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Antidepressant-like effects of *Chaihu-Shugan-San* via c-jun amino-terminal kinase signal transduction in rat models of depression

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Introduction

Depression is a severe common mood disorder with high suicide rate, and the most disabling medical disease (Paykel, 2006). 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 (Sarko, 2000). Accordingly, it is necessary to probe and develop more effective antidepressant with lower adverse-effect. Nature plants, such as *Cissampelos sympodialis* (Almeida et al., 1998), Bacopa monniera (Sairam et al., 2002), Terminalisa Bellirica Roxb (Dhingra and Valecha, 2007), Hypericum perforatum (Caccia, 2005; Sanchez-Mateo et al., 2007), Ginkgo biloba36, *Pueraria lobata* (Yan et al., 2004), may be an important source of new antidepressant drugs, and the safety of nature plants may be better than that of synthetic antidepressants (Schulz, 2006). *Chaihu-Shugan-San*, a traditional Chinese medicinal (TCM) herbal formula, consisting of seven Chinese herbs, recorded in a medical classic *Jingyue Quanshu*, has been used as a remedy for relieving depression symptoms caused by liver-qi stagnation which results from repression of distress and anger to TCM theory. Recently, CHSGS has been showed that be effective in the treatment of depression but it mechanism is not clearly explained (Kim et al., 2005; Li et al., 2009). It is reported that CHSGS may be antidepressant by regulating the MAPKs signal transduction, such as the expression of BNDF, TrkB, and ERK and so on (Wang et al., 2011; Deng et al., 2011). C-Jun amino-terminal kinase (JNK/SAPK), one of the signal transduction on MAPKs, play a key role in the apoptosis of nerve cells, is reported closely correlated with depression (Sun et al., 2007; Brust et al., 2007; Li et al., 2007). In this study, we designed to investigate CHSGS...
antidepressant-like effects in rat models of depression and examined whether it correlated with SAPK/JNK signal transduction.

MATERIALS AND METHODS

Animals and groups

Forty healthy adult male Sprague-Dawley (SD) rats with similar ethological indexes, weighing 180 to 220 g were provided by Experimental Animal Science of Xiangya Medical College of Central South University, and certificate of quality was issued SCXX (Hunan) 4343232.

All rats were housed 5 per cage for one week to adapt to the environment under controlled conditions of 12 h light/dark cycle (lights on from 6:00 am to 6:00 pm), 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 4 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 1st volume 2005. Seven herbs (voucher specimen, No: 20100901-1) of CHSGS, bought from Xiangya Hospital pharmacy, were authenticated by the herbal medicine associate Professor Lei Peng, Department of Pharmacy of Xiangya Hospital affiliated Central South University. CHSGS contained Lovage rhizome 9 g, dried tangerine peel 9 g, Szechwan Lovage rhizome 9 g, nutgrass galangale rhizome 9 g, Fructus auranti 9 g, Paonia 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 1.0 g/ml of liquid when they were applied. Fluoxetine hydrochloride with a form of 20 mg/pellet, and qualifies for health tax credit number of J20080016 was provided by Eli Lilly (Suzhou, China) Pharmaceutical Co., Ltd.; total RNA extraction reagent (TRIzol) was obtained from Invitrogen Corporation in America; 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, Wisconsin, USA) (A3500 29287); primers were synthesized by Shanghai Bio-engineering Technology Co., Ltd.; JNK, p-JNK rabbit monoclonal antibody was purchased from Santa Cruz Biotech (Santa Cruz, California, USA). (SC0564); rabbit IgG horseradish peroxidase (HRP)-conjugated rabbit anti-goat 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). Radio immunoprecipitation 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 CMUS model

All the rats except the normal group were used to establish chronic mild unpredictable stress depression rat model. The animal model was established following modified Willner’s method (Willner et al., 1987; Grippi et al., 2006), giving the rat’s chronic mild unpredictable stress (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 stressor was chosen randomly and done to the rats so that the rats could not expect the stimulus. Every stressor was used for 2 to 3 times in total.

Drugs administration

Rats of the NC were fed normally, and rats in other groups received different stimulus during the 28 d. From the fifteenth 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, 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, 15, and 29th day during the experiment.

Open-field test (OFT)

The OFT was performed according to the improved method recorded in the literature (Song et al., 2007; Yuan et al., 2011) at 7:00 am on the 0, 15, 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) with walls 50 cm high and the floor divided into 10 squares (10 × 10 cm). 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 testing each rat in the OFT, the cage was carefully cleaned with a solution containing 75% ethanol, and 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 (Benelli et al., 1999; Forbes et al., 1996), the test procedure was 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 2 bottles were filled with 1% sucrose solution, and in the following 24 h, one of the bottles was filled with 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 2 bottles of liquid weighed before hand; one was filled with 1% sucrose solution, and the other with pure water. 60 min later, the 2 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 rat brain sample: 5 rats from each group were sacrificed by cutting down their heads on the 29th day of the experiment. Then the brains tissue were immediately taken out on the ice table, and bilateral hippocampuses were isolated, put into RNase free EP tubes and kept first in liquid nitrogen for 24 h and then in -80°C freezer.

RNA was extracted with Trizol. Reverse transcription-PCR (RT-PCR) was accomplished with the Access RT-PCR System. JNK mRNA (211bp; GenBank AB118281.1) was amplified using the forward primer 5'-GGCGGCACCAACAGAAAG-3' and the reverse primer 5’-CTGAGGGCACGGAGGAT-3'. JNK β-actin (201bp) mRNA was amplified as the control using the forward primer 5'-CGTGTGACATCGTGTAAGA-3' and the reverse primer 5’-TGGAGGTTGGACAGTGAG-3’. Each reaction mixture (final volume 25 μl) contained 4 μl MgCl2, 2 μl 10 x RT buffer, 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 depletion 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: in the first denaturation step, the reaction was heated up to 95°C which was held 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 (Ct) value of the samples was analyzed with sequence detection software version 1.2.3 (Applied Bio-systems Corporation). And, it was found that the Ct value decreased as the template concentration increased. Melting curve analysis was carried to assess the characteristics of the PCR. Also, β-actin served as the internal control, 2^-ΔΔCt method was applied to do the relative quantitative analysis of JNK1/2 mRNA. The formula is as follows: ΔΔCt = experimental group (Ct assayed samples - Ct β-actin) – control group (Ct assayed samples - Ct β-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, Illinois, USA). We analyzed the protein expression of JNK, phosphorylated at (p-JNK), using Bio-Rad electrophoresis apparatus and protein transfer equipment (MiniProtean; Bio-Rad, Hercules, California, USA) and Kodak XOMAT AR film (PerkinElmer Life and Analytical Sciences, Inc., Waltham, Massachusetts, USA). The antibodies used to probe the western blot and their dilutions were as follows: JNK 1:1000, p-JNK 1:1500.

Statistical analysis

Statistical analyses were carried out using the SPSS 17.0 (SPSS Inc., Broadfield, Wisconsin, USA) software package. Results are expressed as the mean value ± standard deviation. Dates 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 statistically significant.

RESULTS

Weight, OFT and sucrose-solution consumption

The weight and behaviors of all the rats before the experiment was not statistically significant (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 P < 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 P < 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 single fragment.

The melting temperature of JNK and β-actin was 85.3 and 84.5°C, respectively. The Ct 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^-ΔΔCt. And the results were 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 46-kDa, 54-kDa, and 46-kDa p-JNK from the brain tissue of different groups. 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).
Table 1. Comparison of weight, OFT, and sucrose-solution consumption among pre-duplicating, pre-therapy rats (\(\bar{x} \pm s, n = 10\)).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Scores of crossing</th>
<th>Scores of rears</th>
<th>Total water consumption (ml)</th>
<th>1% sucrose consumption (ml)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>Day 0</td>
<td>65.6 ± 16.3</td>
<td>15.9 ± 6.2</td>
<td>16.3 ± 4.1</td>
<td>203.6 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>Day 15</td>
<td>64.5 ± 14.5</td>
<td>16.1 ± 5.3</td>
<td>17.8 ± 3.7</td>
<td>271.1 ± 16.5</td>
</tr>
<tr>
<td></td>
<td>Day 29</td>
<td>61.5 ± 15.7</td>
<td>15.5 ± 4.9</td>
<td>20.6 ± 5.4</td>
<td>324.5 ± 23.7</td>
</tr>
<tr>
<td>MC</td>
<td>Day 0</td>
<td>66.4 ± 8.2</td>
<td>15.7 ± 5.3</td>
<td>16.6 ± 4.4</td>
<td>204.5 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>Day 15</td>
<td>27.5 ± 8.9(\Delta)</td>
<td>6.8 ± 2.8(\Delta)</td>
<td>15.9 ± 3.6(\Delta)</td>
<td>249.8 ± 13.2*</td>
</tr>
<tr>
<td></td>
<td>Day 29</td>
<td>18.3 ± 6.7</td>
<td>4.9 ± 2.1</td>
<td>12.3 ± 3.3</td>
<td>275.6 ± 15.1</td>
</tr>
<tr>
<td>CHSGS</td>
<td>Day 0</td>
<td>65.8 ± 12.7</td>
<td>16.3 ± 4.8</td>
<td>17.1 ± 4.9</td>
<td>206.9 ± 12.6</td>
</tr>
<tr>
<td></td>
<td>Day 15</td>
<td>26.4 ± 11.3</td>
<td>4.7 ± 2.6</td>
<td>16.3 ± 4.5</td>
<td>252.6 ± 13.4</td>
</tr>
<tr>
<td></td>
<td>Day 29</td>
<td>49.6 ± 15.4(\Delta)</td>
<td>12.3 ± 4.5(\Delta\(\Delta)</td>
<td>19.1 ± 3.8(**)</td>
<td>310.2 ± 20.7**</td>
</tr>
<tr>
<td>FC</td>
<td>Day 0</td>
<td>65.8 ± 20.9</td>
<td>15.8 ± 3.9</td>
<td>16.9 ± 5.1</td>
<td>210.6 ± 15.7</td>
</tr>
<tr>
<td></td>
<td>Day 15</td>
<td>24.9 ± 11.5</td>
<td>5.5 ± 2.7</td>
<td>16.3 ± 4.3</td>
<td>251.9 ± 16.5</td>
</tr>
<tr>
<td></td>
<td>Day 29</td>
<td>45.6 ± 13.4(\Delta)</td>
<td>13.2 ± 4.1(\Delta\(\Delta)</td>
<td>20.3 ± 5.2(\Delta)</td>
<td>308.3 ± 14.7**</td>
</tr>
</tbody>
</table>

*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.

**DISCUSSION**

Currently, 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 (Willner et al., 1987; Song et al., 2011; Isingrini et al., 2012). In the present study, after the establishment of CMUS model, except those in NC group, the rest rats underwent a drop in the increasing speed of weight, their scores in OFT and sucrose solution consumption reduced, showing anhedonia, obviously compared to those in the NC group, loss of appetite, decrease of activity and so on. Those depression-like symptoms improved evidently after treating with the classic antidepressant, fluoxetine, suggesting that the animal models were successfully established (Willner et al., 2002). 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 (Ma et al., 2011; Zhang, 2010; Han et al., 2010). CHSGS consists of seven crude herbal
Figure 2. The expression of c-Jun N-terminal kinase (JNK) and phosphorylated-JNK (p-JNK) in the rat hippocampus in post-treatment of varied groups detected by Western Blot.

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

drugs and is used to treat depression-related syndromes associated with qi stagnation. Qi stagnation has been categorized as a term in TCM that 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 (Wei et al., 2008). 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 (Li et al., 2009), but its molecule mechanism is not very clear.

The hippocampus is one brain structure that has been extensively studied with regard to stress, depression, and antidepressant actions (Drevets et al., 2008). 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 (Bremer et al., 2003; Sapolsky et al., 2000; Sapolsky 2000). Chronic stress alone increased the numbers of apoptotic cells in hippocampal subregions and cortex in the adult tree shrew (Lucassen et al., 2004), while treatment with the antidepressant tianeptine had an antiapoptotic effect in the stressed animals (Lucassen et al., 2001), 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 (Kyriakis et al., 1994; Ichijo, 1999). 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 AP-1 (activator protein-1) transcription factor, which is activated mainly
by the phosphorylation of c-Jun and other related molecules (Gupta et al., 1995). 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-JNK1 (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-imparing effects of stress on contextual fear. This anisomycin-induced amnesia was abolished after co-treatment 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. In a word, currently studies support the possibility that hippocampal JNKs serve as a critical molecular regulator in the formation of contextual fear (Sherrin et al., 2010).

In the present study, our experiment results showed that the expressions of JNK mRNA and the protein of p-JNK in the rat of MG groups were increased significantly, and antidepressant FLU and CHGSG could regulate the expression of JNK. So, our results showed that CHGSs 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.

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