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Synaptic Plasticity, But Not Hippocampal Neurogenesis, Mediated the Counteractive Effect of Wolfberry on Depression in Rats¹

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Depression is a life-threatening psychiatric disorder characterized with a long-term hypercortisolemia in depressed patients. Based on this clinical feature, hypercortisolemia was mimicked in experimental animals to understand the neuropathology of depression and to explore new therapeutic strategies. Wolfberry, also known as *Lycium barbarum*, is a type of common fruit produced in mainland China. Accumulated evidence has shown that the extracts from *Lycium barbarum* (LBP) had a wide range of neuroprotective effects in various neurogenerative models. However, the antidepressant effect of LBP on depression and its mechanism has not yet been explored. In the present study, we investigated the effects of LBP on counteracting depression using an animal model injected with moderate dose (40 mg/kg) or severe dose (50 mg/kg) of corticosterone (CORT) treatments for 14 days. The results showed that CORT significantly increased immobility time and decreased hippocampal cell proliferation. LBP treatment significantly decreased the immobility time in forced swimming test, a test for the intensity of depressive behaviors, both in 40 and 50 mg/kg CORT stressed rats. Moreover, LBP treatment restored the reduced proliferation of neuroprogenitor cells in the hippocampus in 40 mg/kg CORT stressed rats and the neuronal differentiation but not the proliferation in 50 mg/kg CORT stressed rats. After ablation of adult neurogenesis with Ara-c infusion, the beneficial effect of LBP treatment in reducing immobility time was not affected in 40 and 50 mg/kg CORT stressed rats. Golgi staining and Western blotting detection showed that LBP treatment restored the reduced spine density and the decreased level of PSD-95 in the hippocampus caused by 40 and 50 mg/kg CORT, respectively, indicating enhanced synaptic plasticity in the hippocampus. The data showed a novel effect of LBP on reducing depression-like behavior and its antidepressant effect may be mediated by enhanced synaptic plasticity, but not hippocampal neurogenesis.

Key words: Wolfberry; Depression; Corticosterone (CORT); Neurogenesis; Spine density

INTRODUCTION

Depression is a life-threatening psychological disorder worldwide. A large number of clinical observations have shown that a long-term hypercortisolemia existed in the depression patients (2). Based on this clinical feature, repeated corticosterone (CORT, the counterpart hormone of cortisol) injection in rodents has been developed as a preclinical model of depression since this model induces behavioral and neurological changes that correspond to

some core symptoms and brain changes in depression patients (33). It has been shown that chronic exposure to CORT was able to induce “depressive-like” behaviors in several anxiety/depression tests including reduced sucrose consumption (13), elongation of immobility in forced swimming test (FST) (16,40), and in tail suspension test (40). Furthermore, CORT treatment impaired adult neurogenesis in the dentate gyrus and the apical dendritic morphology of pyramidal neurons in the CA3

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(24,36) and the CA1 subfield of hippocampus (1). These observations indicate that the reduced neurogenesis and damaged morphology in pyramidal cells in the hippocampus is associated with the occurrence of depression-like behaviors, which are equivalent to the clinical depressive symptoms. Conversely, numerous studies have shown that administration of antidepressant drugs reduced depression-like behaviors (10,13), restored neurogenesis in the dentate gyrus (30), and induced morphological changes in the apical dendrites and in spine density in pyramidal cells in adult hippocampus (10,35). Therefore, increased hippocampal neurogenesis and the restored neuronal morphology may be involved in the recovery of the experimental depression.

Wolfberry, also known as *Lycium barbarum*, has long been considered to be an antiaging drug in traditional Chinese medicine. One of its active components, polysaccharides in *Lycium barbarum* (LBP), had been shown to improve cognitive functions in the intact hippocampus (28). Furthermore, peritoneal injection of LBP significantly reduced the score of neurological symptoms and stroke index assessment in a mice ischemia–reperfusion model (32). The results suggest that LBP might improve the cerebral recovery against an ischemic challenge. In our laboratory, we have shown that oral feeding of LBP exhibited neuroprotective effects on retinal ganglion cells in a rat model of glaucoma (7,9). Our collaborative study has also shown that wolfberry can protect neurons against acute ischemia (21). Based on the above studies, it is reasonable to investigate the neuroprotective effects of wolfberry in depression. However, the antidepressant effect of LBP has not yet been reported by others so far.

Our previous findings have shown that voluntary wheel running exerted antidepressant effect (i.e., decreased depression-like behavior that associated with restored hippocampal neurogenesis and increased dendritic plasticity) on rats treated with moderate dose (40 mg/kg) of CORT (38). However, it failed to elicit antidepressant effects on rats with severe dose (50 mg/kg). The results indicated that repeated injection of CORT at different doses lead to different depressive stages in rats. In view of the neuroprotective effects of wolfberry, we therefore tested the antidepressant effects of LBP on the performance in FST in depressed rats induced by 40 and 50 mg/kg CORT treatment and investigated the possible molecular mechanisms underlying the neuroprotective effects of LBP.

MATERIALS AND METHODS

Animals and Experimental Grouping

Animals. Adult male Sprague–Dawley (SD) rats weighing 230–250 g were provided by Laboratory Animal Unit, the University of Hong Kong. All experimental animals

were kept in a group of three and raised on 12-h light–dark cycle. Rats were free to obtain food and water and allowed to have 24-h adaptation before the beginning of experiments. All procedures and animal handling were carried out according to guidelines set by the Committee on the Use of Live Animals in Teaching and Research (CULATR), the University of Hong Kong.

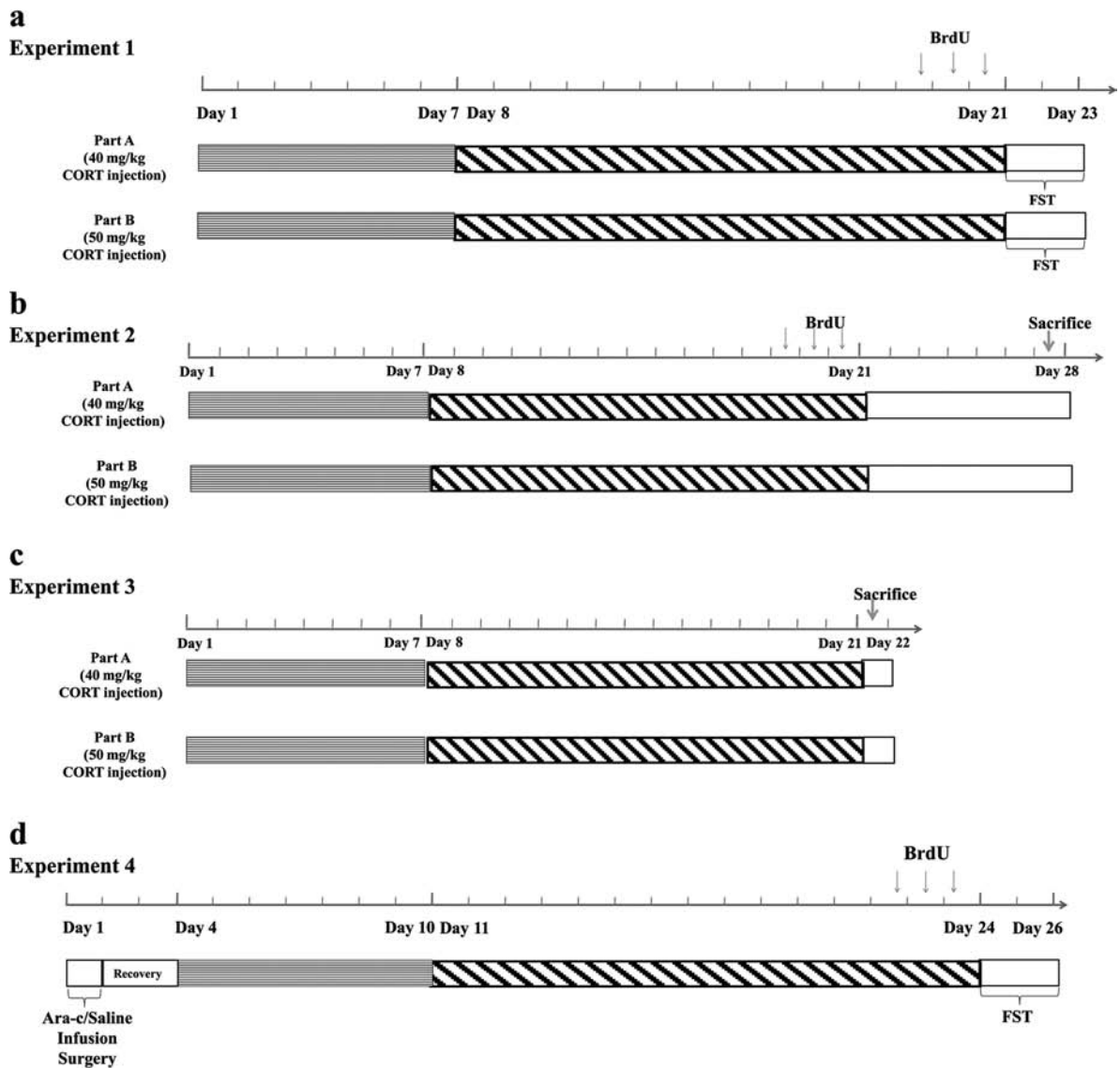
Experimental Grouping. All the experimental animals were divided into four separate experiments (shown in Fig. 1):

In experiment 1 (Fig. 1a), the rats were pretreated with 1 mg/kg LBP in 0.01 M PBS or the equivalent volume 0.01 M PBS prior to the 14-day cotreatment with CORT or vehicle (sesame oil). Thus, all rats were basically divided into four groups: (i) 7-day oral feeding of 0.01 M PBS followed by 14-day oral feeding of PBS plus subcutaneous injection of vehicle; (ii) 7-day oral feeding of PBS followed by 14-day oral feeding of PBS plus subcutaneous daily injection of CORT; (iii) 7-day oral feeding of LBP followed by 14-day oral feeding of LBP plus subcutaneous injection of vehicle; and (iv) 7-day oral feeding of LBP followed by 14-day oral feeding of LBP plus subcutaneous injection of CORT. According to the doses of CORT injection, all rats were injected with either 40 mg/kg CORT in part A or 50 mg/kg CORT in part B. All animals were subjected to forced swimming test individually 24 h after the last injection of CORT. One day after the end of FST, all animals were killed and the brains were dissected out for immunostaining.

In experiment 2, the grouping was the same as in experiment 1. Rats were allowed to survive for 7 days after the last injection of bromodeoxyuridine (BrdU) and euthanatized on day 28 (Fig. 1b).

In experiment 3, the grouping was the same as in experiment 1. However, rats received no BrdU injection and were euthanatized right after the last injection of CORT. Fresh brains were quickly removed. One hemisphere of each brain was used for Golgi staining; the other hemisphere was used for Western blot analysis (Fig. 1c).

In experiment 4, 42 adult male SD rats (weighed 230 ± 20 g) were used and randomly grouped as shown in Figure 1d: (i) animals received saline infusion, 21-day oral feeding with 0.01 M PBS, and 14-day subcutaneous injection of sesame oil ($n=6$); (ii) animals received saline infusion, 21-day oral feeding with 0.01 MPBS, and 14-day subcutaneous injection of 40 mg/kg CORT ($n=6$); (iii) animals received saline infusion, 21-day oral feeding with 1 mg/kg LBP, and 14-day subcutaneous injection of 40 mg/kg CORT ($n=6$); (iv) animals received cytosine-b-d-arabinofuranoside (Ara-c) infusion, 21-day oral feeding with 1 mg/kg LBP, and 14-day subcutaneous injection of 40 mg/kg CORT ($n=6$); (v) animals received saline infusion, 21-day oral feeding of 0.01 M PBS, and



Animal grouping (abbrev. in name):

- Group 1 (Sa+PBS): Saline infusion + 0.01 M PBS + Sesame oil injection;
- Group 2 (Sa+PBS+40CORT): Saline infusion + 0.01 M PBS + 40 mg/kg CORT injection;
- Group 3 (Sa+LBP+40CORT): Saline infusion + 1 mg/kg LBP + 40 mg/kg CORT injection;
- Group 4 (Ara-c+LBP+40CORT): Ara-c infusion + 1 mg/kg LBP + 40 mg/kg CORT injection
- Group 5 (Sa+PBS+50CORT): Saline infusion + 0.01 M PBS + 50 mg/kg CORT injection;
- Group 6 (Sa+LBP+50CORT): Saline infusion + 1 mg/kg LBP + 50 mg/kg CORT injection;
- Group 7 (Ara-c+LBP+50CORT): Ara-c infusion + 1 mg/kg LBP + 40 mg/kg CORT injection.

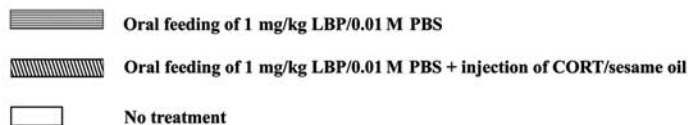


Figure 1. A schematic illumination of the experimental design. FST, forced swimming test; Ara-c, cytosine-b-d-arabinofuranoside; LBP, *Lycium barbarum*; CORT, corticosterone; PBS, phosphate buffered saline.

14-day subcutaneous injection of 50 mg/kg CORT ($n=6$); (vi) animals received saline infusion, 21-day oral feeding with 1 mg/kg LBP, and 14-day subcutaneous injection of 50 mg/kg CORT ($n=6$); (vii) animals received Ara-c infusion, 21-day oral feeding with 1 mg/kg LBP, and 14-day subcutaneous injection of 50 mg/kg CORT ($n=6$). Twenty-four hours after the last injection of CORT or sesame oil, all animals were subjected to FST.

Preparation of Corticosterone and Water Extracts of Polysaccharides From LBP

Corticosterone (Sigma-Aldrich, St. Louis, MO, USA) was suspended in sesame oil as previously described (20,38) and then subcutaneously injected into the neck region of each animal at a dose of 40 or 50 mg/kg daily. A quick and mild massage was done on the injection site to prevent the leakage of CORT following every injection.

The fruit of LBP was collected from NingXia Huizu Autonomous Region, the People's Republic of China. The water extracts of LBP were prepared from the dried fruit of LBP as previously described (39). Briefly, the dried fruit of LBP was grounded into small pieces with a grinder and then extracted twice with distilled hot water for 3 h. The combined extracts were concentrated and deproteinated. The resulting aqueous fractions were extensively dialyzed against running distilled water for 2 days. The retentate was concentrated to 400 ml under reduced pressure and precipitated by addition of three volumes of 95% ethanol. After centrifugation and washing with acetone, the resulting precipitate was vacuum-dried at 40°C to yield a final brown powder product. Generally, 1.7 g powder extracts could be achieved from every 100 g dried fruit. The polysaccharide concentration of the LBP extract was found to be 72% (39). The ready to use powder extracts was then dissolved in 0.01 M PBS. All animals were orally fed with LBP at 1 mg/kg dose daily or the same volume of 0.01 M PBS, starting 7 days prior to subcutaneous injection of CORT.

FST

Forced swimming test is a general behavior test to detect the "learned helplessness" in rodents, initially introduced by Porsolt and coworkers (29). Briefly, this test consists of two sessions with 24-h interval. On the first day, every subject was placed into a transparent plastic cylinder (height: 60 cm; diameter: 25 cm) half filled with warm tap water (25°C) for 15 min as a pretest session. Twenty-four hours later, subjects were placed into the cylinder again for 5 min. This part was the test session. All the activities in the test session were recorded by a video camera and scored in a blinded manner by a trained observer. Three types of behaviors are defined as follows:

Struggling was defined as upward directed movements of the body, with the forepaws quickly scratching the inner wall of the cylinder; swimming behavior was defined as movement (usually horizontal) throughout the swim chamber which includes crossing across quadrants of the cylinder; and immobility, also known as floating behavior, was counted as no additional activity to be observed except the necessary movements to keep the nose above the water.

5-Bromodeoxyuridine (BrdU) Injection and Tissue Preparation

BrdU powder (Sigma) was dissolved in 0.9% saline solution and injected peritoneally to each animal on days 19, 20, and 21 (Fig. 1) at 50 mg/kg in 12-h interval. For the analysis of proliferation, 1 day after the last BrdU injection, one half of the animals was anesthetized with an overdose of sodium pentobarbital and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde. For the analysis of neuronal differentiation, the other half of the animals was anesthetized and perfused in the same way (Fig. 1). All brains were collected and post-fixed in 4% paraformaldehyde at 4°C overnight. Then all brains were immersed in 30% sucrose solution and 48 h later were serially cryosectioned at 40 μ m coronally from bregma -3.30 to -4.52 mm (27). Twelve serial slices were collected in a 24-well plate and stored in cryoprotectant at -20°C .

BrdU Immunohistochemistry and Quantification

Serial slices in one well were used and mounted onto slides precoated with 0.25% gelatin. Sections were then placed in 0.01 M citric acid (pH 6.0) for 30 min at 95°C, followed by 0.01 M PBS rinse. All sections were then denatured in 2 M HCl for 30 min at 37°C, followed by boric acid buffer (pH 8.5) for 15 min at room temperature. After three time rinses of 0.01 M PBS, sections were incubated for 30 min with 3% hydrogen peroxide. After blocking with 10% normal goat serum for 1 h, sections were incubated with mouse anti-BrdU antibody (1:1,000, Roche Molecular Biochemicals, Essex, UK) overnight at room temperature. Following three time rinses of 0.01 M PBS, sections were incubated with biotinylated goat anti-mouse antibody (1:200 Vector Laboratories, Inc., Burlingame, CA) for 1 h at room temperature. Then sections were incubated with an avidin-biotin complex (Vector Laboratories) for 30 min. Following three time rinses of 0.01 M PBS, all sections were incubated with diaminobenzidine. The diaminobenzidine was removed when appropriate color was developed. At last, all sections were counterstained with eosin. Being dehydrated in gradient alcohol and cleared in toluene solution.

The number of BrdU-positive cells, located in the dorsal hippocampus (six sections per animal), was calculated by using the software StereoInvestigator (MicroBrightField, Williston, VT) and the 40× objective lens. Briefly, in every section, the contour of the granular cell layer (GCL) and subgranular zone (SGZ; a two cell-wide area just under GCL) was first outlined under the 10× objective lens. After switching to 40× lens, the software randomly picked out about 30 counting frames in the contour and BrdU-positive cells in each counting frame were calculated by a skilled observer. After counting all the six sections in one animal, the total cell number in this animal was estimated by the software. The whole process of calculation was conducted in a blinded manner.

Fluorescence Staining and Quantification of Neurogenesis

For phenotype analysis, 30 BrdU-positive cells per animal were randomly selected and then were checked for the colabeling of BrdU (1:1,000 Abcam) and doublecortin (DCX, 1:100, Abcam, USA). Ratios of colabeling were counted as the following formula:

Ratio of colabeling = number of BrdU⁺DCX⁺ in 30 BrdU⁺ cells / 30 × 100%.

The whole process of analysis was conducted in a blinded manner.

Protein Extraction and Western Blotting Analysis

Hippocampal tissue in experiment 2 was rapidly dissected out after an appropriate treatment period. Samples were snap-frozen in liquid nitrogen and then stored at -80°C until use. Samples were homogenized in 500 µl lysis buffer containing phosphatase inhibitor (Calbiochem, Cat. No. 524628) and protease inhibitor cocktail (Sigma, Cat. No. P8340). Homogenate were kept on ice for 30 min followed by centrifuge at 14,000 rpm, 4°C for 30 min. The supernatant was then harvested and protein concentration was determined using the protein assay kit (Bio-Rad, Cat. No. 5000116).

To examine the level of synaptophysin (SYP) and post-synaptic density protein-95 (PSD-95), equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (20 µg protein/lane) using 10% polyacrylamide gels. Afterwards, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, followed by blocking with 5% nonfat dry milk in Tris-buffered saline and Tween 20 (TBST). The membrane was then incubated with mouse anti-SYP (1:5,000) (Sigma) and rabbit anti-PSD-95 (1:2,500) (Cell Signaling Technology, CST) for 2 h at room temperature, followed by the incubation of horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 1 h at room temperature. Bands were visualized on a Biomax X-ray film using

the ECL detection kit. The membranes were then stripped with the Re-Blot Western blot recycling kit and reprobed with monoclonal anti-β-actin antibody (1:15,000) as internal control.

Golgi Staining and Analysis

Golgi staining was performed with FD Rapid Golgi Staining Kit (FD NeuroTechnologies, USA, Cat. No. PK401) according to the manufacturer's instructions. In brief, freshly dissected brain was left in impregnation solutions A and B containing potassium dichromate and chromate for 2 weeks at room temperature. After being replaced with solution C for 48 h at 4°C, the brain tissue was sectioned into 150 µm and fixed to gelatin-coated slides. Solutions D and E and distilled water (1:1:2 ratio) were used for staining the pyramidal neurons for 10 min, followed by dehydration in 50%, 75%, 95% ethanol and absolute ethanol and finally cleared in toluene and covered by coverslips in permount. Neurons were analyzed by Neurolucida (MicroBrightField) and selected according to methods described by Woolley et al. (36),

1. relative isolation from neighboring impregnated neurons to avoid interference with analysis;
2. cell bodies located in the middle part of the section thickness to minimize the loss of branch segments;
3. consistent and dark impregnation along the entire extent of all of the dendrites;
4. located in the appropriate subregion of the dorsal hippocampus.

Three CA3 pyramidal cells in each animal were selected and drawn at the 200× lens. Spine density was estimated by randomly selecting high-magnification tracing of a 15-µm long terminal segment of the basal/apical dendritic branch. Three to five tertiary apical dendrites and basal dendrites that had at least one branch point were selected for measurement. Visible spines along the branch segment were counted, and data were expressed as spine number/10 µm.

Intracerebroventricular Infusion of Ara-c

All animals were infused as previously described (18). Briefly, each animal was anesthetized and fixed in a stereotaxic stage. The top of the skull was exposed and a hole over the right lateral ventricle (2.0 mm posterior and 1.5 mm lateral to bregma) was drilled. An osmotic pump (Alzet, model 2004, Palo Alto, CA, USA; flow rate at 0.25 µl/h) containing Ara-c (4%, Sigma) in 0.9% saline or 0.9% saline alone was implanted into the subcutaneous space of the neck of the rat with a 4.0-mm metal cannula crossing through the hole. The osmotic pump was pre-assembled according to the instruction. After the suturing the skin, every animal was put back to a warm cage and allowed for a 48-h recovery.

Statistic Analysis

All data were presented as mean \pm standard error of mean (SEM) and were analyzed using two-way ANOVA, with LBP treatment in the CORT-treated and nontreated groups as between-subjects factors. Following ANOVA analyses, Dunnett's post hoc tests were used via the Statistical Analysis System. Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Effects of LBP on Depression-Like Behavior in the FST

In experiment 1, three types of typical behaviors were recorded in FST. In part A of experiment 1 (Fig. 2a), daily injection of 40 mg/kg CORT for 14 days was administered. In the immobile activity, a two-way ANOVA indicated a significant CORT effect [$F(1, 14) = 14.45, p = 0.002$] and a significant LBP effect [$F(1, 14) = 23.51, p < 0.001$] and CORT \times LBP interaction [$F(1, 14) = 17.24, p = 0.001$]. After the injection of 40 mg/kg CORT for 14 days, a significantly longer immobility was observed when compared to the PBS group ($p < 0.001$). Treatment with 1 mg/kg LBP for 21 days alone showed no effects on immobile time when compared to the PBS group ($p > 0.05$). The LBP+40C group showed significantly shortened immobility time in the LBP+40C group when compared to the CORT group ($p < 0.001$) (Fig. 2a). In a struggling activity, a two-way ANOVA indicated no CORT effect [$F(1, 14) = 1.308, p > 0.05$], no LBP effect [$F(1, 14) = 1.308, p > 0.05$], and no CORT \times LBP interaction

[$F(1, 14) = 0.43, p > 0.05$] (Fig. 2b). In a swimming activity, a two-way ANOVA indicated a significant CORT effect [$F(1, 14) = 10.48, p = 0.006$] and no significant LBP effect [$F(1, 14) = 3.45, p > 0.05$] and CORT \times LBP interaction [$F(1, 14) = 9.33, p = 0.009$]. After injection of 40 mg/kg CORT for 14 days, a significantly shorter swimming activity was observed when compared to the PBS group ($p < 0.001$) (Fig. 2c). Treatment of 1 mg/kg LBP for 21 days alone showed no effects on the swimming activity when compared to the PBS group ($p > 0.05$) (Fig. 2c). The combined LBP+40C showed a significantly longer swimming time when compared to the PBS+40C group ($p = 0.005$) (Fig. 2c).

In the set B of experiment 1, 50 mg/kg CORT was administered. In the immobile activity, two-way ANOVA analysis indicated a significant CORT effect [$F(1, 15) = 26.30, p < 0.001$] and a significant LBP effect [$F(1, 15) = 16.84, p = 0.001$] and CORT \times LBP interaction [$F(1, 15) = 15.46, p = 0.001$]. After injection of 50 mg/kg CORT for 14 days, a significantly longer immobility time was observed when compared to the PBS group ($p < 0.001$) (Fig. 2d). Treatment of 1 mg/kg LBP for 21 days alone showed no effects on the immobile activity when compared to the PBS group ($p = 0.902$). The combined treatment of 1 mg/kg LBP for 21 days plus injection of 40 mg/kg CORT for 14 days showed a significantly shorter immobility time when compared to the CORT group ($p < 0.001$) (Fig. 2d). In struggling activity, two-way ANOVA analysis indicated no CORT effect [$F(1, 13) = 0.839, p > 0.05$], no LBP effect [$F(1, 13) = 0.084, p > 0.05$], and no CORT \times LBP interaction

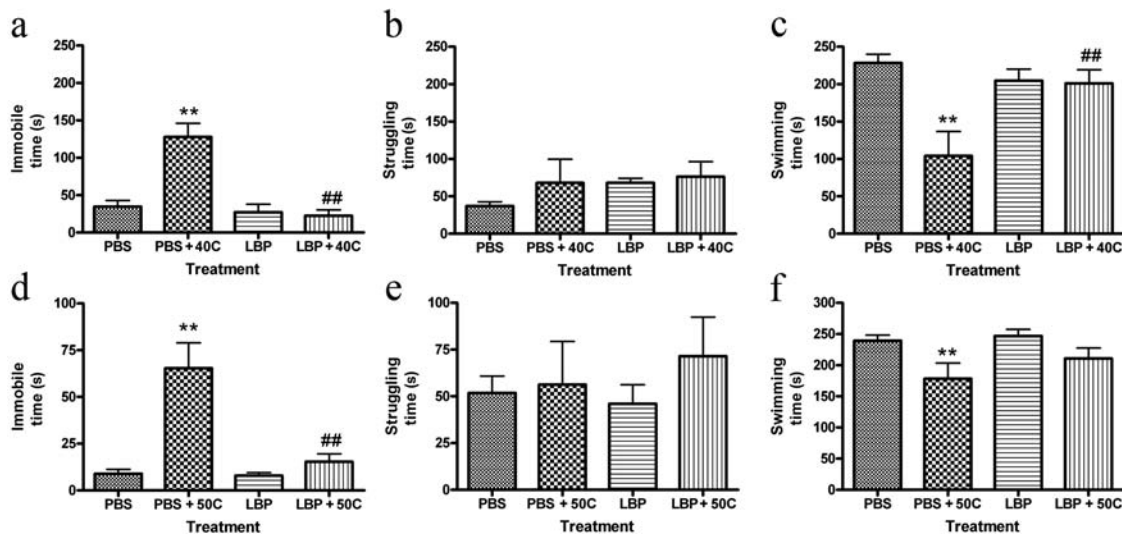


Figure 2. Effects of CORT injection and 1 mg/kg LBP treatment on the FST performance. Quantitative analysis of three types of activities including immobility (a), struggling (b), and swimming (c) of the FST performance in different groups receiving 40 mg/kg CORT injection and 1 mg/kg LBP feeding. Quantitative analysis of immobility (d), struggling (e), and swimming (f) in the FST performance in different groups received 50 mg/kg CORT injection and 1 mg/kg LBP feeding. ** $p < 0.01$ compared to the PBS group; ## $p < 0.01$ compared to the PBS+40C group or the PBS+50C group. FST, forced swimming test; LBP, *Lycium barbarum*; CORT, corticosterone; PBS, phosphate buffer; 40C, 40 mg/kg corticosterone; 50C, 50 mg/kg corticosterone.

[$F(1, 13)=0.412, p>0.05$] (Fig. 2e). In a swimming activity, two-way ANOVA indicated a significant CORT effect [$F(1, 13)=9.445, p=0.009$] and no significant LBP effect [$F(1, 13)=1.621, p>0.05$] and CORT \times LBP interaction [$F(1, 13)=0.619, p=0.009$]. After injection of 50 mg/kg CORT for 14 days, a significantly shorter swimming activity was observed when compared to the PBS group ($p=0.015$). Treatment of 1 mg/kg LBP for 21 days alone showed no effects on the swimming activity when compared to the PBS group ($p>0.05$). Combined treatment of 1 mg/kg LBP for 21 days plus injection of 50 mg/kg CORT for 14 days failed to shorten swimming time when compared to the CORT group ($p>0.05$) (Fig. 2f). All the above data indicated that LBP treatment was able to reduce “depressive-like” behaviors in the FST caused by 40 and 50 mg/kg CORT treatment, respectively.

Effects of LBP on the Hippocampal Neurogenesis

LBP Restored Hippocampal Cell Proliferation in 40 mg/kg CORT-Treated Rats, but Failed to Do so in 50 mg/kg CORT-Treated Rats. Figure 3a showed a coronary brain section containing the dorsal dentate gyrus, and Figure 3b showed that the BrdU-positive cells located beneath the granular cell layer. Compared to the number of BrdU-positive cells ($5,715\pm 850$) in the PBS group, the number of BrdU-positive cells in rats with 40 mg/kg CORT treatment in the PBS+40C group significantly reduced to 48% ($2,746\pm 801, p=0.02$). The number of BrdU-positive cells in the LBP group showed no significant change, indicating that LBP treatment alone did not elicit any effect on the proliferation of neural progenitor cells in the hippocampus. Compared to the cell number in the PBS+40C group, the number of BrdU-positive cells significantly increased ($5,393\pm 687, p=0.033$) in the LBP+40C group, indicating treatment with LBP significantly restored the BrdU-positive cells in rats treated with 40 mg/kg CORT (Fig. 3c).

The results of BrdU staining in part B of experiment 1 (50 mg/kg CORT injection) was shown in Figure 3d–f. Compared to the number of BrdU-positive cells in the PBS group ($6,454\pm 672$), the cell number in the PBS+50C group significantly reduced to $2,867\pm 463$ ($p<0.001$). Compared to the PBS+50C group, there was no difference in the number of BrdU-positive cells ($3,604\pm 642, p>0.05$) in the LBP+50C group, suggesting that LBP treatment was unable to restore the proliferation of neural progenitor cells suppressed by 50 mg/kg CORT injection (Fig. 3e, f).

Effects of LBP on the Differentiation of Neural Progenitor Cells in the Hippocampus in the 40 mg/kg CORT-Injected and 50 mg/kg CORT-Injected Rats. Colabeling of BrdU and DCX was used to estimate the neuronal differentiation of newly born cells. In 40 mg/kg CORT-treated rats, the ratio of DCX⁺/BrdU⁺ cells to

BrdU⁺ cells in the PBS+40C group did not show any difference when compared to that in the PBS group ($p>0.05$). Similarly, the ratio of DCX⁺/BrdU⁺ cells to BrdU⁺ cells in both the LBP group and LBP+40C group did not demonstrate any difference when compared to that in the PBS group ($p>0.05$) (Fig. 4b). However, the ratio of DCX⁺/BrdU⁺ cells to BrdU⁺ cells in the PBS+50C group was significantly lower than that in the PBS group ($p=0.005$). The ratio of DCX⁺/BrdU⁺ cells to BrdU⁺ cells in the LBP+50C group was significantly higher than that in the PBS+50C group ($p=0.028$) and comparable to that in the PBS group ($p>0.05$) (Fig. 4c).

Effects of LBP on Dendritic Morphology of CA3 Pyramidal Cells in the CORT-Treated Rats (Fig. 5)

No Effect of LBP and 40 mg/kg CORT on Soma Size and Dendritic Length. Figure 5a illustrated a qualified Gogli-impregnated CA3 pyramidal cell (left) and the outline of the neurites by NeuroLucida software (right). After a 14-day injection of 40 mg/kg CORT, quantitative analysis of CA3 pyramidal cells did not show any significant decrease neither in either cell body area (Fig. 5b) or in dendritic length of basal dendrites (Fig. 5d) and apical dendrites (Fig. 5d). The data indicated that 14-day injection of 40 mg/kg CORT did not decrease the pyramidal cell body and did not reduce dendrites in pyramidal cells of the CA3 region.

Alteration in Apical Spine Density by 40 mg/kg CORT and LBP Treatment. The spine density in the basal dendrites was not affected by 40 mg/kg CORT or by LBP (Fig. 5f) ($p>0.05$). However, comparing to spine density in apical dendrites in the PBS group, spine density in apical dendrites was significantly reduced in the PBS+40C group ($p<0.05$). In comparison to the PBS+40C group, spine density was significantly increased ($p<0.05$) in the LBP+40C group. The data indicated that LBP restored the reduced spine density caused by 40 mg/kg CORT (Fig. 5f). In addition, LBP alone did not affect spine density in the basal and apical dendrites of pyramidal cells (Fig. 5f) ($p>0.05$).

Effects of LBP and 50 mg/kg CORT on Soma Size and Dendritic Length in CA3 Pyramidal Cells. After 14-day injection of 50 mg/kg CORT, quantitative analysis of cell body area of CA3 pyramidal cells in the PBS+50C group showed a decreasing trend (Fig. 5c) ($p=0.071$) when compared to the cell body area in the PBS group. The cell body area in the LBP+50C group was restored to the baseline and showed no difference in the cell body area when compared with the PBS group. No significant change was detected in the length of basal dendrites (Fig. 5e) and apical dendrites (Fig. 5e). These data indicated that a 14-day injection of 50 mg/kg CORT, somehow, induced an atrophy in the pyramidal cell body, while LBP treatment was able to reverse the atrophy of the pyramidal cell body.

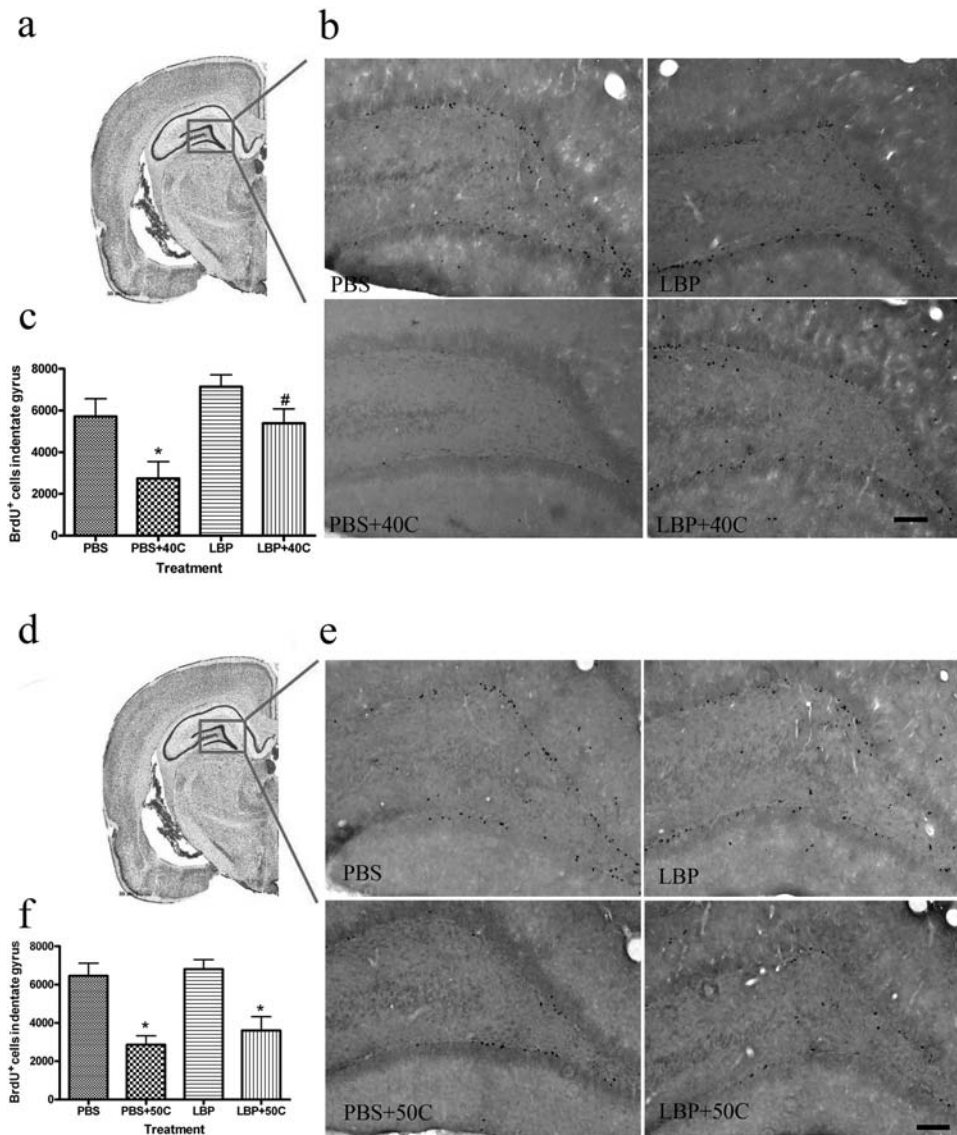


Figure 3. Immunohistochemical staining and quantitative analysis of BrdU-positive cells in dentate gyrus in groups receiving 40 and 50 mg/kg CORT injection. (a) Nissl staining of the half coronary brain section. (b) BrdU immunohistochemistry in different groups receiving 40 mg/kg CORT injection. Scale bar: 100 μ m. (c) Quantitative analysis of BrdU-positive cell number in different groups receiving 40 mg/kg CORT injection. (d) Nissl staining of the half coronary brain section. (e) BrdU immunohistochemistry in different groups receiving 50 mg/kg CORT injection. Scale bar: 100 μ m. (f) Quantitative analysis of BrdU-positive cell number in different group receiving 50 mg/kg CORT injection. * p <0.05 compared to the PBS group; # p <0.05 compared to the PBS+40C group. BrdU, bromodeoxyuridine; PBS, phosphate buffer; LBP, *Lycium barbarum*; 40C, 40 mg/kg corticosterone; 50C, 50 mg/kg corticosterone.

Alteration in the Apical Spine Density by 50 mg/kg CORT and LBP Treatment. Spine density in basal dendrites was unchanged either by 50 mg/kg CORT or LBP treatment (Fig. 5g) (p >0.05). However, comparing to spine density in apical dendrites in the PBS group, spine density in apical dendrites in the PBS+50C group was significantly reduced (p <0.05). Comparing to spine density in the PBS+50C group, spine density significantly increased (p <0.05) in the LBP+50C group. These data suggest that LBP treatment was able to restore the decrease spine density in

apical dendrites caused by 50 mg/kg CORT injection (Fig. 5g). Also, in this part of the experiment, LBP alone did not affect the spine density in the basal and apical dendrites of the pyramidal cells (Fig. 5g).

Effects of LBP on Hippocampal Expression Level of SYP and PSD-95 (Fig. 6)

LBP Increased the Expression of PSD-95 in 40 mg/kg CORT-Treated Rats. Figure 6a showed the results of the expression level of SYP and PSD-95, two dominant synaptic

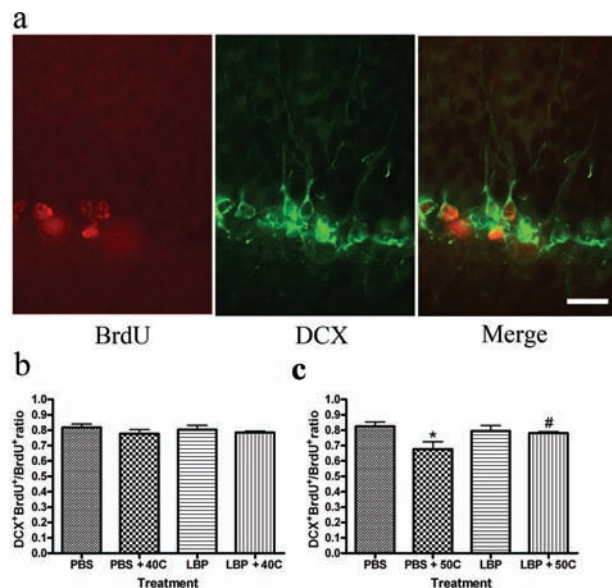


Figure 4. Differentiation of the neural progenitor cells the dentate gyrus in experiment 2. (a) Double-labeling of BrdU (red) and doublecortin (DCX, green) cells in the dentate gyrus. Scale bar: 10 μ m. (b) Quantitative analysis of DCX⁺BrdU⁺ cells to BrdU⁺ cells ratio in part A of experiment 2 (40 mg/kg CORT). (c) Quantitative analysis of DCX⁺BrdU⁺ cells to BrdU⁺ cells ratio in part B of experiment 2 (50 mg/kg CORT). * $p < 0.05$ compared to PBS group; # $p < 0.05$ compared to the PBS+40C group/the PBS+50C group. BrdU, bromodeoxyuridine; PBS, phosphate buffer; LBP, *Lycium barbarum*; 40C, 40 mg/kg corticosterone; 50C, 50 mg/kg corticosterone.

proteins in experiment 3 by Western blotting. Neither 40 mg/kg CORT nor LBP induced an obvious change in the expression of SYP in all four groups (Fig. 6c) ($p > 0.05$). However, the expression level of PSD-95 was significantly decreased in the PBS+40C group when compared to the level in the PBS group ($p = 0.003$). The expression level of PSD-95 was increased in the LBP+40C group when compared to the level in the PBS+40C group (Fig. 6e) ($p = 0.003$).

LBP Also Increased the Expression of PSD-95 in 50 mg/kg CORT-Injected Rats. Figure 6b showed the results of the expression level of SYP and PSD-95 in 50 mg/kg CORT treatment by Western blotting. Neither 50 mg/kg CORT nor LBP induced a change in the expression of SYP in all four groups (Fig. 6d) ($p > 0.05$). Similarly, expression level of PSD-95 did not show any significant decrease in the PBS+50C group when compared to the level in the PBS group (Fig. 6f) ($p > 0.05$). The expression level of PSD-95 robustly increased in the LBP+50C group when compared to the level in the PBS+50C group (Fig. 6f) ($p = 0.046$).

Ara-c Infusion Did Not Block the Counteractive Effect of LBP on CORT-Treated Rats (Fig. 7)

Ara-c Infusion Abolished the BrdU-Positive Cells in the Dentate Gyrus. Figure 7a showed the tract made by

the metal cannula of the osmotic pump. The tract clearly showed that the 4-mm long cannula entirely penetrate the cortex allowing the release of Ara-c or 0.9% saline into the right lateral ventricle near the hippocampal fornix, a part that was just anterior to the dorsal hippocampus.

BrdU-positive cells were detected in the groups with saline infusion (Fig. 7b–d, f–g). However, no BrdU-positive cells were observed in the groups with Ara-c infusion (Fig. 7e, h). Similar to previous findings (20,38), both BrdU-positive cell number was greatly decreased in the Sa+PBS+40C group and Sa+PBS+50C group (Fig. 7c, e) when compared to the number in the Sa+PBS group (control).

Ara-c Infusion Did Not Block the Antidepressant Effect of LBP on 40 and 50 mg/kg CORT-Treated Rats. Figure 8 showed the immobile period time in different groups. Three-way ANOVA analysis revealed a significant CORT effect [$F(1, 21) = 5.865, p = 0.009$] and a significant CORT \times LBP effect [$F(1, 21) = 6.650, p = 0.006$] and no CORT \times LBP \times Ara-c interaction [$F(1, 21) = 0.664, p = 0.525$]. These data indicated that similar to previous findings, compared to the immobile time in the Sa+PBS group, the immobile time significantly increased in both the Sa+PBS+40C ($p < 0.01$) and the Sa+PBS+50C groups ($p < 0.01$). However, 1 mg/kg LBP significantly decreased the immobile time both in the Sa+LBP+40C and the Sa+LBP+50C groups. These data indicated that the infusion of normal saline had no influence on the beneficial effects of LBP treatment. Furthermore, in the Ara-c+LBP+40C and the Ara-c+LBP+50C groups, it was proved that the Ara-c infusion was unable to block the behavioral improvement of LBP treatment as shown in the FST both in 40 and 50 mg/kg CORT-treated rats.

DISCUSSION

In the present study, we investigated whether LBP exhibited a counteractive effect on depression-like behavior in FST induced by a sophisticated stress model by repeated CORT injections (14,38). Also, we further determined whether the beneficial effects of LBP were mediated by hippocampal neurogenesis or were associated with dendritic plasticity in hippocampus. Our results showed that LBP reduced the depression-like behavior in FST both in 40 and 50 mg/kg CORT-treated rats. The effects of LBP were associated with recovered spine density in pyramidal cells as well as with the increased expression of PSD-95 in hippocampus. Ablation of neurogenesis by Ara-c infusion did not block the antidepressant effects of LBP treatment on reducing the depression-like behaviors in FST both in 40 and 50 mg/kg CORT-treated rats. The above data indicate that the antidepressant effects of LBP may not require hippocampal neurogenesis but is associated with enhanced dendritic plasticity and increased expression of certain synaptic proteins.

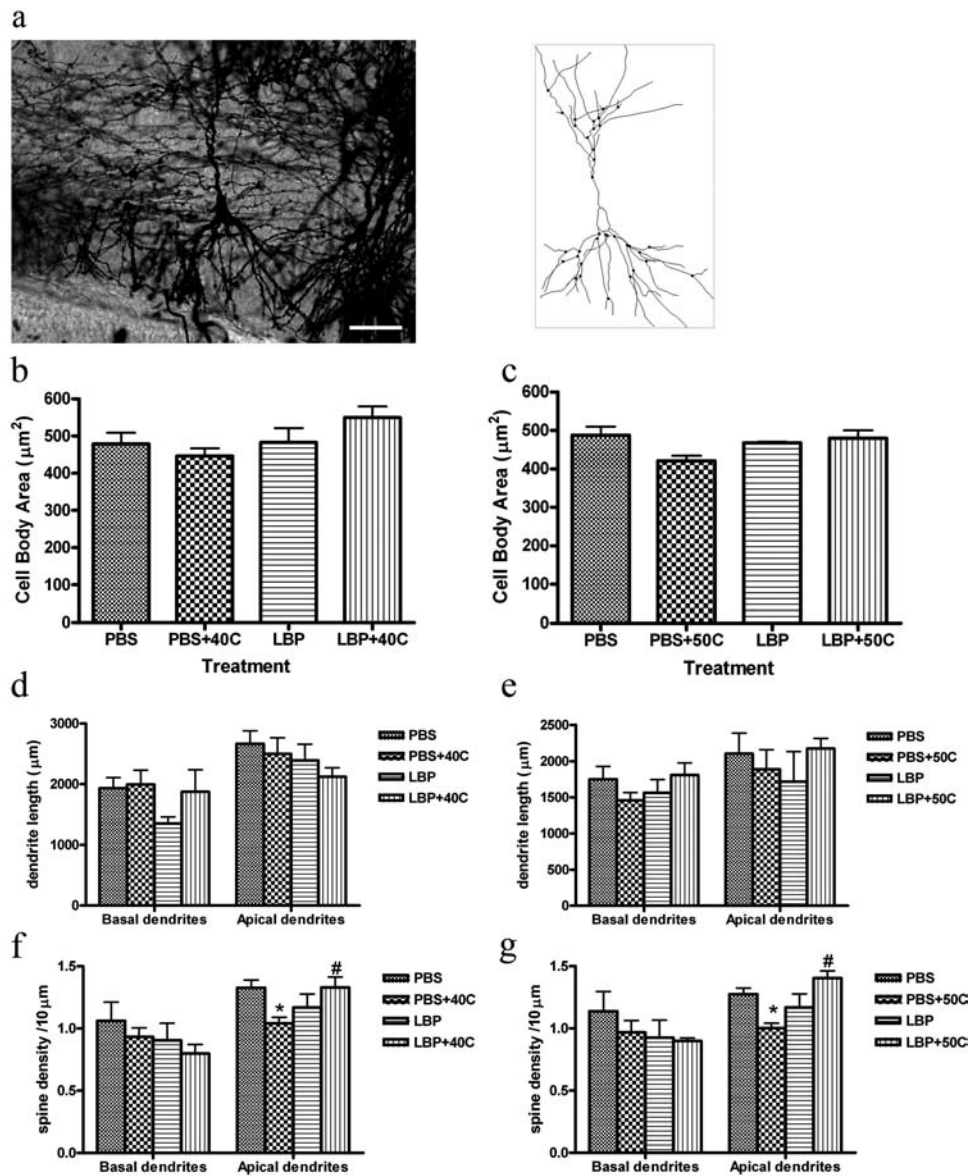


Figure 5. Golgi staining and quantitative analysis data of pyramidal cells after LBP and CORT treatment. (a) Left: representative image of a selected pyramidal neuron for analysis; right: the drawing image created by NeuroLucida. Scale bar: 50 μm . (b, c) Quantitative analysis of soma size in different groups. (d, e) Quantitative analysis of basal dendritic length and apical length in different groups. (f, g) Quantitative analysis of spine density on apical and basal dendrites in different groups. * $p < 0.05$ compared to PBS group; # $p < 0.05$ compared to the PBS+40C/50C group. PBS, phosphate buffer; LBP, *Lycium barbarum*; 40C, 40 mg/kg corticosterone; 50C, 50 mg/kg corticosterone.

LBP Exhibits Antidepressant Effects in Both 40 and 50 mg/kg CORT-Treated Rats

Chronic exposure to high dose of CORT has been commonly adopted to establish stress model in experimental animals (4,22,36). More interestingly, the recent data in our laboratory have proved that 14-day injection of CORT using 30 mg/kg (low), 40 mg/kg (mediate), and 50 mg/kg (high) led to a graded increase in depression-like behavior and impaired spatial learning. Furthermore, volunteer running reversed those behavioral deficits in rats treated

with 30 or 40 mg/kg but not 50 mg/kg of CORT (38). Thus, CORT injection provides a sophisticated control on depression-like behavior in animals in terms of increasing the immobility time. According to the above findings, doses at 40 and 50 mg/kg CORT were chosen to induce the “running-reversible” depression model and the “running-irreversible” depression model in rats, respectively; in this study, LBP was employed to antagonize the depression-like behaviors in FST. The present findings showed that LBP significantly decreased the immobile time and increased

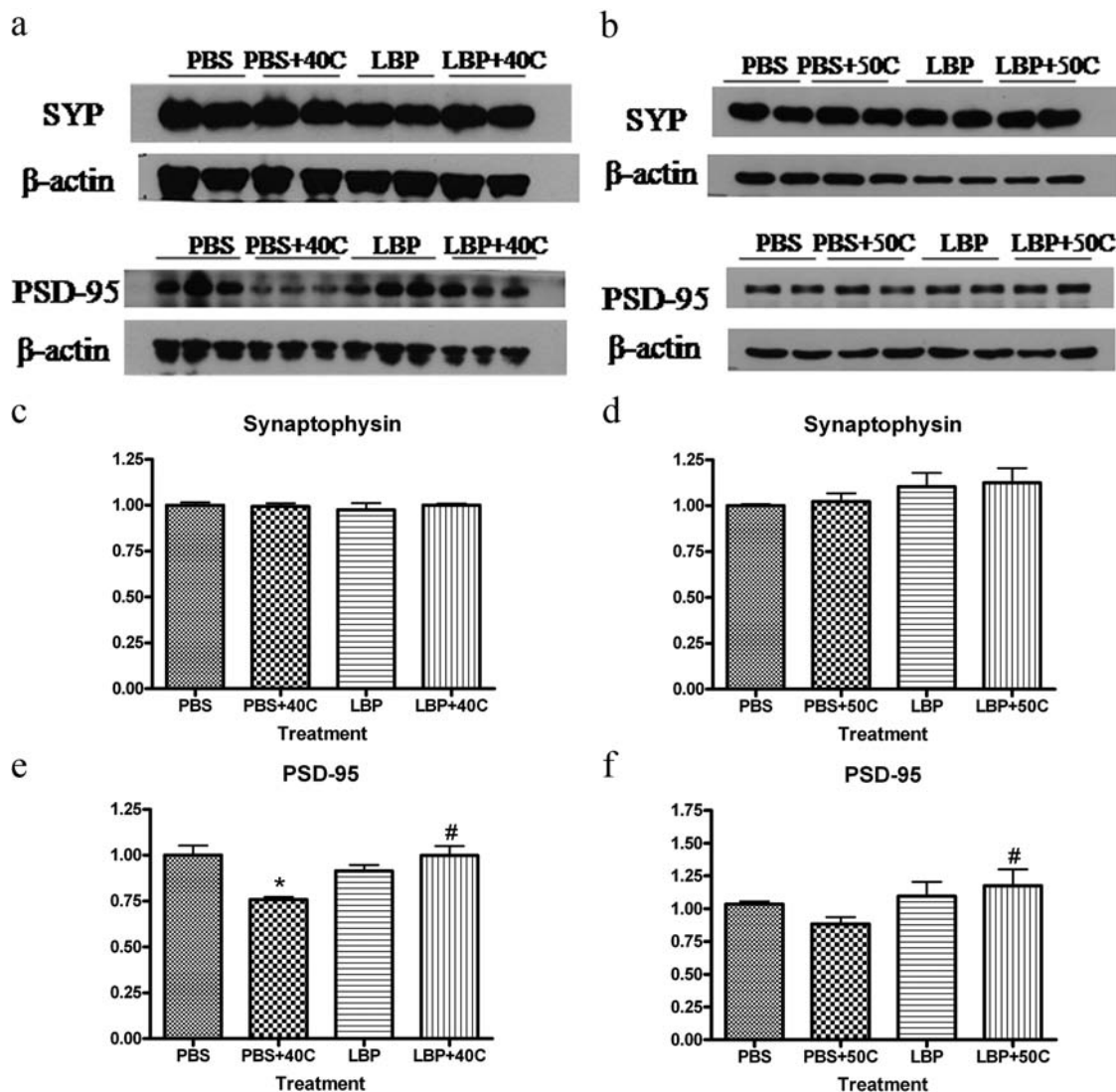


Figure 6. Western blot detection of SYP and PSD-95 in the hippocampus in 40 and 50 mg/kg CORT-treated rats. (a) Positive bands in SYP and PSD-95 detection after 40 mg/kg CORT and LBP treatment. (b) Positive bands in SYP and PSD-95 detection. (c, d) Quantitative analysis of SYP expression in different groups. (e, f) Quantitative analysis of PSD-95 expression in different groups. * $p < 0.05$ compared to PBS group; # $p < 0.05$ compared to the PBS+40C/50C group. PBS, phosphate buffer; LBP, *Lycium barbarum*; 40C, 40 mg/kg corticosterone; 50C, 50 mg/kg corticosterone; SYP, synaptophysin; PSD-95, postsynaptic density protein 95.

the swimming time in FST in both 40 and 50 mg/kg CORT-treated rats. This data indicate that LBP exhibits an antidepressant effect in the CORT-treated rats. Neuroprotective effects of LBP have been extensively proved in various neurodegenerative models (8). Previous studies have found that polysaccharides in LBP are able to improve cognitive function in the intact hippocampus (28). Furthermore, peritoneal injection of LBP can significantly reduce the score of neurological symptoms and stroke index assessment in a mice model of ischemia–reperfusion (32). Therefore, LBP improves the cerebral recovery against an ischemic challenge. Our results showing the antidepressant effects

of LBP treatment are among the first to prove that LBP elicits beneficial effects on psychiatric disorders and added the knowledge in the neuroprotection of LBP in the central nervous system (CNS). Furthermore, the findings showing that LBP reversed the behavioral deficits caused by 50 mg/kg CORT injection indicate that LBP has a more potent therapeutic effect on counteracting depression-like behaviors than running did, since running showed no effects on counteracting the behavioral deficits in 50 mg/kg CORT-treated rats (38). One possible explanation here is that this potent therapeutic effect of LBP may be due to 7-day pre-treatment regimen.

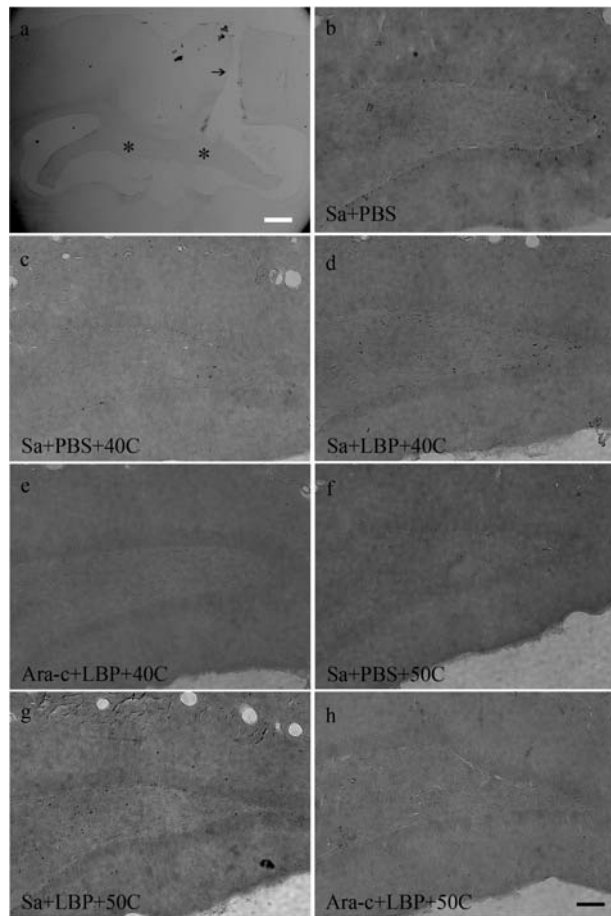


Figure 7. Entire abolishment of BrdU-positive cells in the dentate gyrus by Ara-c infusion. (a) Coronal section of the cerebrum across the penetrate site of the metal cannula of the osmotic pump; arrow, the tract made by the cannula of the osmotic pump; asterisk, bilateral hippocampal fornix. Scale bar: 200 μm . (b–h) Immunohistochemical staining of BrdU in dentate gyrus in different groups. Scale bar: 100 μm . a+PBS, saline infusion+0.01 M PBS+sesame oil injection; Sa+PBS+40C, saline infusion+0.01 MPBS+40 mg/kg CORT injection; Sa+LBP+40C, saline infusion+1 mg/kg LBP+40 mg/kg CORT injection; Ara-c+LBP+40C, Ara-c infusion+1 mg/kg LBP+40 mg/kg CORT injection; Sa+PBS+50C, saline infusion+0.01 MPBS+50 mg/kg CORT injection; Sa+LBP+50C, saline infusion+1 mg/kg LBP+50 mg/kg CORT injection; Ara-c+LBP+50C, Ara-c infusion+1 mg/kg LBP+40 mg/kg CORT injection. Sa, normal saline in the osmotic bump; PBS, phosphates buffer; CORT, corticosterone; Ara-c, cytosine-b-D-arabinofuranoside; LBP, *Lycium barbarum*.

LBP Restores the Hippocampal Neurogenesis in CORT-Treated Rats

The “Neurogenesis hypothesis” in depression has been first proposed by Gage and colleagues (17) since numerous studies have shown that hippocampal neurogenesis was greatly reduced in animal models (6,12,37). Reduction of neurogenesis can be recovered after

treatment of antidepressant drugs or in the healing phase of depression (5). These findings indicate that hippocampal neurogenesis not only responds to antidepressant treatment but also contributes to the recovery of depression. By using BrdU and DCX/BrdU immunohistochemical staining, we detected the effects of CORT and LBP treatment on the process of hippocampal neurogenesis: cell proliferation and neuronal differentiation. Our results showed that the proliferation was greatly impaired by 40 mg/kg CORT and 50 mg/kg CORT injection. In addition, 50 mg/kg CORT injection reduced neuronal differentiation of neuroprogenitor cells in the hippocampus. After cotreatment with LBP, the proliferation was totally restored in the 40 mg/kg CORT-treated rats. However, the proliferation was not restored in the 50 mg/kg CORT-treated rats, and neuronal differentiation was recovered in 50 mg/kg CORT-treated rats. The above data indicate that LBP is likely to elicit its antidepressant effects by improving hippocampal neurogenesis in different aspects. As a result, the recovery of hippocampal neurogenesis may be a potential factor in mediating the antidepressant process of LBP treatment.

The Antidepressant Effects of LBP Treatment Is Not Blocked by the Ara-c Infusion

To better understand whether adult neurogenesis mediates the antidepressant effects of LBP, Ara-c infusion was adopted to inhibit the newly born cells from the pool of neural progenitor cells in the dentate gyrus. It was found that, after Ara-c infusion, LBP could retain its ability to reduce the immobile time in FST in both 40 and 50 mg/kg CORT-treated rats. This finding can be attributed to two possible explanations. First, other factors beyond neurogenesis are underlying

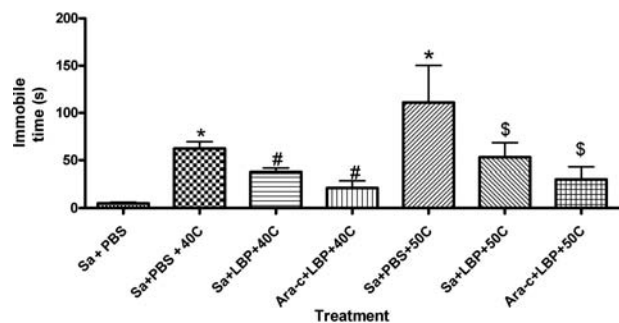


Figure 8. Immobile time in FST in different groups after Ara-c infusion. * $p < 0.05$ compared to the Sa+PBS group; # $p < 0.05$ compared to Sa+PBS+40C group; \$ $p < 0.05$ compared to Sa+PBS+50C group. Sa, normal saline in the osmotic bump; PBS, phosphates buffer; LBP, *Lycium barbarum*; 40C, 40 mg/kg corticosterone; 50C, 50 mg/kg corticosterone; Ara-c, cytosine-b-D-arabinofuranoside; FST, forced swimming test.

the antidepressant action of LBP. It has been found that classic antidepressant drugs such as fluoxetine exhibit antidepressant effects via neurogenesis-dependent and neurogenesis-independent manner (11). Their recent work in a mice model of anxiety/depression has shown that efficacy of fluoxetine disappeared in novelty suppressed feeding test after X-ray irradiation, whereas its efficacy was kept intact in other depression behavior tests including FST and open field test after X-ray irradiation. Another elegant work done by Bessa and his colleagues (3) shows that classic antidepressant drugs such as fluoxetine and imipramine restored the complexity of dendrite trees of neurons in the PFC and hippocampus after chronic mild stress. Intriguingly, the antidepressive effects of these classic drugs were not abolished even when the hippocampal neurogenesis was ablated by methylazoxymethanol, a cytostatic agent. Therefore, our data showing that the efficacy of LBP is not affected by the ablation of adult neurogenesis strongly suggests that other neurogenesis-independent factors may play a more important role than neurogenesis in mediating the antidepressant action of LBP. Second, Ara-c is a widely used chemotherapeutic drug that is able to cause cell cycle arrest by inhibiting DNA synthesis. Hippocampal neurogenesis is a dynamic process that took place throughout the whole lifetime. Generally, adult neurogenesis in the hippocampus undergoes five developmental stages: stage 1 (Proliferation), stem cells located in the subgranular zone give rise to the transient amplifying cells; stage 2 (Differentiation), the transient amplifying cells differentiated into immature neurons; stage 3 (Migration), the immature neurons gradually migrated into the granular cell layer and then extend their axons to target on the neurons in CA3 field (stage 4, Axonal targeting), and finally formed synaptic connections (stage 5, Synaptic integration). This whole process usually takes about 4 weeks to be completed (23). Thus, based on the pharmacological feature of Ara-c, the infusion of this drug is able to affect the proliferative stage (stage 1) alone and leave the rest of stages intact. In other words, newly born neurons earlier than the implantation of the osmotic pump containing Ara-c are nearly intact. A previous study has proved that only long-term administration with fluoxetine enhances the maturation and the synaptic formation of adult-borne hippocampal granular cells and hence improves the behavior outcomes (34). The findings indicate that the efficacy of classic antidepressant drugs rely on the maturation and integration of the newly born granular cells. Since Ara-c infusion in the present study only blocked the proliferation stage of the newly born granular cells, it is possible that the antidepressant effects of LBP did not require the new neurons proliferated from the stem cell pool. Alternatively, whether the antidepressant effect of LBP is mediated by the maturation

and integration of the newly born granular cells needed to be further explored.

The Antidepressive Effect of LBP Is Associated With Enhanced Dendritic Plasticity and Increased Expression of PSD-95

Another feature of the hippocampus in depressed animals is retraction of apical dendrites of pyramidal cells in the CA3 region (26,36). Furthermore, the spine density of pyramidal cells in the CA3 region decreased after exposure to stressful events (31). Bessa and his colleagues (3) found that classic antidepressant drugs such as fluoxetine and imipramine restored the complexity of dendrite trees of neurons in the prefrontal cortex and hippocampus after chronic mild stress. Intriguingly, the antidepressive effects of these classic drugs were not abolished even when the hippocampal neurogenesis was ablated by MAM, a cytostatic agent. These data indicate that the restoration of dendritic morphology is more important than the recovery of hippocampal neurogenesis in mediating the efficacy of the classic antidepressants. Therefore, we studied the dendritic morphology of CA3 pyramidal cells by Golgi staining and also the changes in expression level of two major synaptic proteins: synaptophysin and PSD-95. Our results showed that the major changes caused by 40 mg/kg CORT and 50 mg/kg CORT injection was the robust decrease of spine density in apical dendrites. These findings echoed others' data (4,22,36). LBP not only reversed the slight atrophy of pyramidal cell bodies caused by 50 mg/kg CORT injection but also restored the number of spines in those cells after 40 and 50 mg/kg CORT challenges. These data further confirmed the protective effects of LBP on maintaining the original morphology of mature neurons (39). More importantly, our results provide the first evidence by showing that LBP has the potential to specifically protect synaptic structures. LBP strongly increases the expression level of PSD-95 after 14-day injection of 40 and 50 mg/kg CORT. PSD-95 is abundantly distributed in the postsynaptic part of synapse and plays a key role in maintaining synapses. Overexpression of PSD-95 increases spinogenesis and enhances the synaptic connection with presynaptic terminals (25). Furthermore, targeting the interaction molecules of PSD-95 also directly change the spine density in pyramidal cells. For instance, overexpression of diacylglycerol kinase zeta (DAKzeta), which is known to interact with PSD-95 increases spine density in cultured neurons. Conversely, DGKzeta knockdown reduces spine density (19). Similar results are found in targeting another interacting protein of PSD-95, TANC1 (15). Overexpression of TANC1 increases the spine density in cultured neurons. However, TANC1-deficient mice showed reduced spine density in the CA3 region and impaired spatial memory. All these evidence indicate that PSD-95 is a key player in maintaining spine density and spinogenesis. It is possible

that LBP restores the spine density through upregulating the expression of PSD-95.

CONCLUSION

Depression is a complicated clinical disease lacking efficient therapies. The present study shows that LBP exhibits an antidepressant potential in a rat model of depression. The antidepressant action of LBP may be more potent than the routine exercise intervention. By ablating adult neurogenesis, it indicates that LBP may play its role through a neurogenesis-independent way. Furthermore, the upregulation of the synaptic protein (PSD-95) and the restoration of apical spine density after LBP treatment indicated that the dendritic morphology in the CA3 region may be an important part for supporting the antidepressant effect of LBP. Whether other neurogenesis-independent factors are involved in the antidepressant action of LBP needs to be further explored.

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