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Gabapentin inhibits central sensitization during migraine**

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Research Highlights

- (1) In this novel study, the authors explore the mechanisms and treatment associated with migraine with a focus on central sensitization, and they investigate the effect of gabapentin.
- (2) Excitatory amino acids and protein kinase C are involved in the formation and maintenance of central sensitization during migraine.
- (3) Gabapentin can decrease neuronal excitability in spinal trigeminal nuclei and inhibit the formation of central sensitization during migraine by reducing excitatory amino acid content in the cerebrospinal fluid and by inhibiting protein kinase C activation.

Abstract

Peripheral and central sensitizations are phenomena that occur during migraine. The role of gabapentin, a migraine preventive drug, on central sensitization remains unclear. In this study, a rat model of migraine was established by electrical stimulation of the trigeminal ganglion, and the animals were given intragastric gabapentin. Changes in amino acid content in the cerebrospinal fluid and protein kinase C membrane translocation in the spinal trigeminal nucleus were examined to clarify the mechanisms underlying the efficacy of gabapentin in the treatment of central sensitization during migraine. Electrophysiology, liquid chromatography-mass spectrometry and western blot analysis results revealed that gabapentin reduces neuronal excitability in the spinal nucleus in the trigeminal nerve, decreases excitatory amino acid content and inhibits the activation of protein kinase C. This provides evidence that excitatory amino acids and protein kinase C are involved in the formation and maintenance of central sensitization during migraine. Gabapentin inhibits migraine by reducing excitatory amino acid content in the cerebrospinal fluid and inhibiting protein kinase C activation.

Key Words

neural regeneration; migraine; protein kinase C; excitatory amino acid; ionic excitatory amino acid receptors; neurons; calcitonin gene-related peptide; nitric oxide synthase; nuclei of trigeminal nerve; glutamic acid; gamma-aminobutyric acid; grants-supported paper; neuroregeneration

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Author contributions:

Zhang YB was responsible for the experimental design, collected data and wrote the manuscript. Shao G, Zhang W, Li SJ and Yang MF implemented the experiments. Niu JZ, Hu DM and Ji XM were responsible for results assessment. All authors were well trained, and the experiments were performed using the blind method. Zhang YB was in charge of funding. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Taishan Medical University in China.

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INTRODUCTION

Gabapentin is a gamma-aminobutyric acid derivative, and was approved for the treatment of neuropathic pain by the U.S. Food and Drug Administration in 2002. The drug is also used as an analgesic agent. Since 1996, gabapentin has attracted increasing attention in migraine treatment^[1-6] as it has shown efficacy as a preventive drug. However, little evidence is available on the effects and mechanisms of action of gabapentin during the migraine attack period. Gabapentin's analgesic effect may result from the antagonism of N-methyl-D-aspartate receptors and central nervous system calcium channels^[5-6].

Migraine is a common, multifactorial neurovascular disorder. However, the pathogenesis of migraine is unclear, thereby hindering treatment. Consequently, migraine provides a huge economic burden on society and families, and seriously affects the patient's quality of life^[7-10]. Therefore, understanding the pathogenesis of migraine and developing new treatments are major topics in neuroscience research. Several theories on migraine pathogenesis have emerged in recent years, and migraine pain hypersensitivity is a particularly hot topic in migraine treatment^[10-31].

In 1996, a number of scholars proposed the theory of pain sensitization (including peripheral and central sensitization) in migraine pathogenesis^[7-12]. Peripheral sensitization is characterized by pulsatile headache exacerbation when migraine patients cough, bend or perform other physical activities which may increase intracranial pressure; while central sensitization is reflected by skin paralgnesia at the scalp, periorbit and neck in responses to cold, heat, crush, brush and other non-noxious stimuli.

Clinical treatment of migraine, especially skin allodynia and pain central sensitization, has previously been neglected, and no satisfactory clinical outcome has been identified. Zolmitriptan, a typical anti-migraine drug, can produce a better analgesic effect prior to skin

allodynia formation, but it is ineffective after central sensitization has been established, mainly due to the very limited therapeutic time window^[7-12]. Migraine central sensitization mechanisms affect the efficacy of migraine treatment; thus, it is necessary to investigate the signal transduction pathways involved in central sensitization to develop effective drugs^[7-24].

Excitatory amino acids, signaling through ionic and metabotropic receptors, contribute to the formation of peripheral and central sensitization during migraine. Consequently, excitatory amino acid receptors, especially ionic amino acid receptors, are drug targets for the treatment of migraine^[18]. Protein kinase C is an important pain signal transduction molecule, and functions to regulate neuropathic pain. Protein kinase C inhibitors have been used in the treatment of neuropathic pain^[22-24]. However, while protein kinase C is regarded a promising drug target for migraine treatment, the role of the protein kinase C signaling system in migraine central sensitization remains unclear. An in-depth study of the role of protein kinase C in the pathogenesis of migraine central sensitization may assist the discovery of new drugs for the treatment of migraine.

Previous experiments have focused on the use of gabapentin for the prevention and treatment of migraine; however, little evidence is available on the effect of the drug on central sensitization during migraine attack or its effect on excitatory amino acids and protein kinase C. Therefore, this study aimed to investigate the effect of gabapentin on neuronal excitability in the spinal trigeminal nucleus in a rat model of migraine with central sensitization using electrophysiology, liquid chromatography-mass spectrometry and western blot analysis, in a broader attempt to explore the role of gabapentin in migraine central sensitization. In addition, changes in cerebrospinal fluid excitatory amino acid levels, inhibitory amino acid content and spinal trigeminal nuclear protein kinase C membrane translocation (activation) were also examined.

RESULTS

Quantitative analysis of experimental animals

One hundred and eight rats were equally and randomly divided into six groups: normal group: no treatment was given; sham operation group: the electrodes were placed in the trigeminal ganglion, but no electrical stimulation was given; migraine model group: the electrodes were placed in the trigeminal ganglion and provided electrical stimulation; flunarizine group: 0.25 mg flunarizine (typical drug for migraine) was given *via* intragastric administration; gabapentin group: 50 mg gabapentin was given *via* intragastric administration; H-7 group: protein kinase C inhibitor H-7 (10 μ g) was given *via* intraventricular injection. Flunarizine, gabapentin and H-7 were injected into rats immediately after establishment of the model. All 108 rats entered into the analyses, without any loss.

Behavioral changes in migraine model rats

Successful establishment of the model was defined as the appearance of masticatory muscle contraction, increasing oral and nasal secretions on the stimulated side, frequent scratching of the head, closed eyes, an increase in the number of times the animal climbed the cage, and irritability. The modeling process and behavioral changes in model rats are shown in Figure 1.

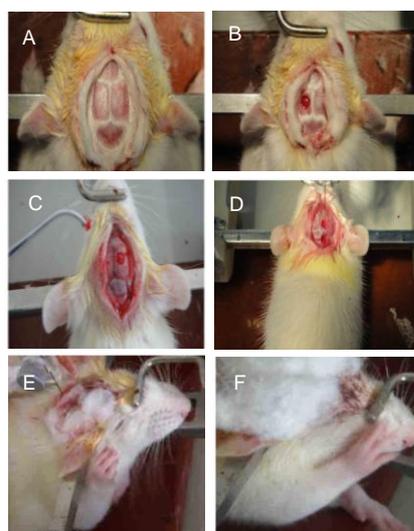


Figure 1 Migraine model establishment process and behavioral changes.

The skull was exposed (A), a window in the skull was opened (B: left; C: right), and the trigeminal ganglion was electrically stimulated (D). (E, F) Frequent scratching of the head, closed eyes, increasing frequency of climbing the cage, and irritability indicated that the migraine model was successfully generated.

Effect of gabapentin on extracellular discharge in the spinal trigeminal nucleus of migraine model rats

Six rats in each group were selected for measurement of extracellular discharge in the spinal nucleus of the trigeminal nerve. The extracellular discharge in the spinal trigeminal nucleus increased after modeling, and the discharge frequency at 2 hours was $325.88 \pm 47.32\%$ of that prior to modeling, suggesting the presence of central sensitization. A comparison of the extracellular discharge frequencies recorded at 2 hours showed that the discharge frequency in the gabapentin group was significantly higher than in the normal group ($P < 0.01$), and significantly lower than in the migraine model and flunarizine groups ($P < 0.01$). There was no significant difference in discharge frequency between the H-7 and gabapentin groups ($P > 0.05$; Table 1, Figures 2–6).

Table 1 Gabapentin inhibits extracellular discharge in the spinal trigeminal nucleus of migraine model rats

Group	Discharge frequency (%)
Normal	100.00 \pm 0.00
Sham operation	108.25 \pm 17.02
Migraine model	325.88 \pm 47.32 ^a
Flunarizine	251.88 \pm 20.35 ^{ab}
Gabapentin	170.83 \pm 8.22 ^{abc}
H-7	139.80 \pm 25.70 ^{bc}

Data represent discharge frequencies recorded 2 hours after modeling, and the values before electrical stimulation served as a reference. Discharge frequency (%) = frequency after stimulation or administration/frequency before stimulation \times 100%. ^a $P < 0.01$, vs. normal group; ^b $P < 0.01$, vs. migraine model group; ^c $P < 0.01$, vs. flunarizine group. Data are expressed as mean \pm SD, $n = 6$, one-way analysis of variance, Tukey's test. H-7: Protein kinase C inhibitor.

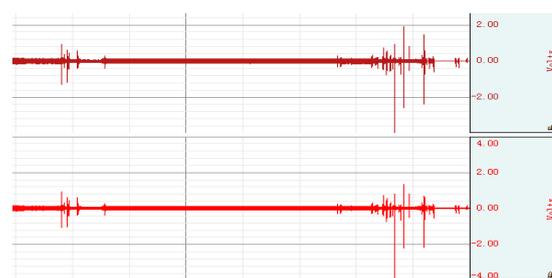


Figure 2 Extracellular discharge recordings from the spinal trigeminal nucleus of a normal rat (no migraine model was established).

A small quantity of neuronal discharge was visible, indicating baseline level of neuronal discharge. The upper picture is the analog input of the neuronal discharge. The lower picture is the neuronal discharge after filtering. Y axis: Voltage (V).

Effect of gabapentin on cerebrospinal fluid amino acid content in migraine model rats

Six rats in each group were selected for measurement of

cerebrospinal fluid amino acid content. The linear equations for the four cerebrospinal fluid amino acids (glutamic acid, aspartic acid, gamma-aminobutyric acid and serine) were determined, and the standard curves were plotted. Results showed good linear relationships (Table 2).

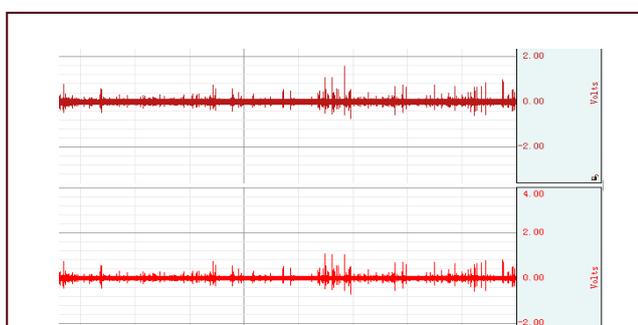


Figure 3 Extracellular discharge recordings from the spinal trigeminal nucleus of migraine model rats.

Compared with the normal group (Figure 2), a large amount of neuronal discharge was observed in the model group, indicating the presence of central sensitization. The upper picture is the analog input of the neuronal discharge. The lower picture is the neuronal discharge after filtering. Y axis: Voltage (V).

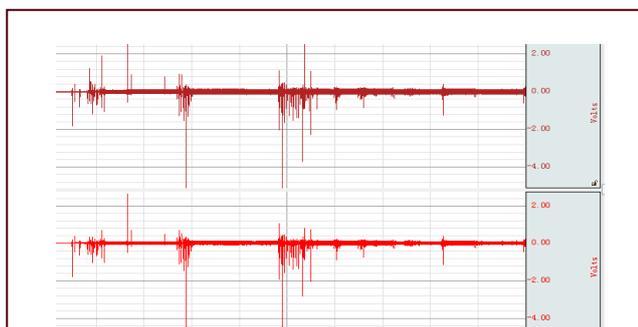


Figure 4 Effect of flunarizine on extracellular discharge in the spinal trigeminal nucleus of migraine model rats 2 hours after modeling.

The level of neuronal discharge after flunarizine administration was examined and compared with the normal group (Figure 2). The upper picture is the analog input of neuronal discharge. The lower picture is the neuronal discharge after filtering. Y axis: Voltage (V).

Liquid chromatography-mass spectrometry was performed to measure amino acid levels. The appearance time for the peaks was 8.71 minutes for glutamate, 8.51 minutes for aspartic acid, 8.56 minutes for serine, and 10.62 minutes for gamma-aminobutyric acid (Figure 7).

The excitatory amino acid levels (glutamic acid and aspartic acid) in the gabapentin group were significantly lower compared with the migraine model and H-7 groups ($P < 0.01$). Excitatory amino acid levels in the gabapentin group were also lower, to a similar extent, in comparison

with the flunarizine group ($P < 0.05$). Compared with the normal group, the inhibitory amino acid levels (gamma-aminobutyric acid and serine) were significantly lower in the gabapentin group ($P < 0.01$), and there were no significant differences between the gabapentin and migraine model groups (Table 3).

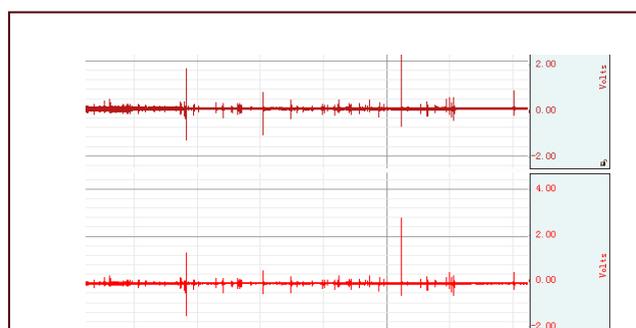


Figure 5 Effect of gabapentin on extracellular discharge in the spinal trigeminal nucleus of migraine model rats 2 hours after modeling.

Compared with the normal group (Figure 2), a larger amount of neuronal discharge was observed. Compared with the migraine model group (Figure 3), neuronal discharge was significantly reduced, indicating an inhibitory effect of gabapentin on sensitization. The upper picture is the analog input of the neuronal discharge. The lower picture is the neuronal discharge after filtering. Y axis: Voltage (V).



Figure 6 Effect of H-7 on extracellular discharge in the spinal trigeminal nucleus of migraine model rats 2 hours after modeling.

Compared with the normal group (Figure 2), neuronal discharge showed no change after H-7 administration. The upper picture is the analog input of the neuronal discharge. The lower picture is the neuronal discharge after filtering. Y axis: Voltage (V).

Effect of gabapentin on protein kinase C membrane translocation in the spinal trigeminal nucleus of migraine model rats

Six rats in each group were selected for examination of protein kinase C membrane translocation. Translocation of protein kinase C to the membrane in the gabapentin group was significantly lower compared with the migraine

model and H-7 groups ($P < 0.01$). Translocation of protein kinase C to the membrane was also similarly lower in the gabapentin group in comparison with the flunarizine group ($P < 0.05$; Table 4, Figure 8).

Table 2 Linear regression analysis of the content of four amino acids in the cerebrospinal fluid of migraine model rats

Amino acid	Equation	R
Aspartic acid	$Y=1\ 680.40X+754.10$	0.999 9
Glutamic acid	$Y=7\ 141.30X-2\ 075.80$	0.999 9
Gamma-aminobutyric acid	$Y=44\ 164.00X-4\ 927.60$	0.999 9
Serine	$Y=2\ 738.80X+1\ 943.50$	1.000 0

Shown are the linear regression equations for the amino acids in each group, which are based on the formula $Y = a + bX$. The a and b values were calculated from the known amino acid concentration and content.

In the simple linear regression equations for the amino acids in each group, X represents the amino acid concentration; Y represents the amino acid content; R represent regression coefficients. Glutamic acid and aspartic acid are excitatory amino acids, while gamma-aminobutyric acid and serine are inhibitory amino acids.

DISCUSSION

The pain sensitization phenomenon during migraine has been confirmed in animal experiments and clinical studies. In previous studies, 5–20 minutes of stimulation with inflammatory mediators, injected into the dura mater of experimental animals, triggered peripheral pain sensitization, and 20–120 minutes later, central sensitization occurred, with a peak at 120–120 minutes.

Migraine patients exhibit similar symptoms; throbbing headache at 5–20 minutes, central sensitization at 20–120 minutes, and peak appearance at 120–240 minutes after migraine attack^[13-17]. Therefore, a migraine model established using the Moskowitz method^[30] was used in this study to observe central sensitization 2 hours after modeling.

Migraine pain sensitization is mediated in large part by neuronal signaling and signal transduction cascades involving numerous molecules.

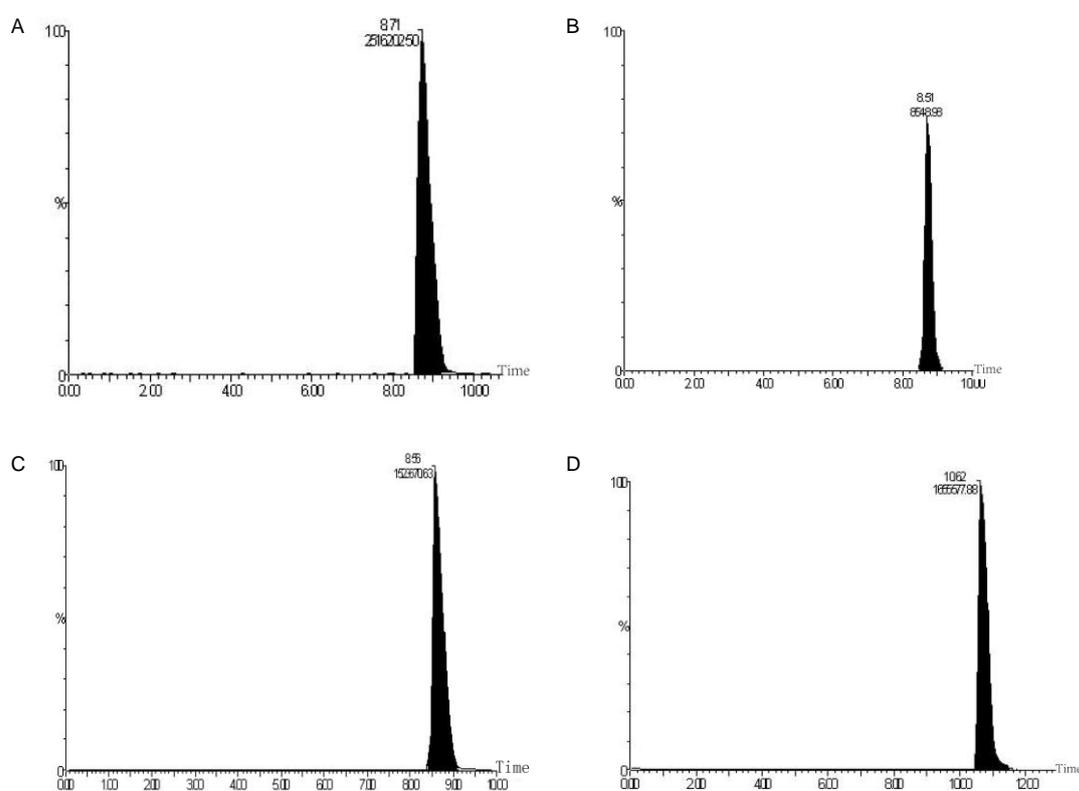


Figure 7 Ion chromatogram of glutamic acid (A), aspartic acid (B), serine (C) and gamma-aminobutyric acid (D; liquid chromatography-mass spectrometry total ion chromatogram).

The glutamic acid, aspartic acid, serine and gamma-aminobutyric acid peak time were at 8.71, 8.51, 8.56, 10.62 minutes respectively. X-axis represents the peak time (minute), Y-axis represents the peak height (%), and the peak area represents the total amount of four amino acids ions.

Table 3 Effect of gabapentin on four amino acid content in migraine model rats (ng/mL)

Group	Excitatory amino acids		Inhibitory amino acids	
	Glutamic acid	Aspartic acid	Serine	Gamma-aminobutyric acid
Normal	9.85±1.09	19.60±1.27	47.60±5.48	6.21±0.68
Sham operation	9.44±0.65	20.20±1.34	45.00±5.35	5.85±0.75
Migraine model	18.10±0.73 ^a	39.30±1.65 ^a	22.50±3.11 ^a	3.40±0.42 ^a
Flunarizine	14.30±0.79 ^{ab}	29.00±1.11 ^{ab}	18.40±2.75 ^a	3.89±0.47 ^a
Gabapentin	12.20±0.47 ^{abc}	26.10±1.49 ^{abc}	20.00±4.11 ^a	3.30±0.49 ^a
H-7	9.58±0.70 ^{bde}	19.00±0.94 ^{bde}	21.60±3.07 ^a	3.85±0.55 ^a

^a*P* < 0.01, vs. normal group; ^b*P* < 0.01, vs. migraine model group; ^c*P* < 0.05, ^d*P* < 0.01, vs. flunarizine group; ^e*P* < 0.01, vs. gabapentin group. Data are expressed as mean ± SD, *n* = 6, one-way analysis of variance, Tukey's test. The amino acid content in each group was calculated using the linear regression equation in Table 2. H-7: Protein kinase C inhibitor.

Table 4 Effect of gabapentin on protein kinase C membrane translocation in the spinal trigeminal nucleus of migraine model rats

Group	Protein kinase C membrane translocation level (%)
Normal	31.21±5.14
Sham operation	32.26±4.89
Migraine model	44.25±9.30 ^a
Flunarizine	40.99±6.38 ^{ab}
Gabapentin	36.12±5.62 ^{abc}
H-7	25.33±3.60 ^{abde}

^a*P* < 0.01, vs. normal group; ^b*P* < 0.01, vs. migraine model group; ^c*P* < 0.05, ^d*P* < 0.01, vs. flunarizine group; ^e*P* < 0.01, vs. gabapentin group. Data are expressed as mean ± SD, *n* = 6, one-way analysis of variance.

The level of membrane translocation = (membrane protein content)/(membrane protein content – cytoplasmic protein content) × 100%. H-7: Protein kinase C inhibitor.

First, primary sensory neurons that convey signals from peripheral receptors (such as facial Aδ fibers) and free endings (such as dura mater and intracranial vascular C fibers) synapse with secondary sensory neurons within the cervical spine complex (from the spinal trigeminal nucleus to the spinal dorsal horn) via the trigeminal nerve. The complex then projects centrally via the spinothalamic tract. The spinal trigeminal nucleus contains second-order neurons in pain transmission and participates in the formation of migraine central sensitization. Therefore, the trigeminal nucleus is a major focus of research on migraine central sensitization^[15, 18].

In this study, cerebrospinal fluid excitatory amino acid content and protein kinase C membrane translocation were increased in rats with migraine central sensitization, suggesting that excitatory amino acids and protein kinase C are involved in the formation of central sensitization. This finding is consistent with those of Galeotti *et al*^[31]. They injected nitroglycerin and sodium

nitroprusside into the dura mater of migraine model rats for 1–4 hours. They found that these rats displayed thermal allodynia and hyperalgesia.

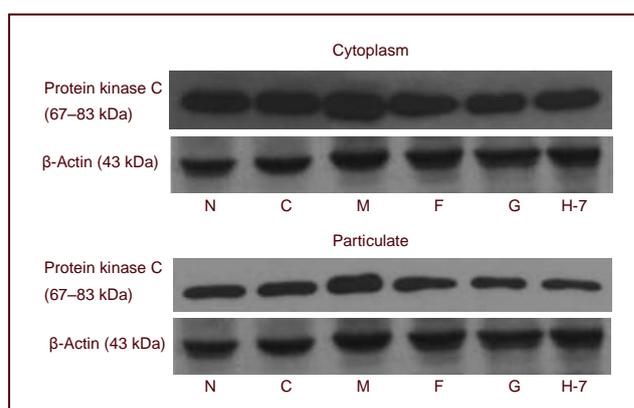


Figure 8 Effect of gabapentin on protein kinase C protein expression in the spinal trigeminal nucleus of migraine model rats.

Protein kinase C is normally inactive in the cytoplasm. After electrical stimulation, some cytoplasmic protein kinase C is translocated to the cell membrane. As shown here, the protein levels in the cell membrane in all groups were lower than those in the cytoplasm.

N: Normal group; C: sham operation group; M: migraine model group; F: flunarizine group; G: gabapentin group; H-7: protein kinase C inhibitor H-7 group.

In addition, intradural protein kinase C γ and C ϵ phosphorylation levels increased, which resulted in increasing phosphorylation of downstream signaling proteins, such as cyclic adenosine monophosphate response element binding protein and signal transducer and transcription activator 1. Administration of the protein kinase C inhibitor calphostin C suppressed the nitroglycerin and sodium nitroprusside-induced hyperalgesia.

The mechanisms underlying the effect of protein kinase C on central sensitization may be as follows: after the trigeminal ganglion is stimulated, afferent stimulation can

enhance the discharge frequency of afferent nerve fibers, and accordingly increase the release of excitatory amino acid neurotransmitters in the spinal trigeminal nucleus. These neurotransmitters signal through postsynaptic ionic and metabotropic excitatory amino acid receptors. Subsequently, intracellular Ca^{2+} and diacylglycerol levels increase, thereby activating pain sensitivity pathways including protein kinase C, and resulting in a positive feedback loop.

The activated protein kinase C may phosphorylate gamma-aminobutyric acid receptor channels, inhibit transient and sustained inhibitory Cl^- currents, and directly phosphorylate N-methyl-D aspartate receptors. As a result, the Mg^{2+} block of N-methyl-D-aspartate receptor channels is eliminated, allowing these channels to open under low electric potential, thereby increasing ion flow. This in turn increases the release of intracellular Ca^{2+} and diacylglycerol, enhancing protein kinase C activation^[29].

In this study, gabapentin reduced neuronal excitability in the spinal nucleus of the trigeminal nerve, diminished protein kinase C membrane translocation and cerebrospinal fluid excitatory amino acid content, and inhibited the formation of central sensitization during migraine. This is evidence that gabapentin can treat central sensitization, and the effect is mainly associated with the reduction of excitatory amino acid content and inhibition of protein kinase C activation.

Maneuf and McKnight^[32] found that a protein kinase C agonist significantly increased K^+ -evoked glutamate release in *ex vivo* slices after continuous perfusion into the spinal tract of the trigeminal nucleus caudalis, while 30 mmol/L gabapentin inhibited glutamate release and then suppressed the activation of protein kinase C, which was activated by substance P and calcitonin gene-related peptide release.

Gabapentin inhibits protein kinase C activation through the following mechanisms: gabapentin, as an N-methyl-D aspartate receptor and voltage-gated calcium channel blocker^[1-6], can decrease intracellular Ca^{2+} and diacylglycerol levels and inhibit the activation of protein kinase C, thereby inhibiting the positive feedback loop and reducing excitatory amino acid release^[29, 32].

Flunarizine can also block calcium ion channels and have a therapeutic effect on migraine central pain sensitization; however, this effect is significantly weaker than that of gabapentin. Gabapentin is inferior to the protein kinase C inhibitor H-7 for the treatment of pain central

sensitization, indicating that the protein kinase C inhibitor is a promising drug for migraine central sensitization.

Galeotti *et al*^[31] found that the nitric oxide donors nitroglycerin and sodium nitroprusside can directly activate protein kinase C and trigger migraine hyperalgesia by promoting intradural protein kinase $\text{C}\gamma$ and $\text{C}\epsilon$ phosphorylation, which contributes to hyperalgesia formation.

Administration of the protein kinase C inhibitor calphostin C significantly inhibited the formation of pain sensitivity, indicating that protein kinase $\text{C}\gamma$ and $\text{C}\epsilon$ are potential therapeutic drug targets for migraine. However, our observations of protein kinase C activation in the spinal trigeminal nucleus, where migraine central sensitization is formed, are inconsistent with the findings of Galeotti *et al*^[31] who investigated the activation of protein kinase C in the dura mater. This discrepancy may result from differences in the sites of pain sensitization and in corresponding differences in the underlying molecular mechanisms. There are at least 12 isoforms within the protein kinase C superfamily^[29]. Identifying which isoforms function in the migraine central sensitization process and in the protein kinase C signaling pathway requires further study.

In summary, gabapentin reduces spinal trigeminal nucleus neuronal excitability and inhibits the formation of central sensitization in rats with migraine by reducing cerebrospinal fluid excitatory amino acid levels and by inhibiting protein kinase C activation.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed from March 2011 to July 2012 in the Institute of Life Sciences, Taishan Medical College, China.

Materials

A total of 108 clean male Sprague-Dawley rats, aged 8–12 weeks, weighing 200–250 g, were provided by the Experimental Animal Center of Shandong University of Traditional Chinese Medicine (license No. SCXK (Lu) 20050015). All rats were housed in the specific pathogen free grade laboratory of Taishan Medical College, and given free access to standard grains and water at

18–25°C.

Experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[33].

Methods

Preparation of drug solutions

The flunarizine group was intragastrically injected with 1 mg/kg flunarizine solution (5 mg flunarizine was dissolved in 20 mL of warm saline). The gabapentin group was intragastrically injected with 200 mg/kg gabapentin solution (200 mg gabapentin was dissolved in 4 mL warm saline). Rats in the H-7 group were injected with 10 µg of H-7 into the lateral cerebral ventricle (5 mg H-7 was dissolved in 500 µL chloroform, for a final concentration of 10 µg/µL. 10 µL H-7 solution was mixed with 5 µL chloroform, for a total injection volume of 15 µL).

Establishment of rat migraine model

A neurogenic inflammation migraine model was established using the Moskowitz method^[30]. In brief, rats were intraperitoneally anesthetized with 10% chloral hydrate, 3.5 mL/kg, and fixed in the brain stereotaxic instrument. An opening in the skull, 2 mm in diameter, was formed 3.2 mm posterior to the bregma and 2.5 mm right of the sagittal suture, exposing the dura mater. The stimulating electrodes were inserted into the calvarial bone to a depth of 9.2–9.8 mm, and the right trigeminal ganglion was stimulated for 5 minutes (3.0 mA, 5 ms, 5 Hz). In the sham operation group, the stimulating electrodes were positioned in the trigeminal ganglia, but the animals were not provided electrical stimulation.

Model success was defined as the appearance of masticatory muscle contraction, increasing oral and nasal secretions on the stimulated side, frequent scratching of the head, closed eyes, increase in the number of times the animal climbed the cage, and irritability^[31].

Drug administration

The syringe was filled with 1 mL of intragastric drug solution, and the stomach feeding tube was inserted to a depth of 5 cm. The drugs were gradually injected into rats in the flunarizine and gabapentin groups, and the syringe was retained for 30 seconds. Rats in the H-7 group were anesthetized with intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg) and fixed in the brain stereotaxic apparatus (type 51600; Stoelting Co., Illinois, IL, USA).

A window, 2 mm in diameter, was opened 1.2 mm posterior to the bregma and 2.0 mm lateral to the sagittal suture. The microinjector was inserted. H-7 solution, 15 µL, was injected into the lateral ventricle 1.2–1.4 mm posterior to the bregma and 2.0 mm right of the sagittal suture. The syringe needle, which was parallel to the skull horizontal plane, was inserted 5 mm. The microinjection pump was started, and injection was given for 5 minutes at a speed 3 µL/min.

Recording of extracellular discharge frequency in the spinal trigeminal nucleus of migraine model rats

Rats were intraperitoneally anesthetized with 10% chloral hydrate (3.5 mL/kg) and fixed in the brain stereotaxic apparatus. A window in the skull, 3 mm in diameter, was formed 13.2 mm posterior to the bregma and 2.5 mm right of the sagittal suture. The tungsten electrodes (3–5 MΩ) were inserted to a depth of 8.8–9.2 mm to record discharge for 30 minutes. The recordings taken before modeling were used as the control. After the right trigeminal ganglion was stimulated (3.0 mA, 5 ms, 5 Hz) for 5 minutes, the continuous discharge frequency was recorded with a multi-channel electrophysiology recorder (type MP150; BIOPAC Systems, Inc., CA, USA) 2 hours after modeling. The recording duration was 30 minutes.

To facilitate comparisons between the different groups and to minimize the effects of cell variation, data were standardized. Taking the discharge frequency of neurons prior to administration as a reference, the relative discharge frequency value after administration was calculated according to the following formula (changes in discharge frequency were expressed as a percentage): Discharge frequency = reaction frequency after stimulation or administration/reaction frequency before stimulation or administration × 100%.

Liquid chromatography-mass spectrometry for cerebrospinal fluid glutamic acid, aspartic acid, gamma-aminobutyric acid and serine content

70–100 µL of cerebrospinal fluid was collected with foramen magnum puncture 2 hours after modeling. The extracted cerebrospinal fluid was centrifuged for 5 minutes at 3 000 × g, and the supernatant was harvested as cerebrospinal fluid samples. The 50 µL samples were transferred to 4-mL centrifuge tubes, mixed with methanol (200 µL) to precipitate protein, sonicated for 10 minutes, and then centrifuged for 5 minutes at 10 000 × g. The supernatants, 10 µL, were sampled into 0.01–1.00 mmol/L amino acid standard solutions and analyzed with a liquid chromatograph-mass spectrometer (Waters Corp., MA, USA).

The ion current was recorded, the selective daughter ion m/z peak area was measured, the concentration-peak area standard curve was plotted, and the regression equation was obtained. The amino acid concentration ($\mu\text{g/mL}$) in samples was calculated, and the sampling volume in each group was $10 \mu\text{L}$. The amino acid content was calculated (amino acid concentration in samples \times sampling volume).

Western blot assay for protein kinase C membrane translocation levels in rat spinal trigeminal nuclei

Rats were killed by decapitation under deep anesthesia 2 hours after modeling. The spinal trigeminal nuclei were harvested and preserved in liquid nitrogen. Then, $10 \mu\text{L/mg}$ tissue was homogenized with Buffer A, sonicated, and centrifuged at 4°C , $30\ 000 \times g$ for 30 minutes. Finally, the supernatant was collected as a cytoplasmic protein component. The remaining precipitate was homogenized with Buffer A and 0.5% NP-40, sonicated and centrifuged at 4°C , $30\ 000 \times g$ for 30 minutes. The supernatant was harvested as a membrane-associated protein component.

Protein quantity was assayed with the BCA method. $45 \mu\text{g}$ total protein was electrophoresed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 4°C , 400 mA current, for 3 hours, and then transferred to a nitrocellulose membrane. Membranes were incubated with rabbit anti-protein kinase C polyclonal antibody (1:1 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-beta-actin polyclonal antibody (1:200; Santa Cruz Biotechnology) and goat anti-rabbit IgG secondary antibody (1:5 000; Santa Cruz Biotechnology) at 4°C . Then, the blots were exposed to X-ray film, which was subsequently developed and fixed in the dark. The X-ray film signal was scanned on a Gel Doc gel imaging analysis system (Bio-Rad, Hercules, CA, USA) and absorbance values were analyzed.

Protein kinase C level is represented as the ratio of protein kinase C absorbance to beta-actin absorbance in the same membrane, and the membrane translocation levels were calculated using the protein content as follows: The level of membrane translocation = membrane protein content/(membrane protein content – cytoplasmic protein content) \times 100%.

Statistical analysis

Data were expressed as mean \pm SD. Statistical analysis was performed using SPSS 10.0 software package (SPSS, Chicago, IL, USA). The values between groups were compared with one-way analysis of variance, and

pairwise comparisons were performed using Tukey's test. The effect of gabapentin on cerebrospinal fluid amino acid content in migraine model rats was determined with a simple linear regression equation. Significance level was defined at $P < 0.05$.

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