Original Article
Roles of acid-sensing ion channel 3 and calcitonin gene-related peptide in the hypersensitivity of dorsal root ganglia neurons in a rat model of visceral pain

Xiao-Ping Niu[1], Chi-Yi He[1], Yun-Dong Wang[1], Shao-Feng Liu[1], Gang Feng[2], Zhen Han[1]

[1]Department of Gastroenterology, Yijishan Hospital, Wannan Medicine College, Wuhu 241001, China; [2]Department of Central Laboratory, Yijishan Hospital, Wannan Medicine College, Wuhu 241001, China

Abstract: This study aimed to investigate the roles of acid-sensing ion channel 3 (ASIC3) and calcitonin gene-related peptide (CGRP) in the hypersensitivity of dorsal root ganglia neurons (DRG) in a rat model of visceral pain. The animal model of visceral pain was established by esophageal distension (ED). The action potentials and currents of the ASIC3 in DRG neurons were recorded using the cell patch-clamp technique. The location of ASIC3 and CGRP in DRG neurons was detected using an immunofluorescence assay. The mRNA and protein expression of ASIC3 and CGRP was measured using quantitative real-time polymerase chain reaction and western blotting, respectively. We found that both the number of action potentials and the currents through ASIC3 ion channels were significantly increased in the ED group compared to the control group. Using immunohistochemical examination, ASIC3 was detected on the cell membranes and in the cytoplasm of DRG neurons, whereas CGRP was seen only in the cytoplasm. mRNA and protein expression levels of ASIC3 and CGRP in the ED group were significantly higher than those in the control group. Both ASIC3 and CGRP participated in the development of hypersensitivity in DRG neurons in the ED model. Change of current in the ASIC3 channels is one of the important mechanisms for DRG neuron hypersensitivity.

Keywords: Acid-sensing ion channel 3, calcitonin gene-related peptide, dorsal root ganglia, esophageal distension, visceral hypersensitivity

Introduction

Gastroesophageal reflux disease (GERD) is a common disease of the digestive system. The prevalence of GERD in China is lower than that in Western countries; however, it does appear to be increasing [1]. However, only about one-third to one-half of patients with GERD have endoscopically positive findings, such as erosions and ulcers, whereas others with GERD symptoms have no obvious mucosal breaks during endoscopic examination. Therefore, GERD includes erosive esophagitis (EE) and endoscopy-negative reflux disease (NERD). At present, the most effective drug therapy for GERD is proton-pump inhibitors (PPIs). Studies have reported that the efficacy of PPIs for NERD is much lower than for EE [2]. Our previous study also showed that about 40% of NERD patients were not responsive to the standard doses of PPIs [3]. PPI failure has become a common clinical dilemma in primary care. Some patients repeatedly utilize healthcare re-

sources by frequent consultations, diagnostic tests, and repeat prescriptions, which severely affect their quality of life and wastes medical resources [4, 5]. It has been suggested that all of the GERD subtypes are independent and each of them has their own specific pathogenic mechanism [6]. The different pathogenic mechanisms may lead to the different responses to PPIs by NERD and EE. Many studies have found that visceral hypersensitivity is a key pathophysiological mechanism of NERD. Although they can be effective in the treatment of acid reflux, PPIs cannot eliminate visceral hypersensitivity, which may partially explain why NERD patients have a poor response to PPI treatment [7]. Further study on the molecular mechanisms of visceral hypersensitivity will provide an important basis for the treatment of NERD.

Visceral hypersensitivity is an important etiology of GERD; however, its mechanisms are unclear. Visceral hypersensitivity may be ca-

used by two factors [8]: peripheral sensitization or central sensitization. Sensitization of the primary afferent neurons is one of the reasons for, and prerequisites of, central sensitization. Understanding the mechanisms underlying the sensitization of primary neurons is particularly important for inhibiting the transmission of pain signals. Pain signals are regulated in the spinal cord before they enter the higher nerve centers. In the spinal cord, the volume, nature, and speed of the pain information is adjusted, converted, or controlled. This function is mainly located in the dorsal root ganglia (DRG) of the spinal cord. There are three types of DRG neurons: small (19-27 μm), medium (33-38 μm), and large (39-50 μm). The medium- and small-sized neurons, which excite unmyelinated type C fibers and thin-myelinated Aδ fibers, are closely related to the transmission of pain. Therefore, as the primary targets for the signaling and regulation of visceral pain signals, the medium- and small-sized DRG neurons have become focuses of visceral hypersensitivity research [9, 10].

Esophageal paresthesia is a complex pathophysiological condition involving multiple factors, including neurotransmitters, ion channels, and receptors [11]. Research on the regulatory mechanisms of visceral hypersensitivity of the esophagus is still at an early stage. Evidence suggests that the increased expression of calcitonin gene-related peptide (CGRP) in esophageal mucosa is associated with esophageal hypersensitivity [12]. However, the specific mechanism of CGRP's involvement in visceral hypersensitivity is unclear. Acid-sensing ion channels (ASICs) are a group of ion-permeable protein complexes commonly found on the cell membrane. They play important roles in sensing body-fluid pH and regulating pain sensation. Matricon J reported that ASIC channels were part of the molecular effectors of central sensitization leading to visceral pain in rat models of irritable bowel syndrome [13]; however, few studies reported on the relationship between ASICs and esophageal visceral hypersensitivity. Acid-sensing ion channel 3 (ASIC3) and CGRP may co-participate in visceral hypersensitivity of the esophagus, but the specific mechanism is unclear.

In the present study, we established a rat model of visceral pain using esophageal dis-tension (ED). The action potentials and currents of ASIC3 in DRG neurons were recorded using the cell patch-clamp technique. Location and expression of ASIC3 and CGRP in DRG neurons were detected using an immunofluorescence assay, quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting, respectively. The aim of this study was to investigate the roles of ASIC3 and CGRP in the hypersensitivity of dorsal root ganglia neurons in the ED model, and to provide a new therapeutic target for visceral hypersensitivity.

Materials and methods

Animals

Forty healthy Sprague-Dawley rats weighing 220-250 g were randomized into 2 groups: the ED group and the control group (20 rats/group). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Wannan Medical College.

Labeling of DRG neurons

The rats were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (10 mg/kg). The chest was opened to find the thoracic esophagus, and DiI (1,1-dioctadecy 1-3-3-3-3-tetramethylindocarbcyanine; 5 mg/ml) was injected. Following the injection at 6-10 sites (1.0 mg/ml/injection), the chest was sutured to complete the operation. The rats were placed on a 37°C thermal blanket to recover.

Establishment of a rat model

Ten days after injection of DiI, the rats were again anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (10 mg/kg). A PE-240 tube with a small latex ball (1.0 cm in length) at its end was inserted into the thoracic esophagus (7-10 cm from the incisor teeth). The tubing that extended from the mouth was attached to the stereotaxic frame with adhesive tape to avoid movement of the balloon during repeated distensions. Warm water was filled into the balloon for repeated ED (0.4 ml each time at a rate of 0.05-0.1 ml/s;

hold 20 s on, 20 s off) [14]. The low volume ED (≤ 0.2 ml) is considered to be an innocuous distension, whereas an ED ≥ 0.4 ml is likely to be noxious or painful in rats [15, 16]. In the control group, the rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg), however they did not undergo ED.

Acutely dissociated DRG neurons

After the rats were killed by cervical dislocation, the thoracic spinal cord was harvested and immediately placed in ice-cold Kreb's solution. The spine was dissected under a microscope to harvest the bilateral DRG, which was placed in ice-cold L-15 solution and gently washed twice. Collagenase (type I-A, 1 mg/ml) and trypsin (type II-S, 0.25%) were added for digestion at 37°C for 50 min. 5 ml of L-15 culture solution (containing 10% fetal bovine serum (FBS)) was added to terminate the digestion, followed by centrifugation at 1000 rpm for 5 min and washing twice with L-15 medium. Cell pellets were suspended in DMEM/F12 supplemented with 10% FBS (Gibco, Grand Island, NY). Then cells were plated on poly-D-Lysine (1 mg/ml; Sigma) coated coverslips in 6-well plates (8×10^5 cells/well). The DRG neurons were ready for patch-clamp recordings after 30 min of attachment on coverslips.

Detection of hypersensitivity

After ED stimulation, the DRG neurons were isolated. The threshold, frequency, and shape of the action potentials were recorded using a patch-clamp technique, and the results were compared with those in the control group. DiI-labeled neurons were orange under fluorescence microscopy. Only DiI-labeled neurons with a capacity of 40 pF were recorded, and the pH value of the internal liquid of the electrodes was adjusted to 7.25 using 1 mM KOH. The extracellular fluid was prepared using Kreb's solution, and the electrodes were set with a P97 micropipette puller (Sutter CA). The electrode resistance was maintained between 4 and 6 MΩ. Signals were acquired using an Axopatch-1D 200B amplifier. Digital-to-analog conversion and data processing were carried out using a Digidata 1322AA/D converter and P Clamp 8 data acquisition and analysis system, respectively. Curve fitting was performed using Origin Pro 7.5.

Cell patch-clamp technique

The ASIC3-specific blocker APETx2 (20 µmol/L) was used to distinguish the ASIC3 from other currents. In the voltage-clamp mode, the current through the ASIC3 ion channels in the DRG neurons was recorded using the whole-cell patch-clamp technique, and the results were compared with those of the control group. In the whole-cell mode, the potential was kept at -80 mV. The pH value of the extracellular fluid was rapidly changed from 7.4 to 6.0 using Tris-OH. The ASIC3-specific blocker APETx2 (20 µmol/L) was added. In some cells, an inward current may be provoked, which is the acid-induced current in rat DRG neurons. Series resistance was compensated by 50-80% to minimize clamping errors and signal distortion. The electrode resistance was maintained at 2-5 MΩ. All the tests were performed at room temperature (22-25°C).

Immunofluorescence assay

ASIC3 and CGRP expressions in DRG neurons were located using an immunofluorescence assay. The DRG cells were collected and fixed in 4% paraformaldehyde for 10 min. After blocking in 10% FBS at room temperature for 1 h, the neurons were incubated overnight with one of the following primary antibodies at 4°C: rabbit anti-mouse CGRP (1:1000; Abcam) or rabbit anti-mouse ASIC3 (1:1000; Abcam). The cells were washed with PBS three times (10 min each). The cells were then incubated with fluorescent-dye-conjugated secondary antibodies at room temperature for 1 h before rinsing with PBS three times (10 min each). After excess secondary antibodies were washed off, pictures were taken using a microscope (Leica, Solms, Germany). The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole).

qRT-PCR

The mRNA expression levels of ASIC3 and CGRP in DRG neurons in the ED animal model were measured using qRT-PCR, and the results were compared with those in the control group. Thoracic DRG samples were collected from the ED and control groups. Total RNA was extracted and purified with TRIzol reagent using the RNeasy kit (Qiagen, Hilden, Germany) and the Turbo DNA-free kit (Ambion, Waltham,

Table 1. Primer sequences of the genes studies for qRT-PCR

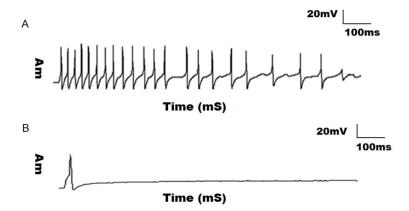| Gene	Sense (5'-3')	Antisense (5'-3')	Product
CGRP	CTC TCA GCA GCA TGT GGG T	TAA CTC ATT TAT ACT TGG TTT CA	554 bp
ASIC3	CCC AGC TCT GGA CGC TAT G	TCT TCC TGG AGC AGA GTG TTG	414 bp
β-actin	TAA AGA CCT CTA TGC CAA CAC AGT	CAC GAT GGA GGG GCC GGA CTC ATC	241 bp

Figure 1. Action potentials of DRG neurons in ED group and control group. A. Action potentials after ED stimulation of DRG neurons (2 times pain threshold) in ED group; B. Action potentials after DRG neurons received stimulation (2 times pain threshold) in the control group.

MA). Then, 400 ng of RNA was used for reverse transcription using the TaqMan reverse transcription reagent kits (Applied Biosystems). The mRNA expression levels were measured using the SYBR Green Master Mix (Shanghai GeneCore). The Ct value of each sample was standardized using β-actin. Changes in mRNA expression of ASIC3 and CGRP were compared between the ED and control groups. The sequences of the primers are shown in **Table 1**.

Western blot

The protein expression levels of ASIC3 and CGRP in DRG neurons in the ED animal model were measured using western blotting, and the results were compared with those in the control group. Thoracic DRG samples were collected from the ED and control groups. They were placed in lysis buffer (containing RIPA, phenylmethylsulfonyl fluoride, and cocktail) on ice to allow ultrasonic homogenization. After the protein concentration was measured using the bicinchoninic acid method (BCA Kit, Thermo Pierce, Rockford, IL), an equal amount of protein was loaded for SDS-PAGE. After the proteins in the gel were transferred to a nitrocellulose membrane (Whatman, Clifton, NJ),

the membrane was treated with 5% skimmed milk and blocked for 1 h at room temperature. The mixture was incubated overnight at 4°C after adding one of the following primary antibodies: rabbit anti-mouse CGRP (1:1000; Abcam) or rabbit anti-rat ASIC3 (1:1000; Abcam). On the second day, the membrane was thoroughly washed with tris-buffered saline-tween (TBST). After incubation at room temperature with horseradish-peroxidase-labeled IgG antibody (1:5000; Huabio, Hangzhou, China) for 1 h, the final lane was recorded and quantified using Quantity One imaging software (Bio-Rad, Hercules, CA).

Statistical analysis

All experiments were performed in triplicate and were repeated at least three times. Representative experiments and mean values ± SD are shown. Statistical analysis was performed using the software SPSS v. 15.0. Statistical differences of the ASIC3 currents and mRNA and protein levels of CGRP and ASIC3 between the ED and control groups were determined by Student's t-test. A *P*-value of < 0.05 was considered statistically significant.

Results

The number of action potentials of the DRG neuron was markedly increased after ED stimulation

In the ED group, the number of action potentials of the DRG neuron was markedly increased by 2 times pain threshold stimulation. However, only one action potential was produced in the DRG neuron of the control group under the same condition (**Figure 1**).
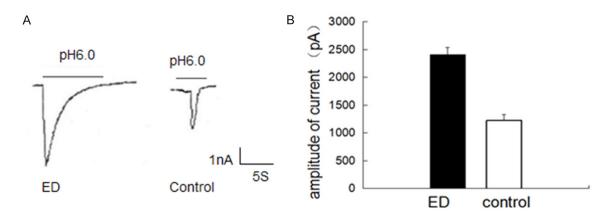
Figure 2. Currents of ASIC3 ion channels of DRG neurons were detected by whole-cell patch-clamp technique. A. Currents through ASIC3 ion channels in DRG neurons when pH of the extracellular fluid was 6.0 (detected by whole-cell patch-clamp technique); B. Data was the current through the ASIC3 ion channels in DRG neurons (pH of the extracellular fluid was 6.0). The current through the ASIC3 ion channels in DRG neurons was markedly increased after ED stimulation. Compared with the control group, the difference was statitistically significant (t = 13.76, $P <$ 0.01). mean ± SEM, n = 20 in each group.
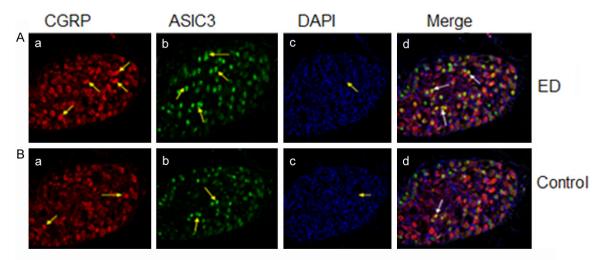

Figure 3. Location of ASIC3 and CGRP in DRG neurons were detected by immunofluorescence assay. A. The Representative immunofluoresence image of ASIC3 (a, red), CGRP (b, green), and DAPI (c, blue) in ED treated DRG neurons (scale bar = 50 μm); B. The Representative immunofluoresence image of ASIC3 (a, red), CGRP (b, green), and DAPI (c, blue) in control DRG neurons (scale bar = 50 μm). a. Expression of ASIC3 (red) in DRG neurons, mainly located in the cell membrane and cytoplasm. b. Expression of CGRP (green) in DRG neurons, mainly located in the cytoplasm. c. DAPI-stained nuclei of DRG neurons. d. Merged immunostaining of CGRP and ASIC was yellow, suggesting the co-expression of CGRP and ASIC in the cytoplasm of DRG neurons.

The current through the ASIC3 ion channels was markedly increased after ED stimulation

ASIC3-specific blocker APETx2 (20 μmol/L) was used to distinguish the ASIC3 and other currents. When the pH value of the extracellular fluid was rapidly changed from 7.4 to 6.0, the acid-induced current in rat DRG neurons can be provoked. We found that the current through the ASIC3 ion channels was markedly increased after ED stimulation (**Figure 2**). Compared with

the control group, the difference was statistically significant (t = 13.76, $P < 0.01$).

Co-expression of CGRP and ASIC in the cytoplasm of DRG neurons

After ED stimulation, the rats were sacrificed by neck dislocation. The thoracic DRG neurons were collected, the expression of ASIC3 and CGRP was detected using an immunofluorescence assay, and the results were compared
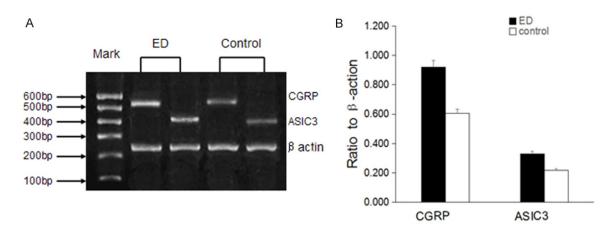
Figure 4. mRNA levels of CGRP and ASIC3 in DRG neurons were detected by qRT-PCR. Data are presented as mean ± SEM. Both CGRP and ASIC3 mRNA levels in ED group were significantly increased compared with the control group (t1 = 13.45, $P < 0.01$; t2 = 15.98, $P < 0.01$).
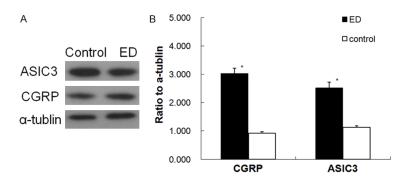

Figure 5. Protein levels of CGRP and ASIC3 in DRG neurons were detected by Western blot. Data are presented as mean ± SEM. The protein levels of CGRP and ASIC3 in ED group were significantly increased compared with the control group (t1 = 21.01, $P < 0.01$; t2 = 17.13, $P < 0.01$).

with those in the control group. **Figure 3** is the representative immunofluorescence image of ASIC3 (red), CGRP (green), and DAPI (Blue) in DRG neurons. Merged immunostaining of CGRP and ASIC is yellow, which suggests the co-expression of CGRP and ASIC in the cytoplasm of DRG neurons.

mRNA expression of CGRP and ASIC3 in DRG neurons was significantly increased after ED stimulation

qRT-PCR showed that mRNA expression of CGRP and ASIC3 in rat DRG neurons was significantly increased compared with the control group (t1 = 13.45, $P < 0.01$; t2 = 15.98, $P < 0.01$) (**Figure 4**).

The protein levels of CGRP and ASIC3 were significantly increased after ED stimulation

Protein levels of CGRP and ASIC3 were detected by western blot in rat DRG neurons. The pro-

tein levels of CGRP and ASIC3 in the ED group were significantly increased compared to the control group (t1 = 20.52, $P < 0.01$; t2 = 22.63, $P < 0.01$) (**Figure 5**).

Discussion

Visceral hypersensitivity is a key pathophysiological mechanism for NERD, and one of the reasons why NERD responds poorly to PPIs. Studies have reported that some ion channels and neurotransmitters are involved in the pathogenesis of visceral hypersensitivity in NERD; however, the exact mechanisms remain unclear. Further research of visceral pain and its molecular mechanisms will provide an important basis for the treatment of NERD. In the present study, we established an animal model of ED-induced esophageal hypersensitivity. After ED stimulation, the DRG neurons were sensitized and the number of action potentials following depolarization markedly increased, which suggests that sensitization of primary neurons is involved in the development of esophageal hypersensitivity.

ASICs, first described by Waldman in 1997 [17], are a group of ion-permeable protein complexes commonly found on the cell membrane. They play important roles in sensing body-fluid pH and regulating pain sensation. ASICs are homopolymer or heteromer channels formed by ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3,

and ASIC4 subunit proteins, with similar features, including that they are: (1) activated directly by extracellular H^+; (2) mainly Na^+-permeable; and (3) permeable for other positive ions, such as K^+ and Ca^{2+}. ASICs are also known as acid-sensitive sodium channels. Since the discovery of ASICs, their regulatory roles in pain sensation have been recognized. In particular, because ASIC3 can rapidly activate and mediate the steady-state current, it has become a hot research topic in the regulation of inflammatory pain sensitivity [18-20]. Research has shown that ASIC3 is expressed in DRG and peripheral nociceptive chemoreceptors, and the expression of ASIC3 is markedly upregulated with decreased pain threshold and the occurrence of hyperalgesia [21]. Further research found that mice with ASIC3 knockout do not develop pain hypersensitivity, which suggests that ASIC3 is an essential component in the development of inflammatory hyperalgesia [22]. The mechanisms of ASIC3's regulation of visceral hypersensitivity are unclear [23]. Furthermore, there are few reports about ASICs and esophageal visceral hypersensitivity at present. In our study, after ED stimulation in the rat model, the acid-induced currents in the ASIC3 channels in DRG neurons markedly increased compared with the control group, which suggests that the change of currents in the ASIC3 channels are the important mechanism for DRG neurons hypersensitivity development.

CGRP is a 37-amino-acid vasoactive neuropeptide that is released by sensory nerve endings and widely distributed in the central and peripheral nervous systems. As a major excitatory neurotransmitter, it is involved in nociceptive information transmission and pain sensitization [24]. In a rat model of chronic arthritis pain, the biosynthesis of CGRP increased in DRG neurons, however after treatment with the CGRP receptor antagonist, CGRP 8-37, the pain was alleviated [25]. Many experiments have confirmed that CGRP can up-regulate cyclic adenosine monophosphate (cAMP) concentration, and thus activate cAMP-dependent protein kinase A (PKA). After specific binding with its receptor (calcitonin receptor-like receptor; CRLR) [26-28], CGRP showed a variety of biological activities to affect pain sensation. The role of CGRP in the development of gastrointestinal hypersensitivity was initially described

by Mönnikes in a study of functional dyspepsia [29]. A further study found that CGRP expression was markedly increased in the lumbosacral spinal cord DRG in a rat model of visceral hypersensitivity due to neonatal maternal separation [30]. Xu found that CGRP expression is significantly increased in the esophageal mucosa of NERD patients [31], which is consistent with one of our previous studies [12]. Meanwhile, we found that CGRP expression was positively correlated with GERD-Q score, and this result suggests that the expression of CGRP is associated with pain sensation. It is therefore speculated that CGRP might participate in visceral hypersensitivity in NERD patients. In the present study, mRNA and protein expression of CGRP was significantly higher in the ED group than in the control group. These results further demonstrated that CGRP is involved in the development of esophageal hypersensitivity.

Ichikawa and Sugimoto found that CGRP and ASIC3 were co-expressed in the trigeminal ganglion [32]. Further studies found that CGRP was co-expressed with ASIC3 in rat models of acute arthritis pain [33]. Meanwhile, the expression of ASIC3 increased along with upregulation of CGRP expression, although the exact mechanism was unclear. We found co-expression of ASIC3 and CGRP in the cytoplasm of DRG neurons. After ED stimulation, mRNA and protein expression of ASIC3 and CGRP significantly increased compared with the control group, and this result indicates that both ASIC3 and CGRP participate in the hypersensitivity of DRG neurons in ED-stimulated animal models. Therefore, we speculate that ED can increase CGRP and CRLR expression in DRG neurons. Through autocrine and paracrine mechanisms, CGRP can bind to CRLR to activate adenylate cyclase, degrade ATP, produce cAMP, and activate PKA, thus showing highly diverse biological activities, such as inducing changes in ASIC3 expression and function, inducing cell depolarization, increasing excitability of DRG neurons, and affecting transmission of pain signals. CGRP may regulate ASIC3 via the CGRP/CRLR/PKA pathway and participate in the development of the hypersensitivity of DRG neurons in ED models. However, such conclusions need to be validated in further experiments.

Address correspondence to: Chi-Yi He, Department of Gastroenterology, Yijishan Hospital, Wannan Medicine College, Wuhu 241001, China. Tel: +86 553 5739316; Fax: +86 553 5739107; E-mail: chiyihe@126.com

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