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Piper sarmentosum Roxb. produces antidepressant-like effects in rodents, associated with activation of the CREB-BDNF-ERK signalling pathway and reversal of HPA axis hyperactivity

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Abstract

Ethnopharmacological relevance: There are many plants of genus *Piper* which have been reported to induce antidepressant-like effects, *Piper sarmentosum* (PS) is one of them. PS is a Chinese herbal medicine and a traditional edible vegetable.

Materials and Methods: In the present study, the antidepressant-like effects of PS extracts and the ethyl acetate fraction of PS extracts (PSY) were assessed using the open field test (OFT), forced swimming test (FST), and tail suspension test (TST) in mice. Furthermore, we applied a 4 consecutive weeks of chronic unpredictable mild stress (CUMS) as a model of depression in rats, followed by a sucrose preference test. Then we examined the possible mechanisms of this action. The activity of the hypothalamic–pituitary–adrenal (HPA) axis was evaluated by detecting the serum corticosterone (CORT) concentrations, and the protein expression levels of brain-derived neurotrophic factor (BDNF), the phosphorylated form CREB and ERK1/2 were detected by qRT-PCR or Western blot.

Results: The results showed that PS extracts (100, 200 mg/kg) and PSY (12.5, 25, 50 mg/kg) treatment produced antidepressant-like effects in mice similar to fluoxetine (20 mg/kg), indicated by the reduced immobility time in the FST and TST, while both had no influence on the locomotor activity in the OFT. PSY treatment significantly increased sucrose preference and reduced serum CORT levels in CUMS rats. Moreover, PSY up-regulated BDNF protein levels, and increased CREB and ERK phosphorylation levels in the hippocampus on CUMS rats.

Conclusions: These findings suggest that the antidepressant-like effects of PS extracts and PSY are mediated, at least in part, by modulating HPA axis, BDNF, CREB and ERK phosphorylation and expression in the hippocampus.

KEYWORDS

Piper sarmentosum; Antidepressant; FST; BDNF; CREB-ERK

1. Introduction

Depression is a syndrome of abnormal mood and a widespread mental disorder with a lifetime prevalence of 16.2% (Kessler et al., 2003). Despite decades of clinical experience, the definite mechanism underlying depression development is still poorly understood. Nowadays, the current antidepressants used clinically are chemical synthetic compounds with significant side effects and have low rates of remission and response (Sarko, 2000). Thus, better antidepressants with higher efficacy and safety are urgently needed. At present, numerous herbal medicines with antidepressant effects have become the focus of attention for the treatment of depression because the natural plant extracts have higher safety, such as *Hypericum perforatum* L. (HP, also called St John's Wort), which is a worldwide well-known herbal antidepressant (Sarris et al., 2011), and the randomized clinical studies have shown that HP extracts are significantly superior to placebo (Lecrubier et al., 2002) and have similarly effects as standard antidepressants (Szegedi et al., 2005) in the treatment of depression.. Several human clinical trials have also provided preliminary positive evidence of antidepressant effects of some herbs, such as *Echiumamoenum*, *Crocus sativus*, *Rhodiolarosea* and *Piper methysticum* (Sarris et al., 2011).Therefore, herbal medicines have become a compelling part of pharmacotherapy in the treatment of depression (Thachil et al., 2007).

The genus *Piper* is an important member of the family–*Piperaceae* in medicine, consisting of about 2000 species and approximately 60 species are distributed in the tropical areas of China. However, several recent researches have indicated that some plants of genus *Piper*, for instance, *Piper methysticum* (Sarris et al., 2011) (commonly known as Kava–Kava), *Piper laetispicum* C.DC. (Xie et al., 2011) and *Piper sarmentosum* (PS) (Wu, 2002) have antidepressant effects. Kava-Kava used to be a popular antidepressant used all around the world, but is now banned because of hepatotoxicity (Teschke et al., 2009). PS, one of the genus *Piper* plants distributed in the India, Vietnam, Indonesia, Philippines, Malaysia and southern part of China, such as Yun nan, Guang xi, Fu jian, and Hai nan Province. It is reported that PS has biological activities, including: antioxidant (Ugusman et al., 2010), anti–tuberculosis (Hussain et al., 2008), anti–atherosclerosis (Amran et al., 2011), fracture healing (Estai et al., 2011), osteoporotic (Suhana Mohd Ramli et al., 2013). In China, it has been used for treating toothache, beriberi, and abdominal distension by the local population (SATCM, 1999). PS also plays an important role in dealing with insomnia, anxiety and depression by indigene in the minority national areas of Yun nan, China (Huang et al., 2005). The local people grind the steam and leaf of PS into a powder, then they soak the powder into Chinese liquor for a month. It is reported that indigene treat insomnia, anxiety and depression by drinking such liquor. Moreover, it is worth mentioning that, PS plays an important role as a traditional edible vegetable in the southern part of China (Zheng et al., 2013) suggesting low toxicity.

Several researches have indicated a critical role of monoaminergic hypothesis in the response to depression and modulation of stress (Hou et al., 2006). However, clinical experience over the years indicates that not all patients respond to existing monoamine–based antidepressants

(Cassano and Fava, 2004; Trivedi et al., 2006). This suggests that the reduction in monoamine levels might not be the only pathogenic mechanism of depression. So, in recent years, investigations have focused on non–monoamine–based antidepressants (Berton and Nestler, 2006). A leading hypothesis of depression which has received a lot of attention is that the hyperactivity of hypothalamic–pituitary–adrenal (HPA) axis plays an important role in response to stress (Hofman and Swaab, 2010). It is well known that the corticosterone (CORT) level in blood rise during persistently stressful situation or depression (Sapolsky, 2000). Besides the hypothesis of HPA, numbers of studies suggest that neurotrophic factors and adult neurogenesis also play an important role in mediating the responses to antidepressants (Krishnan and Nestler, 2008). The brain-derived neurotrophic factor (BDNF) has been demonstrated to have significant involvement in the modification of the hippocampus due to stress-causing stimuli (Nestler et al., 2002), and its overexpression elicits cellular and behavioral effects of antidepressant treatments (Shirayama et al., 2002). It has also been known that cAMP response element-binding protein (CREB) is a transcription factor that mediates gene expressions activated by cAMP cascade, CREB mediates neurogenesis, survival of neurons, and response to antidepressant-like effects (Duman et al., 2000). Antidepressant drugs counteract the cascade of cAMP–CREB in the pathophysiology of depression (Gass and Riva, 2007), and CREB has been suggested to be a potential molecular mechanism of antidepressants that maintains a balance to stressful disturbance by phosphorylation of CREB (pCREB) (Kwon et al., 2008). In addition, it has been demonstrated that CREB acts as an upstream transcription factor of BDNF and permits a transcriptional alternation in BDNF expression following antidepressant treatment (Conti et al., 2002). ERK (extracellular signal-regulated kinase) is one of the most-researched member of the MAPK (mitogen-activated

protein kinase) family, and in previous studies it has been suggested that the ERK may take part in the molecular mechanism of depression (Qi et al., 2006). Moreover, ERK pathway is a down stream signal transduction protein activated by BDNF (Mebratu and Tesfaigzi, 2009) and the antidepressant treatment can induce the phosphorylation of ERK (phospho-ERK1/2, p-ERK1/2) (Mattson et al., 2004). Taken together, these results indicate that BDNF, CREB and ERK could be potential molecular targets in the treatment of depression.

In this study, we explored the antidepressant-like effect of PS, and its behavioral effects in mice were evaluated in the open field test (OFT), forced swim test (FST) and tail suspension test (TST).This was followed by a CUMS (chronic unpredictable mild stress) model to establish a depression situation followed by sucrose preference test. Furthermore, serum corticosterone, BDNF protein levels, and the phosphorylated levels of CREB and ERK were also assessed in the hippocampus of rats to investigate the potential mechanisms of action.

2. Method

2.1 Animals and Overall experimental plan

Male ICR (Institute of Cancer Research) mice weighing between18–22 g and male SD (Sprague Dawley) rats weighing between 230–260 g were obtained from Second Military Medical University (SMMU) Animal Center. The animals were housed under standard conditions (12 h light/dark cycle; 24–26℃ ambient temperature; 55 ± 10% relative humidity) for one week with free access to food and water. All experimental procedures were approved by the Animal Care and Use Committee at SMMU and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985). The overall experimental plan is shown in Figure 1A.

2.2 Drug administration and experimental groups

The aerial parts of PS (15 kg) were collected from Xishuangbanna of Yunnan Province, PR China, and identified by one of the authors (Lu-Ping Qin). A voucher specimen (No.SYX20131217) was deposited at the Department of Pharmacognosy, School of Pharmacy, SMMU. The air–dried powder of the plant material (15 kg) was exhaustively extracted with ethanol. The resulting ethanol extract of PS (1.5 kg) was suspended in H_2O and further partitioned into four fractions: petroleum ether (375 g, PSS), dichloromethane (150 g, PSR), ethyl acetate (150 g, PSY), butyl alcohol (225 g, PSZ). Fluoxetine hydrochloride was purchased from Lilly Suzhou Pharmaceutical Co., LTD. HP extract was purchased from Zhejiang HUISONG Pharmaceutical Co., LTD. All drugs were dissolved in H_2O and diluted to the desired concentration on the day of experiment, and all of the drugs were administered by intragastric administration. Drugs and H_2O as a vehicle were administered between 8:30 a.m. and 9:30 a.m. once a day for 7 consecutive days. The observers of behavior test were unaware of the treatment of the animals.

2.2.1 Experiment groups of the PS extracts in mice

After one-week of adaptation, the mice were randomly assigned to the following 5 groups (n=10 each): control group (received H_2O as a vehicle), fluoxetine (FLX) group (20 mg/kg fluoxetine), PSL (50 mg/kg PS extracts), PSM (100 mg/kg PS extracts), PSH (200 mg/kg PS extracts) groups .

2.2.2 Experiment groups of the different fractions of PS extracts in mice

After the one–week adaptation period, the mice were randomized into 15 groups (n=10 each), including: control group (received H_2O as a vehicle); fluoxetine group (20 mg/kg fluoxetine); HP

group (250 mg/kg extracts of HP); 3 PSS treatment groups (30, 60,120 mg/kg PSS); 3 PSR treatment groups (12.5, 25,50 mg/kg PSR); 3 PSY treatment groups (12.5, 25,50 mg/kg PSY); 3 PSZ treatment groups (20, 40,80 mg/kg PSZ).

2.2.3 Experiment groups of the PSY in rats

At the end of the one week adaption period, the rats were randomly assigned into 6 groups (n=10 each), including an unhandled control group (received H_2O as a vehicle); a CUMS group (received H_2O as a vehicle, CUMS); a fluoxetine (FLX) treatment group (12 mg/kg fluoxetine + CUMS); and 3 PSY treatment groups (7.5, 15, 30mg/kg PSY + CUMS).

2.3 Open field test (OFT)

Spontaneous activity was measured by an OFT performed as described previously (Liu et al., 2013) with slight modification. For OFT, 1 h after the last treatment, mice were individually placed in a wooden box (50×50×30 cm) with the floor divided into 49 equal squares marked with black lines. The mice were placed in the central square and observed for 5 min. The following behaviors were recorded: the number of crossing (the number of squares crossed); rearing (the frequency of standing on hind limbs); and grooming (number of times the animal made like grooming of the face, licking/cleaning and scratching the various parts of the body). The OFT arena was thoroughly cleaned between each test.

2.4 Forced swimming test (FST)

FST was carried out according to a previously described method (Porsolt et al., 1978) with minor modifications. In briefly,1 h after the last drug administration, mice were individually placed into a glass cylinder (30 cm in height, 15 cm in diameter) filled with 12 cm high water (24–26℃), which ensured that the mice could not support themselves by touching the bottom. All

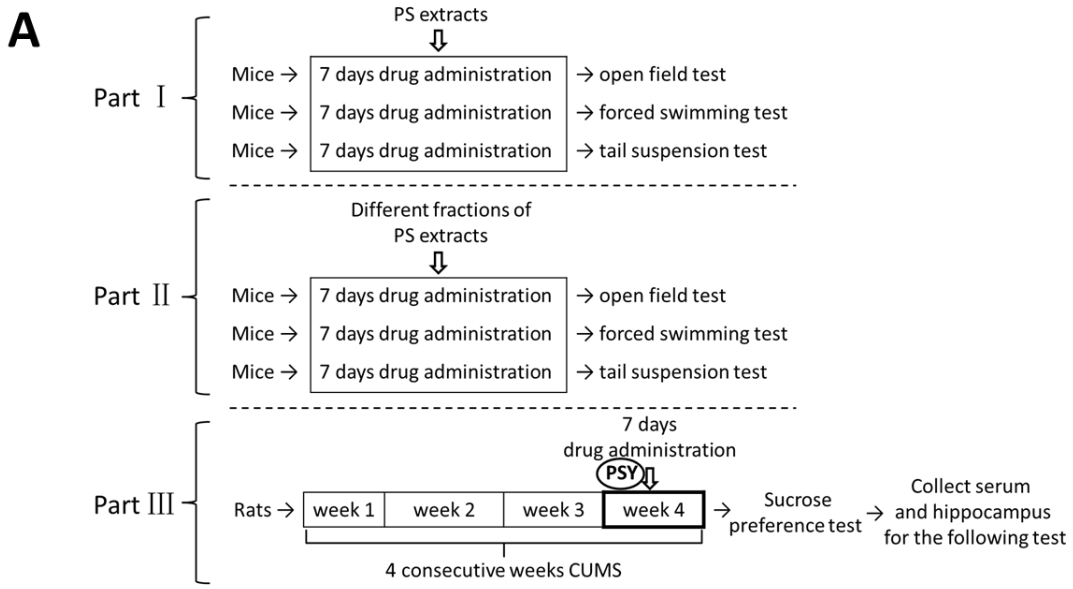
mice were forced to swim for 6 min, and the immobility time during the final 4 min interval of the test was recorded. Immobility time was defined as the time spent by the mouse floating in the water without struggling (climbing walls or diving), and making only those small movements necessary to keep its head above the water.

2.5 Tail suspension test (TST)

The TST is an another well-known animal model for assessing antidepressant activity. The total duration of immobility induced by tail suspension was measured according to the method described previously (Steru et al., 1985). Briefly, 1 h after the last drug administration, mice were suspended 50 cm above the floor for 6 min by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time during the final 4 min of the testing period was recorded. Mice were considered immobile only when they hung passively and completely motionless.

2.6 Chronic unpredictable mild stress (CUMS) procedure

The CUMS procedures were performed as described previously (Willner et al., 1992) with minor modifications. Briefly, rats in stressed groups were exposed to the two of the stressors daily for 4 consecutive weeks (Figure 1B). These stressors were randomly scheduled over a one-week period and repeated with unpredictable sequence during the following 3 weeks. Non-stressed animals were left undisturbed in their home cages except during housekeeping procedures such as cage cleaning. Rats in the treatment group received treatment daily at 30 min before the stress exposure on days 21–28; the control and CUMS group rats were given water as a vehicle-only equivalent to the drug treatment.



B

Date	Stressor 1	Stressor 2
Day 1	15 min forced swimming (25℃) 9:00~10:00	1h exposure to an empty bottle 10:00~11:00
Day 2	24 h of social crowding 9:30~9:30(next day)	4h odor exposure 14:00~18:00
Day 3	5 min tail suspension 10:00~10:30	5 min forced swimming (4℃) 14:00~15:00
Day 4	24 h water deprivation 9:30~9:30(next day)	4 h immobilization 11:00~15:00
Day 5	1 min tail pinch (1 cm from the tip of the tail) 10:00~10:30	12 h overnight illumination 19:00~7:00(next day)
Day 6	24 h wet bedding 10:00~10:00(next day)	24 h exposure to a foreign object 10:00~10:00(next day)
Day 7	24 h food deprivation 9:30~9:30(next day)	7 h cage tilt (45°) 11:00~18:00

Figure 1: The overall experimental plan of our research about the antidepressant-like effect of PS (*Piper sarmentosum* Roxb.) and PSY (ethyl acetate fraction of PS) (A). Schedule of CUMS procedure (B). The CUMS protocol consisted of the sequential application of a variety of mild stressors. These stressors were randomly scheduled over a one-week period from Day 1 to 7 and repeated for 3 weeks.

2.7 Sucrose preference test

The sucrose preference test was performed as described previously (Luo et al., 2008) with minor modifications. Test was carried out at the end of 4–week CUMS exposure. Briefly, 72 h before the test, rats were trained to drink 1% sucrose solution (w/v): two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water for 24 hours. At the end of adaptation, rats were deprived of water and food for 24 hours. Sucrose preference test was conducted at 9:00 a.m., in which rats were housed in individual cages and were permitted to access to two bottles containing 100 mL of sucrose solution (1%, w/v) and 100 mL of water, respectively. After 1 h, the volumes of consumed sucrose solution and water were measured, and sucrose preference was calculated by the following formula:

$$\text{Sucrose preference} = \frac{\text{Sucrose consumption}}{\text{Water consumption + sucrose consumption}} \times 100\%.$$

2.8 Measurement of serum corticosterone (CORT) concentration

24 h after the sucrose preference test, rats were deeply anesthetized with chloral hydrate, and blood was collected from the abdominal aorta and placed on ice. Serum was separated by centrifugation at 3500 × g for 10 min at 4℃ and stored at –80℃ until assay. The concentration of serum CORT was measured using a commercially available ELISA (enzyme-linked immunosorbent assay) kit (WUHAN BEINGLAY BIOTECH Co., LTD., Wuhan, Hubei, China). The ELISA was performed according to the manufacturer's instruction.

2.9 Quantitative real-time PCR

After the blood samples had been collected, the rats were sacrificed by decapitation. The brains were rapidly removed and the hippocampus was carefully dissected on ice and quickly

frozen at –80℃. Five hippocampi in each group were selected randomly to homogenize, and total RNA was extracted from the hippocampus with TRIzol reagent (Invitrogen, USA). The cDNA was synthesized using a commercial RT-PCR (reverse transcription-polymerase chain reaction) kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Real-time PCR was performed on an ABI Prism 7300 Sequence Detector system using a SYBR Green PCR kit (Thermo, USA) and normalized to GAPDH under the following conditions: initial denaturation at 95℃ for 10 min, followed by 40 cycles at 95℃ for 15 s, 60℃ for 45 s. At the end of the PCR reaction, a melting curve was obtained by holding at 95℃ for 15 s, cooling to 60℃ for 1 min, and then 95℃ for 15 s, 60℃ for 15 s. The primers used were as follows: BDNF: forward, 5'-TAGGCAGAATGAGCAATGTC-3'; reverse, 5'-CCCAAGAGGTAAAGTGTAGAAG-3'; GAPDH: forward, 5'-GTCGGTGTGAACGGATTTG-3'; reverse, 5'-TCCCATTCTCAGCCTTGAC-3'. The relative quantity was calculated according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.10 Western blot analysis

Rats were decapitated after blood collection, and the brain of each animal was quickly removed and the hippocampus was carefully dissected on ice and rapidly frozen at −80℃. Frozen hippocampus tissues (n=5/group) were homogenized in RIPA buffer containing protein inhibitors and phosphatase inhibitors with an ice bath. The homogenate was centrifuged at 12,000 × g (4℃) for 10 min and supernatants were collected. Protein concentration in the supernatants of hippocampus tissue extracts was estimated by a BCA protein assay kit (Thermo, USA). A same quantity (35μg) of protein from each hippocampus was loaded and separated by 10% SDS/PAGE gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore,

USA). Transferred membranes were blocked with 5% skim milk (or with BSA for phosphorylated protein) for 1 h, incubated overnight at 4℃ with primary antibodies: BDNF (1:500,Santa Cruz Biotechnology, USA), ERK1/2 (1:1000, Santa Cruz Biotechnology, USA), phospho-ERK1/2 (1:1000, Santa Cruz Biotechnology, USA), CREB (1:500, Santa Cruz Biotechnology, USA), phosphor-CREB (1:500, Santa Cruz Biotechnology, USA), GAPDH (1:1000, Santa Cruz Biotechnology, USA), and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology, USA) for 1h at 37℃. The blots were developed using enhanced chemiluminescence (ECL) (Pierce, USA). The optical density of the protein bands was scanned and analyzed using ImageJ2x software (National Institutes of Health, USA).

2.11 Statistical analysis

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) software version 19.0. The results were expressed as mean ± SEM. All data were statistically analyzed by one-way analysis of variance (ANOVA), followed by a post hoc LSD (least-significant difference) test. The differences were considered statistically significant when $P<0.05$.

3. Results

3.1 Antidepressant-like effects of PS extracts in the FST and TST in mice

In the FST, the results showed that PS extracts produced a strong antidepressant-like effect as well as fluoxetine (Figure 2A).The data were analyzed by a one-way ANOVA with drug treatment as the factor and revealed a significant effect of drug treatment ($F[4,45]=26.325$, $P<0.001$). Subsequent LSD as post hoc analysis indicated that doses of PS extracts from 50 to 200 mg/kg decreased the immobility time in the FST in a dose-dependent manner, as immobility time was

significantly reduced at the dose of 50, 100, 200 mg/kg of PS extracts (P<0.05; P<0.01; P<0.001, vs. control), fluoxetine also produced a significant reduction in immobility time (P<0.001, vs. control). Data from TST (Figure 2B) also revealed a significant effect of drug treatment (F[4,45]=3.006, P＜0.05). The LSD showed that a significant reduction in immobility time has been found in the PS treatment groups (100, 200 mg/kg; P＜0.01, P＜0.01) and fluoxetine group (P＜0.01) compared with controls. A dose-dependent effect in PS treatment groups has also been showed.

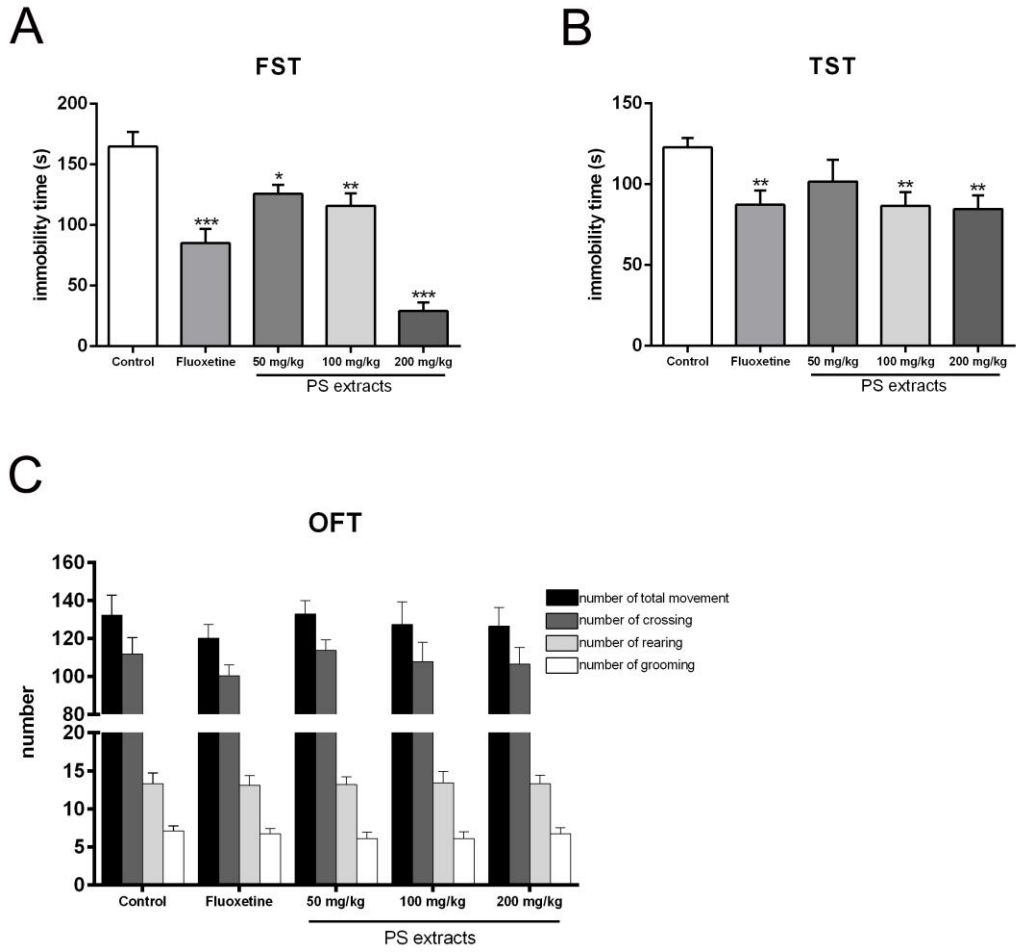


Figure 2 Antidepressant-like effect of PS (*Piper sarmentosum* Roxb.) in mice in the FST (forced swimming test) and TST (tail suspension test). The mice were treated with water (control), fluoxetine and PS. PS decreased the immobility time in the FST in a dose-dependent manner (A). PS decreased the immobility time in the TST in a dose-dependent manner (B). PS had no effect on the spontaneous activity in the OFT (open field test) (C). The data are presented as the mean ± SEM (n=10). *P < 0.05, **P < 0.01 and ***P

< 0.001 compared with the control group.

3.2 Effects of PS extracts on the spontaneous activity in the OFT

To exclude the possibility that the antidepressant-like effects in these two tests might be due to differences in locomotor activity (e.g. increases in locomotor activity causing a reduction in immobility), mice were subjected to OFT. The results (Figure 2C) showed that there was no significant difference in the number of crossing (F[4,45]=0.426, P=0.789), rearing (F[4,45]=0.008, P=1.000), grooming (F[4,45]=0.306, P=0.873) and total movement (F[4,45]=0.305, P=0.873) in all groups. Furthermore, it was demonstrated that PS extracts had no effect on locomotor activity, indicating that the reduction of immobility time observed in the FST and TST after PS treatment was not due to spontaneous locomotor hyperactivity.

3.3 Antidepressant-like effects of different fractions of PS extracts in the FST and TST in mice

To further explore the antidepressant-like effects of PS, the extracts were partitioned into four fractions (PSS, PSR, PSY and PSZ) according to the polarity of constituents. The data in FST (Figure 3A) showed a significant difference in immobility time reduction of different drug treatments (F[14,135]=8.022, P＜0.001). The further LSD test indicated that, compared with controls, FLX group (P <0.001), HP group (P <0.001), PSS groups (30, 60, 120 mg/kg; P＜0.05, P＜0.05, P＜0.01), PSR group (50 mg/kg; P＜0.01) and PSY groups (12.5, 25, 50 mg/kg; P＜ 0.01, P＜0.001, P＜0.001) significantly reduced the immobility time. In TST (Figure 3B), a significant reduction of immobility time (F[14,135]=7.711, P＜0.001) has also been discovered among treatment groups. The result of LSD showed that PSY (12.5, 25, 50 mg/kg; P＜0.05, P＜ 0.001, P＜0.001) made a significant reduction in immobility time as well as FLX (P＜0.001) and

HP ($P<0.001$) compared to the control group.

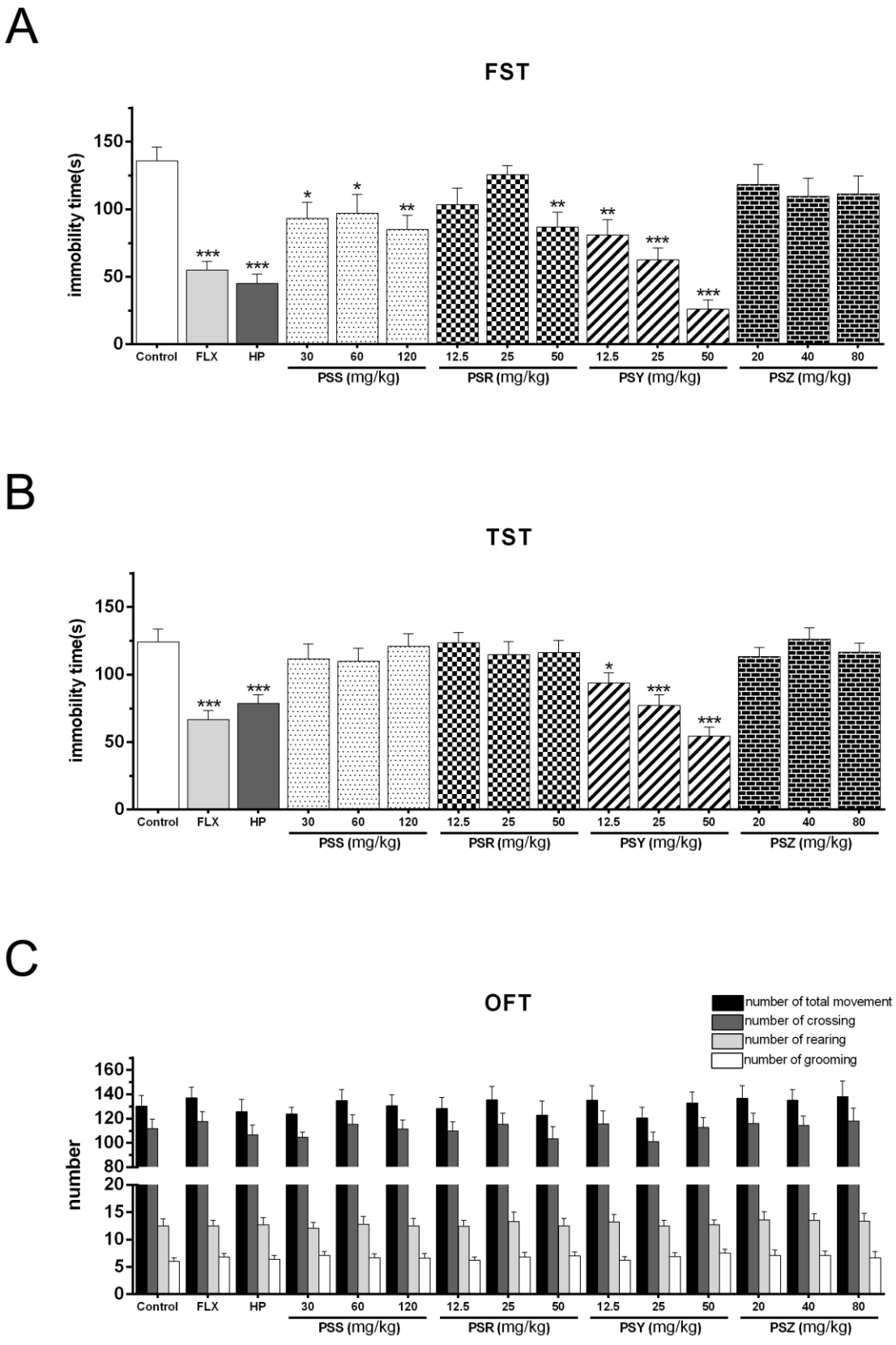


Figure 3 Different fractions of PS extracts produced antidepressant-like effect in mice in the FST and TST. The mice were treated

with water (control), FLX, HP extracts, PSS, PSR, PSY and PSZ. PSS (30, 60, 120 mg/kg), PSR (50 mg/kg) and PSY (12.5, 25, 50 mg/kg) decreased the immobility time in the FST (A). PSY (12.5, 25, 50 mg/kg) decreased the immobility time in the TST in a dose-dependent manner (B). Different fractions of PS extracts had no effect on the spontaneous activity in the OFT (C). The data are presented as the mean ± SEM (n=10). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the control group.

3.4 Effects of different fractions of PS extracts on the spontaneous activity in the OFT

In the OFT, the results (Figure 3C) showed that there was no difference in the number of crossing (F[14,135]=0.423, P=0.965), rearing (F[14,135]=0.129, P=1.000), grooming (F[14,135]=0.273, P=0.996) and total movement (F[14,135]=0.341, P=0.987) in all groups, proving that the drug treatments had no effect on spontaneous activity. This indicates that the reduction in immobility time observed both in FST and TST after PS administration was not related to locomotor hyperactivity.

3.5 PSY treatment reverses the CUMS-induced depressive behavior in rats

The work with mice has indicated that PS extracts may have antidepressant-like effects, and constituents of the PSY may also display similar properties. Therefore, we decided to focus on PSY in the following studies. To make further efforts on characterizing the antidepressant-like effects of PSY, we applied CUMS, which is currently considered as one of the most predictive animal models of depression (Forbes et al., 1996). In present study, we examined the effects of PSY on the sucrose preference and bodyweight as indexes of stress-induced responses. In sucrose preference test, the data (Figure 4A) showed that CUMS procedure resulted in a significant decrease in the sucrose consumption compared with controls, while treatment groups dramatically reversed the CUMS-induced reduction (F[5,54]=2.759, P＜0.05). Post hoc analysis indicated that

CUMS performed a significant reduction of sucrose consumption than control group ($P<0.01$), and compared with CUMS group, FLX ($P<0.01$) and PSY treatment groups (7.5, 15, 30 mg/kg; $P<0.05$, $P<0.05$, $P<0.01$) showed an increase in sucrose preference. The results not only demonstrated that CUMS model progressed successfully, but also indicated that PSY administration could reversed the CUMS-induced depressive behavior. The data of body weight gain was analyzed by ANOVA, and the results showed (Figure 4B) that the CUMS treatment significantly influenced body weight gain compared with controls ($F[1,58]=105.818$, $P<0.001$), demonstrating that CUMS reduces the growth in body weight seen in controls. Then we analyzed the body weight gain of all groups except controls by ANOVA, the results showed that there was no significant difference ($F[4,45]=0.686$, $P=0.606$) in any of the CUMS treatment groups (with or without drug administration).

3.6 PSY administration decreased the hyperactivity of the HPA axis induced by CUMS

Secretion of corticosterone by the hyperactivity of HPA axis is one of the most important neuroendocrine response to stress-caused stimuli (Stokes, 1995). Accordingly, we assessed the effects of PSY on serum corticosterone levels. In our studies, the results revealed (Figure 4C) that there was a significant effect of drug treatment on serum corticosterone levels ($F[5,54]=47.845$, $P<0.001$) by one-way ANOVA. Post hoc LSD analysis revealed that CUMS significantly increased the concentration of corticosterone in serum compared to control group ($P<0.001$). PSY (7.5, 15, 30 mg/kg; $P<0.01$, $P<0.001$, $P<0.001$) and FLX ($P<0.001$) treatment groups had a significant reduction in serum corticosterone levels compared with CUMS group. The results indicated that PSY reduced the CUMS-induced increase of serum corticosterone as fluoxetine did.

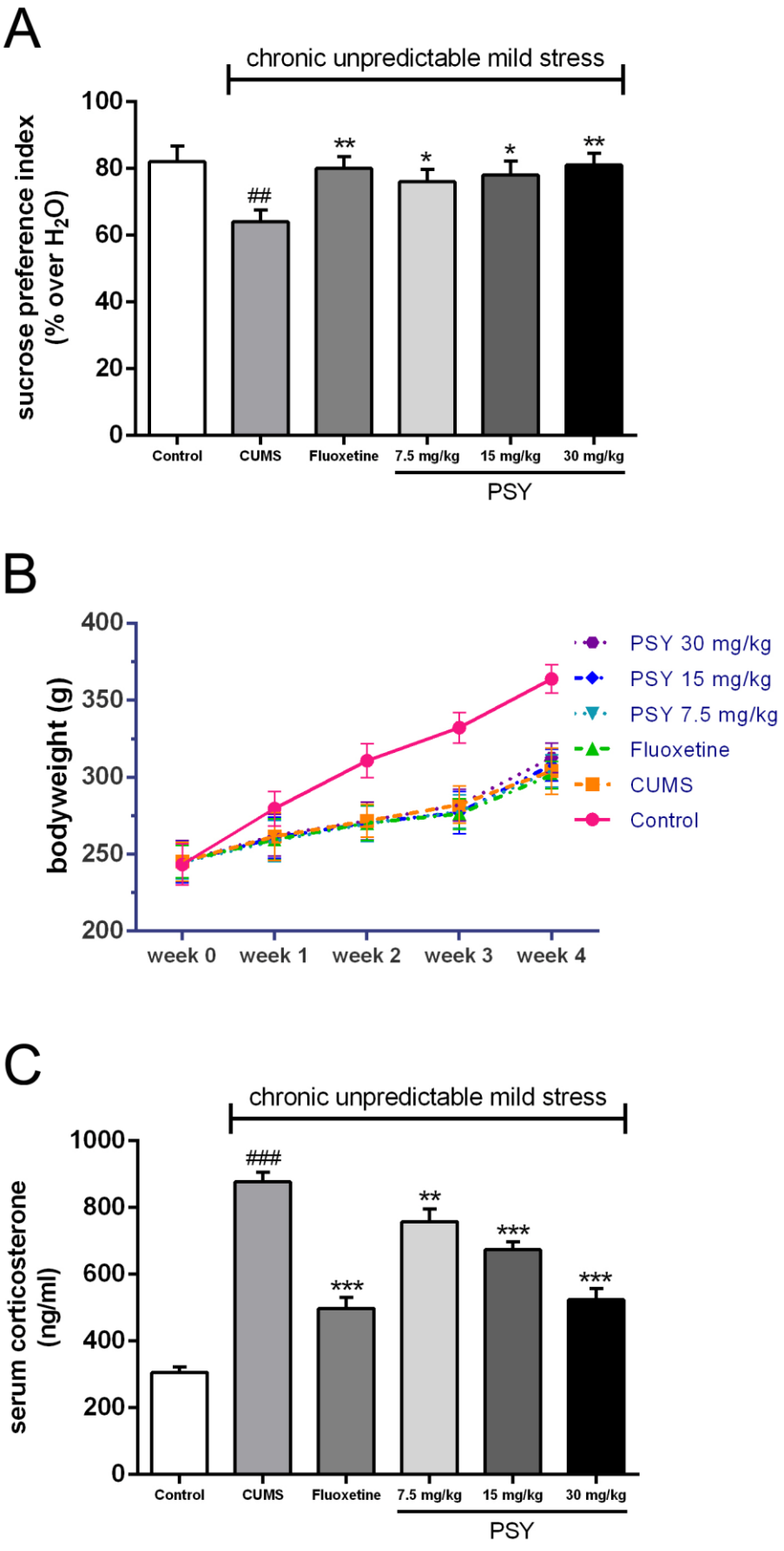


Figure 4 PSY reverses depressive-like behaviour in the CUMS rats. Rats were exposed to CUMS for 4 weeks and received a daily

treatment during the last week. PSY treatment increased the sucrose preference index compared to CUMS rats (A). The body weights of control rats were significantly higher than those of CUMS-exposed rats during the 4 consecutive weeks of CUMS; there was no significant difference between CUMS group and drug treatment groups (B). PSY treatment significantly reversed the CUMS-induced elevation of serum CORT levels (C). The data are presented as the mean ± SEM (n=10). $^{\#\#}P< 0.01$ and $^{\#\#\#}P < 0.001$ compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the CUMS group.

3.7 PSY restored the CUMS-induced decrease in the mRNA expression and protein levels of BDNF in the hippocampus of rats

The mRNA expression and protein levels of BDNF in the hippocampus is important for neurogenesis in the brain and plays a critical role in current hypothesis of depression (Dranovsky and Hen, 2006). Accordingly, we measured the mRNA expression of BDNF in the hippocampus following CUMS. In our studies, the BDNF mRNA expression was expressed as a ratio of GAPDH mRNA expression. The results (Figure 5A) were analyzed with one-way ANOVA and demonstrated that there was a significant difference in the expression of BDNF mRNA (F[5,54]=70.006,P＜0.001). The following LSD test showed that the BDNF mRNA expression in hippocampus of CUMS significantly decreased than controls (P＜0.001), and compared with CUMS group, PSY(7.5, 15, 30 mg/kg; P＜0.05, P＜0.001, P＜0.001) and FLX (P＜0.001) treatment groups had a significant increase in BDNF mRNA levels. Moreover, we also measured the BDNF protein levels with Western blot method, and the data were expressed as a ratio to GAPDH protein levels. The results (Figure 5B) showed that there was a significant difference in BDNF protein levels among the different treatment groups(F[5,24]=5.400, P＜0. 01). LSD test demonstrated that there was an obvious decrease of BDNF protein levels in CUMS group (P＜

0.001) compared with control group. While the BDNF protein levels of PSY (15, 30 mg/kg; $P<$ 0.05, $P<0.01$) and FLX ($P<0.05$) treatment groups were significantly increased than CUMS group. These results indicated that PSY treatment reversed the ability of stress effect to decrease BDNF in the hippocampus.

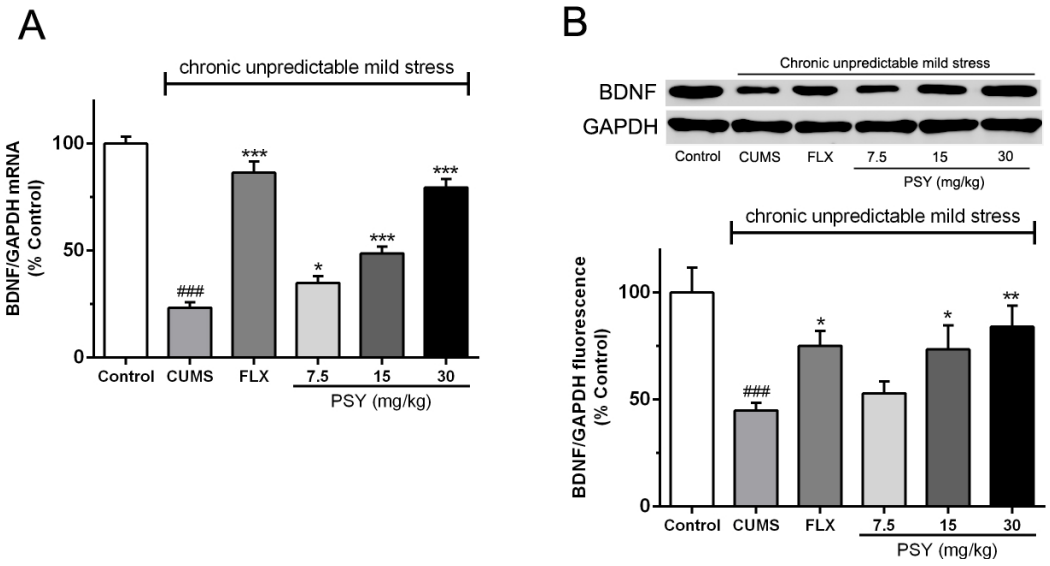


Figure 5 PSY treatment increases BDNF in the hippocampus of stressed rats. RT-PCR results showed that treatment with PSY reversed the decrease of BDNF mRNA expression in the hippocampus induced by CUMS (A). Western blotting results showed that PSY treatment increased the CUMS-induced reduction of BDNF protein in hippocampus (B). The data are presented as the mean ± SEM (n=10). $^{###}P < 0.001$ compared with the control group; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the CUMS group.

3.8 PSY increased the phosphorylation of CREB and ERK in the hippocampus of rats

CREB is an important transcription factor for BDNF in hippocampus, and it has also been involved in the actions of antidepressants (Lane-Ladd et al., 1997).The ERK is another key signal pathway of BDNF, and the phosphorylation of both CREB and ERK1/2 has been proposed as an intracellular common mechanism of antidepressant (Gourley et al., 2008). Therefore, we examined the phosphorylation of CREB and ERK among the treatment groups in the hippocampus, the

phosphorylation of CREB and ERK being expressed as a ratio of the pCREB/CREB and pERK/ERK. The results of CREB phosphorylation ratio (Figure 6A) showed that there was a significant effect among the treatment groups (F[5,24]=9.287, P＜0. 001) by one-way ANOVA. The following LSD test indicated that the ratio of CREB phosphorylation in CUMS group was significant lower than control group (P＜0. 001),and an obvious effect of increasing CREB phosphorylation was found in PSY (7.5, 15, 30 mg/kg; P＜0.05, P＜0.05, P＜0.01) and FLX (P ＜0. 001) treatment groups compared to CUMS. The same situation appeared in ERK phosphorylation, as the results shown (Figure 6B), there was a significant difference of ERK phosphorylation in treatment groups (F[5,24]=6.587, P＜0. 01). The post hoc LSD test demonstrated that, compared to controls, CUMS group had a significant decrease of ERK phosphorylation in hippocampus (P＜0. 001), while in PSY (7.5, 15, 30 mg/kg; P＜0.05, P＜0.01, P＜0.001) and FLX (P＜0.01) groups, the reduction of ERK phosphorylation levels induced by CUMS was completely reversed. These results indicated that PSY increased the phosphorylation level of CREB and ERK, which was obviously decreased by this model of depression.

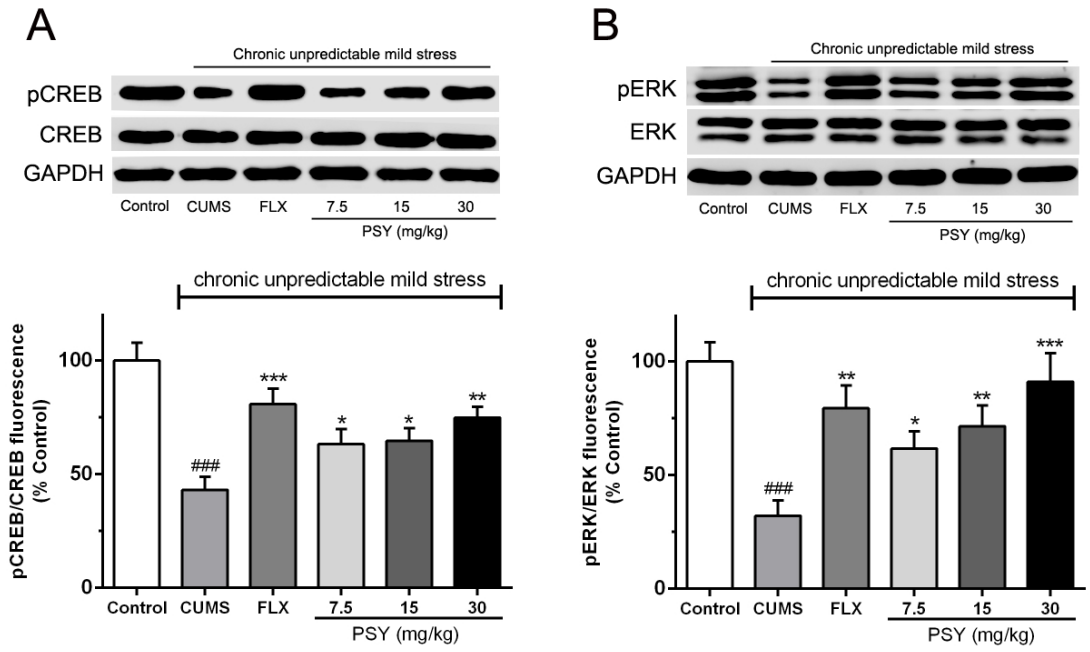


Figure 6 Effect of PSY on the protein phosphorylation of CREB and ERK1/2 in the hippocampus of CUMS rats. PSY reversed the reduction of CREB phosphorylation caused by CUMS in hippocampus (A). PSY restored the CUMS-induced inhibition of ERK1/2 phosphorylation in hippocampus (B). The data are presented as the mean ± SEM (n=10). $^{###}P < 0.001$ compared with the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the CUMS group.

4. Discussion

In our present study, we have demonstrated that PS and PSY produced powerful antidepressant-like effects in animal depression models, with comparable profiles to that observed for the established antidepressants, fluoxetine and HP. The results showed that administration of PS and PSY reduced immobility time in FST and TST, without the effect on spontaneous locomotor which was proved in OFT. PSY administration alleviated the depression-like behavior caused by CUMS, as indicated by an increased sucrose preference in rats. In addition, PSY treatment significantly reduced the serum corticosterone levels in rats, which had been elevated by CUMS. Moreover, PSY induced an increase of BDNF and up-regulated phosphorylated CREB and ERK protein levels in the hippocampus of rats similar to that obtained with fluoxetine.

Immobility time is the characteristic behavior index in FST and TST, which reflects behavioral despair as seen in human depression (Steru et al., 1985). OFT is usually used to make sure that antidepressant-like effect is independent of spontaneous activity. Our result indicated that treatment with PS or PSY produced significant antidepressant-like effects, when assessed in FST and TST, and the date of OFT demonstrated that PS and PSY had no effect on locomotor activity, indicating that the antidepressant-like activity of PS and PSY is specific. What is noteworthy is that, a dose-dependent effect of both PS (dose from 50 mg/kg to 200 mg/kg) and PSY (dose from

12.5 mg/kg to 50 mg/kg) has been found in FST and TST. More importantly, the antidepressant-like effects of PS and PSY at high dose (PS: 200 mg/kg, PSY: 50 mg/kg) were similar to that of fluoxetine or HP, suggesting that PS may have the potential of being developed as a novel antidepressant.

CUMS is a well-known effective model to imitate the pathogenesis of depression (Bhutani et al., 2009). In this study, rats in CUMS displayed representative anhedonia behavior, as indicated by their decreased sucrose preference. Anhedonia is a prominent symptom of depression. The preclinical analogues of the anhedonia scales and the procedures most commonly used to assess depression-like behaviour in rodents are the sucrose intake and preference tests (Der-Avakian and Markou, 2012). In the present study, our results confirmed that similar to fluoxetine, PSY treatment could reverse the decreased sucrose preference index, indicating a positive antidepressant-like effect of PSY.

It is reported that the response to stress is mediated by the HPA axis, which is considered an important pathway in the pathogenesis of depression (Swaab et al., 2005). A potent stressor can activate the HPA axis hyperactivity, leading to the release of CORT into the blood. Treatment with some antidepressant drugs, such as fluoxetine attenuated the stress-induced elevation in serum CORT levels. Consistent with this hypothesis, in our present study, the CUMS caused hyperactivity of the HPA axis in rats, as indicated by the elevation of serum CORT, and administration of PSY and fluoxetine significantly reversed the CMUS-induced increase of serum CORT levels. Moreover, there is also a dose-dependent effect of PSY (from 12.5 mg/kg to 50 mg/kg) to reduce serum CORT. It is indicated that the antidepressant-like effects of PSY were accompanied by alterations in the HPA axis.

There is increasing evidence which suggests that high corticosterone concentration and chronic stress can result in the down-regulation of BDNF (Kunugi et al., 2010). Infusion of BDNF into the hippocampus area produced antidepressant-like behavioral effects in rats (Shirayama et al., 2002). Therefore, it is indicated that BDNF is an important factor in the pathogenesis of depression, and increasing BDNF levels might be a common pathway for antidepressants to exert therapeutic functions (Swaab et al., 2005). In the present study, the results demonstrated that treatment with PSY reversed the CUMS-induced reduction of BDNF mRNA expression and protein levels in the hippocampus of rats. This effect of PSY was similar to the established antidepressant drug fluoxetine, which has been reported previously with effect of increasing BDNF (Ubhi et al., 2012). Our results indicated that antidepressant-like effects of PSY were related to the alteration of BNDF levels.

We also examined the changes in CREB and ERK activities. The cAMP signaling pathway has been implicated in antidepressant-like effect after chronic treatment and there is an obvious connection to BDNF. As a transcription factor, CREB plays a critical role in neuronal plasticity and neurogenesis which enhance stress response and etiology of depression, and it is activated through phosphorylation. Increasing evidence points to the fact that stress exposure is associated with the reduction of CREB expression in hippocampal (Alfonso et al., 2006). Previous studies suggest that decreasing CREB function may contribute to clinical depression pathophysiology, and the increasing of it may also be an important part of the antidepressant response in humans (Pittenger and Duman, 2008). Additionally, it has been reported that CREB up-regulation may activate down stream targets, one of these targets being BDNF (Tao et al., 1998). The expression of BDNF is dependent on CREB activation and this procedure may be a key point of the

therapeutic responses to antidepressants (Nair and Vaidya, 2006). MAPK is a major signal system that regulates cellular responses and activation of the MAPK cascade plays an important role in the pathophysiology of depression (Roux and Blenis, 2004). It has been indicated that inhibition of MAPK produces a depressive phenotype and blocks behavioral effects of antidepressants. ERK including ERK1 and ERK2 belongs to the MAPKs family, and accumulating evidences suggest that ERK signal path way may take part in pathogenesis of depression and the mechanism of antidepressant-like action (Gourley et al., 2008). ERK is one of the most-studied intracellular signaling pathways and it is highly sensitive to stress and closely associated with mood processing (Gerrits et al., 2006; Rubinfeld and Seger, 2005). Moreover, recent studies have demonstrated a positive modulation of ERK1/2 by antidepressant drugs such as fluoxetine (Kuo et al., 2013). Additionally, accumulating evidences suggest that activation of ERK might be attributable to BDNF-mediated neuronal function. ERK1/2 was persistently activated by a long-lasting induction of BDNF, in a social defeat stress model, suggesting a key role for ERK1/2 in BDNF expression (Berton and Nestler, 2006; Leem et al., 2014). In the present study, the results further confirm that exposure to CUMS was able to inactivate the CREB and ERK 1/2 as signaling and transcription factors in the hippocampus through phosphorylation, and it was also demonstrated that administration of PSY was able to reverse CUMS-induced decrease in phosphorylation of CREB and ERK 1/2, as well as classic antidepressant fluoxetine. These results suggest the involvement of CREB and ERK in the antidepressant-like effects of PSY. Furthermore, a growing number of studies suggests that CREB is a transcription factor and downstream target of ERK signal pathway (Xing et al., 1996). It is reported that the increasing of CREB phosphorylation by fluoxetine is accompanied by the up-regulation of ERK activation, suggesting an important role of the ERK

signal pathway in the increase of CREB phosphorylation (Qi et al., 2008). ERK activity is able to induce phosphorylation of CREB at a specific serine residue, serine133, producing an active transcription complex enabling target gene activation (Conkright et al., 2003), and it is demonstrated that ERK-CREB signal system may play a critical role in the molecular mechanism of depression (Guan et al., 2013). In summary, ERK, CREB and BDNF-mediated signal pathways are indicated to be implicated in the neuroplasticity alterations induced by antidepressants (Covington et al., 2010; Vialou et al., 2013). These previous studies suggest that a CREB-BDNF-ERK circle signal pathway may be involved in the pathogenic mechanism of depression. So, combined with our results, we suggest that PSY induces antidepressant-like effect either on one or multi-target systems in CREB-BDNF-ERK circle signal pathway.

Nowadays, it is well-known that many chronic and complex diseases have a complicated pathophysiology mechanism. These diseases are not regulated by a single molecular target but caused by multi-factorial, and there are often multiple ways or alternate processes that may be switched on in response to the inhibition of a specific target (Home et al., 2009; Rather et al., 2013), for which a new technical term polypharmacology has been proposed (Efferth and Koch, 2011; Xie et al., 2012). Depression is one of these multi-target diseases, and various hypothesis or protein factors participate in its pathogenesis, such as monoaminergic hypothesis, HPA axis hypothesis, BDNF, CREB and ERK signaling pathways. It is suggested that a balanced modulation of several targets can offer a better therapeutic effect with less side effects compared to a single selective ligand, significantly in the treatment of chronic and complex diseases (Morphy et al., 2004). In recent years, the development of new drugs aimed at a single molecular target has showed a down trend (Firman et al., 2012). At present, efforts are devoted to finding

new therapeutics focus on multiple targets, which has become a novel direction in drug discovery (Zhang, 2005). There are two available strategies to meet the multiple targets implicated in complex diseases such as depression. The first one makes an attempt on utilizing a single compound to hit the multiple targets, another one is employing two or more active ingredients being constituted in one drug to hit the multiple targets. Therefore, it is worthy to pay attention to that some natural products can affect multiple targets to generate multiple pharmacological activities, producing significant physiological effects, and providing benefits due to the synergistic action that cannot be observed with a single compound (Chan and Loscalzo, 2012). These characteristics, along with their reliable clinical application, have thrust traditional Chinese herbal medicines into the limelight again, and scientists worldwide are becoming increasingly interested in the evaluation of herbal medicines products. PS is one of the Chinese herbal medicines, and in present studies, the antidepressant-like effect of PS extract and PSY may due to the action of their bioactive ingredients on multiple targets, such as HPA axis, BDNF, CREB and ERK.

In conclusion, the present studies show that PS and PSY extracts exerted antidepressant-like effect in animal models of depression. Moreover, it is indicated that the increased expression of BDNF, phosphorylated CREB, phosphorylated ERK1/2 and the hyperactivity of HPA axis may be involved in the mechanism by which PS is effective as an antidepressant. However, further work is required to make PS into a novel antidepressant with higher efficacy and fewer side effects.

Contributors

Prof. Lu-Ping Qin and Prof. Ting Han designed the study, and wrote the protocol and the first draft of the manuscript. Yue Gao managed the literature searches. Qing Li performed animal

model experiments and managed the statistical analyses. Fa-Lin Qu performed the gene expression experiments. Yi-Ping Jiang, Khalid Rahman and Kuo-Hsiung Lee wrote parts of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

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