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Editorial Board Member of *World Journal of Gastroenterology*, Kádima N Teixeira, PhD, Professor, Medicine School - Campus Toledo, Federal University of Paraná (UFPR), Toledo 85919-899, Paraná, Brazil. kadimateixeira@ufpr.br

AIMS AND SCOPE

The primary aim of *World Journal of Gastroenterology* (*WJG*, *World J Gastroenterol*) is to provide scholars and readers from various fields of gastroenterology and hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJG* mainly publishes articles reporting research results and findings obtained in the field of gastroenterology and hepatology and covering a wide range of topics including gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, gastrointestinal oncology, and pediatric gastroenterology.

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ORIGINAL ARTICLE

Basic Study

NMDAR2B/PKA/CREB signaling pathway contributes to esophageal neuropathic pain in gastroesophageal reflux disease

Yi Wang, Guan-Wu Li, Sheng-Liang Zhu, Ting-Ting Xu, Yi-Wen Qin, Chuan-Qi Cheng, Qin-Wei Zheng, Cong He, Bing-Duo Zhou, Sheng-Quan Fang

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Yi Wang, Sheng-Liang Zhu, Ting-Ting Xu, Yi-Wen Qin, Chuan-Qi Cheng, Qin-Wei Zheng, Cong He, Bing-Duo Zhou, Sheng-Quan Fang, Department of Gastroenterology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China

Guan-Wu Li, Department of Radiology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China

Co-corresponding authors: Bing-Duo Zhou and Sheng-Quan Fang.

Corresponding author: Bing-Duo Zhou, PhD, Chief Doctor, Department of Gastroenterology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, No. 110 Ganhe Road, Hongkou District, Shanghai 200437, China. bingduozhou@126.com

Abstract

BACKGROUND

Esophageal hypersensitivity is an important cause of refractory gastroesophageal reflux disease, in which patients do not respond to standard acid-suppressive therapy and suffer from continuous noncardiac chest pain and regurgitation. The N-methyl-D-aspartate receptor (NMDAR) may play a crucial role in the development of visceral hypersensitivity in functional gastrointestinal disorders. However, the specific mechanisms of visceral hypersensitivity in upper digestive tract diseases remain poorly understood.

AIM

To investigate the role of the NMDAR2B/protein kinase A (PKA)/cAMP-response element binding protein (CREB) signaling pathway in the development of esophageal neuropathic pain associated with gastroesophageal reflux disease (GERD).

METHODS

Thirty-six 6-week-old specific pathogen free rats were randomly assigned to six groups: the control, model, model + NMDAR agonist, model + NMDAR antagonist, model + PKA antagonist, and model + NMDAR antagonist + PKA agonist groups, with six rats in each group. The model was induced *via* an intraperitoneal

injection of ovalbumin for sensitization along with local esophageal stimulation. Immunohistochemistry and Western blotting were utilized to assess the expression levels of NMDAR2B signaling pathway-related proteins in the cingulate gyrus, dorsal thalamus, spinal dorsal horn, and peripheral esophageal tissues. RT-PCR was used to measure the corresponding mRNA expression, and ELISA was used to determine the serum brain-derived neurotrophic factor (BDNF) concentration. Behavioral scoring was performed during balloon distention and acid perfusion of the lower esophagus.

RESULTS

Compared with the control group, the model group presented significantly increased expression levels of the NMDAR2B, PKA, CREB, BDNF, substance P, and calcitonin gene-related peptide proteins and mRNAs in the cingulate gyrus, dorsal thalamus, spinal dorsal horn, and lower esophagus ($P < 0.05$). Compared with the model group, the model + NMDAR agonist group exhibited even higher expression levels of these proteins and mRNAs ($P < 0.05$), whereas the model + NMDAR antagonist and model + PKA antagonist groups presented lower expression levels ($P < 0.05$). The model + NMDAR antagonist + PKA agonist group presented higher expression levels than did the model + NMDAR antagonist group ($P < 0.05$). The changes in the serum BDNF concentration and behavioral score during balloon distention and acid perfusion were consistent with these changes in expression.

CONCLUSION

The NMDAR2B signaling pathway plays a critical role in the development of neuropathic pain in GERD through the PKA/CREB/BDNF pathway.

Key Words: Gastroesophageal reflux disease; Esophageal hypersensitivity; N-methyl-D-aspartate; Brain-derived neurotrophic factor

Core Tip: Esophageal hypersensitivity is a major contributor to refractory gastroesophageal reflux disease (GERD), meaning that minor distension and physiological acid reflux can cause significant discomfort. Given that N-methyl-D-aspartate receptors (NMDARs) may play a crucial role in visceral hypersensitivity in functional gastrointestinal disorders, this study aimed to investigate whether NMDAR2B contributes to esophageal neuropathic pain. Immunohistochemistry, Western blot, RT-PCR and ELISA were used to measure the corresponding protein and mRNA expression levels in the cingulate gyrus, dorsal thalamus, spinal dorsal horn, esophageal tissues and serum of a rat model of esophageal hypersensitivity. Our findings suggest that the NMDAR2B/ protein kinase A/cAMP-response element binding protein signaling pathway contributes to esophageal neuropathic pain in GERD patients.

Citation: Wang Y, Li GW, Zhu SL, Xu TT, Qin YW, Cheng CQ, Zheng QW, He C, Zhou BD, Fang SQ. NMDAR2B/PKA/CREB signaling pathway contributes to esophageal neuropathic pain in gastroesophageal reflux disease. *World J Gastroenterol* 2025; 31(11): 98974

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common digestive disorder that is characterized mainly by reflux and heartburn. However, approximately 30%-40% of patients do not respond to standard acid-suppressive therapy, leading to refractory GERD (rGERD)[1,2]. According to the latest international guidelines[3,4], esophageal hypersensitivity plays a significant role in the refractory nature of this condition, although the precise mechanisms are still not well understood.

At the spinal segment, afferent nerves conveying peripheral visceral sensations terminate in the posterior horn of the spinal cord. Once activated, the sensory neurons in the posterior horn maintain a prolonged activation state. This persistent activation can result in a phenomenon known as "wind up", characterized by enduring pain memory even after the cessation of peripheral stimuli, due to neurosensitization in the posterior horn[5]. It is currently believed that the mechanism of spinal sensitization for this visceral sensation involves the activation of N-methyl-D-aspartate (NMDA) receptor in the posterior horn of the spinal cord[6].

Animal studies on irritable bowel syndrome, a functional gastrointestinal disorder affecting the lower digestive tract, have shown that the activation of the NMDA receptor (NMDAR)2B subunit can induce the expression of brain-derived neurotrophic factor (BDNF)[7,8], which plays a crucial role in the development of visceral hypersensitivity. However, the specific mechanisms of visceral hypersensitivity in upper digestive tract diseases remain poorly understood.

Our group has previously demonstrated through animal experiments that the NMDAR subunit is activated in the dorsal horn of the spinal cord in rats with esophageal hypersensitivity[9]. Therefore, this study aimed to investigate whether NMDAR2B regulates esophageal sensation *via* the protein kinase A (PKA)/cAMP-response element binding protein (CREB)-BDNF signaling pathway in GERD rats. By elucidating the mechanisms underlying esophageal visceral hypersensitivity, we hope to identify novel therapeutic targets for the treatment of rGERD.

MATERIALS AND METHODS

Experimental materials

Experimental animals: Thirty-six healthy male Sprague-Dawley (SD) rats, aged 6 weeks and weighing 200 ± 20 g, were used for this experiment. The rats were obtained from the Shanghai Laboratory Animal Quality Supervision and Testing Center. The animals were housed under controlled conditions: a temperature of 20 ± 2 °C, humidity of 50% ± 10%, and a 12-hour light/dark cycle. Sterilized bedding was provided, and the animals had ad libitum access to standard feed and water. Following a one-week acclimatization period, the experiments began.

Experimental antibodies and reagents: The experimental antibodies and reagents used are listed in the supplementary materials (Supplementary Table 1).

Major experimental instruments: The major experimental instruments used are detailed in the supplementary materials (Supplementary Table 2).

Experimental methods

Animal model preparation: A rat model of GERD with esophageal visceral hypersensitivity was prepared using a combination of intraperitoneal sensitization with ovalbumin and localized esophageal stimulation, as described previously[10,11].

Modeling procedure: Healthy adult male SD rats were used for this experiment. On the first day, each rat received an intraperitoneal injection of 1.5 mL of a mixture containing 100 mg of ovalbumin and 200 mg of aluminum hydroxide adjuvant. Fourteen days after the initial sensitization, the rats were fasted for 12 hours before being subjected to the subsequent procedures under anesthesia induced by an intraperitoneal injection of 2% sodium pentobarbital (1 mL/kg).

Esophageal balloon dilation: The rats were placed in a supine position and immobilized on the experimental table. Using a mouth opener, the pharynx was fully exposed, and a PTCA balloon catheter was inserted orally and positioned approximately 8 cm from the incisors. A medical balloon inflation pressure pump was used to gradually inflate the balloon to a diameter of 3 mm, ensuring a steady increase in intraballoon pressure.

Esophageal acid perfusion: A drainage tube was inserted at the gastric cardia through incisions in the abdominal and gastric walls to collect the infused esophageal fluid. The anesthetized rats' heads were elevated by 20-30°. A single-lumen perfusion tube (inner diameter 0.50 mm, outer diameter 0.80 mm) was placed orally into the esophagus, with the catheter tip positioned 2-3 cm above the esophagogastric junction and secured in place. The other end of the tube was connected to a continuous perfusion pump. A 0.1 N hydrochloric acid solution, maintained at 37 °C, was perfused at a rate of 10 mL/hour for a total of 50 minutes.

Animal grouping and treatment: Thirty-six SD rats were randomly divided into six groups, each containing six rats: control, model, model + NMDAR agonist (NR agonist), model + NMDAR antagonist (NR antagonist), model + PKA antagonist, and model + NMDAR antagonist + PKA agonist (NR antagonist + PKA agonist). (1) Model + NMDAR agonist group: Rats received an NMDAR agonist (75 mg/kg, intraperitoneally) 30 minutes before anesthesia was induced by an intraperitoneal injection of 2% sodium pentobarbital (1 mL/kg); (2) Model + NMDAR antagonist group: Rats received an NMDAR antagonist (1 mg/kg, intraperitoneally) 30 minutes before anesthesia; (3) Model + PKA antagonist group: Rats received a PKA antagonist (2 mg/kg, intraperitoneally) 30 minutes before anesthesia; (4) Model + NMDAR antagonist + PKA agonist group: Rats received a PKA agonist (10 mg/kg, subcutaneously) 60 minutes before anesthesia and an NMDAR antagonist (1 mg/kg, intraperitoneally) 30 minutes before anesthesia; and (5) Control and model groups: Rats received the same volume of saline.

Sample collection: Blood collection from the aorta: Under deep anesthesia, a thoracotomy was performed to expose the heart. Blood (0.8-1.2 mL) was drawn from the left ventricle. The samples were left to stand for 30 minutes and then centrifuged at 3000 rpm for 15 minutes. The resulting serum was collected and stored at -20 °C.

Pathological tissue collection: Following blood collection, a cannula was inserted into the ascending aorta *via* the left ventricle. The circulatory system was perfused with 200 mL of 0.9% saline solution to clear the blood, followed by 500 mL of 4% paraformaldehyde solution (pH 7.4) at 4 °C for 1 hour. Once the limbs and spine became rigid, the spinal cord (T1-T6) and whole brain were collected, fixed in paraformaldehyde, and embedded in paraffin.

Frozen tissue collection: The lower esophagus, brain, and spinal cord were harvested, rinsed with 4 °C saline, dried with filter paper, placed in RNase-free EP tubes, and stored at -80 °C.

Observation indicators: The quantitative analysis of NMDA pathway-related gene and protein expression involves several techniques. Immunohistochemistry was used to semiquantitatively analyze the expression of NMDAR2B, PKA, CREB, and BDNF in the cingulate cortex, dorsal thalamus, and spinal cord dorsal horn. Additionally, substance P (SP) and calcitonin gene-related peptide (CGRP) expression in the lower esophageal mucosa was examined using immunohis-

Table 1 mRNA primer sequences

mRNA	Primers	Product length (bp)
NMDAR2B	Primer F 5′ AGCTCAGCGACCTGTATG 3′	204
	Primer R 5′ ACTCCCTCCTCGATTTGG 3′	
PKA	Primer F 5' TCCTTTGGGCGAGTGATG 3'	283
	Primer R 5' CGTAGAAACGGGCGTGGG 3'	
CREB	Primer F 5' CAGGGAGGAGCAATACAG 3'	148
	Primer R 5' GCACTAGAATCTGCTGTCC 3'	
BDNF	Primer F 5' TGGATGAGGACCAGAAGG 3'	115
	Primer R 5' AGAAAGAGCAGAGGAGGC 3'	
GAPDH	Primer F 5' GGAGTCTACTGGCGTCTTCAC 3'	237
	Primer R 5' ATGAGCCCTTCCACGATGC 3'	

NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

tochemistry. The expression of related proteins in 5 fields of vision at random was observed by the high-magnification microscopy. The average optical density (AOD), *i.e.*, integrated optical density/area, of the positive reaction site of each field was calculated. The average value of the AOD in 5 fields represented the quantity of antigen. The larger the value was, the more the antigen was expressed. For quantitative protein analysis, Western blotting was conducted to measure the protein expression of NMDAR2B, PKA, CREB, and BDNF in the aforementioned brain regions, as well as SP and CGRP expression in the lower esophageal mucosa.

Furthermore, RT-PCR was used to measure the mRNA expression of NMDAR2B, PKA, CREB, and BDNF in the cingulate cortex, dorsal thalamus, and spinal cord, along with SP and CGRP mRNA expression in the lower esophageal mucosa. The sequences of primers used for RT-PCR are provided in Table 1. Experimental procedure: After the extraction of total RNA, DNA elimination (reaction conditions: 37 °C, 30 minutes; Hold, add 1 μL of EDTA; 65 °C, 10 minutes), reverse transcription (reaction conditions: 37 °C, 60 minutes; 85 °C, 5 minutes; 4 °C, 5 minutes; storage at -20 °C), real-time PCR amplification (reaction conditions: 95 °C, 10 minutes; 95 °C, 15 seconds; 60 °C, 45 seconds) and 40 cycles, melting curves: 95 °C, 15 seconds; 60 °C, 1 minute; 95 °C, 15 seconds; 60 °C, 15 seconds) were performed in turn.

Serum BDNF levels were measured using ELISA. To each well, 40 μL of the sample was added, followed by the addition of 10 μL of biotin-labeled BDNF antibody. The mixture was then incubated at 37 °C for 30 minutes. Subsequently, 50 μL of enzyme-labeled reagent was added to each well, and the plate was further incubated at 37 °C for another 30 minutes. Next, color-developing agent was added, and the plate was incubated at 37 °C in the dark for 15 minutes. The reaction was halted by adding 50 μL of stop solution. The absorbance (OD value) was measured using a microplate reader within 15 minutes.

Behavioral scoring involved the description and scoring of SD rats' responses to acid stimulation and balloon distension of the lower esophagus as follows: 1 point for no obvious response, 2 points for rapid breathing, 3 points for groaning, 4 points for mild struggle, and 5 points for severe struggle.

Statistical analysis

Statistical analysis was conducted using SPSS 20.0 software. Normally distributed data are presented as the mean ± SD, whereas data not adhering to a normal distribution are expressed as the median (interquartile range), denoted as M (P25, P75). For normally distributed data with homogeneous variance, one-way ANOVA followed by the least significant difference test was used. In cases where a normal distribution or homogeneity of variance assumption was violated, the Kruskal-Wallis test was used. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemistry of NMDA pathway proteins in the cingulate cortex

The protein expression levels of NMDAR2B, PKA, CREB, and BDNF in the cingulate cortex of model rats were significantly greater than those in the control group ($P < 0.05$). Additionally, the model + NR agonist group exhibited significantly greater expression of these proteins than did the model group ($P < 0.05$). Conversely, the model + NR antagonist group and the model + PKA antagonist group presented significantly lower expression levels of these proteins than did the model group ($P < 0.05$). Notably, the model + NR antagonist + PKA agonist group presented significantly higher expression levels of these proteins than did the model + NR antagonist group, except for NMDAR2B (Figure 1).

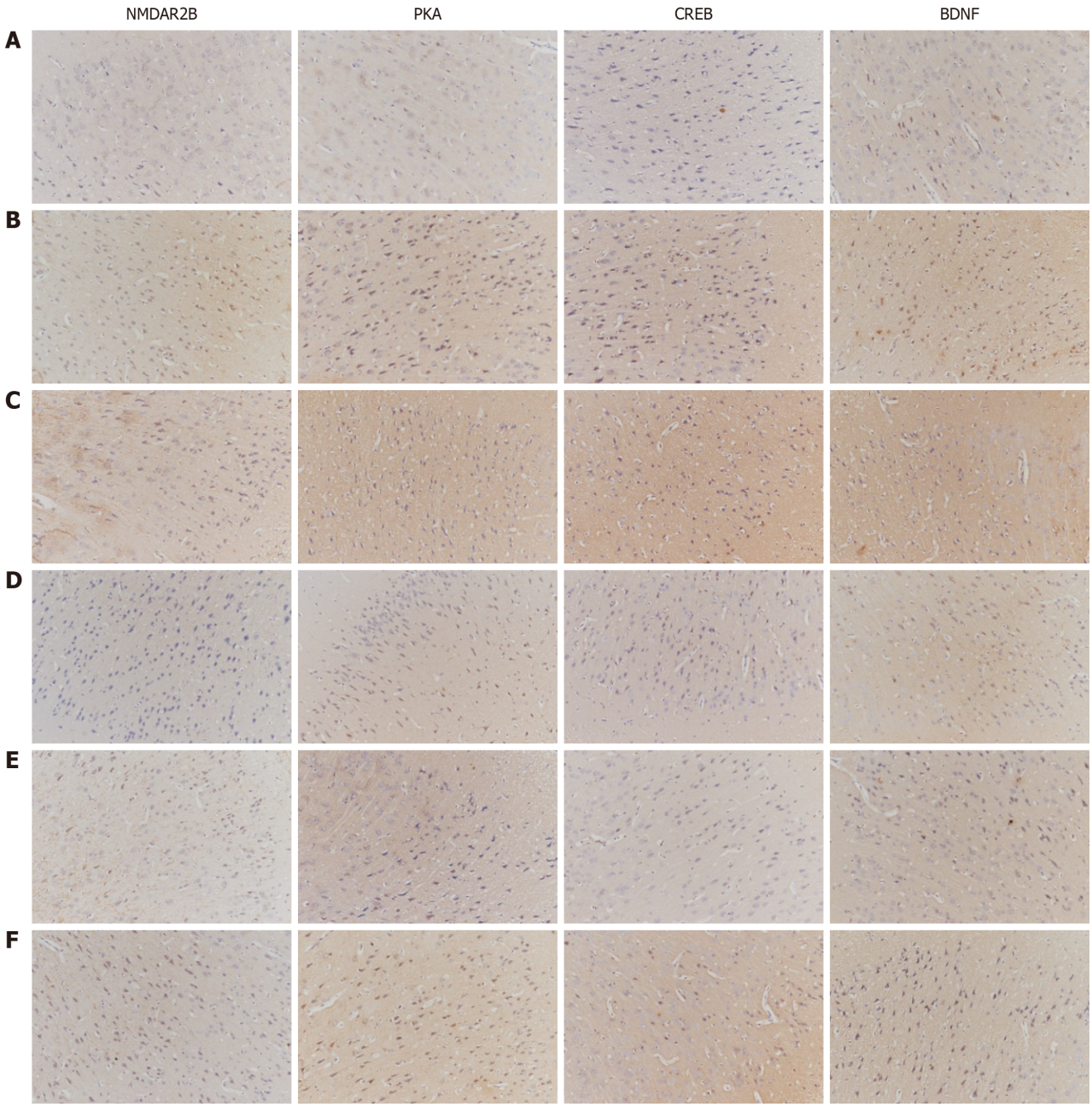

Figure 1 Immunopositive product expression of N-methyl-D-aspartate pathway-related proteins in the cingulate gyrus of rats in each group (original magnification: × 200). A: Control; B: Model; C: Model + N-methyl-D-aspartate receptor (NMDAR) agonist; D: Model + NMDAR antagonist; E: Model + protein kinase A (PKA) antagonist; F: Model + NMDAR antagonist + PKA agonist. NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

Immunohistochemistry of NMDA pathway proteins in the dorsal thalamus

The protein expression levels of NMDAR2B, PKA, CREB, and BDNF in the dorsal thalamus of model rats were significantly greater than those in the control group ($P < 0.05$). The model + NR agonist group also presented significantly greater expression of these proteins compared to the model group ($P < 0.05$). In contrast, the model + NR antagonist group and the model + PKA antagonist group presented significantly lower expression levels of these proteins than did the model group ($P < 0.05$). The model + NR antagonist + PKA agonist group also presented significantly greater expression levels of all these proteins except for NMDAR2B than did the model + NR antagonist group (Figure 2).

Immunohistochemistry of NMDA pathway proteins in the posterior horn of the spinal cord

The protein expression levels of NMDAR2B, PKA, CREB, and BDNF in the posterior horn of the spinal cord of model rats were significantly greater than those in the control group ($P < 0.05$). Furthermore, the model + NR agonist group exhibited significantly greater expression of these proteins than did the model group ($P < 0.05$). In contrast, the model + NR antagonist group and the model + PKA antagonist group presented significantly lower expression levels than did the model group ($P < 0.05$). The model + NR antagonist + PKA agonist group presented significantly higher expression levels (except for NMDAR2B) compared to the model + NR antagonist group (Figure 3).

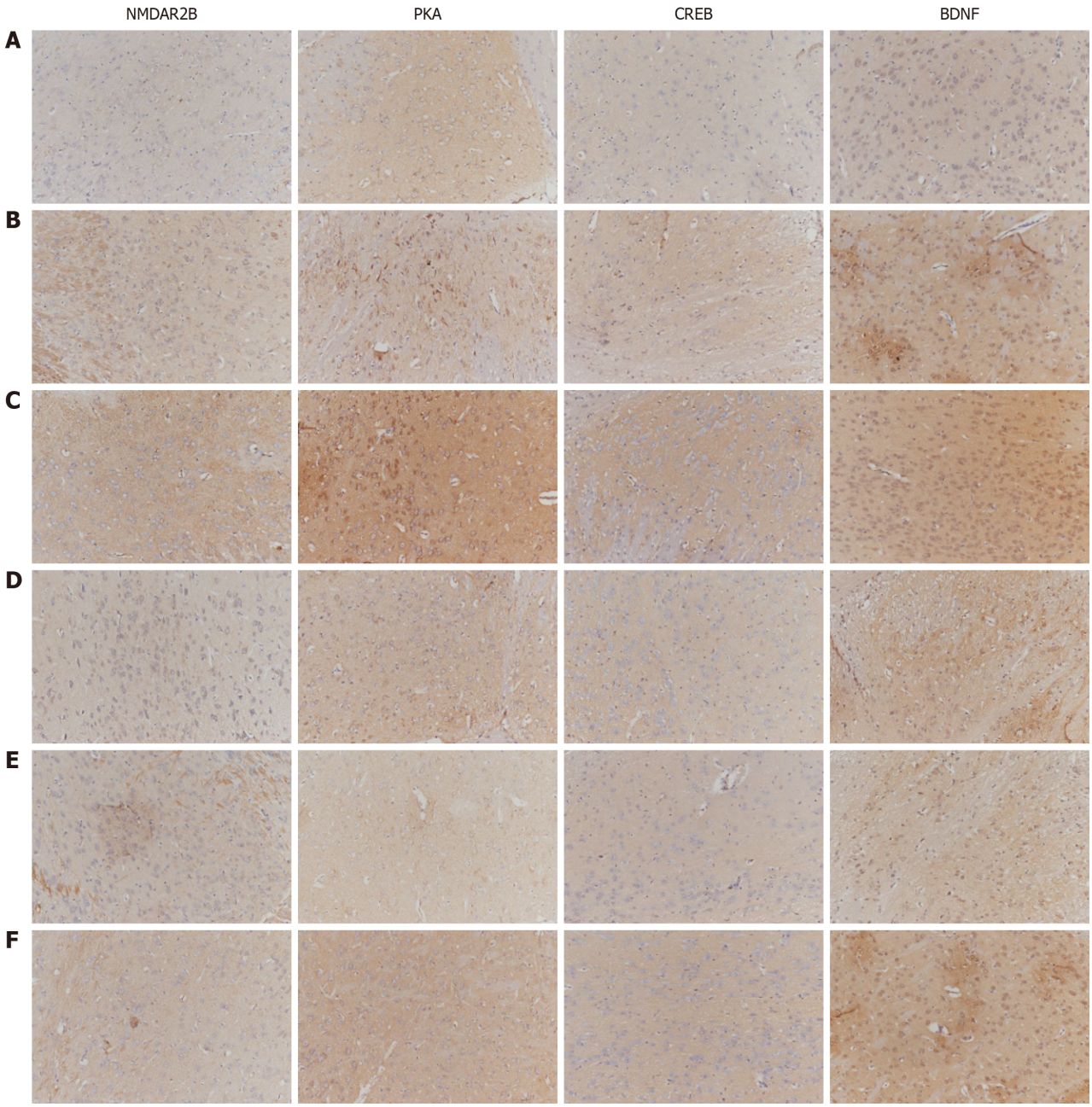

Figure 2 Immunopositive product expression of N-methyl-D-aspartate pathway-related proteins in the dorsal thalamus of rats in each group (original magnification: × 200). A: Control; B: Model; C: Model + N-methyl-D-aspartate receptor (NMDAR) agonist; D: Model + NMDAR antagonist; E: Model + protein kinase A (PKA) antagonist; F: Model + NMDAR antagonist + PKA agonist. NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

Immunohistochemistry of proteins in the lower esophageal mucosa

The protein expression levels of SP and CGRP in the lower esophageal mucosa of model rats were significantly greater than those in the control group ($P < 0.05$). The model + NR agonist group presented significantly greater expression of these proteins than did the model group ($P < 0.05$). Conversely, the model + NR antagonist group and the model + PKA antagonist group exhibited significantly lower expression levels than did the model group ($P < 0.05$). The model + NR antagonist + PKA agonist group presented significantly higher expression levels compared to the model + NR antagonist group (Figure 4).

Western blot of NMDA pathway proteins in the cingulate cortex

Western blot analysis, in which GAPDH was used as an internal control, revealed that the protein expression levels of NMDAR2B, PKA, CREB, and BDNF in the cingulate cortex of model rats were significantly greater than those in the control group ($P < 0.05$). The model + NR agonist group presented significantly greater expression of these proteins than did the model group ($P < 0.05$). NMAR2B, PKA, CREB, and BDNF proteins in both the model + NR antagonist group and the model + PKA antagonist group were significantly downregulated compared with those in the model group ($P < 0.05$). The expression of related proteins (with the exception of NMDAR2B) in the model + NR antagonist + PKA agonist group

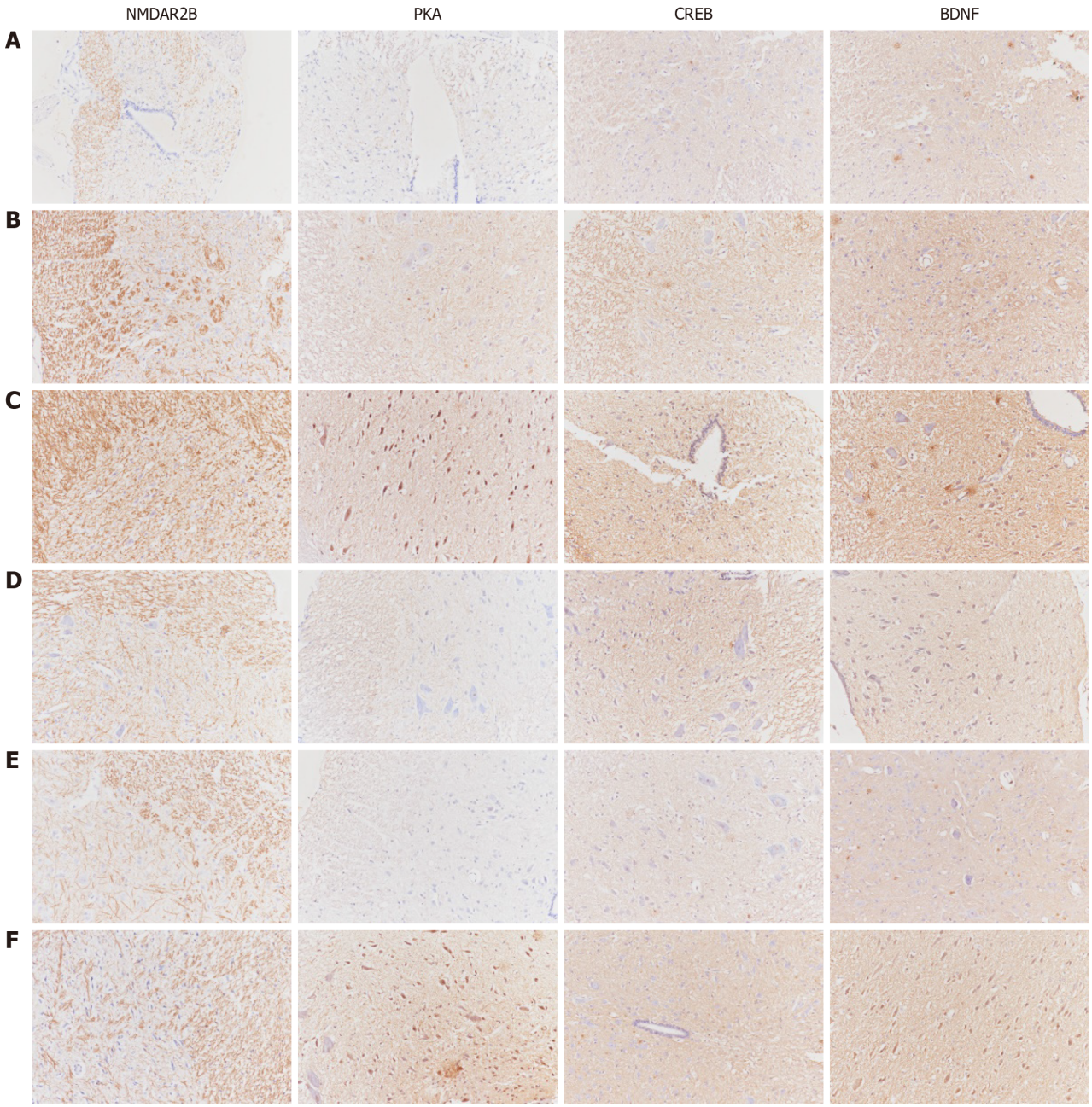

Figure 3 Immunopositive product expression of N-methyl-D-aspartate pathway-related proteins in the dorsal horn of the spinal cord of rats in each group (original magnification: × 200). A: Control; B: Model; C: Model + N-methyl-D-aspartate receptor (NMDAR) agonist; D: Model + NMDAR antagonist; E: Model + protein kinase A (PKA) antagonist; F: Model + NMDAR antagonist + PKA agonist. NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

was significantly greater than that in the model + NR antagonist group ($P < 0.05$; Figure 5).

Western blot of NMDA pathway proteins in the dorsal thalamus

Western blot analysis, in which GAPDH was used as a control, revealed significantly increased expression of the NMDAR2B, PKA, CREB, and BDNF proteins in the dorsal thalamus of model rats compared with those in the control group ($P < 0.05$). The model + NR agonist group presented a significantly greater expression of these proteins compared to the model group ($P < 0.05$). Conversely, the model + NR antagonist group and the model + PKA antagonist group presented significantly lower expression levels than did the model group ($P < 0.05$). Notably, the model + NR antagonist + PKA agonist group exhibited significantly greater expression of these proteins (with the exception of NMDAR2B) than did the model + NR antagonist group ($P < 0.05$; Figure 6).

Western blot of NMDA pathway proteins in the posterior horn of the spinal cord

Western blot analysis, in which GAPDH was used as a control, revealed significantly elevated protein levels of NMDAR2B, PKA, CREB, and BDNF in the posterior horn of the spinal cord in model rats compared with those in the control group ($P < 0.05$). The model + NR agonist group presented significantly higher levels of these proteins compared

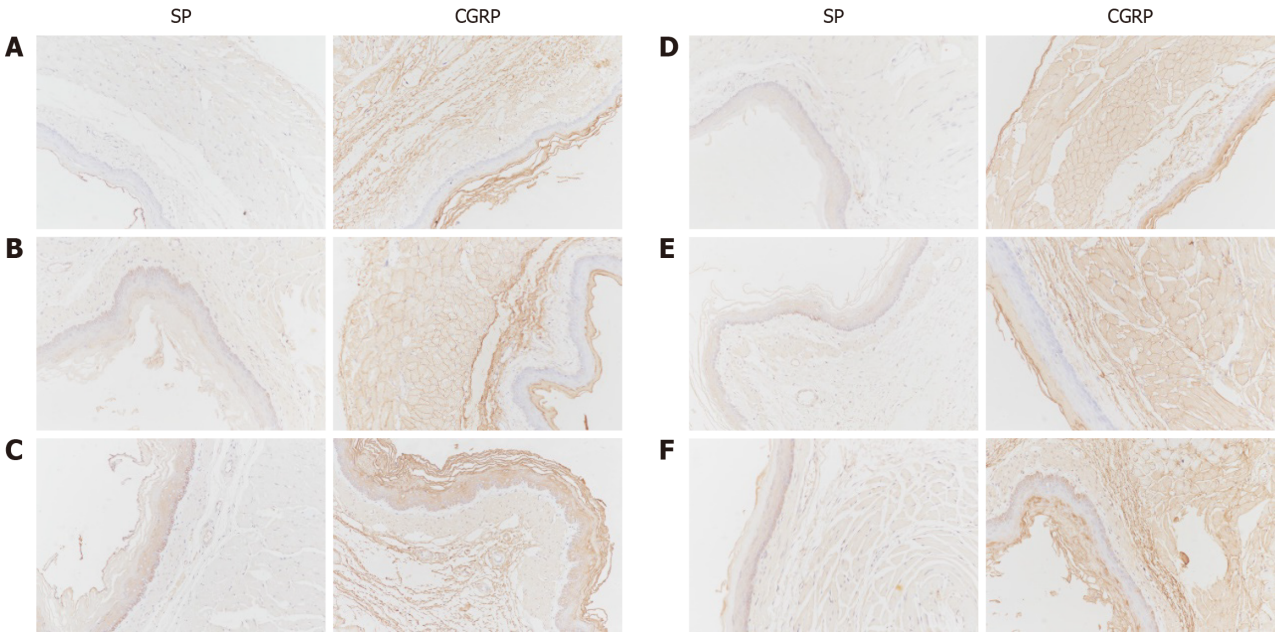

Figure 4 Immunopositive product expression of N-methyl-D-aspartate pathway-related proteins in the distal esophageal mucosa of rats in each group (original magnification: × 200). A: Control; B: Model; C: Model + N-methyl-D-aspartate receptor (NMDAR) agonist; D: Model + NMDAR antagonist; E: Model + protein kinase A (PKA) antagonist; F: Model + NMDAR antagonist + PKA agonist. SP: Substance P; CGRP: Calcitonin gene-related peptide.

to the model group ($P < 0.05$). Conversely, the protein levels of NMDAR2B, PKA, and BDNF in the model + NR antagonist group and the protein levels of NMDAR2B, PKA, CREB, and BDNF in the model + PKA antagonist group were significantly lower than those in the model group ($P < 0.05$). Furthermore, the model + NR antagonist + PKA agonist group presented significantly higher protein levels (with the exception of NMDAR2B) than did the model + NR antagonist group ($P < 0.05$; Figure 7).

Western blot of proteins in the lower esophageal mucosa

Western blot analysis, in which GAPDH was used as a control, revealed significantly increased levels of the SP and CGRP proteins in the lower esophageal mucosa of model rats compared with those in the control group ($P < 0.05$). The model + NR agonist group exhibited significantly greater levels of these proteins compared to the model group ($P < 0.05$). Conversely, the model + NR antagonist group and the model + PKA antagonist group presented significantly lower protein levels than did the model group ($P < 0.05$). Additionally, the model + NR antagonist + PKA agonist group exhibited significantly higher SP and CGRP protein levels than did the model + NR antagonist group ($P < 0.05$; Figure 8).

RT-PCR analysis of NMDA pathway mRNAs in the cingulate cortex

The RT-PCR results revealed significantly elevated expression of NMDAR2B, PKA, CREB, and BDNF mRNAs in the cingulate cortex of model rats compared with the control group ($P < 0.05$). The model + NR agonist group presented significantly greater levels of these mRNAs compared to the model group ($P < 0.05$). NMDAR2B, CREB, and BDNF mRNAs in the model + NR antagonist group and PKA, CREB, and BDNF mRNAs in the model + PKA antagonist group were significantly downregulated compared with those in the model group ($P < 0.05$). The model + NR antagonist + PKA agonist group exhibited significantly greater levels of PKA, CREB, and BDNF mRNAs compared to the model + NR antagonist group ($P < 0.05$; Figure 9).

RT-PCR analysis of NMDA pathway mRNAs in the thalamus

The RT-PCR results revealed significantly increased expression of the NMDAR2B, PKA, CREB, and BDNF mRNAs in the thalamus of model rats compared to the control group ($P < 0.05$). The model + NR agonist group exhibited significantly greater levels of these mRNAs compared to the model group ($P < 0.05$). NMDAR2B, PKA, CREB, and BDNF mRNAs in the model + NR antagonist group and PKA, CREB, and BDNF mRNAs in the model + PKA antagonist group were significantly downregulated compared with those in the model group ($P < 0.05$). The model + NR antagonist + PKA agonist group presented significantly greater levels of PKA, CREB, and BDNF mRNAs compared to the model + NR antagonist group ($P < 0.05$; Figure 10).

RT-PCR analysis of NMDA pathway mRNAs in the spinal cord

The RT-PCR results revealed significantly greater expression of the NMDAR2B, PKA, CREB, and BDNF mRNAs in the posterior horn of the spinal cord in model rats than in control rats ($P < 0.05$). The model + NR agonist group exhibited significantly greater levels of these mRNAs compared to the model group ($P < 0.05$). NMDAR2B, PKA, CREB, and BDNF mRNAs in the model + NR antagonist group and PKA, CREB, and BDNF mRNAs in the model + PKA antagonist group

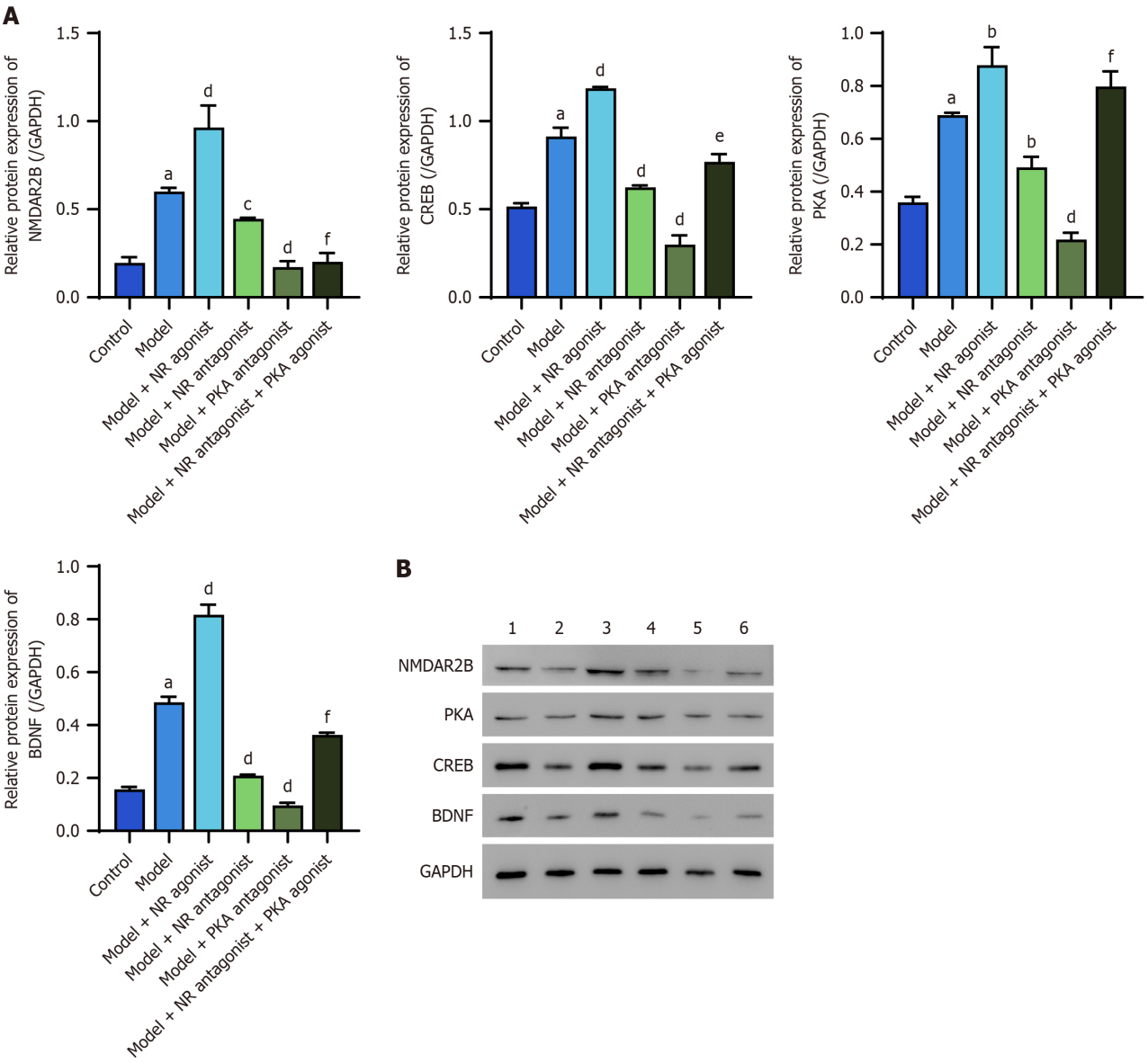

Figure 5 Results of Western blot of N-methyl-D-aspartate pathway-related proteins in the cingulate gyrus of rats in each group. A: Relative expression of N-methyl-D-aspartate (NMDA) pathway proteins in the cingulate cortex among different groups of rats (n = 6). Data are presented as mean ± SD; B: Relative expression of NMDA pathway proteins in the cingulate gyrus of rats in each group [1: Model; 2: Control; 3: Model + NMDA receptor (NMDAR) agonist; 4: Model + NMDAR antagonist; 5: Model + protein kinase A (PKA) antagonist; 6: Model + NMDAR antagonist + PKA agonist]. $^{a}P < 0.001$, model group *vs* control group. $^{b}P < 0.05$, $^{c}P < 0.01$, $^{d}P < 0.001$, model + NMDAR agonist group, model + NMDAR antagonist group, model + PKA antagonist group *vs* model group. $^{e}P < 0.05$, $^{f}P < 0.001$, model + NMDAR antagonist + PKA agonist group *vs* model + NMDAR antagonist group. NR/NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

were significantly downregulated compared with those in the model group ($P < 0.05$). The model + NR antagonist + PKA agonist group presented significantly greater levels of PKA mRNAs compared to the model + NR antagonist group ($P < 0.05$; Figure 11).

RT-PCR analysis of mRNAs in the lower esophageal mucosa

The RT-PCR results revealed significantly increased expression of the SP and CGRP mRNAs in the lower esophageal mucosa of model rats compared with the control group ($P < 0.05$). The model + NR agonist group exhibited significantly greater levels of these mRNAs compared to the model group ($P < 0.05$). The model + NR antagonist group and the model + PKA antagonist group presented significantly lower levels of these mRNAs than did the model group ($P < 0.05$). Additionally, the model + NR antagonist + PKA agonist group exhibited significantly greater levels of SP and CGRP mRNAs than did the model + NR antagonist group ($P < 0.05$; Figure 12).

Serum BDNF concentration

The ELISA results revealed that the serum BDNF level in the model group was significantly greater than that in the control group ($P < 0.05$). The model + NR agonist group exhibited higher serum BDNF level compared to the model group ($P < 0.05$). The serum BDNF levels in the model + NR antagonist group and the model + PKA antagonist group

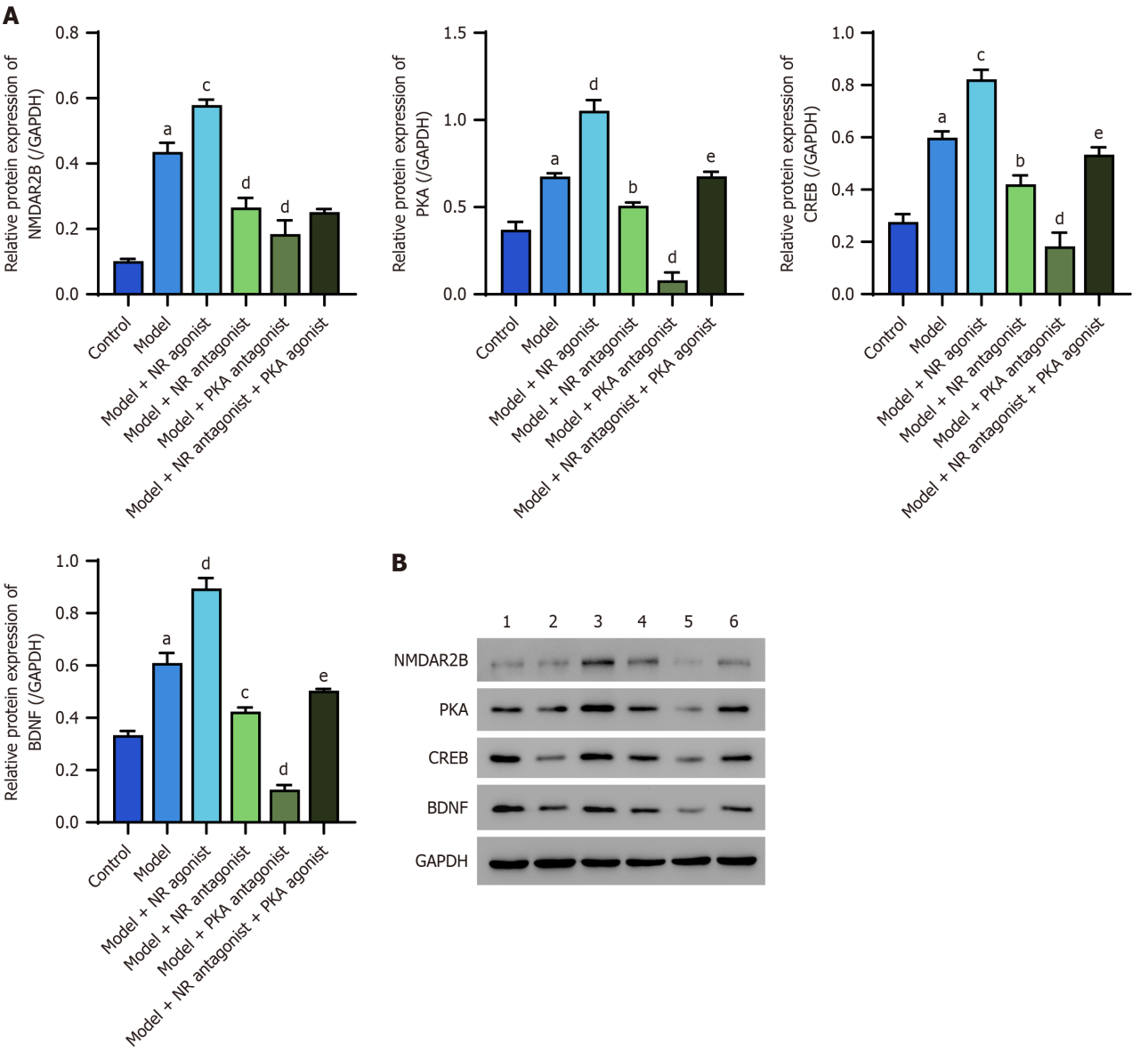

Figure 6 Results of Western blot of N-methyl-D-aspartate pathway-related proteins in the dorsal thalamus of rats in each group. A: Relative expression of N-methyl-D-aspartate (NMDA) pathway proteins in the dorsal thalamus among different groups of rats. Data are presented as mean ± SD; B: Relative expression of NMDA pathway proteins in the dorsal thalamus of rats in each group [1: Model; 2: Control; 3: Model + NMDA receptor (NMDAR) agonist; 4: Model + NMDAR antagonist; 5: Model + protein kinase A (PKA) antagonist; 6: Model + NMDAR antagonist + PKA agonist].. NR/NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor. $^{a}P < 0.001$, model group *vs* control group. $^{b}P < 0.05$, $^{c}P < 0.01$, $^{d}P < 0.001$, model + NMDAR agonist group, model + NMDAR antagonist group, model + PKA antagonist group *vs* model group. $^{e}P < 0.05$, model + NMDAR antagonist + PKA agonist group *vs* model + NMDAR antagonist group. NR/NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

were significantly lower than that in the model group ($P < 0.05$; Table 2, Figure 13).

Behavioral scores for esophageal acid infusion and balloon distension

The behavioral scores for balloon distension and acid infusion were significantly greater in the model group than in the control group ($P < 0.05$). The behavioral scores of the model + NR agonist group, the model + NR antagonist group and the model + PKA antagonist group were significantly different from those of the model group ($P < 0.05$). Additionally, the behavioral scores of the model + NR antagonist + PKA agonist group were significantly greater than those of the model + NR antagonist group ($P < 0.05$; Table 3).

DISCUSSION

GERD is characterized by the backflow of stomach contents into the esophagus, causing discomfort and potential complications[12,13]. In 2013, the American Gastroenterological Association expanded the definition of GERD to include

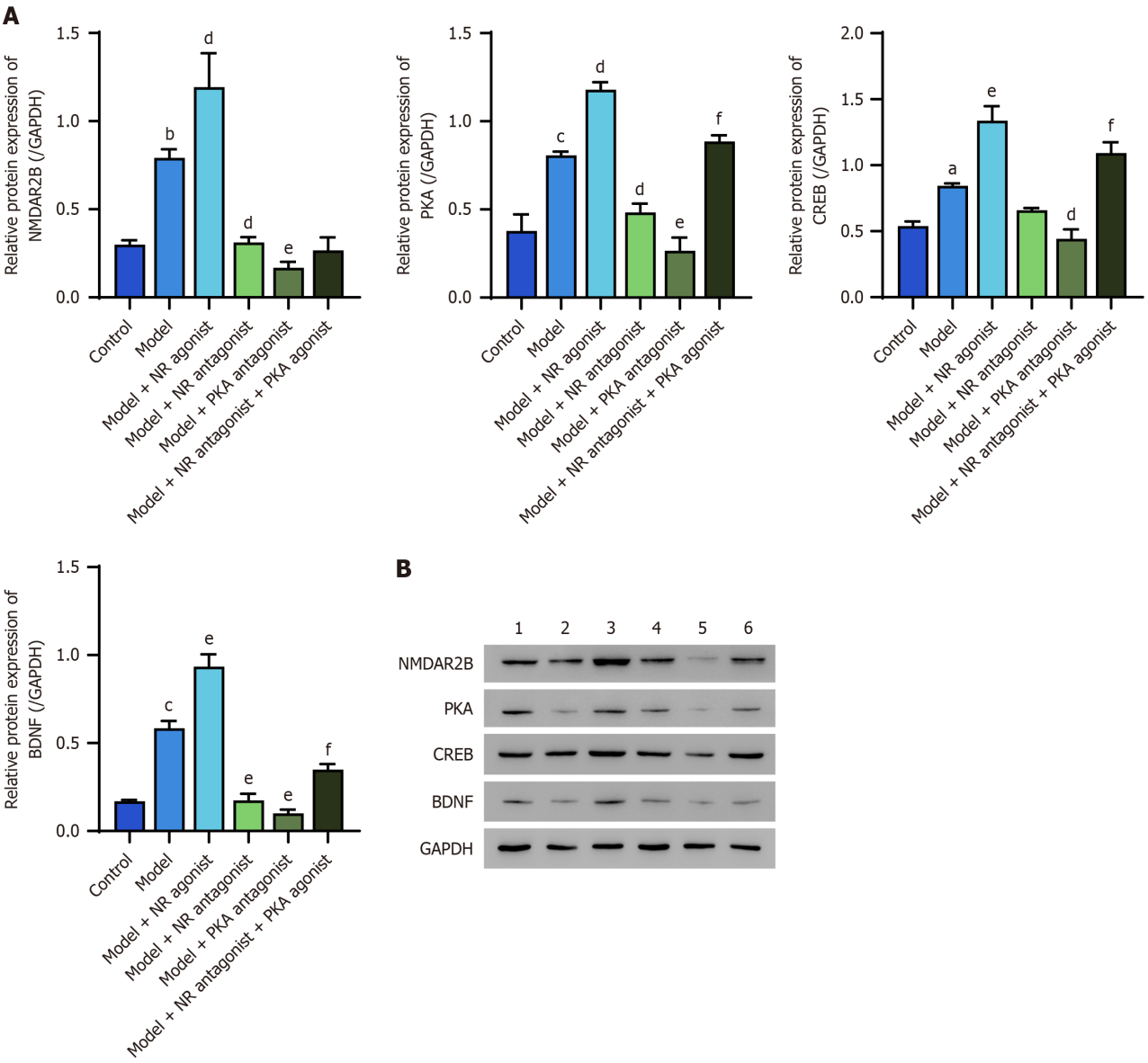

Figure 7 Results of Western blot of N-methyl-D-aspartate pathway-related proteins in the dorsal horn of the spinal cord of rats in each group. A: Relative expression of N-methyl-D-aspartate (NMDA) pathway proteins in the dorsal horn of the spinal cord among different groups of rats. Data are presented as mean ± SD; B: Relative expression of NMDA pathway proteins in the dorsal horn of the spinal cord of rats in each group [1: Model; 2: Control; 3: Model + NMDA receptor (NMDAR) agonist; 4: Model + NMDAR antagonist; 5: Model + protein kinase A (PKA) antagonist; 6: Model + NMDAR antagonist + PKA agonist]. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, model group *vs* control group. $^{d}P < 0.01$, $^{e}P < 0.001$, model + NR agonist group, model + NR antagonist group, model + PKA antagonist group *vs* model group. $^{f}P < 0.01$, model + NR antagonist + PKA agonist group *vs* model + NR antagonist group. NR/NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; CREB: cAMP-response element binding protein; BDNF: Brain-derived neurotrophic factor.

extraesophageal manifestations, covering a spectrum of symptoms, end-organ effects, and complications affecting the esophagus, mouth (including the throat), and lung[14]. Consequently, GERD presents with both typical symptoms such as reflux and heartburn, and atypical symptoms including chest pain, upper abdominal burning, bloating, belching, asthma, chronic cough, pharyngitis, and dental erosion, necessitating a multidisciplinary approach.

Globally, the prevalence of GERD ranges from 8% to 33%[15,16], with higher rates observed in Europe and North America than in Asia. A recent meta-analysis of 70 studies reported an overall GERD prevalence of 8.7% in mainland China. Over the past two decades, the prevalence of GERD in China has increased from 6.0% to 10.6%, with higher rates in the western and eastern regions than in the central region, influenced by regional variations in diet and lifestyle habits [17]. Although the prevalence of GERD in China is lower than that in Western countries, the increasing trend has garnered significant attention from researchers, particularly regarding rGERD, where symptoms persist after 8 weeks of treatment with double-dose proton pump inhibitors[18]. Refractory cases constitute approximately 30%-40% of the GERD population[1,2], presenting a substantial clinical challenge.

Esophageal hypersensitivity is a major contributor to rGERD and is characterized by increased sensitivity to chemical and mechanical stimuli within the esophagus. This heightened sensitivity means that minor distension and physiological acid reflux can cause significant discomfort, such as acid reflux and heartburn, severely impacting patients' quality of life. In the visceral sensory transmission pathway, impulses are transmitted from sensory nerve endings to the dorsal horn of

Table 2 Serum brain-derived neurotrophic factor concentration

Groups	BDNF (pg/mL)
Control	179.19 ± 13.42
Model	498.74 ± 69.92[a]
Model + NR agonist	774.58 ± 113.18[c]
Model + NR antagonist	322.79 ± 44.83[b]
Model + PKA antagonist	265.63 ± 35.42[c]
Model + NR antagonist + PKA agonist	384.03 ± 68.42

[a]$P < 0.001$ *vs* control group.
[b]$P < 0.01$ *vs* model group.
[c]$P < 0.001$ *vs* model group.
NR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; BDNF: Brain-derived neurotrophic factor.

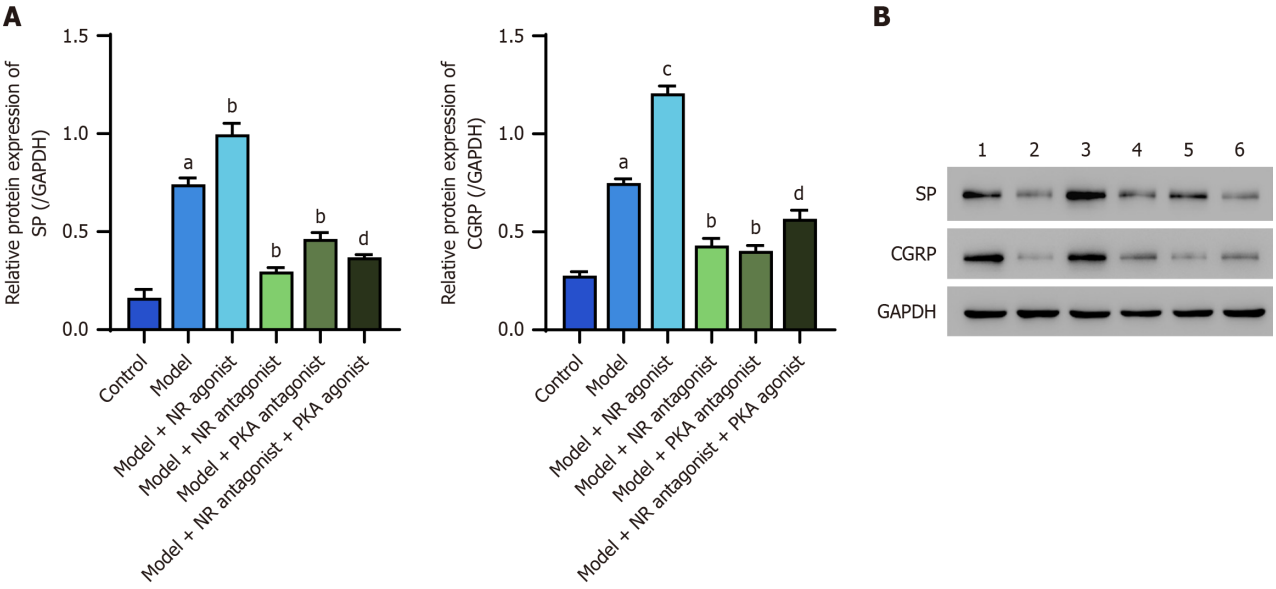

Figure 8 Results of Western blot of substance P and calcitonin gene-related peptide in the distal esophageal mucosa of rats in each group. A: Relative expression of substance P and calcitonin gene-related peptide in the distal esophageal mucosa among different groups of rats. Data are presented as mean ± SD; B: Relative expression of N-methyl-D-aspartate (NMDA) pathway proteins in the distal esophageal mucosa of rats in each group [1: Model; 2: Control; 3: Model + NMDA receptor (NMDAR) agonist; 4: Model + NMDAR antagonist; 5: Model + protein kinase A (PKA) antagonist; 6: Model + NMDAR antagonist + PKA agonist]. [a]$P < 0.001$, model group *vs* control group. [b]$P < 0.01$, [c]$P < 0.001$, model + NR agonist group, model + NR antagonist group, model + PKA antagonist group *vs* model group. [d]$P < 0.01$, model + NR antagonist + PKA agonist group *vs* model + NR antagonist group. NR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; SP: Substance P; CGRP: Calcitonin gene-related peptide.

the spinal cord *via* the dorsal root ganglia. The dorsal horn directly regulates pain feedback and receives descending regulatory signals from higher neural centers, making it a crucial hub for pain signal processing[19].

NMDAR, an ionotropic glutamate receptor, is extensively expressed in both the nervous system and peripheral tissues, including gastrointestinal mucosal epithelial cells[20,21]. Although NMDARs are distributed throughout the brain, the highest density is in the hippocampal CA1 region, with moderate levels in the CA3 region and dentate gyrus; the areas with the highest density in the cerebral cortex are the prefrontal cortex, anterior cingulate gyrus, and piriform cortex; in addition, there is high expression in the striatum, thalamus, and cerebellar granule cell layers. There is a reciprocal fiber connection between the anterior thalamic nucleus and the posterior cingulate cortex, and glutamate neurotransmitters play important roles in these two regions[22]. NMDARs also play a crucial role in visceral nociception, and its expression in the dorsal horn of the spinal cord influences visceral pain transmission[6]. Functional NMDARs consist of two NR1 subunits and two NR2 subunits, with the NR2 subunits playing a key role in visceral hypersensitivity, particularly in the dorsal horn[23]. There are four isoforms of NR2 (NR2A, NR2B, NR2C, and NR2D), each with distinct functions[24,25].

BDNF is an alkalinous protein synthesized in the nervous system. Its molecular monomer is a polypeptide composed of more than 100 amino acid residues and is composed mainly of beta-folding and random N-level structures. BDNF can regulate synaptic plasticity, change the morphology of neurons in the brain, promote the growth of dendrites and axons, and is abundant in areas such as the hippocampus, cingulate gyrus, thalamus, and spinal cord. Its role in nerve

Table 3 Behavioral scores for esophageal acid infusion and balloon distension

Groups	Balloon distension	Acid infusion
Control	1.00 (1.00, 2.00)	2.00 ± 0.89
Model	3.00 ± 0.89[a]	3.00 ± 0.89[a]
Model + NR agonist	4.17 ± 0.75[b]	4.17 ± 0.75[b]
Model + NR antagonist	1.83 ± 0.75[b]	2.00 ± 0.63[b]
Model + PKA antagonist	1.83 ± 0.75[b]	2.00 ± 0.89[b]
Model + NR antagonist + PKA agonist	3.50 (2.75, 4.00)[c]	3.50 (2.00, 4.00)[c]

[a]$P < 0.05$ *vs* control groups.
[b]$P < 0.05$ *vs* model group.
[c]$P < 0.05$ *vs* model + N-methyl-D-aspartate receptor antagonist group.
NR: N-methyl-D-aspartate receptor; PKA: Protein kinase A.

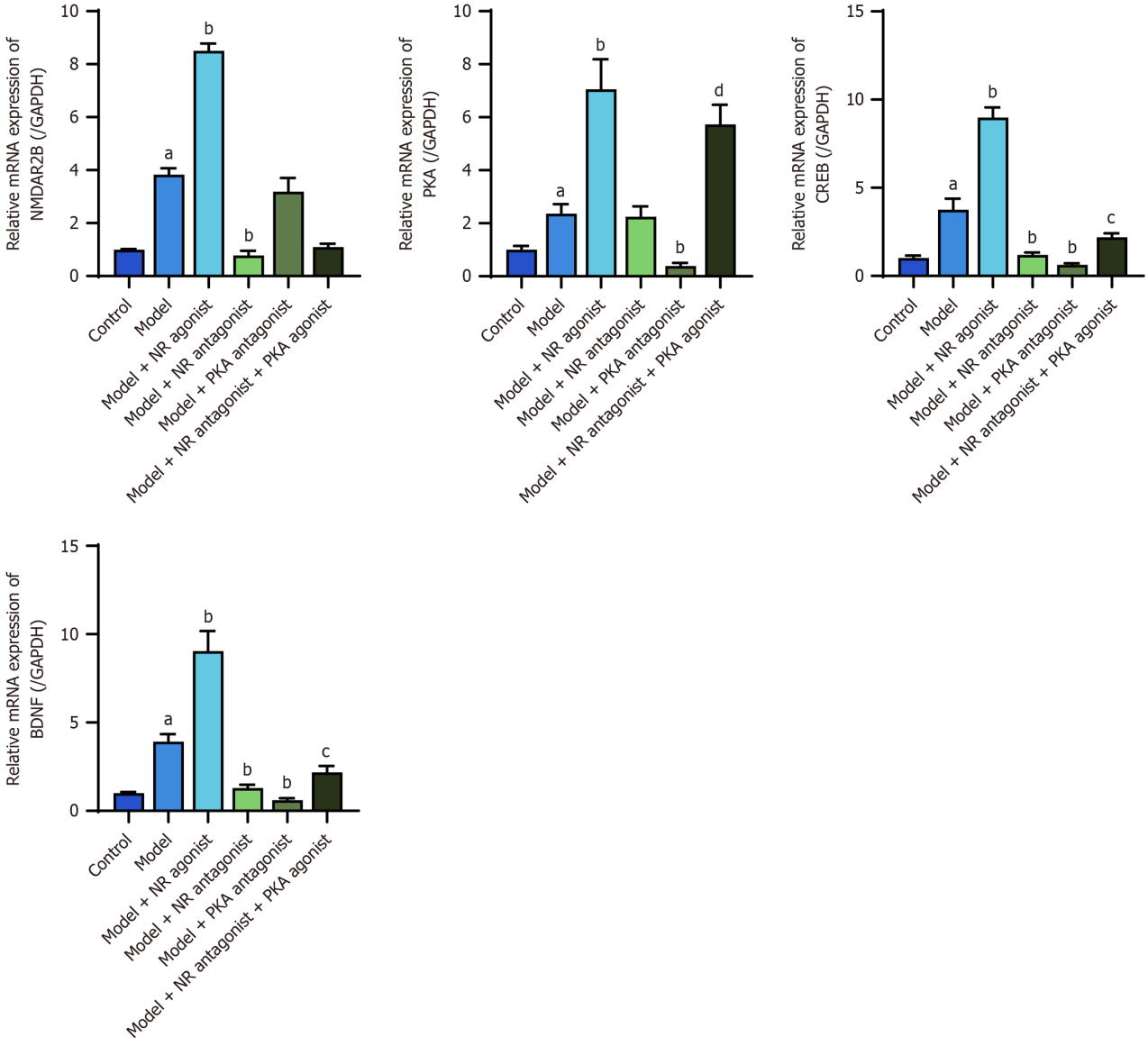

Figure 9 Expression of mRNA for N-methyl-D-aspartate pathway-related proteins in the cingulate cortex among different groups of rats.
Data are presented as mean ± SD. [a]$P < 0.001$, model group *vs* control group. [b]$P < 0.001$, model + N-methyl-D-aspartate receptor (NMDAR) agonist group, model + NMDAR antagonist group, model + protein kinase A (PKA) antagonist group *vs* model group. [c]$P < 0.01$, [d]$P < 0.001$, model + NMDAR antagonist + PKA agonist group *vs* model + NR antagonist group. NR/NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A.

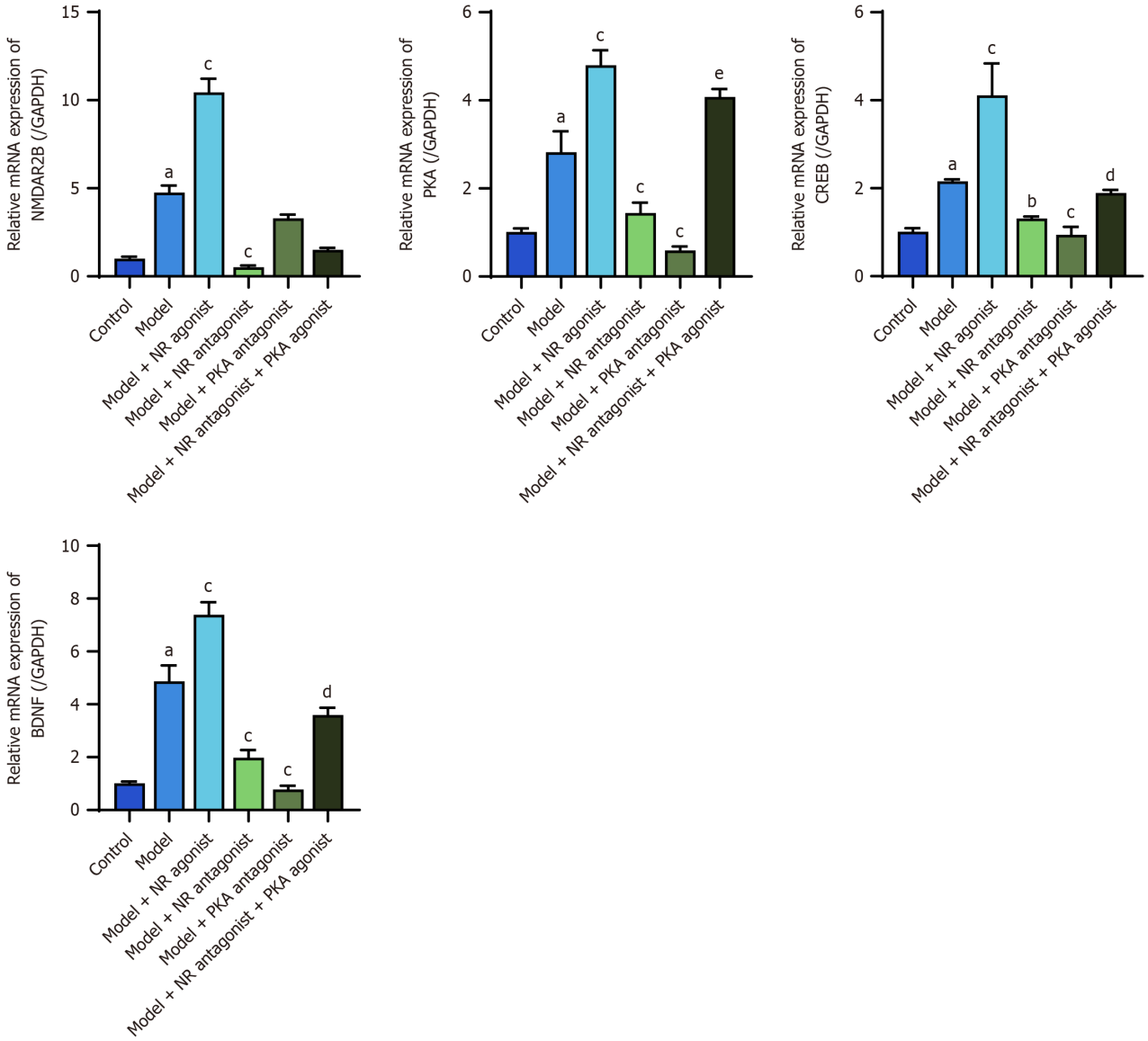

Figure 10 Expression of mRNA for N-methyl-D-aspartate pathway-related proteins in the dorsal thalamus among different groups of rats.
Data are presented as mean ± SD.

conduction has been a hot research topic in recent years. In neonatal models, visceral pain hypersensitivity is associated with the overexpression of BDNF in the dorsal horn[26]. In the mature nervous system, BDNF is closely linked to sensory hypersensitivity, enhancing the release of excitatory neurotransmitters and participating in the formation of long-term synaptic plasticity. It is also associated with regulating factors of sensation and motility such as SP and CGRP. Abnormally increased BDNF can lead to visceral hypersensitivity either by directly acting on sensory nerves or through interactions with factors such as CGRP[27,28].

In patients with irritable bowel syndrome, NMDAR2B induces intestinal hypersensitivity by promoting BDNF expression in the intestinal mucosa, an effect that can be blocked by NR2B-specific antagonists[29]. Tian *et al*[30] reported that NMDAR activation could regulate the expression of BDNF genes *via* CREB/CREB-binding protein in synapses, affecting sensory input and synaptic plasticity. These observations indicate that activation of the PKA pathway can increase the phosphorylation levels of kinases to induce downstream BDNF expression by regulating CREB expression and transcriptional activity, whereas blocking NMDAR can lead to downregulation of BDNF mRNA levels[31]. Researchers subsequently reported that BDNF also had a reverse effect on NMDARs, promoting glutamate synaptic transmission by regulating NMDAR phosphorylation, increasing NMDAR expression and activity, and increasing postsynaptic NMDAR enrichment, thereby regulating synaptic plasticity in a manner dependent on its activity[32]. We hypothesized that BDNF and NMDAR regulated each other in the central nervous system (spinal cord and brain), maintaining the normal response of the body to mechanical and chemical stimuli. Disruption of the dynamic balance between the two may lead to sensitization at the central level.

This study aimed to verify the existence of the NMDAR2B-PKA-CREB-BDNF pathway in a rat model of esophageal hypersensitivity and to determine whether PKA is an important kinase downstream of NMDAR2B. Our preliminary experiments confirmed that NMDAR was activated in the rat model, so comparisons among the 6 groups were performed: comparisons among the model + NR agonist, model + NR antagonist and model groups were used to clarify

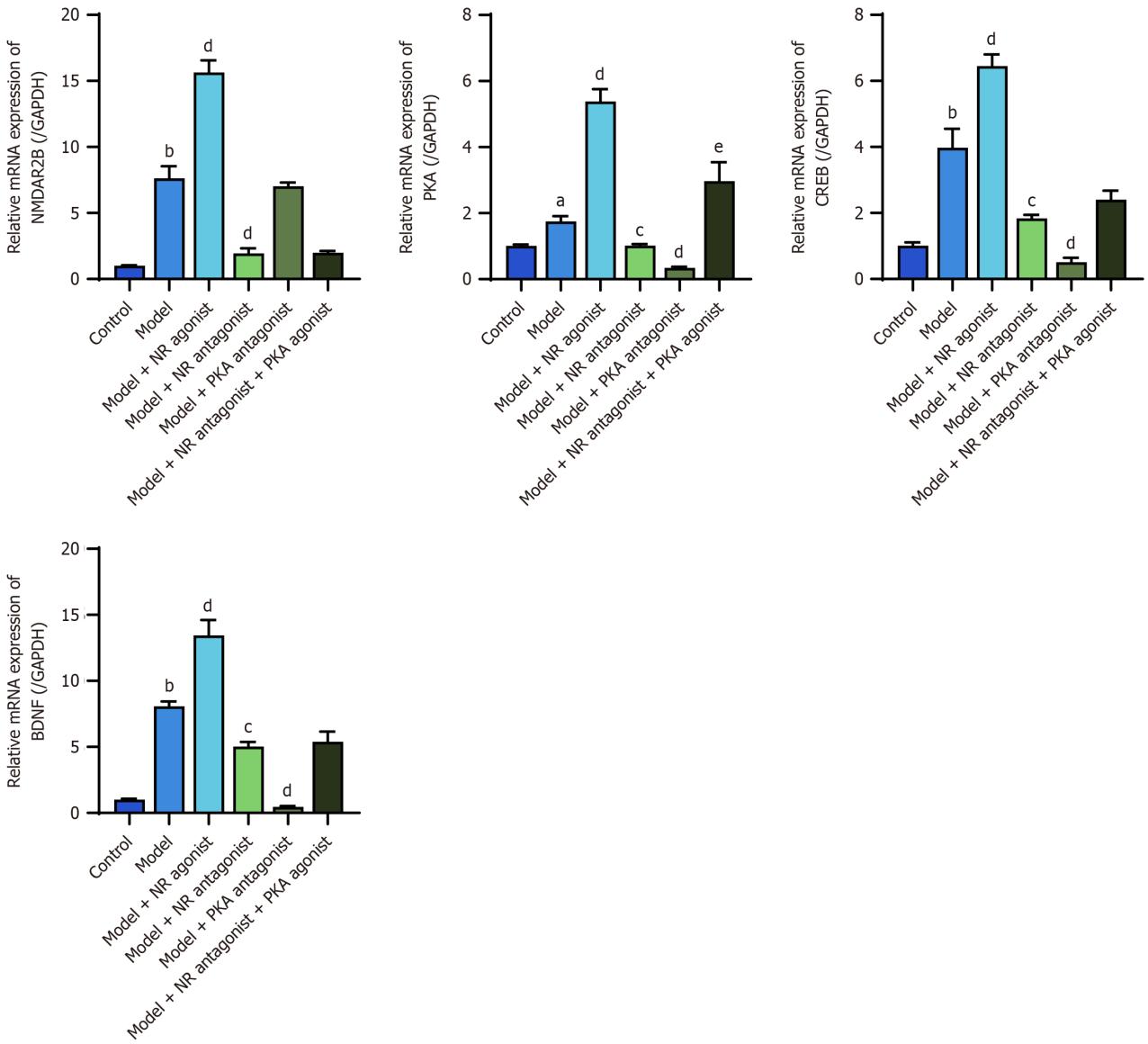

Figure 11 Expression of mRNA for N-methyl-D-aspartate pathway-related proteins in the dorsal horn of the spinal cord among different groups of rats. Data are presented as mean ± SD. $^{a}P < 0.01$, $^{b}P < 0.001$, model group *vs* control group. $^{c}P < 0.01$, $^{d}P < 0.001$, model + N-methyl-D-aspartate receptor (NMDAR) agonist group, model + NMDAR antagonist group, model + protein kinase A (PKA) antagonist group *vs* model group. $^{e}P < 0.001$, model + NMDAR antagonist + PKA agonist group *vs* model + NMDAR antagonist group. NR/NMDAR: N-methyl-D-aspartate receptor; PKA: Protein kinase A.

whether PKA and downstream gene expression were affected by NMDAR activation/antagonism; comparisons between the model + PKA antagonist and model groups were used to determine whether the expression of downstream genes was inhibited after PKA antagonism; and comparisons between the model + NR antagonist + PKA agonist and model + NR antagonist groups were used to reveal, after NMDAR antagonism following PKA activation, whether downstream gene expression was reactivated.

Our study demonstrated that in rats with esophageal hypersensitivity, the expression of NMDAR2B-PKA-CREB-BDNF pathway-related proteins and mRNAs was significantly greater than that in the control group. Further activation or inhibition of the NMDAR2B receptor resulted in corresponding increases or decreases in the expression of downstream PKA, CREB, and BDNF proteins and mRNAs, accompanied by changes in the peripheral esophageal sensitizers SP and CGRP. The behavioral scores for esophageal acid infusion and balloon distension in rats also correspondingly increased or decreased. Although the results of this study elucidate the effect of NMDAR2B on esophageal neuropathic pain, the corresponding relationship between the PKA-CREB-BDNF pathway and esophageal hypersensitivity has not been shown. It is unclear whether there are other pathways (such as the MAPK/ERK pathway) in addition to the PKA-CREB pathway that jointly intervene and interact with each other in the formation of visceral hypersensitivity. Moreover, in addition to the posterior horn of the spinal cord, the cingulate gyrus and the dorsal thalamus, the hippocampus and striatum, which are parts of the limbic system, may collectively participate in the central nervous system sensitization process, which was not assessed in this study. The functional NMDAR is composed of the NR1 and NR2 subunits. While not explored in this study, the relationship between the two and the regularity of their distribution will be the starting point for our next study.

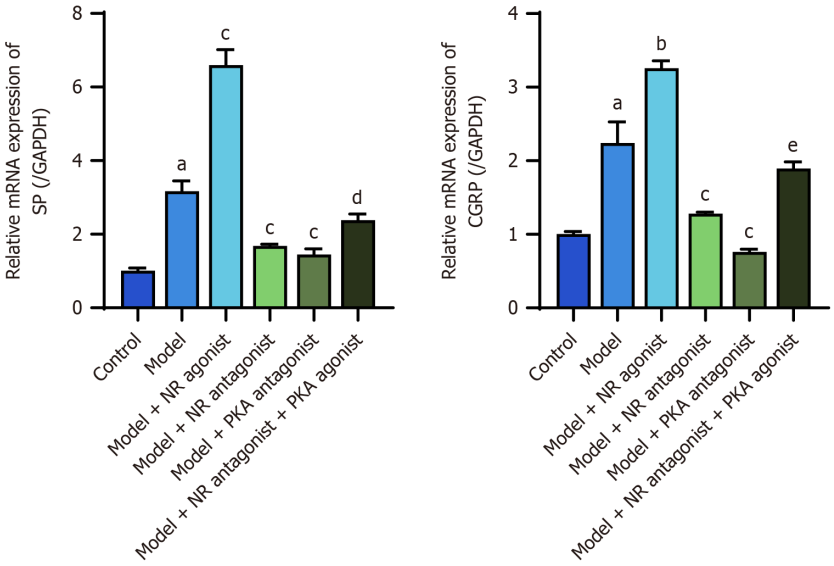

Figure 12 Expression of mRNA for substance P and calcitonin gene-related peptide in the distal esophageal mucosa among different groups of rats. Data are presented as mean ± SD. [a]*P* < 0.001, model group *vs* control group. [b]*P* < 0.01, [c]*P* < 0.001, model + N-methyl-D-aspartate receptor (NMDAR) agonist group, model + NMDAR antagonist group, model + protein kinase A (PKA) antagonist group *vs* model group. [d]*P* < 0.05, [e]*P* < 0.01, model + NMDAR antagonist + PKA agonist group *vs* model + NMDAR antagonist group. NR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; SP: Substance P; CGRP: Calcitonin gene-related peptide.

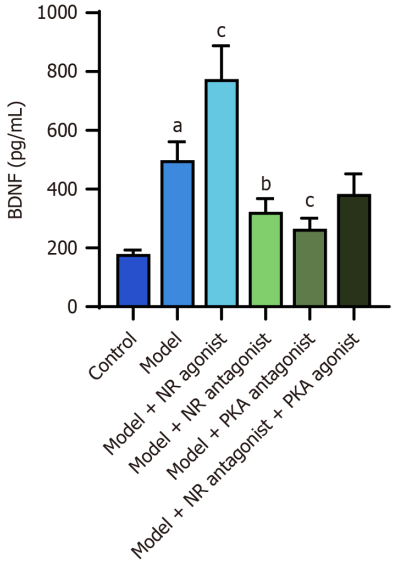

Figure 13 Serum brain-derived neurotrophic factor concentration among different groups of rats. Data are presented as mean ± SD. [a]*P* < 0.001, model group *vs* control group. [b]*P* < 0.01, [c]*P* < 0.001, model + N-methyl-D-aspartate receptor (NMDAR) agonist group, model + NMDAR antagonist group, model + protein kinase A antagonist group *vs* model group. NR: N-methyl-D-aspartate receptor; PKA: Protein kinase A; BDNF: Brain-derived neurotrophic factor.

CONCLUSION

In conclusion, these findings suggest that NMDAR2B plays a significant role in the development of neuropathic pain in GERD, which is mediated through the PKA-CREB-BDNF pathway. This result identifies a reliable molecular target for the clinical treatment of esophageal hypersensitivity and offers new therapeutic strategies and insights for the management of rGERD.

FOOTNOTES

Author contributions: The work presented here was performed in collaboration among all the authors. Zhou BD and Fang SQ identified the research topic; Li GW and Zhu SL designed the methods and experiments; Wang Y, Xu TT and Qin YW performed the laboratory experiments, analyzed the data and interpreted the results; Wang Y wrote the paper; Cheng CQ, Zheng QW and He C drafted and

revised the manuscript; all the authors contributed to, read and approved the manuscript. Zhou BD and Fang SQ contributed equally to this work as co-corresponding authors. The reasons for designating Zhou BD and Fang SQ as co-corresponding authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resulting paper. This designation also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team included authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This designation also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Zhou BD and Fang SQ both provided financial support for the research. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution while recognizing the spirit of teamwork and collaboration in this study. In summary, we believe that designating Zhou BD and Fang SQ as co-corresponding authors is appropriate for our manuscript, as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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Country of origin: China

ORCID number: Bing-Duo Zhou 0000-0001-7036-4721.

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