Shugan Jiangni Hewei Granule regulates synaptic plasticity in central sensitization via NGF/TrkA Signaling Pathways in psychological stress rats with nonerosive reflux disease

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Research Article

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Abstract

Objective. We sought to investigate the mechanism of visceral hypersensitivity improvement of Shugan Jiangni Hewei Granules (SJHG) treatment of non-erosive reflux disease (NERD) and provide an experimental basis for its clinical application.

Methods. Fifty healthy male Sprague-Dawley (SD) rats were divided into 5 groups, with 10 rats in each group, including normal group (healthy rats), model group (model rats without any treatment), SJHG group (model rats with SJHG treatment), Omeprazole group (model rats with Omeprazole treatment), and k252a group (model rats treated with 100μmol/kg NGF/TrkA pathway inhibitor).The esophageal mucosa and synaptic vesicles in spinal cord were observed by EMT, NGF, TrkA, PSD95, and GAP43 proteins expression in spinal cord were detected by western blot, and GluA1 protein expression in spinal cord was detected by immunohistochemistry among the normal group, model group, SJHG group and Omeprazole group, PSD95 protein expression in spinal cord was detected by immunofluorescence.

Results. In the model group, the intercellular spaces of epithelia of esophageal mucosa widened, and the synaptic vesicles in spinal cord increased compared with the normal group. NGF amd TrkA proteins of model group in the spinal cord were significantly increased compared with the normal group ($P<0.01$), After the treatment of SJHG or Omeprazole or k252a, the expression of NGF amd TrkA were decreased compared with the model rats. ($P<0.01$). GAP43, PSD95 proeins of model group in the spinal cord were higher than that of the normal group.($P<0.01$). Compared with the normal group, the expression of NGF increased ($P<0.05$), but no significant difference of TrkA and CREB mRNA expression. The content of GluA1 in spinal cord of rats in normal group was low. According to the integrated optical density (IOD), GluA1 protein in the model group was higher than that in the normal group ($P<0.01$). The expression of GluA1 of both the SJHG group and the Omeprazole group decreased signifcantly ($P<0.01$).

Conclusions. SJHG can ameliorate visceral hypersensitivity and decrease central hypersensitivity in mental stress rats with non-erosive reflux disease, which is partially due to the regulation of NGF/TrkA signaling pathways. Psychological factor is also an important cause of visceral hypersensitivity. These findings provided a new target for Traditional Chinese medicine treatment of NERD to improve the hypersensitivity of esophageal viscera.

1. Background

Gastroesophageal reflux disease (GERD) is a symptom and complication caused by reflux of gastric contents into the esophagus, with typical symptoms including heartburn and extra-esophageal reflux, and extra-esophageal symptoms including ear and nose, in severe cases, esophageal stenosis Esophageal carcinoma, which seriously affects the physical and mental health of patients. [(1)] GERD is classified into non-erosive gastroesophageal reflux disease (NERD), reflux esophagitis (RE) and Barrett's esophagus (BE). In China, the rate of GERD is 8.2%-17.3%, [(2; 3)] of which the incidence of NERD is about 70%.[(4)] After treatment with conventional drugs, only 39.0%-45.0% of NERD patients could improve symptoms,[(5)]

which may not only due to the damage of esophageal mucosa anti reflux barrier and defense function, but also due to esophageal visceral hypersensitivity.[1]

At present, Esophageal visceral hypersensitivity caused by psychological stress is the main cause of NERD.[6] Psychological stress regulates the expression of related neurotransmitters and their receptors to sensitize the spinal cord, which plays an important role in the maintenance of NERD visceral hypersensitivity. Traditional Chinese medicine (TCM) is an important part in the treatment of NERD. Our previous study found that TCM can relief the symptoms of acid reflux, abdominal distension, heartburn and regurgitation. Among those patients, most of them had anxiety and depression. However, the nature of signals that CM regulates the visceral hypersensitivity remains unclear.

Shugan Jiangni Hewei Granule (SJHG) is a Chinese herbal formula which is based on Chinese medicine theory. TCM maintains that the liver regulates mental state, and is essential for the controlling of psychological stress. Hepatic and gastric incompatibility is a classic and common syndrome in TCM in NERD caused by psychological stress. In clinical research, Shugan-Hewei recipes are often used in the treatment of NERD. There was no significant difference between the TCM group and western medicine group. The current studies suggest that the pathogenesis of NERD may be related to visceral hypersensitivity, esophageal motility, esophageal acid exposure, and esophageal mucosal barrier changes. NGF/TrkA signaling plays a crucial role in neuronal development, function, survival, and growth. Nerve growth factor (NGF) is one of the most important members of the neurotrophic factor family.[7; 8] Its binding to receptors can promote neuronal growth and development and play a key role in regulating synaptic plasticity.[9] In the central nervous systems of adults, increased expression of NGF may regulate synaptic plasticity and cause hyperalgesia.[10] Synaptic plasticity, the activity-dependent change in nerve connections, has long been recognized as a significant component of learning and memory.[11] The postsynaptic density protein-95 (PSD95) is a major biomarker of synaptic, which plays an important role in synaptic plasticity.[12] The growth-associated protein-43 (GAP43) is an important newborn nerve fiber biomarker.[13] AMPA-type glutamate receptor (GluA) targeting and trafficking is central to synaptic transmission and plasticity.[14] Those molecules plays an important role in synaptic plasticity.

In this study, to explore the effect of SJHG, we investigated the role of NGF-TrkA/AMPAR signaling pathways,and examined the synaptic plasticity associated proteins.

2. Methods

2.1 Experimental Animals

Fifty specific pathogen-free male Sprague-Dawley (SD) rats(250±30g,6-8weeks) were purchased from Experimental Animal Center of Liaoning University of Traditional Chinese Medicine（No. 210726211100566367,Liaoning, China), The rats were housed under 12h light-dark cycle in separate plastic cages under standard laboratory conditions, and had free access to food and water in Animal

Laboratory Center of Liaoning University of Traditional Chinese Medicine. Adapting to the environment for 1 week.

2.2 Drugs

SJHG comprises 10 Chinese herbs: Reddle 6g, Paeoniae radix alba 10g, Radix bupleuri 12g, Paeoniae radix alba 12g, Ligusticum wallichii 12g, Fructus aurantii 1

2g, Tangerine peel 12g, Liquorice root 6g, Rhizoma cyperi 10g, inula japonica 12g. SJHG was prepared by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (2109333). The inspection report certificates of all the herbs were provided by the company, and all the herbs met the requirements of the Chinese Pharmacopoeia (2020). Omeprazole Rat to human drug dosage conversion ratio is 6.3, So the granules needed were 11.07g/kg per day.The granules were mixed into the solution and stored at 4℃ for further use. Omeprazole Enteric Capsules, produced by Shandong Luoxin Pharmaceutical Group Stock Co., Ltd was 1.80mg/kg per day according to the same ratio of conversion.

2.3 Animal Grouping

Rats were randomly divided into five groups by a random number table(10 rats in each group): including normal (healthy rats), model (model rats without any treatment), SJHG (model rats with SJHG treatment), Omeprazole(model rats with Omeprazole treatment), k252a (model rats treated with 100μmol/kg NGF/TrkA pathway inhibitor) groups, After the injection of mixture of ovalbumin and aluminum hydroxide. Rats in SJHG group and Omeprazole group were treated with drug gavage. The other groups were given 10 mL/kg of distilled water by gavaged at the same time once a day for two weeks, and K252a group were intraperitoneally injected with 100μmol/kg K252a once a day for 7 days.

2.4 Establishment of psychological stress with non-erosive reflux rats models

Other than the normal group, the other groups were subjected to chronic random stress for 21 days in the second week. One of the following eight stimuli was given randomly every day.The specific stress stimuli were as follows: ① Fasting for less than 20 hours; ②Water-deprived for 17hours; ③4°Cswimming for 5minutes; ④45 degree angle cage tilt for 17hours; ⑤Trembling stress (high-speed shaking) for 10minutes; ⑥Restraint stress for 2hours; ⑦Wet litter (100g sawdust with 200ml water, 5h); ⑧Tail pinching for 2minutes. After each stressing, the rats were kept alone under normal conditions. The sequence and timing of stress should follow a random and unpredictable principle, and the same stress stimuli should not be repeated every week.

In the third week, the other groups were intraperitoneally injected with 1.5 mL of a mixture of ovalbumin and aluminum hydroxide (ovalbumin 100 mg/aluminum hydroxide 200 mg), and the rats in the normal group were intraperitoneally injected with the same volume of normal saline simultaneously. In the 29th day, except for the normal group, the other groups were treated with esophageal acid perfusion.Then the rats in the experiment were then sacrificed.ovalbumin(A8040) was supplied by Solarbio Technology Co., Ltd, Beijing, China.Aluminum hydroxide(77161) was supplied by Thermo Fisher Scientific Co., Ltd, Shanghai, China.

2.5 Esophageal Hydrochloric acid Perfusion Approach

After the rat was completely anesthetized, the abdominal and gastric walls were incised, the gastric cardia was inserted with a drainage cannula for collecting runoff solution from the esophagus. A single lumen clear vinyl tube was passed through the mouth into the esophagus. The tip of the tube was located 2cm-3cm above the esophagogastric junction. The anesthetized rats was positioned with its head elevated at a slight angle (20°-30°). Then, the tube was connected to a continuous perfusion pump. Hydrochloric acid (0.1 mol/L HCl, supplied by Yida Technology Co., Ltd., Quanzhou, China.) was perfused continuously at a rate of 10 mL/h for 50 minutes.

2.6 Sample Collection

After 10% chloral hydrate (0.3ml per100g in weight) for deep anaesthesia, the normal group rats were directly sacrificed, the other group rats were sacrificed after the acid perfusion. Then we took their spinal segments (L4-L6), and collected the lower third of esophagus (from 15mm above and 2mm below the esophageal sphincter). Some of the samples were put into fixative for TEM (Transmission EM), and some were taken into paraformaldehyde for immunohistochemistry (IHC), others were frozen for western blot (WB) and quantitative PCR (qPCR) and immunofluorescence (IF). The experiments were approved by Animal Experiment Ethics Committee of Liaoning University of Traditional Chinese Medicine (Ethical Approval Number:21000042021049) and flowchart of this experiment is presented in the supplemental file.

2.7 Testing Indexes and Methods

2.7.1 Observation of General Condition of rats.

Throughout the whole experiment, all rats' general condition was observed daily, including their mental state, activity, and fur appearance.

2.7.2 Transmission Electron Microscopy (TEM) staining of esophageal mucosa and synaptic vesicles in spinal cord.

The tissues were cut quickly within 1-3 minutes by a sharp blade.The size of tissue block should be no more than 1 mm^3. Then the tissue blocks were transferred into an EP tube with fresh TEM fixative for further fixation, which was fixed at 4℃ for preservation and transportation. The embedding models with resin and samples were cut to 60nm-80nm thin on the ultra microtome, and the tissues were fished out onto the 150 meshes cuprum grids with formvar film. The esophagus and vesicles from spinal were observed under TEM.

2.7.3 Western Blot Analysis.

k252a(Lot.104529) were supplied by MedChemExpress Co.,Ltd.,Shanghai,China), Target protein was detected by antibodies: anti-NGF (Cat No.ab52918,1:1000, abeam), anti-TrkA (Cat No.Ab109010, 1:1000,abeam), anti-GAP43(Cat No.GB11095, 1:1000, Servicebio), anti-PSD95 (Cat No.GB11277,1:1000, Servicebio) subsequently incubated with secondary antibodies (Cat No. GB25301,1:5000,Servicebio). The values were corrected by reference to the value of β-actin (Cat No. GB15001, 1:2000, Servicebio) and the levels of target protein were analyzed by using Image J. The values were corrected by reference to the value of and the levels of target protein were analyzed by using AlphaEase FC.

2.7.4 Quantitative real-time PCR analysis.

RT-qPCR was conducted as previously described [(10)]. Briefly, the RNA was reverse transcribed to cDNA using the Servicebio® RT First Strand cDNA Synthesis Kit according to the manufacturer's protocol. qPCR was performed using heat-activated SYBR Premix EX Taq DNA polymerase in a TaqMan ABI 5700 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). β-actin served as internal control. The sequences of the primers are listed in able1. PCR amplification was performed in three stages: Pre-denaturation at 95℃ for 10 min, denaturation at 95℃ for 15 sec and annealing, extension at 60℃ for 30 sec for 40 cycles. A melting curve analysis was performed between 65℃and 95℃, with a 0.5℃ raise in temperature every 5 s. The fluorescence signal was recorded every 0.5 ℃. Primer sequences were listed in Table 1.

Table 1

Primer sequences in q-PCR analysis

2.7.5 Immunohistochemical Analysis.

Primer name	Primer sequences（5'-3')	Fragment length
β-actin	Forward:TGCTATGTTGCCCTAGACTTCG	240
	Reverse:GTTGGCATAGAGGTCTTTACGG	
NGF	Forward:CATCACTGTGGACCCCAAACTGT	247
	Reverse:GTCCGTGGCTGTGGTCTTATCTC	
TrkA	Forward:AACAAGAAGAATGTGACGTGCTG	119
	Reverse:TGATGCTGTTCCACGGCTT	
CREB	Forward:CATTGCCCCTGGAGTTGTTAT	113
	Reverse:CTCTTGCTGCTTCCCTGTTCTT	

4 μm-sections were cut from the paraffin tissue block, and the GluA1 (Cat.No. 381355,1:200,Cellsignal). Antibody drips were prepared in a certain proportion with phosphate buffered solution (PBS) on the sections, incubated at 4°C overnight. HRP-labelled corresponding source of the secondary antibody (HRP-labeled Goat Anti-Rabbit, Cat No. G23303. 1:200, Servicebio) covered the tissue. The scanned files were collected by a tissue slice digital scanner on the immunohistochemical slices, then the image analysis system (Servicebio, Wuhan, China) automatically read the tissue measurement area, and first divide the positive grade (i): negative without staining, 0 points ; Weak positive light yellow, 1 point; medium positive tan, 2 points; strong positive tan, 3 points.

2.7.6 Immunofluorescence Analysis.

Frozen sections were used for immunofluorescence staining.The expressions of PSD95 were detected by immunofluorescence staining. Sections were incubated overnight with primary antibodies in PBS at 4℃. After washes with PBS, the sections were incubated with the fluorescent secondary conjugated Alexa Fluor-488 at room temperature for 2 hours. The cell nuclei were counterstained with DAPI (G1012,Servicebio). The slides were dried and sealed with anti-fluorescence quenching sealing tablets (G1401,Servicebio). Stained sections were examined and photographed with a fluorescence microscope (Nikon Fluorescence Microscope, Tokyo, Japan).

2.8.Statistic Analysis

The measurement data were presented as mean ± standard deviation ($\bar{x}\pm s$) standard deviation and plotted using Graphpad Prism 8 software. The Shapiro-Wilk test was used to determine normal distribution and F test was used to evaluate the homogeneity of variance. Differences between two groups were compared using t tests. Differences between multiple groups were performed using analysis of one-way analysis of variance (ANOVA), or nonparametric test was used. All data were processed with

SPSS 25.0 (provided by IBM, USA) software. P<0.05 was considered as statistically difference. P<0.01 was considered as significant difference.

3. Results

3.1 The general state of rats

During the experiment, no deaths occurred in control group, but two rats died in model group, three deaths occurred in SHJG group, two death occurred in K252a group. Before modeling, the rats in each group had good mental state, glossy hair and normal drinking water. After the stress, except for the normal group, the other groups had waker mental state, less diet and water, yellow and withered hair, and were vulnerable to irritability. The weights of rats between the normal and model groups were statistically significant at the end of 28^{th} day and the normal group was heavier. (P<0.05, Table 2).

Table 2

Weight (g) of rats in the normal group and the model group ($\bar{x}\pm s$)

Group	0^{th} day	14^{th} day	28^{th} day
Normal	270.33±13.05	325.67±12.00	365.25±16.98
Model	275.00±9.682#	327.09±10.45#	333.8±16.61*

Notes *P<0.05; #P>0.05, compared with the normal group.

3.2 Comparison of the intercellular spaces of epithelia of esophageal mucosa and synaptic vesicles between the Normal group and the Model group

The intercellular spaces of epithelia of esophageal mucosa and synaptic vesicles in spinal cord was observed under TEM. In the model group,the intercellular spaces of epithelia of esophageal mucosa was widen under 1.5k electron microscopy. Under 15k electron microscopy, in the model group, the synaptic vesicles in spinal cord were more than the normal group.(Figure 1). These results also showed that the NERD model induced by stress was successfully established.

3.3 Comparison of the Expression of NGF, TrkA protein in spinal cord in each group

As shown in Figure 2, compared with rats in the normal group, NGF protein in the spinal cord were significantly increased in the model group (P<0.01), After the treatment of SJHG or Omeprazole or k252a, the expression of NGF were decreased compared with the model rats. (P<0.01). Compared with the model group, TrkA (Tyrosine Receptor Kinase A) protein in the spinal cord were also obviously increased in the model group (P<0.01), After the treatment of SJHG or Omeprazole, the expression of TrkA were decreased compared with the model rats. (P<0.05). Compared with the model group, TrkA protein showed no significant difference in the k252a group. (P>0.05).

3.4 Comparison of the Expression of GAP43, PSD95 protein in spinal cord between the Normal group and the Model group

As shown in Figure 3, compared with rats in the normal group, GAP43, PSD95 in the spinal cord were significantly increased in the model group.(P<0.01)

3.5Comparison of the Expression of NGF, TrkA,CREB mRNA in spinal cord in each group.

Compared with the normal group, the expression of NGF increased (P□0.05)□No significant difference of TrkA and CREB mRNA expression. The inconsistency between qPCR and Western blot results takes into account the problem of post-translational modification, that is, there are active sites of unknown activators in the advanced structure of the protein, which vary with the concentration of activators in the cell.

3.6 Comparison of the Expression of GluA1 in spinal cord among the Normal group, the Model group, the SHJG group,and the Omeprazole group

As shown in the Figure 7-8. The content of GluA1 in spinal cord of rats in normal group was low. According to the integrated optical density (IOD), GluA1 protein in the model group was higher than that in the normal group (P<0.01).The expression of GluA1 of both the SJHG group and the Omeprazole group decreased signifcantly, compared with the model group, and the decrease was more obvious in Omeprazole group (P<0.01).

3.7 Comparison of the Expression of PSD95 in spinal cord between the Normal group, the Model group

Immunofluorescence analysis showed that the immunofuorescent positive product of PSD95 was very little and scattered in spinal cord of normal group.In the model group PSD95 increased. (Figure 9).

4. Discussion

Current studies suggest that the pathogenesis of NERD may be related to visceral allergy, esophageal movement, esophageal acid exposure and changes in esophageal mucosal barrier.[1] In addition, there may be psychiatric factors involved in NERD, some of patients complain of sleep deprivation and are easy to get depression, which may eventually lead them to NERD. [15] Thus, among the pathogenesis factors, the visceral hypersensitivity are widely studied. In clinic, it's always considered to be caused by hyperalgesia. The decrease of esophageal neuron in the peripheral or central layer of the esophageal visceral hypersensitivity enhances the perception of esophageal stimulation.[16] Wang et al.[17] found that, in the model of NERD rats, the SGHW formula might improve the visceral hypersensitivity, reduce the expression of CRF protein of hypothalamus and ACC, lower the levels of NMDAR1 in spinal dorsal horn and SP inesophageal mucosa in lower third of esophagus.Trimble and Rodriguez Stanley[18; 19] reported that NERD esophagus is allergic to mechanical stimulation. However, it is controversial that some scholars argue that the design of the experiments above are not perfect.

In clinic, a study have pointed that psychological factors can aggravate symptoms and increase visit frequency of patients with NERD.[20] Antidepressants can increase the content of dopamine, norepinephrine and 5-hydroxytryptamine in synaptic space, regulate central pain pathway and improve visceral sensory threshold, and also help to improve the function of lower esophageal sphincter, effectively improve mental perception disorders. Xu et al.[21] used the flupentixol melitoxin combined with proton pump inhibitor (PPI) in the NERD with anxiety and depressive symptoms. The results showed that on the basis of routine treatment of PPI, flupentixton melitracine combined with treatment of NERD patients with anxiety and depression can improve physical discomfort symptoms, relieve anxiety and depression and other negative emotions and sleep status, and reduce recurrence.

Lori A Orlando et al.[22]confirmed by biopsy that the esophageal intraepithelial dilatation intercellular space (DIS) was widened in patients with heartburn and PPI reactive NERD. The intercellular space of esophageal mucosa was increased regardless of acid exposure.With the change of ultrastructure of esophageal mucosa, the amount of H^+ entering and remaining in the intercellular space under esophageal mucosa increases, which makes the weak acid reflux that does not cause symptoms may cause heartburn, chest pain and other typical reflux symptoms.[23] At the same time, the abnormal accumulation of H^+ in esophageal mucosa also leads to the increase of inflammatory factors and the strengthening of inflammatory responses, which jointly cause the visceral hypersensitivity of esophageal mucosa.[24]

Central sensitization is the abnormal activation of the central brain areas and the activation of the spinal cord conduction pathway, which changes the processing and integration process of the visceral sensory

afferent information, thus amplifying the sensory afferent signals and leading to high visceral sensitivity.[25] The essence of central sensitization is altered synaptic plasticity triggered by visceral sensory transmission in the CNS, and the resulting hyperalgesia is a key cause of visceral persensitivity.In the adult CNS, increased NGF expression in the spinal cord can regulate synaptic plasticity to cause hyperalgesia.[26] Nerve growth factor (NGF), the first member of the NT family discovered, is a polymeric protein composed of α, β and γ subunits, with β being the active subunitis, involved in the formation of visceral pain sensitivity.[27] NGF is distributed in various organs, such as the brain, skeletal muscle, intestine, bladder, etc. NGF exerts its functions via two membrane receptors: a high-affinity receptor TrkA and a low-affinity receptor p75NTR.The main biological effects of NGF include promoting neuronal differentiation, stimulating the growth of dendrites and cell bodies, and influencing nerve fiber density. The NGF / TrkA pathway promotes intracellular Ca^{2+} release, generating sustained excitatory postsynaptic potential by changing postsynaptic mobility, promoting the transmission of nociceptive signaling and increasing sensitivity of internal tissues and organs.[28]

The AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor, AMPAR) is the most important postsynaptic membrane glutamate receptor and plays an important role in stress induced high visceral sensitivity. [29] AMPAR is a tetrameric proionic glutamatergic receptor, and its most abundant subunits are Ca^{2+} permeable GluA1 subunits and non-Ca^{2+} permeable GluA2 subunits. [30] It has been shown that acute binding stress promotes the formation of fear memory in mice, and its mechanism may be related to GluA1 subunit phosphorylation.[31] AMPAR- mediated depolarization of postsynaptic neurons influx Na^{2+} and Ca^{2+} into cells, enabling rapid increase in intracellular Ca^{2+} content, altert synaptic plasticity and activate many intracellular pathways to maintain central sensitization.[32] In addition, Cyclic AMP response element-binding protein (CREB) has been previously implicated in the expression of the NGF gene.[33]In the rat model of neuropathic pain, spinal cord phosphorylated CREB expression increased after nerve injury surgery, and after intrathecal injection of higher concentrations of CREB antagonist, bilateral limb mechanical pain and nerve injury were significantly reduced, suggesting that CREB is involved in the formation of central sensitization of chronic pain.[34]

In traditional Chinese medicine, NERD in mind factor is closely related to the liver, heart, brain, and stomach in the viscera.When the regulation of emotion is abnormal, it affects the drainage of the liver and the balance of qi, which directly affects stomach function. When liver soul is disturbed, new thinking memories are formed, that is, the new neural pathway, leading to central sensitization, and directly acting on the stomach and esophagus, form habitual reflux. SJHG was made up of Reddle 6g, Paeoniae radix alba 10g, Radix bupleuri 12g, Paeoniae radix alba 12g, Ligusticum wallichii 12g, Fructus aurantii 12g, Tangerine peel 12g, Liquorice root 6g, Rhizoma cyperi 10g, inula japonica 12g. The prescription of SJHG complies with the pathogenesis of NERD to Sooth liver, lower the adverse Qi and harmonize stomach by the combination of spicy and bitter herbs, cold,and warmth herbs, regulating Qi and blood, so that gastric Qi go down, the liver and gastric keep balance not to traverse, interior harmonize,ascent and descent cooperate well, and the body will be self healing. Modern pharmacological studies showed that Traditional Chinese medicine can reduce depression by enhancing hippocampal neurogenesis such as

Albiflorin, Baicalein, Berberine chloride, beta-Asarone. [35; 36] Terefore, according to the theory of traditional Chinese medicine and modern pharmacology, it is well-grounded that SGHW prescription can play a role in improving the depressive behavior and visceral hypersensitivity of NERD patients.

In this study, we found that in electron microscopy, the gap between esophageal mucosa cells widened and the number of synaptic vesicles in spinal cord increased, therefore, psychological stimulation combined with esophageal hydrochloric acid perfusion can well simulate the pathophysiological state of NERD. At the same time, the expressions of NGF,TrkA, GAP43, PSD95,GluA1 protein in spinal cord of model group were signifcantly higher than that of the normal group, indicating that there was visceral hypersensitivity in model group and synaptic plasticity were changed.Compared with the model group,NGF,TrkA,GluA1 decreaed in the SJHG and omeprazole group, therefore, we believe that SJHG can effectively relieve visceral hypersensitivity and reverse central sensitization in NERD rats. SJHG might reduce the sensitization of the spinal cord in NERD rats by reducing the expression of GluA1 protein in it. Regulating the expression of NGF/TrkA protein in the spinal cord might be the intervention mechanism of SJHG on the central nervous system of rat model of NERD.

5. Conclusion

We confirmed that Central sensitization is an important pathological mechanism of NERD visceral hypersensitivity, and it also provided an evidence that SJHG can ameliorate visceral hypersensitivity and decrease central hypersensitivity in psychological stress rats with non-erosive reflux disease, which is partially due to the regulation of NGF-TrkA/AMPAR signaling pathways. These findings provided a new target for Traditional Chinese medicine treatment of NERD to improve the hypersensitivity of esophageal viscera.

Abbreviations

SJHG: Shugan Jiangni Hewei Granule

NERD: Nonerosive refux disease

IOD: Integrated optical density

GERD: Gastroesophageal refux disease

PPI: Proton pump inhibitor

GAP43: Growth-associated protein-43

PSD95: Postsynaptic density protein-95

TrkA: Tyrosine Receptor Kinase A

NGF: Nerve growth factor

CREB:cAMP-response element binding protein

AMPAR:α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor

Declarations

Data Availability

The data used to support the fndings of this study are included within the article.

Ethical Approval

Animal welfare and experimental procedures were strictly carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council,Washington, DC, 1996) and were approved by the Animal Experiment Ethics Committee of Liaoning University of Traditional Chinese Medicine (Ethical Approval Number:21000042021049)

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Guang Bai and Jing Li have made substantial intellectual contributions to the conception and design of this study. Yuebo Jia, Tianzuo Wang, Jiaqi Zhao carried out the experiments. Yuebo Jia, Jiaqi Zhao contributed to the experiment conduction and data analysis of this study. Yuebo Jia and Tianzuo Zhao participated in the manuscript writing and approved the final version. All authors havecontributed to, seen, and approved the manuscript.

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Figures

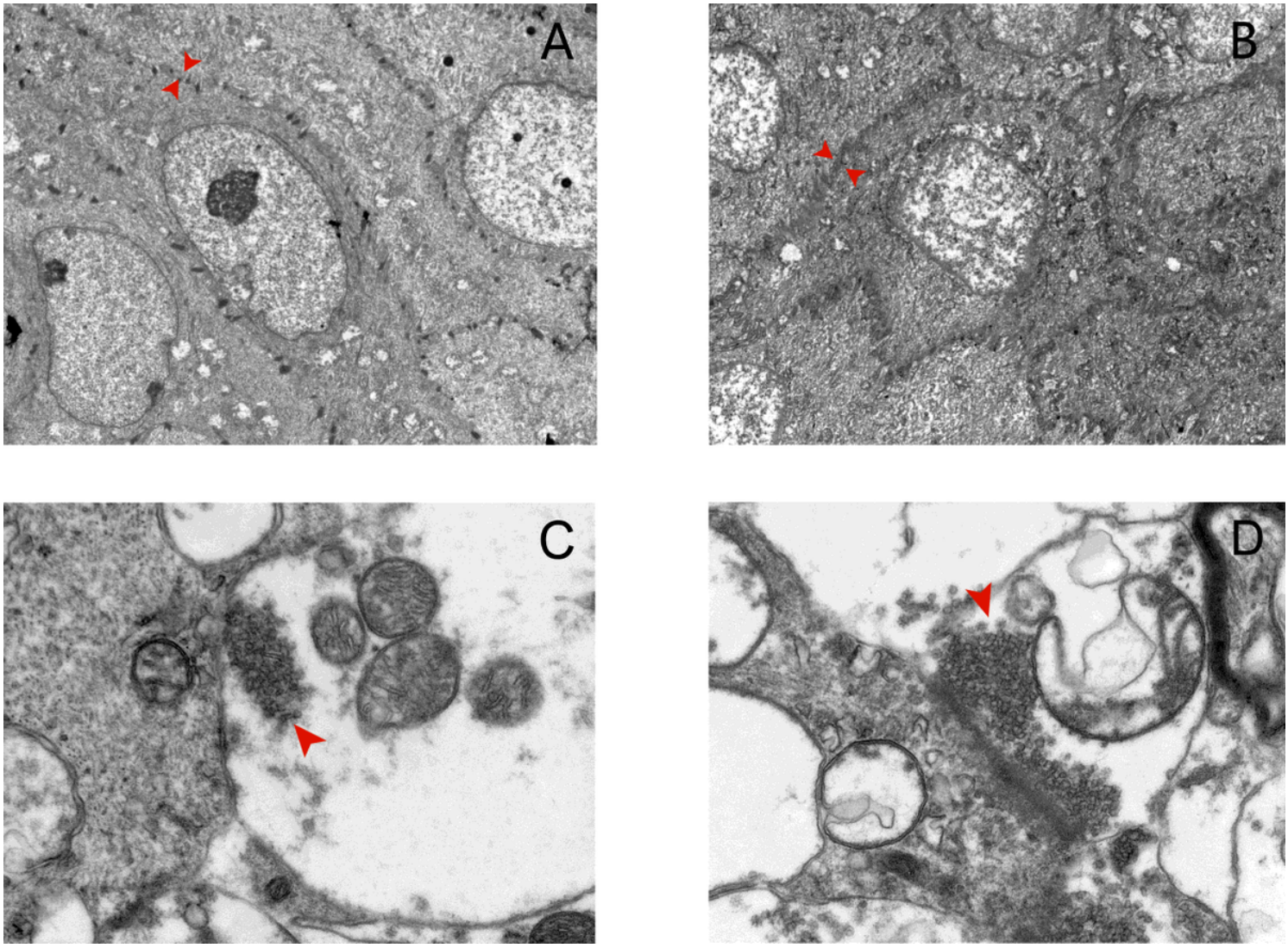

Figure 1

Electron microscopy of esophageal mucosa and synaptic vesicles in spinal cord

Notes: A,B: intercellular spaces of epithelia of esophageal mucosa as observed by TEM (×1.5k). A.Normal group, B.Model group. Red arrows indicate the interstitial space; C,D:synaptic vesicles as observed by TEM (×15k). C.Normal group, D.Model group. Red arrows indicate synaptic vesicles).

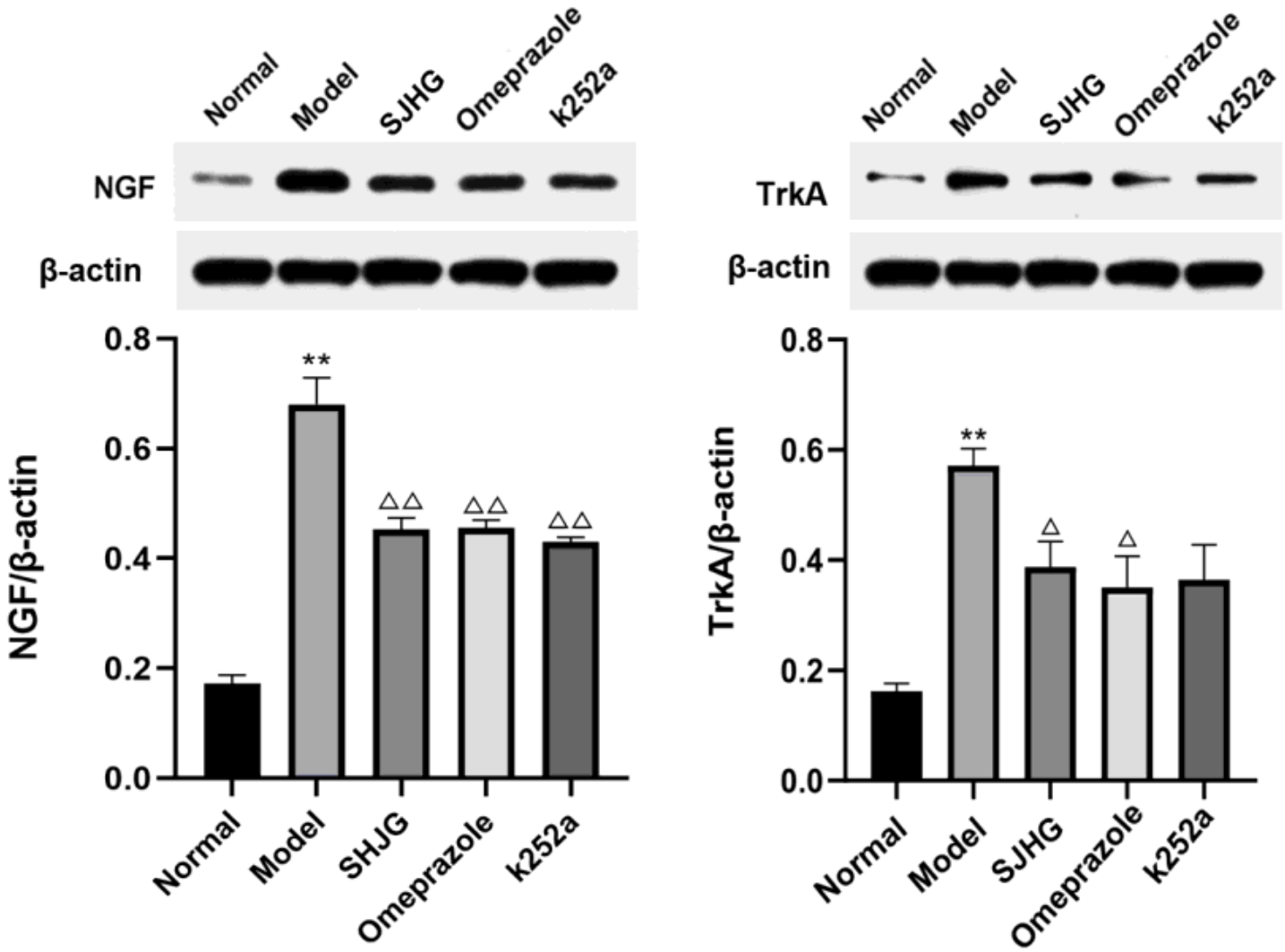

Figure 2

Protein Expression of NGF,TrkA among each groups

Notes: Relative expressions of NGF and TrkA in the spinal cord were determined using western blot analysis.**P<0.01, compared with the normal group; △P<0.05, △△P<0.01, compared with the model group

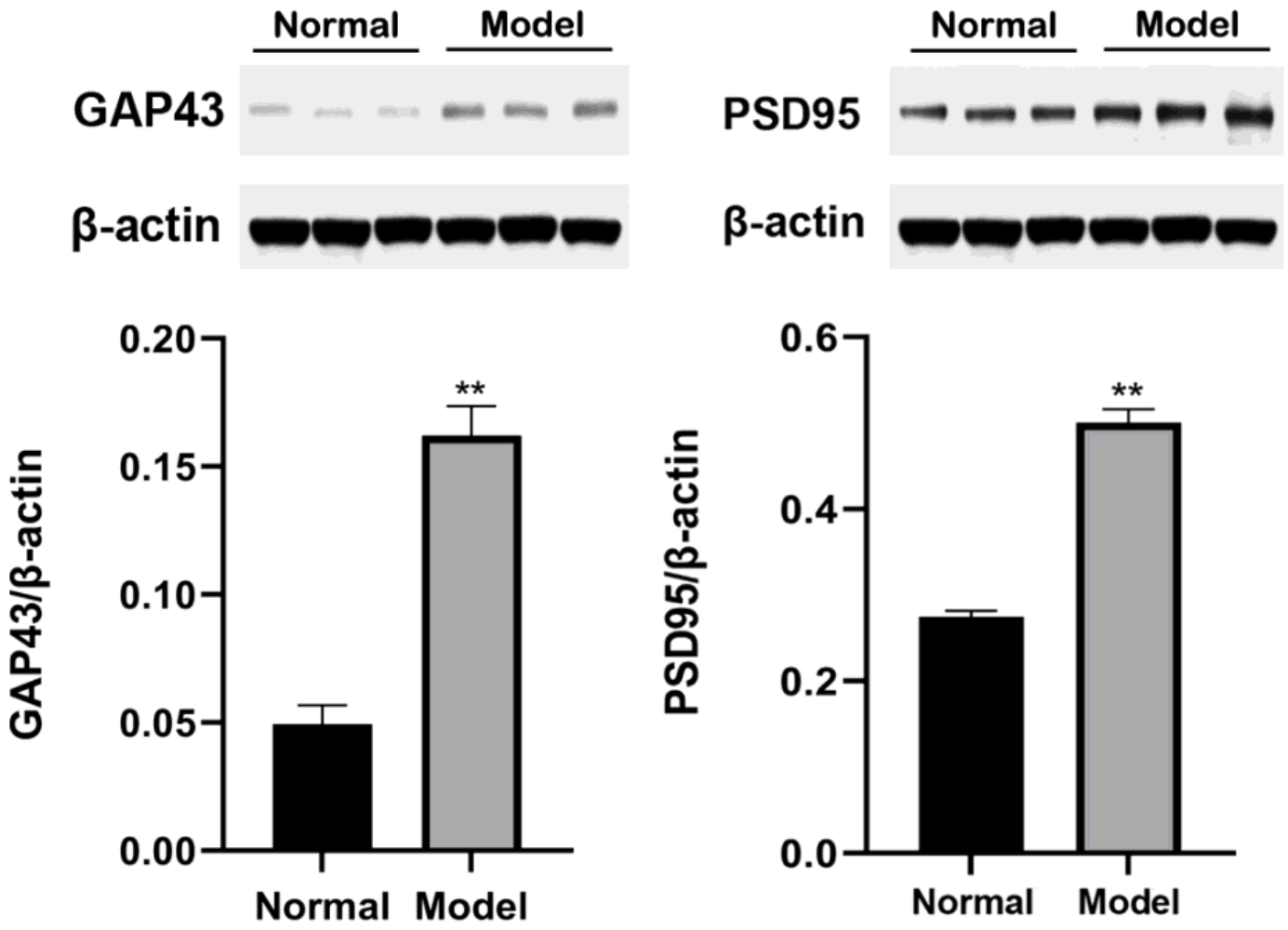

Figure 3

Protein Expression of GAP43, PSD95 between the normal group and the model group

Notes: Relative expressions of GAP43 and PSD95 in the spinal cord were determined using western blot analysis.**P<0.01, compared with the normal group.

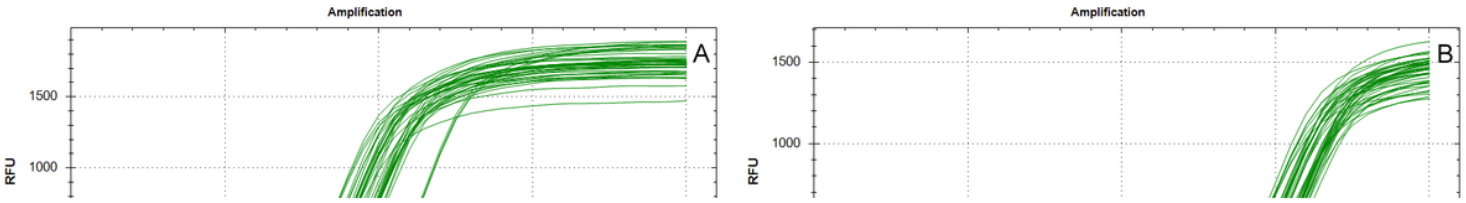

Figure 4

Amplification curve dissolution curve of RT-PCR

Notes:A.β-actin, B.NGF, C.TrkA,D.CREB

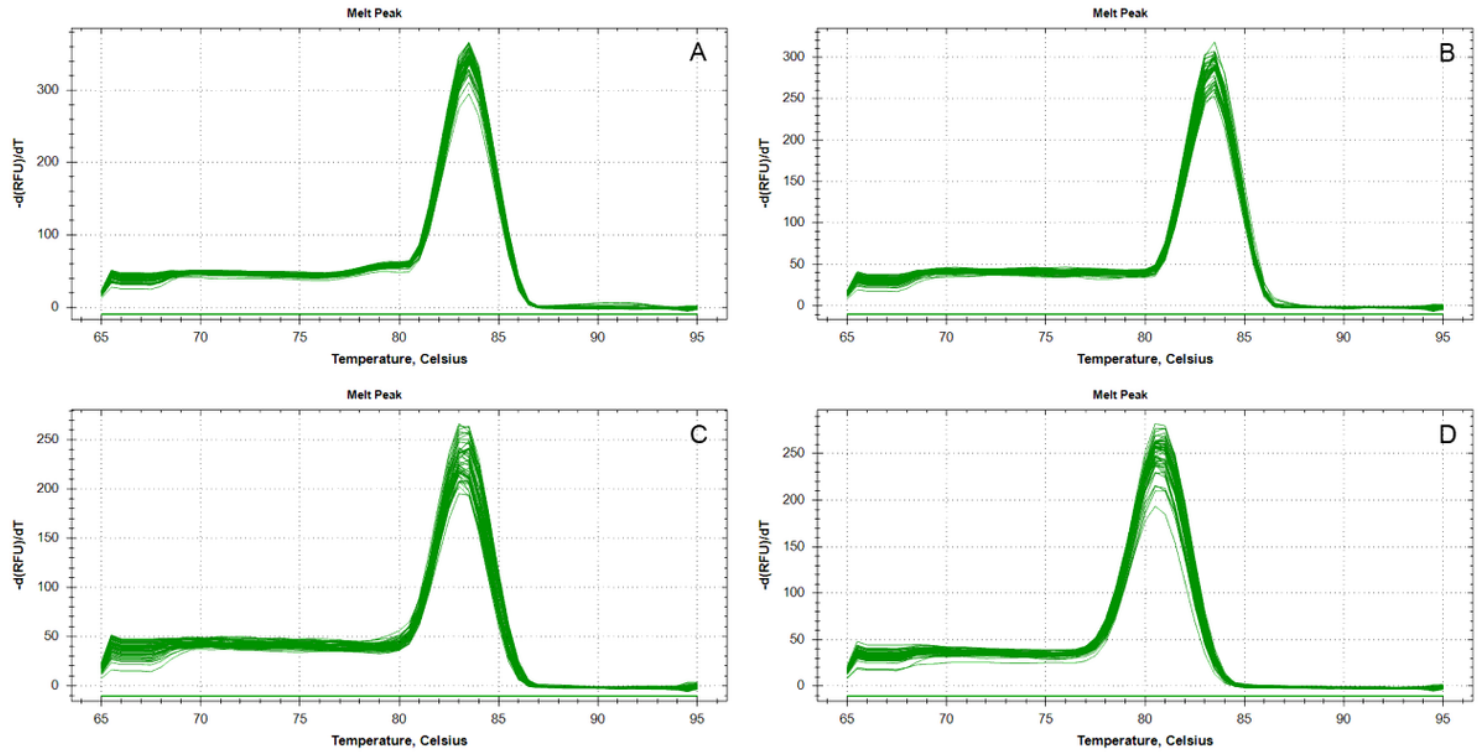

Figure 5

Dissolution curve of RT-PCR

Notes:A.β-actin, B.NGF, C.TrkA,D.CREB

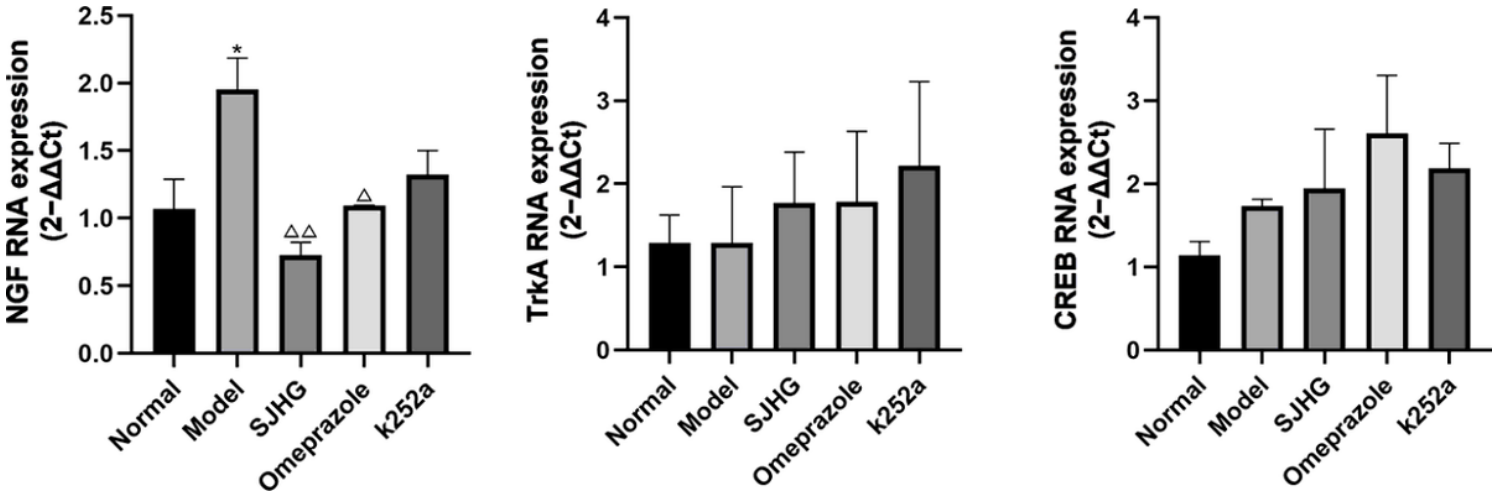

Figure 6

qPCR analysis of NGF, TrkA and CREB expression in each group. Results are presented as 2-ΔCT values.

Notes:*P□0.05,compared with the nomal group; △P□0.05, △△P□0.01□compared with the model group.

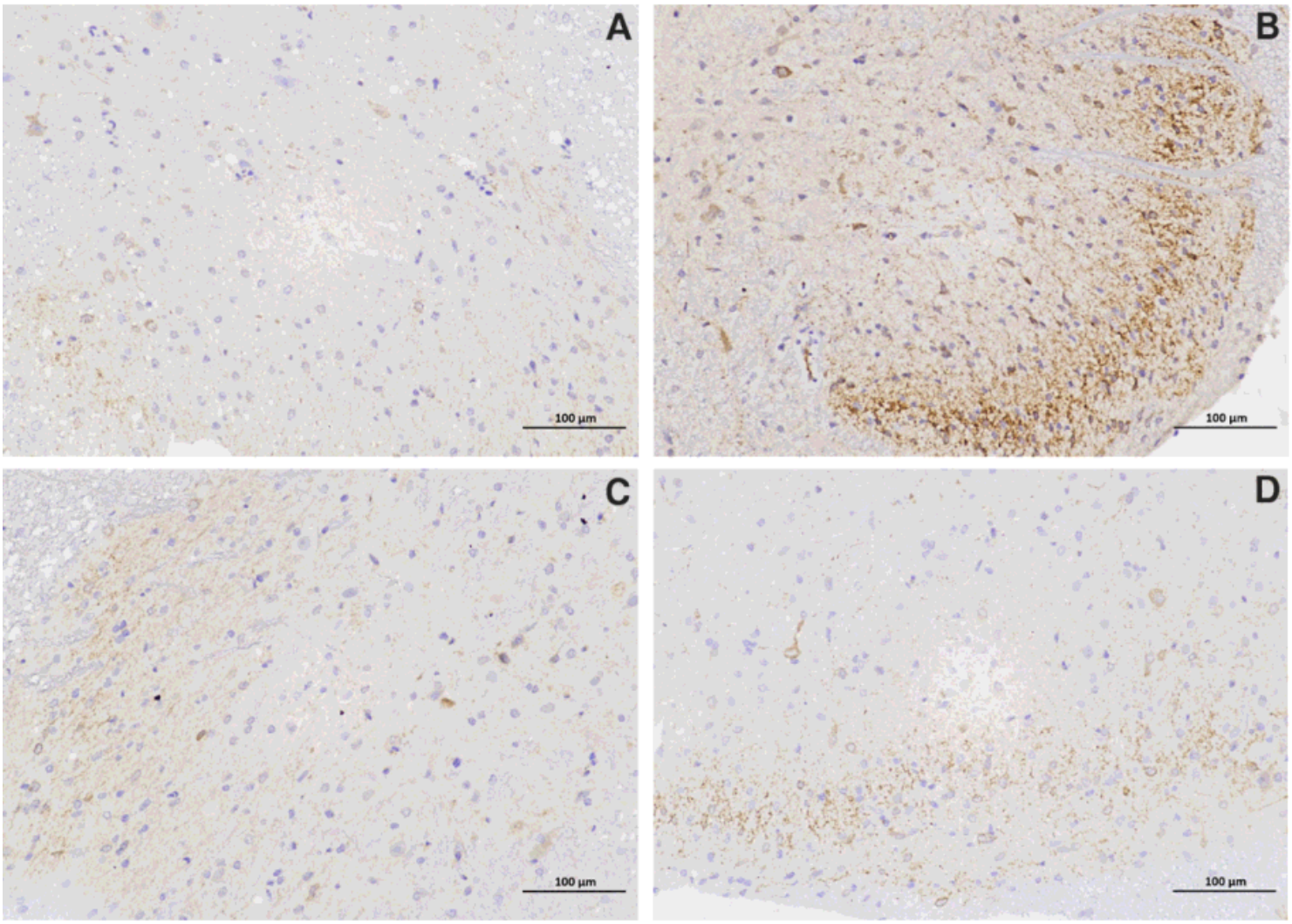

Figure 7

Expression of GluA1 protein in spinal cord of rats.

Notes: (A) normal group, (B) model group, (C) SJHG group, (D) Omeprazole group. (immunohistochemical staining,×200).

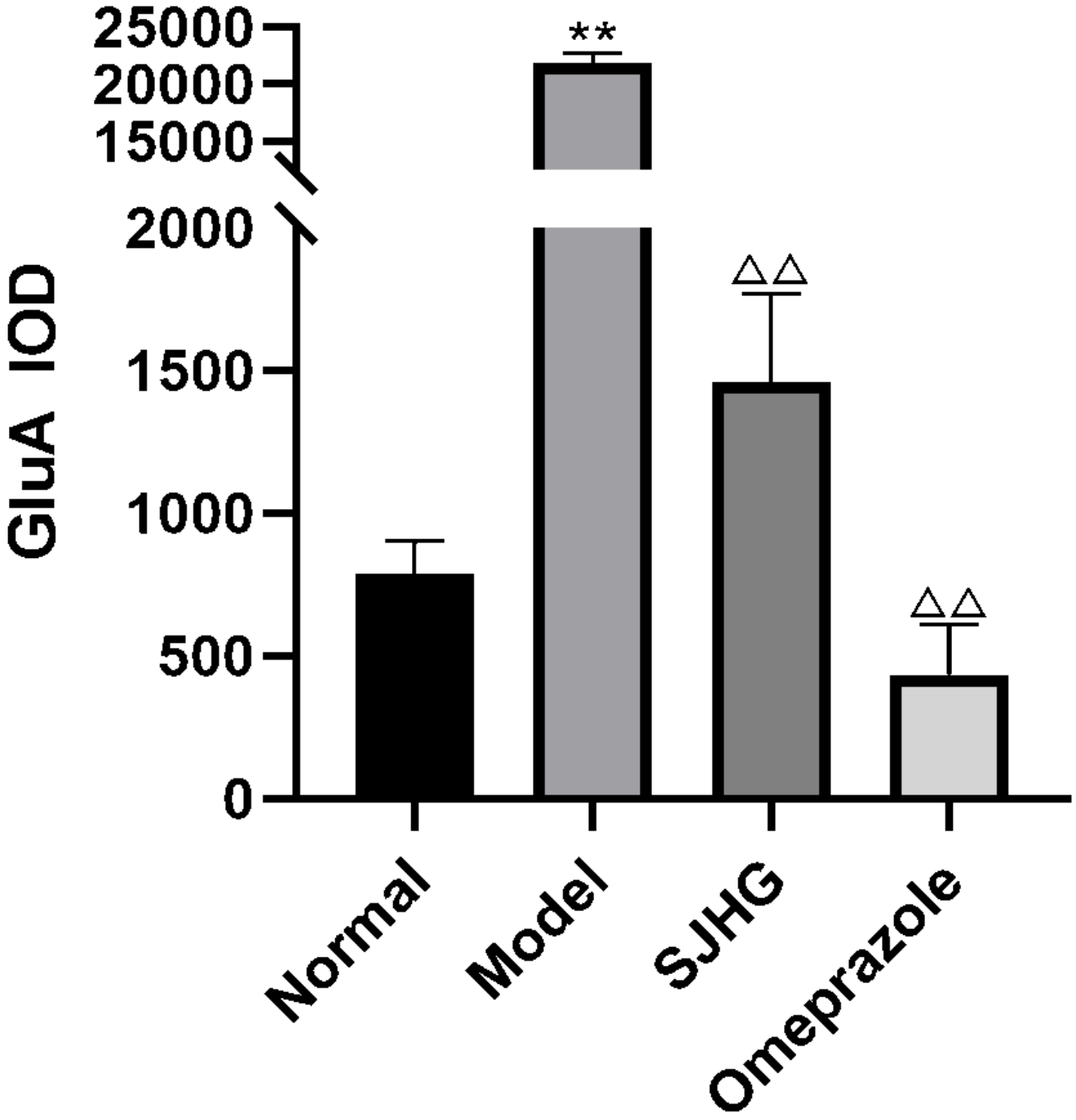

Figure 8

IOD values of GluA1 protein in the spinal cord of rats in the 4 groups

Notes: **P<0.01, compared with the normal group; △△P<0.01,compared with the model group

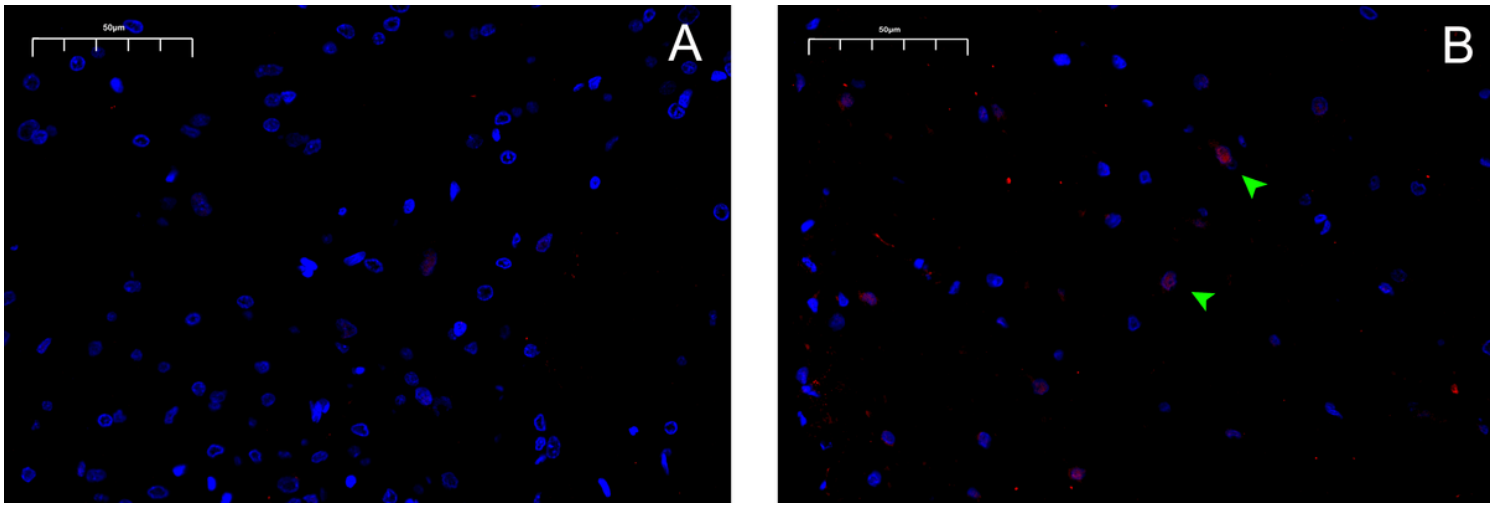

Figure 9

Immunofluorescence analysis for PSD95 on cryosections

Notes: (A) normal group, (B) model group(The green arrow indicates a positive PSD95 marker,immunofuorescence staining, ×400.)

Supplementary Files

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- Technologyroadmapping.png