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The effects of *Xingnao Jieyu* capsules on post-stroke depression are similar to those of fluoxetine

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Research Highlights

(1) Synaptotagmin promotes neurotransmitter release, regulates the transfer of synaptic vesicle to synaptic active zones, and is a key factor in information transfer among neurons.
(2) A rat model of post-stroke depression was established using left middle cerebral artery occlusions in combination of chronic unpredictable stress and solitary housing during development. Experimental rats received intragastric perfusion of *Xingnao Jieyu* capsules dissolved in distilled water.
(3) The *Xingnao Jieyu* capsules upregulated synaptotagmin expression in the hippocampi of rats with post-stroke depression, and improved depression symptoms.

Abstract

The *Xingnao Jieyu* capsule has been shown to effectively relieve neurologic impairments and lessen depression. It remains poorly understood whether this capsule can be used to treat post-stroke depression. Thus, in the present study, we established a rat model of post-stroke depression using left middle cerebral artery occlusions in combination of chronic unpredictable stress and solitary housing during development. Experimental rats received intragastric perfusion with 0.82, 0.41, and 0.20 g/kg *Xingnao Jieyu* capsules separately dissolved in 2 mL distilled water. Fluoxetine served as a positive control. The treatment was conducted over 28 days. Sugar water consumption test, open-field test, real-time fluorescent quantitative PCR and immunohistochemical staining results demonstrated that intragastric perfusion with various doses of *Xingnao Jieyu* capsules increased sugar water consumption, voluntary behaviors and synaptotagmin mRNA and protein expression in rats with post-stroke depression. These therapeutic effects were similar to those of fluoxetine. These results indicate that *Xingnao Jieyu* capsules upregulate synaptotagmin expression in hippocampi of rats with post-stroke depression, and exert antidepressant effects.

Key Words

neural regeneration; traditional Chinese medicine; *Xingnao Jieyu* capsule; stroke; post-stroke depression; synapsin; neurologic impairment; chronic stress; depression; hippocampus; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

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INTRODUCTION

The treatment of post-stroke depression is based on the treatment of stroke in combination with antidepressant drugs. In accordance with their chemistry and mechanisms of action, common antidepressant drugs are categorized into tricyclic antidepressants, tetracyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, and serotonin-norepinephrine reuptake inhibitors. These antidepressant drugs have similar therapeutic effects, but also have toxic and adverse effects on the heart and brain, which frequently lead to allergies and drug withdrawal syndromes, therefore limiting their clinical application. Traditional Chinese medicine with definite therapeutic effects, multiple targets, and negligible toxic and adverse effects have become a focus for clinical development.

Post-stroke depression is a frequent complication of cerebrovascular disease, but its underlying causes remain unclear. The occurrence of post-stroke depression results from the effects of biological, psychological, and social factors[1], likely involving neurotransmitters, neuroendocrine effects, nerve anatomy, neurotrophic factors, neural regeneration, inflammatory reactions, and social psyche factors[2-4]. Theories about primary endogenous and reactive mechanism have been investigated in the study of the pathogenesis of post-stroke depression[3-8]. On the basis of the recent study of neurotransmitters, information transmission among neurons is mainly mediated by synaptic vesicles from nerve endings, which are particles that release transmitters[9-12]. There are specific vesicle-associated proteins on synaptic vesicles that store transmitters such as synaptophysin. Ca^{2+} influx induces synaptic vesicle endocytosis and exocytosis. Ca^{2+} binds to Ca^{2+} sensors and synergically controls the release of vesicle exocytosis, and synaptotagmin likely acts as a Ca^{2+} sensor for neurotransmitter release[13-14]. It is also a synaptic protein involved in learning and memory[15-17]. Synaptotagmin regulates the transfer of synaptic vesicle to synaptic active zones[18].

Previous studies using imaging and other techniques have confirmed that the onset of post-stroke depression is associated with the disturbance of hippocampal neural plasticity, but few studies have addressed the precise pathogenesis. Therefore, in this study, we established a rat model of post-stroke depression using left middle cerebral artery occlusions in combination with chronic unpredictable stress and solitary housing during development. The experimental rats were administered *Xingnao Jieyu* capsules. Immunohistochemical methods were used to detect synaptotagmin expression in the hippocampi of post-stroke depression rats following treatment with *Xingnao Jieyu* capsules.

RESULTS

Quantitative analysis of experimental animals

A total of 60 Sprague-Dawley rats were equally and randomly assigned into six groups: blank control group (normal raising), model group (rat model of post-stroke depression), fluoxetine group (model of post-stroke depression + receipt of 2.08 mg/kg of fluoxetine dissolved in 2 mL distilled water by intragastric perfusion), high-, moderate-, and low-dose *Xingnao Jieyu* capsule groups (rat model of post-stroke depression + receipt of 0.82, 0.41, or 0.20 g/kg medicine powder dissolved in 2 mL distilled water by intragastric perfusion). All administrations were conducted for 28 consecutive days. One rat died in the blank control group, two died in the model group, one died in the fluoxetine group, and two died in each *Xingnao Jieyu* capsule group. The causes of these deaths were attributable to model establishment, anesthetic accidents, improper intragastric administrations, and spontaneous disease. A total of 50 rats were included in the final analysis, including nine in the blank control group, nine in the fluoxetine group, eight in the model group, eight in the high-dose *Xingnao Jieyu* capsule group, eight in the moderate-dose *Xingnao Jieyu* capsule group, and eight in the low-dose *Xingnao Jieyu* capsule group.

Effects of *Xingnao Jieyu* capsule on synaptotagmin expression in the hippocampal CA1 region of rats with post-stroke depression

At 1 day after administration (28 days of the experiment), synaptotagmin expression in the rat hippocampus was measured using immunohistochemical staining. Compared with the model group, synaptotagmin expression in the rat hippocampus was significantly higher in the various *Xingnao Jieyu* capsule groups and fluoxetine group ($P < 0.01$; Figure 1, Table 1).

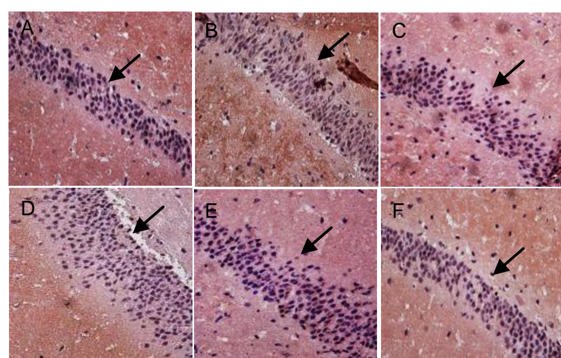

Figure 1 Effects of the *Xingnao Jieyu* capsule on synaptotagmin expression in the hippocampal CA1 region of rats with post-stroke depression (immunohistochemical staining, optical microscope, × 20).

Compared with the model group (A), the number of synaptotagmin-positive cells (arrows) was significantly higher in the high-, moderate-, and low-dose *Xingnao Jieyu* capsule groups (D–F) and the fluoxetine group (C). No significant changes in the number of synaptotagmin-positive cells were visible in the blank control group (B). High-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups: 0.82, 0.41, 0.20 g/kg *Xingnao Jieyu* capsule medicine powder and 2.08 g/kg fluoxetine medicine powder were dissolved in 2 mL distilled water for intragastric administration.

Effects of *Xingnao Jieyu* capsule on synaptotagmin mRNA expression in the hippocampal CA1 region of rats with post-stroke depression

Compared with the model group, relative expression of synaptotagmin mRNA of rat hippocampi in the fluoxetine, high-, moderate-, and low-dose *Xingnao Jieyu* capsule groups was increased to different degrees ($P < 0.01$; Table 2).

Effects of *Xingnao Jieyu* capsule on sugar water consumption in rats with post-stroke depression

At 1 day of treatment, sugar water consumption (sucrose preference) was reduced in rats with post-stroke depression compared with the blank control group ($P < 0.01$). At 15 days, sugar water consumption was higher in the high- and moderate-dose *Xingnao Jieyu* capsule and fluoxetine groups compared with the model group ($P < 0.01$), indicating that these drugs exerted effects after 2 weeks of treatment. At 28 days, sugar water consumption significantly increased in each treatment group compared with the model group ($P < 0.01$ or $P < 0.05$), especially in the high- and moderate-dose *Xingnao Jieyu* capsule and fluoxetine groups ($P < 0.01$). Therapeutic effects in these groups were better than that in the low-dose *Xingnao Jieyu* capsule group (Table 3).

Table 1 Effects of the *Xingnao Jieyu* capsule on synaptotagmin expression in the hippocampal CA1 region of rats with post-stroke depression

Group	*n*	Synaptotagmin	
		Number of positive cells	Mean absorbance
Blank control	9	198.1±12.8	0.53±0.01
Model	8	154.9±13.3[b]	0.28±0.01[b]
Fluoxetine	9	182.5±16.7[c]	0.42±0.03[bc]
Xingnao Jieyu capsule			
High-dose	8	179.5±18.3[ac]	0.35±0.01[bc]
Moderate-dose	8	167.4±17.4[b]	0.33±0.02[bc]
Low-dose	8	161.8±16.9[b]	0.31±0.01[bc]

Five sections of the same region were selected from each rat, and five non-overlapping fields were randomly selected from the hippocampus. The number of synaptotagmin-positive cells and their absorbance values were measured using a Motic image analysis system. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* blank control group; [c]$P < 0.01$, *vs.* model group. The data are expressed as mean ± SD and were analyzed by one-way analysis of variance and Student-Newman-Keuls test. High-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups: 0.82, 0.41, 0.20 g/kg *Xingnao Jieyu* capsule medicine powder and 2.08 g/kg fluoxetine medicine powder were dissolved in 2 mL distilled water for intragastric administration.

Table 2 Effects of the *Xingnao Jieyu* capsule on synaptotagmin mRNA expression in the hippocampal CA1 region of rats with post-stroke depression

Group	*n*	Relative expression of synaptotagmin mRNA
Blank control	9	0.82±0.15
Model	8	0.04±0.00[a]
Fluoxetine	9	0.35±0.02[ab]
Xingnao Jieyu capsule		
High-dose	8	0.36±0.02[ab]
Moderate-dose	8	0.20±0.00[ab]
Low-dose	8	0.10±0.00[ab]

[a]$P < 0.01$, *vs.* blank control group; [b]$P < 0.01$, *vs.* model group. The data are expressed as mean ± SD and were analyzed by one-way analysis of variance and Student-Newman-Keuls test. Real-time PCR was used to measure C_T values in each specimen. Relative expression of the target gene was expressed as the ratio of copy number of detected gene (the number of detected genes in the genome) to the copy number of internal reference GAPDH gene. High-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups: 0.82, 0.41, 0.20 g/kg *Xingnao Jieyu* capsule medicine powder and 2.08 g/kg fluoxetine medicine powder were dissolved in 2 mL distilled water for intragastric administration. GAPDH: Glyceraldehyde-3-phosphatedehydrogenase.

Table 3 Effects of the *Xingnao Jieyu* capsule on sugar water consumption in rats with post-stroke depression (%)

Group	*n*	Time after treatment (day)		
		1	15	28
Blank control	9	30.2±2.4	29.2±1.5	31.4±1.3
Model	8	22.3±1.5[b]	16.2±1.0[b]	15.2±1.4[b]
Fluoxetine	9	20.8±1.4[b]	18.5±1.6[bc]	24.5±1.8[ac]
Xingnao Jieyu capsule				
High-dose	8	22.1±1.5[b]	17.8±0.9[bc]	23.8±1.2[ac]
Moderate-dose	8	21.5±2.1[b]	17.2±2.4[bc]	22.9±2.6[ac]
Low-dose	8	20.2±2.5[b]	17.8±1.6[ad]	21.6±2.6[ad]

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* blank control group; [c]$P < 0.01$, [d]$P < 0.05$, *vs.* model group. The data are expressed as mean ± SD and were analyzed by one-way analysis of variance and Student-Newman-Keuls test. High-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups: 0.82, 0.41, 0.20 g/kg *Xingnao Jieyu* capsule medicine powder and 2.08 g/kg fluoxetine medicine powder were dissolved in 2 mL distilled water for intragastric administration. Sucrose preference (%) was calculated as sucrose water intake/(sucrose water consumption + water intake) × 100%.

Voluntary behavior of rats with post-stroke depression following treatment with *Xingnao Jieyu* capsule

Open-field test results revealed that horizontal movement and vertical movement decreased compared with the blank control group at 1 day of treatment ($P < 0.01$). Compared with the model group, horizontal movement and vertical movement significantly increased in each treatment group, and voluntary behavior increased in the high-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups at 15 and 28 days ($P < 0.05$ or $P < 0.01$; Tables 4, 5).

Table 4 Effects of the *Xingnao Jieyu* capsule on horizontal movement in rats with post-stroke depression (open-field test, number of movement/3 minutes)

Group	*n*	Time after treatment (day)		
		1	15	28
Blank control	9	46.5±4.6	44.2±5.2	41.5±4.2
Model	8	30.1±3.4[b]	20.3±2.5[b]	17.5±1.6[b]
Fluoxetine	9	28.4±4.2[b]	34.7±2.2[bc]	38.5±1.8[ac]
Xingnao Jieyu capsule		27.8±3.9[b]	33.6±1.5[bc]	35.3±2.7[bc]
High-dose	8	27.2±2.9[b]	32.1±3.7[bc]	34.0±1.6[bc]
Moderate-dose	8	25.6±2.4[b]	30.3±4.4[bc]	31.3±3.2[bc]
Low-dose	8	46.5±4.6[b]	44.2±5.2[bc]	41.5±4.2[bc]

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* blank control group; [c]$P < 0.01$, *vs.* model group. The data are expressed as mean ± SD and were analyzed by one-way analysis of variance and Student-Newman-Keuls test. High-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups: 0.82, 0.41, 0.20 g/kg *Xingnao Jieyu* capsule medicine powder and 2.08 g/kg fluoxetine medicine powder were dissolved in 2 mL distilled water for intragastric administration.

Table 5 Effects of the *Xingnao Jieyu* capsule on vertical movements in rats with post-stroke depression (open-field test, number of movements/3 minutes)

Group	*n*	Time after treatment (day)		
		1	15	28
Blank control	9	15.6±1.6	13.4±1.7	13.0±2.5
Model	8	9.8±1.8[b]	6.8±1.4[b]	5.7±2.8[b]
Fluoxetine	9	9.6±2.1[b]	11.6±2.4[c]	12.8±2.8[c]
Xingnao Jieyu capsule				
High-dose	8	9.0±1.5[b]	9.8±1.5[bc]	11.0±2.3[c]
Moderate-dose	8	8.8±1.6[b]	9.3±1.5[bc]	9.8±2.1[ac]
Low-dose	8	8.5±2.8[b]	8.8±2.3[bd]	9.1±1.0[bc]

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* blank control group; [c]$P < 0.01$, [d]$P < 0.05$, *vs.* model group. The data are expressed as mean ± SD and were analyzed by one-way analysis of variance and Student-Newman-Keuls test. High-, moderate-, and low-dose *Xingnao Jieyu* capsule and fluoxetine groups: 0.82, 0.41, 0.20 g/kg *Xingnao Jieyu* capsule medicine powder and 2.08 g/kg fluoxetine medicine powder were dissolved in 2 mL distilled water for intragastric administration.

DISCUSSION

The main goal of the treatment of post-stroke depression is to delay the progress of disease, to control symptoms, and to elevate patients' quality of life[19-24]. Drug therapy for post-stroke depression mainly consists of four kinds of antidepressants in combination with psychotherapy. These drugs have some therapeutic effects in the clinic, but they also engender toxic and adverse effects on the heart and brain to different degrees, and have the disadvantages of poor compliance and high price[25-26]. These drugs can lead to allergies and drug withdrawal syndromes, which limits their clinical application[25-26]. We urgently need to develop effective treatments with no toxic or adverse effects for patients with post-stroke depression.

Previous studies show that depression is improved mainly by elevating monoamine neurotransmitter contents in patients with post-stroke depression. The *Xingnao Jieyu* capsule effectively contributed to the recovery of neurological function, regulated neurotrophic factors, hormones and corresponding signaling pathways, and further promoted neuron regeneration in the hippocampal CA3 region and frontal lobe[27-30]. Simultaneously, *Xingnao Jieyu* capsule increased the content of *in vivo* monoamine neurotransmitters and improved depression. Synaptotagmin, a synaptic vesicle protein, is involved in neurotransmitter release and learning and memory[30-31]. The release of transmitters at nerve synapse is an important physiological process of the nervous system.

Synaptotagmin plays a key role in regulating nerves, endocrine cells, and other cell secretions. Synaptotagmin is strongly associated with post-stroke depression. *Xingnao Jieyu* capsule interferes with the mechanism of action of synaptotagmin, providing evidence for multiple pathways and multiple targets of Chinese compound preparations, and providing new ideas for treatment of post-stroke depression[32-33].

This study demonstrated that synaptotagmin was extensively distributed in hippocampal neuronal cells. Synaptotagmin expression decreased in the hippocampi of rats with depression, and its expression could be upregulated by treatment, indicating that synaptotagmin is essential to the regulation of synaptic plasticity. This study investigated the mechanisms of action of synaptotagmin in the release of neurotransmitters. Immunohistochemistry was used to detect synaptotagmin expression in the rat hippocampi following post-stroke depression. Reverse-transcription PCR results demonstrated that the *Xingnao Jieyu* capsule obviously increased synaptotagmin and synaptotagmin mRNA expression in the hippocampi of rats with post-stroke depression, suggesting that the *Xingnao Jieyu* capsule may protect against depression probably by upregulating synaptotagmin expression, which may be a mechanism underlying the effects of the *Xingnao Jieyu* capsule against depression. We conclude that the *Xingnao Jieyu* capsule has antidepressant effects, possibly mediated by intervening with synaptic plasticity. This study provides a scientific and experimental basis for determining the pathogenesis of and treatments for post-stroke depression.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

Experiments were performed at the Experimental Animal Center, Shaanxi University of Chinese Medicine, China from August to December 2011.

Materials

Animals

A total of 60 healthy adult clean Sprague-Dawley rats, of equal numbers of males and females, weighing 175 ± 20 g, were supplied by the Experimental Animal Center, Health Science Center, Xi’an Jiaotong University, China with animal license No. SCXK (Shaan) 2007-001. The rats were housed at 22 ± 1°C, 50–70% humidity, in 150–200 lx, with a 12-hour light/dark cycle, and an ambient noise level < 50 dB. The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[34].

Drugs

Xingnao Jieyu capsule consisted of 10 g *Rhizoma Acori Talarinowii*, 10 g *Radix Polygalae*, 8 g *Rhizoma Pinelliae*, 10 g *Radix Curcumae*, 10 g *radix bupleuri*, 10 g *Concretio Silicea Bambusae*, 12 g *Radix Salviae Miltiorrhiae*, 12 g *Caulis Spatholobi*, and 10 g *Radix Morindae Officinalis* (all purchased from Xi’an Traditional Chinese Medicine Decoction Pieces Factory, Xi’an, Shaanxi Province, China). They were decocted at 100°C in water 10 times their total weight for 1–2 hours, filtered by gauze, followed by decoction at 100°C in water eight times their total weight for 1–2 hours. The solution was filtered using gauze. The filtrates of the two solutions were mixed, condensed to thick cream at 50°C with a relative density of 1.25–1.30 and mixed with starch to prepare the particles. These particles were packaged into capsules, and the capsule was produced by the Preparation Center, Affiliated Hospital of Shaanxi University of Chinese Medicine. Each capsule (0.3 g/capsule) contained 3.5 g of crude drug.

Fluoxetine capsules (20 mg per capsule) were purchased from Eli Lilly and Company, Suzhou, China (Approval No. GYZZ J20080016; batch No. 204362). Administration dose was 2.08 mg/kg in accordance with *Pharmacology Experiment*[35].

Methods

Establishment of a post-stroke depression model and administration of the test compounds

During 1 week of adaptation, the rats were trained to drink sucrose water and to receive behavioral detection. In the blank control group, five rats were housed in a cage (males and females in separate cages). Rats in the remaining groups were separately housed in individual cages.

The stroke model was established by unilateral internal carotid artery ligation in accordance with a previously published method[16]. At 24 hours after the establishment of the model of stroke, neurological deficits were scored according to Zea Longa’s method[17]. A score of ≥ 1 represented a successful induction. Ten experimental rats underwent the post-stroke depression model in combination with solitary housing and chronic unpredictable stress in accordance with the method described

by Willner *et al*[8]. All rats were separately housed and randomly subjected to 11 kinds of stimuli for 14 days or 21 days, including behavioral restriction for 2 hours, ice water swimming for 5 minutes at 4°C, fasting for 24 hours, loss of access to water for 24 hours, tail clamping for 1 minute, light-dark reverse for 24 hours, electric shock on a footplate for10 minutes (1 mA electric current, 30 V voltage, once every 10 seconds, each for 1 second, total 60 times), the home cage inclined at 45° for 24 hours, moist padding for 24 hours, an empty bottle for 2 hours and shaking (50 Hz) at 45°C for 5 minutes. One kind of stimulus was administered every day, and each kind of stimulus appeared once or twice, so the rats could not predict the stimulus. Establishment of a successful model was demonstrated when rats exhibited evident depression, low emotion, dark hair, decreased horizontal movement, loss of sugar water consumption, decreased vertical movement, and loss of body mass and appetite[16-18]. After establishment of post-stroke depression, the rats were intragastrically perfused with fluoxetine or *Xingnao Jieyu* capsules of 45, 15, 7.5 mg/100 g, once a day.

Real-time PCR for synaptotagmin mRNA expression in rat hippocampi

At 28 days of treatment, the rat hippocampus was triturated in liquid nitrogen, and placed in a homogenization tube (the operation was performed on ice). A 1 mL Trizol solution was added to each 50–100 mg of tissue, and homogenized in an electric homogenization machine (without ever letting the sample volume exceed 10% of the Trizol volume). Homogenate samples were placed on ice for 10 minutes until nucleic acid and protein complex was completely separated. Lysate was centrifuged at 12 000 r/min in a high-speed refrigerated centrifuge at 2–8°C for 10 minutes, and then the supernatant containing RNA was obtained. The sediment containing pericellular membranes, high-molecular-weight DNA, and polysaccharides was placed at room temperature for 5 minutes. Then, 0.2 mL chloroform was added to each 1 mL Trizol solution, followed by shaking on a mixer for 20 seconds and placement on ice for 5 minutes. The specimens were centrifuged at 12 000 r/min in the high-speed refrigerated centrifuge at 4°C for 15 minutes. The specimen presented three layers: an upper layer aqueous phase containing RNA with a volume of about 60% of the RNA extract; a middle layer containing protein, genomic DNA, polysaccharides and other impurities; and a lower layer, which was the organic layer. For RNA precipitation, the upper layer was transferred into an Ependorf tube, with an addition of isopropanol whose volume was half of the RNA extract, because RNA could be precipitated by isopropanol, followed by blending at room temperature and standing for 10 minutes. The specimens were centrifuged at 12 000 r/min in a refrigerated centrifuge at 4°C for 10 minutes, and then white or yellow sediment was visible in the bottom of the tube, *i.e.* RNA precipitation. For RNA washing, after removal of the supernatant, the sediment was obtained and washed with at least 1 mL 75% alcohol (prepared with diethyl pyrocarbonate-treated water) in a 1 mL Trizol solution, and centrifuged at 8 000 r/min at 2–8°C for 10 minutes. The supernatant was then discarded. In accordance with the two-step PCR amplification procedures of the TP800 PCR system (Takara), each tube was placed in a DNA thermal cycler. Amplifications were carried out using the SYBR Green assay. Synaptotagmin amplification conditions were as follows: 40 cycles of 95°C for 3 minutes, 95°C for 30 seconds, 60°C for 30 seconds and 68°C for 30 seconds, followed by 72°C for 10 minutes. The dissolution temperature was adjusted according to the solubility curve, with complete degeneration at 95 °C for 10 seconds, renaturation at 60°C for 30 seconds, and then maintenance at 90°C for 15 seconds. Ct value of each sample was detected using the TP800 PCR system. The relative gene expression was represented by the copy number ratio of the detected gene to GAPDH.

In accordance with Oligo 6.62 software (Shanghai GenePharma Co., Ltd., Shanghai, China), primers were designed and synthesized by Invitrogen, Xi'an, Shaanxi Province, China. The primer sequence is listed as follows:

Gene	Primer sequence (5'–3')
Synaptotagmin	Upstream: GTA CTT GGG TAC CTC TGC AG
	Downstream: GAC CAG CAC TCT ACC TCC TA
GAPDH	Upstream: GTA ACC CGT TGA ACC CCA TT
	Downstream: CCA TCC AAT CGG TAG TAG CG

Open-field test for voluntary behavior

At 1, 15, and 28 days after treatment, an open-field test was conducted[27-28]. A custom-made open box with black walls (40 cm high, 80 cm long and 80 cm wide) was used. The floor area contained 25 blocks of equal size. Horizontal movements represented the range of motion of animals and vertical motion reflected curiosity to the new environment. The number of blocks that rats passed through represented the horizontal movement score (1 square represented 1 score). The rats received a score of 1 every 10 cm if they walked along the lines. The frequency of straight upward movements represented vertical movement scores (rearing). This was operationally defined as both feet of the rats off the ground, and when they were put down, it was regarded as one movement and scored 1. Every rat was observed for 3 minutes.

Sugar water consumption test for rat depression symptoms

At 1, 15, and 28 days after intervention, rats of each group were allowed free access to two kinds of water: a bottle of tap water and a bottle of tap water containing 1% sucrose. Tap water and sucrose water consumption was measured from 7:00 a.m. to 7:00 a.m. the next day by weighing the bottles. Sucrose preference was compared before and after the experiment. Sucrose preference (%) was equal to sucrose water intake/(sucrose water consumption + tap water intake) × 100%. The average value was calculated as the percentage of sugar water consumption.

Immunohistochemistry for synaptotagmin expression in the rat hippocampus

At 28 days of the experiment (after the end of administration), the rats were anesthetized with 10% chloral hydrate, perfused with saline and 4% neutral paraformaldehyde, and then decapitated. Brain tissues were fixed in 4% neutral paraformaldehyde. Approximately 4-mm-thick samples were obtained anterior and posterior to the injection site of the hippocampus, dehydrated, permeabilized, embedded in paraffin, and then sliced into 4 μm coronal sections. These sections were placed in a constant-temperature oven overnight, and then baked at 68°C for 20 minutes. Subsequently, the sections were dewaxed three times in xylene, each for 10 minutes, and dehydrated through a graded alcohol series. The sections were then placed twice in 100% alcohol, each for 10 minutes, hydrated through a graded alcohol series, and then washed with running water for 2 minutes. Residual wax oil was removed from the sections. The sections were incubated in 3% H_2O_2 at room temperature for 10 minutes to inactivate endogenous peroxidases, and washed three times with PBS, each for 5 minutes. The sections were immersed in 0.01 mol/L citrate buffer (pH 6.0), heated with an electric stove, and boiled for 15 minutes to retrieve the antigen. The sections were washed with running water to cool them to the ambient temperature, washed three times with PBS, each for 15 minutes, and blocked with normal goat serum at room temperature for 10 minutes. After removing any excess liquid, each section was incubated with 50 μL of a primary antibody (rabbit anti-rat synaptotagmin I monoclonal antibody, 1:100; Shanghai Tongpai Biotechnology Co., Ltd., Shanghai, China) at 4°C overnight. The sections were resuscitated to room temperature, and washed three times with PBS, each for 5 minutes. PBS buffer served as a negative control for comparison to the primary antibody. Each section was incubated with biotin-labeled anti-rabbit IgG (1:100; Shanghai Tongpai Biotechnology Co., Ltd.) at 37°C for 30 minutes, followed by three washes with PBS, each for 5 minutes. Each section was incubated with horseradish peroxidase-labeled streptavidin at 37°C for 30 minutes, followed by three washes with PBS, each for 5 minutes. Each section was treated with a drop of 3,3′-diaminobenzidine, and observed under a microscope. After a wash with running water, the sections were counterstained with hematoxylin, dehydrated through a graded alcohol series, permeabilized twice by xylene, each for 10 minutes, dried, mounted in neutral resin, and then stored at 4°C.

The number of synaptotagmin-positive cells was quantified by measuring absorbance values using a Motic image analysis system (Shenzhen Mashide Instrument Co., Ltd., Shenzhen, Guangdong Province, China). Five sections from each rat hippocampus were selected, and five non-overlapping fields were randomly selected under a 200× light microscope.

Statistical analysis

The data were expressed as mean ± SD, and were analyzed using SPSS 18.0 software (SPSS, Chicago, IL, USA). Differences in the mean among multiple groups were compared using one-way analysis of variance. Paired comparison among groups was completed using Student-Newman-Keuls test. A value of $P < 0.05$ was considered statistically significant.

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