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Original Article

A novel positive modulator of $GABA_A$ receptor exhibiting antidepressive properties

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Abstract: γ-Aminobutyric acid (GABA) neurotransmission alterations have been implicated to play a role in depression pathogenesis. While $GABA_A$ receptor positive allosteric modulators are emerging as promising in clinical practice, their precise antidepressant mechanism remains to be further elucidated. The aim of the present study was to investigate the effects of LY-02, a novel compound derived from the metabolite of timosaponin, on depression in animals and its mechanism. The results of behavioral tests showed that LY-02 exhibited better antidepressant effects in both male C57BL/6 mice and Sprague Dawley (SD) rats. The results of cellular voltage clamp experiments showed that LY-02 enhanced GABA-mediated currents in HEK293T cells expressing recombinant α6β3δ subunit-containing $GABA_A$ receptors. Electrophysiological recording from brain slices showed that LY-02 decreased the amplitude of spontaneous inhibitory postsynaptic current (sIPSC) and increased action potentials of pyramidal neurons in the medial prefrontal cortex (mPFC) of C57BL/6 mice. Western blot results showed that LY-02 dose-dependently up-regulated the protein expression levels of brain-derived neurotrophic factor (BDNF), tropomyosin related kinase B (TrkB) and postsynaptic density protein 95 (PSD-95) in mPFC of mice. The above results suggest that LY-02, as a positive modulator of $GABA_A$ receptors, reduces inhibitory neurotransmission in pyramidal neurons. It further activates the BDNF/TrkB signaling pathway, thus exerting antidepressant effects. It suggests that LY-02 is a potential novel therapeutic agent for depression treatment.

Key words: antidepressant; $GABA_A$ receptor; positive modulator; neurotransmission

一种具有抗抑郁特性的$GABA_A$受体新型正调节剂

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摘 要：γ-氨基丁酸(γ-aminobutyric acid, GABA)神经递质传递的失调是抑郁症发病的重要机制。虽然临床试验表明$GABA_A$受体正变构调节剂有积极的抗抑郁作用，但其确切的抗抑郁机制仍有待进一步阐明。本研究旨在探讨一种来源于知母皂苷代谢物的新型化合物LY-02对动物抑郁的作用及其机制。行为学试验结果显示，LY-02在雄性C57BL/6小鼠和Sprague Dawley

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(SD)大鼠体内均表现较好的抗抑郁效果。细胞电压钳实验结果显示，LY-02增强表达含重组α6β3δ亚基的$GABA_A$受体的HEK293T细胞GABA所介导的电流；脑切片电生理记录显示，LY-02降低C57BL/6小鼠内侧前额叶皮层(medial prefrontal cortex, mPFC)锥体神经元自发抑制性突触后电流(spontaneous inhibitory postsynaptic current, sIPSC)振幅，并增加其动作电位。Western blot结果显示，LY-02剂量依赖性上调小鼠mPFC脑源性神经营养因子(brain-derived neurotrophic factor, BDNF)、原肌球蛋白相关激酶B (tropomyosin related kinase B, TrkB)和突触后密度蛋白-95 (postsynaptic density protein 95, PSD-95)蛋白表达水平。以上结果提示，LY-02作为$GABA_A$受体的正向调节剂，可减少锥体神经元的抑制性神经递质，进一步激活BDNF/TrkB信号通路，从而发挥抗抑郁作用，表明LY-02是一种潜在的新型抑郁症治疗药物。

关键词：抗抑郁药物；$GABA_A$受体；正调节剂；神经传递

Major depressive disorder (MDD) is a serious mental illness. Comparing the pooled prevalence from 2001 to 2010 with that from studies published from 2011 to 2020, global adolescent depressive symptom exacerbations increased from 24% to 38% [1]. The rate of major depressive episodes also increased from 8.7% to 13.2% [2]. The causes of depression are complex and include psychosocial and biological factors. In terms of psychosocial factors, studies have shown that MDD is associated with several sociodemographic factors, including reduced work difficulty role functioning, persistence of severe secondary illness, and physical illness with early death [3]. Previous studies on the neurobiology of MDD have focused on the "monoaminergic hypothesis", concentrating on the monoamine neurotransmitter system [4]. However, it is estimated that such medications are still ineffective in the treatment of 40% of patients [5]. Moreover, these drugs have a slow onset of action and require several weeks of administration [6, 7]. Therefore, there is an urgent need to explore more targets and find fast and effective antidepressant drugs.

In recent years, the role of γ-aminobutyric acid (GABA) receptor in psychiatric disorders has gradually attracted the attention of many researchers. In a study of the ventral medial prefrontal cortex/anterior cingulate cortex (vmPFC/ACC), it was found that the more severe the degree of depressive symptoms, the higher the Glx (combined glutamate + glutamine) levels and the lower the GABA levels [8]. By using widely validated transcranial magnetic stimulation (TMS) assays to indicate $GABA_A$ receptors and $GABA_B$ receptor-mediated inhibitory neurotransmission, results showed that major depression is associated with a deficit in GABA inhibitory neurotransmission [9]. In addition, Brexanolone (Zulresso), a positive allosteric modulator of $GABA_A$ receptors, was approved in March 2019 for the treatment of postpartum depression in adult women [10]. And Zuranolone, another allopregnenolone analogue, is also a positive variant modulator of $GABA_A$ receptors. This has also led to an in-depth investigation of the antidepressant mechanisms of this class of drugs.

Both Brexanolone and Zuranolone are allopregnenolone analogs in neurosteroids. The most widely studied endogenous neurosteroids include allopregnenolone, androstenediol and allotetrahydrodeoxycorticosterone (THDOC) [11]. Among these, studies have shown that reduced levels of allopregnenolone are associated with both MDD and postpartum depression [12]. Furthermore, antidepressant medication can significantly increase allopregnenolone levels [13, 14]. The current study showed that, Brexanolone, an exogenous analogue of allopregnenolone, can rapidly improve postpartum depressive symptoms and alleviate insomnia and anxiety [15], which confirms that this drug class has value in antidepressant research.

Timosaponin, derived from *Rhizoma Anemarrhenae* in traditional Chinese medicine, possesses a neurosteroid structure. The low toxic side effects coupled with its antidepressant properties have piqued the interest of many researchers. In our previous study, we identified a novel chemical structure, LY-02, a derivative of timosaponin B-III (TB-III), which is modified from a metabolite found in timosaponin (Fig. 1) [16]. In the

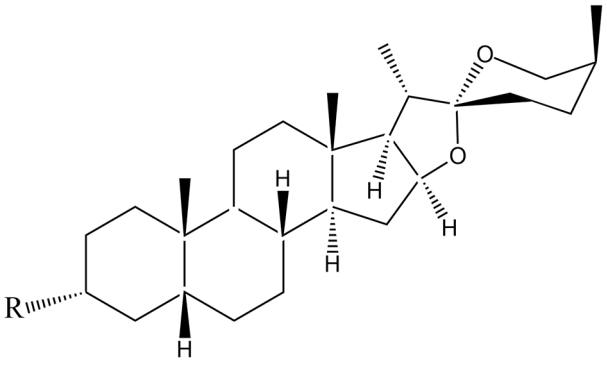

Fig. 1. The chemical structure of LY-02.

present study, we employed behavioral methods, electrophysiological experiments, and Western blotting to delve into the antidepressant effects of LY-02 and the underlying mechanisms.

1 MATERIAL AND METHODS

1.1 *Animals*

All animal studies and experimental procedures were approved by the Animal Care Committees of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and experiments were carried out in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purpose. All Sprague Dawley (SD) rats and C57BL/6 mice used in our experiments were purchased from Shanghai SLAC Laboratory. All experimental wild-type mice (C57BL/6) were housed under a 12/12 h light/dark cycle with free access to food and water in groups of 5. C57BL/6 mice, 20–25 days old, were used for electrophysiological experiments. Male C57BL/6 mice (18–22 g) and male SD rats (6 weeks old) were used for behavioral testing after acute administration.

1.2 *Forced swimming test (FST)*

FST was performed according to a previously reported method [17], and drug efficacy was assessed by measuring resting time. Twenty-four hours prior to drug administration, mice were placed in a clear cylindrical glass cylinder and acclimated to the water environment for 15 min. On the day of the behavioral test, the animals were administered with corresponding drugs by gavage 4 h and 1 h prior to the behavioral test, respectively. The animals were individually placed in a cylindrical glass tank of 30 cm height and 20 cm diameter with a water depth of 15 cm for mice (20 cm for rats), so that the animals could not escape from the glass tank without their feet and tails touching the bottom of the tank. The water temperature was 23–25 °C. The mice were filmed for 6 min after entering the water, and since most of the animals were very active in the first 2 min, the 4 min of immobility were calculated (criteria for determining immobility: the mice stopped struggling in the water, did not move and made small limb movements to maintain balance or floating). Groups of mice were operated in parallel. The rats were divided into three states in the water: immobility, swimming and climbing. The equivalence scores of these three states were counted (every 5 s, 5 min, 60 times in total).

1.3 *Tail suspension test (TST)*

TST is a classical and rapid method to evaluate the efficacy of antidepressants, euphoric drugs and sedative drugs. On the day of the behavioral test, the animals were orally administered the drug twice, 4 h and 1 h before the behavioral test, respectively. During the experiment, the mice were suspended upside down by tape or clips from a tail test stand about 15 cm above the ground. The mice struggled to move in order to overcome the abnormal body position, but after a period of activity, intermittent immobility occurred, showing a state of disappointment. The duration of the experiment was 6 min for each group. Since most of the mice struggled frequently in the first 2 min due to excitement, the subsequent 4 min of immobility were counted. The tail suspension experiment in rats was the same as that in mice.

1.4 *Open field test (OFT)*

The mice were placed into the arena (40 cm × 40 cm × 35 cm) and allowed to explore freely for 10 min. Locomotor activity was recorded and analyzed using a mouse spontaneous activity video analysis system (JL-Behv-LAG-4, Shanghai Jiliang Software Technology Co., Ltd.). The total move distance in the central square (20 cm × 20 cm) was automatically calculated.

1.5 *Expression of recombinant $GABA_A$ receptors*

We expressed recombinant human $GABA_A$ receptors containing α6β3δ receptor subunit in HEK293T cells (cell lineage: ATCC, Cat. No. CRL-3216). HEK293T cells were preserved in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin, at 37 °C in 5% CO_2/95% air. Small dishes of HEK293T cells with approximately 70%–90% of the growth area were taken. Then, the cells were transfected with α6, β3, δ, and green fluorescent protein (GFP) cDNA (2∶0.3∶0.25∶1) using Lipofectamine LTX and Plus TM reagents (Thermo Fisher Scientific Inc.) [18]. The cells were placed back in the incubator for 6 h, followed by a fluid change and continued incubation for 24–48 h. The cells were ready for whole-cell voltage-clamp recording.

1.6 *Whole-cell voltage-clamp recording*

Whole-cell recordings were performed on HEK293T cells immersed in an external solution consisting of (in mmol/L): NaCl 145, KCl 5, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1 and glucose 10, pH 7.4 (pH adjusted with NaOH). The electrodes were drawn from borosilicate glass tubes with a tip resistance of approximately 5–7 MΩ.

The filled electrode internal solution contained (in mmol/L): KCl 140, HEPES 10, EGTA 1, $MgCl_2$ 2, and Mg-ATP 2, pH 7.3 (pH adjusted with KOH). The experiment was conducted with 1 μmol/L GABA-induced GABA current as a control. The changes of GABA current induced by LY-02 at different concentrations of 0.3, 1, 3, 10 and 30 μmol/L were recorded. Current responses were recorded under a whole-cell voltage-clamp configuration using an Axonpatch 200B amplifier (Axon Instruments, USA). Membrane potential was maintained at −60 mV. Clamp voltage commands and current recording were controlled by Clampex 10.3 (Molecular devices) via the Digidata-1440A conversion interface.

1.7 *Slice preparation and electrophysiology*

Mice (20–25 days old) were taken and anesthetized. Then frozen oxygenated artificial cerebrospinal fluid (ACSF, in mmol/L: 120 NaCl, 2.5 KCl_2, 2.5 $CaCl_2$, 1.3 magnesium sulfate, 1.0 sodium dihydrogen phosphate, 26 sodium bicarbonate, 10 glucose, aerated with 95% O_2 and 5% CO_2) was injected into the heart. After perfusion, the brain was rapidly removed and placed in chilled oxygenated modified artificial cerebrospinal fluid (mACSF, in mmol/L: 25.0 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl_2, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 25.0 glucose, 110 choline chloride, 11.6 ascorbic acid, and 3.1 sodium pyruvate, gassed with 95% O_2 and 5% CO_2). Coronal brain slices (300-μm thick) of medial prefrotal cortex (mPFC) were made using a vibratome (Leica VT1000s, USA) and incubated in oxygenated (95% O_2, 5% CO_2) ACSF solution for at least 1 h for recovery.

Subsequently, the slices were placed in a dosing tank and continuously perfused with ACSF in a bath temperature of 32 °C. All drugs were dissolved in ACSF and administered by bath perfusion. The electrode tip resistance was between 4 and 8 MΩ. To record spontaneous inhibitory postsynaptic currents (sIPSCs), the electrode was filled with electrode internal solution including (in mmol/L): CsCl 110, K-gluconic acid 30, $CaCl_2$ 0.1, Mg-ATP 4, Na_3-GTP 0.4, EGTA 1.1, HEPES 10 (pH 7.3). sIPSCs were recorded in voltage clamp mode with a clamp voltage of −65 mV. To record the action potential (AP), the electrode was filled with the internal fluid including (in mmol/L): K-glucose acid 120, KCl 20, ATP-Na_2 4, Na-GTP 0.3, Na-phosphocreatine 5, EGTA 0.1, HEPES 10 (pH 7.3). In the current-clamp mode, the slices were stimulated with depolarizing currents of 1 200 ms in duration, increasing by 30 pA for a total of 8 stimulations. The number of APs generated was also analyzed, as well as the relationship between the intensity of stimulation and the number of APs output.

The mPFC V-layer pyramidal neurons were imaged and recorded through a 40 × 40 water immersion lens and a MultiCLAMP 700B amplifier (Molecular Devices, USA).

1.8 *Western blotting*

Twenty C57BL/6 mice (6 weeks old) were divided into four groups, including vehicle (Veh) group, fluoxetine (10 mg/kg FLX) group, high-dose (5 mg/kg) LY-02 group, and low-dose (2.5 mg/kg) LY-02 group. The animals were dosed twice, orally 4 h and 1 h before the collection of mPFC tissue from the brain. After sectioning, the tissues were placed in frozen homogenization buffer and blended on ice using an ultrasonic homogenizer. Tissue lysates were centrifuged at 14 000 r/min for 15 min at 4 °C, and supernatants were collected. Total protein concentration was determined by the BCA method (EpiZyme). The primary antibodies used were: postsynaptic density protein 95 (PSD-95, 1∶1 000, Cell Signaling), brain-derived neurotrophic factor (BDNF, 1∶1 000, Cell Signaling), tropomyosin related kinase B (TrkB) (80E3) (1∶1 000, Cell Signaling) and β-tubulin (1∶1 000, Cell Signaling) antibodies. The bands were developed using a fully automated chemiluminescence imaging analysis system (Tanon 5200), and the density of each band on Western blots was determined using Image Processing and Analysis in Java (ImageJ) software.

1.9 *Drugs*

LY-02 was provided by the Phytochemistry Research Laboratory, Shanghai Institute of Pharmaceutical Sciences. In animal behavioral experiments and Western blot, fluoxetine (FLX) hydrochloride (TCI Shanghai) was diluted with 0.9% physiological saline, LY-02 was diluted with 0.5% sodium carboxymethylcellulose (CMC-Na) to the desired concentrations for gavage administration, and 0.5% CMC-Na was used to treat vehicle group. For electrophysiology experiments, LY-02 was dissolved in dimethyl sulfoxide (DMSO, Sigma) and then diluted to a range of concentrations by ACSF or extracellular fluid. GABA (Sigma) was dissolved in water and then diluted to 1 μmol/L by extracellular fluid for experiments.

1.10 *Statistical analysis*

All data are expressed as mean ± SEM. GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analyses. We used paired

t-tests to analyze the changes of APs and sIPSC. And one-way analysis of variance (ANOVA) was used for multiple-groups comparsion of both animal behavior and Western blot results. Differences were considered statistically significant at $P < 0.05$.

2 RESULTS

2.1 LY-02 produced antidepressant-like effects

To access the antidepressant effects of LY-02, we compared its dose regiment for oral administration to mice and rats by using TST and FST. FLX served as a reference drug. Our findings showed that 5 mg/kg LY-02 and 10 mg/kg FLX significantly reduced immobility duration of mice in the FST and TST compared to the Veh group (Fig. 2*c*, *d*). This shows that LY-02 has a dose effect. In the OFT, neither LY-02 nor FLX impacted the total distance travelled (Fig. 2*a*) and percent distance in the central area (Fig. 2*b*) in mice, suggesting LY-02 doesn't affect the locomotor activity in mice.

To further confirm the antidepressant effects of LY-02, we used the rats to evaluate its efficacy in the TST and FST. We first adjusted the mouse doses for rat, with equivalent dose for the rats being nearly half of that for mice [19]. Based on conversation, the rats were administered FLX at 6.92 mg/kg and LY-02 at dosages 1.73 and 3.46 mg/kg. The results revealed that both 3.46 mg/kg LY-02 and 6.92 mg/kg FLX notably decreased

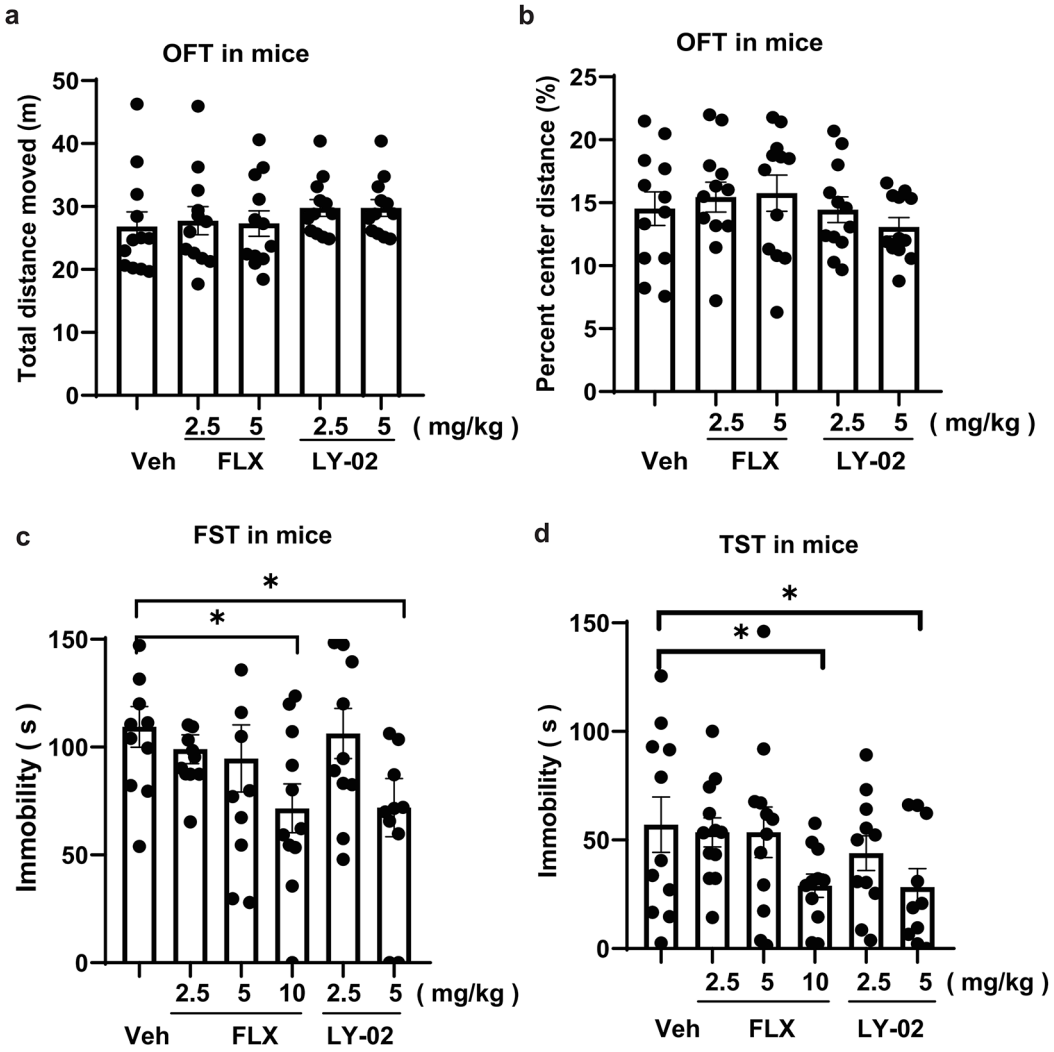

Fig. 2. LY-02 exhibited antidepressant-like effects in mice. *a*, *b*: Locomotor activities remained normal in mice, as demonstrated in the open field test (OFT) at 1 h after drugs administration. *c*, *d*: Both 10 mg/kg fluoxetine (FLX) and 5 mg/kg LY-02 notably decreased immobility time in the forced swimming test (FST, *c*) and tail suspension test (TST, *d*). Analysis by one-way ANOVA followed by uncorrected Fisher's LSD. Mean ± SEM, $n = 9–12$. $^*P < 0.05$. Veh, vehicle.

immobility time in the TST. Similarly, a marked reduction in immobility time was observed with 3.46 mg/kg LY-02 and 6.92 mg/kg FLX in the FST. Moreover, 1.73 mg/kg YL-02 was also able to significantly reduce the immobilization time in the FST (Fig. 3). These finding confirm that LY-02 has antidepressant-like effects on both mice and rats, with the efficacious oral dose of LY-02 being lower than that of FLX.

2.2 LY-02 enhanced $GABA_A$ receptor channel currents

We then investigated the pharmacological target of LY-02, initially examining its effect on $GABA_A$ receptors. $GABA_A$ receptors were first expressed by transfecting plasmids of α6, β3, and δ, and also transfecting GFP plasmid to facilitate the localization of transfected HEK293 cells (Fig. 4). Using whole-cell patch-clamp recording with clamping potential, we found that LY-02 augmented GABA-evoked currents in a dose dependent manner. The EC_{50} (concentration for 50% of maximal effect) was approximately 0.5 μmol/L (Fig. 5), indicating that LY-02 acted as an allosteric modulator for $GABA_A$ receptor. We then explored whether LY-02 impacted NMDA receptors by conducting electrophysiological recordings on recombinant GluN1-GluN2 receptor subtypes expressed in HEK293T cells. However, there was no significant effect of LY-02 on NMDA receptor currents, regardless of the GluN subunit types, including GluN2A, GluN2B, GluN2C and GluN2D subunits (Fig. S1, https://actaps.sinh.ac.cn/supplement/EN-2023-0005Sfig1.pdf). These finding suggest that LY-02 primarily enhances $GABA_A$ receptor activity and could have antidepressant effects.

2.3 LY-02 augmented prefrontal neuronal activity by reducing inhibitory synaptic transmission

As LY-02 enhanced $GABA_A$ receptor, we explored its

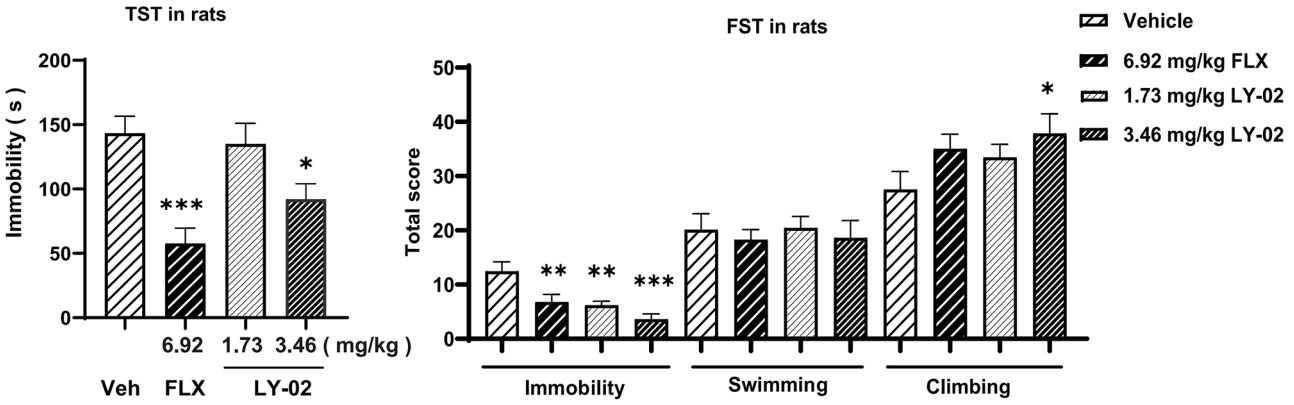

Fig. 3. LY-02 demonstrated antidepressant-like effects in rats. *a*: One hour post oral administration, both fluoxetine (FLX, 6.92 mg/kg) and LY-02 (3.46 mg/kg) significantly decreased immobility time in the tail suspension test (TST, *a*) and total score of immobility in the forced swimming test (FST, *b*). Analysis by one-way ANOVA followed by uncorrected Fisher's LSD. Mean ± SEM, $n = 8–12$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* vehicle (Veh).

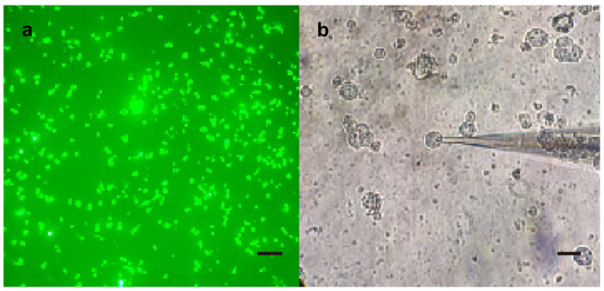

Fig. 4. Expression of recombinant $GABA_A$ receptors. *a*: HEK293T cells expressed with $GABA_A$ receptor under fluorescent light. Scale bar, 80 μm. *b*: Figure of whole-cell membrane clamp sealing operation under microscope. Scale bar, 20 μm.

effect on inhibitory synaptic transmission by recording sIPSC in pyramidal neurons of mPFC. We observed that 30 μmol/L LY-02 significantly reduced the sIPSC amplitude but not frequency (Fig. 6*e*, *f*), whereas 10 μmol/L LY-02 did not affect either sIPSC amplitude or frequency (Fig. 6*b*, *c*). Subsequently, we investigated whether the reduction of sIPSC could amplify neuronal activity by recording APs in the pyramidal neurons. As expected, 30 μmol/L LY-02 but not 10 μmol/L LY-02, increased discharge rate (Fig. 7). This suggests that LY-02 enhances pyramidal neurons activity by reducing inhibitory neurotransmission.

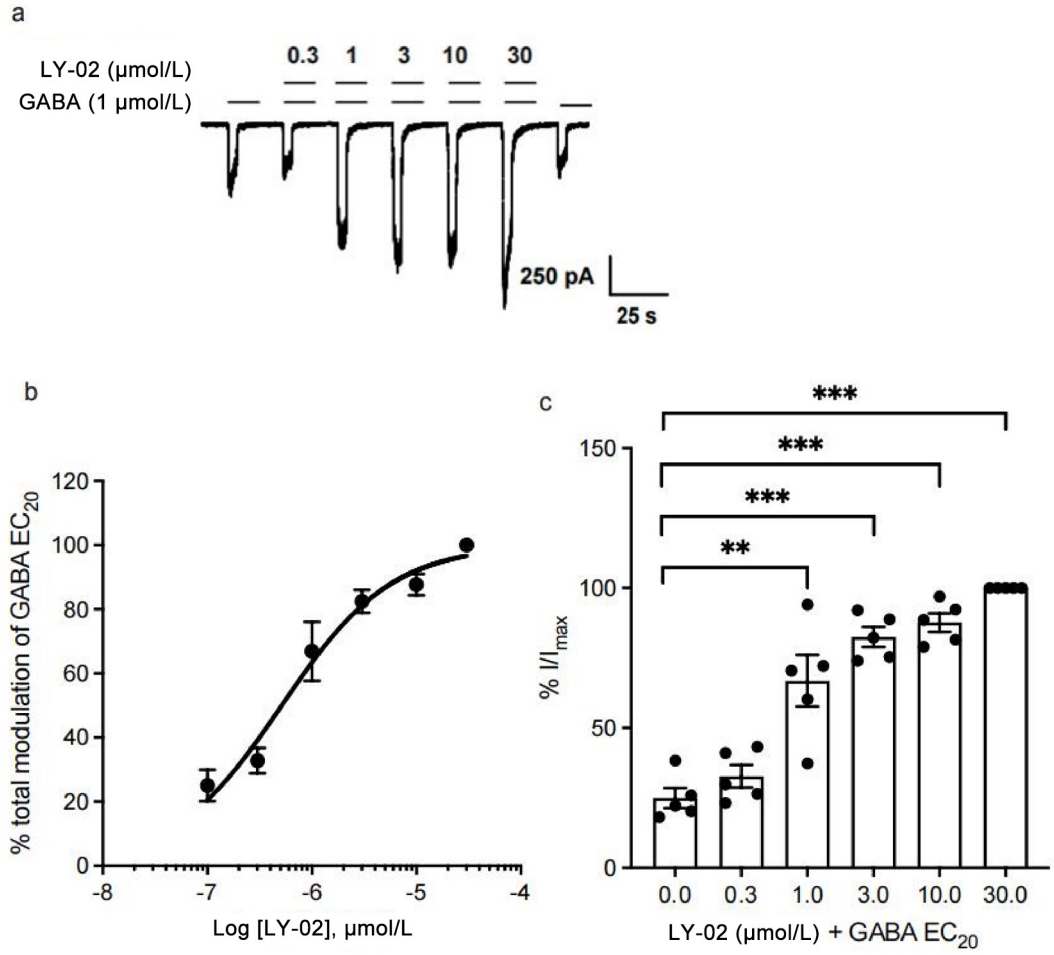

Fig. 5. LY-02 enhanced GABA-evoked current. *a*: Sigle-cell representative current traces from whole-cell patch-clamp electrophysiological recording of the modulation of GABA EC_{20} (1 μmol/L)-induced currents by LY-02 at α6β3δ-containing $GABA_A$ receptor. *b*: Concentration-response curves for modulation of GABA-evoked responses and direct activation of $GABA_A$ receptors by LY-02. EC_{20}, concentration for 20% maximal effect. *c*: Bar diagram summarizing the LY-02 modulation of α6β3δ-containing $GABA_A$ receptor. Mean ± SEM, $n = 5$. $^{**}P < 0.01$, $^{***}P < 0.001$.

2.4 LY-02 activated synaptogenesis-related signaling pathway

Previous research revealed an association between synaptogenesis-related proteins and antidepressant effects [20, 21]. In our current investigation, following 1 h of drug administration to mice, there was a significant, dose-dependent up-regulation in the expression levels of BDNF, its receptor TrkB, and the synaptogenesis-associated protein, PSD-95, upon treatment with 5 mg/kg LY-02 (Fig. 8). In contrast, 10 mg/kg FLX elevated the expression of PSD-95, but did not exhibit similar effects on BDNF and TrkB. This result suggests that the BDNF/TrkB signaling pathway may play a role in the antidepressant-like properties of LY-02.

3 DISCUSSION

In the present study, we assessed the antidepressant potential of LY-02 using FST, TST, and OFT. Our findings suggest that LY-02 exhibits antidepressant properties. When compared to the conventional antidepressant FLX, LY-02 demonstrated a notable advantage in dosage, with efficacy evident even at low starting doses. This can potentially be attributed to LY-02's pronounced lipid solubility, given its structural resemblance to neurosteroids. Previous research has indicated that the lipophilic nature of neurosteroids bolsters receptor binding potency and overall efficacy [22]. Additionally, our research showed that LY-02 potentiated

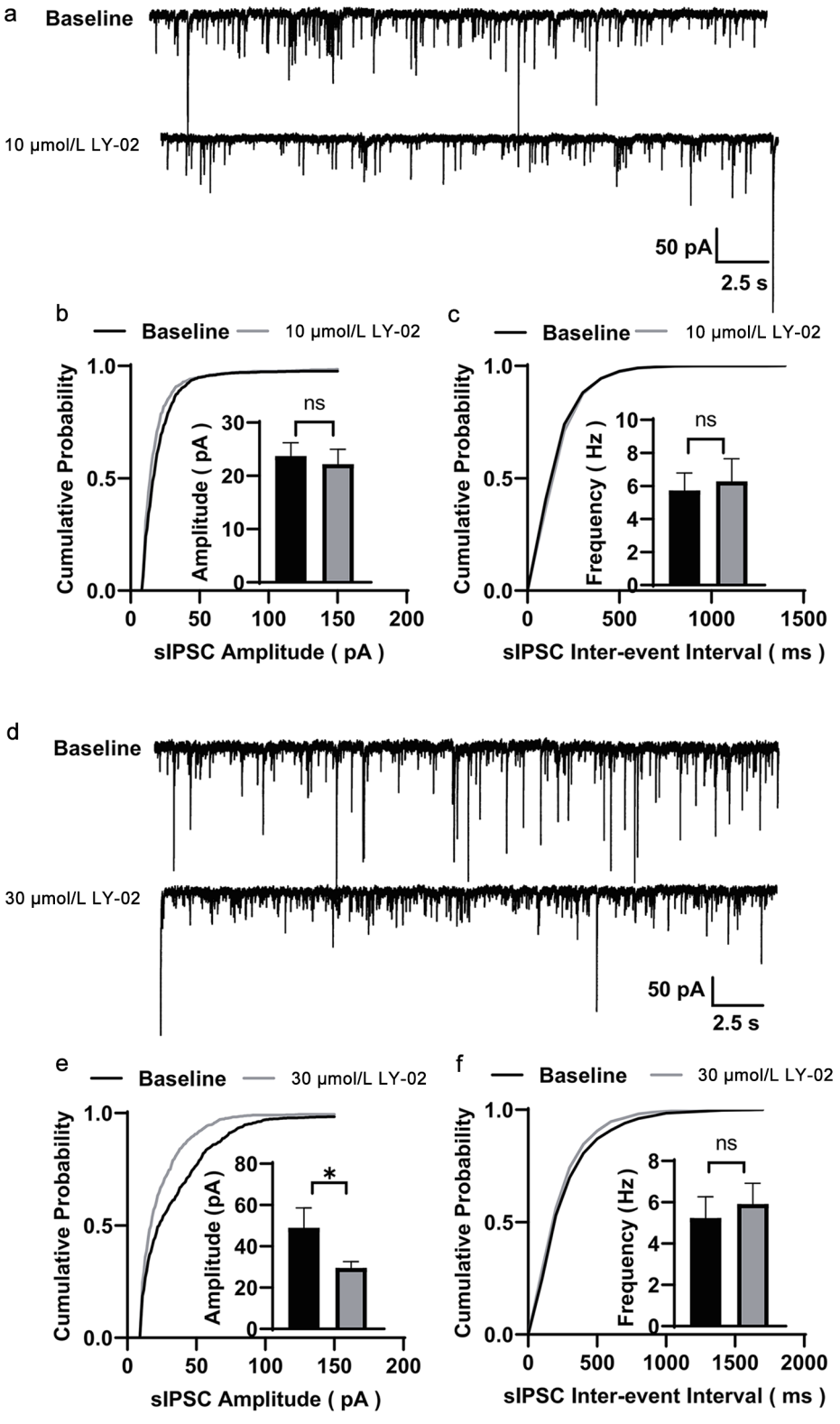

Fig. 6. LY-02 reduced inhibitory synaptic transmission of pyramidal neurons in the mPFC. *a*, *d*: An example of spontaneous inhibitory postsynaptic current (sIPSC) trace, showing the effects of 10 and 30 μmol/L LY-02 after 10 min of baseline recording. The data were measured in a whole-cell configuration, from mPFC pyramidal neurons. *b*, *c*: Insets showing changes in sIPSC amplitude and average frequency at a concentration of 10 μmol/L LY-02, as well as the cumulative distributions of sIPSC inter-event intervals and amplitudes. *e*, *f*: Insets showing changes in sIPSC amplitude and average frequency at a concentration of 30 μmol/L LY-02, as well as the cumulative distributions of sIPSC inter-event intervals and amplitudes. Mean ± SEM, n = 7–9. ns: not significant. $^{*}P < 0.05$.

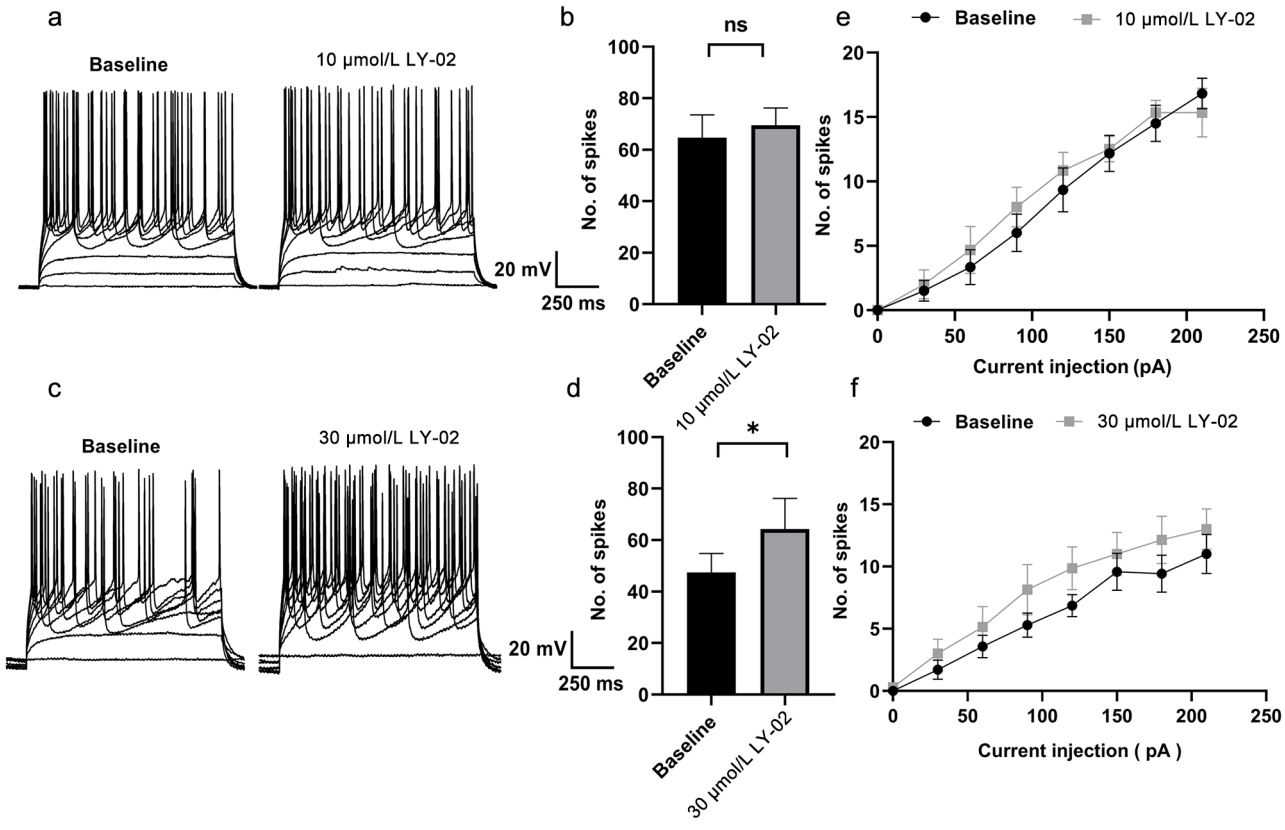

Fig. 7. LY-02 increased the action potential frequency of pyramidal neuron in the mPFC. *a*, *c*: Sample traces of current-evoked action potentials following 10 min of baseline recording, observed in the presence of LY-02 at 10 and 30 μmol/L. *b*, *d*: Numbers of spikes observed in the presence of LY-02 at 10 and 30 μmol/L. *e*, *f*: Graphical representations depicting the mean number of action potentials evoked at each stimulus intensity, comparing baseline with post-LY-02 application. Mean ± SEM, $n = 6–7$. ns: not significant. $^*P < 0.05$.

α6β3δ-incorporated $GABA_A$ receptor activity and diminished inhibitory neurotransmission in the pyramidal neurons of the mPFC. This might play a role in enhancing neural activities within the mPFC. Moreover, LY-02 was found to stimulate the BDNF/TrkB signaling pathway, which is widely recognized for its association with antidepressant effects [21, 23].

3.1 LY-02 exerted antidepressant effect via $GABA_A$ receptors

GABA receptors include $GABA_A$, $GABA_B$, and $GABA_C$. Among them, $GABA_A$ receptors are heteropentameric receptors formed by the combination of 19 known subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3). They can be divided into synaptic receptors (mainly γ subunit-containing) and extrasynaptic receptors (mainly δ subunit-containing) [24]. Previously, it was found that the binding sites of neurosteroids include metastable sites within the transmembrane domain of the α subunit and direct activation sites between the α and β subunits [25, 26]. In some cases, researchers identified the hippocampal extrasynaptic δ$GABA_A$ receptor structure as a key structure in the efficacy of neurosteroids [27]. Zuranolone enhanced $GABA_A$ receptor current, including representative receptors for both synaptic (γ subunit-containing) and extrasynaptic (δ subunit-containing) $GABA_A$ receptors [28].

In the current study, we also observed the effect of LY-02 on $GABA_A$ receptor channel currents by whole-cell electrophysiological recordings (Fig. 5). The results revealed that LY-02 has an agonistic effect on the currents of $GABA_A$ receptor channels expressing α6, β3, and δ subunits. At a dose of 1 μmol/L, LY-02 induced currents two to three times higher than those induced by GABA alone. It also has a concentration-dependent effect, with the higher the concentration the stronger the ability to induce currents. In the brain, δ$GABA_A$ receptor expression (and tonic currents) correlated significantly with neurosteroid levels in the brain [29, 30]. Inadequate expression of δ$GABA_A$ receptors decreases sensitivity to neurosteroids [31]. This research is consistent

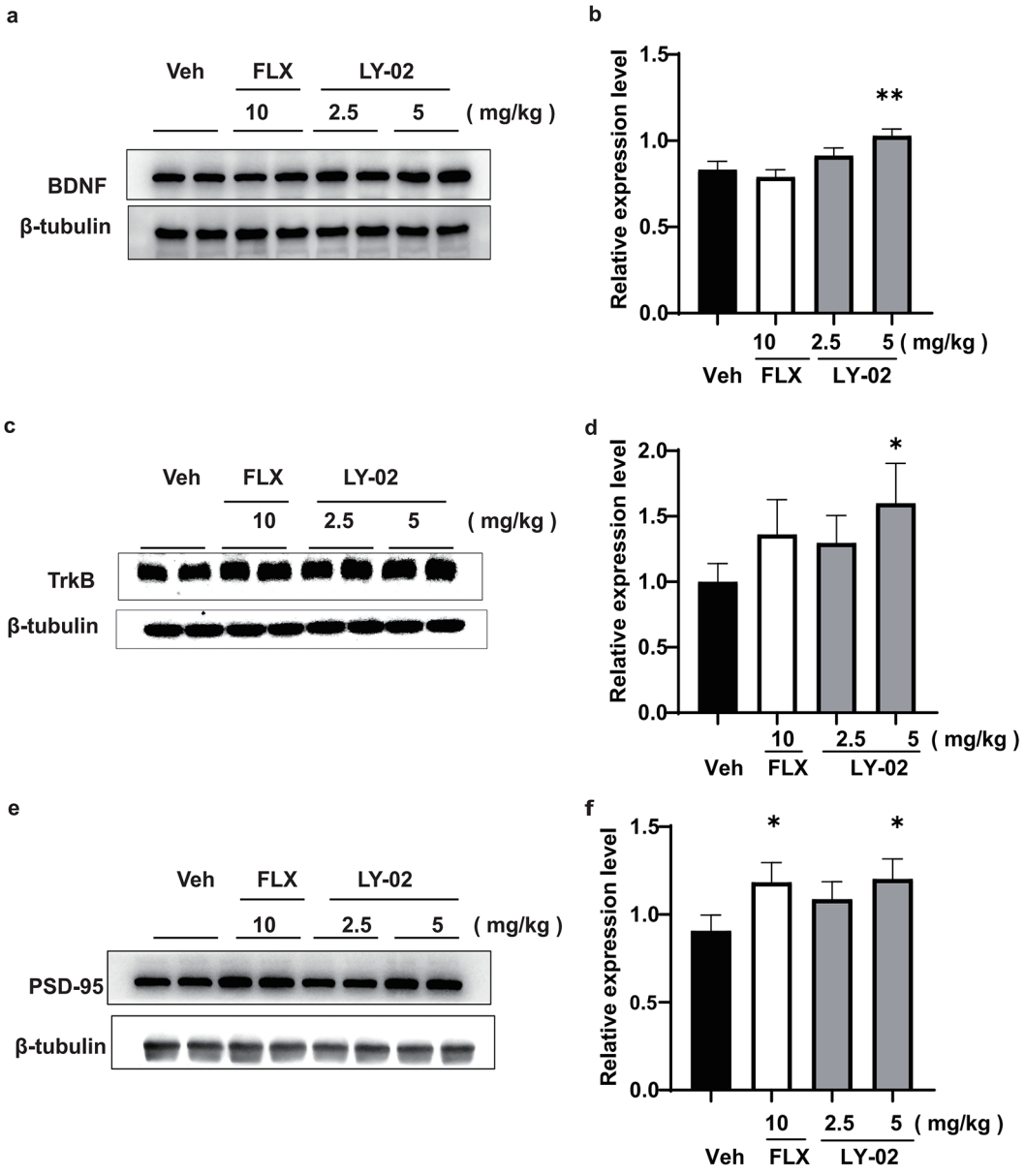

Fig. 8. LY-02 dose-dependently increased the expression of BDNF, TrkB and synapse-associated proteins. *a*, *c* and *e*: Western blotting images depicting BDNF, TrkB, PSD-95 and β-tubulin at 1 h post drug administrations. *b*, *d* and *f*: Relative protein expression levels of BDNF (*b*), TrkB (*d*) and PSD-95 (*f*) in the PFC. One-way ANOVA by Dunnett's multiple comparisons test. Mean ± SEM, $n = 6–8$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* vehicle (Veh).

with our electrophysiological experiments that LY-02 enhanced $GABA_A$ receptor channel currents containing α6, β3, and δ subunits.

Furthermore, in the context of neural circuits, especially within cortical areas, the interplay between different types of interneurons and pyramidal neurons is crucial for maintaining a balanced excitation-inhibition (E/I) dynamic, which is essential for proper brain function. Vasoactive intestinal peptide (VIP) and parvalbumin (PV) expressing interneurons play significant roles in this balance, influencing the activity of pyramidal neurons and, by extension, cognitive processes and behaviors.

VIP-expressing neurons are a subclass of GABAergic interneurons that, intriguingly, often act to disinhibit pyramidal neurons. This disinhibition occurs through VIP neurons' inhibition of other interneurons, such as SST (somatostatin-expressing) and PV neurons, which directly inhibit pyramidal neurons. By inhibiting these inhibitory interneurons, VIP neurons indirectly increase the activity of pyramidal neurons, thus playing a crucial

role in modulating the E/I balance towards excitation in certain contexts. This mechanism allows for a nuanced regulation of neural circuits, enabling the enhancement of signal-to-noise ratio in neural representations and the facilitation of cortical processing necessary for complex behaviors [32].

In the current study, LY-02 may directly enhance GABAergic transmission onto interneurons and inhibit SST or PV interneurons. By enhancing the inhibition of these GABAergic neurons, the molecule would effectively reduce their inhibitory output, potentially leading to less inhibition of pyramidal neurons. Even though, the effects of molecules on neuronal activity can be nuanced and multi-faceted, depending on their mechanisms of action, the specific neuronal circuits they target, and the balance between excitatory and inhibitory signaling in those circuits.

3.2 LY-02 excited the mPFC pyramidal neurons by diminishing inhibitory synaptic input

The prefrontal cortex (PFC) plays a pivotal role in orchestrating various behavioral functions, encompassing cognition, emotion, and both learning and memory process [33]. Neuronal compositions within the PFC predominantly include glutamatergic pyramidal neurons and GABA inhibitory neurons. Notably, GABA interneurons facilitate connections in approximately one-third of synapses throughout the central nervous system (CNS) and constitute 20%–30% of neocortical neurons, underscoring their significance in the inhibitory circuitry [34, 35].

Extensive research has elucidated that diminished GABA concentrations and deficits in $GABA_A$ receptor functionality correlate with diverse neurological pathologies. In the current analysis, LY-02 was observed to enhance $GABA_A$ receptor currents (Fig. 5). Intriguingly, it simultaneously diminished inhibitory synaptic transmission (Fig. 6) and increased the APs within the mPFC pyramidal neurons (Fig. 7). This presents a seeming incongruence with respect to its modulatory effects on inhibitory neurotransmission. Based on our findings, we postulate that LY-02 directly potentiated $GABA_A$ receptors in interneurons, leading to diminished excitability of these interneurons. This, in turn, resulted in reduced inhibitory feedback on pyramidal neurons, manifesting as increased APs within these neurons. Although the specific GABAergic interneurons directly involved in mediating inhibitory synaptic neurotransmission have yet to be identified, additional experiments will be essential in future research endeavors.

3.3 LY-02 activated the antidepressant-related BDNF/TrkB signaling pathway

Proteins associated with neurological function may also be important for the treatment of depression. We found that an acute administration of LY-02 led to elevated expression of BDNF, TrkB, and PSD-95 in the mPFC. This indicates that the antidepressant characteristics of LY-02 might be mediated by the BDNF/TrkB signaling axis, complemented by the synaptogenic protein, PSD-95.

The fundamental role of BDNF in depression is well-established [21]. Direct BDNF infusion into the brain [36], or intravenous Neotrofin (a neurotrophic factor agonist) administration, has displayed potent antidepressant outcomes in animal depression paradigms [37]. Notably, compounds like TB-III and allopregnanolone have been shown to restore BDNF levels in mouse brains modeled for depression [38–40]. The latest study found that antidepressant drugs could directly bind to TrkB, the receptor of BDNF, and enhance the signaling pathway of BDNF by increasing the expression of TrkB on the membrane surface [23]. Interestingly, the therapeutic influence of psychedelics on neurotrophic signaling and antidepressant-like behaviors in mice seems contingent on TrkB association. Yet, their convulsive side effects don't appear TrkB-dependent [41]. This insight offers a novel trajectory for crafting antidepressants with minimized side effects. LY-02's potential mechanism might encompass enhancing BDNF expression and its signaling pathway via TrkB binding, subsequently escalating TrkB membrane expression, thereby modulating neuroplasticity to achieve its antidepressant efficacy.

Synaptic plasticity also plays an integral role in the pathophysiology of depression. PSD-95 protein, a member of the Discs-large (DLG) scaffolding protein family, recruits a variety of proteins to synaptic adhesion sites, promotes inter-synaptic signaling, and is an essential modifier of synaptic strength and plasticity [42, 43]. A previous study showed that PSD-95 deficiency leads to a significant elevation of the indicated $GABA_A$ receptor subunit α1 and an increase in the frequency and amplitude of sIPSCs in pyramidal neurons in the mPFC [44]. This suggests that the regulation of PSD-95 is able to influence the excitatory transmission between synapses. It also indicates that the pharmacological mechanisms of antidepressant drugs may be relevant for PSD-95 protein-related regulation of signaling pathways. In several studies of antidepressants, it has been

found that antidepressants raise the expression level of PSD-95 along with the level of BDNF [20, 37]. Injection of the neurotrophic factor agonist Neotrofin increased not only BDNF levels, but also PSD-95 levels [37, 45]. LY-02 may also regulate synaptic plasticity through BDNF protein.

3.4 Conclusion

This research probed the properties of a newly identified antidepressant compound, LY-02. Our results indicate that the agonistic action of LY-02 on GABA receptor currents is comparable to that of Zuranolone [46]. Moreover, LY-02's enhancement of the AP frequency in pyramidal neurons could be attributed to the attenuation of inhibitory synaptic transmission. Furthermore, LY-02 appears to bolster the BDNF signaling pathway through its binding with the TrkB receptor. This association may influence synaptic protein expression, potentially contributing to its antidepressant efficacy.

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