J Acupunct Meridian Stud 2017;10(6):402—408

Available online at www.sciencedirect.com

Journal of Acupuncture and Meridian Studies

journal homepage: www.jams-kpi.com

RESEARCH ARTICLE

# Antidepressant Effects of Pharmacopuncture on Behavior and Brain-Derived Neurotrophic Factor (BDNF) Expression in Chronic Stress Model of Mice

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Available online 30 August 2017

Received: Jun 12, 2017
Revised: Aug 22, 2017
Accepted: Aug 24, 2017

**KEYWORDS**
amygdala;
brain-derived neurotrophic factor;
chronic immobilization stress;
depression;
hippocampus;
pharmacopuncture

**Abstract**
*Objectives:* The present study aimed to investigate the antidepressant effect of the traditional Korean medical pharmacopuncture, *Liver Qi Depression* (HJ11), in a mouse model of depression induced by exposure to chronic immobilization stress (CIS).
*Methods:* Mice were subjected to 2 hours of immobilization stress daily for 14 days. They were also injected with distilled water (DW) (CIS + DW) or HJ11 at the acupoints HT7, SP6, and GV20 (CIS + HJ11) an hour before stress. The positive control group (CIS + paroxetine) was intraperitoneally injected with paroxetine (10 mg/kg, 14 days). The tail suspension test and the forced swimming test were performed to assess depression-like behaviors. Western blotting was also conducted to seek the change in brain.
*Results:* CIS + DW mice showed significantly longer immobile times in the tail suspension test and forced swimming test than sham mice that did not go through daily restraint. Immobility of CIS + HJ11 and that of CIS + paroxetine mice was significantly decreased compared with immobility of CIS + DW mice. Immunoblotting showed that HJ11 increased the expression of brain-derived neurotrophic factor both in the hippocampus and the amygdala.

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pISSN 2005-2901 eISSN 2093-8152
http://dx.doi.org/10.1016/j.jams.2017.08.007
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*Conclusion:* HJ11 improves depressive-like behaviors in the stress-induced mouse model of depression, and the results indicate that the neuroprotective effect of HJ11, identified by brain-derived neurotrophic factor expression, may play a critical role in its antidepressant effect.

## 1. Introduction

According to the report of the World Health Organization, depression turned out to be the third major disease of global disease burden in 2004, and was expected to impose the heaviest burden by 2030 [1]. It is predicted that 350 million people are going to be influenced by depression, and nearly 5% of the whole population reported having an episode of depression in 2011 in the World Mental Health Survey [2].

Pharmacopuncture is a treatment that combines acupuncture and herbal medicine. Herb extract is inserted into acupoints using syringes, based on qi/flavor theory and meridian theories [3]. This method is widely used in a diverse array of diseases such as Bell's palsy, pain, dyspepsia, and tinnitus. In Korea and China, there have been attempts to ameliorate neuropsychiatric diseases and their symptoms by pharmacopuncture. There have been continuous studies reporting efficacy in depression [4–9]. *Liver Qi Depression* (HJ11) used in this study was developed under the theory of eight principle pharmacopuncture. HJ11, of which the main ingredients are derived from *Paeoniae Radix*, *Salviae miltiorrhizae Radix*, and *Leonuri Herba*, is usually prescribed for "liver qi depression pattern", hence the name 'HJ11'. Features of liver qi depression pattern include mental stress, hypochondriac pain, distension, or lumps in the breast and string-like pulses [10,11], some of which overlap with the symptoms of depression.

Based on the data of behavior and tissue characteristics, this study aimed to investigate the effect of HJ11 as a representative of pharmacopuncture on depression and its underlying change in the brain using a mouse model induced by chronic immobilization. The neuroprotective effect of HJ11 was assessed by brain-derived neurotrophic factor (BDNF) in the hippocampus and amygdala, which play a key role in stress-related disorders. Injecting distilled water (DW) on acupoints and oral administration of paroxetine was used for comparison to ascertain the genuine effect of HJ11.

## 2. Materials and methods

### 2.1. Animals

Male C57Bl/6 mice (Orient Bio Inc., Seongnam, Korea), weighing 20–22 g (8 weeks old), were used in the experiments. The mice were maintained under standard laboratory conditions, and were housed in acrylic cages (20 cm × 27 cm × 12 cm) under an artificial 12-hour light/dark cycle at a controlled temperature (22 ± 2°C) and humidity (60 ± 10%), with free access to water and food unless specified otherwise. The cages were kept in the departmental room for 7 days to ensure adaptation to the new environment. All animal procedures were conducted under the regulations of the Kyung Hee University Medical Center Institutional Animal Care and Use Committee, Seoul, Korea. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Mice were randomly assigned to the following groups with different treatments: sham control (sham), negative control chronic immobilization stress (CIS + DW), positive control (CIS + paroxetine), and pharmacopuncture (CIS + HJ11). Sham mice did not undergo any stress or treatments, and were housed under standard conditions prior to sacrifice. The CIS + DW, CIS + paroxetine, and CIS + HJ11 groups of mice underwent 14 days of CIS (See Fig. 1). They were weighed at 7:00 AM every second day.

### 2.2. Induction of stress

Drugs and DW were administered 1 hour prior to stress. The mice were immobilized for 2 hours (from 14:00 PM to 16:00 PM) in specially designed plastic restraint tubes (dimensions: 2 cm × 2 cm × 10 cm), which completely restricted their movement. Sham mice were not exposed to stress. These procedures were repeated once daily for 2 weeks. Mice were then returned to their respective home cages.

### 2.3. Drug treatment

HJ11 is the extract of *Paeoniae Radix*, *Salviae miltiorrhizae Radix*, *Leonuri herba*, *Ligustri fructus*, *Zizyphi Semen*, *Cynanchi Wilfordii Radix*, *Cyperi Rhizoma*, *Bupleuri Radix*, *Cnidii Rhizoma*, *Curcumae longae Radix*, *Glycyrrhizae Radix*, and *Citrii Unshiu Immaturi Pericarpium*. HJ11 was supplied by Kyung Hee Medical Center, Seoul, Korea and was stored at 4°C in a refrigerator until use. CIS + DW or CIS + HJ11 mice were injected with 20 μL DW or HJ11, respectively, at the acupoints HT7, SP6, and GV20, which have been adopted in previous animal experiments of depression [12–16]. Paroxetine (10 mg/kg, GlaxoSmithKline Inc., Mississauga, Ontario, Canada) was used as a positive control (CIS + paroxetine). Paroxetine was dissolved in sterile water and injected intraperitoneally in a volume of 10 mL/kg. The dosage of these drugs was chosen by referral to previous relevant animal studies [12,17].

### 2.4. Behavioral tests

#### 2.4.1. Open field test

The experiments were performed in a dark quiet room. The apparatus included four acrylic boxes (50 cm × 50 cm × 50 cm) with a video camera installed above to record the activity of the mice. One mouse at a

time was placed gently into the central grid and allowed to freely explore the apparatus. Locomotor activity (total distance traveled) and anxiety-related behaviors (distance travelled in the central grid and entries into the central grid) were analyzed for the last 5 minutes of the recorded movements in the video files. The effects of HJ11 on mice were evaluated automatically using an open field test (OFT) with a computer-aided control system (SMART, Panlab Harvard Apparatus, Barcelona, Spain).

#### 2.4.2. Tail suspension test

The tail suspension test (TST) is a frequently used test to assess antidepressant activity. In a quiet room, acoustically and visually isolated animals were suspended 50 cm above the floor by adhesive tape that was placed approximately one third away from the tip of the tail. Immobility time, which was defined as the duration of being hung passively and completely motionlessness, was analyzed during the last 4 minutes of the 6-minute period.

#### 2.4.3. Forced swimming test

In the forced swimming test (FST), mice were individually forced to swim in an open cylinder (diameter 20 cm, height 35 cm) with 19 cm of water at 25°C. The total time that mice ceased to struggle and remained floating, motionless in the water was calculated during the last 4 minutes out of each 6-minute-long experiment. Decreased immobile time was regarded as a result of antidepressant-like effects.

### 2.5. Tissue preparation

When the behavioral tests were finished, tissue for western blotting was prepared. Mice in each group were euthanized by cervical dislocation, and the brains were rapidly removed. The hippocampus and amygdala was dissected and stored at −80°C until western blotting analysis.

### 2.6. Western blotting

Mouse monoclonal antibodies against β-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the rabbit polyclonal antibody against BDNF was purchased from Alomone Labs (Jerusalem, Israel). The horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL, USA).

Brain tissues were homogenized in 1x RIPA buffer. The homogenate was centrifuged at 12,000*g* for 10 minutes at 4°C, and the supernatant was collected. Total protein level in the supernatant was assayed using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The protein extracts (30 μg per lane) were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked in blocking buffer for 1 hour and then incubated overnight at 4°C with specific primary antibodies. The following day, the membranes were incubated with secondary antibodies for 1 hour at room temperature. Immunoreactive bands were developed using an enhanced chemiluminescent kit (Pierce Biotechnology; Amersham Biosciences, Amersham, UK). The blots were captured using a Davinch-Chemi (Celltagen, Seoul, Korea). Quantification of bands was carried out using ImageJ version 1.49 software (NIH, Bethesda, Maryland, USA).

### 2.7. Statistical analysis

Results, including those from the behavioral tests and western blotting, are expressed as the mean ± standard errors of the mean (SEM). Data were analyzed using the one-way analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. Comparison of body weight on Day 14 and Day 16 was analyzed using Games-Howell's *post hoc* test. Results are presented as the mean ± SEM. The level of significance was defined as $p < 0.05$. The analysis was conducted using SPSS 22.0 (IBM Inc., Armonk, NY, USA).

## 3. Results

### 3.1. Body weight

The body weight increment of stress-treated mice began to diminish compared with that of the sham group immediately following application of CIS, and the body weight of the CIS + DW mice remained significantly lower. These changes were significantly improved in mice treated with HJ11 and paroxetine, which were not significantly different from the sham mice, with the exception of Day 10 and Day 20 (See Fig. 2).

### 3.2. HJ11 ameliorates depressive-like behavior in CIS-treated mice

With respect to the total distance travelled in the OFT, CIS + DW and CIS + paroxetine mice showed hypoactivity compared with the sham mice. CIS + HJ11 mice moved longer distances in the OFT than CIS + DW and CIS + paroxetine mice, and showed no significant difference compared with sham mice. Regarding the ratio of distance travelled in the central grid, CIS + HJ11 mice showed no significant difference compared with sham mice, while CIS + DW and CIS + paroxetine mice moved a significantly shorter distance than sham mice. CIS + HJ11 mice entered the central grid more frequently than CIS + DW mice. However, CIS + paroxetine mice travelled a shorter distance in both the whole and central grids, and entered the central grid less than CIS + DW mice, with the difference in the total distance travelled being statistically significant (Fig. 3).

The changes of the duration of immobility in the FST and TST after injection of HJ11 are presented in Figs. 4 and 5, respectively. Treating mice with HJ11 for 14 days significantly reduced the immobile time in the FST ($p < 0.01$). The duration of immobility in the TST also significantly decreased after the same treatment in mice with HJ11. As a positive control, mice treated with the classical antidepressant paroxetine at a daily dose of 10 mg/kg presented a marked reduction in immobility time in both the FST and TST.

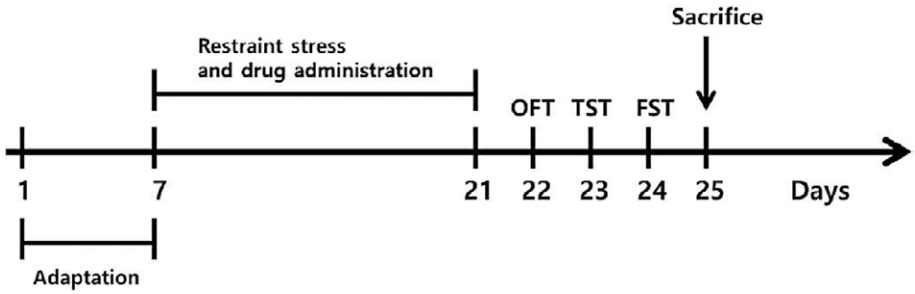


**Figure 1** Animal experimental protocol. Behavioral tests were performed 2 hours following drug administration. The animals were then sacrificed the following day. FST = forced swimming test; OFT = open field test; TST = tail suspension test.

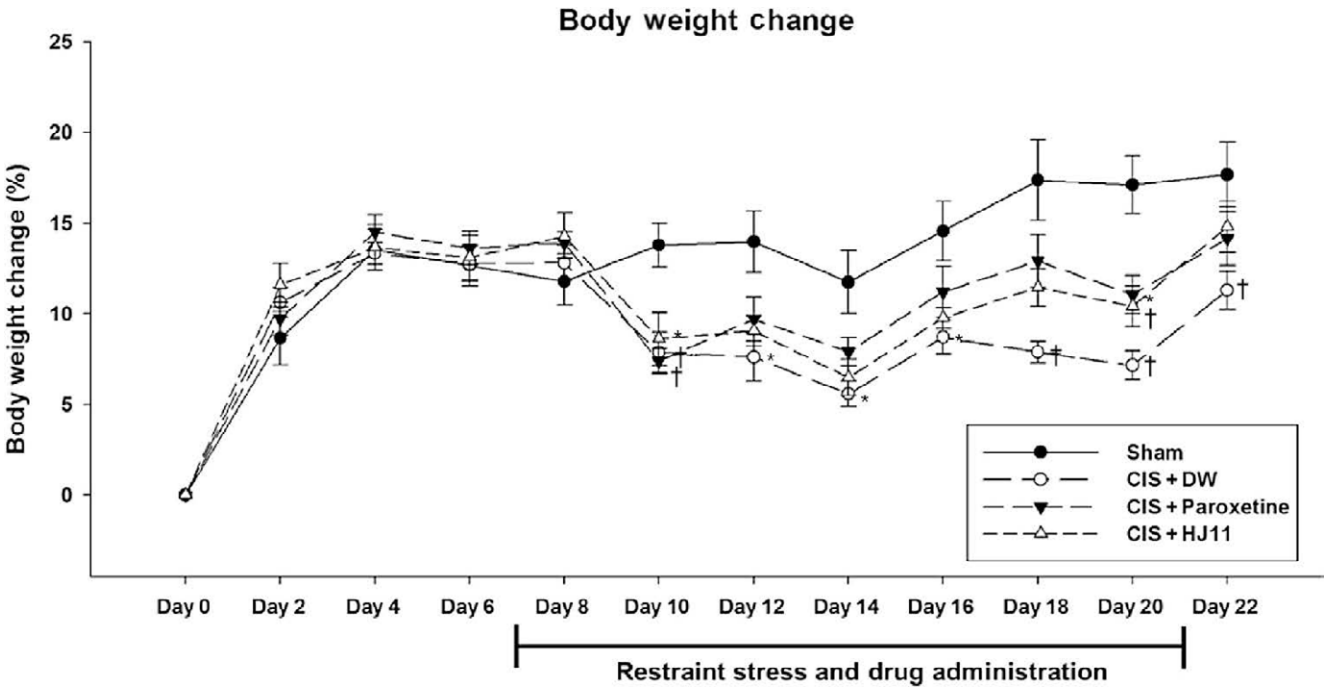


**Figure 2** Effects of restraint stress and drug administration on the body weight of mice. There was a significant increase in body weight in the group receiving HJ11 and paroxetine treatment compared with the vehicle treatment group. $^{*}p < 0.05$ versus the sham. $^{\dagger}p < 0.01$ versus the sham. CIS = chronic immobilization stress; DW = distilled water; HJ11 = *Liver Qi Depression* pharmacopuncture.

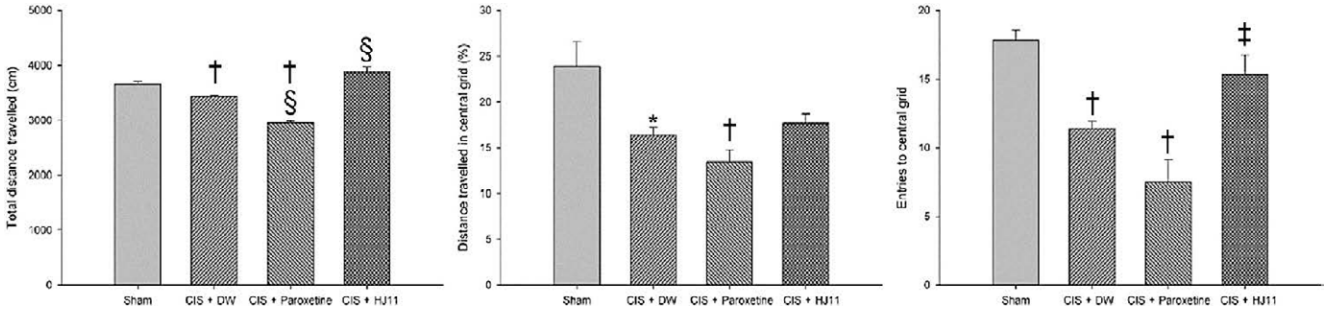


**Figure 3** Effect of HJ11 on locomotor activity and anxiety in the open field test (OFT). Data represent the mean ± standard error of the mean (SEM). $^{*}p < 0.05$ versus the sham. $^{\dagger}p < 0.01$ versus the sham. $^{\ddagger}p < 0.05$ versus CIS + DW. $^{\S}p < 0.01$ versus CIS + DW. CIS = chronic immobilization stress; DW = distilled water; HJ11 = *Liver Qi Depression* pharmacopuncture.

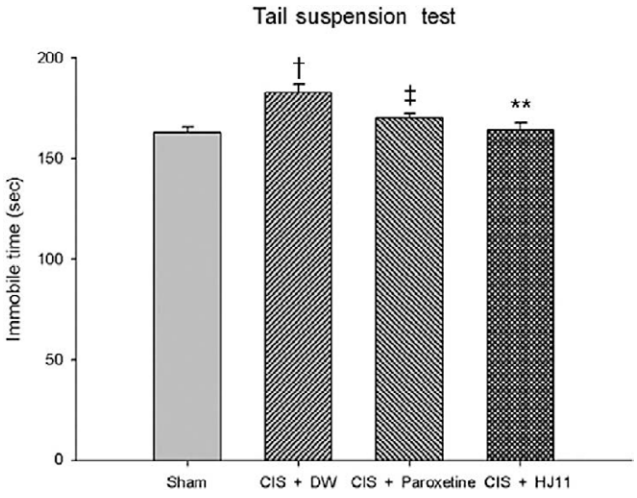


**Figure 4** Effect of HJ11 on the immobility of mice in the tail suspension test (TST). Data represent the mean ± standard error of the mean (SEM). The duration of immobility was measured. $^{\dagger}p < 0.01$ versus the sham. $^{\ddagger}p < 0.05$ versus CIS + DW. $^{\S}p < 0.01$ versus CIS + DW. CIS = chronic immobilization stress; DW = distilled water; HJ11 = *Liver Qi Depression* pharmacopuncture.

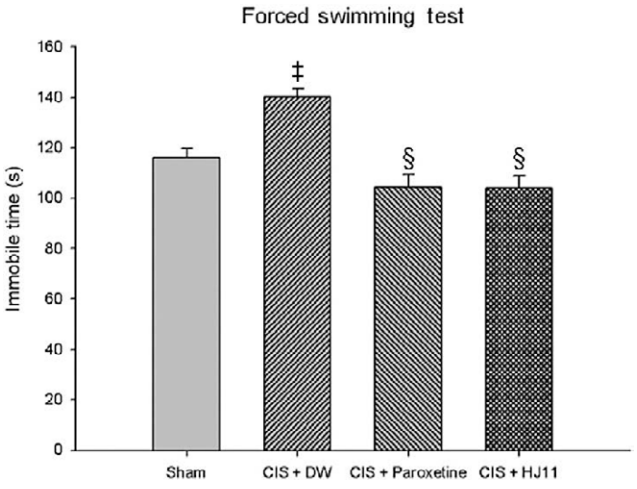


**Figure 5** Effect of HJ11 on the immobility of mice in the forced swimming test (FST). Data represent the mean ± standard error of the mean (SEM). The duration of immobility was measured. $^{\dagger}p < 0.01$ versus the sham. $^{\S}p < 0.01$ versus CIS + DW. CIS = chronic immobilization stress; DW = distilled water; HJ11 = *Liver Qi Depression* pharmacopuncture.

### 3.3. HJ11 improves changes in hippocampus and amygdala tissue induced by CIS

The effect of stress on BDNF expression was determined by immunoblotting of hippocampus and amygdala tissue. The level of BDNF expression in the hippocampus of CIS + DW mice was decreased by half compared with that of the sham group. However, the reduced level of BDNF expression following stress was enhanced in the HJ-treated and paroxetine-treated groups (Fig. 6). It was found that the expression of BDNF in amygdala tissue was decreased by immobilization, while administration of HJ11 increased the expression of BDNF (Fig. 7). Paroxetine did not significantly elevate BDNF level in the amygdala compared with the CIS + DW group.

## 4. Discussion

In the present study, antidepressant and anxiolytic effects of HJ11, a pharmacopuncture prescription, were observed in behavioral tests and immunoblotting analysis in mice. As a novel therapy, pharmacopuncture can be introduced to treat depression, which has the double effect of acupuncture and herbal medicine. In several clinical trials and observational studies, some of these ingredients in the form of pharmacopuncture had positive neuropsychiatric effects in humans. *Salviae Miltiorrhizae Radix*, one of the main ingredients of HJ11, has been proven to be effective in neuropsychiatric diseases [18—20], in particular for schizophrenia [21—24]. *Zizyphi Semen* has traditionally been used for insomnia, which is also supported by several pharmacopuncture studies [25—27]. There have also been several studies indicating that *Cnidii Rhizoma* pharmacopuncture is effective in headaches and dizziness [19,28,29]. There are yet to be studies directly relevant to antidepressant or anxiolytic effects, however, these previous studies support a series of behavioral and tissue effects. Nevertheless, the majority of the ingredients of HJ11 have been revealed to have antidepressant or anxiolytic effects

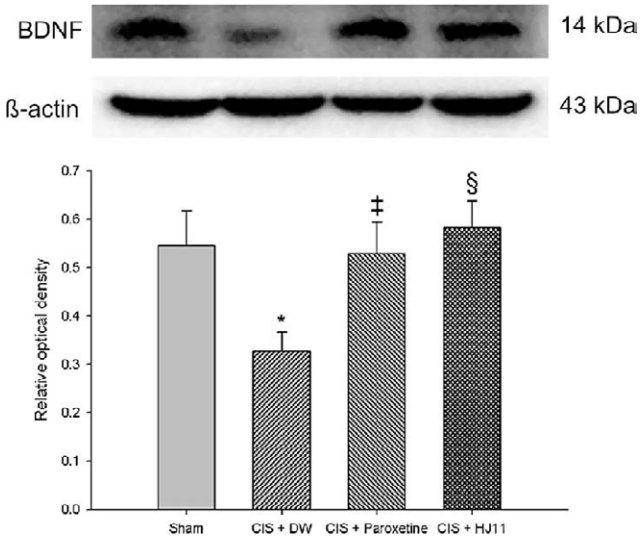


**Figure 6** Representative western blot and densitometric analysis of brain-derived neurotrophic factor (BDNF) compared with β-actin in the hippocampus. *Liver Qi Depression* pharmacopuncture-treated mice showed an increased level of BDNF compared with DW-treated and paroxetine-treated mice. The values are the mean ± standard error of the mean (SEM). $^{*}p < 0.05$ versus the sham. $^{\ddagger}p < 0.05$ versus CIS + DW. $^{\S}p < 0.01$ versus CIS + DW. CIS = chronic immobilization stress; DW = distilled water; HJ11 = *Liver Qi Depression* pharmacopuncture.

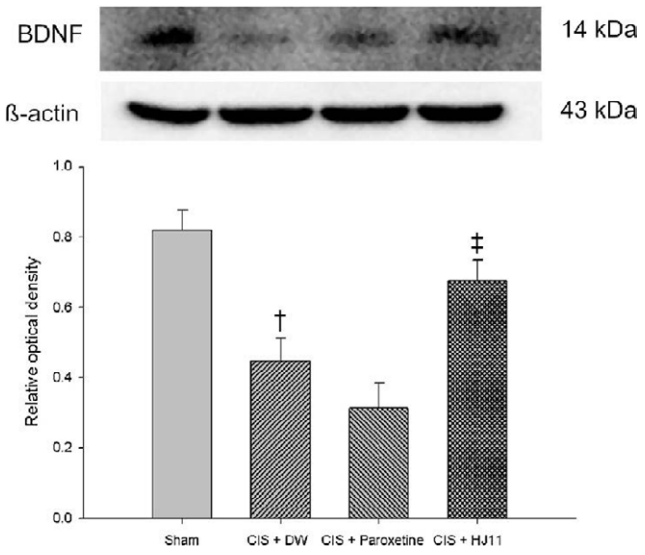


**Figure 7** Representative western blot and densitometric analysis of brain-derived neurotrophic factor (BDNF) compared with β-actin in the amygdala. *Liver Qi Depression* pharmacopuncture-treated mice showed an increased level of BDNF compared with DW-treated and paroxetine-treated mice. The values are the mean ± standard error of the mean (SEM). $^{\dagger}p < 0.01$ versus the sham. CIS = chronic immobilization stress; DW = distilled water; HJ11 = *Liver Qi Depression* pharmacopuncture.

in animal studies performed by intraperitoneal or oral administration. Further study regarding the potential effects of the respective ingredients is necessary.

Mice were treated with HJ11 by injection at acupoints to investigate the antidepressant-like effects, and the duration of immobility was markedly reduced, similar to that seen with paroxetine. By contrast, in the OFT, HJ11-treated mice showed increased activity and decreased anxiety compared with CIS + DW and CIS + paroxetine mice, indicating that HJ11 may alleviate lassitude derived from chronic stress. CIS induced a decrease in body weight and an elevation in anxiety and learned helplessness, as described in previous studies. Learned helplessness induces a decrease in activity [30,31], and HJ11 may be a candidate for the treatment of stress-induced depression.

In the OFT, CIS + DW mice also showed increased activity and decreased anxiety, which were more enhanced than in the CIS + paroxetine mice. Injection of DW or HJ11 at acupoints can cause stimulation. Owing to this stimulatory effect on acupoints, CIS + DW mice had a potentially insignificant difference compared with CIS + paroxetine mice in behavioral tests with respect to inactivity, anxiety, and learned helplessness. This tendency was consistent in the tissue analysis. Simply injecting at acupoints may produce a baseline effect, as acupoints are thought to be related to the sites where external stimuli result in a greater sensory stimulus [32]. Even though the dose of paroxetine was determined in accordance with previous studies [12,17], the effect was analogous to the effect of simply injecting DW in acupoints. What is clear is that a significant difference between CIS + HJ11 and CIS + DW mice indicates that certain effects were oriented by the ingredients of the pharmacopuncture as shown in behavior tests and immunoblotting.

Recent studies have reported that chronic stress induces depressive behaviors and that the symptoms are related to changes in diverse regions of the brain including the hippocampus and amygdala [33,34], which can be recovered by treatments [35,36]. As a mediator in survival of neurons from neuronal damage resulting from stress, BDNF is the important factor for neuroprotection and participates in dendritic remodeling in the hippocampus and amygdala [34]. It is closely associated with psychiatric disorders such as depression, schizophrenia, and dementia [37–39] and is also proven to be affected by CIS [33]. Structural and functional plasticity of the hippocampus is initially affected by stress and it consequently extends to change in the amygdala and prefrontal cortex, the key areas that shows malfunction after stress [34,40]. The alteration of BDNF expression after administration of HJ11 suggests that HJ11 reduces neurodegeneration induced by stress. The improvement of different regions of the brain suggests a potential effect of HJ11 on overall recovery in the brain influenced by neural damage.

There exist limitations in this study. In terms of insufficient effect of positive control to represent current usual treatment, the comparison with a higher dose of selective serotonin reuptake inhibitor agents or other prevalently used antidepressants can be considered. This study was limited to analyzing BDNF in the hippocampus and amygdala. By investigating changes in other components in the brain and other brain regions such as the amygdala and prefrontal cortex, the overall effect and precise mechanism of action of HJ11 on depressive-like behavior could be clearly identified.

In conclusion, the results of this study indicate that HJ11 suppresses both anxiety and depression caused by stress. It was also observed that the expression of BDNF was increased in hippocampus and amygdala tissue. Thus, these neuroprotective changes may be involved in the mechanism of the effect of HJ11 pharmacopuncture and should be investigated in the future. The use of pharmacopuncture can be considered in stress-related diseases including depression.

## Disclosure statement

The authors declare that they have no conflicts of interest.

## Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2013R1A1A1063477).

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