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Antidepressant-like effects of Chaihu-Shugan-San via SAPK/JNK signal transduction in rat models of depression

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ABSTRACT

Background: Chaihu-Shugan-San (CHSGS), a traditional Chinese medicinal herbal formula, registered in Jingyue Quanshu, has been indicated that oral administration of the extract from it can remit depressive disorder. C-Jun amino-terminal kinase (JNK/SAPK) signal transduction plays a key role in the apoptosis of nerve cells, be reported closely correlated with depression. This study was designed to investigate CHSGS antidepressant-like effects in rat models of depression and probe its possible mechanism. **Materials and Methods:** The classical experimental depression model chronic mild unpredictable stress (CMUS) was used to evaluate the antidepressant-like effects of CHSGS. The extracts were administered orally for 14 days, while the parallel positive control was given at the same time using fluoxetine hydrochloride. The expressions of JNK in the hippocampus were detected by real-time fluorescent quantitation PCR and Western blot assay. **Results:** Intragastric administration of CHSGS for 14 days caused a significant improvement of weight and locomotor activity in the open-field test. In addition, CHSGS treatment inhibited the expressions of JNK in the hippocampus tissue in CMUS rats. **Conclusion:** CHSGS could obviously improve the depressive state of the model rats and its mechanism may be correlated with regulating the expressions of JNK in the hippocampus.

Key words: Antidepressant, C-Jun amino-terminal kinase, Chaihu-Shugan-San, fluoxetine, open field test, signal transduction

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INTRODUCTION

Depression is a severe, common mood disorder with high suicide rate and the most disabling medical disease.[1] Because the mechanism of depression is quite complex and is not explicit, many currently available synthetic chemical antidepressants have low rates of response and remission and even severe adverse-effect.[2] Accordingly, it is necessary to probe and develop more effective antidepressant with lover adverse-effect. Nature plants, such as *Cissampelos sympodialis*,[3] *Bacopa monniera*,[4] *Terminalia Bellirica* Roxb,[5] *Hypericum perforatum*,[6,7] *Ginkgo biloba* 36, and *Pueraria lobata*,[8] are an important source of new antidepressant drugs and the safety of nature plants may be better than that of synthetic antidepressants.[9] Chaihu-Shugan-San, a traditional Chinese medicinal (TCM) herbal formula, which consisted of seven Chinese herbs and recorded in a medical classic Jingyue Quanshu, has been used as a remedy for reliving depression symptoms caused by liver-Qi stagnation, which results from repression of distress and anger to TCM theory. Recently, it has been reported that CHSGS is effective in the treatment of depression. However, its mechanism is not clear.[10,11] It is reported that CHSGS may be antidepressant by regulating the MAPKs signal transduction, such as the expression of BNDF, TrkB, ERK, etc.[12,13]. C-Jun amino-terminal kinase (JNK/SAPK), one of the signal transduction on MAPKs, plays a key role in the apoptosis of nerve cells and is closely correlated with depression.[14-16] This was designed to investigate CHSGS antidepressant-like effects in rat models of depression and examined whether they are correlated with JNK/SAPK signal transduction.

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MATERIALS AND METHODS

Animals and groups

Forty healthy adult male Sprague-Dawley (SD) rats with similar ethological indexes, weighing 180-220 g, were provided by Experimental Animal Science of Xiangya Medical College of Central South University, and certificate of quality was SCXX (Hunan) 4343232. All rats were housed 5 per cage for 1 week to adapt to the environment under controlled conditions of 12 h light to 12 h dark cycle (lights on from 6:00 a.m. to 6:00 p.m.), background noise (40 ± 10) db, 10% relative humidity and temperature (20 ± 3) °C with food and water available *ad libitum*. Then, the rats were randomly divided into four groups: The normal control group (NC), the model control group (MC), the CHSGS group, and the fluoxetine control group (FC) group, with 10 rats in each group. Except those in the NC, the rest rats were singly housed and exposed on an unpredicted sequence of mild stressor. The experimental procedures were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988) and approved by the Animal Experimental Center for Central South University.

Drugs and reagents

The recipe and dosage of CHSGS used in this study followed the Chinese Pharmacopoeia, first volume 2005. Seven herbs (voucher specimen, no: 20100901-1) of CHSGS were obtained from Xiangya Hospital pharmacy, which were authenticated by the herbal medicine associate Professor Lei Peng, Department of Pharmacy of Xiangya Hospital affiliated Central South University. CHSGS contained Chinese thorowax root 9 g, dried tangerine peel 9 g, Szechwan Lovage Rhizome 9 g, Nutgrass Galingale Rhizome 9 g, Fructus Aurantii 9 g, Paeonia 15 g, and Radix Glycyrrhizae 5 g. CHSGS was boiled twice in distilled water (1:12, w/v) for 30 min. The blended supernatants were then lyophilized (yield = 19.13% (w/w)). The dried extract containing 8 g of crude drugs per gram were made into 1 g/mL of liquid when they were applied. Fluoxetine hydrochloride (FLU) was taken with a form of 20 mg/pellet, and qualified for health-tax credit number of J20080016 which was provided by Eli Lilly (Suzhou, China) Pharmaceutical Co., Ltd. The total RNA extraction reagent (TRIzol) was obtained from Invitrogen Corporation in America and SYBR Premix Ex Tap (perfect real time) kit was bought from Takara Co. Ltd.. The Revert Aid First Strand cDNA Synthesis kit was obtained from Promega (Madison, WI, USA) (A3500 29287) and primers were synthesized by Shanghai Bio-engineering Technology Co., Ltd. JNK (p-JNK) rabbit monoclonal antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA) (SC0564). Rabbit IgG horseradish peroxidase (HRP)-conjugated rabbit antigoat polyclonal antibody was purchased from KPL (Maryland, USA), and mouse IgG HRP-conjugated rabbit anti-mouse polyclonal antibody was purchased from Upstate (New York, USA). Radioimmunoprecipitation assay protein lysis buffer was obtained from Santa Cruz Biotechnology. Hybond-Polyvinylidene difluoride was obtained from Amersham (Uppsala, Sweden), and phenylmethyl sulfonylfluoride was obtained from Sigma.

Establishment of the CMUS model

All the rats except the normal group were used to establish chronic mild unpredictable stress (CMUS) depression rat model. The animal model was established following modified Willner's method,[17,18] which provide the rats CMUS and housed by single cage. The stress regimen consisted of the following stressors: 24 h reversed light/dark, electric shock in foot (10 mA electricity was given every other minute, which last 10 s per time for 30 times), 48 h food deprivation, shaking (once a second, lasting for 15 min), noise (85 db), clipping the tail (clipping last 5 s per time for 10 times), and strange smell. During a period of 28 days, one of the stressors was chosen randomly and done to the rats so that the rats could not expect the stimulus. Every stressor was used for two to three times in total.

Drugs administration

Rats of the NC group were fed normally, and rats in other groups received different stimulus during the 28 days. From the 15th day, all rats were administered with equal volume of normal saline (to the NC group and the MC group) and of corresponding medicinal liquid (5.9 g/kg to the CHSGS group, and 1.8 mg/kg to the FC group) by gastrogavage for 2 successive weeks. The dosage of medicines administered to the rats equaled to that of a 70 kg adult.

Weights and behavioral tests

Weight change

All the rats were weighed on the 0, 15th, and 29th day during the experiment.

Open-field test

The open-field test (OFT) was performed according to the improved method recorded in the literature[19] at 7:00 a.m. on the 0, 15th, and 29th day of the experiment in a quiet room. The procedure of the test was as follows: The rats were individually placed in the central wood cage (100 × 100 cm^2) with walls 50 cm high and the floor divided into 10 squares (10 × 10 cm^2). When the hind legs crossed the line of the squares, the rat was considered to have crossed from one square to another (crossing), when the forelegs lift from the floor the rat was considered to have gotten one point (scores of rears). The rats' scores during 3 min were recorded. After each rat was tested in the OFT, the cage was carefully cleaned with a solution containing 75% ethanol, purified water to remove the scent of the previously evaluated rat, which could modify the spontaneous behavior of the rat.

Sucrose-solution consumption test

According to the literature,[20,21] the test procedures were as follows: In a quiet room 72 h before each OFT. Before the test, the rats were trained to adapt to drinking water with sugar in it. Two bottles were placed in every cage. In the first 24 h, the two bottles were filled with 1% sucrose solution, and in the following 24 h, one of the bottles was filled 1% sucrose solution, and the other was filled with pure water. Then, the basic energy expenditure test and water consumption test were done after 24 h of food and water forbidden. In the same time, the rats were given two bottles of liquid weighed beforehand, one was filled with 1% sucrose solution, and the other with pure water. Further 60 min later, the two bottles were weighed. Then, the total liquid consumption, sucrose solution consumption, and pure water consumption were calculated.

Fluorescence real-time quantitative transcription-PCR analysis of JNK mRNA

Preparation of the rat brain sample

Five rats from each group were killed by cutting down their heads on the 29th day of the experiment. Then, the brain tissues were immediately taken out on the ice table, and bilateral hippocampuses were isolated. They were placed into RNase-free EP tubes and kept first in liquid nitrogen for 24 h and then at -80°C in a freezer.

RNA was extracted with Trizol. Reverse transcription-poly merase chain reaction (RT-PCR) was accomplished with the Access RT-PCR System. JNK mRNA (211bp; GenBank AB118218.1) was amplified using the forward primer 5'-GGCGGCCAAACAGAAAG-3' and the reverse primer 5'-CTGAGGGCACGGAGGAT-3'. JNK β-actin (201 bp) mRNA was amplified as the control using the forward primer 5'-CGTTGACATCCGTAAAGA-3' and the reverse primer 5'-TGGAAGGTGGACAGTGAG -3'. Each reaction mixture (final volume 25 μL) contained 4 μL $MgCl_2$, 2 μL 10× RT butter, 2 μL dNTP mixture, 0.5 μL recombinant Rnasin, 0.7 μL AMV, 1 μL Oligo dT, 2 μL RNA, and 12.8 μL DEPC-treated water. The reaction parameters were: The reverse transcription was performed at 42°C for 15 min, followed by deactivation of reverse transcriptase at 95°C for 5 s.

The real-time quantitative PCR reaction was carried out with ABI 7300 Fluorescence quantitative PCR using reagents of the SYBR Premix Ex Taq (perfect real time) kit, in a reaction volume of 25 μL, consisting of 10 μL SYBR fluorescent dye, 2 μL PCR forward primer and PCR reverse primer, 2 μL RT reactive fluid (cDNA), and 11 μL DEPC-treated water. The reaction conditions were are as follows: In the first denaturation step, the reaction was heated up to 95°C which was kept for 10 s, and followed by the PCR reaction step consisting of 40 circles, each circle composed of denaturation at the temperature of 95°C for 5 s and annealing at the temperature of 56°C for 30 s. In the whole process of the reaction, the threshold cycle (C_T) value of the samples was analyzed with Sequence Detection software version 1.2.3 (Applied Bio-systems Corporation). Further, it was found that the C_T value decreased as the template concentration increased. Melting curve analysis was carried to assess the characteristics of the PCR. Further, β-actin served as the internal control, the $2^{-\Delta\Delta CT}$ method [11] was applied to do the relative quantitative analysis of JNK1/2 mRNA. The formula was as follows: $\Delta\Delta C_T$ = experimental group (C_T assayed samples-C_T β-actin)-control group(C_T assayed samples-C_Tβ-actin).

Protein extraction and Western blot analysis of JNK

Radio immunoprecipitation assay protein lysis buffer was used to separate the brain tissue proteins, followed by quantification by the BCA Protein Assay kit (Pierce, Rockford, IL, USA). We analyzed the protein expression of JNK, phosphorylated at p-JNK, using a Bio-Rad electrophoresis apparatus and a protein transfer equipment (MiniProtean; Bio-Rad, Hercules, CA, USA) and Kodak XOMAT AR film (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA). The antibodies used to probe the Western blot and their dilution was as follows: JNK 1:1000, p-JNK 1:1500.

Statistical methods

Statistical analyses were carried out using the SPSS 17.0 (SPSS Inc., Brookfield, WI, USA) software package. The results are expressed as the mean value ± standard deviation. Data were analyzed two-way repeated measures analysis of variance (ANOVA), because the same rats were subjected to several test sessions. A two-tailed $P < 0.05$ was considered to be statistical significance.

RESULTS

Weight, OFT, and sucrose-solution consumption

The weight and behaviors of all the rats before the experiment was no statistical significance ($P > 0.05$). After the stress of 14 days chronic mild unpredictable stress, all the rats in the experiment groups had depressed behaviors. Compared with the rats in the NC group, their scores in the OFT decreased, the sucrose solution consumption reduced and the increasing of weight lowered, and the difference was of statistical significance ($P < 0.05$ or < 0.01). After

14 days of intragastric administration, the depression-like behaviors of the rats in the CHSGS group and the FC group had improved, scores of crossing and rears of the OFT increased obviously, the volume of the sucrose solution consumption increased, the increasing of their weight became evident, and the differences between them and the rats in the NC group were of statistical significance ($P < 0.05$ or < 0.01) [Table 1].

Effects of CHSGS on mRNA of JNK

The molecular cloning of human JNK and rat JNK (SAPK) led to identification of JNK as a member of the MAP kinase group of protein kinases. The melting curves of PCR produces of JNK and β-actin had only one peak, which indicated that the amplified gene was a single fragment . The melting temperature of JNK and β-actin was 85.3°C and 84.5°C, respectively. The C_T values of JNK in different groups and encephalic regions were detected by the amplification of β-actin. With β-actin as internal control, and the JNK mRNA expression of model was supposed to be 1, the relative JNK mRNA expression was calculated by $2^{-\Delta\Delta C_T}$. Further, the results are presented in Figure 1.

Effects of CHSGS on protein expression of JNK and p-JNK

To confirm that the results obtain at the mRNA level corresponded at the protein level, Western blot analysis was carried out. JNK is activated when phosphorylated. To determine the effect of CHSGS on the activation status of JNK, we evaluated its phosphorylation state in the rat brain tissue treated with CHSGS. We detected the total JNK, including the 46-KDa and 54-KDa from the brain tissue of different groups, simultaneously, the 46-KDa p-JNK was detected. Digital image analysis of the Western blot signals demonstrated significantly higher levels of p-JNK in the MC group relative to NC, and compared with the MC group, the expression of p-JNK was obviously lower in the CHSGS and FC group, the differences between them were of statistical significance ($P < 0.05$ or $P < 0.01$) [Figures 2 and 3].

DISCUSSION

Currently, the chronic mild unpredictable stress depression model has become a widely used animal model for studying the pathogenesis of depression and mechanism of antidepressant's action.[17,22] In this study, after the establishment of the CMUS model, except those in the NC group, the rest rats underwent a drop in the increasing speed of weight, their scores in OFT and sucrose solution consumption reduced, showed that anhedonia, obviously compared to those in the NC group, loss of appetite, decrease of activity, etc., Those depression-like symptoms improved evidently after treated by the classic antidepressant fluoxetine, suggesting that the animal models were successfully established.[23] Meanwhile, after the treatment with administration of CHSGS for 14 days, the depressive-like symptoms were obviously improved.

Depression is one of the major mental disorders associated with symptoms such as regular negative moods, decreased physical activity, feelings of helplessness, sluggish thought, and cognitive function. It has been regarded as "depression syndrome" and "liver qi stagnation" in TCM.[24-26] CHSGS consists of seven crude herbal drugs and is used to treat depression-related syndromes associated with qi stagnation. Qi stagnation has been categorized as a term in TCM that

Table 1: Comparison of weight, OFT, and sucrose-solution consumption among preduplicating, pretherapy rats (±s, *n*=10)

Groups	Scores of crossing	Scores of rears	Total water consumption/ml	1% sucrose consumption/ml	Weight/g
NC					
Day 0	65.6±16.3	15.9±6.2	16.3±4.1	13.3±4.1	203.6±10.8
Day 15	64.5±14.5	16.1±5.3	17.8±3.7	14.5±3.9	271.1±16.5
Day 29	61.5±15.7	15.5±4.9	20.6±5.4	17.2±4.8	324.5±23.7
MC					
Day 0	66.4±8.2	15.7±5.3	16.6±4.4	14.1±3.5	204.5±10.3
Day 15	27.5±8.9$^{\Delta}$	6.8±2.8$^{\Delta}$	15.9±3.6$^{\Delta}$	6.6±2.3$^{\Delta}$	249.8±13.2*
Day 29	18.3±6.7	4.9±2.1	12.3±3.3	7.1±2.8	275.6±15.1
CHSGS					
Day 0	65.8±12.7	16.3±4.8	17.1±4.9	13.2±3.7	206.9±12.6
Day 15	26.4±11.3	4.7±2.6	16.3±4.5	5.6±2.3	252.6±13.4
Day 29	49.6±15.4$^{\Delta\Delta}$	12.3±4.5$^{\Delta\Delta}$	19.1±3.8**	15.3±3.9$^{\Delta\Delta}$	310.2±20.7**
FC					
Day 0	65.8±20.9	15.8±3.9	16.9±5.1	13.9±3.5	210.6±15.7
Day 15	24.9±11.5	5.5±2.7	16.3±4.3	6.3±3.1	251.9±16.5
Day 29	45.6±13.4$^{\Delta\Delta}$	13.2±4.1$^{\Delta\Delta}$	20.3±5.2$^{\Delta\Delta}$	16.2±5.4$^{\Delta\Delta}$	308.3±14.7**

*P<0.05, $^{\Delta}P$<0.01:Compared with the normal at the same time point: **P<0.05, $^{\Delta\Delta}P$<0.01: Compared with model control group at the same time point; OFT: Open-field test

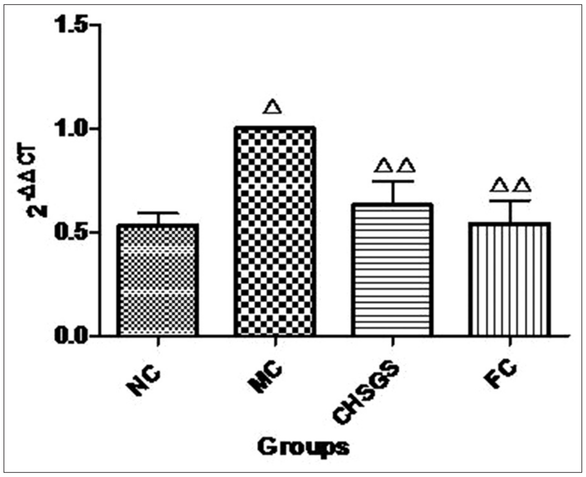

Figure 1: The expression of JNK mRNA in the hippocampus tissue in post-treatment of varied groups detected by fluorescence real-time quantitative transcription-PCR. △P < 0.01, compared with the NC group; △△P < 0.01, compared with MC group

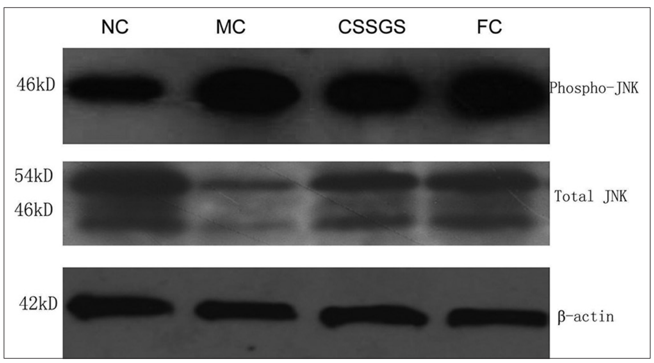

Figure 2: The expression of c-Jun N-terminal kinase (JNK) and phosphorylated-JNK (p-JNK) in the rat hippocampus in posttreatment of varied groups detected by Western blot

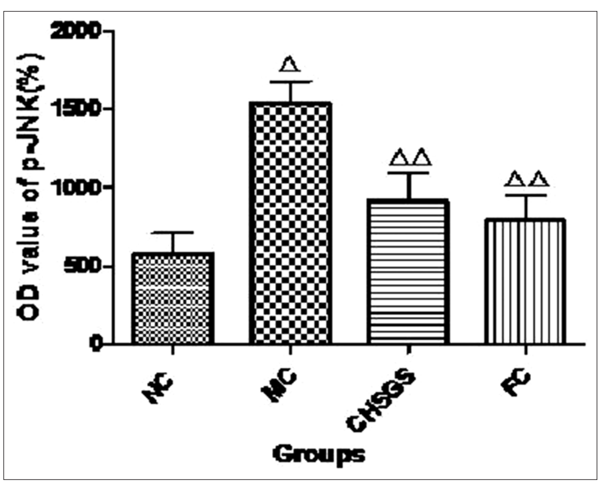

Figure 3: The expression of p-JNK in the hippocampus tissue in posttreatment of varied groups detected by Western blot. △P < 0.01, compared with the NC group; △△P < 0.01, compared with the MC group

refers to insidious, long-standing, serious discomfort that is projected into the body and is manifested by numerous symptoms, such as insomnia, fatigue, panic, and dyspnea.[27] In previous studies, the antidepressant effect of CHSGS was revealed using the CMUS model and it was shown that the mechanism may be by regulating the neuroendocrine,[11] but its molecule mechanism is not very clearly.

The hippocampus is one brain structure that has been extensively studied with regard to stress, depression, and antidepressant actions.[28] It was reported that volumes of the double-side hippocampus were reduced in patients with major depression compared to healthy controls, and there was a positive correlation between hippocampus atrophy and the time course of the depression.[29-31] Chronic stress alone increased the numbers of apoptotic cells in hippocampal subregions and cortex in the adult tree shrew,[32] while treatment with the antidepressant tianeptine had an antiapoptotic effect in the stressed animals,[33] suggesting that chronic antidepressant treatment may have neuroprotective effect. Some previous studies found that classical antidepressants also protected rat hippocampal neurons in primary culture from the lesion induced by corticosterone.

JNK, initially described as stress-activated protein kinase (SAPK), was identified by their ability to phosphorylate specific sites on the amino terminal transactivation domain of the c-Jun, a transcription factor, following exposure to UV irradiation, growth factors, cytokines, or expression of transforming oncogenes. By phosphorylating these sites, the JNKs stimulate c-Jun's transcriptional activity.[34,35] The JNK pathway's major role, involvement in stress responses, has been widely studied. The JNK cascade follows the typical MAPK signaling arrangement. The major target of the JNK pathway is activator protein-1 (AP-1) transcription factor, which is activated mainly by the phosphorylation of c-Jun and other related molecules.[36] In the adult mouse, signaling through c-Jun N-terminal kinases (JNKs) links exposure to acute stress to various physiological responses. Inflammatory cytokines, brain injury and ischemic insult, or exposure to psychological acute stressors induce activation of hippocampal JNKs. Acute stress caused activation of JNKs in the hippocampal CA1 and CA3 subfields, and impaired contextual fear conditioning. Conversely, intrahippocampal injection of JNKs inhibitors sp600125 (30 μM) or D-JNKI1 (8 μM) reduced activity of hippocampal JNKs and rescued stress-induced deficits in contextual fear. In addition, intrahippocampal administration of anisomycin (100 μg/μL), a potent JNKs activator, mimicked memory-impairing effects of stress on contextual fear. This anisomycin-induced amnesia was abolished after cotreatment with JNKs selective inhibitor sp600125 without affecting anisomycin's ability to effectively inhibit protein synthesis as measured by c-Fos immunoreactivity. Studies showed milder and transient activation of the JNKs pathway in the CA1 subfield of the hippocampus during contextual fear conditioning and an enhancement of contextual fear after pharmacological inhibition of JNKs under baseline conditions. Using combined biochemical and transgenic approaches with mutant mice lacking different members of the JNK family (Jnk1, Jnk2, and Jnk3), evidence suggested that JNK2 and JNK3 are critically involved in stress-induced deficit of contextual fear, while JNK1 mainly regulates baseline learning in this behavioral task. Currently studies support the possibility that hippocampal JNKs serve as a critical molecular regulator in the formation of contextual fear.[37]

In this study, our experiment results showed that the expressions of JNK mRNA and the protein of p-JNK in the rat of MC groups were increased significantly, and antidepressant Flu and CHGSG could regulate the expression of JNK. Therefore, our results showed that CHSGS could obviously improve the depressive state of the model rats and its mechanism may be correlated with regulating the expressions of JNK in the hippocampus.

CONCLUSIONS

CHSGS could obviously improve the depressive behavior of the model rats and its mechanism may be correlated with regulating the expressions of JNK in the hippocampus.

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