# Treatment with Shuyu capsule increases 5-HT1AR level and activation of cAMP-PKA-CREB pathway in hippocampal neurons treated with serum from a rat model of depression

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**Abstract.** Depressive disorder (DD) is one of the typical affective disorders with a high morbidity, high suicide rate and high recurrence rate. Dysfunction of the 5-hydroxytryptamine 1A receptor (5-HT1AR) in the brain may serve an important role in the pathogenesis of DD. Currently, selective serotonin reuptake inhibitors are the first line antidepressants with 60-70% efficacy and severe adverse effects. Previous studies have demonstrated that Chinese herbal medicines, including the Shuyu capsule (SYC), are effective antidepressants with few side effects. However, the mechanism remains unclear. In the present study, the effects of the SYC on the 5-HT1AR level and the activation of adenylyl cyclase-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA)-cAMP response element-binding (CREB) signaling pathway that 5-HT1AR mediates in the cells of hippocampal neurons were investigated *in vitro*. The SYC demonstrated an antidepressant effect similar to that of fluoxetine in a rat depression model. Treatment of hippocampal neurons with the serum of depressive rats resulted in a decrease in the 5-HT1AR protein level and the activation of the cAMP-PKA-CREB signaling pathway in hippocampal neurons. Exposure to the serum of rats that received chronic mild stress plus SYC treatment led to no alterations in the 5-HT1AR level or the activation of the cAMP-PKA-CREB signaling pathway compared with those of cells exposed to normal rat serum. This effect is similar to the effects of 5-HT1AR antagonist WAY-100635. In addition, the 5-HT1A agonist 8-hydroxy-(dipropylamino) tetralin did not antagonize the effects of the SYC. Furthermore, the SYC exhibited an increased effect compared with fluoxetine on 5-HT1AR levels and CREB activation. The present study suggested that the SYC functions by increasing 5-HT1AR protein levels and the activation of the 5-HT1AR-mediated cAMP-PKA-CREB signaling pathway in hippocampal neurons.

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*Key words:* depressive disorder, Chinese herbal medicine, Shuyu capsule, 5-hydroxytryptamine 1A receptor, signaling pathway, hippocampal neuron

## Introduction

Depressive disorder (DD) imposes a severe burden on the afflicted individuals and is becoming increasingly prevalent in modern society (1). The most generally accepted hypothesized pathogenesis of DD entails a depletion in levels of monoamines including 5-hydroxytryptamine (5-HT), norepinephrine and dopamine in the central nervous system (2). Therefore, selective serotonin reuptake inhibitors (SSRIs) including fluoxetine, paroxetine, fluvoxamine, sertraline and citalopram are currently the first line antidepressants (3,4). Monoaminergic antidepressants serve crucial roles in depression therapy, however their efficacy is only 60-70% and the side effects include sexual problems, drowsiness, fatigue, sleep difficulties, nausea, weight gain, nervousness, dry mouth and blurred vision (3,4) indicating that alternative treatments are necessary (5).

Chinese herbal formulas have advantages in antidepressant treatment with broad prospects for development (6). The classical Chinese herbal formula Xiao Yao San (XYS) has been verified to have an antidepressive effect with few side effects in clinical studies (7,8). However, its composition of six Chinese herbs leads to difficulties in identifying its pharmacological mechanism.

The Shuyu capsule (SYC), a novel Chinese herbal formula preparation developed by the authors' research team based on a modification of XYS, was used in a clinical therapy for depression approved by the China Food and Drug Administration (Approval Number: 2008L11169). The SYC is composed of active ingredients extracted from four Chinese herbs, namely *Bupleurum chinensis*, *Paeonia sufruticosa*, *Cyperus rotundus* and *Glycyrrhiza uralensis* Fisch. Although previous pharmacological studies have revealed that components of the SYC

have antidepressant effects, the antidepressant mechanism of the SYC remains unclear (9-12). The authors' previous study demonstrated that levels of the 5-HT1A receptor (5-HT1AR) decrease in the hippocampus of rats with chronic mild stress (CMS)-induced depression and increase with use of the SYC (13).

5-HT1AR is of primary research interest, due to its involvement in depression and anxiety states (14). The therapeutic effect of the most commonly prescribed antidepressants, including SSRIs and the partial 5-HT1AR agonist buspirone, may in part be associated with the enhancement of 5-HT neurotransmission in the hippocampus, involving the 5-HT1AR (15-17). In line with these results, a reduced binding potential and mRNA level of the 5-HT1AR have been observed in the hippocampus of major DD patients (18,19).

The 5-HT1AR has been tied to a variety of physiological and pathological processes as it is involved in a number of signal transduction pathways. A previous study observed that 5-HT1AR is an inhibitory G-protein coupled receptor (20). Agonist binding to 5-HT1ARs exchanges GDP for GTP on the subunit of Gi/o and then inhibits adenylyl cyclase (AC), resulting in decreased intracellular cyclic adenosine monophosphate (cAMP) production (21). In turn, cAMP, as an important second messenger, mediates a number of intracellular signaling cascades, including the protein kinase A (PKA)-cAMP response element-binding (CREB) signaling pathway (22). A number of animal tests have indicated that the cAMP-PKA-CREB signaling pathway in hippocampus is closely related to depression and the pathogenesis of cognitive function impairments (23). Chronic unpredictable stress reduced the expression of cAMP, PKA, CREB in the hippocampus of model rats (24). This signaling pathway contributes to impaired neurogenesis, similar to depressive-like behaviors (25).

To determine whether the SYC serves antidepressive roles partly by acting on 5-HT1ARs in the brain, the effects of the SYC on the expression level of the 5-HT1AR and activation of the 5-HT1AR-mediated AC-cAMP-PKA-CREB signal transduction pathway were investigated in hippocampal neurons *in vitro*, using a serum pharmacological method.

## Materials and methods

*Animals and primary reagents*. A total of 40 male adult Wistar rats [Charles River Laboratories, Beijing, China, License No. SCXK (Beijing, China) 2012-0001], weighing 110-130 g and 6-8 weeks old, were used in the experiment. The rats were given 1 week upon arrival to adjust to the novel environment (21±1˚C, 50-60% humidity, white noise (40±10 dB) and a 12-h light/dark cycle with light from 8:00 p.m. to 8:00 a.m.). Food and water were available freely prior to the experimental procedures. The rats were housed separately according to the chronic mild stress (CMS) procedure. Wistar rats born within 24 h were purchased from the Laboratory Animal Center, Shandong University of Traditional Chinese Medicine, Shandong China, [SCXK (Lu) 20120003] and sacrificed upon arrival. All animal care procedures were carried out in accordance with the National Institutes of Health Guide for Care and were approved by the Institutional Committee for Animal Care and Use of Shandong University of Traditional Chinese Medicine (Approval ID: DWSY201206102).

The SYC was purchased from the Haichuan Research Center for Innovative Biological Natural Drug Discovery (Qingdao, China). The quality and stability of the SYC were the same as those in a previous study (26). Neurobasal A media, L-glutamine, a B27 supplement and fetal bovine serum were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The rat cAMP ELISA kit (cat. no. F15181) was purchased from Xi Tang Biotechnology Co., Ltd., (Shanghai, China). The 5-HT1AR primary antibody (cat. no. ab44635) and 5-HT1AR antagonist WAY-100635 (cat. no. ab120550) were purchased from Abcam (Cambridge, UK). Primary antibodies against PKA (cat. no. 5842) and CREB (cat. no. 9197) in addition to the activated (phosphorylated) versions of these proteins (cat. nos. 4781 and 9191) were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Mouse monoclonal antibodies against β-actin (cat. no. A1978) and the 5-HT1AR agonist (R)-(+)-8-hydroxy-DPAT hydrobromide (cat. no. H140) were obtained from Sigma-Aldrich; Merck KGaA, (Darmstadt, Germany).

*Animal treatments*

*Experimental procedure and grouping*. The depression rat model was established using CMS as described below. Prior to stress, all adult rats were randomly divided into the following four groups (n=10 per group): i) Control group [received intragastric (ig) administration daily with normal saline at 5 ml/kg·d]; ii) model group (received stress plus normal saline at 5 ml/kg·d, ig, daily); iii) SYC group (received stress and SYC at 0.408 g/kg·d ig, daily); and iv) fluoxetine group (received stress and fluoxetine 2.67 mg/kg·d, ig, daily). The treatments lasted for 4 weeks. Prior to treatments and at the end of every week during treatments, a sucrose preference test and body weight measurement were performed. Finally, 1.5 h following the last dose, sodium pentobarbital anesthesia at 45 mg/kg, intraperitoneally, was administered to the rats. Abdominal aorta blood (as much as possible) was collected, left for 1 h and then centrifuged at 800 x g for 15 min at 4˚C; subsequently, the serum was obtained from all rats in all groups and inactivated in a 56˚C water bath for 30 min, filter sterilized through a 0.45-$\mu$m membrane, sealed, and preserved at -70˚C for cell treatment.

*CMS procedure*. The CMS protocol consisted of water deprivation for 24 h, food deprivation for 24 h, cage rotation for 30 min, a cage tilt of 45˚C for 16 h, white noise (100 dB) for 3 h, wet bedding (100 ml water/individual cage) for 17 h, empty water bottles for 1 h, restricted access to food for 2 h, gauze bundling for 1 h, continuous lighting for 24 h, mothball odor for 24 h, a foreign object in the cage for 17 h and stroboscopic lighting (100 frequency/min) for 1 h. These stressors were sequential and mild, as described by Willner *et al* (27). The stressors were applied in a random sequence. The CMS procedure was administered to the stressed rats once per day for 4 weeks.

*Sucrose preference test (SPT)*. The sucrose preference test was performed on days 0, 7, 14, 21 and 28. Prior to the start of the test, the rats were trained to consume 1% sucrose solution. They

were habituated for 48 h to two bottles, one with 1% sucrose and the other with tap water, followed by a period of 24 h with no food or water available, and a 1 h exposure to the two identical bottles again for testing fluid consumption. To have a concordance measure for all groups, each rat in a control group was randomly selected and housed individually at the beginning of this test. Two-bottle tests for each cage were adopted throughout the procedure. Sucrose solution consumption was recorded by calculating the volume of the test solution. Sucrose preference=sucrose consumption (g)/[sucrose consumption (g) + tap water consumption (g)]x100%.

*Hippocampal neuron culture and treatments*

*Cell culture and appraisal*. Primary cultures of dissociated hippocampal neurons were performed as previously described (28). Hippocampi were dissected from neonatal rats within 24 h and collected in Hank's balanced salt solution (HBSS) containing 1% penicillin/streptomycin and 14 isolated hippocampi were then dissociated at 37˚C for 15 min in 0.25% trypsin-EDTA. The digestion medium was then replaced with a dissection buffer (HBSS solution containing 1% penicillin/streptomycin) and they were centrifuged at 200 x g for 1 min at room temperature twice. Pelleted cells were centrifuged at 300 x g for 5 min at room temperature and washed with inoculated culture medium (Neurobasal A media plus 0.5 mM L-glutamine, the B27 supplement and 10% fetal bovine serum). Hippocampus cells ($5x10^6$ cells/$cm^2$) were plated into six-well plates precoated with poly-L-lysine in inoculated culture medium followed by incubation at 37˚C in a humidified atmosphere containing 5% $CO_2$. The entire medium was replaced with fresh inoculated culture medium following 12 h. Half of the medium was then replaced with fresh serum-free neuronal maintenance medium (Neurobasal A media plus 0.5 mM L-glutamine and the B27 supplement) twice weekly. The cell viability was measured using 5 mg/ml MTT with the formazan dissolved in dimethyl sulfoxide and absorbance read at 490 nm.

*Cell treatment*. To detect the effect of the SYC on 5-HT1AR protein levels in primary cultured hippocampal neurons, cells were randomly divided into the following groups: i) Control group (with no serum treatment), ii) normal group (treatment with serum from normal rats), iii) model group (treatment with serum from CMS rats), iv) SYC group (treatment with serum from SYC rats) and v) fluoxetine group (treatment with serum from fluoxetine rats). All serum treatments were administered for 48 h at 10% concentration.

To investigate the role of 5-HT1AR and the AC-cAMP-PKA-CREB signaling cascade activity in the antidepressive effect of SYC treatment on hippocampal neurons, primary cultured hippocampal neurons were divided randomly into seven groups: i) Control group (with no serum treatment); ii) normal group (treatment with serum from normal rats); iii) model group (treatment with serum from CMS rats); iv) SYC group (treatment with serum from SYC rats); v) fluoxetine group (treatment with serum from fluoxetine rats); vi) model plus 5-HT1AR agonist group (treatment with serum from CMS rats plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min); and vii) model plus 5-HT1AR antagonist group (treatment with serum from CMS rats plus 0.5 $\mu$M WAY-100635 stimulation for 60 min). All serum treatments lasted for 24 h at 10% concentration.

To further verify the role of the 5-HT1AR-mediated AC-cAMP-PKA-CREB postreceptor signal transduction pathway in the antidepressive effect of the SYC, hippocampal neurons that underwent primary culture *in vitro* were divided into four groups: i) Normal group (with no serum treatment); ii) model plus 5-HT1AR agonist group (treatment with serum from CMS rats plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min); iii) SYC plus the agonist group (treatment with serum from SYC rats plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min); and iv) fluoxetine plus the agonist group (treatment with serum from fluoxetine rats plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min). All serum treatments lasted for 24 h at 10% concentration.

*Cell protein extraction*. Following treatments, all cell groups ($5x10^6$ cells/group) were washed twice with HBSS and then lysed in 100 $\mu$l radioimmunoprecipitation lysis buffer (containing 1 $\mu$l phenylmethylsulfonyl fluoride and 10 $\mu$l phosphatase inhibitors). Following incubation on ice for 30 min, cell lysates were centrifuged at 13,000 x g for 15 min at 4˚C. The supernatants were collected and protein concentrations were determined using a commercial bicinchoninic Protein Assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology, Haimen, China).

*ELISA assay*. The concentration of cAMP in the cell lysate of hippocampal neurons in each group was measured using a rat cAMP Elisa kit (cat. no. F15181; Westang Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol.

*Western blot analysis*. Equal denatured cell lysates (45 $\mu$g) were separated using 10% SDS-PAGE and then electrotransferred onto nitrocellulose membranes. Following being blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, the membranes were incubated overnight at 4˚C with a respective primary antibody in TBST containing 5% nonfat milk. The following primary antibodies were used: 5-HT1AR (1:1,200), PKA (1:700), phosphorylated (p)-PKA (1:800), CREB (1:1,500), p-CREB (1:1,500) and β-actin (1:3,000). All antibodies were dissolved in 0.5% blocking reagent. Following being washed three times with TBST, the membranes were incubated for 70 min at room temperature with a secondary antibody, either goat antirabbit immunoglobulin (Ig)G-horseradish peroxidase (HRP; Jingmei, cat. no. SB200; 1:4,000) or goat antimouse IgG-HRP (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-2005; 1:4,000). The protein bands were detected by enhanced chemiluminescence (EMD Millipore, Billerica, MA). ImageJ software version 2.1.4.7 (National Institutes of Health, Bethesda, MD, USA) was used for quantification.

*Statistical analysis*. All experiments were performed at least three times and the values represent the means + standard deviations. The significance of differences was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. When two factors were assessed, the significance of the differences was determined using two-way

ANOVA followed by Bonferroni post hoc tests. Prism software 5.0 (GraphPad software, Inc., La Jolla, CA, USA) for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Effect of SYC on depression-like behavior and body weight of CMS-induced model rats*. Rat body weight in all groups increased throughout the experiment (Fig. 1A). The model group had a significantly decreased body weight compared with the control group following the implementation of 2 weeks of stress (P<0.05). The SYC group and fluoxetine group had similar body weight gain to that of the control group and the two groups had significant weight gain in the third week (P<0.05) and in the fourth week (P<0.05) compared with the model group (Fig. 1A).

Anhedonia, defined as the inability to feel pleasure from usually enjoyable activities, is a core syndrome of depression (29). The SPT is a common depression-like behavior test for anhedonia in rats (30). At the 28th day, sucrose solution intake was significantly lower in the CMS-induced model group compared with the control group (P<0.05; Fig. 1B). SYC and fluoxetine treatment led to an increase in sucrose intake in the fourth week, indicating a significant difference compared with the model group (P<0.05). However, there were no significant differences among the SYC group, fluoxetine group and the control group, suggesting that the SYC and fluoxetine groups did not increase the sucrose intake compared with control. They simply prevented it from decreasing (keeping it at control levels) as happened in the model group. There was no substantial difference between the SYC group and fluoxetine group (Fig. 1B).

*Effect of SYC on 5-HT1AR expression in cultured hippocampal neurons treated with serum from depressive rats*. To investigate the effect of the SYC on 5-HT1AR protein levels in hippocampal neurons *in vitro*, western blotting was performed following exposure of cultured hippocampal neurons to serum from rats in the different groups for 48 h. There was no significant difference in 5-HT1AR protein levels between cultured neurons with no serum treatment and those with serum from normal rats (Fig. 2). A one-way ANOVA revealed that the serum of the depression model rats induced a significant reduction of 5-HT1AR expression in hippocampal neurons compared with the control (P<0.05). Compared with the serum from the model rats, the sera of the rats in the SYC and fluoxetine groups elicited a significant increase in 5-HT1AR expression (P<0.05). Additionally, there was a significant difference between the SYC group and the normal group (P<0.05). Compared with the fluoxetine group, the expression of the 5-HT1AR in the SYC group increased significantly (P<0.05). These results indicated that treatment with the depression model rat serum for 48 h resulted in a decrease of the 5-HT1AR in hippocampal neurons, whereas the SYC and fluoxetine effectively prevented 5-HT1AR reduction, demonstrating that the 5-HT1AR was the target of SYC and fluoxetine. The effect of the SYC on 5-HT1AR protein expression was increased compared with fluoxetine, with the expression being greater than normal levels, suggesting that SYC serves a stronger regulation effect on the expression of 5-HT1AR.

*Effect of SYC on cAMP-PKA-CREB pathway in cultured hippocampal neurons*. The cAMP-PKA-CREB signaling pathway is the primary second messenger cascade mediated by the 5-HT1AR, in addition to other receptors. To investigate the effect of the SYC on 5-HT1AR activity in hippocampal neurons, the activation of this cascade was measured. The concentration of cAMP was first measured in cultured hippocampal neurons (Fig. 3A). There was no significant difference between the control group and the normal group. The model group exhibited a significant decrease in the concentration of cAMP compared with the normal group (P<0.05), however SYC and fluoxetine treatment significantly increased the cAMP compared with the model group (P<0.05). Treatment with model rat serum plus 8-hydroxy-DPAT resulted in a significantly decreased cAMP concentration compared with the normal group (P<0.05) however there was no significant difference with the model group, whereas treatment with WAY-100635 was able to return the concentration to normal levels compared with the model group. Alterations in phosphorylation levels of PKA and CREB were similar to those in cAMP concentrations (Fig. 3B and C). These results demonstrated that treatment with the serum of depressive rats led to reduced cAMP-PKA-CREB signaling pathway activity mediated by the 5-HT1AR, whereas the SYC and fluoxetine reversed the reduction, having similar effects to those of the 5HT1AR antagonist.

*Effect of SYC on 5-HT1AR-mediated cAMP-PKA-CREB signaling pathway in cultured hippocampal neurons*. To further investigate the effect of the SYC on the 5-HT1AR-mediated cAMP-PKA-CREB cascade, the activities of the components of the cascade were measured again in hippocampal neurons treated with the serum of SYC or fluoxetine rats plus the 5-HT1AR agonist 8-hydroxy-DPAT. As exhibited in Fig. 4, the treatment resulted in a significant increase in the concentration of cAMP to normal levels (P<0.05) as well as the phosphorylation levels of PKA and CREB. The SYC had a stronger effect on CREB phosphorylation compared with fluoxetine. These results suggest that the SYC effectively restores the 5-HT1AR-mediated cAMP-PKA-CREB signaling pathway in primary cultured hippocampal neurons treated with the serum of DD rats.

## Discussion

The 5-HT1AR and its linked signaling pathways are known to serve crucial roles in depression. In the present study, it was demonstrated that the 5-HT1AR protein level and activation of the 5-HT1AR-AC-cAMP-PKA-CREB signaling pathway in hippocampal neurons are antidepressant targets of the SYC, a traditional Chinese herbal compound composed of four herbs (*Bupleurum chinensis, Paeonia sufruticosa, Cyperus rotundus* and *Glycyrrhiza uralensis* Fisch). Although previous reports have demonstrated the antidepressant effects of these herbs and formulas with these herbs have been employed as primary constituents in animal experiments, the 5-HT1AR and the signaling pathway that it mediates have not been

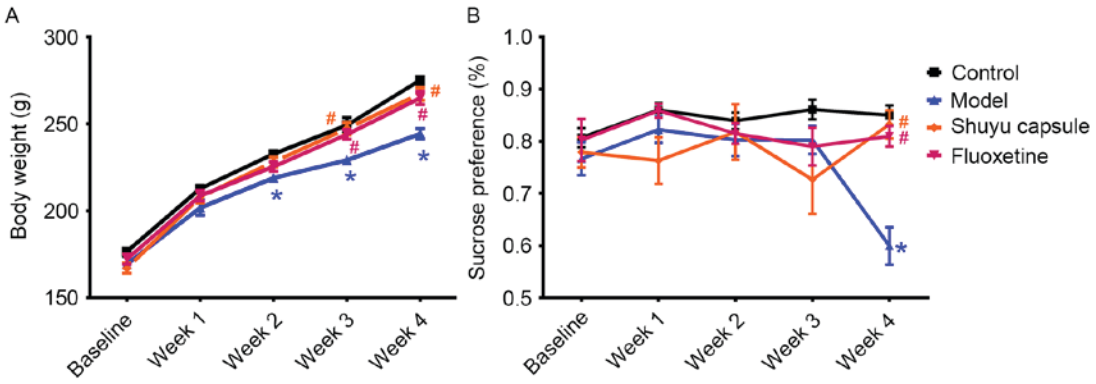


Figure 1. Sucrose preference test and body weight measurement of rats in all groups. (A) Body weight of rats and (B) sucrose preference in groups prior to treatments (baseline) and at the end of every week during treatments for 4 weeks. The rats were exposed to different treatments as follows: Control group (received ig administration daily with normal saline at 5 ml/kg·d), model group (received stress plus normal saline at 5 ml/kg·d, ig, daily), SYC group (received stress and SYC at 0.408 g/kg·d, ig, daily) and fluoxetine group (received stress and fluoxetine 2.67 mg/kg·d, ig, daily). *P<0.01 vs. the control. #P<0.01 vs. the model group. SYC, Shuyu capsule; ig, intragastric; daily, four times daily.

implicated in the antidepressant mechanism of these natural products (9-12). The present study, to the best of the author's knowledge, was the first to provide evidence of the aforementioned herbal antidepressant effects, with the 5-HT1AR and its linked AC-cAMP-PKA-CREB signaling pathway as targets.

It is generally believed that chronic exposure to stress serves a primary role in the onset and relapse of DD. Therefore, chronic stress paradigms in laboratory animals constitute important tools in this field. The CMS model, originally developed by Willner, has been extensively used to study the pathophysiology of and therapy for DD due to its high predictive, face and construct validity (27,31). Animals have been reported to exhibit a persistent reduction in responsiveness to pleasurable stimuli, measured by a decrease in their consumption of 1% sucrose solution and decreases in sucrose consumption have been validated as a reliable behavioral measure that may be associated with the anhedonia involved in depression (32-34). Consistent with other reports, the present study demonstrated that CMS induced a decrease in SPT, which was reversed by the SYC and fluoxetine respectively, suggesting that the SYC may be as effective as SSRIs in treating depression.

In addition, alterations were observed in the body weight of rats. The results of the present study demonstrated that the model group had lower body weight compared with control group following implementation of 2 weeks' stress, which was not demonstrated by the SYC or fluoxetine groups. The SYC treatment and fluoxetine treatment groups demonstrated similar body weight gain to that of the control group, suggesting that the SYC or fluoxetine had an effect on body weight in CMS-induced DD model rats. In a previous DD study, a number of behavioral and physiological alterations were reported, including an ~0-10% loss of body weight (27). However, certain studies have demonstrated that antidepressants, including fluoxetine, elicit even lower body weight compared with CMS group and that CMS rats gain body weight (35,36). In the present study, the serum pharmacological method was used, to the best of the author's knowledge, for the first time, to investigate effects of the SYC on the 5-HT1AR-mediated AC-cAMP-PKA-CREB postreceptor signal transduction pathway *in vitro*. The serum pharmacological method was developed in the 1980s and is now frequently

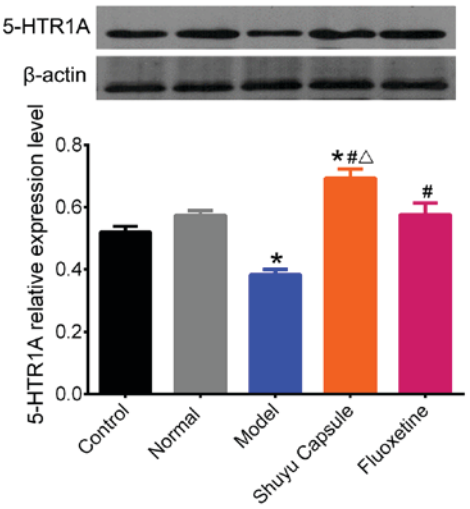


Figure 2. 5-HT1AR protein levels in primary cultured hippocampal neurons treated with the serum of rats in different groups. Western blotting analysis was performed following the exposure of cells to different serum treatments at a concentration of 10% for 48 h. The cells were divided into the following groups: Control group (no serum), normal group (treatment with serum of normal rats), model group (treatment with serum of rats in chronic mild stress group), SYC group (treatment with serum of rats in SYC group) and fluoxetine group (treatment with serum of rats in fluoxetine group). *P<0.05 vs. the normal group. #P<0.01 vs. the model group. △P<0.05 vs. the fluoxetine group. SYC, Shuyu capsule; 5-HT1AR, 5-hydroxytryptamine 1A receptor.

used for herbal mixture pharmacological studies *in vitro* (37). To ensure that certain active components of the SYC in serum pass through the blood-brain barrier, the authors previously measured the serum and cerebrospinal fluid from rats treated orally with the SYC by liquid chromatography electrospray ionization tandem mass spectrometry (38). According to the results, 13 prototype compounds were absorbed into the rat serum in the prototype and identified as quinic acid, dibenzoyl paeoniflorin, 6'-O-galloyldibenzoyl paeoniflorin, paeoniflorin sulfonate, albiflorin, paeoniflorin, liquiritin apioside, liquiritin, hydrated-liquiritin, galloylpaeoniflorin, galloylalbiflorin, apigenin-7-O-β-D-glucuronide and wogonoside.

Downregulation of the 5-HT1AR has been proposed as a general hypothesis to explain alterations in the 5-HTR function

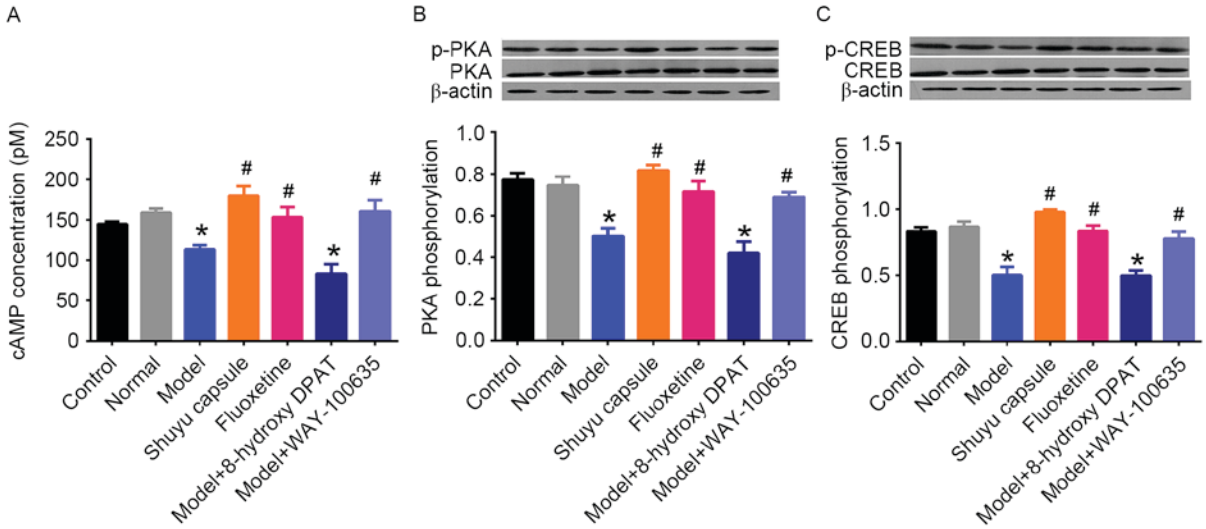


Figure 3. Activation of cAMP-PKA-CREB pathway in primary cultured hippocampal neurons treated with serum of rats in different groups. ELISA and western blotting analyses were performed to detect the intracellular (A) cAMP concentration and phosphorylation of (B) PKA and (C) CREB following exposure of cells to different serum treatments at the concentration of 10% for 24 h. The cells were divided into the following groups: Control (no serum), normal (treatment with serum of normal rats), model (treatment with serum of rats in CMS group), SYC (treatment with serum of rats in SYC group), fluoxetine (treatment with serum of rats in fluoxetine group), model plus 5-hydroxytryptamine 1A receptor agonist (treatment with serum of rats in CMS group plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min) and model plus 5-HT1AR antagonist (treatment with serum of CMS rats plus 0.5 $\mu$M WAY-100635 stimulation for 60 min). $^{*}$P<0.05 vs. the normal group. $^{\#}$P<0.05 vs. the model group. CMS, chronic mild stress; SYC, Shuyu capsule; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP response element-binding; p, phosphorylated.

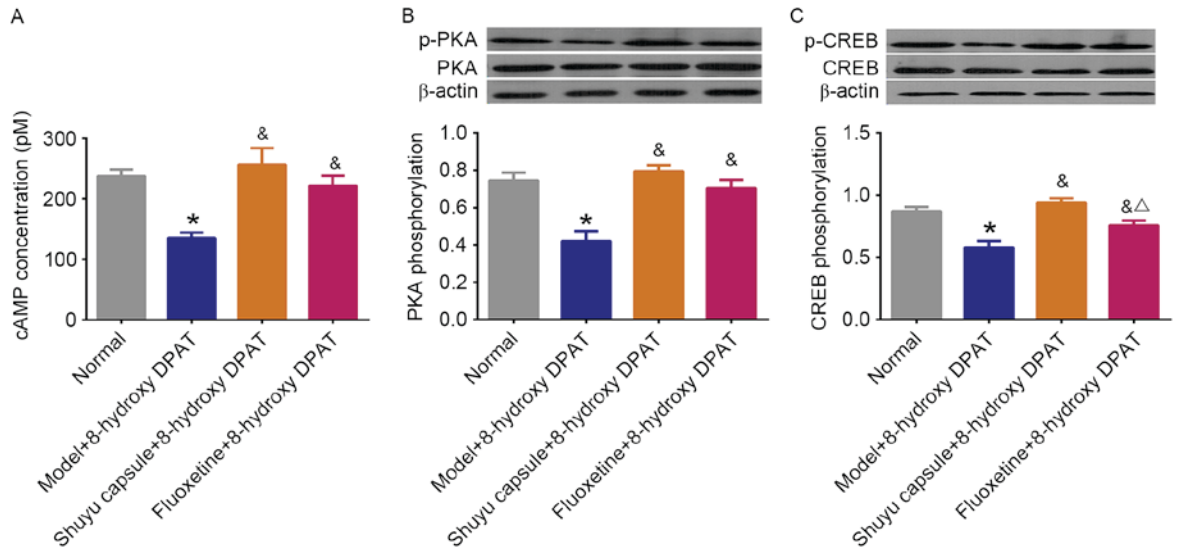


Figure 4. Activation of 5-HT1AR-mediated cAMP-PKA-CREB pathway in primary cultured hippocampal neurons treated with serum of rats in different groups. ELISA and western blotting analyses were performed to detect the intracellular (A) cAMP concentration and phosphorylation of (B) PKA and (C) CREB following the exposure of cells to different serum treatments at the concentration of 10% for 24 h. The cells were divided into the following groups: Normal (treatment with serum of normal rats), model plus 8-hydroxy-DPAT (treatment with serum of rats in Chronic mild stress group plus 1 $\mu$M 5-HT1AR agonist (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min), SYC plus 8-hydroxy-DPAT (treatment with serum of SYC rats plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min) and fluoxetine plus 8-hydroxy-DPAT (treatment with serum of fluoxetine rats plus 1 $\mu$M (R)-(+)-8-hydroxy-DPAT hydrobromide stimulation for 45 min). $^{*}$P<0.05 vs. the normal group. $^{\&}$P<0.05 vs. the model plus 8-hydroxy-DPAT group. $^{\Delta}$P<0.05 vs. the SYC plus 8-hydroxy-DPAT group. SYC, Shuyu capsule; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP response element-binding; 5-HT1AR, 5-hydroxytryptamine 1A receptor.

in subjects with depressive symptoms (39). The results of the present study demonstrated that the levels of 5-HT1AR protein expression in primary cultured hippocampal neurons were decreased in the CMS model group compared with the control group, which is in line with previous studies (40). It has been reported that fluoxetine has an antidepressant function partially mediated by the 5-HT1AR and is involved in brain region-dependent 5-HT1AR gene transcription alterations (41,42). The serum from fluoxetine-treated rats was observed to reverse the decrease of 5-HT1AR protein levels in the cultured hippocampal neurons. Furthermore, the SYC demonstrated a similar role to that of fluoxetine, which implied that the beneficial action of the SYC or fluoxetine on the CMS-induced depressive state may in part be based on

5-HT1AR dysfunction in the hippocampal neurons. Notably, the increasing effect of the SYC on 5-HT1AR protein levels was statistically greater compared with fluoxetine, indicating that the receptor is involved more in the antidepressant-like effect of the SYC than in that of fluoxetine.

The AC-cAMP-PKA-CREB cascade is regulated by serotonin and norepinephrine. Dysfunction of the AC-cAMP-PKA cascade, including decreased G protein and cAMP levels, reduced AC and PKA activity and altered PKA-mediated phosphorylation, have been observed in DD patients (43,44). Chronic antidepressant treatment upregulates cAMP signal transduction and PKA activity in the brain (45). Previous studies have demonstrated that the pathophysiology of major depression may involve abnormalities within critical effector systems responsible for neurotransmitter effects on cells, including PKA and CREB, and conventional antidepressants may ameliorate depression in part thorough effects on the cAMP-PKA pathway (46-48). In addition, chronic fluoxetine treatment exerts a marked effect on phosphorylated-CREB in the hippocampus. Furthermore, various studies have demonstrated that the cAMP-PKA cascade is involved in SSRI-induced antidepressant actions (49,50).

The present study evaluated the association between the 5-HT1AR and the AC-cAMP-PKA-CREB cascade in the context of SYC treatment. Studies have demonstrated that CMS induced downregulation of the AC-cAMP-PKA cascade, which was reversed by SYC and fluoxetine treatment. The treatments plus 8-hydroxy-DPAT and WAY-100635, the specific agonist and antagonist of the 5-HT1AR (51,52), verified that the alterations in the AC-cAMP-PKA cascade in primary cultured hippocampal neurons were mediated by the 5-HT1AR. Furthermore, the results of the present study indicated that the SYC or fluoxetine were able to restore downregulation of the AC-cAMP-PKA cascade induced by the 5-HT1AR agonist. Additionally, the present study revealed that the effect of the SYC is superior to that of fluoxetine on the upregulation of the 5-HT1AR and improvement of the CREB phosphorylation level.

In conclusion, the SYC is an effective antidepressant treatment in CMS-induced depression model rats, as effective as fluoxetine, and the AC-cAMP-PKA-CREB signal transduction pathway mediated by the 5-HT1AR is crucial for its antidepressant mechanism.

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