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Authors: Yan-Jiao Li, Yu-Jiao Li, Liu-Di Yang, Kun Zhang, Kai-Yin Zheng, Xin-Miao Wei, Qi Yang, Wen-Min Niu, Ming-Gao Zhao, Yu-Mei Wu

PII: S0166-4328(18)30157-8
DOI: https://doi.org/10.1016/j.bbr.2018.04.025
Reference: BBR 11391

To appear in: *Behavioural Brain Research*

Received date: 30-1-2018
Revised date: 20-3-2018
Accepted date: 17-4-2018

Please cite this article as: Li Y-Jiao, Li Y-Jiao, Yang L-Di, Zhang K, Zheng K-Yin, Wei X-Miao, Yang Q, Niu W-Min, Zhao M-Gao, Wu Y-Mei, Silibinin exerts antidepressant effects by improving neurogenesis through BDNF/TrkB pathway, *Behavioural Brain Research* (2010), https://doi.org/10.1016/j.bbr.2018.04.025

Silibinin exerts antidepressant effects by improving neurogenesis through BDNF/TrkB pathway

Yan-Jiao Li[1,2,3†], Yu-Jiao Li[2†], Liu-Di Yang[2,3†], Kun Zhang[2], Kai-Yin Zheng[1,2], Xin-Miao Wei[4], Qi Yang[2], Wen-Min Niu[3], Ming-Gao Zhao[1,2], Yu-Mei Wu[1,2,3¶]

[1]Precision Pharmacy & Drug Development Center, Department of Pharmacy, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province, P.R.China, 710038

[2]Department of Pharmacology, School of Pharmacy, the Fourth Military Medical University, Xi'an, Shaanxi Province, P.R.China, 710032

[3]Department of Acupuncture-moxibustion-massage, Shaanxi University of Chinese Medicine, Xi'an, Shaanxi Province, P.R.China, 712000

[4]Student Brigade, the Fourth Military Medical University, Xi'an, Shaanxi Province, P.R.China, 710032

[†]These authors contributed equally to this study

[¶]Corresponding Author:

Associate Prof. Yu-Mei Wu

Department of Pharmacology, School of Pharmacy

The Fourth Military Medical University

Xi'an, Shaanxi Province, P.R.China, 710032

Phone: 86-29-84774555

Fax: 86-29-84774552

E-mail: yumeiwu@fmmu.edu.cn

Abstract

Classic antidepressants benefit depression patients partially by improving neurogenesis and/or brain-derived neurotrophic factor (BDNF)/TrkB pathway which were impaired in depression. In this study, we demonstrated that Silibinin (SLB), a polyphenolic flavanoid from *Silybum marianum*, ameliorated reserpinized mouse depressant-like behaviors. The antidepressants of SLB administration was associated with increased neural stem cells (NSCs) proliferation and further confirmed in BDNF/TrkB signaling transduction. SLB treatment reversed the decreased expression levels of BDNF and its receptor TrkB, and the reduced activation of downstream target proteins including phosphorylated extracellular-regulated protein kinase (p-ERK) and phosphorylated cAMP-response element binding protein (p-CREB) in depressived hippocampus. Furthermore, intracerebroventricular injection of GNF5837, a TrkB antagonist, abrogated antidepressant-like effects of SLB in mice along with the improved NSC proliferation, as well as enhanced levels of p-ERK and p-CREB in mice hippocampus. Taken together, these results suggest that SLB may exert antidepressant effects through BDNF/TrkB signaling pathway to improve NSC proliferation in acute depression.

Keywords: Silibinin, acute depression, brain-derived neurotrophic factor, TrkB, neurogenesis, neural stem cell

Introduction

Major depressive disorder (MDD) constitutes an enormous medical, individual, societal and economical challenge and has become the second most prevalent cause of illness-induced disability worldwide[1]. Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for depression treatment, while displayed various side effects and high failure rate that limit clinical applications[2]. Therefore, more efforts should be put into exploring more efficient and secure antidepressants against MDD.

Evidence showed that neuroplasticity altered due to the noxious or long-lasting stress, characterized as abnormality in long-term potentiation, dendritic shrinkage and functional changes, axonal sprouting, and impaired neurogenesis. Abnormal neuroplasticity leads to maladaptive and abnormal state of neuronal responses, resulting in depression. Adult hippocampal neurogenesis, manifesting as the generation of new neurons from neural stem cells (NSCs) in dentate gyrus (DG), is impaired and researched in the etiology of depression in recent years[3, 4]. Research work has put efforts to elucidate the association of neurogenesis, neuroplasticity in hippocampus with depression. The newborn neurons undergo synaptic connections through proliferation, migration, differentiation and integration into the existing neuronal circuits, thus completing hippocampal neural plasticity[5, 6]. The destruction of neurogenesis in depression leads to an aberrant hippocampal plasticity, and plays an important role in the pathophysiology of depression. Classical antidepressants benefit patients mainly due to the upregulation of neurotrophic factors, correcting and improving hippocampal neural remodeling, and activating the neural protective

pathways by increasing neurogenesis in DG[7]. This can also explain the clinical lag effect of antidepressants (usually 2-3 weeks after the onset of drug), coinciding with the essential time needed for neurogenesis, and this has been verified by ablating hippocampal neurogenesis by X-ray irradiation[8]. All the evidence suggests that neurogenesis is critical for antidepressant treatment, and depression is closely associated with impaired neurogenesis and neuroplasticity.

Numberous factors affect neuroplasticity in brain, which has a profound impact on the occurrence of depression. Insufficient signaling by neurotrophic factors has been recognized as a potential underlying factor for depression besides neurogenesis, and promotion of neurotrophin signaling has been linked with antidepressant responses in the last decades[9]. Brain-derived neurotrophic factor (BDNF) is a most abundant and widely distributed neurotrophin in brain, responsible for neuronal development, function, survival, and modulating neural and behavioral plasticity to injury[10]. Reduced BDNF levels were confirmed in the postmortem samples of brains from depressed patients[11, 12], as well as in suicide victims, many of which suffer from severe depression[13]. Moreover, the levels of both BDNF receptor TrkB and the active (phosphorylated) form of TrkB, were downregulated in the brains of depressed patients and suicide victims[9, 14], indicating that the BDNF signaling mediated through receptor TrkB reduced in depression. Increased BDNF/TrkB signaling by antidepressant treatment[15, 16], suggested that neurotrophins promote neuronal plasticity that then translates into antidepressant responses in depression patients[17], thus, this signaling pathway could become a useful tool for screening novel

antidepressant drugs.

Silymarin (flavonoid complex from *silybum marianum*--milk thistle seeds) has been used in China and Europe for thousands of years in patients suffering from liver diseases of different etiology[18, 19]. Silibinin (SLB), also known as silybin, is a natural polyphenolic flavonoid forming up to 80% of standardized extracts[20] in silymarin. Previous studies have suggested that the therapeutic effect of SLB is mainly related to antioxidation, radical-scavenging, immunoregulation and antifibrosis. However, the effectiveness of SLB in CNS diseases, especially in depression, is relatively unknown. In the present study, we evaluated the antidepressant effect of SLB in reserpinized mice and its possible mechanisms of action. Data demonstrated that SLB administration ameliorated depressive symptoms induced by reserpine insult against decreased BDNF/TrkB signaling transduction to improve hippocampal neurogenesis, and this effect was further confirmed using TrkB antagonist GNF5837. These results are suggestive of the involvement of neurogenesis through BDNF/TrkB signaling transduction in the rapid-acting antidepressant-like behavioral effects of SLB.

EXPERIMENTAL PROCEDURES

Materials

Reserpine (RESP), fluoxetine (FLXT), hoechst 33258, bromodeoxyuridine (BrdU), and antibody for β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for BrdU was obtained from Abcam (Cambridge, UK, USA), antibodies for BDNF and TrkB were obtained from BBI Life Sciences Corporation (Shanghai, China),

antibodies for phosphorylated extracellular-regulated protein kinase (p-ERK), total ERK, phosphorylated cAMP-response element binding protein (p-CREB), and total CREB were from Cell Signaling Technology (Danvers, MA, USA). All secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz (CA, USA). Alexa Fluro 594 goat IgG was purchased from Molecular Probes (Eugene, OR, USA). BCA Kit, M-PER Protein Extraction Buffer and enhanced chemiluminescent solution (ECL) were obtained from Pierce (Rockford, IL, USA). PVDF membrane was purchased from Roche (Mannheim, Germany). GNF5837 was purchased from Selleckchem (Shanghai, China) and dissolved in sterile 0.1% DMSO to desire concentrations before use. SLB (purity ≥ 98%) was purchased from Yuanye Biotechnology Company Limited (Shanghai, China). All chemicals were obtained from Sigma unless otherwise stated.

Acute depression model and drug treatment

Six- to eight-week-old male C57BL/6 mice (body weight 20-25 g) were obtained from the Fourth Military Medical University Experimental Animal Center (certificate No. 201000082, Grade II). Mice were housed in groups of 5-6 at room temperature (25 ± 1°C) with *ad libitum* access to food and water. The animals were housed with a 12-h light/dark cycle. All experimental procedures were approved by the Ethics Committee of Fourth Military Medical University. Every effort was made to minimize the number of animals used and their suffering. Mice were adapted to laboratory conditions for at least 2 d before the procedure. The model of acute depression was established by RESP (1 mg/kg) injected intraperitoneally (*i.p.*) once daily for 3 d [day (d) 1-d3] as described

previously. The mice received SLB (0, 100, 200, 400 mg/kg) or vehicle (saline, 10 ml/kg) by intragastric administration (*i.g.*) once daily for 3 d consecutively before RESP insult, then followed by an additional 4 d-administration (d4-d7). A selective serotonin reuptake inhibitor (SSRI), fluoxitine (20 mg/kg), served as a positive antidepressant. To determine the roles of BDNF/TrkB signaling transduction in antidepressant-like effects of SLB, GNF5837, an antagonist for receptor TrkB, was injected into intracerebroventricular (*i.c.v.*) 30 min before RESP insult. All experiments were performed at d 7 between 8:00 a.m and 2:00 p.m to minimize circadian influences after the last treatment. Animal depression-like behaviors were tested after habituation for 15 min in the testing room. The brain tissues were collected for immunohistochemstry staining and Western blot analysis after behavior tests.

Intracerebroventricular cannulation and microinjection

Male mice received an *i.p.* injection of a mixture of ketamine (30 mg/ml) and xylazine (3 mg/ml) and were mounted on a stereotaxic apparatus (RWD68001, Shenzhen Ruiwode Life Science, China). A 24-gauge guide cannula was bilaterally implanted into the cerebroventricle, and the stereotaxic coordinates for cerebroventricle were 0.3 mm posterior to Bregma, 1.2 mm lateral from the midline, and 2.5 mm beneath the skull surface. The mice were given at least 2 weeks to recover after cannula implantation. For *i.c.v.* injection, the animals were placed individually in an induction chamber, and anaesthesia was induced and maintained with 2.5% isoflurane (JiuPai, Shijiazhuang, China) in 100% oxygen with a delivery rate of 0.5 L min^{-1}. To block the effects of BDNF/TrkB involved in antidepressant-like effects of SLB, a TrkB antagonist

GNF5837 (1 µg in 1µl) or vehicle (saline, 1 µl) was bilaterally delivered using a syringe driven by an infusion pump (Harvard Apparatus, Inc., South Natick, MA), 30 min before RESP insult. After each infusion, the cannula was left in place for an additional 2 min to allow the solution to diffuse away from the cannula tip.

Behavioral Tests

Tail suspension test (TST)

Tail suspension test was performed in a quiet room as described previously[21]. Each mouse was suspended 50 cm above the floor and a small piece of adhesive taped to a wooden stick near the end of the mice tail about 2 cm, and the mice views were surrounded by a barrier. The mice tail test lasted 6 min, and the last 4 min was its immobility time. Mice were considered immobile when they showed hopelessness and were nearly immobile or completely motionless.

Forced swimming test (FST)

Forced swimming test (FST) is used to assess the desperate behavior and is one of the most widely used tests of antidepressant actions[22]. The forced swimming procedure was carried out according to the slightly modified method as previously described[23]. FST was conducted 30 min after the OFT. Mice were placed individually in a glass cylinder (height 25 cm, diameter 10 cm) filled with water to a depth of 11 cm at temperature 23-25°C and forced to swim for 6 min, the lighting level (lux) around the glass cylinders is ≤ 0.005 lux. After the initial 2 min of vigorous activity, the total duration of immobility was recorded during the last 4 min of the test. Immobility was

considered as maintaining floating passively, making no attempts to escape, with only minimal movements to keep their heads above the water. Water in the beakers was regularly changed between subjects. The immobility time was recorded by a trained observer with the help of cumulative stopwatches. Data obtained in groups were expressed as means (in s) ± the standard error of the mean (SEM).

Open field test (OFT)

To assess the effects of SLB on spontaneous locomotor activities, each mouse was placed into the center of an open field apparatus (50 × 50 × 60 cm) and allowed to explore freely around the open field. The light level < 50 lux was chosen because of little influence on mice behavioral. The number of crossings (squares crossed), and time spent in central area were recorded during a test period of 15 min. The paths of the animal were recorded by a video camera above the arena and analyzed by a video tracking system (DigBehav system, yishu Co., Ltd.). The arena was carefully cleaned with 70% alcohol and rinsed with water after each test[24]. Mice received behavior tests 30 min after SLB or FLXT treatment.

Bromodeoxyuridine incorporation and immunohistochemistry staining

Bromodeoxyuridine incorporation assay was used to evaluate NSC proliferation since BrdU is a thymidine analog that incorporates DNA of dividing cells during S-phase of cell cycle. BrdU (100 mg/kg) was injected *i.p.* once daily, 3 d consecutively. After the last behavioral test, the animals were anesthetized with chloral hydrate (3.5 mg/kg, *i.p.*) and transcardially perfused with 150 ml pre-cooled saline followed by 400 ml of 4%

paraformaldehyde solution. The brains were removed and post-fixed in paraformaldehyde for 24 h prior to being placed in a 15% sucrose solution overnight. The brains were transferred to a 30% sucrose solution and incubated for 72 h prior to embedding in optimal cutting temperature matrix. Thirty μm-thick coronal sections containing DG were collected and incubated in 30% H_2O_2 in methanol (1:50) for 30 min at 24°C to block endogenous peroxidase. After PBS rinses, sections were incubated in 0.1% Triton X-100 for 10 min at 4°C and then denatured by incubation in 2 N HCl for 30 min at 37°C. After washing in 0.1 M borate buffer (pH = 8.5) for 30 min, the sections were incubated with 3% BSA for 1 h. Sections were then incubated with primary antibody for BrdU at 4°C overnight followed by Alexa Fluor secondary antibody. Photomicrographs were obtained with a FluoView FV1000 microscope (Olympus, Japan). BrdU positive cells were separately counted in both SGZs of DG. Immunohistochemical controls were performed as above except for the omission of the primary antibodies.

Western blot analysis

Immediately after the last behavioral test from various treatments, mice were deeply anesthetized with 2% pentobarbital sodium and sacrificed, then the brains were removed and bilateral hippocampi were dissected. Total proteins were lysed by M-PER Protein Extraction Buffer according to the manufacturer's instructions and protein concentrations were determined using a BCA Kit. Equal amounts of protein aliquots were analyzed *via* sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to check the expression levels of BDNF, TrkB, p-ERK, ERK, p-CREB and

CREB, β-actin served as a loading control. The dilutions for primary antibodies were used as followings: BDNF (1:500), TrkB (1:500), p-ERK (1:1000), ERK (1:1000), p-CREB (1:1000), CREB (1:1000) and β-actin (1:10,000). The target protein signal was detected and digitized using ECL solution and Image J program. The band intensity of each blot was calculated as ratio relative to respective total proteins and β-actin.

Statistical analysis

Results were presented as the mean and SEM (mean ± SEM). The data were analyzed by one way ANOVA followed by a *post hoc* Tukey test to compare the various groups. In all cases, $p < 0.05$ was considered statistically significant. All statistical analysis was performed using GraphPad Prism 7.03 and SPSS statistical software package version 17.0.

Results

Antidepressant effects of SLB treatment on RESP-induced depression mice

Reserpine, a monoamine reuptake blocker, depletes monoamines in the brain, resulting in symptoms of major depression in both humans and rodents[25, 26]. Reserpinized mouse is a well-accepted model of acute depression to evaluate the antidepressants. In this study, we found *i.p.* injection of RESP (1 mg/kg) for 3 d induced behavioral despair in mice, characterized as an increase in total immobility duration (200.7 ± 13.6 s) compared with control group (Fig. 1B, 97.1 ± 7.3 s, $p < 0.01$) in TST, and increased immobility time (189.2 ± 10.2 s) compared with control group (Fig. 1C, 108.1 ± 11.4 s, $p < 0.01$) in FST, indicating that RESP injection could lead

to depression-like behaviors of mice. We further investigated the potential antidepressant effects of SLB, and the results showed that SLB administration ameliorated depression-like behaviors by reducing the immobility duration (Fig. 1B, 141.3 ± 11.2 s in 200 mg/kg group, 139.5 ± 15.1 s in 400 mg/kg group, $p < 0.05$ vs. RESP insult group) in TST, as well as decreasing immobility time in FST (Fig. 1C, 142.1 ± 10.8 s in 200 mg/kg group, 138.3 ± 13.4 s in 400 mg/kg, $p < 0.05$ vs. RESP insult group) in reserpinized mice. These behavioral data are consistent with Liu B's report, in which SLB could ameliorate depression-like behaviors[21]. There were no behavioral differences observed in control group after SLB treatment (Fig. 1B, C). FLXT (20 mg/kg), a classic SSRI, served as a positive antidepressant and reduced the immobility time of reserpinized mice to 123.7 ± 14.2 s in TST, 128.2 ± 13.1 s in FST as expected (Fig. 1B, C, $p < 0.01$ vs. RESP insult group).

At the same time, spontaneous locomotor activity of mice from each group was determined in open field. Reserpinized mice showed depressive symptoms as evidenced by decreased locomotor activity, the total distance in RESP insult group decreased to 10.9 ± 3.1 m compared with 36.8 ± 1.8 m in control group (Fig. 1D, $p < 0.01$). Accordingly, time spent in the central area in reserpinized mice reduced to 17.4 ± 4.4 s compared with 73.4 ± 5.6 s in control mice (Fig. 1E, $p < 0.01$). SLB administration increased the total distance to 24.6 ± 1.9 m in 200 mg/kg group, 25.5 ± 2.2 in 400 mg/kg group (Fig. 1D $p < 0.05$ vs. RESP insult group); and the time spent in the central area increased to 53.7 ± 3.1 s in 200 mg/kg group, 56.5 ± 4.9 s in 400 mg/kg group after SLB treatment (Fig. 1E, $p < 0.05$ vs. RESP insult group). While,

no significant differences were observed in total distance and time spent in central area in control mice treated with SLB (100, 200, 400 mg/kg) groups (Fig. 1D, E).

SLB administration blocked/rescued the impaired NSC proliferation in reserpinized hippocampus

Impaired hippocampal neurogenesis contributes to the etiology of depression, and antidepressant like FLXT benefits patients due to the rescued neurogenesis. We found that SLB ameliorated the depression-like behaviors in reserpinized mice, then, we evaluated the ability of SLB to recover the impaired neurogenesis in RESP-induced depression model. NSC proliferation incorporated by BrdU was observed in DG region of Hipp 7 d post RESP insult. The number of proliferating NSCs labeled as BrdU positive decreased to 2000 ± 243 in DG after RESP injection compared with 4000 ± 475 in control group (Fig. 2A-D and M, $p < 0.01$ vs. control group), while SLB treatment recovered it to 3750 ± 465 (Fig. 2E-F and M, $p < 0.01$ vs. RESP insult group), which was similar to FLXT-treated reserpinized mice (3800 ± 479, Fig. 2G-H and M, $p < 0.01$ vs. RESP insult group), suggesting that SLB rescued the impaired NSC proliferation in reserpinized DG. These data indicated that SLB could reverse the decreased hippocampal neurogenesis induced by RESP insult, thus, providing antidepressant effects.

SLB administration reversed the impaired BDNF/TrkB signaling transduction

Insufficient or impaired signaling transducted by BDNF/TrkB is an underlying critical mechanism responsible for depression development besides neurogenesis. We further

investigated the changes of BDNF/TrkB signaling upon SLB stimulation in RESP-induced acute depression. Western blot results showed that RESP insult robustly reduced the expression levels of BDNF (Fig. 3A-B, 66% ± 5.5% of control group, $p < 0.01$ vs. control group) and TrkB (Fig. 3C-D, 68% ± 1.6% of control group, $p < 0.01$ vs. control group) in hippocampus; while the levels of hippocampal BDNF (Fig. 3A-B, 93% ± 1.8% of control group, $p < 0.01$ vs. RESP insult group) and TrkB (Fig. 3C-D, 91% ± 3.9% of control group, $p < 0.01$ vs. RESP insult group) increased after SLB treatment (200 mg/kg) for 6 d, which was very similar to FLXT administration (Fig. 3A-D, 92% ± 2.4% and 95% ± 2.1% of control group, $p < 0.01$ vs. RESP insult group). This result indicated that SLB administration blocked RESP-induced decrease in BDNF/TrkB signaling.

To address whether BDNF/TrkB signaling was effectively transducted, we further checked the activation of ERK (phosphorylated, p-ERK), an important downstream signaling target of BDNF/TrkB. A significant decrease of p-ERK was observed in reserpinized hippocampus by Western assay (Fig. 3E-F, 69.8% ± 7.6% of control group, $p < 0.01$ vs. control group), while SLB treatment recovered p-ERK level to 95.9% ± 2.5% of control group (Fig. 3E-F, $p < 0.01$ vs. RESP insult group) which was pretty comparative to that of FLXT-treated group. At the same time, we checked the changes of activated CREB (phosphorylated, p-CREB), a critical transcription factor responsible for BDNF expression. We found that RESP insult led to a significant decrease of hippocampal p-CREB level (Fig. 3G-H, 62% ± 6.2% of control group, $p < 0.01$ vs. control group), and p-CREB levels increased to 92.9% ± 1.1% of control

group upon SLB stimulation ($p < 0.01$ vs. RESP insult group), 101.7% ± 1.8% of control group after FLXT administration ($p < 0.01$, Fig. 3G, H). The levels of total ERK and CREB were not altered in each group (Fig. 3E-H, $p > 0.05$). Taken together, improved BDNF/TrkB, up- and down-stream signaling pathways were involved in antidepressant-like effects of SLB effects.

TrkB inhibitor GNF5837 abolished the recoveries of p-ERK and p-CREB mediated by SLB treatment

To check whether the activation ERK and CREB was mediated by BDNF/TrkB upon SLB administration, reserpinized mice were pretreated with TrkB inhibitor, GNF5837 (1 μg for each, *i.c.v.*), with or without SLB treatment. The rescued p-ERK1/2 (Fig. 4B, C) and p-CREB (Fig. 4D, E) upon SLB stimulation were blocked in reserpinized hippocampus pretreated with GNF5837. While, the total levels of ERK and CREB were kept unchanged in each group (Fig. 4B-E). More importantly, GNF5837 pretreatment significantly blocked the effects of SLB on improved NSC proliferation evaluated by BrdU incoporation. Data showed that BrdU positive cells decreased to 1900 ± 256 in RESP + SLB + GNF5837 group (Fig. 2I-J and M, $p < 0.01$ vs. RESP + SLB group), while GNF5837 treatment alone did not affect the numbers of proliferating NSCs in RESP insult group (Fig. 2K-L and M, 1880 ± 228, $p > 0.05$ vs. RESP group). The proliferation analysis indicated that SLB treatment reversed the impaired neurogenesis upon RESP insult, and GNF5837 blocked the proliferative effects of SLB in DG. Taken together, these data indicated that the antidepressant effects of SLB may through BDNF/TrkB pathway.

SLB ameliorated depression-like behaviors depending on BDNF/TrkB signaling transduction

To further investigate that BDNF/TrkB is responsible for antidepressant-like effects of SLB, reserpinized mice were pretreated with TrkB inhibitor, GNF5837 (1 μg for each, *i.c.v.*), with or without SLB treatment. We found that antidepressant-like effects of SLB effects in reserpinized mice, characterized as reduced immobility time (Fig. 5A, 122.2 ± 17.2 s, $p < 0.01$ vs. RESP insult group) in TST, was prevented by GNF5837 pretreatment (Fig. 5A, 193.5 ± 12.1 s, $p < 0.01$ vs. RESP + SLB group). SLB-mediated decreased immobility duration (Fig. 5B, 132.7 ± 14.1 s) in reserpinized mice was also significantly prevented by GNF5837 pretreatment (Fig. 5B, 169.7 ± 15.8 s, $p < 0.01$ vs. RESP + SLB group) in FST. There was no difference between RESP insult group and RESP + GNF5837 with or without SLB treatment.

At the same time, the spontaneous locomotor activities of mice pretreated with GNF5837 were also evaluated. Data indicated that GNF5837 pretreatment abolished SLB-mediated increase of the total distances in reserpinized mice (Fig. 5C, 9.1 ± 2.7 m, $p < 0.01$ vs. 28.9 ± 2.8 m in RESP + SLB group), GNF5837 alone didn't affect that of reserpinized mice (Fig. 5C, 10.1 ± 2.8 m). Also, the enhanced time spent in the central area in SLB-treated reserpinized mice (Fig. 5D, 47.1 ± 5.6 s) was reduced after GNF5837 pretreatment (Fig. 5D, 13.5 ± 3.4 s) using OFT, GNF5837 alone didn't affect the central time of reserpinized mice (Fig. 5D, 15.4 ± 2.9 s, $p > 0.05$ vs. 17.4 ± 1.7 s in RESP insult group). Collectively, antidepressant-like effects of SLB in reserpinized mice were blocked by TrkB inhibitor GNF5837, indicating BDNF/TrkB may be

responsible for antidepressant-like effects of SLB.

Discussion

We employed an acute depression mouse model induced by RESP insult and analyzed the antidepressant effects of SLB, one of the active ingredients of silybinisus, and the potential mechanisms involved in this study. We observed that SLB administration ameliorated depression-like behaviors in reserpinized mice, restored the hippocampal neurogenesis, and the expression levels of BDNF/TrkB and related signaling proteins including p-ERK and p-CREB. While, TrkB antagonist GNF5837 blocked antidepressant-like effects of SLB and the recoveries of neurogenesis, as well as p-ERK and p-CREB in reserpinized mice, indicating that antidepressant-like effects of SLB was through BDNF/TrkB signaling pathway. These results provided evidence the underlying mechanism of antidepressant-like effects of SLB appeared to involve the neurogenesis through BDNF/TrkB.

There is an accumulating body of evidence linking impaired neurogenesis and insufficient signaling of neurotrophic factors to the development of depression. Studies revealed that classic antidepressant drugs benefit depression patients partially by improving neurogenesis and/or BDNF/TrkB pathway. BDNF is a most abundant and widely distributed neurotrophin in the brain which plays critical roles in neuronal development, function, and survival and modulates neural and behavioral plasticity to injury[27]. Reduced levels of BDNF mRNA and protein were shown in the brains of Alzheimer’s disease patients[28] and Parkinson’s disease patients[29] as well as in

depression[15]. Neurons and astrocytes are responsible for BDNF production in the brain[30]. BDNF expression was upregulated by antidepressant treatment[15, 31], suggesting that BDNF contributes to therapeutic action.

Neurogenesis is a process of new-born neurons including NSC proliferation, migration, differentiation into new neurons, and integration into existing neuronal circuits. Neurogenesis is an important form of neuroplasticity and involved in learning and memory[32], and disrupted neurogenesis is implicated in depression. Therefore, it is important for the recovery of neurogenesis to correct despair behaviors. In this study, we observed the depressed behavior was correlated with reduction of NSC proliferation labeled as BrdU positive in reserpinized mice (Fig. 2). Moreover, SLB administration ameliorated the depression-like behaviors (Fig. 1) which was associated with the improved NSC proliferation (Fig. 2), indicating that the antidepressant effect of SLB maybe through restoring neurogenesis.

Evidence also showed that the microenvironment in the neurogenic niche, especially SVZ and SGZ of the hippocampus[33], is important for neurogenesis and is mediated by a range of critical factors, including the oxygen supply, nutrition, hormones, and trophic factors. Lots of detrimental changes induced by depression challenge resulted in unhealthy microenvironment for neurogenesis. Accordingly, it is also critical to improve neurogenesis by correcting the detrimental microenvironment. Silibinin is a natural polyphenolic flavonoid that constitutes the major active component in silymarin, which is isolated from the *Silybum marianum* (milk thistle). The exact target or receptor in CNS for SLB is not clear up to date. Previous studies have suggested that the

therapeutic effect of SLB is mainly related to antioxidation, radical-scavenging, immunoregulation and antifibrosis. Silibinin attenuated Aβ-induced memory impairment in mice through ameliorating inflammatory response and oxidative stress[34, 35], and thus improved behavioral abnormalities in Alzheimer's disease (AD) model. Our previous studies also showed that SLB protected hippocampal neurons from cerebral ischemia by inhibiting both the mitochondrial and autophagic cell death pathways[36]. The beneficial effects of SLB for CNS disorders treatment is based on its bioavailability. The concentration of silibinin in cerebrospinal fluid was detected by HPLC analysis, and the data showed that SLB could pass BBB effectively, therefore, SLB could exert the protective effects in CNS directly. Of course, more researches need to be carried out to investigate the exact target or receptor for SLB in future.

BDNF/TrkB signaling provides beneficial microenvironment for neuronal survival, neurodevelopment, and neurogenesis. Thus, we hypothesized that one of underlying mechanisms of antidepressant-like effects of SLB may be due to its capacity to restore BDNF/TrkB signaling under depression status. Data showed in this study that BDNF/TrkB pathway was impaired upon RESP insult, and SLB treatment restored the expression levels of BDNF/TrkB (Fig 3A-D). At the same time, we found the required downstream of BDNF/TrkB, phosphorylation of ERK and CREB was impaired in reserpinized hippocampus, while SLB administration corrected the reduced expression levels of p-ERK and p-CREB. Moreover, GNF5837, a TrkB antagonist pretreatment blocked antidepressant-like effects of SLB (Fig 5) and recoveries of neurogenesis (Fig 2), as well as p-ERK and p-CREB (Fig 4) in reserpinized mice, suggesting

antidepressant-like effects of SLB was through BDNF/TrkB signaling pathway.

Taken together, the present study indicates that SLB might exert antidepressant effects which appeared to involve the neurogenesis through BDNF/TrkB. These findings have partially revealed the molecular mechanisms underlying antidepressant of SLB. However, we could not exclude the possibility that SLB may undergo antioxidation and/or antiinflammation activities through other pathways. Other supportive experiments need to be performed for further investigation. The present results, along with other studies, might broaden the application of SLB in the treatment, mitigation mental disorder and disorders involving BDNF/TrkB pathway.

Abbreviations: MDD Major depressive disorder; CNS Central nervous system; BDNF Brain-derived neurotrophic factor; OFT Open field test; FST Forced swim test; 5-HT 5-hydroxytryptamine (serotonin); SSRIs Selective serotonin reuptake inhibitors; BrdU Bromodeoxyuridine; *i.p.* intraperitoneally; *i.g.* intragastric administration; *i.c.v.* intracerebroventricular; PVDF Polyvinylidene difluoride; TBST Tris-buffered saline and Tween-20; SEM Standard error of the mean; ANOVA Analysis of variance.

Author Contributions

Associate professor Yu-Mei Wu designed the study and wrote the protocol. Professor Ming-Gao Zhao, Wen-Min Niu, Dr. Yu-Jiao Li and Master Yan-Jiao Li managed the literature searches. Dr. Kun Zhang and Dr. Qi Yang managed the statistical analyses. Master Yan-Jiao Li, Liu-Di Yang, Kai-Yin Zheng and Xin-Miao Wei performed animal model experiments. All authors have approved the final manuscript.

Funding Sources

This work was financially supported by National Natural Science Foundation of China (No. 81571328 and No. 81371322 for Prof Wu), and Research Foundation from Social development science and technology project of Shaanxi Province (2016SF-186).

Conflict Of Interest Statement

The authors declare no competing financial interest.

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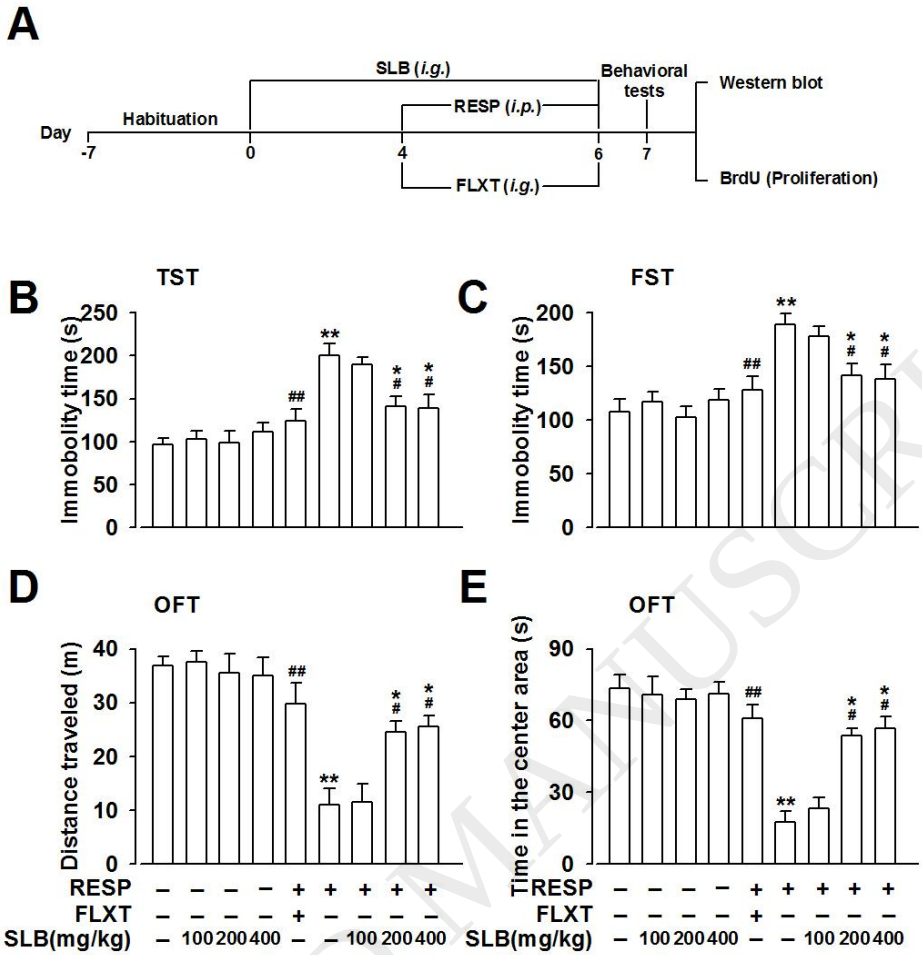
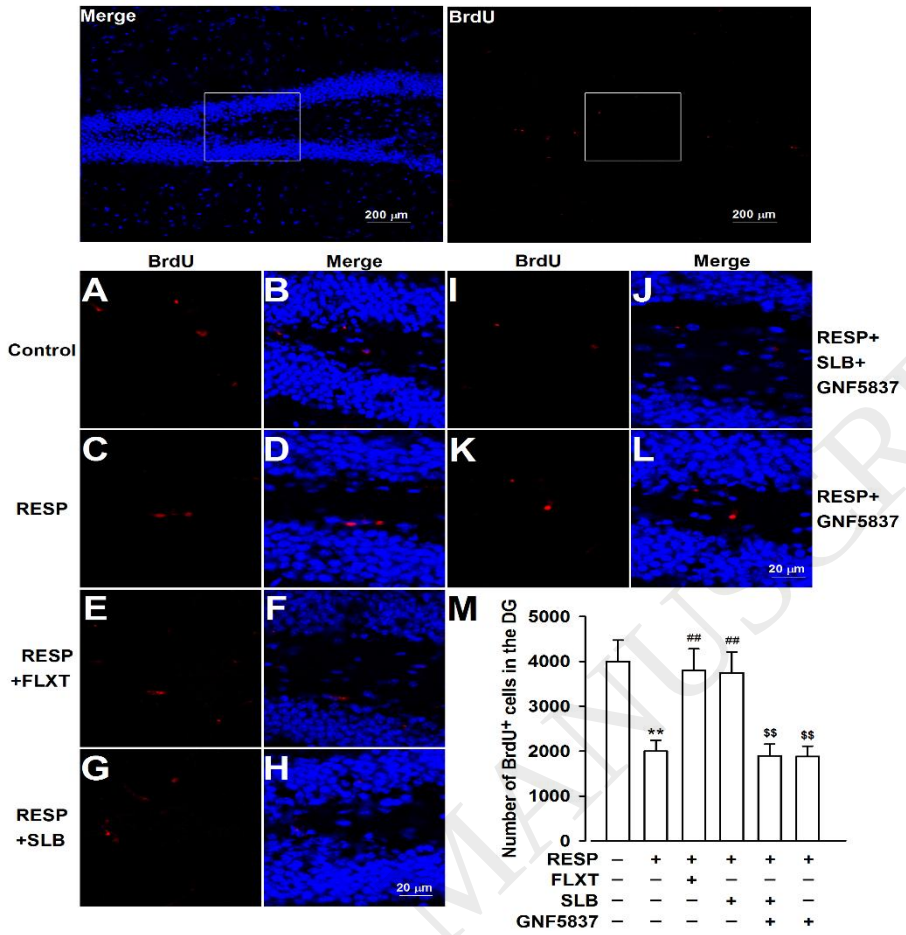
Figure 1



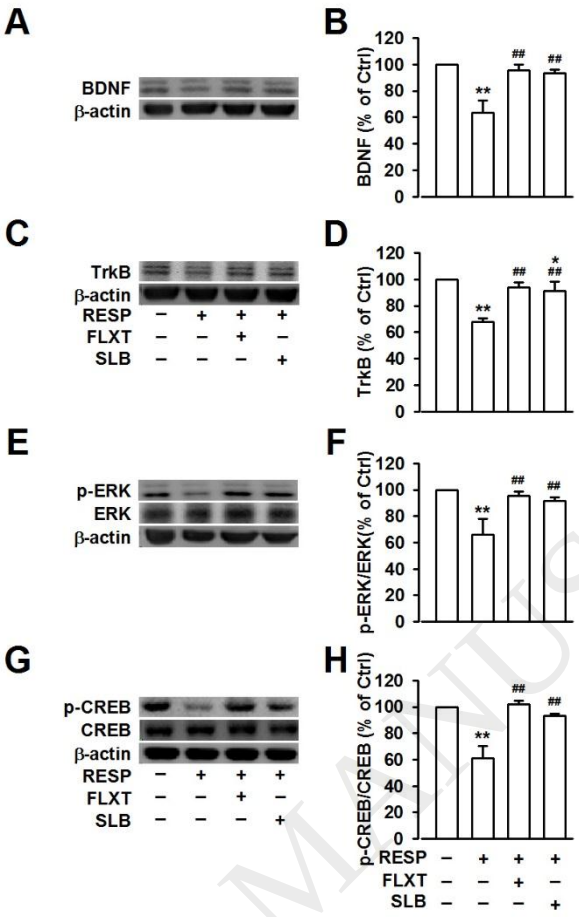
Silibinin (SLB) administration ameliorated depression-like behaviors in reserpinized mice. (A) Schematic illustration of the procedure for the experiment (detailed description in the Materials and Methods part). (B) The immobility time for control or reserpine (RESP)-insulted mice treated with or without SLB (0, 100, 200, 400 mg/kg, *i.g.*) for 7 d, and fluoxetine (FLXT, 20 mg/kg) treatment served as positive antidepressant in forced swimming test (FST). (C) The immobility time for each group in tail suspension test (TST). (D) The total distance and (E) Time in the central area in each group in open field test (OFT). Each value represents the mean ± SEM of three independent experiments (n = 6, $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus RESP insult mice).

Figure 2



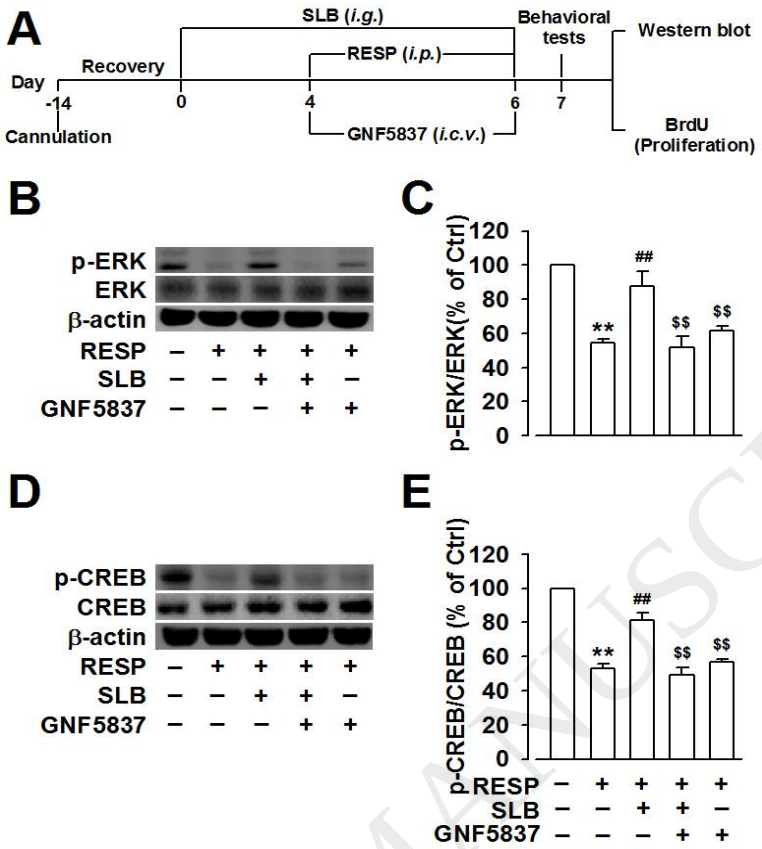
Silibinin (SLB) administration reversed the RESP-induced suppression of hippocampal neurogenesis. (A-L) The proliferating neural stem cells (NSCs) in dentate gyrus were stained after bromodeoxyuridine (BrdU) incorporating from control or reserpine (RESP) insult mice treated with or without SLB (0, 200 mg/kg, *i.g.*) for 7 d, and fluoxetine (FLXT, 20 mg/kg) treatment served as positive control; for GNF5837 (a TrkB antagonist) pretreatment, reserpinized mice were pretreated with or without GNF5837 (1 μg in 1 μl) injection into cerebroventricle (*i.c.v.*) 30 min before RESP insult followed by SLB administration. BrdU showed in red and total cells were labeled with Hoechst in blue. (M) The number of BrdU incorporation in NSCs was determined in each group. Each value represents the mean ± SEM of three independent experiments (n = 6, $^{**}p < 0.01$ versus control group; $^{\#\#}p < 0.01$ versus RESP insult mice; $^{\$\$}p < 0.01$ versus SLB-treated reserpinized mice).

Figure 3



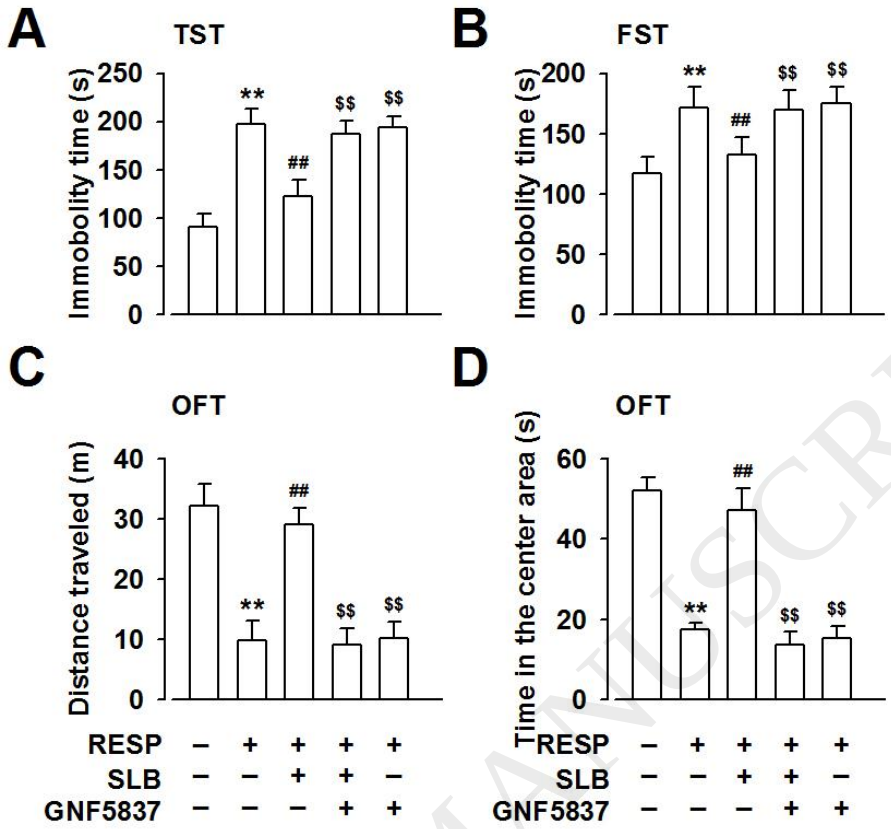
Silibinin (SLB) administration reversed the impaired BDNF/TrkB signaling transduction upon RESP insult. Control and reserpine (RESP) insulted mice treated with or without SLB (0, 200 mg/kg, *i.g.*) for 7 d, and fluoxetine (FLXT, 20 mg/kg) treatment served as positive control. The expression levels of (A) BDNF, (C) TrkB, (E) the activation (phosphorylation) of ERK (p-ERK), and (G) phosphorylation CREB (p-CREB) in hippocampus from each group were determined by Western blot, β-actin served as a loading control. (B, D, F, H) Summary of the expression levels of BDNF, TrkB, p-ERK and p-CREB. Each value represents the mean ± SEM of three independent experiments (n = 6, $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group; $^{\#\#}p < 0.01$ versus RESP insult mice).

Figure 4



TrkB inhibitor GNF5837 abolished the recoveries of p-ERK and p-CREB mediated by Silibinin (SLB). (A) Schematic illustration of the procedure for the experiment (detailed description in the Materials and Methods part). Control and reserpine (RESP)-insulted mice treated with or without SLB (0, 200 mg/kg, *i.g.*) for 7 d. For GNF5837 (a TrkB antagonist) pretreatment, reserpinized mice were pretreated with or without GNF5837 (1 μg in 1μl) injection into cerebroventricle (*i.c.v.*) 30 min before RESP insult followed by SLB administration. The expression levels of (B) the activation (phosphorylation) of ERK (p-ERK), and (D) phosphorylation CREB (p-CREB) in hippocampus from each group were determined by Western blot, β-actin served as a loading control. (C, E) Summary of the expression levels of p-ERK and p-CREB. Each value represents the mean ± SEM of three independent experiments (n = 6, $^{**}p < 0.01$ versus control group; $^{\#\#}p < 0.01$ versus RESP insult mice; $^{\$\$}p < 0.01$ versus SLB-treated reserpinized mice).

Figure 5



Silibinin (SLB) ameliorated depression-like behaviors in reserpinized-mice through BDNF/TrkB signaling transduction. (A) The immobility time for control and reserpine (RESP)-insulted mice treated with or without SLB (0, 200 mg/kg, *i.g.*) for 7 d, and fluoxetine (FLXT, 20 mg/kg) treatment served as positive control in forced swiming test (FST); for GNF5837 (a TrkB antagonist) pretreatment, reserpinized mice were pretreated with or without GNF5837 (1 μg in 1μl) injection into cerebroventricle (*i.c.v.*) 30 min before RESP insult followed by SLB administration. (B) The immobility time for each group in tail suspension test (TST). (C) The total distance and (D) Time in the central area in each group in open field test (OFT). Each value represents the mean ± SEM of three independent experiments (n = 6, $^{**}p < 0.01$ versus control group; $^{\#\#}p < 0.01$ versus RESP insult mice; $^{\$\$}p < 0.01$ versus SLB-treated reserpinized mice).