that licorisoflavan A exhibited anti-depressive property. In the present study, we conducted the murine model of depression to verify the antidepressant-like effect of licorisoflavan A and explore its underlying molecular mechanism.

Materials and Methods

Animals

Male Kunming mice (6 to 8 weeks old) weighing 20–25 g were used in the present study. Animals were obtained from the China Academy of Military Medical Sciences (Beijing) and kept under a 12:12 h light/dark cycle at a constant temperature (22 ± 2 °C) and humidity (50 ± 10%) with free access to water and food. Before behavioral testing, mice were habituated to animal facilities for 1 week. All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee at Guangdong Medical University (permission number 20160510).

Isolation and Purification of Licorisoflavan A

Licorisoflavan A was isolated and purified according to the procedures described in our previous study [21]. Briefly, air-dried roots and rhizomes of *G. uralensis* (40 kg) were extracted with 95% ethanol under reflux and repeated for 3 h thrice. All ethanol-extracts were combined, filtrated, and concentrated under vacuum to dryness. The mixture was suspended in distilled water, and extracted with EtOAc. Then, EtOAc extract (1112 g) was subjected to a silica gel column eluted with gradient (PE:EtOAc, 5:1 → PE:EtOAc, 2:1 → PE:EtOAc, 1:2 → EtOAc → EtOAc:MeOH, 10:1) affording five fractions (Fr.1–Fr.5). Fr.1 (220 g) was further subjected to a silica gel column eluted with PE:EtOAc (30:1 → 5:1) to afford compound licorisoflavan A (80 mg). On the basis of UV, NMR and MS analysis, the structure of isolated reference standard was confirmed (Fig. 1a), and its purity determined using UPLC-PDA-MS was over 98.0%.

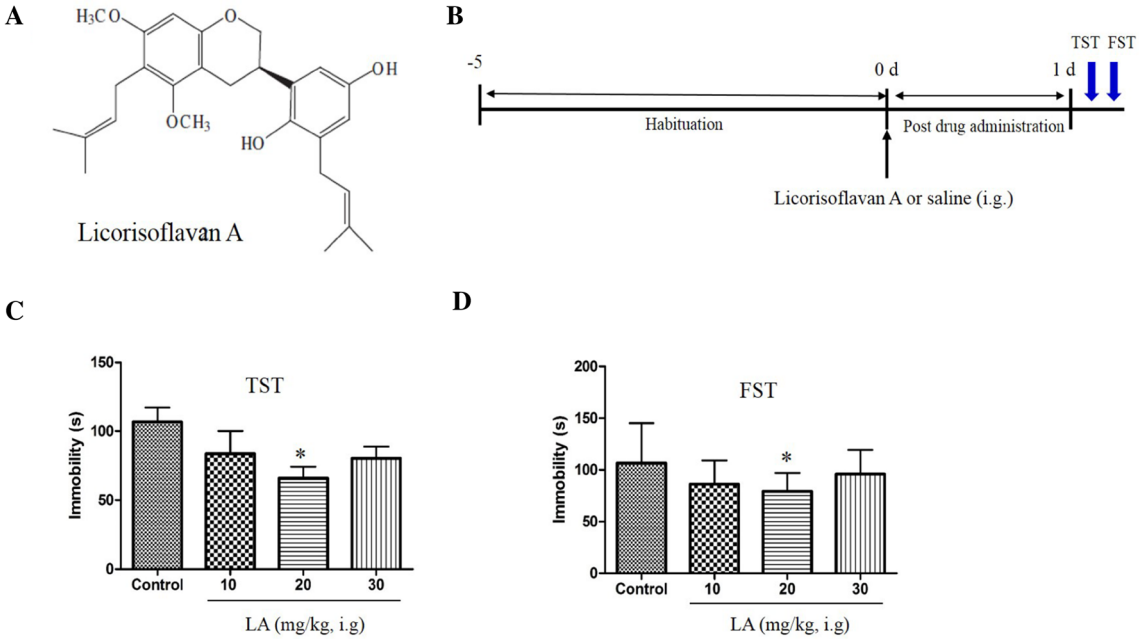

Fig. 1 Licorisoflavan A administration exerted antidepressant-like effect in acutely stressed mice. *$p < 0.05$ versus the control, n = 10–11

Drugs and Materials

Ketamine HCl was purchased from Gutian Pharmaceuticals (Fujian, China). Licorisoflavan A and ketamine were dissolved in normal saline. Vehicle solvent served as a negative control while ketamine was taken as a positive control. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F) quinoxaline(NBQX) was supplied from sigma. Licorisoflavan A was administered by via gastric intubation route 24 h before the forced swimming test, tail suspension test or open-field test. Except otherwise noted, all other drugs were given intraperitoneally in a volume of 10 ml kg^{-1} of body weight.

Experimental Design

Experiment 1: Effects of Licorisoflavan A Administration on Depressive-Like Behaviors in Acutely Stressed Mice

As shown in Fig. 1b, experiment 1 was conducted to determine the effects of licorisoflavan A on depressive-like behaviors in mice responded to behavioral despair. After a 7-day habituation, mice were randomly divided into four groups and were intragastrically treated with single dose of saline or licorisoflavan A (10, 20, or 30 mg kg^{-1}). Then the behavioral tests including TST and FST were conducted 24 h after the drug treatment.

Experiment 2: Effects of Licorisoflavan A Administration on Depressive-Like Behaviors in Chronically Stressed Mice

To further assess the effects of licorisoflavan A on behavior changes in mice after chronic stress, CMS procedure was applied in this work. The new mice were purchased and divided into 4 groups (n = 6–8 per group): Control + saline, CMS + saline, CMS + licorisoflavan A, and CMS + ketamine. After a 7-day habituation, mice in CMS groups were treated with a consecutive 3-week chronic stress procedure (Table 1), and then were intragastrically given with single dose of saline (10 ml kg^{-1}, i.g.), licorisoflavan A (20 mg kg^{-1}, i.g.) or ketamine (30 mg kg^{-1}, i.p.), respectively. Mice in control group were left in their homecages with only normal saline treatment. Behavioral tests, including OFT, FST and TST were conducted 24 h after the drug treatment, while SPT was performed at 2 day post drug administration (Fig. 2a).

Additionally, new mice were purchased for detecting the underlying mechanism of AMPA receptor antagonist NBQX in the pathogenesis of licorisoflavan A-involved antidepressant effect. The mice were randmonly divided into four groups (n = 6 per group): control + saline, licorisoflavan A + saline, Ket + saline, control + NBQX, licorisoflavan A + NBQX, ketamine + NBQX. After a 7-day habituation, the mice were given with single dose of saline (10 ml·kg^{-1}, i.g.) and NBQX (30 mg·kg^{-1}, i.p.). Two hours later, the animals were treated with licorisoflavan A (20 mg kg^{-1}, i.g.) or

Table 1 Chronic mild stress (CMS) procedure

Monday	
9:00	Closed light
11:00	Remove food and water, 20 h cold–wet cage (200 ml water (4 °C)/cage)
Tuesday	
9:00	Change dry cage, restore food and water, and 40 min of cage shaking (200 rpm)
9:40	Stop cage shaking, continuous light for 24 h
Wednesday	
9:00	Closed light, record animal weight
10:00	24 h of tilted cage (45°), and remove water
Thursday	
9:00	Stop tilted cage (45°), restore water, and change to 5 mice /cage
15:00	Change to single cage, remove food
Friday	
9:00	Restore food, 40 min of cage shaking (200 rpm)
9:40	Stop cage shaking, 20 h hot–wet cage (200 ml water (45 °C)/cage)
Saturday	
9:00	Change dry cage
10:00	24 h of tilted cage (45°), and remove water
Sunday	
9:00	Stop tilted cage (45°), restore water, and change to five mice/cage
15:00	Change to single cage, continuous light for 20 h

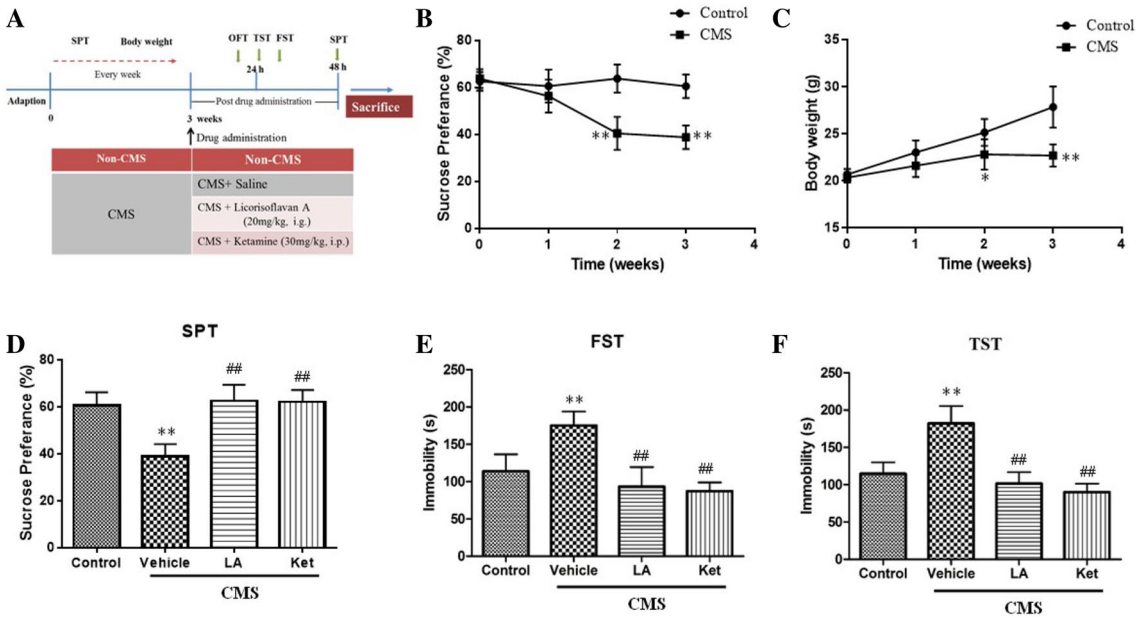

Fig. 2 Licorisoflavan A administration reversed depressive-like behaviors in chronically stressed mice (**a**); Effects of licorisoflavan A on the percentages of sucrose consumption (**b–d**); Effects of licorisoflavan A on immobility time in the forced swim test (**e**); Effects of licorisoflavan A on immobility time in the tail suspension test (**f**), n = 8. $^{**}p < 0.01$ versus the control group, $^{\#\#}p < 0.01$ versus the vehicle group

ketamine (30 mg kg^{-1}, i.p.), respectively. Meanwhile, mice in control group were left in their homecages with only normal saline treatment. Behavioral tests including FST and TST were conducted 24 h after the drug treatment.

Chronic Mild Stress (CMS) Paradigm

In the present study, the procedures for CMS were followed as described in our previous research [22]. After the seven

days habituation period, all mice (except the normal) were subjected to CMS for 3 weeks. As shown in Table 1, The CMS procedure consisted of a variety of unpredictable mild stressors including 6 h of paired caging, 24 h of tilted cage (45°), 40 min of case shaking (200 rpm), 24 h of food and water deprivation, 20 h of soiled bedding (200 ml), and overnight illumination (12 h). Mice received a stress with the order of the randomly scheduled stressors over a one-week period and repeated throughout the 3-week experiment. Overnight illumination was administrated twice a week, 3–4 days apart. The non-stressed control animals were housed under normal conditions.

Behavioral Testing

Open Field Test (OFT)

The locomotor activity and anxiety-like behavior were assessed in an open-field test. The open field test was performed 24 h after the drug exposure. A well-illuminated (~300 lx) transparent acrylic cage ($40 \times 40 \times 15$ cm^3) was applied for the testing. All tests were carried out in a temperature, noise, and light controlled room to avoid anxiety behavior. At the start of each trial, a mouse was gently placed in the center and left to freely explore the area for 5 min. The path taken by each mouse was recorded by camera, and parameters like total crossing and time spent at the center of the open field were scored using ANY-maze software.

Forced Swim Test (FST)

The FST was conducted as previously described [23]. Briefly, the mouse was individually forced to swim for 6 min in a clear glass tank (diameter 20 cm, height 40 cm), containing 30 cm of water (depth) at 22–23 °C. The duration of immobility was recorded for each animal. A mouse was judged to be immobile when it floated motionless in the water with necessary small movements to keep its head above the water. At the end of swim test, mice were removed from the water and dried with a paper towel. A decrease in the immobility duration within the last 4 min is indicative of an antidepressant-like response.

Tail Suspension Test (TST)

TST was performed to assess depression-like behavior and antidepressant-like response in mice. Each mouse was suspended 50 cm above the floor with an adhesive tape placed approximately 1 cm from the tip of the tail. A camera was mounted facing the tail suspension test arena, and the total immobility time was recorded during the final 4 min of a 6-min-test. Mice were considered immobile only when they hung passively and completely motionless.

Sucrose Preference Test (SPT) and Body Weight Gain

Sucrose intake and body weight were measured once a week on separate days. The SPT, including training and testing sections, was performed as described previously with minor modifications [24]. During training, each mouse was individually housed and trained to consume 1% (w/v) sucrose solution (Sigma, St Louis, MO, USA) without giving food and water. After 36 h, mice were again deprived of food and water for 12 h, then had free access to two pre-weighed bottles respectively containing 1% sucrose solution and water. The volumes of consumed sucrose solution and water were recorded for 2 h exposure. Sucrose preference was defined as the sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption), normalized to each individual animals' body weight.

Western Blot

After the behavioral tests, mice were sacrificed by deep anaesthesia with halothane. The brain tissues were immediately removed on ice plate and the whole hippocampal tissues were harvested for western blot. The murine hippocampus was homogenized in ice-cold RIPA buffer containing protease and/or phosphatase inhibitors. After centrifugation at 12,000×*g* for 20 min, the supernatant was collected, and the dissolved protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA). Then, protein lysates were separated by an SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% BSA for 1 h, the membranes were incubated at 4 °C overnight with respective primary antibodies for PSD-95 (Millipore, #04–1066, 1:1000), GluR1 (Cell Signal Inc., CA, USA, #13,185, 1:1000), p-mTOR (Cell Signal Inc., CA, USA, #2971, 1:1000), mTOR (Cell Signal Inc., CA, USA, #2972, 1:1000), p-4EBP-1 (Cell Signal Inc., CA, USA, #2855, 1:1000), 4E-BP-1 (Cell Signal Inc., CA, USA, #2855, 1:1000), p-p70S6K (Cell Signal Inc., CA, USA, #9324, 1:1000), p70S6K (Cell Signal Inc., CA, USA, #2708, 1:1000), β-tubulin (Abcam, ab151318, 1:2000), p-ERK (Cell Signal Inc., CA, USA, #4370, 1:1000), ERK (Cell Signal Inc., CA, USA, #9120, 1:1000), BDNF (Santa Cruz, Sc-546, 1:200), p-CREB (Cell Signal Inc., CA, USA, #9198S, 1:500), CREB (Cell Signal Inc., CA, USA, #9197, 1:1000), Trk-B(Cell Signal Inc., CA, USA, #4603, 1:1000), eEf2 (Cell Signal Inc., CA, USA, #2332, 1:1000), p-eEF2 (Cell Signal Inc., CA, USA, 2331S, 1:1000). After washing with tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (1:12,000) for 1 h at room

temperature. The blots were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). For analysis, BDNF, Trk-B, PSD-95, and GluR1 were normalized to β-tubulin bands, and p-eEF2, p-ERK, p-CREB, p-mTOR, p-p70S6K, and p-4E-BP-1 bands were normalized to correlated non-phosphorylated protein levels. All experiments were performed in triplicate.

Statistical Analyses

The statistical analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA by Graphpad 5.0, followed by a Bonferroni post hoc analysis if appropriate. P values less than 0.05 were considered to be statistically significant.

Results

licorisoflavan A Administration Exerted Antidepressant-Like Effect in Acutely Stressed Mice

In the present study, the possible antidepressant-like activity of licorisoflavan A (Fig. 1a) was first tested using TST and FST (Fig. 1b), two widely used behavioral assays for detecting antidepressant drugs. TST and FST were conducted 24 h after the drug treatment, and the behavioral effects were presented in Fig. 1c, d. The results showed that a single administration of licorisoflavan A at dose of 20 mg kg^{-1} significantly reduced immobility time in the TST as well as FST ($F(3, 39) = 2.863$, $p = 0.049$, $p < 0.05$ vs. control for TST; $F(3, 39) = 3.327$, $p = 0.029$, $p < 0.05$ vs. control for FST,). However, the 10 or 30 mg kg^{-1} dose showed no significant effects on the immobility time compared with normal saline-treated control mice. In addition, the licorisoflavan A (10, 20, 30 mg kg^{-1}) did not significantly altered the distance traveled and the time spent in the central area, which excluded the possibility that licorisoflavan A changed the locomotion or cause anxiety in mice. These findings elicited that licorisoflavan A at 20 mg kg^{-1} could produce significant antidepressant-like effect in mice responded to behavioral despair. Hence, the medium dose (20 mg kg^{-1}) was selected in following work for studying the antidepressant-like effect of licorisoflavan A in CMS mice.

Licorisoflavan A Administration Reversed Depressive-Like Behaviors in Chronically Stressed Mice

To further characterize the antidepressant-like effects of licorisoflavan A, CMS, one of the most predictive animal models of depression, was employed in our work (Fig. 2a). Following long-term exposure to a series of mild unpredictable stressors, animals would exhibit key depressive-like phenotypes such as anhedonia and despair. The effects of licorisoflavan A on the depressive-like behaviors in chronically stressed mice were summarized as follows.

Effects of Licorisoflavan A on the Percentages of Sucrose Consumption

Anhedonia is an important symptom of depression, and the decrease of sucrose solution consumption was used as the marker for depressive-like behavior in rodents. The sucrose preference in the CMS procedure was shown in Fig. 2b. At the beginning of the CMS procedure, there was no significant difference among the groups. After 3-week stress stimulation, sucrose consumption of CMS mice was significantly reduced compared with that of the negative control group ($n = 8$, t-test, $p < 0.01$ vs. CMS). Additionally, the weight gain in CMS-treated mice was less than that of control mice ($n = 8$, t-test, $p < 0.05$ vs. CMS) (Fig. 2c). By contrast, as shown in Fig. 2d, the sucrose consumption was increased with the administration of 20 mg kg^{-1} licorisoflavan A ($p < 0.01$ vs. CMS), which was similar to that of ketamine (30 mg kg^{-1}) ($p < 0.01$ vs. CMS). These results suggested that licorisoflavan A might increase hedonic states in mice ($F(3, 28) = 31.63$, $p < 0.001$).

Effects of Licorisoflavan A on Immobility Time in the Forced Swim Test

The effect of licorisoflavan A on immobility time in the FST was shown in Fig. 2e ($F(3, 28) = 30.7$, $p < 0.001$). Licorisoflavan A at dose of 20 mg kg^{-1} or the positive control ketamine at dose of 30 mg kg^{-1} remarkably reduced immobility time in the FST versus CMS-vehicle group. For further understanding of the antidepressant-like activity of licorisoflavan A, the percentage decrease in the immobility duration (% DID) was calculated. The results showed that licorisoflavan A reduced the duration of immobility and gave high % DID values (46.81%). The % DID for licorisoflavan A at 20 mg·kg^{-1} was similar to that of ketamine (50.21%) in the FST.

Effects of Licorisoflavan A on Immobility Time in the Tail Suspension Test

Figure 2f presented the potential antidepressant-like effect of licorisoflavan A in mouse tail suspension test ($F(3, 28) = 47.63$, $p < 0.001$). Licorisoflavan A treatment showed a marked reduction of the immobility duration after acute drug administration (44.30% immobility reduction), indicating that Licorisoflavan A effectively exhibited an antidepressant-like effect in TST. Ketamine (30 mg kg^{-1}, i.p.) also significantly decreased the immobility time (reached 50.59% reduction of the immobility time).

Effect of Licorisoflavan A on Locomotor Activity

To determine whether the observed reductions in immobility were associated with alterations in motor activity, the mice were further exposed to the open-field apparatus for 5 min. We found that, the administrations of licorisoflavan A and ketamine did not change either locomotor activity (distance traveled) (Fig. 3a, f (3, 28) = 1.661, p = 0.198) or time spent on the center part of open field (Fig. 3b, f (3, 28) = 1.64, p = 0.202). These data indicated that the reduction of immobility induced by acute licorisoflavan A treatment in the FST and TST was not due to locomotor hyperactivity.

The Effects of Licorisoflavan A on Hippocampal BDNF, Trkb and Phosphorylated CREB Expression

To investigate the possible mechanism underlying rapid antidepressant-like effect of licorisoflavan A, we first examined the level of BDNF protein in the hippocampus. There were significant differences in the level of BDNF among the four groups (F (3, 20) = 3.238, p = 0.043). As depicted in Fig. 4a, the levels of BDNF proteins was decreased after chronic exposure to CMS compared with that in the vehicle control group. The administration of licorisoflavan A by gavage significantly lessened this decrease in BDNF level in the chronically stressed mice (p = 0.0176, vs. CMS). There was also a significant difference in the level of the BDNF receptor TrkB protein among the four groups (F (3, 20) = 3.622, p = 0.031). The decreased level of TrkB protein in the hippocampus of chronically stressed mice was attenuated by licorisoflavan A (Fig. 4B). The cAMP response element-binding protein (CREB) is a critical mediator of neural plasticity and the transcription factor for BDNF. In this study, p-CREB was significantly decreased after CMS exposure (p < 0.001 vs. control; Fig. 4c), and licorisoflavan A treatment inhibited this reduction without causing any change in the total level of CREB (F (3, 24) = 5.922, p = 0.004). Positive drug ketamine presented similar effect on BDNF, phosphorylated CREB and TrkB expression as licorisoflavan A. Thus, licorisoflavan A might exert the antidepressant-like effect at least in part through the activation of BDNF-TrkB signaling in the hippocampus.

Licorisoflavan A Did Not Alter Eef2 Expression But Decreased Its Phosphorylation

The deactivation of eukaryotic elongation factor 2 kinase (eEF2K) can lead to reduced eEF2 phosphorylation and desuppression of translation of BDNF. Consequently, licorisoflavan A treatment triggered a significant decrease in phosphorylation of eEF2 compared with vehicle group following intragestrical administration (Fig. 4d, f (3, 20) = 9.664, p = 0.004). Licorisoflavan A did not have any significant effect on total eEF2 protein level. Of note, ketamine showed similar effect on eEF2 phosphorylation as licorisoflavan A.

Licorisoflavan A Treatment Attenuated the CMS-Induced mTOR, 4E-BP-1 and p70S6K Phosphorylation Reduction

We performed a western blot to examine the influence of licorisoflavan A on the phosphorylated forms of mTOR, and its two down-stream effectors, p70S6K and 4E-BP-1 in the hippocampus. As shown in Fig. 5, CMS procedure significantly reduced the phosphorylation of mTOR, p70S6K and 4E-BP-1 in the hippocampus of mice (all p < 0.05). By contrast, licorisoflavan A (20 mg kg^{-1}) induced a significant increase in the phosphorylated forms of mTOR (p < 0.05), p70S6K (p < 0.01) and 4E-BP-1 (p < 0.05) compared with control group, by 156% 139%, and 143%, respectively (F (3, 24) = 3.915, p = 0.021 for mTOR; F (3, 20) = 3.132, p = 0.049 for 4E-BP-1; F (3, 20) = 3.829, p = 0.028 for p70S6K). The results suggested that acute administration of licorisoflavan A could stimulate the mTOR signaling pathway in the hippocampus.

Licorisoflavan A Increased the Level of Phosphorylated ERK in Hippocampus of CMS Mice

As mTOR signaling pathway was activated by licorisoflavan A, we tested whether this compound regulate

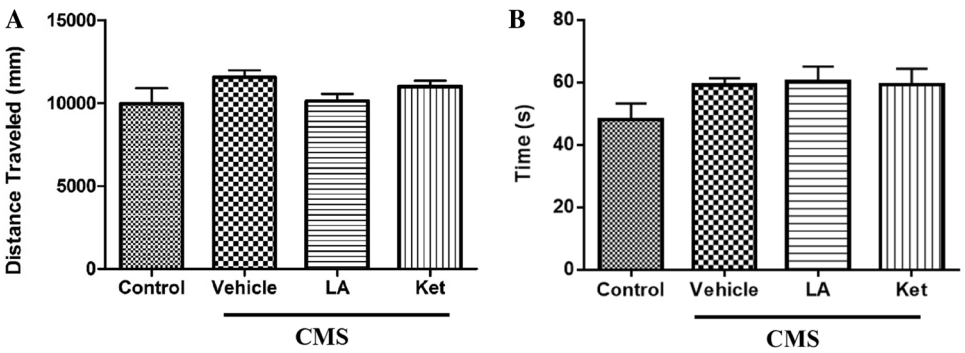

Fig. 3 Effect of licorisoflavan A on locomotor activity (n = 8)

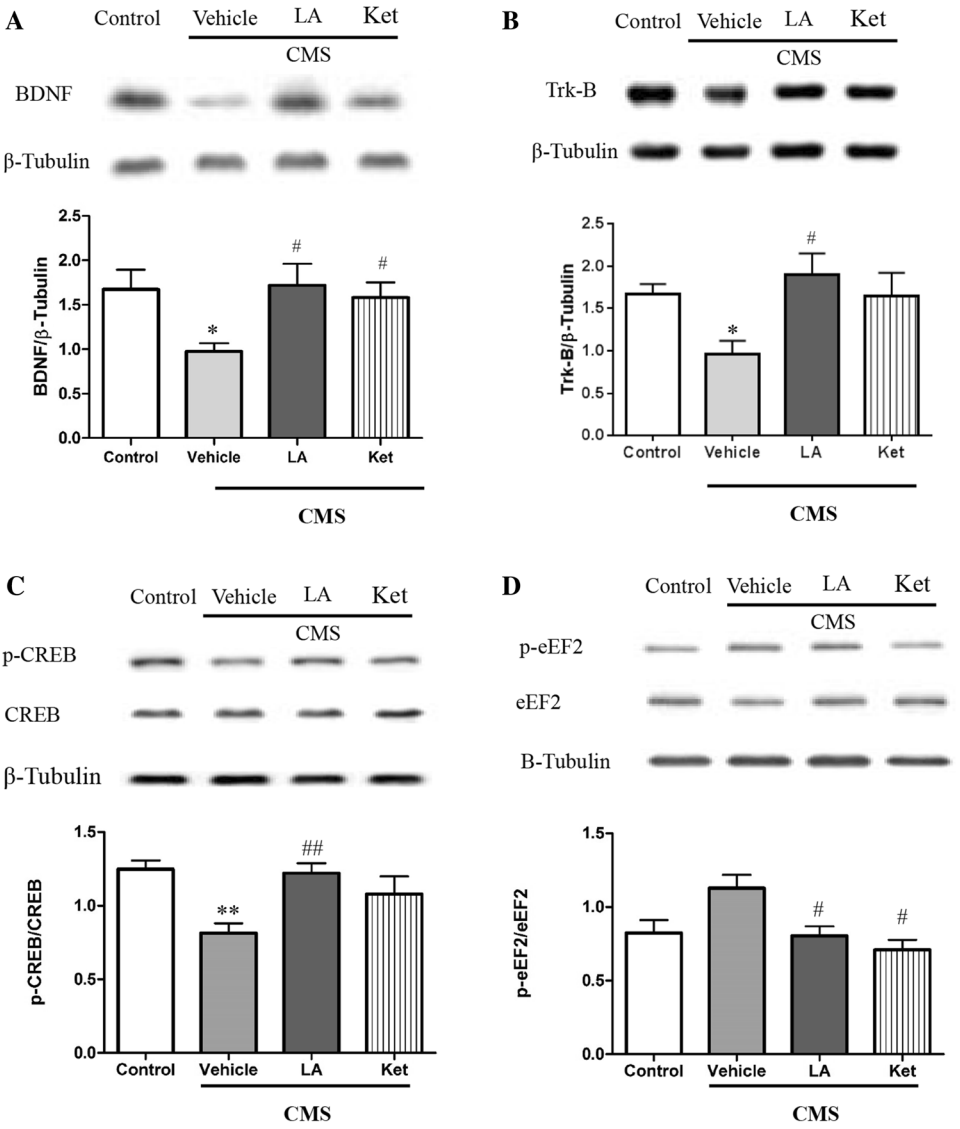

Fig. 4 Licorisoflavan A increased hippocampal BDNF, TrkB and phosphorylated CREB expression (**a–c**); Licorisoflavan A did not alter eEF2 expression but decreased its phosphorylation (**d**). $^{**}p < 0.01$, $^{*}p < 0.05$ versus the control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ versus the vehicle group, n = 6

phosphorylation of ERK, 4E-BP1 and p70S6K in hippocampus. In a pattern consistent with 4E-BP1 or p70S6K, there were significant differences in the levels of p-ERK among different groups (Fig. 5d, $f\ (3, 22) = 6.323$, $p = 0.003$). Chronic exposure to CMS decreased the level of p-ERK in the hippocampus of mice. Compared with the CMS group, ketamine restored the hippocampal p-ERK expression compared with that in control group ($p = 0.001$ vs. CMS). Similarly, acute treatment with licorisoflavan A at the dose of 20 $mg{\cdot}kg^{-1}$ also increased p-ERK in the hippocampus of CMS mice.

Licorisoflavan A Treatment Normalized the Synaptic Protein Expression in the Hippocampus of CMS Mice

It was further examined whether licorisoflavan A altered hippocampal expression of proteins associated with synapse structure and strength, namely postsynaptic density protein 95 (PSD-95), and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) subunit GluR1, respectively. Western blotting analysis revealed that chronic mild stress significantly reduced the expression of the AMPA receptor subunit GluR1 (Fig. 6a, $F\ (3, 20) = 12.43$, $p = 0.001$) as well as post-synaptic marker PSD-95 (Fig. 6b, $F\ (3, 20) = 10.68$, $p < 0.001$). Licorisoflavan A reversed the decreased expression of GluR1 ($p < 0.05$) and PSD-95 ($p < 0.01$). Similar effects were observed in response to ketamine treatment (both $p < 0.001$). These data indicated that licorisoflavan A could normalize the levels of synaptic proteins in hippocampal neurons mediated by mTOR signaling.

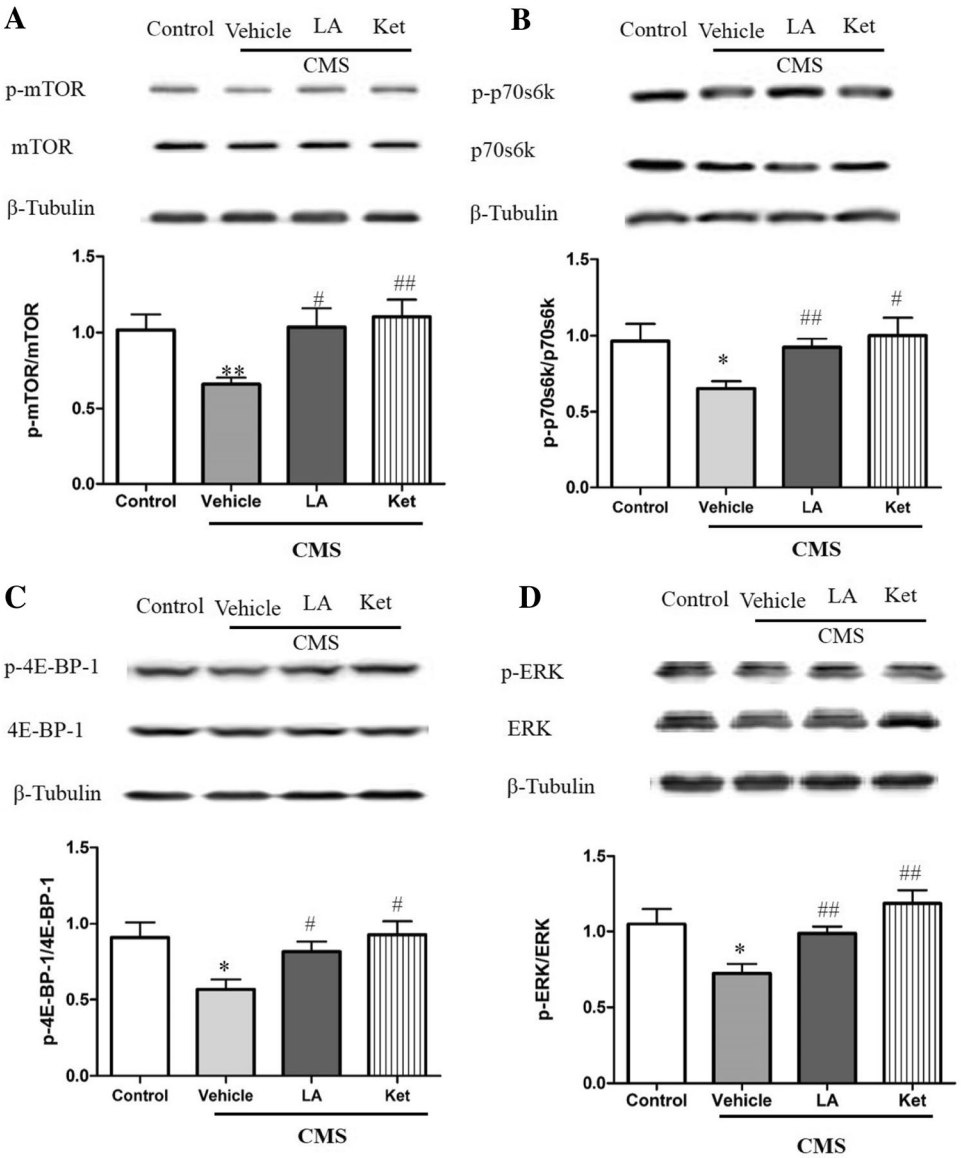

Fig. 5 Licorisoflavan A treatment attenuated the CMS-induced mTOR, 4E-BP-1 and p70S6K phosphorylation reduction (**a–c**). Licorisoflavan A increased the level of phosphorylated ERK in hippocampus of CMS mice (**d**). $^{**}p < 0.01$, $^{*}p < 0.05$ versus the control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ versus the vehicle group, n = 6

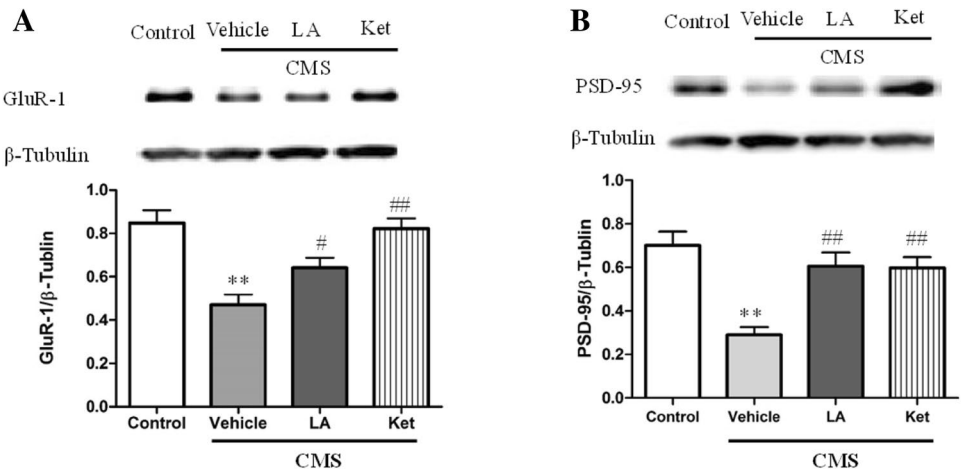

Fig. 6 Licorisoflavan A treatment normalized the synaptic protein expression in the hippocampus of CMS mice. $^{**}p < 0.01$ versus the control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ versus the vehicle group, n = 6

Antidepressant-Like Effects of Licorisoflavan A were Reversed by AMPA Receptor Antagonist

To estimate whether the increase in AMPA receptor-mediated synaptic transmission is required for the antidepressant-like effects of licorisoflavan A, we further treated the depressed mice with NBQX, an AMPA receptor antagonist. As shown in Fig. 7, two-way ANOVA showed significant effects of NBQX ($F(1, 30) = 125.1$, $p < 0.001$), drug treatment ($F(2, 30) = 30.39$, $p < 0.001$) and interaction between NBQX and drug treatment ($2, 30) = 35.33$, $p < 0.001$) in FST, and NBQX ($F(1, 30) = 178.8$, $p < 0.001$), drug treatment ($F(2, 30) = 37.69$, $P < 0.01$) and interaction between NBQX and drug treatment ($2, 30) = 46.81$, $p < 0.001$) in TST. The treatments with licorisoflavan A and ketamine notably reduced the immobility time in both TST (both $p < 0.001$). While the NBQX treatment significantly reversed the immobility duration of licorisoflavan A ($p < 0.001$) and ketamine ($p < 0.001$). It was noteworthy that NBQX treatment had no obvious effect on the immobility time compared with that in control group. The abolishment of licorisoflavan A's antidepressant-like effect by NBQX suggested the role of AMPA receptor activation in licorisoflavan A's antidepressant-like effects.

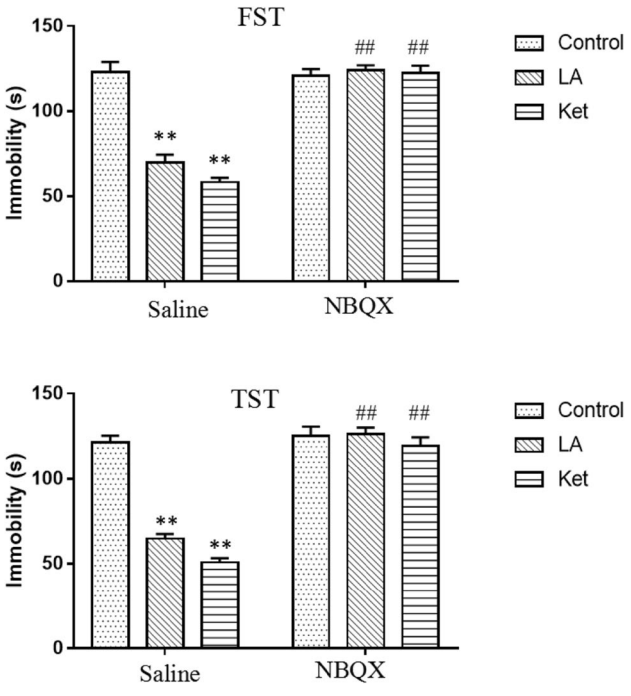

Fig. 7 Antidepressant-like effects of licorisoflavan A were reversed by AMPA receptor antagonist. **$p < 0.01$ vs. the control group, ##$p < 0.01$ versus the control group, $n = 6$

Discussion

Current conventional antidepressants act slowly, and fast acting antidepressants would be an important breakthrough in the therapy for depression. In the current study, we explored whether licorisoflavan A, an isoflavane isolated from Glycyrrhizae Radix, exerted antidepressant-like effects in acute and chronic stress murine model. We found that licorisoflavan A possessed powerful antidepressant-like effect, and its neuroprotective action might be associated with the regulation on BDNF-TrkB and mTOR signaling pathways. Furthermore, antidepressant effects of licorisoflavan A were reversed by AMPA receptor antagonist, NBQX. The results of the present work provided a new sight in the therapeutic effect of licorisoflavan A and its potential mechanism.

Accumulating evidence proved that stress, especially chronic stress was the key factor to induce depression in rodents and human [25]. Chronic stress could lead to ahedonia, despair, and anxiety behaviors in animals, as resemble the human depressive-and anxiety-like phenotypes. SPT was the most used parameter to evaluate the ahedonia of depressed animals [26]. The FST and TST are two standard tests which can mimic human depressive symptom. They are usually used to assess depressive-like behaviors and screen bioactive compounds with antidepressant-like activity [27, 28]. The tests in Fig. 1 were carried out to evaluate the anti-depressive effect of Licorisoflavan A at different dosage. With the examination of immobility duration in FST and TST, the Licorisoflavan A (20 mg/kg) was chosen for the following detection. Next, the single administration of Licorisoflavan A effectively attenuated the behavior alteration owing to CMS, without the stimulation of any locomotor activity in OFT.

Licorisoflavan A produced a quick antidepressant effect similar to ketamine. Since ketamine showed rapid antidepressant effect, we used it as a positive control to investigate the molecular mechanism underlying the antidepressant-like effect of licorisoflavan A. Although multiple mechanisms are reported to be responsible for the development of depression, it is well accepted that neuroplasticity, including synaptic and neurotrophic mechanism, have a close relationship with depression [29]. BDNF is an important neurotrophic factor implicated in the development and treatment of depression. It modulates neuronal plasticity, and affects the proliferation and maintenance of neurons in the central nervous system [30]. Growing experimental studies found that BDNF expression in the hippocampus of animals exposed to chronic stress was decreased, and this change could be reversed by antidepressant treatment [31, 32]. Our findings revealed that licorisoflavan A and ketamine upregulated the BDNF

and TrkB expressions in CMS-induced mice. Classical monoamine based antidepressants like imipramine, escitalopram or reboxetine also up-regulate BDNF, but only after chronic drug administration [33]. The fast induction of BDNF may account for the fast action of licorisoflavan A. CREB is a key target of antidepressant drugs activation and leads to increased expression and secretion of BDNF, which acts on TrkB receptors [34]. BDNF plays a pivotal role in regulating plasticity, which is implicated in the antidepressant behavior of ketamine. The ketamine-caused behavioral alteration are blocked in BDNF mutant mice [35]. BDNF Met polymorphism also blocks the process and release of BDNF, suggesting that the antidepressant actions of ketamine require BDNF release, not just synthesis [36]. Various factors including P2X4, p38-MAPK, intracellular Ca^{2+}, exogenous neurotrophin is required for the induction of BDNF release [37]. Despite this evidence, the dowregulation BDNF acts and drives the phosphorylations of TrkB and CREB in depressive etiology [38]. The binding between BDNF and TrkB protects hippocampal neurons against glutamate-mediated toxicity [39]. Furthermore, the bioactive ingredient of Radix glycyrrhizae promoted the BDNF protein levels [40, 41]. Our previous work also proved that Liquiritigenin elevated BDNF expression in depression [42]. Thus, we assumed that the alteration of BDNF in the present study was due to the change of protein expression rather than release. The effect on BDNF by licorisoflavan A may also be regulated by eEF2, since phosphorylation of eEF2 can suppress translation of BDNF. Our data presented that the increase of BDNF after licorisoflavan A treatment was also accompanied by the activation of phosphorylated CREB. Additionally, licorisoflavan A rapidly downregulated the eEF2 phosphorylation and augmented BDNF expression.

The activation of BDNF-induced TrkB stimulates downstream signaling including the activation of MEK/ERK [43, 44]. ERK is the upstream regulator of mTOR signaling, and its phosphorylation has been used as an additional biomarker of mTOR cascade activation. The MEK/ERK pathway regulates the phosphorylation of mTOR and its down-stream signaling components (e.g. p70S6K, 4E-BP-1), which is crucial for rapid antidepressant effects. In our study, mTOR signaling via 4E-BP-1 and p70S6K was found to be suppressed in the hippocampus of mice after CMS exposure, which was consistent with previous reports [45]. Our findings showed for the first time that in the chronically stressed mice, a single dose of licorisoflavan A contributed to increases in the phosphorylated mTOR, 4E-BP-1 and p70S6K, which might be required for its rapid antidepressant-like action via promoting synaptic protein synthesis. CMS exposure might also lead to the deficiency in ERK activation which would suppress 4E-BP-1 and p70S6K activation. In our work, ERK activation returned to normal level after licorisoflavan A administration, which was consistent with increased 4E-BP1 and p70S6K activities. Normalization of ERK paralleled the antidepressant-like effect of licorisoflavan A, indicating that mTOR signaling regulated by ERK pathway might be crucially involved in producing rapid antidepressant response.

The mTOR signaling is a downstream pathway that transmits information after the direct activation of AMPA and neurotrophic factor receptors. Activation of these receptors promote local protein synthesis of synaptic proteins underlying synaptic plasticity, which is critical to the rapid effects exerted by classic antidepressants. In this work, considering the activation of mTOR signaling pathway, we also studied the effect of licorisoflavan A on synaptic protein expression in the hippocampus of CMS mice [46]. PSD-95, a scaffold protein regulating the clustering of glutamate receptors in dendritic spines, is indispensable in synaptic maturation, strengthening and plasticity [47]. Increased levels of PSD-95 protein may reflect an increase in the size and number of dendritic spines, and result in a greater number of synapses. Previous study showed that stress might reduce AMPAR activities [48]. Although AMPAR activities apparently are not required for mediating stress induced dendritic atrophy, the AMPA receptor subunit GluR1 containing synapses are resistant to stress. Furthermore, there are much evidence supporting the idea of the association between increased AMPAR and antidepressant responses [49]. Considering that CMS suppressed mTOR activation, we suspected that the mTOR-regulated synaptic proteins were also disturbed. Consistent with our hypothesis, we observed that CMS procures obviously reduced the protein levels of synaptic plasticity marker proteins PSD-95 and AMPA-GluR1 in hippocampus were ameliorated by licorisoflavan A treatment. Moreover, the immobility reductions in FST and TST induced by licorisoflavan A were blocked by the AMPA receptor antagonist NBQX, a pattern previously demonstrated by ketamine and

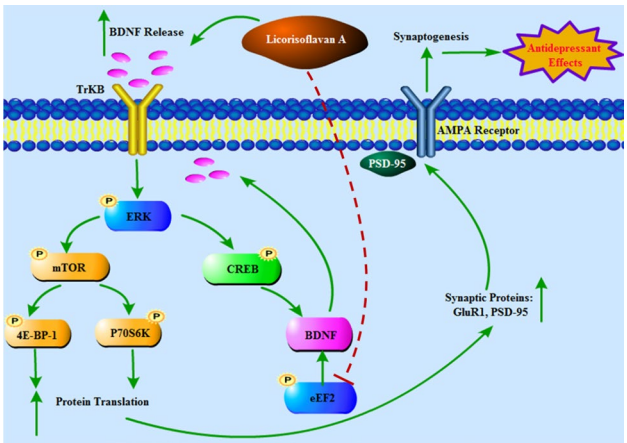

Fig. 8 Framework for the therapeutic mechanisms of licorisoflavan A

other ketamine-like rapid-onset antidepressants. The mechanism illustration was presented in Fig. 8.

There are some limitations for the present work: Numerous studies showed that there are huge differences between males and females in behavioral responses. As more females suffer from depression, we should test new treatment strategies in female rodents as well. However, the present work confirmed only that single licorisoflavan A administration could alleviate the depressive symptoms in the male mice subjected to acute or chronic stress. The effect of the licorisoflavan A on female mice is still unknown. Therefore, to get more evidence for the present researches, further work is still needed in the future.

In summary, our findings provided preclinical evidence that licorisoflavan A exhibited a potent rapid antidepressant-like effect in animal models of depression, without apparent motor alterative effects. The action of licorisoflavan A appeared to be mediated through regulation of the hippocampal BDNF-TrkB and AMPA receptor. Based on our results, a framework for the therapeutic mechanisms of licorisoflavan A is proposed in Fig. 8. The newly discovered effect of licorisoflavan A provides a new insight to understand its pharmacological effects, and more importantly, sheds light on the development of new fast-acting antidepressant with fewer side effects. However, the antidepressant-like mechanisms of licorisoflavan A is worth further testing in more details in future studies.

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Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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