Received: 4 June 2021 | Revised: 3 November 2021 | Accepted: 22 November 2021

DOI: 10.1096/fj.202100606R

THE FASEB JOURNAL

RESEARCH ARTICLE

Upregulation of acid sensing ion channels is associated with esophageal hypersensitivity in GERD

Xu Han[1] | **Yawen Zhang**[2] | **Allen Lee**[2] | **Zhaoshen Li**[3] | **Jun Gao**[3] | **Xiaoyin Wu**[2] | **Jiulong Zhao**[3] | **Hui Wang**[3] | **Di Chen**[3] | **Duowu Zou**[4] | **Chung Owyang**[2]

[1]Department of Gastroenterology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

[2]Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

[3]Division of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai, China

[4]Department of Gastroenterology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Correspondence
Duowu Zou, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, No. 197 Ruijin Second Road, Huangpu District, Shanghai 200025, China.
Email: zdw_pi@163.com
Chung Owyang, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-5362, USA.
Email: cowyang@med.umich.edu

Funding information
The studies were supported by grants from the National Natural Science Foundation of China (Grant nos. 81370493 and 81670485) (DZ), the National Institutes of Health (NIH) 1K23 DK124567 (AL) as well as NIH 2R01 DK058913 (CO), P30 DK 34933 (CO) and Shanghai Pujiang Program (Grant nos.20PJ1409700)

Abstract

Proton pump inhibitors (PPIs) are the mainstay of therapy for gastroesophageal reflux disease (GERD) but up to 60% of patients have inadequate response to therapy. Acid sensing ion channels (ASICs) play important roles in nociception. This study aimed to investigate whether the increased expression of ASICs results in neuronal hyperexcitability in GERD. Esophageal biopsies were taken from GERD patients and healthy subjects to compare expression of ASIC1 and 3. Next, gene and protein expression of ASIC1 and 3 from esophageal mucosa and dorsal root ganglia (DRG) neurons were measured by qPCR, Western-blot and immunofluorescence in rodent models of reflux esophagitis (RE), non-erosive reflux disease (NERD), and sham operated groups. Excitability of DRG neurons in the GERD and sham groups were also tested by whole-cell patch-clamp recordings. We demonstrated that ASIC1 and 3 expression were significantly increased in patients with RE compared with healthy controls. This correlated positively with symptom severity of heartburn and regurgitation ($p < .001$). Next, ASIC1 and 3 gene and protein expression in rodent models of RE and NERD were similarly increased in esophageal mucosa as well as T3–T5 DRG neurons compared with sham operation. DRG neurons from RE animals showed hyperexcitability compared with sham group. However, intrathecal injection of ASIC specific inhibitors, PcTx1 and APTEx-2, as well as silencing ASIC1 and 3 genes with specific siRNAs prevented visceral hypersensitivity. Overall, upregulation of ASIC1 and 3 may lead to visceral hypersensitivity in RE and NERD and may be a potential therapeutic target for PPI non-responsive patients.

Abbreviations: ASICs, acid sensing ion channels; DRG, dorsal root ganglia; ED, esophageal distention; EMG, electromyography; GERD, gastroesophageal reflux disease; IBS, irritable bowel syndrome; NERD, nonerosive reflux disease; PPIs, proton pump inhibitors; RE, reflux esophagitis; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; VMR, visceromotor response.

KEYWORDS
acid sensing ion channel 1 and 3 (ASIC1 and 3), gastroesophageal reflux disease (GERD), neuronal hyperexcitability, visceral hypersensitivity

1 | INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder characterized by reflux of gastric contents leading to chronic symptoms, such as heartburn, regurgitation, and/or chest pain.[1] Population-based studies suggest that GERD is a common disorder and the prevalence appears to be growing worldwide, particularly in North America and East Asia.[2]

The spectrum of GERD includes erosive reflux disease (ERD) characterized by the presence of esophagitis and non-erosive reflux disease (NERD) characterized by the absence of endoscopically visible lesions and the presence of abnormal pH monitoring.[3] NERD represents up to 60% of all patients with reflux symptoms. It has been increasingly recognized as the most common cause of reflux symptoms in community populations with significant impact on quality of life. Compared to patients with erosive esophagitis, NERD patients have been shown to be less responsive to proton pump inhibitors.[4]

The mainstay of treatment for GERD typically involves acid suppressive therapies, such as proton pump inhibitors (PPIs). A meta-analysis showed that PPIs were effective in healing erosive esophagitis in 86% of cases.[5] However, symptom response is much more variable. Studies have documented that 20%–60% of GERD patients are either partial or complete non-responders to PPI therapy, particularly in NERD.[6–8] This represents a substantial and growing population of patients who are inadequately treated.

Visceral hypersensitivity is believed to be an important pathogenic factor in the development of reflux symptoms with one study documenting hypersensitivity to both acid and mechanical stimuli in 30% of patients.[9] Previous studies have documented that several mechanisms contribute to the development of visceral hypersensitivity, including peripheral and central sensitization.[10] However, the molecular mechanisms leading to visceral hypersensitivity in GERD remain unclear.

The acid sensing ion channels (ASICs 1–3) are a family of voltage-insensitive epithelial Na^+ channels.[11] ASICs are heavily expressed in small and medium sensory neurons which point to their importance in modulating nociception. Animal models have confirmed their role in transmitting nociceptive and mechanosensory signals.[12–14] In humans, ASIC3 was found to be overexpressed in inflamed intestines from patients with Crohn's disease.[15] Although these findings as well as the possibility that a pH-dependent channel may be involved in nociception in an acid-related disorder are intriguing, little is known about the role of ASICs in GERD.

We hypothesize that inflammation due to chronic acid reflux leads to upregulation of ASIC1 and 3 resulting in visceral hypersensitivity in GERD. In this study, we aimed to investigate expression of ASIC1 and 3 in esophageal biopsies in patients with erosive esophagitis compared with healthy controls. To determine if upregulation of ASIC1 and 3 are related to chronic acid reflux and mucosal inflammation, we employed a rodent model of reflux esophagitis (RE) as well as a rodent model of NERD to quantify expression of ASIC1 and 3 in esophageal mucosa as well as esophageal-specific dorsal root ganglion (DRG) neurons (T3–T5). Furthermore, to demonstrate whether altered expression of ASIC1 and 3 are involved in esophageal hypersensitivity in GERD, we applied a whole-cell patch-clamp and acid perfusion as well as esophageal distension (ED) techniques to study the electrophysiology of DRG neurons and esophageal motor and sensory functions in these two rodent models of GERD.

2 | MATERIALS AND METHODS

2.1 | Esophageal biopsies in GERD subjects and healthy controls

Esophageal biopsies were obtained from subjects undergoing upper endoscopy at the Changhai Hospital, Second Military Medical University. During endoscopy, biopsy specimens were obtained from esophageal mucosa 3 cm proximal to the gastroesophageal junction and preserved in liquid nitrogen. All human experiments were approved by the institutional review board at the Second Military Medical University. Informed consent was obtained from all subjects prior to acquisition of tissue.

2.1.1 | Symptom assessment

Prior to endoscopy, all patients and healthy controls completed the GerdQ questionnaire to characterize the presence of GERD as well as frequency and severity of GERD symptoms (score ranges from 0 to 18).[16]

2.1.2 | GERD subjects

Patients (age 18–70) with typical symptoms of GERD, including heartburn, regurgitation, and/or chest pain for at least 1 month as well as findings of Los Angeles (LA) grade A or grade B erosive esophagitis on upper endoscopy were enrolled in this study into Group A and Group B, respectively.[17]

2.1.3 | Healthy controls

Esophageal biopsies were also obtained from healthy subjects (age 18–70) without GERD symptoms undergoing upper endoscopy for routine screening of gastric cancer.

2.2 | Rodent model of GERD

2.2.1 | Animals

Male Sprague Dawley rats (200–250 g; Sino-British SIPPR/BK Lab Animal Ltd, Shanghai, China) were used for all animal experiments. The rats were fed standard laboratory diet and maintained on a 12:12 h light-dark cycle (lights on at 6:00 a.m. to 6:00 p.m.). Temperature and humidity were maintained at constant levels.

Rodents were randomly divided into two groups in this study: Group 1 constituted the RE ($n = 5$) or NERD rodent group ($n = 5$) while Group 2 ($n = 5$) consisted of a sham-operated rodent group. Preoperative and postoperative care of the GERD and sham rodent groups were identical. All experimental procedures were performed in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

2.2.2 | RE rodent model

Rodents were housed singly in cages and deprived of food for 24 h before and after surgery. The rodent model of GERD was based off a prior method devised by Omura et al. (Supporting Information Figure S1).[18] Rodents were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The abdomen was opened by using a 3-cm upper midline incision. The transitional region between the forestomach and glandular stomach was ligated with 2-0 silk thread to restrict the compliance of the stomach. A latex ring made from 18-Fr Nelaton catheter (width, 2 mm) was placed around the area proximal to the pyloric sphincter in order to restrict gastric emptying. For rodents in the control group, the abdomen was opened without ligation and restriction. Rodents were subcutaneously injected with Rimadyl (5 mg/kg) for analgesia.

2.2.3 | NERD rodent model

A NERD rodent model was established using hyperglycemia combined with mental stress as previously described.[19] Briefly, rats were given free access to fructose-water (200 g/L) for 28 days while the control group was provided free water without fructose. The NERD model rats were also placed in plastic restraint devices for 2 h/day for 14 days. Control rats were kept in cages without restraint in the same room. The veracity of this model was confirmed by typical histological changes of NERD in the esophageal mucosa, including dilated intercellular spaces (DIS), basal cell layer hyperplasia, papillary elongation, and intraepithelial inflammatory cell infiltration.[19] A semi-quantitative assessment of DIS was assessed by light microscopy under 40× magnification as described previously.[20] Briefly, a small intercellular space was defined as diameter less than one lymphocyte while large was defined as diameter greater than or equal to one lymphocyte. The severity of DIS was scored in one high-power field as follows: 0 (≤5 small intercellular spaces); 1 (≥6 small intercellular spaces and ≤5 large intercellular spaces); 2 (≥6 large intercellular spaces). The individual and total histological scores were assessed based on published studies.[20–22]

2.3 | Histology

All rodents were euthanized on postoperative day 15. The esophagus was removed en bloc quickly and opened longitudinally, pinned flat on a corkboard, then fixed in 10% buffered formalin overnight. The esophagus was then dehydrated before paraffin fixation and staining with hematoxylin and eosin (H&E). Histological examination was carried out on serial sections with 5 μm thickness. Mucosal integrity and inflammatory changes within the mucosa and deeper layers were assessed histologically.

2.4 | Real-time quantitative polymerase chain reaction

Cellular RNA was extracted from rodents' esophageal mucosa, DRG as well as human biopsy specimens by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and Qiagen (Hilden, Germany) RNeasy mini columns

according to the manufacturer's recommendations. Total extracted RNA were reverse-transcribed into first-strand cDNA by using 100 U/ml of reverse transcriptase (Takara Biomedicals, Shiga, Japan) and 0.1 μM of oligo (dT)-adapter primer (Takara) in a 50-μl reaction mixture. Real-time RT-PCR was carried out with a Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) using the DNA-binding dye SYBER Green I for the detection of PCR products. Relative gene expression by RT-PCR was calculated using the $2^{-\Delta\Delta CT}$ method as described previously.[23] Fold changes in gene expression were calculated relative to the average of the control group. Standard curves were used to evaluate the efficiency of PCR amplification.

2.5 | Western blot analysis

Frozen specimens from rodents were homogenized in 100 μl lysis buffer containing a mixture of proteinase and phosphatase inhibitors and then centrifuged at 15 000 rpm for 15 min at 4°C. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). A total of 50 μg of protein was resolved on 12% precasted SDS-PAGE gels, then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was blocked with 5% non-fat milk in PBS containing 0.1% Tween 20 for 2 h at room temperature and then incubated overnight at 4°C with primary antibodies. The following antibodies were used in this study: GAPDH, anti TNF-α, anti-ASIC1, and anti-ASIC3 antibody (1:1000, abcam, USA). After washing with Tris-Buffered Saline, 0.1% Tween 20 Detergent (TBST), the blots were incubated for 2 h at room temperature with HRP-conjugated secondary antibody (1:5000; Amersham Biosciences, San Francisco, CA, USA), visualized by using Electro-Chemi-Luminescence (ECL) chemiluminescent detection system (Amersham Biosciences).

2.6 | Immunofluorescent staining

2.6.1 | Dorsal root ganglia

After the rats were sacrificed, the chest was opened, and the ascending aorta was then infused with ice-cold saline and 4% paraformaldehyde. The T3–T5 DRGs were removed and incubated with 4% paraformaldehyde for 3 h at room temperature and then replaced with 30% sucrose for 24 h at 4°C. The DRGs were then embedded in Histoprep and were cut at a thickness of 10 μm on a cryostat.

For double immunofluorescence, the DRG sections were incubated with a mixture of anti-ASIC1 (1:1000, abcam, USA), monoclonal neuronal-specific nuclear protein (NeuN) (1:500, Millipore, USA), and anti-ASIC3 (1:1000, abcam, USA) overnight at 4°C. The sections were washed with PBS and then incubated with Alexa 488-conjugated goal anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, USA) for 2 h at room temperature.

2.6.2 | Lower esophageal submucosal-specific dorsal root ganglia neurons

For experiments involving patch clamp recordings, six to eight injections were made at different sites into the lower esophageal submucosa of control and experimental rodents with the lipid-soluble fluorescence dye, 1,19-dioleyl-3,3,39,3 tetramethyl-lindocarbocyanin methanesulfonate (DiI; Invitrogen, Carlsbad, CA, USA). After injection, rodents were euthanized on day 15. Only fluorescence dye labeled DRG neurons from T3–T5 were used for patch clamp studies.

2.7 | Primary DRG neuron preparation

Rodents were decapitated immediately after euthanization, and bilateral thoracic dorsal root ganglia neurons (T3–T5) were acutely dissected out. DRG neurons were then incubated in dissecting solution (NaCl 130 mM, KCl 5 mM, KH_2PO_4 2 mM, $CaCl_2$ 1.5 mM, $MgSO_4$ 6 mM, glucose 10 mM, and HEPES 10 mM, pH 7.2, osmolarity 305 mOsm) with trypsin (1.2 mg/ml, Sigma, St. Louis, MO, USA) and collagenase D (1.5–1.8 mg/ml, Roche, Mannhein, Baden-Württemberg, Germany) for 1.5 h at 34.5°C. DRGs were harvested from the enzyme solution, washed with external solution, and then transferred to 2 ml of the dissecting solution containing DNase (0.5 mg/ml). Single cell suspensions were then harvested by repeat trituration through flame-polished glass pipettes and put onto acid-cleaned glass coverslips.

2.8 | Whole-cell patch-clamp recordings

A coverslip containing the adherent DRG neurons was placed in a recording chamber and attached to the stage of an inverted microscope (IX71; Olympus, Tokyo, Japan) equipped for both fluorescence and phase objectives. The external solution contained NaCl 130 mM, KCl 5 mM, KH_2PO_4 2 mM, $CaCl_2$ 2.5 mM, MgCl2 1 mM, glucose 10 mM, and HEPES 10 mM, pH 7.2, adjusted by NaOH,

osmolarity 290–300 mOsm. The patch pipettes had a resistance of 3–5 MΩ when they were filled with the solution containing potassium gluconate 140 mM, NaCl 10 mM, HEPES 10 mM, glucose 10 mM, BAPTA 5 mM, and $CaCl_2$ 0.01 mM. Pipette solutions were adjusted to pH 7.25 by KOH, osmolarity 295 mOsm. Cells labeled by DiI were held at −60 mV. Up to 80% of series resistance was compensated. The potential and current recordings were filtered at 2–5 kHz and sampled at 50 or 100 μs/point. Electrophysiological recordings were performed at room temperature. Data were stored through HEKA EPC10 (HEKA Instruments, Lambrecht, Germany) and analyzed by Patch Master Software (HEKA Instruments).

2.9 | Intrathecal injection of specific inhibitors or siRNA of ASIC1 and 3

In the rodent model of RE, PcTx1, APTEx-2, or vehicle (saline) (1 nmol per rat) was administered intrathecally 15 min before the ED test.[24,25] In separate studies, 10 ml of a siRNA (ASIC1 or ASIC3) (2 μg)/i-Fect (Neuromics) mix was injected intrathecally using a Hamilton syringe and a 25-gauge needle. Animals received one injection per day for 3 days before the ED test. SiRNA ASIC1, SiRNA ASIC3 and the corresponding scramble siRNAs were ordered from Sigma. Intrathecal injections were performed freehand under isoflurane anesthesia (2% isoflurane inhalation) between spinal T3 and T5 vertebrae of rats as previously described.[26] RT-PCR studies showed that intrathecal administration of specific ASIC1 and ASIC3 siRNAs resulted in >70% reduction of ASIC1 and 3 expression in the T3-T5 thoracic dorsal root ganglia.

2.10 | Esophageal HCl perfusion test

To demonstrate development of visceral hypersensitivity, we used hydrogen chloride (HCl) perfusion and mechanical distension to evoke pain response. Acid-evoked pain responses were performed according to a previous report.[27] Briefly, intraesophageal HCl was administered using a polyethelyene (PE) tube inserted into the lower esophagus of rats (8 cm from the incisors). The PE tube was attached to a needle connected to a 1 ml syringe. Different concentrations of 0.2 ml HCl solutions (0.001, 0.01 and 0.1 N) were used for perfusion. Normal saline (0.2 ml) was used as control. Both HCl and normal saline were infused over 1 min and the EMG activities of the trapezius muscle were recorded. Four flushes of 0.2 ml of warm saline were injected after each HCl infusion to rinse away the residual acid in the esophagus. To avoid de-sensitization, each HCl injection was separated by 30 min intervals. Each test solution was repeated 3 to 4 times for each rat.

2.11 | ED test

We next measured sensitivity to esophageal distention (ED), which is a recognized model of GERD in rats[27] and also has evidence of clinical relevance in the pathogenesis of GERD in humans as well.[28] Low-threshold mechanoreceptors modulate spinal nociceptive pathways while high-threshold mechanoreceptors mediate esophageal nociception.[29] Briefly, Teflon-coated, silver wires were implanted into the trapezius muscle in the neck 4–5 days before the beginning of the experimental procedures. ED was produced using a 1 cm length latex balloon, ligated to the end of PE-240 tubing. The balloon was placed orally in the thoracic esophagus (8 cm from the incisors) in anesthetized rats (2% isoflurane inhalation; Baxter, France).[27] After recovery from anesthesia, rats were placed in the middle of a 40 × 40 cm polymethyl acrylate box and the catheter was connected to an electronic barostat apparatus (Synectics Visceral Stimulator, Medtronic, France). ED was produced by an increase of pressure using an esophageal balloon. Balloon pressure was increased to 20, 40, 60, 80 mmHg and held for 20 s. Graded-intensity stimulation trials were conducted to establish stimulus response curves. Each distention trial consisted of 3 segments: a 20-s predistention baseline period, a 20-s distention period, and a 20-s post-distention termination period with a 5-min interstimulus interval. EMG activity was amplified and digitized using a SPIKE2/CED 1401 data acquisition interface (Cambridge Electronic Design, Cambridge, UK). The responses were considered stable if there was <20% variability between 2 consecutive trials of each ED. The increase in the area under the curve of EMG amplitude during ED from the baseline period before ED was recorded as the response.

2.12 | Statistical analysis

All data are presented as mean ± SEM. Continuous data were compared using *t*-tests while one-way ANOVA with Bonferroni's correction for multiple comparisons were applied for continuous data from more than two groups. For continuous variables that were not normally distributed, comparison between groups was performed using Wilcoxon tests. A nominal value of $p < .05$ was considered statistically significant. Statistical analyses were performed using SPSS 11.0.0 software (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

3.1 | Expression of ASIC1, 3 in human esophageal mucosa

A total of 28 patients with LA grade A esophagitis (group A), 15 patients with LA grade B esophagitis (group B), and 16 healthy controls (group C) were recruited (Table 1). ASIC1 and 3 mRNA subunits were identified in human esophageal biopsies by RT-PCR and normalized against expression of B-actin. Compared with healthy controls (group C), there was a significant increase in gene expression of ASIC1 (group A vs. group C [5.3 ± 1.0 vs. 1.1 ± 0.2, $p < .01$], group B vs. group C [9.2 ± 2.4 vs. 1.1 ± 0.2, $p < .01$]) and ASIC3 (group A vs. group C [3.4 ± 0.6 vs. 1.1 ± 0.2, $p < .001$], group B vs. group C [14.8 ± 3.8 vs. 1.1 ± 0.2, $p < .01$]) in the esophageal mucosa of GERD subjects (Figure 1).

We next determined whether ASIC1 and 3 gene expressions were correlated with changes in symptom scores by GerdQ. In patients with LA grade A esophagitis, ASIC1 and 3 expressions were strongly correlated with symptoms of heartburn, while ASIC3 expression showed a positive correlation with regurgitation (Table 2). Meanwhile, a trend was observed for ASIC1 and 3 expression and symptoms of regurgitation in patients with LA grade B esophagitis (Table 2).

3.2 | Esophageal mucosal histology in RE rodent model

After surgical induction of GERD, large, excavated ulcerations with inflammatory cell infiltrates of neutrophils, eosinophils, and other inflammatory cells within the submucosa were observed in the esophagus after 15 days (Figure 2E,F). Additionally, marked thickening of the esophageal epithelium, elongation of lamina propria papillae, and basal cell hyperplasia were detected in this model of RE (Figure 2E,F, Table 3).

TABLE 1 Baseline demographic information for GERD patients and healthy controls

	Healthy controls	LA grade A esophagitis	LA grade B esophagitis
Number of subjects	16	28	15
Gender			
Male, *N* (%)	10 (62.5)	19 (67.9)	10 (66.7)
Female, *N* (%)	6 (37.5)	9 (32.1)	5 (33.3)
Age, mean (SD)	50.3 (11.6)	52.8 (11.2)	57.9 (10.7)
GerdQ score, median	1.5	6.5	9
Use of PPIs, *N* (%)	0 (0)	10 (35.7)	2 (13.3)

Abbreviations: GerdQ, gastroesophageal reflux disease questionnaire (ranging from 0 to 18); LA, Los Angeles classification; PPIs, proton pump inhibitors.

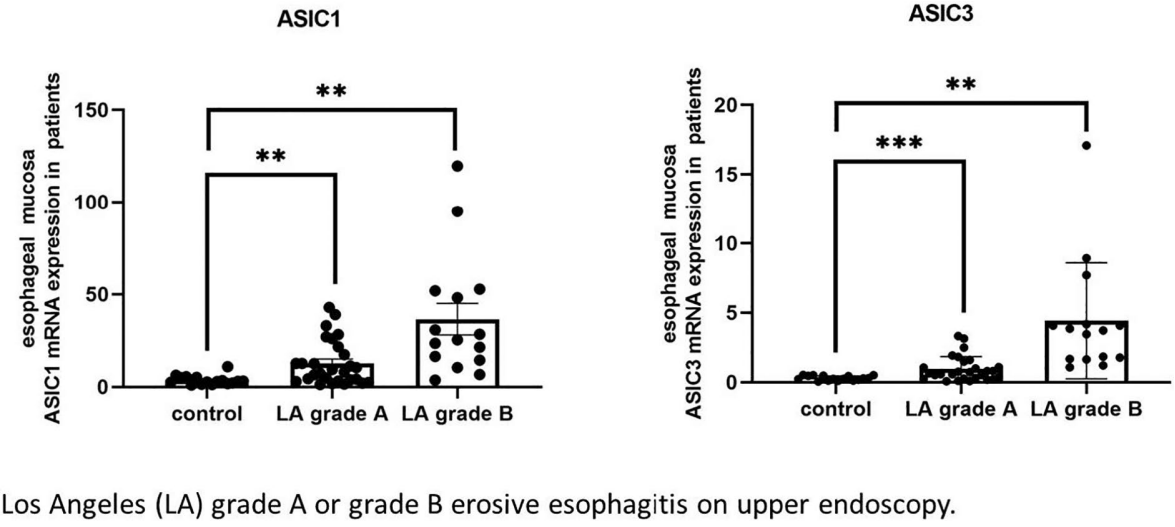

FIGURE 1 ASIC1&3 levels were up-regulated in group A and group B patients with GERD. The relationship between ASIC1 and 3 gene levels and endoscopic grading of reflux esophagitis based on the Los Angeles classification. Both ASIC1 and 3 gene expressions were correlated with the severity (grade) of esophagitis. $^{**}p < .001$; $^{***}p < .0001$ for GERD patients compared with healthy controls

TABLE 2 Increased ASIC1 and 3 expression is associated with severity of esophagitis and GERD symptoms

		LA grade A esophagitis		LA grade B esophagitis	
		Regurgitation	**Heartburn**	**Regurgitation**	**Heartburn**
ASIC1	Pearson correlation	.515	.798**	.572	−.204
	Sig. (2-tailed)	.105	.003	.052	.524
	N	11	11	12	12
ASIC3	Pearson correlation	.663*	.732**	.520	−.208
	Sig. (2-tailed)	.014	.004	.083	.516
	N	13	13	12	12

Note: Increased ASIC1 and 3 expression was associated with symptoms of GERD as well as LA grade esophagitis. For patients with LA grade A esophagitis, there was a positive correlation between ASIC 1 gene expression and reflux symptoms, such as heartburn ($r = .798$, $p < .01$). There was also a positive correlation between the ASIC 3 gene expression and GERD symptoms including heartburn ($r = .732$, $p < .01$) and regurgitation ($r = .663$, $p < .05$). For patients with LA grade B esophagitis, a trend towards positive correlation was observed between regurgitation and ASIC1 ($r = .572$, $p = .05$) and ASIC3 expression ($r = .52$, $p = .083$).

*$p < .05$; **$p < .01$.

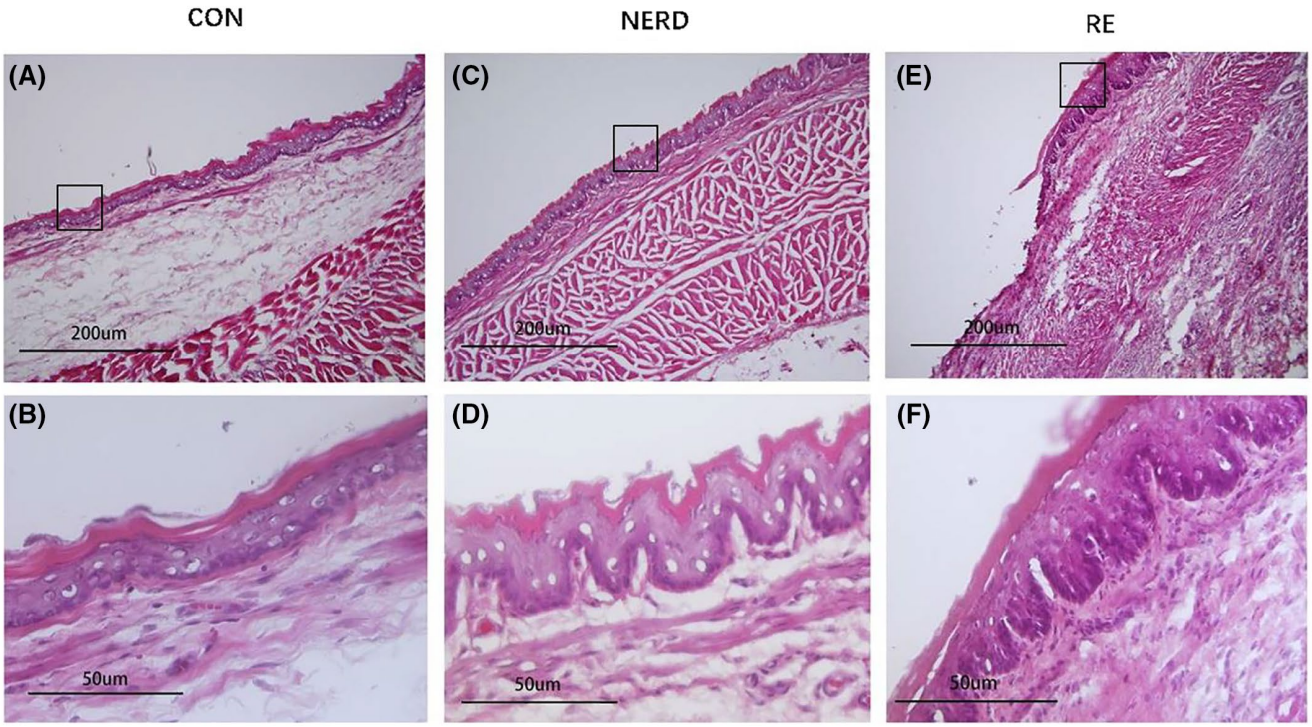

FIGURE 2 Histology of esophageal mucosal injury in GERD rodent model. Representative images of esophageal lesions (H&E stain) under 10× (A, C and E) and 40× (B, D and F) magnification. (A, B) sham-operated (CON) group ($n = 5$); (C, D) non-erosive reflux disease (NERD) group ($n = 5$) with mild esophagitis characterized by basal cell hyperplasia, papillary elongation and several dilated intracellular spaces. Reflux esophagitis (RE) group ($n = 5$) with (E) severe esophagitis characterized by basal cell hyperplasia, papillary elongation, obvious dilated intracellular spaces; and (F) inflammatory cells infiltration and erosion. $n = 3$ to 5 rats/group

3.3 | Esophageal mucosal histology in NERD rodent model

After induction of NERD, there were changes in the mucosal layer, including basal cell hyperplasia, papillary elongation and DIS (Table 3). However, there were little or no inflammatory cells or erosions (Figure 2C,D). In addition, the total histological scores were significantly milder compared to the scores observed in the RE rats (Table 3).

3.4 | Increased esophageal expression of TNF-α in RE rodent model (mucosa and DRG)

As TNF-α has previously been shown to rapidly enhance functional activity of ASICs in primary sensory neurons,[30] we measured TNF-α gene and protein expression in rodent esophageal mucosa by qPCR and western blot. Compared with the sham-operated group, the RE group showed

TABLE 3 Histological changes of esophageal mucosa between control and GERD rodent models

Group	Basal cell hyperplasia (proportion, %)	Papillary elongation (proportion, %)	Dilated intercellular spaces (DIS)		Score of inflammatory cells	Score of erosions	Total score
			Number of small DIS (diameter <1 lymphocyte)	Number of large DIS (diameter ≥1 lymphocyte)			
Control	23.89 ± 0.04	33.49 ± 0.04	2.33 ± 1.37	0.33 ± 0.82	0	0	1.19 ± 0.31
NERD	32.92 ± 0.04*	52.63 ± 0.02**	4.33 ± 1.16	2.33 ± 1.53*	0	0	2.20 ± 0.26**
RE	31.81 ± 0.03*	55.91 ± 0.11*	12.50 ± 5.57**	15.00 ± 8.17***	1.33 ± 1.52	0.75 ± 0.5	6.43 ± 1.29****

Note: Results were expressed as Mean ± SD. Score of inflammatory cells was calculated by the counts of intraepithelial eosinophils, neutrophils and mononuclear cells. Score of erosions was assessed according to the present of erosions and healed erosions. Total score was calculated by the sum score of each microscopic lesions according to published paper.[20] $n = 3$ to 5 rats/group.

Abbreviations: NERD, nonerosive reflux disease; RE, reflux esophagitis.

*$p < .05$ compared with control group; **$p < .01$ compared with control group; ***$p < .05$ compared with NERD group; ****$p < .01$ compared with NERD group.

significantly higher TNF-α gene (4.2 ± 1.5 vs. 1.0 ± 0.2, $p < .05$) and protein expression (1.96 ± 0.2 vs. 1.0 ± 0.01, $p < .01$), respectively (Supporting Information Figure S2).

3.5 | Upregulation of ASIC1 and 3 expressions in esophageal mucosa and DRGs in RE model

We next measured gene and protein expression of ASIC1 and 3 by qPCR and western blot. In the esophageal mucosa of the rodent RE group, there was significantly higher gene expression of ASIC1 (1.5 ± 0.6 vs. 1.0 ± 0.1, $p = .05$) and ASIC3 (4.2 ± 1.1 vs. 1.0 ± 0.1, $p < .05$) compared with the sham-operated group (Figure 3A). Western-blot analysis confirmed higher protein expression of ASIC1 (2.7 ± 0.8 vs. 1.0 ± 0.01, $p < .05$) and ASIC3 (2.4 ± 0.6 vs. 1.0 ± 0.01, $p < .05$) in the esophageal mucosa of the RE group compared with sham-operated group (Figure 3B).

ASIC1 and 3 gene expressions in rodent DRG neurons were also measured. On postoperative day 15, T3–T5 DRG neurons were dissected. Rodents in the RE group exhibited significantly higher gene expression of ASIC1 (2.0 ± 0.2 vs. 1.0 ± 0.1, $p < .05$) and ASIC3 (2.2 ± 0.1 vs. 1.0 ± 0.1, $p < .01$) compared with the sham-group (Figure 3C). Similarly, the rodents in the RE group showed significantly higher protein expression of ASIC1 (1.4 ± 0.1 vs. 1.0 ± 0.1, $p = .05$) and ASIC3 (2.7 ± 0.1 vs. 1.0 ± 0.1, $p < .01$) compared with the sham-operated group by Western blot (Figure 3D).

A population of sensory neurons in T3–T5 DRGs which innervate the esophagus were identified by co-localization of ASIC1 or 3 (green) and NeuN, a biomarker for neurons. In these sensory neurons, rodents from the RE group exhibited a significantly higher percentage of ASIC1 (45.5% ± 4.1 vs. 25.0% ± 2.0, $p < .05$) and ASIC3 (56.5 ± 3.9 vs. 27.8% ± 1.2, $p < .01$) positive DRG neurons compared with the sham-operated rodents (Figure 4A–D).

As ASIC1 and 3 are expressed by both sensory neurons and esophageal epithelial cells,[31] we next examined whether the increases in ASIC1 and 3 expression in the DRG parallel the increases in the esophageal mucosa in this rodent model of GERD. We found that gene expression of ASIC1 and ASIC3 in DRG neurons correlated positively with ASIC1 ($r = .805$, $p = .005$) and ASIC3 ($r = .852$, $p < .005$) expression in esophageal epithelium, respectively (Table 4). Similarly, we found ASIC1 and 3 protein expression in DRG neurons correlated positively with ASIC1 ($r = .801$, $p = .005$) and ASIC3 ($r = .719$, $p = .02$) expression in esophageal epithelium, respectively (Table 5). These data support the increased expression of ASIC1 and 3 occurring at the neuronal level paralleled the increases in the esophageal mucosa.

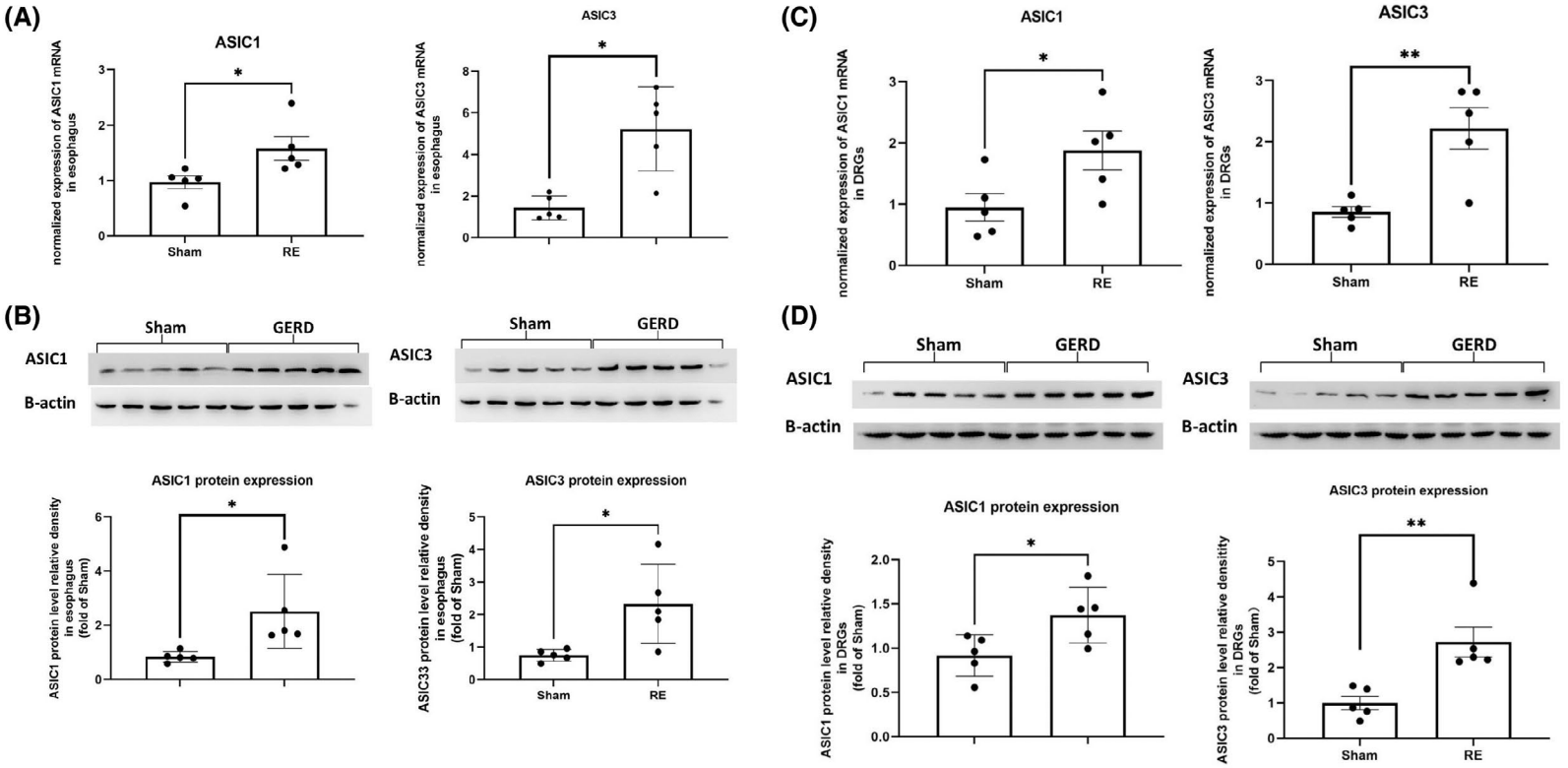

FIGURE 3 Upregulation of acid sensing ion channel 1&3 expression in the esophagus and DRG after gastric ligation. (A) Expression of ASIC transcripts in esophagus in rats detected by RT-PCR. Summary of quantitative PCR results to compare relative expression of ASIC1&3 genes in RE model and sham group. Bars for each gene assay represent the mean relative value normalized to GAPDH level from the same tissue. Gene expression was expressed as fold of sham group. Data in histograms represent means ± SE; $n = 5$ in each group (*$p < .05$). (B) Western blot assay was employed to examine esophageal ASIC 1&3 protein levels in RE model and sham group. Representative Western blot indicated the detection of ASIC1&3 in the esophagus. β-Actin control levels are shown below the corresponding ASIC1&3 blot. Protein expression of ASIC1 and 3 was expressed as fold of sham group. Bar represents the mean relative value normalized to β-actin. Data in histograms represents mean ± SE; $n = 5$ in each group (*$p < .05$). (C) qPCR analysis of ASIC 1 and 3 gene expressions in rat dorsal root ganglion (DRG) neurons 2 weeks following gastric ligation. Summary of quantitative PCR results to compare relative ASIC 1&3 gene expressions in the RE model and sham group. Bars for each gene assay represent the mean relative value normalized to GAPDH level from the same tissue. Gene expression was expressed as fold of sham group. Data in histograms represent mean ± SE, $n = 5$ in each group (*$p < .05$; **$p < .01$). (D) Western blot assay was employed to examine ASIC1&3 protein expression in T3–T5 DRG at day 15 following gastric ligation. Representative bands of ASIC1 and ASIC3 expression. Bands of β-actin are used as control for an equal protein loading. Bars represent the mean relative value normalized to β-actin. Data in histograms represent mean ± SE; $n = 5$ in each group. *$p < .05$; **$p < .001$ for RE group compared with sham group

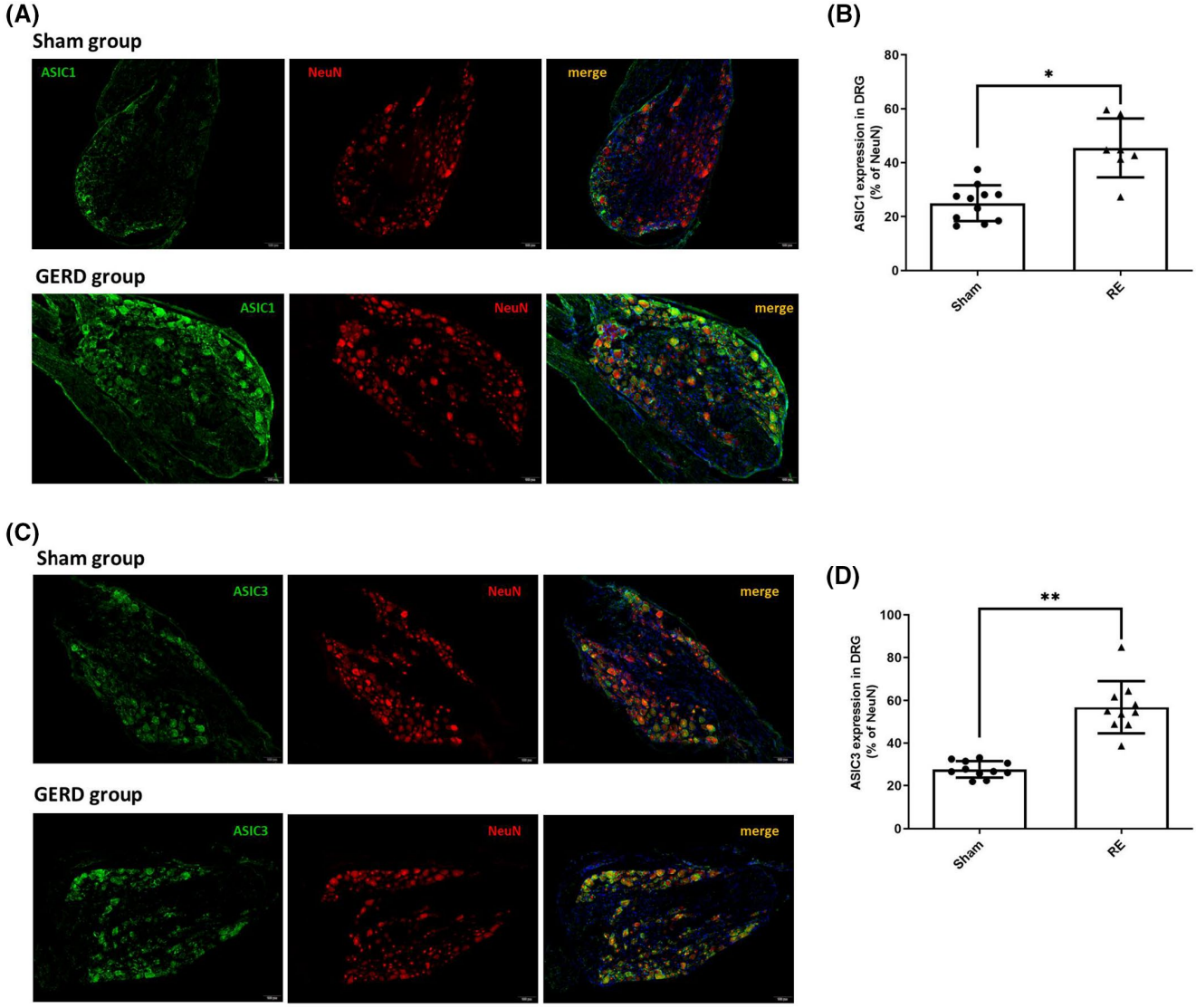

FIGURE 4 ASIC1&3 expression is upregulated in DRG neurons innervating the esophagus in RE. (A) Analysis of ASIC1 expression in dorsal root ganglion (DRG) neurons innervating the esophagus (T3–T5). Colocalization of ASIC1 (*green*) and NeuN (*red*) in the T3–T5 in both RE model ($n = 5$) and sham group ($n = 5$). Double staining of ASIC1 with NeuN, as a biomarker for neurons, showed colocalization in DRG. Merged image shows ASIC1-positive neurons expressing or not expressing NeuN. (B) Histograms represent the percentage of the total population of ASIC1-positive neurons colocalized with NeuN in sham-operated and RE model (*$p < .05$). (C) Representative images show the distribution of ASIC3-positive and NeuN-immunoreactive neurons in the DRG. Double immunocytochemical labeling was performed on the T3–T5 DRG removed from surgically-induced RE rats and sham-operated. Merged image shows ASIC3-positive neurons expressing or not expressing NeuN. (D) The histograms represent the percentage of ASIC3-positive neurons expressing NeuN in the RE and sham groups (**$p < .001$)

TABLE 4 Increased gene expression of ASIC1 and 3 in DRG neurons correlates with ASIC1 and 3 expression from esophageal mucosa in a rat model of RE

	ASIC1 gene expression		ASIC3 gene expression	
	DRG neurons	Esophageal epithelial cells	DRG neurons	Esophageal epithelial cells
Pearson correlation coefficient (r)	1	.805	1	.852
N	10	10	10	10
Significance (2-tailed)		.005		.002

TABLE 5 Increased protein expression of ASIC1 and 3 in DRG neurons correlates with ASIC1 and 3 expression from esophageal mucosa in a rat model of NERD

	ASIC1 protein expression		ASIC3 protein expression	
	DRG neurons	Esophageal epithelial cells	DRG neurons	Esophageal epithelial cells
Pearson correlation coefficient (*r*)	1	.801	1	.719
N	10	10	10	10
Significance (2-tailed)		.005		.019

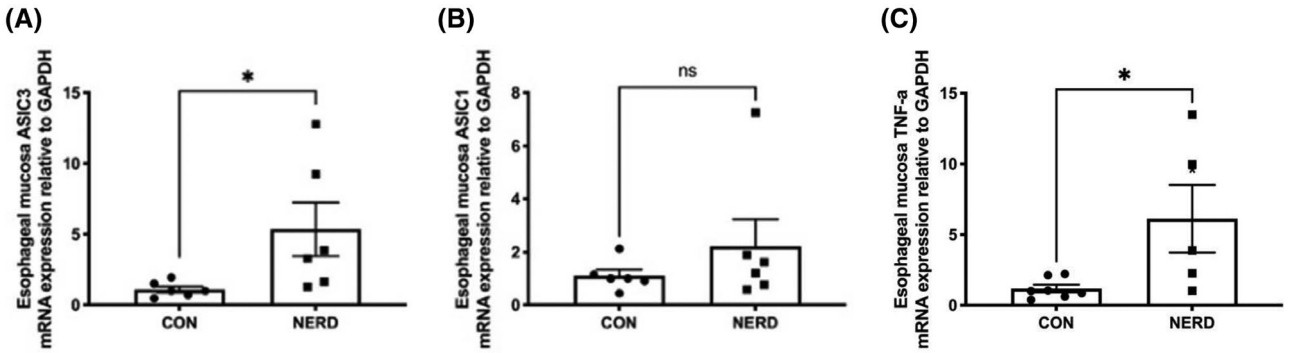

FIGURE 5 ASIC3, ASIC1 and TNF-α gene expression levels in esophageal mucosa of control and NERD rodents. (A) Comparison of esophageal mucosa ASIC3 mRNA expression level relative to GAPDH between control and NERD rodents. (B) Comparison of esophageal mucosa ASIC1 mRNA expression level relative to GAPDH between control and NERD rodents. (C) Comparison of esophageal mucosa TNF-α mRNA expression level relative to GAPDH between control and NERD rodents. $n = 6$ to 8/group. Data were expressed by mean ± SEM. *$p < .05$ for NERD compared with control group. CON, control; NERD, nonerosive reflux disease

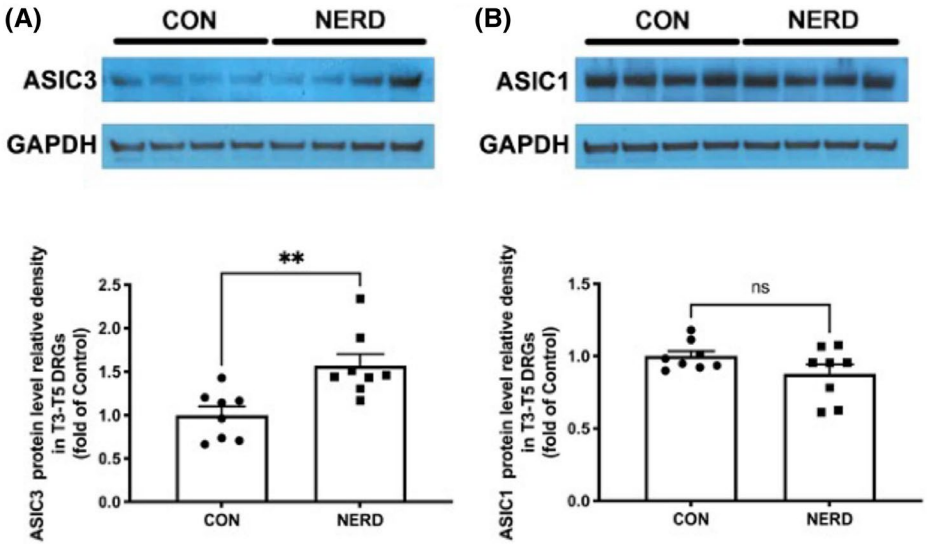

FIGURE 6 ASIC3 and ASIC1 protein expression levels in DRG neurons of control and NERD rodents. (A) Comparison of T3–T5 DRG neurons ASIC3 protein expression level measured by western blot between control and NERD rodents. (B) Comparison of T3–T5 DRG neurons ASIC1 protein expression level measured by western blot between control and NERD rodents. $n = 6$ to 8/group. Data were expressed by mean ± SEM. *$p < .05$ for NERD compared with control group. CON, control; NERD, nonerosive reflux disease

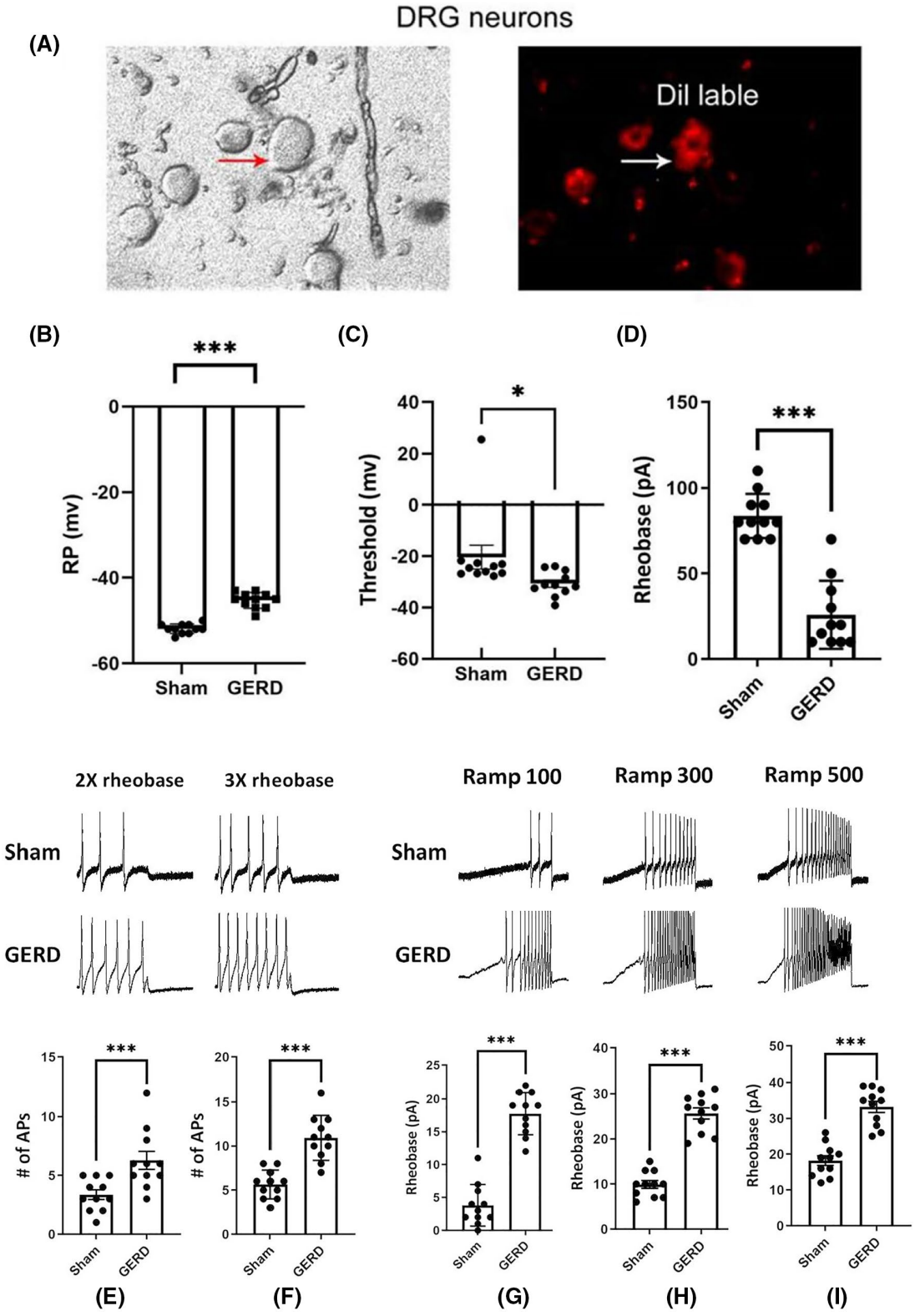

FIGURE 7 Enhanced excitability of dorsal root ganglion neurons in RE model. (A) DiI-fluorescence (right) and bright-field (left) images of acutely isolated DRG neurons innervating the esophagus are shown in red in DiI-fluorescence image. (B) Resting membrane potentials (RPs) in DRG in sham-operated ($n = 5$) and surgically induced RE rats ($n = 5$) (***$p < .0001$). (C) DRGs in surgical RE group showed a markedly decreased action potential (AP) threshold compared with sham group (*$p < .05$). (D) DRGs in RE model displayed a significantly reduced rheobase compared with sham group (***$p < .0001$). Typical traces of APs evoked by 2 times (E) and 3 times (F) rheobase current stimulation (**$p < .01$; ***$p < .0001$ for RE compared with sham group). Typical traces of APs evoked by 100 pA (G), 300 pA (H) and 500 pA (I) ramp current stimulation (***$p < .0001$ for RE compared with sham group)

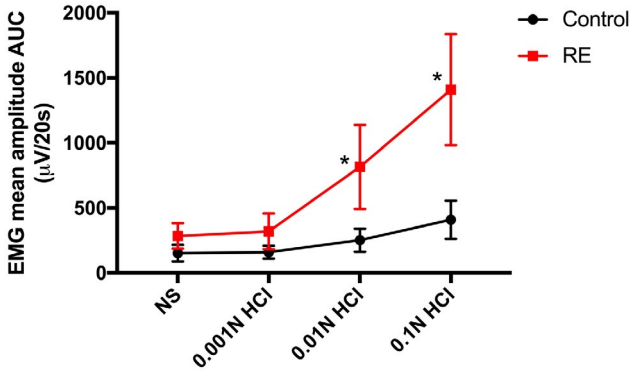

FIGURE 8 Visceral hypersensitivity induced by acid perfusion in RE rodents. Different concentrations of 0.2 ml HCl solutions (0.001, 0.01 and 0.1 N) or normal saline (NS) were injected into the lower esophagus of the rats and maintained for 1 min. Four flushes of 0.2 ml warm saline were used to rinse residual acid in the esophagus and 30 min were allowed to elapse between each injection. The EMG activities from trapezius during perfusion were recorded. $n = 3$ to 4 rats/group. Data was compared by EMG mean amplitude AUC in 20 s and expressed by mean ± SEM. *$p < .05$ for RE group compared with control group at each concentration of HCl. RE, reflux esophagitis

3.6 | Upregulation of ASIC 3 expression in esophageal mucosa and DRGs in NERD model

In the NERD rodent group, gene expression of ASIC3 in the esophageal mucosa was significantly higher compared to the control group (5.36 ± 1.89 vs. 1.11 ± 0.22, $p < .05$, Figure 5A). The gene expression of ASIC1 in the NERD group also showed an increase although it failed to reach statistical significance (2.22 ± 1.03 vs. 1.11 ± 0.23, Figure 5B). We also measured TNFα gene expression in the esophageal mucosa. Compared with the control group, TNFα gene expression in the NERD group was significantly elevated (6.13 ± 2.40 vs. 1.18 ± 0.27, $p < .05$, Figure 5C).

Similar to the esophageal mucosa, there was upregulation in the protein expression of ASIC3 in the DRG neurons in T3–T5 (1.57 ± 0.13 vs. 1.00 ± 0.10, $p < .05$; Figure 6A). However, we did not detect any significant change in ASIC1 protein expression in this group of DRG neurons (Figure 6B). Furthermore, we did not find any difference in ASIC2, 4, or 5 mRNA expression in the esophageal mucosa or T3–T5 DRG neurons between NERD and control groups (Supporting Information Figure S3).

3.7 | Increased excitability of DRG neurons in RE rodent model

To investigate the potential role of ASIC1 and 3 in the activation of peripheral nociceptors, electrophysiological recordings were performed on DRG neurons. Fluorescent dye DiI-labeled lower esophageal submucosal-specific DRG (including T3–T5 DRGs) neurons were recorded (Figure 7A). The passive and active membrane properties of DRG neurons in sham and RE groups were measured. Under whole-cell current clamp recordings, the resting membrane potentials (RPs) in sham and RE groups were −51.9 ± 0.3 and −45.1 ± 0.5 mV ($p < .0001$), respectively (Figure 7B).

We next compared the provoked excitability of DRG neurons. The action potential (AP) threshold, the minimal voltage at which the AP was generated, was markedly decreased in the RE group compared to sham group (−30.7 ± 1.5 mV vs. −20.4 ± 4.6 mV, $n = 9$, $p = .0032$) (Figure 7C). Rheobase, the minimal stimulation current required to evoke APs, was significantly decreased in the RE group compared with sham group (22.8 ± 4.8 pA vs. 82.2 ± 3.2 pA, $n = 9$, $p < .0001$) (Figure 7D). In addition, the number of APs evoked by 300-ms 2 times (2×) and 3 times (3×) rheobase current stimulation was significantly higher in the RE group compared with sham group (2×, 6 ± 0.9 vs. 3.2 ± 0.5, $n = 9$, $p = .0132$; 3×, 10.8 ± 0.9 vs. 5.6 ± 0.6, $n = 9$, $p = .0002$) (Figure 7E,F). The number of APs evoked by 100, 300 and 500 pA current ramps was significantly increased in the RE group compared with sham group (100 pA ramp, 17.7 ± 1.0 vs. 3.8 ± 1.0, $n = 9$, $p < .0001$; 300 pA ramp, 25.7 ± 1.2 vs. 9.9 ± 0.9, $p < .001$; 500 pA ramp, 33.2 ± 1.5 vs. 18.2 ± 1.4, $p < .0001$) (Figure 7G–I). These findings indicate that RE results in hyperexcitability of esophagus-specific DRG neurons.

3.8 | Visceral hypersensitivity induced by chronic acid reflux is mediated by upregulation of ASIC1 and 3 in RE model

Both control and RE rats showed pH-dependent increases in the VMR to acid perfusion (Figure 8). These responses were significantly enhanced in RE rats. The mean amplitude of the electromyogram (AUC in microvolts per 20 s) was 3.24-fold ($p < .05$) and 3.44-fold ($p = .05$) higher in the RE rats than the control group in response to perfusion of 0.01 N and 0.1 NHCl respectively (Figure 8). These findings provide evidence of visceral hypersensitivity in RE rats.

We next examined whether RE rats showed visceral hypersensitivity to pressure distension and determined if this was mediated by upregulation of ASIC. While both control and RE rats showed pressure dependent increases in VMR to ED these responses were significantly enhanced in RE rats (Figure 9A) indicating development of visceral hypersensitivity to mechanical stimulation. We then determined if ASIC1 and ASIC3 contribute to the transmission of the

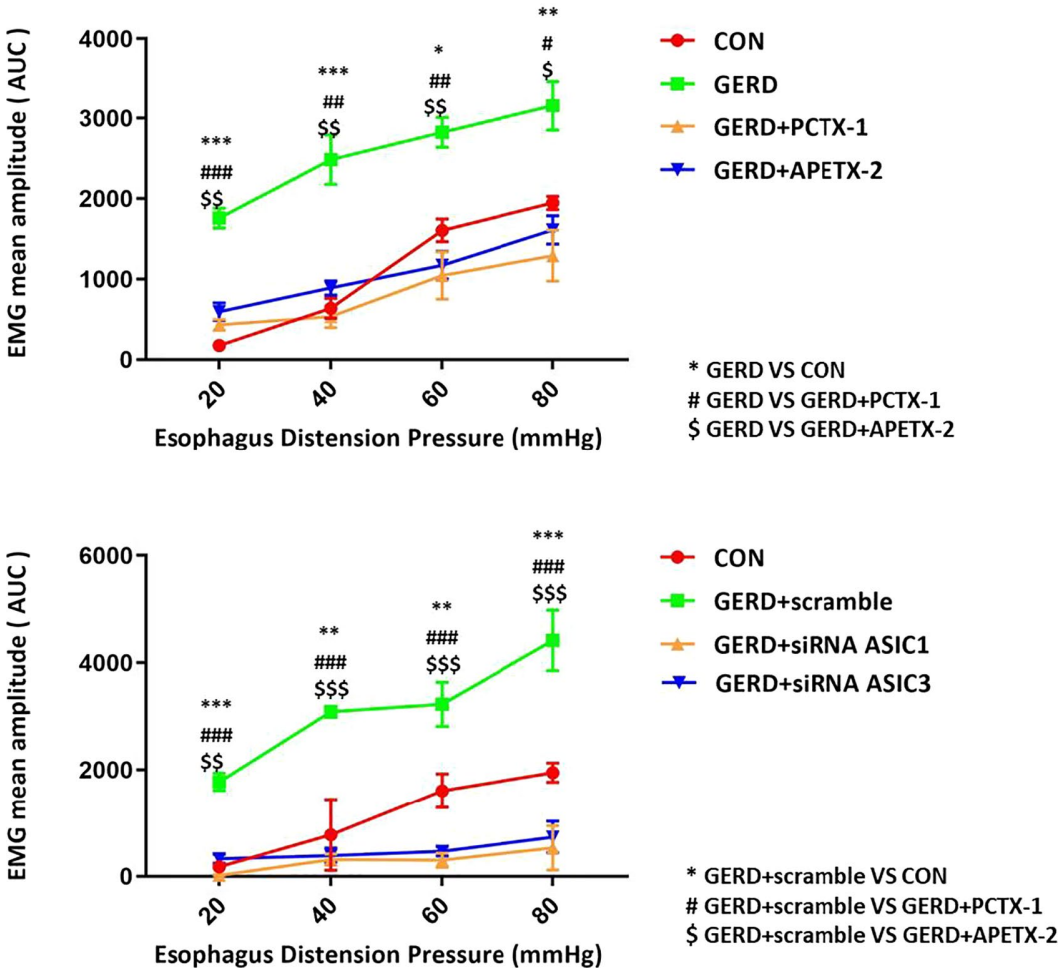

FIGURE 9 Visceral hypersensitivity induced by chronic acid reflux is reversed by inhibiting or silencing ASIC1 and 3. Mean amplitude of abdominal muscle contractions are expressed as area under the curve (AUC) after baseline subtraction ($n = 5$ in each group). (A) Effect of intrathecal injection of selective ASIC1 blocker PcTx1 or ASIC3 blocker APETx2 on pain behavior in response to esophageal distension in the RE rodent model. Intrathecal injection of selective ASIC1 blocker PcTx1 or ASIC3 blocker APETx2 normalized pain response to esophageal distension in RE rodent model. *$p < .05$; **$p < .01$; ***$p < .0001$ for RE compared with controls (CON); $^{\#}p < .05$; $^{\#\#}p < .01$; $^{\#\#\#}p < .001$ for RE compared with RE and PcTx1; while $^{\$}p < .05$; $^{\$\$}p < .01$; $^{\$\$\$}p < .001$ for RE compared with RE and APETx2 at each esophageal distention pressure. (B) Effect of siRNAs (ASIC1si, ASIC3si and scramble) on behavior pain measured as EMG response. Silencing ASIC1 or ASIC3 by specific siRNAs but not scramble siRNA prevented the development of visceral hyperalgesia in response to chronic acid reflux. *$p < .05$; **$p < .01$; ***$p < .0001$ for RE and scramble compared with controls (CON); $^{\#}p < .05$; $^{\#\#}p < .01$; $^{\#\#\#}p < .001$ for RE and scramble compared with RE and PcTx1; while $^{\$}p < .05$; $^{\$\$}p < .01$; $^{\$\$\$}p < .001$ for RE and scramble compared with RE and APETx2 at each esophageal distention pressure. CON, control group; EMG, electromyographic activity

visceral nociceptive message within the spinal cord in RE rats by administering selective ASIC1a blocker PcTx1 and ASIC3 blocker APETx2 intrathecally and then challenging with esophageal distention from 0 to 80 mmHg. PcTx1 injection significantly increased behavioral pain thresholds from 20 ± 6 mmHg ($p < .05$) to 80 ± 3 mmHg ($p < .05$) in the RE model (Figure 9A). Similar to PcTx1, intrathecal injection of APET2 also reversed the mechanical hyperalgesia in the RE group (Figure 9A). To confirm these observations, we showed that in vivo knockdown of ASIC1 and 3 gene expressions in the RE rodent group through intrathecal injection of specific siRNA targeting ASIC1 and 3 mRNA caused a significant decrease in pain perception in response to ED compared to rats treated with scramble siRNA (Figure 9B).

3.9 | Visceral hypersensitivity is mediated by upregulation of ASIC 3 in NERD model

Rats in the NERD group also developed signs of visceral hypersensitivity. While both control and NERD rats showed pressure dependent increases in the VMR to ED,

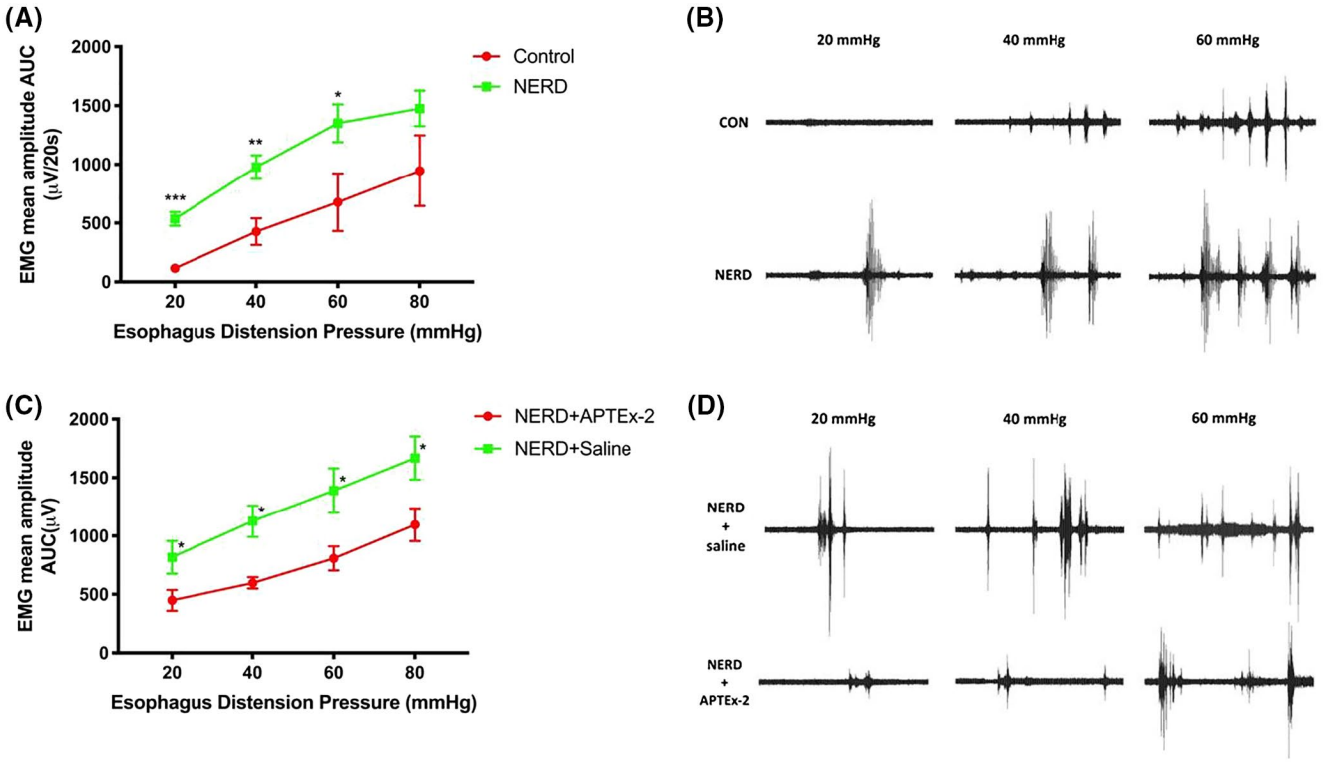

FIGURE 10 Visceral hypersensitivity induced by esophageal distension regulated by ASIC3. (A) EMG activities from trapezius after esophageal distension (20, 40, 60, 80 mmHg) in control and NERD rodents. (B) Representative images of EMG activities from trapezius after esophageal distension (20, 40, 60 mmHg) in control and NERD rodents. (C) EMG activities from trapezius after esophageal distension (20, 40, 60, 80 mmHg) in NERD rodents with or without ASIC3 inhibitor administration. APTEx-2 (1 nmol/rat), an ASIC3 inhibitor, was injected intrathecally (T3–T5) into the NERD rodents 15 min before the esophageal distension. (D) Representative images of EMG activities from trapezius after esophageal distension (20, 40, 60 mmHg) in NERD rodents with or without ASIC3 inhibitor administration. $n = 3$ to 9 rats/group. Data was compared by EMG mean amplitude AUC relative to self-baseline in 20s and expressed by mean ± SEM. *$p < .05$; **$p < .01$; ***$p < .001$ for NERD compared with control group at each esophageal distension pressure. CON, control; NERD, nonerosive reflux disease

these responses were significantly enhanced in NERD rats (Figure 10A,B). The mean amplitude of the EMG (AUC in microvolts per 20 s) was 1.75-fold ($p < .001$), 1.55-fold ($p < .01$) and 1.45-fold ($p < .05$) higher than the control group in response to 20-, 40-, and 60-mmHg distension in the esophagus, respectively. These changes were prevented by intrathecal injection of APETx-2, an ASIC3 inhibitor ($p < .05$) (Figure 10C,D) indicating visceral hypersensitivity in the NERD group was mediated by upregulation of ASIC3.

4 | DISCUSSION

GERD is the most common outpatient diagnosis leading to gastroenterology consultation and its prevalence is increasing worldwide.[32] PPIs remain the mainstay of treatment for GERD but there is a growing number of subjects who are either partial or complete non-responders to PPI therapy. Given the disease burden as well as the health care related costs of this patient population, understanding the mechanisms by which subjects continue to have symptoms despite adequate acid suppression remains a vital and important mission. One important factor that has been consistently demonstrated in prior studies is the development of visceral hypersensitivity in GERD.[33–35] This likely involves both peripheral and central sensitization but the neurophysiological basis driving visceral hypersensitivity remains unclear.

Several pain-causing stimuli, such as inflammation, ischemia, or tumorigenesis, are associated with tissue acidosis.[36] This hints at the possibility of pH-sensitive receptors on nociceptive neurons. However, the molecular mechanisms by which sensory neurons detect changes in extracellular pH were unknown until recently when ASICs were discovered.[37] ASICs are particularly sensitive to extracellular acidification and are expressed by both peripheral nociceptive neurons as well as areas of the central nervous system involved in pain processing.[38] This suggests that ASICs are critical in nociception and transmission of pain signals. Among ASICs, ASIC1 and 3 are the most sensitive to protons and are activated by small fluctuations in pH.[39] These ASIC receptors also appear

to be sensitive to mechanical pressure as we showed that in both the RE and NERD model rats, the visceral hypersensitivity to pressure distension could be blocked by ASIC inhibitors or intrathecal silencing of the ASIC 1 and 3 gene expressions.

The contribution of ASICs to pain perception in GERD, an acid-related disorder, is tantalizing but still unknown. Animal models using ASIC3 knockout mice have shown altered acid-evoked pain responses.[12,13] There is upregulation of ASIC1a in DRG neurons as well as ASIC1 and 2 in spinal cord in a rodent model of IBS.[24,40] However, this is the first study to demonstrate that expression of ASIC1 and 3 are upregulated in GERD. Furthermore, ASIC1 and 3 expressions correlated positively with the severity of symptoms in patients with GERD. Although the increased expression of ASIC1 and 3 from esophageal mucosal biopsies in our human studies is most likely reflecting ASIC expression in epithelial cells, it was not feasible to measure ASIC expression in DRG neurons in patients. We subsequently demonstrated that RE and NERD groups in rodents have higher expression of ASIC- and NeuN-expressing sensory neurons innervating the esophagus compared with control rats. While we do not know whether all NeuN positive neurons were projecting to the esophagus, we further observed that ASIC1 and 3 expressions in DRG neurons strongly correlated with ASIC expression in esophageal epithelial cells in our rodent models of GERD. A prior study by Akiba et al. employing immunofluorescence demonstrated that ASIC1-3 were expressed in the esophageal epithelium, muscularis mucosa, as well as in the DRGs.[31] These findings suggest that the increased expression of ASICs in the esophageal mucosa may be used as a surrogate to reflect the increased expression of ASICs in the DRG neurons. As such, upregulation of ASIC expression in the esophageal mucosa may be a relevant marker of peripheral sensitization and visceral hypersensitivity in GERD.

It is of interest to note that ASIC3 was upregulated in patients with Crohn's disease.[15] We demonstrated that expression of a proinflammatory cytokine, TNF-α, in esophageal mucosa was increased in animal models of RE and NERD. This was associated with the increased expression of ASIC1 and 3 in esophageal-specific DRGs. Prior studies have demonstrated that pro-inflammatory mediators, such as nerve growth factor and serotonin, lead to the increased expression of ASIC1a, ASIC2b, and ASIC3 in the DRGs.[41,42] While these data suggest that inflammation from GERD results in increased ASIC1 and 3 expressions in sensory neurons which may regulate central pain pathways leading to central sensitization, further confirmatory studies detailing the effect of TNF inhibition and ASIC expression are required.

We then performed whole cell patch clamp studies which showed reduced rheobase, markedly decreased action potential threshold, and increased number of action potentials in esophageal-specific DRG neurons obtained from a RE rodent model. The esophagus, similar to other visceral organs, receives dual sensory innervation from both vagal and spinal neurons.[43] Vagal afferent neurons have cell bodies in the nodose ganglia and likely are sensitive to mechanical distention but do not play a direct role in nociception.[44] In contrast, spinal afferent neurons with cell bodies located in the DRG are essential in transmission of nociception.[45] A rodent study using whole-cell voltage-clamp recordings from DRG and nodose ganglion neurons innervating the stomach noted that acid-elicited currents were at least partly gated by ASICs.[46] In addition, prior studies have demonstrated that hyperexcitability of spinal afferent neurons likely mediate visceral hypersensitivity in GERD.[33,35] However, the mechanisms leading to neuronal hyperexcitability remain unclear. Our model suggests that inflammation from GERD leads to upregulation of ASIC1 and 3 expression both in the esophagus as well as in esophageal-specific DRG neurons. This is associated with hyperexcitability of DRG neurons involved in esophageal nociception. These findings may explain both peripheral and central sensitization of pain pathways leading to esophageal hypersensitivity which is the most consistent finding in GERD.[47]

To confirm the possibility that upregulation of ASIC1 and 3 contributes to enhanced pain perception in GERD, we examined the effects of specific inhibitors of ASIC1 and 3, PcTx1 and APETx2, on esophageal hyperalgesia. In our rodent RE model, visceral hypersensitivity was observed 15 days following induction of acid reflux. This was accompanied by upregulation of ASIC1 and 3 in the esophageal mucosa and DRG neurons innervating the esophagus. Intrathecal administration of inhibitors or specific siRNA of ASIC1 and 3 prevented the development of visceral hypersensitivity and normalized the VMR to esophageal acid perfusion as well as ED. Together, these data show that peripheral inactivation of ASIC1 and 3 produces significant analgesia in a rat model of RE, and therefore support the involvement of ASIC1 and 3-containing channels in visceral hypersensitivity in GERD.

NERD represents the common phenotype seen in GERD. Indeed, most of the community-based GERD patients appear to have NERD,[48] but is less responsive to PPI therapy compared to erosive esophagitis. Interestingly, although NERD shows less mucosal injury induced by acid reflux, these patients appear to be less responsive to proton pump inhibitors as compared with patients with erosive esophagitis.[4] In addition, many NERD patients are more sensitive to weak acid reflux than those with erosive esophagitis,[49] suggesting the presence of visceral hypersensitivity in these patients. This is confirmed by our pain behavior studies using a rodent model of NERD.

Furthermore, we showed that this was accompanied by an increased expression of ASIC3 in the esophageal mucosa as well as T3–T5 DRG neurons innervating the esophagus. The exaggerated pain responses were prevented by pretreatment with intrathecal injection of an ASIC3 inhibitor, APETx-2, suggesting visceral hypersensitivity in NERD is mediated by upregulation of ASIC3. It is conceivable that acid exposure disrupts intercellular connections in the esophageal mucosa, producing DIS and increasing esophageal permeability, allowing refluxed acid to penetrate the submucosa and reach chemosensitive nociceptors such as ASIC3. Further studies may investigate whether acid suppression may lead to downregulation of ASIC expression and subsequent normalization of neuronal activity.

While our study demonstrates several strengths, including identification of a novel pathway for visceral hypersensitivity in GERD in both animal models and human patients, there are limitations as well. First, human studies were performed only in a Chinese population. Given the large differences in the prevalence of GERD between Asian and Western countries, there may be regional differences in GERD pathophysiology, including dietary, lifestyle, and genetics, that we were not able to demonstrate. Secondly, while we used common rodent models for RE and NERD, neither are perfect models with the RE model employing delayed gastric emptying to induce GERD while the NERD model is more consistent with chemical esophagitis. Development of animal models that more closely mirror the pathophysiology of GERD in humans is needed. Thirdly, it is not clear whether changes in ASIC expression are local or systemic phenomena in GERD. Future studies may determine whether downregulation of ASIC expression by specific inhibitors may improve outcomes in GERD.

In conclusion, this study demonstrated that symptom severity and esophageal inflammation in patients with GERD are associated with upregulation of ASIC1 and 3 expressions. Similarly, in an animal model of RE and NERD, we also showed that inflammation leads to upregulation of ASIC expression in both esophageal mucosa as well as esophageal-specific DRG neurons. Furthermore, this was associated with hyperexcitability of DRG neurons. We further demonstrated that in vivo knockdown of ASIC1 and 3 expression or intrathecal administration of ASIC inhibitors normalized pain response to esophageal distention or acid perfusion. Our data support a role for ASICs in the mediation of peripheral and central sensitization leading to visceral hypersensitivity which is a key mechanism in the pathogenesis of GERD symptoms. Identification and development of ASIC-specific antagonists may provide an effective pharmacological strategy for treating patients with GERD who are unresponsive to conventional acid suppression therapies. This is especially important in patients with NERD who are less responsive to proton pump inhibitors.

ACKNOWLEDGMENTS

DZ receives funding from the National Natural Science Foundation of China (Grant nos. 81370493 and 81670485). AL receives funding from the National Institutes of Health (NIH) 1K23 DK124567. CO receives funding from the NIH 2R01 DK058913 and P30 DK 34933. XH receives funding from the Shanghai Pujiang Program 20PJ1409700.

DISCLOSURES

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

Conception and design: Xu Han, Zhaoshen Li, Duowu Zou and Chung Owyang. *Acquisition of data*: Xu Han, Yawen Zhang, Jun Gao, Xiaoyin Wu, Jiulong Zhao, Hui Wang, Di Chen, and Duowu Zou. *Data analysis and interpretation*: Xu Han, Allen Lee, Chung Owyang, and Zhaoshen Li. *Manuscript drafting and critical revision*: Xu Han, Allen Lee, Duowu Zou and Chung Owyang.

ORCID

Chung Owyang https://orcid.org/0000-0002-1746-2370

REFERENCES

1. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R, Global Consensus Group. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol*. 2006;101:1900-1920.
2. El-Serag HB, Sweet S, Winchester CC, Dent J. Update on the epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut*. 2014;63:871-880.
3. Savarino E, Bredenoord AJ, Fox M, Pandolfino JE, Roman S, Gyawali CP, International Working Group for Disorders of Gastrointestinal Motility and Function. Expert consensus document: advances in the physiological assessment and diagnosis of GERD. *Nat Rev Gastroenterol Hepatol*. 2017;14:665-676.
4. Dean BB, Gano AD, Knight K, Ofman JJ, Fass R. Effectiveness of proton pump inhibitors in nonerosive reflux disease. *Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc*. 2004;2:656-664.
5. Khan M, Santana J, Donnellan C, Preston C, Moayyedi P. Medical treatments in the short term management of reflux oesophagitis. *Cochrane Database Syst Rev*. 2007;2:CD003244.
6. Cicala M, Emerenziani S, Guarino MPL, Ribolsi M. Proton pump inhibitor resistance, the real challenge in gastro-esophageal reflux disease. *World J Gastroenterol*. 2013;19:6529-6535.
7. Hussain ZH, Henderson EE, Maradey-Romerao C, George N, Fass R, Lacy BE. The proton pump inhibitor non-responder: a clinical conundrum. *Clin Transl Gastroenterol*. 2015;6:e106.

8. El-Serag H, Becher A, Jones R. Systematic review: persistent reflux symptoms on proton pump inhibitor therapy in primary care and community studies. *Aliment Pharmacol Ther*. 2010;32:720-737.
9. Rodriguez-Stanley S, Robinson M, Earnest DL, Greenwood-Van Meerveld B, Miner PB. Esophageal hypersensitivity may be a major cause of heartburn. *Am J Gastroenterol*. 1999;94:628-631.
10. Knowles CH, Aziz Q. Visceral hypersensitivity in non-erosive reflux disease. *Gut*. 2008;57:674-683.
11. Holzer P. Taste receptors in the gastrointestinal tract. V. Acid sensing in the gastrointestinal tract. *Am J Physiol - Gastrointest Liver Physiol*. 2007;292:G699-G705.
12. Price MP, McIlwrath SL, Xie J, et al. The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. *Neuron*. 2001;32:1071-1083.
13. Chen C-C, Zimmer A, Sun W-H, Hall J, Brownstein MJ, Zimmer A. A role for ASIC3 in the modulation of high-intensity pain stimuli. *Proc Natl Acad Sci*. 2002;99:8992-8997.
14. Page AJ, Brierley SM, Martin CM, et al. Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. *Gut*. 2005;54:1408-1415.
15. Yiangou Y, Facer P, Smith JA, et al. Increased acid-sensing ion channel ASIC-3 in inflamed human intestine. *Eur J Gastroenterol Hepatol*. 2001;13:891-896.
16. Jones R, Junghard O, Dent J, et al. Development of the GerdQ, a tool for the diagnosis and management of gastro-oesophageal reflux disease in primary care. *Aliment Pharmacol Ther*. 2009;30:1030-1038.
17. Lundell LR, Dent J, Bennett JR, et al. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut*. 1999;45:172-180.
18. Omura N, Kashiwagi H, Chen G, Suzuki Y, Yano F, Aoki T. Establishment of surgically induced chronic acid reflux esophagitis in rats. *Scand J Gastroenterol*. 1999;34:948-953.
19. Zayachkivska O, Havryluk O, Hrycevych N, Bula N, Grushka O, Wallace JL. Cytoprotective effects of hydrogen sulfide in novel rat models of non-erosive esophagitis. *PLoS One*. 2014;9:e110688.
20. Yerian L, Fiocca R, Mastracci L, et al. Refinement and reproducibility of histologic criteria for the assessment of microscopic lesions in patients with gastroesophageal reflux disease: the Esohisto project. *Dig Dis Sci*. 2011;56:2656-2665.
21. Triantos C, Koukias N, Karamanolis G, Thomopoulos K. Changes in the esophageal mucosa of patients with non erosive reflux disease: how far have we gone? *World J Gastroenterol*. 2015;21:5762-5767.
22. Wang Y, Li G, Wang X, Zhu S. Effects of Shugan Hewei granule on depressive behavior and protein expression related to visceral sensitivity in a rat model of nonerosive reflux disease. *Evid-Based Complement Altern Med*. 2019;2019:1505693.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods San Diego Calif*. 2001;25:402-408.
24. Matricon J, Gelot A, Etienne M, Lazdunski M, Muller E, Ardid D. Spinal cord plasticity and acid-sensing ion channels involvement in a rodent model of irritable bowel syndrome. *Eur J Pain Lond Engl*. 2011;15:335-343.
25. Mazzuca M, Heurteaux C, Alloui A, et al. A tarantula peptide against pain via ASIC1a channels and opioid mechanisms. *Nat Neurosci*. 2007;10:943-945.
26. Mestre C, Pélissier T, Fialip J, Wilcox G, Eschalier A. A method to perform direct transcutaneous intrathecal injection in rats. *J Pharmacol Toxicol Methods*. 1994;32:197-200.
27. Qin C, Farber JP, Foreman RD. Intraesophageal chemicals enhance responsiveness of upper thoracic spinal neurons to mechanical stimulation of esophagus in rats. *Am J Physiol Gastrointest Liver Physiol*. 2008;294:G708-G716.
28. Hu WH, Martin CJ, Talley NJ. Intraesophageal acid perfusion sensitizes the esophagus to mechanical distension: a Barostat study. *Am J Gastroenterol*. 2000;95:2189-2194.
29. Mashimo H, Goyal RK. Physiology of esophageal motility. *GI Motil Online*. 2006. doi:10.1038/gimo3
30. Wei S, Qiu C-Y, Jin Y, Liu T-T, Hu W-P. TNF-α acutely enhances acid-sensing ion channel currents in rat dorsal root ganglion neurons via a p38 MAPK pathway. *J Neuroinflammation*. 2021;18:92.
31. Akiba Y, Mizumori M, Kuo M, et al. CO2 chemosensing in rat oesophagus. *Gut*. 2008;57:1654-1664.
32. Peery AF, Dellon ES, Lund J, et al. Burden of gastrointestinal disease in the United States: 2012 update. *Gastroenterology*. 2012;143:1179-1187.e1-3.
33. Hobson AR, Furlong PL, Aziz Q. Oesophageal afferent pathway sensitivity in non-erosive reflux disease. *Neurogastroenterol Motil*. 2008;20:877-883.
34. Sarkar S, Aziz Q, Woolf CJ, Hobson AR, Thompson DG. Contribution of central sensitisation to the development of non-cardiac chest pain. *Lancet Lond Engl*. 2000;356:1154-1159.
35. Sarkar S, Hobson AR, Furlong PL, Woolf CJ, Thompson DG, Aziz Q. Central neural mechanisms mediating human visceral hypersensitivity. *Am J Physiol - Gastrointest Liver Physiol*. 2001;281:G1196-G1202.
36. Reeh PW, Steen KH. Tissue acidosis in nociception and pain. *Prog Brain Res*. 1996;113:143-151.
37. Wemmie JA, Taugher RJ, Kreple CJ. Acid-sensing ion channels in pain and disease. *Nat Rev Neurosci*. 2013;14:461-471.
38. Deval E, Gasull X, Noël J, et al. Acid-Sensing Ion Channels (ASICs): pharmacology and implication in pain. *Pharmacol Ther*. 2010;128:549-558.
39. Yagi J, Wenk HN, Naves LA, McCleskey EW. Sustained currents through ASIC3 ion channels at the modest pH changes that occur during myocardial ischemia. *Circ Res*. 2006;99:501-509.
40. Matricon J, Muller E, Accarie A, et al. Peripheral contribution of NGF and ASIC1a to colonic hypersensitivity in a rat model of irritable bowel syndrome. *Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc*. 2013;25:e740-e754.
41. Wu L-J, Duan B, Mei Y-D, et al. Characterization of acid-sensing ion channels in dorsal Horn neurons of rat spinal cord. *J Biol Chem*. 2004;279:43716-43724.
42. Mamet J, Baron A, Lazdunski M, Voilley N. Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. *J Neurosci Off J Soc Neurosci*. 2002;22:10662-10670.
43. Goyal RK, Hirano I. The enteric nervous system. *N Engl J Med*. 1996;334:1106-1115.
44. Fass R, Tougas G. Functional heartburn: the stimulus, the pain, and the brain. *Gut*. 2002;51:885-892.

45. Mayer EA, Gebhart GF. Basic and clinical aspects of visceral hyperalgesia. *Gastroenterology*. 1994;107:271-293.
46. Sugiura T, Dang K, Lamb K, Bielefeldt K, Gebhart GF. Acid-sensing properties in rat gastric sensory neurons from normal and ulcerated stomach. *J Neurosci*. 2005;25:2617-2627.
47. Richter JE, Barish CF, Castell DO. Abnormal sensory perception in patients with esophageal chest pain. *Gastroenterology*. 1986;91:845-852.
48. Lind T, Havelund T, Carlsson R, et al. Heartburn without oesophagitis: efficacy of omeprazole therapy and features determining therapeutic response. *Scand J Gastroenterol*. 1997;32:974-979.
49. Emerenziani S, Sifrim D, Habib FI, et al. Presence of gas in the refluxate enhances reflux perception in non-erosive patients with physiological acid exposure of the oesophagus. *Gut*. 2008;57:443-447.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Han X, Zhang Y, Lee A, et al. Upregulation of acid sensing ion channels is associated with esophageal hypersensitivity in GERD. *FASEB J*. 2022;36:e22083. doi:10.1096/fj.202100606R