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CORRESPONDENCE

Functional Upregulation of TRPM3 Channels Contributes to Acute Pancreatitis-associated Pain and Inflammation

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Abstract

Transient receptor potential melastatin M3 (TRPM3) channels have been recognized as a pain transducer in dorsal root ganglion (DRG) neurons in recent years. TRPM3 activation initiates neurogenic inflammation and is required for the development of inflammatory hyperalgesia. We aimed to evaluate the role of TRPM3 in pancreas sensory afferents in pancreatic nociception, neurogenic inflammation, and acute pancreatitis (AP)-associated pain. AP was induced by intraperitoneal (i.p.) injection of L-arginine in rats. TRPM3 expression in pancreatic DRG neurons, spontaneous or mechanical-stimulation-evoked pain behaviors, and the extent of inflammation were evaluated. We found that TRPM3 channels were expressed on pancreatic primary afferent nerve terminals containing calcitonin gene-related peptide (CGRP). Activation of TRPM3 in the pancreas by injection of its specific agonist CIM0216 (10 µM) induced pain, CGRP and substance P release, and neurogenic inflammation, as evidenced by edema, plasma extravasation, and inflammatory cell accumulation in the pancreas. Increased TRPM3 functional expression was detected in pancreatic DRG neurons from AP rats, and blocking TRPM3 activity with its antagonist (Primidone, 5 mg/kg, i.p.) attenuated AP-associated pain behaviors and pancreatic inflammation. Pre-incubation of pancreatic DRG neurons with nerve growth factor (NGF) enhanced the increase in intracellular Ca^{2+} induced by the TRPM3 agonist (CIM0216, 1 µM). Our findings indicate that, in addition to TRPV1 and TRPA1 channels, TRPM3 is another pain channel that has a critical role in pancreatic nociception, neurogenic inflammation, and AP-associated pain behaviors. TRPM3 may be a promising pharmaceutical target for AP pain treatment.

KEY WORDS Acute pancreatitis; hyperalgesia; neurogenic inflammation; TRPM3 channel

ABBREVIATIONS

ANOVA	Analysis of variance
AP	Acute pancreatitis
BIBN	Olcegepant hydrochloride
CGRP	Calcitonin gene-related peptide
CIM	CIM0216
DRG	Dorsal root ganglion
ELISA	Enzyme-linked immunosorbent assay
H&E	Hematoxylin and eosin
NGF	Nerve growth factor
OFT	Open field test
PregS	Pregnenolone sulfate
Prim	Primidone
SEM	Standard error of the mean
SP	Substance P

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INTRODUCTION

Acute pancreatitis (AP), which is an inflammatory disorder of the pancreas, is a common cause of hospitalization for gastrointestinal diseases in the USA and many other countries. Although severe abdominal pain is the cardinal feature of AP, the underlying mechanisms of AP-associated pain are poorly understood and its treatment remains difficult [1–3]. Many studies have suggested that sensitization of pancreatic sensory afferents by inflammatory mediators contributes to the development of pain [4–6]. Activation of pancreatic sensory afferents transmit the pain signal to the central nervous system to cause painful sensation. In addition, their excitation can result in the release of substance

P (SP) and calcitonin gene-related peptide (CGRP) from the nerve terminals to induce neurogenic inflammation, which presents as vasodilation, neutrophil infiltration, and mast cell degranulation [7]. Neurogenic inflammation in turn sensitizes the sensory afferents, thus aggravating pancreatic pain [4, 5, 8].

Transient receptor potential V1 (TRPV1) and transient receptor potential ankyrin-1 (TRPA1) channels are the two important pain transducers expressed on primary sensory neurons. Activation of TRPV1 and TRPA1 channels in pancreatic sensory afferents can cause pancreatic pain, and lead to the release of neuropeptides that subsequently induce neurogenic inflammation [6, 9–11]. Upregulation of TRPV1 and TRPA1 in pancreatic sensory afferents participates in afferent sensitization and thus the hyperalgesia in AP animal models [6]. These channels are also instrumental in the transition from AP to chronic pancreatitis [12].

Transient receptor potential melastatin 3 (TRPM3) is another pain transducer that has been recognized in recent years. TRPM3 is a calcium-permeable channel prevalently expressed on nociceptive sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia from rodents and humans [13, 14]. TRPM3 could be activated by heat, endogenous neurosteroid pregnenolone sulfate (PregS), or the potent synthetic ligand 3, 4-dihydro-N-(5-methyl-3-isoxazolyl)-a-phenyl-1(2H)-quinolineacetamide (CIM0216) [13, 14]. In mice, intradermal injection of the TRPM3 agonist CIM0216 led to nociceptive behavior as well as neurogenic inflammation via inducing CGRP release from nerve endings [15]. Recent studies, including one of our investigations, found that upregulation of TRPM3 in DRG neurons contributes to spontaneous pain or heat hyperalgesia in animal models of inflammatory or neuropathic pain [16–18]. Pharmacological inhibition of TRPM3 with its antagonists or genetic deletion of TRPM3 expression in sensory neurons could alleviate chronic pain behaviors [18, 19]. Thus, TRPM3 has been suggested as a therapeutic target for inflammatory or neuropathic pain [20].

To our knowledge, no studies have examined the contribution of TRPM3 in AP-associated pain. In this study, we aimed to investigate the participation of TRPM3 in AP-associated pain and inflammation by examining: (1) TRPM3 expression in primary afferents innervating the rat pancreas and the role of TRPM3 in nociception by injecting TRPM3 agonist into the rat pancreas to acutely activate TRPM3; (2) changes in TRPM3 functional expression in pancreatic sensory afferents and its role in AP-associated pain in an L-arginine-induced AP model rat; and (3) the role of nerve growth factor (NGF), which is an important factor for AP-associated pain, in the regulation of TRPM3 function.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (2–3 months old, 220-250 g) were obtained from Pengyue Animal Company (Jinan, China). All animal studies were performed in accordance with Shandong University Animal Care and Use Committee, and this study was approved by the Research Ethics Committee of the Second Hospital of Shandong University (KYLL-2023–483).

Immunofluorescence Staining of Pancreatic Sensory Nerves

The pancreas was removed under urethane anesthesia and fixed in 4% paraformaldehyde. Paraffin sections of pancreas (6 μm) were incubated with TRPM3 antibody (1:100; ACC-050; Alomone Labs, Jerusalem, Israel) and CGRP antibody (1:100; ab81887; Abcam, Cambridge, UK) overnight at 4 °C followed by 594-conjugated Goat Anti-Mouse IgG (H + L, 1:100; ABclonal, China) and FITC Goat Anti-Rabbit IgG (H + L, 1:100; ABclonal, China) for 1 h at room temperature. To test the specificity of TRPM3 antibody, pancreas sections were pre-incubated with a synthetic blocking peptide (1:200; BLP-CC050) before adding TRPM3 antibody. The immunofluorescence staining was analyzed using Olympus BX53 positive fluorescence microscope (Olympus Corporation, Japan).

Retrograde Labeling of DRG Neuron and Neuron Isolation

To label DRG neurons innervating the pancreas, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Invitrogen, Carlsbad, CA, USA) was injected into the head and tail of the pancreas (five sites at 2 μL/site) with a 30-g needle under isoflurane anesthesia 10–14 days prior to Ca^{2+} imaging.

Isolation of DRG neurons was conducted as described in our previous study [21]. Briefly, T9–T12 DRGs were removed and digested at 37 °C for 30 min in minimum essential medium (MEM) containing collagenase 4 (2 mg/mL) and trypsin (1 mg/mL), and then the ganglia were mechanically dissociated. The disassociated neurons were plated on poly-L-lysine–coated glass coverslips and were incubated in MEM with 10% fetal bovine serum for 2–4 h before Ca^{2+} imaging. To observe nerve growth factor (NGF) effect, isolated DRG neurons were cultured for

2 h in MEM supplemented with NGF (50 ng/L, Sigma, St Louis, MO, USA) and 10% FBS.

Ca^{2+} Imaging

To examine the functional expression of TRPM3 channels, Ca^{2+} imaging was performed on isolated DRG neurons as described in our previous study [21]. Briefly, DRG neurons were incubated with Fura 2-AM (2 μM; Dojindo laboratories, Tongren, Japan) for 30 min at 37 °C. Coverslips with cells were transferred to a recording chamber (RC-26, Warner instruments, USA) and were perfused with Hank's balanced salt solution containing (HBSS, in mM): 138 NaCl, 5 KCl, 0.3 KH2PO4, 4 NaHCO3, 2 CaCl2, 1 MgCl2, 10 Hepes, and 5.6 glucose, pH 7.4. The neurons were excited at 340 and 380 nm alternatively and the fluorescence emission was detected at 510 nm using a computer-controlled monochromator. Image pairs were acquired every 1–30 s using illumination periods between 20 and 50 ms. Image acquisition was controlled using a dynamic image analysis system (MetaFluor® imaging software; Molecular Devices, San Jose, CA, USA). The agonist and antagonist were applied by a gravity driven perfusion system with the flow rate at 3–4 ml/min.

Data were analyzed using program C-Imaging (Compix). Background was subtracted to minimize camera dark noise and tissue autofluorescence. An area of interest was drawn around each cell, and the average value of all pixels included in this area was taken as one measurement. The ratio of fluorescence signal measured at 340 nm divided by the fluorescence signal measured at 380 nm was used to measure the increase of intracellular Ca^{2+}. Baseline intracellular Ca^{2+} concentration was determined from the average of five to eight measurements obtained before drug application. Amplitudes of peak Ca^{2+} responses were computed as the difference between the peak value and the baseline value. Only the cells that are responsive to KCl (30 mM) are included for analysis.

Injection of CIM0216 (CIM) into the Pancreas

To examine TRPM3 activation induced nociceptive responses in the pancreas, intra-pancreas injection of CIM0216, a specific TRPM3 agonist [15], was conducted. Under isoflurane anesthesia, the abdominal incision was made to expose the pancreas and CIM0216 (CIM, 10 μM, Tocris, CA, USA) solution was injected into the head and tail of the pancreas (30 μL total volume, six sites) with a microinjector. In two other groups of rats, a TRPM3 antagonist Primidone (Prim, 10 μM, Selleck Company, TX, USA) [22] or a CGRP receptor antagonist olcegepant hydrochloride (BIBN, 100 μM, MCE, NJ, USA) was co-injected with CIM. For vehicle control rats, the procedures are same to CIM injection, just rats received an equal volume of vehicle solution (0.1% dimethyl sulfoxide (DMSO) in normal saline) into the pancreas. The abdominal incision was then sutured and smeared with a layer of tetracaine ointment for skin pain relief. Pain behaviors were measured at 1 h after the injection, and then the pancreas was removed for histological examination, measurements of MPO activity and release of SP or CGRP.

Evaluation of CIM Injection Induced Pain Behavior

Spontaneous pain behaviors include frequent eye closing, abdomen contraction, and hypo-locomotion. Eye closing was scored based on the methods described in our previous study, with observation for 5 min [16]. The number of abdomen contractions was counted for 5 min. Hypo-locomotion was measured with the open field test (OFT) as in a previous study [6]. Briefly, rats were placed in the OFT apparatus, which consisted of a square arena 100 cm × 100 cm × 40 cm (depth). The distance traveled (meters) during 5 min was recorded using the VisuTrack system (Xinruan Corporation, China).

Pain evoked by mechanical stimulation was assessed in the abdominal area with Von Frey filament testing [23]. Briefly, rats were placed in a plastic cage with a mesh floor, Von Frey filaments (Stoelting, Wood Dale, IL, USA) of various caliber/strengths were applied to the shaved belly of the animals in ascending order 10 times each for 1–2 s with a 10-s interval between applications. A positive response consisted of lifting the belly and/or scratching and licking the abdomen.

Evaluation of CIM Injection Induced Inflammation

Pancreas histopathology scoring Hematoxylin and eosin (H&E) staining of the pancreas was conducted according to a standard protocol. Pancreas inflammation exhibits neutrophile infiltration, edema, necrosis and hemorrhage. And the severity of inflammation was graded using scoring criteria previously described by Vigna SR et al. [9]. and performed by an investigator who are blinded to the research design. The results were expressed in increments of 0.5 as a score of 0 to 3 for edema and neutrophil infiltration and as a score of 0 to 7 for tissue necrosis and hemorrhage. The total histopathology score is the mean of the combined scores for edema, neutrophil infiltration, necrosis, and hemorrhage. 5 slides were evaluated for each rat.

MPO activity MPO is a marker of inflammation associated with neutrophil infiltration [6]. Under isoflurane anesthesia, the pancreas was harvested and immediately frozen in liquid nitrogen and then stored until use. Pancreas tissues were homogenized with phosphate-buffered saline and underwent two cycles of freezing and thawing. The

homogenates were then centrifuged at 12,000 g for 5 min, and the liquid supernatant was transferred into plates. MPO activity were determined photometrically using commercial by Enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's instruction (KYY-0337R2, Research Cloud Biology, China).

Substance P and CGRP release Pancreas tissues were homogenized with phosphate-buffered saline and underwent two cycles of freezing and thawing. The homogenates were then centrifuged at 12,000 g for 5 min, and the liquid supernatant was transferred into plates. The content of CGRP and Substance P (SP) in the liquid supernatant were measured by ELISA kit specific to SP and CGRP (KYY-0490R2, KYY-0444R2, Research Cloud Biology, China).

Evans blue extravasation Increased vascular permeability in inflammation conditions was usually assessed by Evans blue dye extravasation [24]. Measurement of Evans blue extravasation was conducted as described in our previous study [16]. Briefly, Evans blue was injected intravenously (65 mg/kg) in the rats 30 min prior to removal of the pancreas. The removed pancreas was placed in formamide (3 mL) for 72 h. Subsequently, the absorbance of the solution was measured spectrophotometrically (620 nm) and the concentration of extracted Evans blue was determined.

c-Fos Expression

Increased c-Fos expression in spinal dorsal horn has been widely used as an indicator of nociceptive sensory nerve activation [25, 26]. To assess CIM0216 injection induced activation of nociceptive nerves, c-Fos expression in T9-T12 spinal dorsal horn was conducted by immunohistochemistry as described by Ceppa E, et al. [26]. Briefly, segments of spinal cord (T9–T12) were paraffin and sectioned (6 µm) in the transverse plane. Sections were incubated with c-Fos antibody (1:200, 4℃ overnight, A24620, ABclonal Technology Co.,Ltd.). Then were washed and incubated with HRP-conjugated Goat anti-Rabbit IgG (H + L) (1:200, 1 h, room temperature; AS014, ABclonal Technology Co.,Ltd.). Slides were examined by investigators blinded to the experimental purpose. Fos-stained nuclei in laminae I and II of the spinal cord were counted in three sections per spinal cord segment in each rat.

Animal Model of AP

AP was induced by the procedure described in previous studies [27]. Briefly, each rat received two intraperitoneal (i.p) injections of L-arginine (250 mg/100 g of body weight) (Sigma-Aldrich, Inc. MO, USA) as a 20% solution in 0.15 mol/L NaCl, with an interval of 1 h. Control rats received an equal volume of 0.15 mol/L NaCl alone (i.p). To evaluate the blocking effect of TRPM3 antagonist, primidone (5 mg/Kg, i.p) was administrated 30 min before L-arginine.

Evaluation of AP Associated Pain and Pancreatic Inflammation

At 24 h after the second injection of L-arginine, pain behaviors were evaluated as described in previous method description. Then, rats were killed under isoflurane anesthesia, and pancreatic tissues (fixed tissue) were taken for histopathology scoring as described in previous method description. Fresh pancreas tissue was taken for MPO, trypsin (KYY-0595R2, Research Cloud Biology, China) activity and NGF (KYY-0187R2, Research Cloud Biology, China) measurement with ELISA described in previous method description. Mixed arteriovenous blood was taken and centrifuged at 4℃ for 10 min at 2500 g for measurement of serum amylase and lipase according to the manufacturer's instructions (A054-2–1, C016-1–2, Nanjing Jiancheng Bioengineering Institute).

Western Blotting

To examine AP associated change in TRPM3 protein expression in sensory afferents, western blotting experiments were conducted as previously described [16]. Briefly, T9–T12 DRGs were collected under isoflurane anesthesia. Proteins were extracted, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; EC0022-B, Shandong Sparkjade Biotechnology Co., Ltd. China), and then transferred onto polyvinylidene fluoride membranes. After blocking with 5% skim milk, the membranes were incubated overnight at 4°C with primary antibodies against the following proteins: TRPM3 (1:200; ACC-050; Alomone Labs, Jerusalem, Israel) and GAPDH (1:10,000; 10,494–1-AP; Proteintech Group, WuHan, China). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies. Protein bands were detected using an enhanced chemiluminescence kit (Millipore; Burlington, VT). The band density was quantified using a computer-assisted imaging analysis system (ImageJ).

Statistical Analysis

Data are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired two-tailed Student's *t* test for comparisons between two groups, and one- or two-way analysis of variance (ANOVA) followed by the Holm–Sidak method for comparisons of multiple groups (GraphPad Prism version 8.4.0, SanDiego, CA, USA). For unnormal distributed data, Mann–Whitney tests were conducted. $P < 0.05$ was considered statistically significant.

RESULTS

TRPM3 Channels are Expressed on Pancreatic Sensory Afferents

TRPM3 in sensory afferents contributes to somatic and visceral pain [13, 16]. Activation of pancreatic sensory nerves contribute to the pancreas nociceptive responses and AP-associated pain [9, 26, 28]. To examine the role of sensory TRPM3 in the nociceptive response of the pancreas and AP-associated pain, its expression in pancreatic sensory nerves was examined through immunohistochemistry experiments. TRPM3 expression was examined in CGRP-containing nerves, which account for most of the sensory afferents in the rat pancreas[28]. As previously reported [28], CGRP-containing nerves were found in the pancreas (Fig. 1A, middle panel). Double labeling revealed that TRPM3 was expressed in most of the CGRP-positive nerve fibers (Fig. 1A, left and right panels).

The cell bodies of the sensory neurons innervating the rat pancreas are primarily in T9–T12 DRGs [28]. To determine whether TRPM3 channels are functional in pancreatic DRG neurons, Ca^{2+} imaging was conducted in primary cultured DRG neurons from T9–T12 (Fig. 1B). Pancreatic DRG neurons were labeled through microinjection of DiI into the pancreas 10–14 days prior to Ca^{2+} imaging (red, Fig. 1Ba). CIM0216 (CIM,1 μM), which is a specific TRPM3 agonist whose potency and apparent affinity greatly exceeds that of the canonical TRPM3 agonist, pregnenolone sulfate (PS) [15], induced an increase in intracellular Ca^{2+} in 56.98% (49 of 86) of DiI^+ neurons (Fig. 1C). Be consistent with previous reports [13], DRG neurons that are responsive to CIM are small to medium sized with the cell body diameter ≤ 30 μm. And there is no difference in the cell size between DiI^+CIM^+ and DiI^- CIM^+ neurons (25.2 ± 1.8 vs 24.6 ± 1.3 μm).

The CIM-induced Ca^{2+} increase was significantly attenuated by pretreatment with Primdone (Prim, 10 μM),

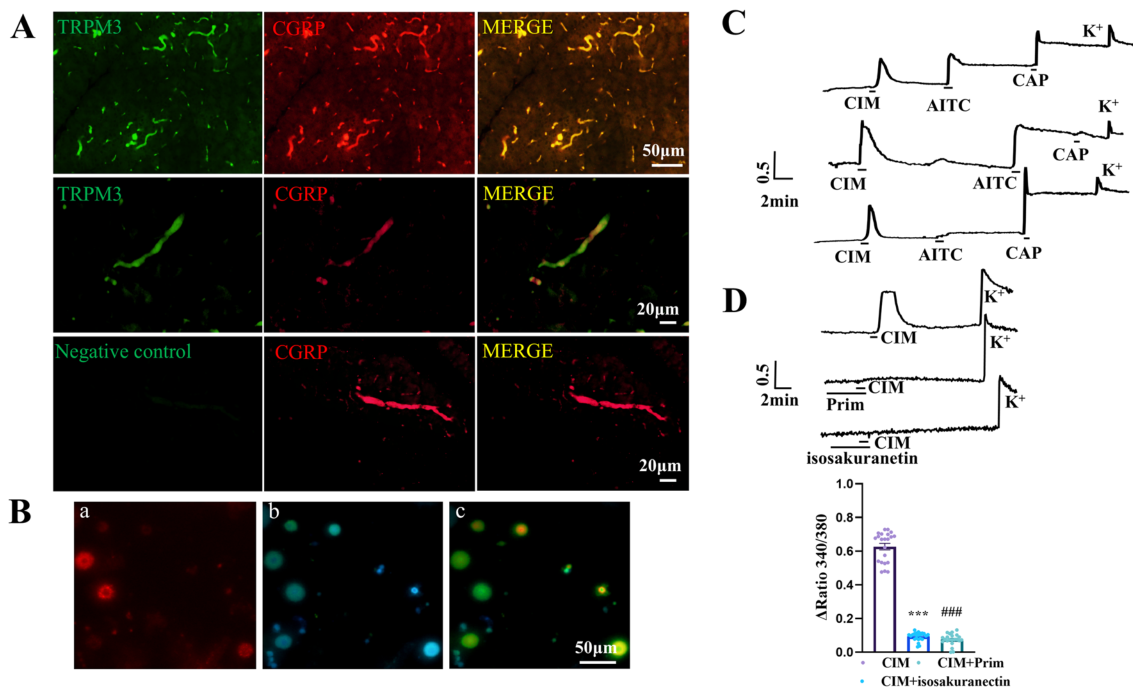

Fig. 1 Expression of TRPM3 channels in pancreatic primary sensory afferents (**A**) TRPM3 (green, left) and CGRP (red, middle) immunofluorescence and their co-localization (yellow, right) in nerve terminals of the pancreas. Scale bar = 50 μm. Specificity of the TRPM3 antibody was tested by pre-incubating the tissue section with the blocking peptide before adding TRPM3 antibody (bottom images). Scale bar = 20 μm. (**B**) Under epifluorescence illumination, three DiI-labeled (DiI +) DRG neurons were identified in the field (a, red). The TRPM3 agonist CIM-induced response was assessed with fura-2 microfluorimetry. Fura-2 fluorescence ratio is low at rest (b) and markedly increased in a subpopulation of neurons (c) following application of CIM (1 μM). (**C**) Representative traces showing intracellular Ca^{2+} increases induced by CIM (1 μM for 30 s), TRPA1 agonist allyl isothiocyanate (AITC; 100 μM for 1 min), and TRPV1 agonist capsaicin (CAP; 500 nM for 10 s) in three DiI^+ DRG neurons. KCl (30 mM, K^+) was applied at the end of the experiment to prove the cells were neurons. (**D**) Representative traces and summary data showing that the CIM-induced Ca^{2+} increase was blocked by the specific TRPM3 antagonist, Primidone (Prim, 10 μM) and another TRPM3 antagonist (isosakuranetin, 10 μM). Ca^{2+} increase is expressed as the net change of the ratio of fluorescence at 340 and 380 nm from the baseline (ΔF340/F380). Values are presented as mean ± SEM. The summary data were analyzed with unpaired Student's *t* test, *** $P < 0.001$

a specific antagonist of TRPM3 with a half maximal inhibitory concentration (IC50) of 0.6 ± 0.15 μM [22] (Fig. 1D). The peak amplitude of the CIM-evoked Ca^{2+} increase was reduced by 88.3% (n = 22 neurons) in the presence of Prim (Fig. 1D). CIM-evoked Ca^{2+} increase was also reduced by 84.1% (n = 24 neurons) in the presence of another TRPM3 antagonist isosakuranectin (10 μM) [29] (Fig. 1D).

Almost all of the CIM-responsive neurons, 95.9% (47 of 49) were responsive to the TRPV1 agonist capsaicin (CAP, 500 nM, Sigma-Aldrich, Inc.), and 63.27% (31 of 49) of CIM-responsive neurons were responsive to the TRPA1 agonist allyl isothiocyanate (AITC, 100 μM, Sigma-Aldrich, Inc.) (Fig. 1C). Since previous report [15] and the finding in this study showed that CIM is more potent than PregS, it has been used as the TRPM3 agonist in our following experiments.

Injection of a TRPM3 Agonist into the Pancreas Evoked Pain Responses

Injection of the TRPM3 selective agonist PregS into rat or mouse hind paws can induce nociceptive responses [13, 15]. To examine whether TRPM3 activation could induce similar responses in the pancreas, CIM (10 μM) was injected into the pancreas, and pain behaviors were evaluated. Since our preliminary observations indicate that CIM induced more severe pain than PregS and pain response reached the peak at 1 h after injection (supplementary Fig. 1), all of the following observations were conducted at 1 h after CIM injection.

Compared with the vehicle control (0.1% DMSO in saline), CIM injection induced prominent spontaneous pain behaviors, demonstrated by eye closing and abdomen contraction (Fig. 2A and supplementary video file).

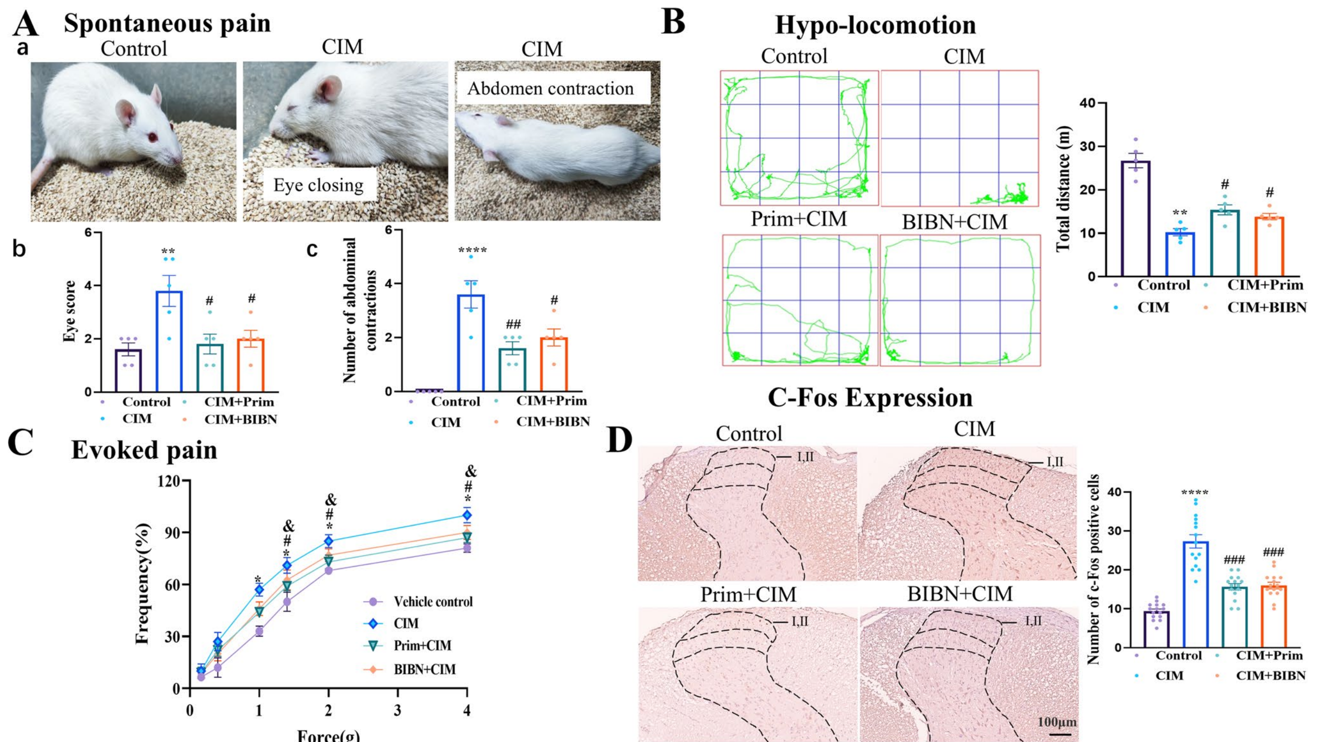

Fig. 2 Injection of CIM0216 into the pancreas induced pain responses (**A**) Rats with CIM0216 (CIM, 10 μM) injection into the pancreas showed frequent eye closing (Aa middle) and abdomen contraction (Aa right) compared with rats with vehicle control (0.1% DMSO) injection that exhibited no eye closing and abdomen contraction (Aa left). (Ab) and (Ac) are summary data. (**B**) Typical recordings (left) and summary data (right) from the open field test demonstrate that rats with CIM injection showed hypo-locomotion, which was attenuated by co-injection with Primidone (Prim, 10 μM, a TRPM3 antagonist) or olcegepant hydrochloride (BIBN, 100 μM, a CGRP receptor antagonist). (**C**) Rats with CIM injection showed enhanced responses to Von Frey filaments stimulation, which were attenuated by co-injection with Prim (10 μM) and BIBN (100 μM). (**D**) Rats with CIM injection showed increased c-Fos expression in T10 dorsal horn of the spinal cord, which were reduced by co-injection with Prim (10 μM) and BIBN (100 μM). Fos-stained nuclei were only counted in laminae I and II of the spinal cord. Scale bar = 100 μm. All measurements were conducted at 1 h after injection of CIM. The summary data were analyzed with 2-way repeated-measures ANOVA: * CIM effect; #, Prim or BIBN treatment effect. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Spontaneous pain was also demonstrated by the hypo-locomotion evaluated in the OFT (Fig. 2B).

CIM injection also induced an enhanced evoked pain response in the abdomen (an assay for referred somatic hyperalgesia [23]), which could be revealed by the increased responses to Von Frey filament stimulation in CIM injection rats compared with rats injected with the vehicle control (Fig. 2C). CIM injection also induced an increase in c-Fos expression in the dorsal horn of T10 spinal cord (Fig. 2D), an indicator of nociceptive sensory nerve activation [25, 26].

In addition, CIM injection induced prominent edema, neutrophil cell infiltration (Fig. 3Aa and 3Ab), increased MPO activity (Fig. 3Ac), SP and CGRP release (Fig. 3B), and plasma extravasation (Fig. 3C) in the pancreas, suggesting that neurogenic inflammation occurs in the pancreas [4, 9].

The pain responses and the neurogenic inflammation were significantly reduced when the selective TRPM3 antagonist Prim (10 μM) or a CGRP receptor antagonist olcegepant hydrochloride (BIBN, 100 μM) was simultaneously injected with CIM (Fig. 2–3).

The TRPM3 Antagonist Attenuated Inflammation and Pain Behaviors Associated with AP

The above observations indicate that directly activating TRPM3 could induce nociceptive response and inflammation in the pancreas. To assess whether TRPM3 is involved in AP-associated pain and inflammation, we investigated the effect of a TRPM3 antagonist on AP-associated pain behaviors and inflammation response in L-arginine induced AP model rats [30, 31]. L-arginine injection induced marked pain behaviors including spontaneous pain revealed by eye closing, frequent abdomen contractions (Fig. 4A), and hypo-locomotion (Fig. 4B). Moreover, in agreement with a previous report [32], L-arginine-induced AP resulted in hypersensitivity to Von Frey mechanical stimuli (Fig. 4C). As reported by others [33], injection of L-arginine induced significant inflammation and histopathology, as revealed by edema, increased neutrophil cell infiltration, necrosis or hemorrhage (Fig. 5Aa and 5Ab) and increased MPO activity (Fig. 5Ac). In addition, injection of L-arginine also produced significant increase in CGRP and SP release (Fig. 5B), and in the level of

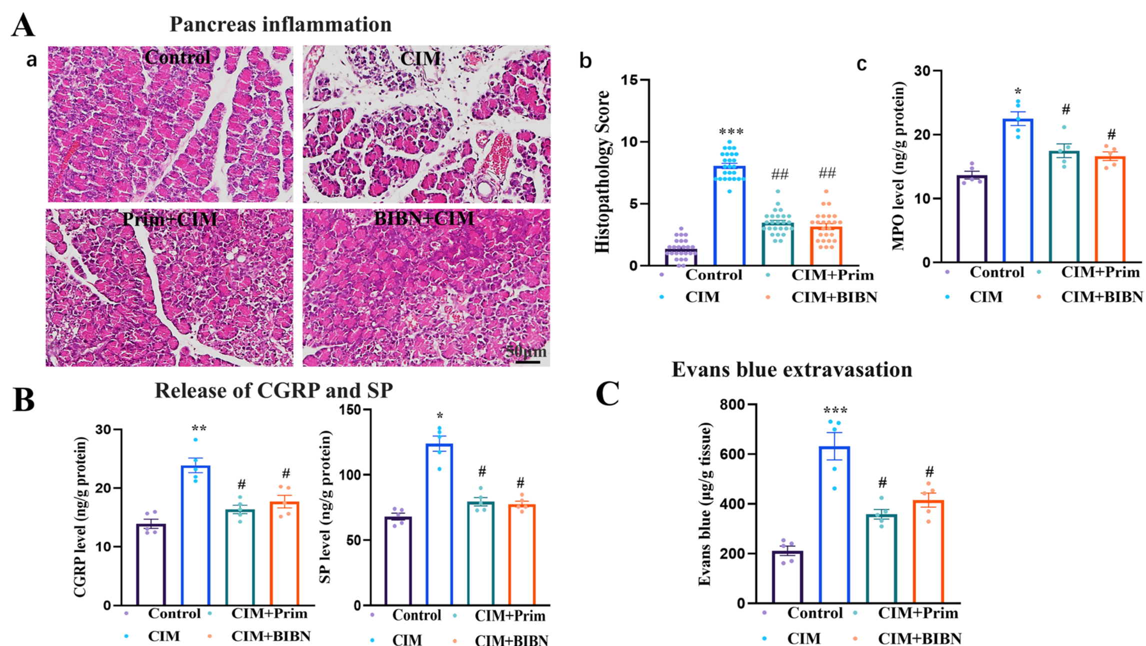

Fig. 3 Injection of CIM0216 into the pancreas induced neurogenic inflammation (**A**) Hematoxylin and eosin staining showing that CIM0216 (CIM, 10 μM) injection into the pancreas induced significant inflammation responses which exhibit edema, significant increases in neutrophil infiltration and hemorrhage in the pancreas compared with the vehicle control injection. Edema was evidenced by widening of the interstitial space. The above histopathological changes were scored and summarized in (Ab). The extent of neutrophil infiltration was further assessed with MPO activity and summarized in (Ac). (**B-C**) CIM injection also induced SP and CGRP release (B), and Evans blue extravasation (C). The above changes (A–C) were significantly alleviated when CIM was co-injected with Primidone (10 μM, a TRPM3 antagonist) or BIBN (100 μM, a CGRP receptor antagonist). All experiments were conducted at 1 h after injection of CIM. Scale bar = 50 μm in the images in (A). The summary data were analyzed with 2-way repeated-measures ANOVA: * CIM effect; # Prim or BIBN treatment effect. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

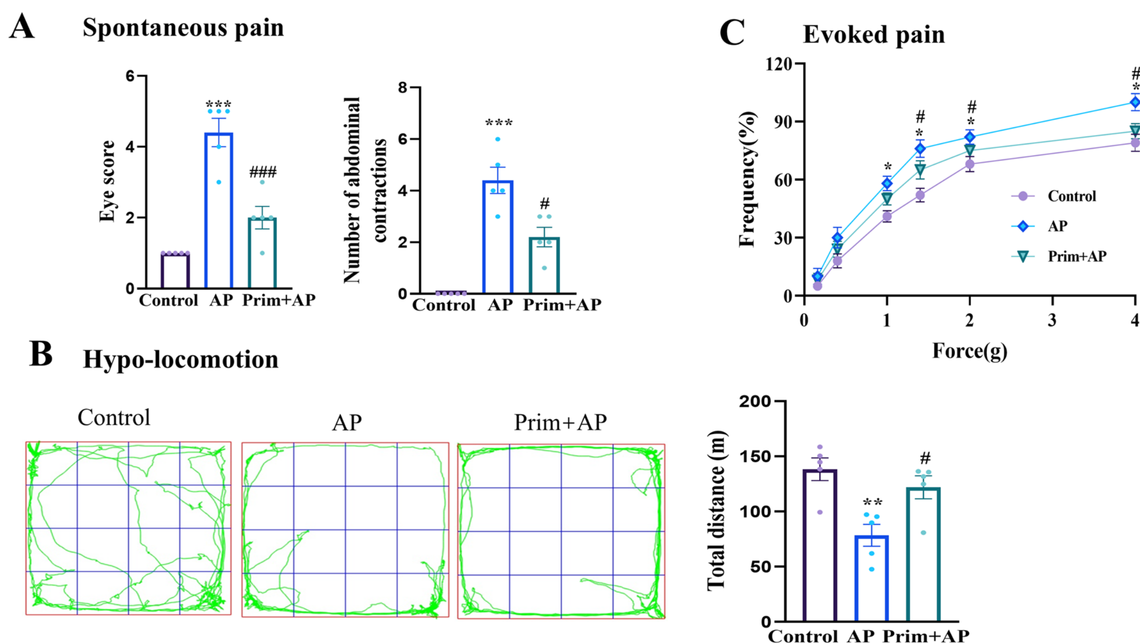

Fig. 4 The TRPM3 antagonist attenuated pain behaviors in AP rats (**A**) AP was induced by intraperitoneal (i.p.) injection of L-arginine. The TRPM3 antagonist Primidone (Prim, 5 mg/Kg) was injected 30 min prior to L-arginine. AP associated spontaneous pain behaviors reflected by the eye score and number of abdominal contractions were significantly attenuated by pretreatment with Prim. (**B**) Representative (left) and summary data (right) of open field tests showing that hypo-locomotion in AP rats was improved by pretreatment with Prim, signifying reduced spontaneous pain behavior. (**C**) Evoked pain behaviors in the abdomen, evaluated with the mechanical hypersensitivity to Von Frey filaments, in AP rats were significantly attenuated by pretreatment with Prim. The summary data were analyzed with 2-way repeated-measures ANOVA: * L-arginine effect; # Prim treatment effect. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

pancreatic trypsin, serum amylase and lipase (Fig. 5C). However, when the TRPM3 antagonist Prim (5 mg/Kg, i.p) was injected 30 min before L-arginine, the above AP-associated responses were markedly reduced (Fig. 4–5)

Functional Upregulation of TRPM3 in Pancreatic Sensory Neurons from AP Rats

To further assess the involvement of TRPM3 channels in AP-associated pain, the functional expression of TRPM3 in DRG neurons innervating the pancreas was investigated with Ca^{2+} imaging in control and AP model rats. The intracellular Ca^{2+} increase induced by CIM (1 μM) in DiI^{+} DRG neurons was compared between control ($n=5$) and AP ($n=5$) rats. The amplitude of the CIM-induced Ca^{2+} increase was significantly increased in AP rats (0.33 ± 0.02 vs 0.56 ± 0.02; $P<0.001$, Fig. 6A and 6B). The fraction of CIM-responsive neurons in AP rats was also increased from 58.74% (84 of 143) to 71.43% (95 of 133). Furthermore, Ca^{2+} increases induced by capsaicin or AITC were also enhanced in DiI^{+} DRG neurons (Fig. 6B). However, there was no difference in the cell size of CIM-responsive neurons between control and AP rats (25.6 ± 1.3 vs 26.4 ± 1.8 μm), and AP did not change the baseline level of intracellular Ca^{2+} (0.603 ± 0.014 vs 0.619 ± 0.009, p > 0.05).

In addition to the functional upregulation, our western blotting experiments indicate an increase in TRPM3 protein expression in T9–T12 DRGs from AP rats (Fig. 6C).

NGF Increase may Contribute to the Enhanced TRPM3 Function in AP Model Rats

Nerve growth factor (NGF) is a crucial factor for AP- or chronic pancreatitis-associated sensory afferent sensitization and pain [23, 27]. Consistent with these previous reports, an increase in NGF concentration were observed in the pancreas of AP model rats (Fig. 7A). To explore whether NGF increase could mediate the functional upregulation of TRPM3 channels, DRG neurons were incubated in culture medium with NGF (50 ng/L) for 2 h and then the CIM-induced intracellular Ca^{2+} increase was examined. As shown in Fig. 7B, pretreatment with NGF significantly enhanced the CIM-induced intracellular Ca^{2+} increase in DiI^{+} DRG neurons compared to NGF untreated neurons, which indicates an upregulation of TRPM3 function.

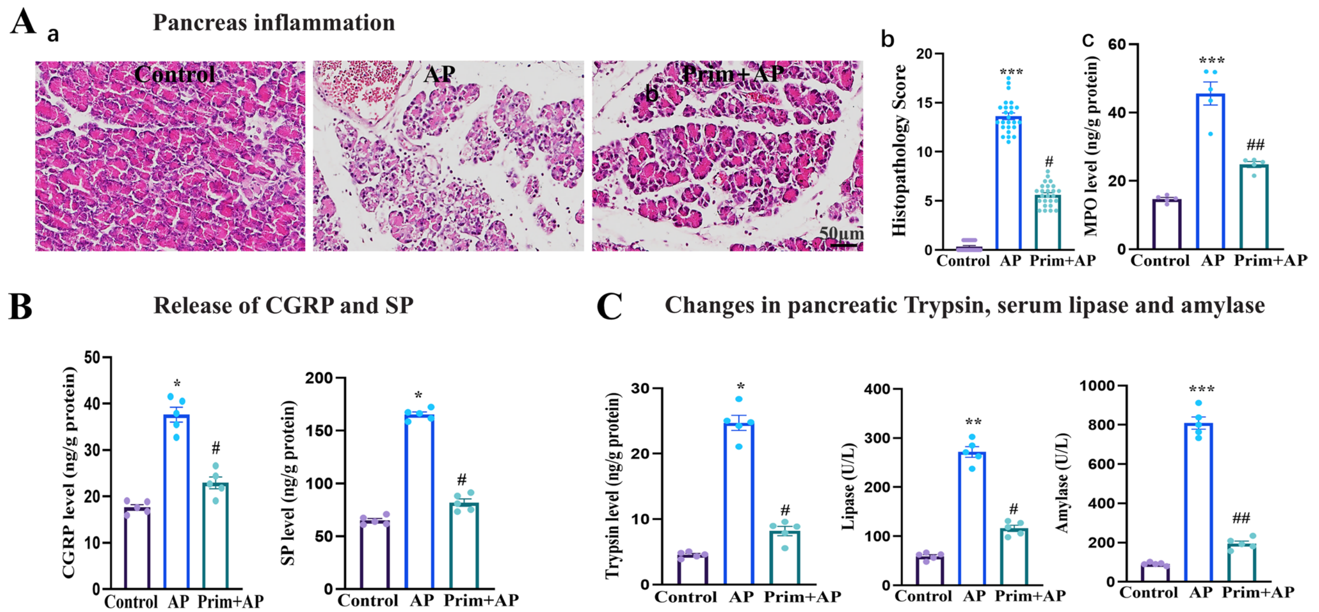

Fig. 5 The TRPM3 antagonist attenuated inflammation in AP rats (**A**) AP was induced by intraperitoneal (i.p.) injection of L-arginine. The TRPM3 antagonist Primidone (Prim, 5 mg/Kg, i.p) was injected 30 min prior to L-arginine. Hematoxylin and eosin staining (Aa) show that there was prominent edema in AP rats (middle) compared with control rats (left). Scale bar = 50 µm. However, the edema was significantly reduced in AP rats that were pretreated with Prim (right). AP-associated histopathological changes and MPO activity were summarized, and they were significantly attenuated by pretreatment with Prim (Aa and Ab). (**B-C**) AP is also associated an increased release of SP and CGRP release (B), increased pancreatic trypsin and serum lipase or amylase (C). The above changes (B-C) were significantly improved by pretreatment with Prim. The summary data were analyzed with 2-way repeated-measures ANOVA: * L-arginine effect; # Prim treatment effect. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

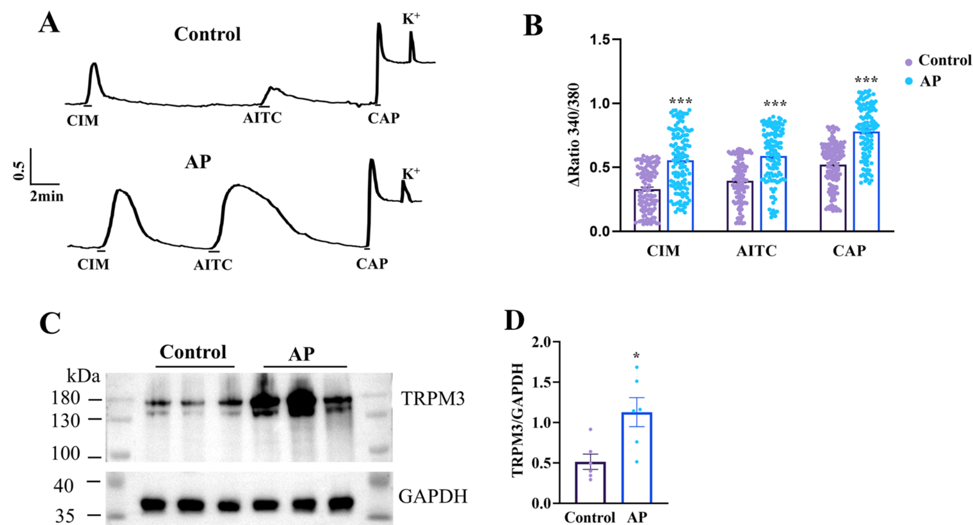

Fig. 6 AP is associated with the upregulation of TRPM3 function and protein expression (**A**) Representative traces showing intracellular Ca^{2+} increases induced by the TRPM3 agonist (CIM, 1 µM applied for 30 s), the TRPA1 agonist allyl isothiocyanate (AITC; 100 µM for 1 min), and the TRPV1 agonist capsaicin (CAP, 500 nM for 10 s) in DiI^+ DRG neurons from control (upper) and AP (lower) rats. The neurons from AP rats showed an enhanced response to the three TRP channel agonists. (**B**) Comparisons of the amplitude of the Ca^{2+} increase in DiI^+ neurons induced by the three agonists between control ($n=5$) and AP ($n=5$) rats. A significant increase was observed for the three agonists in AP rats. (**C-D**) Representative (C) and summary (D) data showed AP is associated with an increase in TRPM3 protein expression in T9-T12 DRG neurons. TRPM3 protein expression are quantitated by densitometry and normalized to GAPDH. Values are presented as mean ± SEM. The summary data were analyzed with unpaired Student's t test, * $P<0.05$, *** $P<0.001$ compared with control

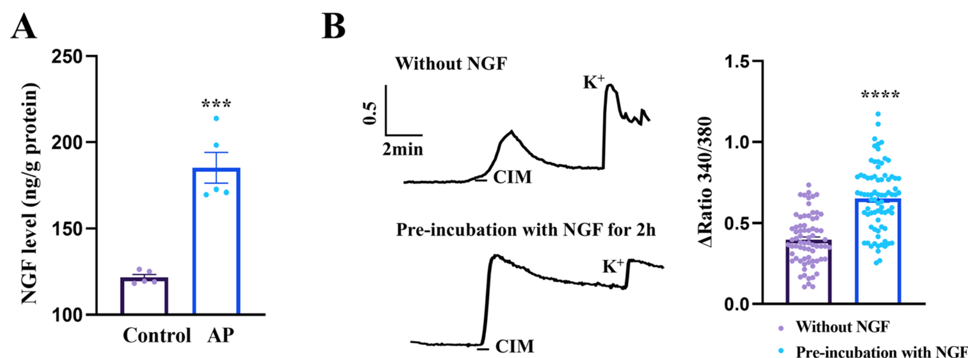

Fig. 7 NGF increase contributes to the upregulation of TRPM3 function in AP rats (**A**) Comparison of NGF concentration in pancreatic tissue from control ($n = 5$) and AP rats ($n = 5$). Pancreatic tissue was harvested at 24 h after L-arginine injection. (**B**) Representative traces (left) and summary data (right) showing the amplitude of intracellular Ca^{2+} increases induced by the TRPM3 agonist (CIM, 1 μM) in DiI^{+} DRG neurons was significantly enhanced after pretreatment with NGF (50 ng/L) for 2 h. Values are presented as mean ± SEM. The summary data were analyzed with unpaired Student's t test, ***$P < 0.001$, ****$P < 0.0001$ compared with control

DISCUSSION

Abdominal pain is the hallmark symptom of patients suffering from AP, however, the underlying mechanisms of AP-associated pain are poorly understood and its treatment remains difficult. To facilitate the development of new pharmaceutical treatments for AP, new receptors/ion channels on pancreatic sensory neurons need to be characterized. In this study, we provided the following evidence to demonstrate that activation of TRPM3 contributes to AP-associated pain: (1) injection of a TRPM3 selective agonist (CIM) to activate TRPM3 produced prominent nociceptive responses (Fig. 2); (2) there is functional upregulation of TRPM3 channels in pancreatic sensory neurons from AP model rats (Fig. 6); and (3) blocking TRPM3 activity with its specific antagonist (Prim) significantly attenuated pain behaviors and inflammation in AP model rats (Fig. 4 and Fig. 5). These findings indicate that in addition to the well-known pain channels TRPV1 and TRPA1, TRPM3 is another pain channel with a critical role in AP-associated pain. Thus, TRPM3 may be a promising pharmaceutical target for AP pain treatment.

Previous studies in somatic primary sensory neurons from rodents showed that activation of TRPM3 channels produced nociceptive responses [20]. In this study, we found that TRPM3 channels are expressed in almost all CGRP-expressing pancreatic sensory afferent nerve terminals and most of the pancreatic DRG neuron cell bodies (Fig. 1A and 1C). Furthermore, activation of TRPM3 produced prominent nociceptive responses evidenced by the spontaneous and the evoked referred pain behaviors (Fig. 2). Nociceptive nerve activation was further confirmed by increased c-Fos expression in the dorsal horn of T10 spinal cord (Fig. 2D). These results suggest that TRPM3 has a similar role in pancreatic nociception as in somatic nociception [13]. These findings set the foundations for TRPM3 channel involvement in acute and chronic pancreatitis-associated pain.

Neurogenic inflammation is the inflammatory response elicited by the release of neuropeptides such SP and CGRP from afferent nerve endings. The release of SP and CGRP contribute to edema, neutrophil infiltration, and vasodilation in the organ. Although the severity of pancreatitis and tissue necrosis varies among different models of AP, the fundamental features of plasma extravasation, edema, and neutrophil infiltration within the pancreas are common to all of these models [10]. This suggests that sensitization of pancreatic sensory neurons and neurogenic inflammation are instrumental in inflammation and pain-related behaviors [12]. In this study, we found that injection of the TRPM3 agonist CIM into the rat pancreas to activate TRPM3 channels evoked considerable neurogenic inflammation, evidenced by the increased CGRP and SP release, increased vascular permeability, increased MPO activity and edema (Fig. 3). These changes could be blocked by treatment with TRPM3 antagonist (Prim) or the CGRP receptor antagonist BIBN (100 μM) (Fig. 3). Our results indicate that TRPM3 mediated sensory afferent hypersensitivity and the resulting neurogenic inflammation may contribute to AP-related inflammation and pain behaviors. This idea is further supported by the finding that application of specific TRPM3 antagonist (Prim) significantly alleviated pain behavior and inflammatory responses in AP model rats (Fig. 4 and 5). However, it should be noted that Prim could not completely block AP associated pain behavior and inflammation responses (Fig. 4 and 5). This may indicate that TRPM3 activation and the resulting neurogenic inflammation is only one of the pathophysiological mechanisms. Other factors such as increase in oxygen free radicals and inflammatory mediators may also contribute to AP associated pain [31].

In addition to the activation of TRPM3 on sensory afferents, injections of CIM into the pancreas may also induce activation of TRPM3 on beta-cells. Reports showed that activation of beta-cell TRPM3 could induce insulin increase and are involved in glucose induced insulin release [34, 35]. In agree with these reports, a decrease in blood glucose and an increase in blood insulin were found at 1 h after CIM injection (Supplementary Fig. 2). However, CIM injection induced nociceptive responses may not be triggered by activation of beta-cell TRPM3. To our knowledge, there are no reports indicating decreased blood glucose or increased insulin could evoke nociceptive responses.

The critical role of TRPM3 in AP-associated pain mimics another two recognized pain channels (TRPV1 and TRPA1) with such roles proven in mice and rats [10, 36, 37]. In our study, we did not examine whether these three pain channels have a synergistical role or whether they work separately. Our Ca^{2+}-imaging experiments indicate that there is an overlap in the functional expression of these three pain channels in pancreatic sensory neurons (Fig. 1C) and in the functional upregulation of TRPV1 and TRPA1 channels (Fig. 6B). Using Ca^{2+} imaging to assess TRPM3 function, we showed that TRPM3 function is upregulated in pancreatic DRG neurons from AP rats (Fig. 6). Functional upregulation of TRPM3 in one DRG sensory neuron was previously reported to potentially increase nociceptor excitability and contribute to augmented responses to TRPA1 and TRPV1 agonists [18]. If the above idea also occurs in pancreatic sensory neurons, TRPM3 upregulation should enhance the function of TRPA1 and TRPV1 and the three pain channels may work synergistically in neurogenic inflammation and the resulting pain in AP.

One of the most crucial findings in our study is that AP is associated with functional upregulation of TRPM3 channels in pancreatic sensory neurons. This enhanced functional expression of TRPM3 may have arisen from changes at post-translational level, which could be confirmed by our western blotting experiments [Fig. 6C]. TRPM3 functional upregulation may also arise from increased NGF. Pancreatitis was reported to be associated with an increase in the concentration of NGF in pancreatic tissues and NGF was believed to play a vital role in the pathogenesis of pancreatic pain [27]. NGF could enhance TRPV1 expression and activity in pancreatic sensory neurons by post-translational or transcriptional mechanisms [23, 32]. Congruent with a previous study [27], we found an increased concentration of NGF in the pancreas tissue from AP rats (Fig. 7A). Furthermore, we showed that pre-incubation of pancreatic DRG neurons with NGF enhanced the TRPM3 agonist-induced increase in intracellular Ca^{2+} (Fig. 7B), suggesting a sensitization role of NGF on TRPM3 function. This enhancing effect of NGF on TRPM3 function has been reported in a recent study in rat DRG neurons from thoracic and lumbar segments [38]. However, we did not explore the cellular mechanism(s) underlying the enhancement action of NGF. It may be that NGF binds to tropomyosin receptor kinase A (TrkA) and subsequently activates the phosphoinositide 3-kinase (PI3K)-signaling pathway, which promotes the generation of phosphatidylinositol 3, 4, 5-trisphosphate, the most potent phosphoinositide for TRPM3-gating, as suggested by other researchers [39]. It should be noted that in addition to TRP channels, NGF have been reported to enhance the function of voltage activated calcium channels (CaV) [40]. Moreover, one study on pancreatic beta-cells indicated that membrane depolarization secondary to TRPM3 activation can further activate CaV [35]. Thus, further experiments like patch-clamp recordings are needed to exclude the contribution of CaV channels.

In summary, we provide evidence for the critical role of TRPM3 in pancreatic nociception, neurogenic inflammation, and AP-associated pain. However, given the vital roles of TRPV1 and TRPA1 in the sensitization of sensory nerves, TRPM3 activation should be regarded as only one of many mechanisms that may contribute to AP-associated pain. Nevertheless, our findings present the possibility of an additional target for the treatment of AP-associated pain.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10753-024-02138-8.

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Author Contributions L. L., H. L., M. Z., X. Z. and L.G. designed research studies and wrote the manuscript. L. L., H. L., M. Z., J. W., J.L., G. L., Z. X., W. W., S.Z., W. S., X. Z. and L. G. were involved in acquisition, analysis, and interpretation of data for the work. L. L., H. L., M. Z., J. W. and G. L. prepared figures. All authors reviewed the manuscript.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Competing Interest The authors declare no competing interests.

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