Study on the protective effect of TRPM8 against indomethacin-induced small intestinal injury in mice via the release of calcitonin gene-related peptide

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Preface

Transient receptor potential melastatin 8 (TRPM8) is a non-selective cation channel activated by mild cooling and chemical agents, including menthol. TRPM8 is expressed in sensory neurons and afferent nerve terminals in somatic and visceral sensory nervous systems. Nonsteroidal anti-inflammatory drugs (NSAIDs), including indomethacin, are the most prescribed drugs for their antipyretic, analgesic, and anti-inflammatory properties. However, they could injure both the stomach and small intestine. Therefore, a clear understanding of the pathogenesis of NSAID-induced intestinal injury is a crucial point for effective treatment and prevention of the adverse effects. As the gastrointestinal (GI) tract has both intrinsic enteric neurons and extrinsic efferent and afferent nerves, the TRP channels expressed in extrinsic sensory neurons are considered a way of neuro-immune communication within the GI tract. The mechanism, which involves releasing neuropeptides from the sensory neuron after activating TRP channels, is responsible for maintaining homeostasis and modulating inflammatory responses. The roles of TRP channels have already been proven in the initiation and regulation of intestinal inflammation. Besides, TRPM8 has been shown the anti-inflammatory effects, including those in the colitis model. However, the role of TRPM8 in intestinal inflammation has not been fully elucidated. Therefore, I was interested in explaining the effect of TRPM8 on the indomethacin-induced intestinal injury, especially in relation to the neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) released from sensory afferent neurons.

This study is divided into two chapters. The first chapter is entitled "Role of TRPM8 in the pathogenesis of indomethacin-induced small intestinal injury." The second chapter is entitled " TRPM8 expression profiles in the small intestine based on the role of CGRP and SP in the pathogenesis of indomethacin-induced intestinal injury". Both chapters have been published in the ***Biological and Pharmaceutical Bulletin 2021, 44 (7), 947-957.***

This study suggests that TRPM8 should be a potential target for the treatment of indomethacin-induced intestinal injury and will significantly contribute to the treatment of NSAID-induced enteropathy and inflammatory bowel diseases.

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Abbreviations

CMC	Carboxymethylcellulose
CGRP	Calcitonin gene-related peptide
DRG	Dorsal root ganglia
DSS	Dextran sulfate sodium
EIA	Enzyme immunoassay
FG	Fluorogold
GI	Gastrointestinal tract
IL-1β	Interleukin-1β
KO	Knockout
MPO	Myeloperoxidase
NGF	Nerve growth factor
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction
SP	Substance P
TBP	TATA-binding protein
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor-α
TRPA1	Transient receptor potential ankyrin 1
TrkA	Tropomyosin receptor kinase A
TRPM8	Transient receptor potential melastatin 8
TRPV1	Transient receptor potential vanilloid 1
WT	Wild type

Introduction

Transient receptor potential channels

Ion channels are pore-forming transmembrane proteins found in nearly all living cells and allow ions to permeate biological membranes [1]. Thus, they are responsible for controlling intracellular signaling pathways, making them attractive drug targets. Transient receptor potential (TRP) channels are diverse proteins expressed in many organisms, tissues, and cell types [2]. TRP channels received their names as there were first discovered in the "transient receptor potential" mutant (TRP-mutant) strain of the fruit fly *Drosophila* where TRP was responsible for a phototransduction process [3, 4]. All TRP channels are permeable to monovalent cations, and most are also permeable to Ca^{2+}. Thus, TRP channels are cellular sensors for a broad spectrum of physical and chemical stimuli by integrating multiple stimuli and coupling their activity to downstream cellular signal amplification via calcium permeation and membrane depolarization [5]. TRP channel superfamily has been divided into seven subfamilies (6 subfamilies in humans) (Figure.1). Based on sequence homologies, TRP channels are divided into TRPV (vanilloid), TRPM (melastatin), TRPC (canonical), TRPA (ankyrin), TRPML (mucolipin), TRPP (polycystin) and, TRPN. TRPN gene was named no mechanoreceptor potential C (nompC) when first discovered, that's why got the letter N [6]. According to variations in their sequences and topologies, TRP subfamilies are sorted into two groups: group 1 (TRPC, TRPM, TRPV, TRPN, and TRPA) and group 2 (TRPP and TRPML). Then subfamilies of TRP channels are divided into groups and subtypes with a total of 30 members [7].

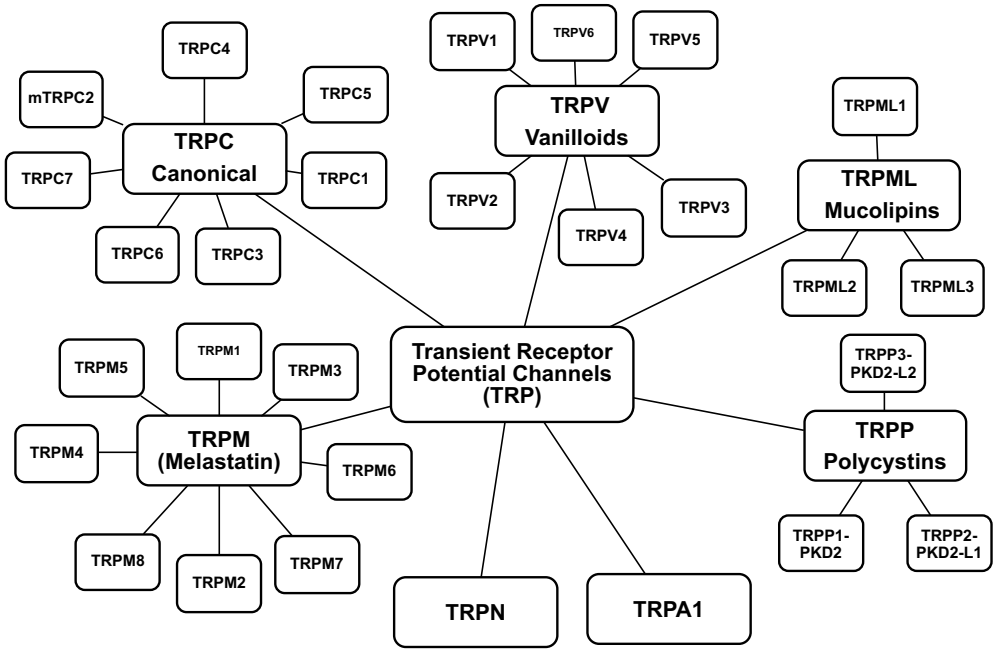

Figure 1. Homology classification of TRP channels

Even though TRP channels differ widely in their sequence homology, they still have almost similar structure which consists of identical tetramers. Each of the tetramers is composed of a six transmembrane (TM) domain, each of the six TM domains is called a segment, and it is structure contains long intracellular carboxy (C-) and amino (N-) termini, including many regulatory modules that distinguish between subfamilies. Recent cryo-electron microscopy studies revealed a high-resolution view of the structures of these channels. A pore-forming region is located between the segments S5–S6 cytoplasmic amino (N) and carboxyl carboxy (C) termini [7].

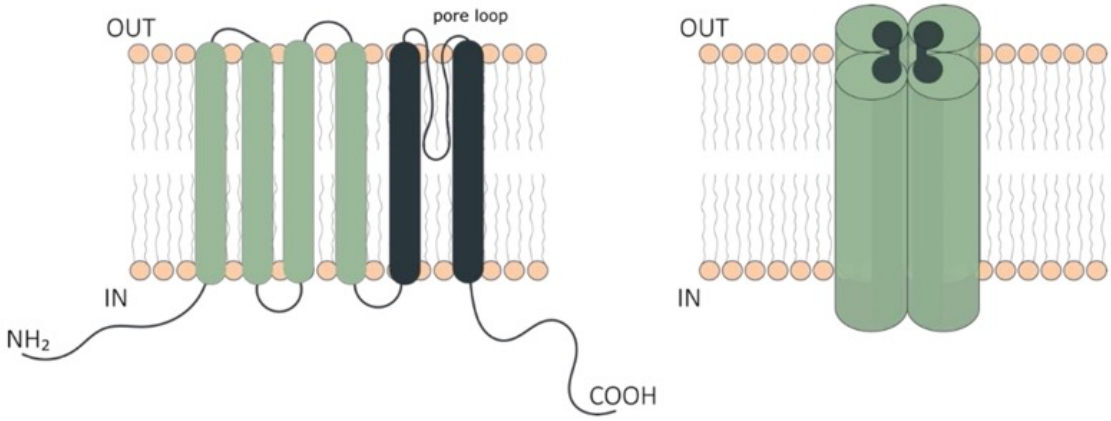

Figure 2. TRP ion channel structure. The six-transmembrane segment topology of the monomer (left) and tetrameric functional unit of TRP channels (right). The cytoplasmic NH2- and COOH-terminal domains and the transmembrane domain constituting the pore region are indicated. [Boonen et al. *Toxins* **2018,** *10* (8), 326]

Although the homology classification is the most relevant and accepted classification, other classification methods are based on different perspectives (Figure 3). For example, functional classification of TRP channels is based on physiological function and endogenous activation mechanism, leading to three subgroups [8, 9].

- Metabotropic: This group is activated by extracellular neuroendocrine signals such as phospholipase-dependent G-protein-coupled receptors [8].
- Sensory: This group is activated by physical and chemical environmental signals [10].
- Organellar: This group is activated by cellular signals generated in the cytoplasm and other cellular compartments. Organellar TRPs can be predominantly localized in the organellar membranes or at the plasma membrane [11].

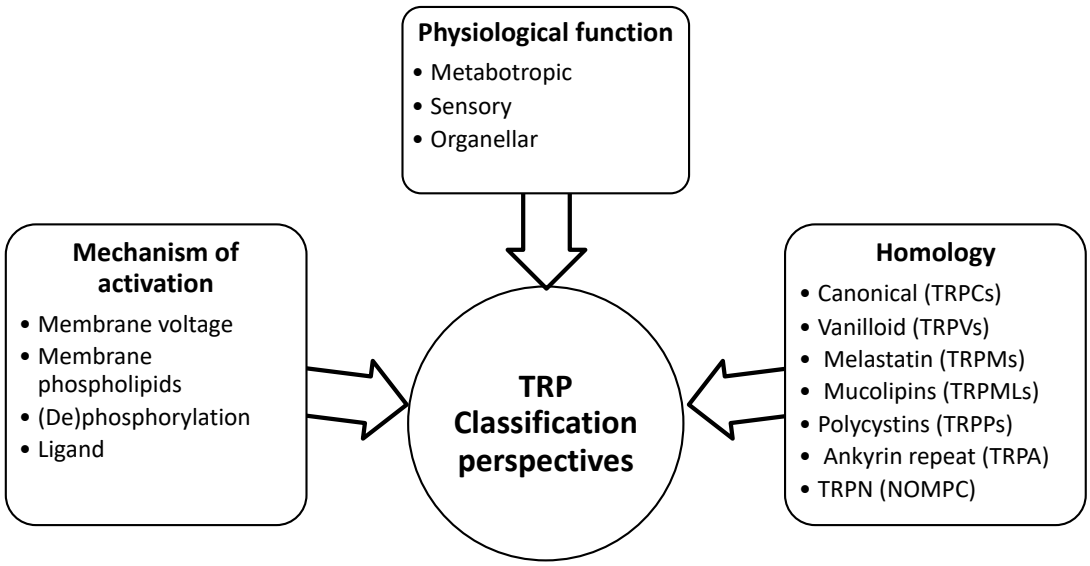

Figure 3. TRP channels classification's methods

Another theoretical classification is based on the mechanism of TRP channel activation; different mechanisms manage the activity of TRP channels; generally, these processes require set of biological processes, including G-protein-coupled receptor-related mechanisms. Thus, mechanisms of TRP channels activation are described as follows.

- Membrane voltage: These TRP channels activation is dependent on the intrinsic voltage, where TRP gating either opening or closing depends on the position of charged groups that move in response to changed membrane potential [12].
- Membrane phospholipids: In these TRP channels, membrane phospholipids were found to regulate their activity directly. Lipid modulators of TRP channels comprise lipids from a variety of metabolic pathways, including metabolites of the cyclooxygenase, lipoxygenase, cytochrome-P450 pathways, phospholipids, and lysophospholipids [13, 14].
- Phosphorylation: Protein kinase C-dependent phosphorylation might activate or sensitize the channels for other stimuli; furthermore, activation of protein kinase A by prostaglandin E_2 stimulation potentiates TRP responses and counteracts channel desensitization [15, 16].

- Ligands: Either exogenous or endogenous ligands regulate TRP channel activities. For example, the heat-sensitive TRPV1, activated by endovanilloids such as N-arachidonoyldopamine endogenously and by capsaicin as exogenous ligand, TRPM8, activated by Pirt (phosphoinositide interacting regulator of TRP) protein as an endogenous ligand and exogenously activated by menthol, and TRPA1, activated by oxidized lipids such as 4-hydroxy-2-nonenal (4-HNE) as an endogenous ligand and exogenously by mustard oil [17, 18].

Transient receptor potential melastatin 8

The genetically and functionally diverse TRPM subfamily has eight members (TRPM1-8). It is considered the largest and most diverse subfamily of the TRP channel superfamily [19] and divided into four groups: TRPM1/3, TRPM4/5, TRPM6/7, and TRPM2/8 [19, 20]. TRPM channels are known for their involvement in sensing temperature and oxidative stress, controlling cellular death, and taste transduction [21-23].

TRPM8 is a non-selective Ca^{2+}-permeable cation channel. This channel is activated with thermal stimulation at 25 to 28°C, cooling agents as menthol, eucalyptol, and icilin [18, 24]. On the other hand, endogenous TRPM8 ligands or modulators may play an essential role in maintaining mucosal homeostasis [25]. Furthermore, endogenous TRPM8 signaling normally protects against mucosal inflammatory damage associated with colitis [25]. The endogenous activation of TRPM8 may explain the anti-inflammatory actions resembling that of icilin [26]. Initially, TRPM8 transcripts were identified in testis and prostate tissues. TRPM8 was strongly upregulated in the prostate and several other tumors [27, 28]. In addition to prostate and testis, primary afferent neurons and their peripheral endings that express TRPM8 were found to innervate vascular tissues, lungs, uterus, placenta, bladder, liver, skin, and eye with different physiological functions [29-32]. TRPM8 was also identified in primary sensory neurons within the dorsal root and trigeminal ganglia [18]. TRPM8 activation correlated with multiple diseases, indicating a specific pharmacological antagonist would be a valuable candidate for therapy, and TRPM8 would be considered a valuable prognostic marker [33-35].

TRPM8 is expressed in the human distal colon in the gastrointestinal (GI) tract, where TRPM8 activation can affect symptoms such as pain, inflammation, and motility discomfort [36]. In addition, TRPM8 is expressed in the sensory neurons and afferent nerve terminals in somatic and visceral sensory nervous systems to maintain homeostasis and modulate inflammatory responses [37]. It was also found that TRPM8 activation may have a role in the menthol-induced relaxation in human colon [38]. However, the precise role of TRPM8 in intestinal inflammation has not been fully understood.

Role of TRP channels in sensory neurons in intestinal inflammation

TRP channels are expressed in somatic and visceral sensory nervous systems, although there is a considerable difference in the expression and function of the TRP channels between visceral and somatic types. Around 20 of the 30-mammalian TRP channel subunits are expressed by specific neurons and cells within the GI tract. TRPV1, TRPA1, and TRPM8 are predominantly expressed in sensory neurons in the somatic and visceral nervous systems [39]. These channels are susceptible to noxious stimuli relating to pungent compounds, temperature, acid, and inflammation. Together, these TRP channels serve to integrate many noxious stimuli that lead to action potentials in visceral afferent nerves at their nerve endings in the GI tract with different regulatory functions [40, 41].

The initial use of capsaicin as a neurotoxin before exploring its mechanism via TRPV1 was known. Also, mustard oil was an inflammatory stimulus before its role as a TRPA1 agonist was discovered. Again, the widespread use of menthol as a cooling agent can relieve abdominal pain and inflammation before investigating its primary target, the TRPM8 [42,43]. These traditional uses were the beginning to confirm the essential role of these TRP channels before knowing the exact mechanism.

Studies indicate that TRPV1 may play a key role in colonic inflammatory nociception [44, 45]. Our previous studies showed clear contribution of TRPV1 in the colitis model [46]. Clinical studies suggest that TRPV1 may play an important role in visceral pain symptoms in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [47]. Furthermore, deletion of TRPV1 was beneficial at the first stage of the indomethacin-induced pathological process to maintain gastric mucosa integrity. However, it may be harmful and aggravate indomethacin-induced pathological actions at the late stages of the pathological process in the small intestine [48]. TRP channels in the GI tract can mediate the crosstalk among the nervous and immune systems by modulating neuropeptide releases. A countless number of neuropeptides have been described in the enteric nervous system, including calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide, somatostatin, gastrin-releasing peptide, and substance P (SP) [49]. TRPV1 and TRPA1 were found to colocalize with CGRP and SP [50, 51].

Neuropeptides are essential mediators between the nervous system and among neurons and other cell types. CGRP and SP are the two predominant neuropeptides in sensory neurons that innervate the colon, and they seem to have two-sided effects on colitis [52]. CGRP is a sensory and vasodilator neuropeptide that consists of 37-amino acid and is expressed in small neurons within the dorsal root ganglia (DRG), trigeminal, and vagal ganglia, which responsible for responding to noxious, thermal, or visceral input [53]. SP is an 11-amino acid neuropeptide that belongs to the tachykinin/neurokinin family encoded by the preprotachykinin I gene, located in the trigeminal and dorsal root of the spinal cord [54, 55]. In the

GI tract, CGRP regulates motor activity, blood flow, gastric acid secretion and exerts protective effects on gastric mucosa [56-58].

Furthermore, CGRP is highly co-expressed with some TRP channels such as TRPA1 and TRPV1. Activation of TRPV1 induces the release of CGRP that acts protectively in colonic inflammation in mice [59, 60]. In comparison, SP has a defined role in the peripheral nervous system as a pro-inflammatory agent in the GI tract or other organs [61].

Although TRPV1 appears to be sensitized by inflammatory mediators, studies on the expression of TRPV1 in human intestinal inflammation, mainly colon tissue, resulted in contradictory results [62]. Thus, TRPV1 and TRPA1 expression and their role in colitis appear to be inconsistent. Activation of these ion channels on sensory nerve terminals mediates neurogenic inflammation via the release of SP and CGRP, which result in increased vascular permeability, plasma protein extravasation, and inflammatory cell activation. Furthermore, these channels can mediate both pro-and anti-inflammatory functions [62]. Therefore, the role of TRPV1 and TRPA1 in experimental colitis is dependent on many factors. These factors include their expression profiles, animal model, and the complex mechanism of the injury [62]. By the same talking, TRPA1 was highly co-expressed in the stomach with TRPV1, SP, and CGRP. Furthermore, TRPA1 mediated mechanosensory transduction in the colon participated in bradykinin-induced mechanical hypersensitivity in colonic afferents [63].

TRPM8 have been identified, plays an essential role in cold detection by the somatosensory peripheral system [64]. However, the lower GI tract is maintained at constant body temperature by blood flow, confirming a different pharmacological role of visceral TRPM8 rather than thermo-sensation and nociception. In the lower GI tract, TRPM8 is supposed to be a part of the immu.ne system that surveys and guards the epithelial cells against foreign substances in the gut. TRPM8 is involved in the immune responses, particularly inflammation, as TRPM8 activation enables crosstalk between neurons, immune cells, and epithelial cells to regulate a wide range of inflammatory actions [65]. Thus, TRPM8 has a potential anti-inflammatory role in IBS patients to relieve intestinal inflammation [66]. Moreover, TRPM8 expression was demonstrated to be upregulated in IBD patients and dextran sulfate sodium (DSS)- or trinitrobenzene sulfonic acid (TNBS)-treated mice with anti-colitogenic functions. Activation of TRPM8 was found to reduce inflammation; this action is mediated by reducing inflammatory neuropeptide release and attenuating proinflammatory cytokines/chemokine release and suppressing leukocyte recruitment to the colon [26]. Furthermore, TRPM8 may serve an anti-inflammatory function to balance the pro-inflammatory responses of TRPV1 and TRPA1, suppress pro-inflammatory cytokine release, and diminishes TRPV1- mediated CGRP release [65].

Pathophysiology of NSAIDs-induced intestinal injury

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used for their antipyretic, analgesic, and anti-inflammatory effects. NSAIDs are also prescribed to treat immunological and rheumatological disorders [67, 68]. NSAIDs inhibit cyclooxygenase, which results in the inhibition of prostaglandin production at inflamed sites. Prostaglandins are involved in maintaining the homeostasis of GI mucosa via the regulation of GI blood flow and various mucosal functions such as increased mucus secretion. Thereby, a decrease in prostaglandins production represents the leading cause of intestinal injuries [69, 70]. Local actions also affect intestinal injury, starting with the damage of cell membrane and mitochondria, which causes a reduction of energy and free radical generation, increasing intestinal permeability. Through the increased intestinal permeability, various materials such as bile acids, intestinal bacteria, and proteolytic enzymes damage the intestinal barrier, and secondary inflammation occurs by activating neutrophils (Figure 4) [71].

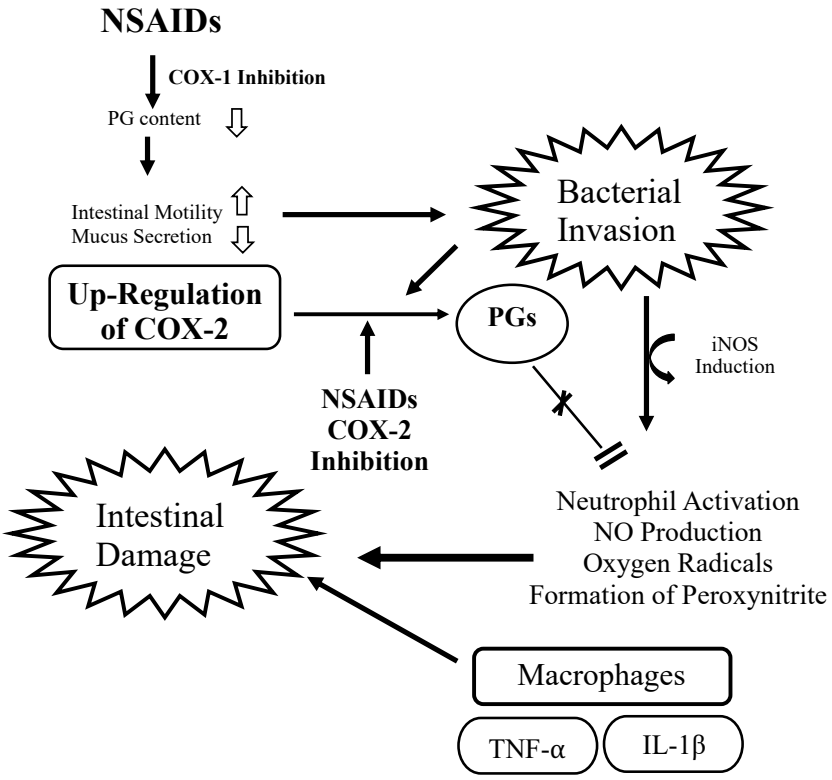

Figure 4. Pathophysiology of NSAIDs-induced intestinal damage [Takeuchi et al., *Clin. Chim. Acta.* **2010,** *411* (7), 459-466] (Partly modified).

NSAIDs-induced enteropathy is asymptomatic. Consequently, intestinal injuries became more obvious only after developing diagnostic methods like capsule and double-balloon endoscopes. Injuries start from bleeding up to complications such as intestinal perforation, stricture, and obstruction [72-74]. Concerning injury prevalence, more than 50% of patients taking NSAIDs might encounter intestinal

mucosal damage [75], increasing to 70% with chronic NSAIDs use [76, 77]. Although acid suppressants such as proton pump inhibitors and histamine H_2 receptor antagonists prevent NSAIDs-induced damages to the upper GI tract, these agents fail to prevent small intestinal injury, as NSAIDs-induced small intestinal injury is independent of gastric acid [78, 79]. On the contrary, proton pump inhibitors may exaggerate NSAIDs-induced small intestinal injury [80, 81], and there is still no proven method of limiting or curing NSAIDs-induced small intestine damage [82, 83]. Among NSAIDs, indomethacin, which is an indole acetic acid derivative, was first described in 1963. Indomethacin is a commonly prescribed drug to reduce fever, pain, and stiffness and treat gout and arthritis. However, indomethacin was confirmed to cause damage in the stomach and the small intestine in both experimental animal studies and humans by the exact mechanism [84, 85].

Previously mentioned roles of TRP channels in GI tract physiology and pathology with the broad involvement of TRPM8 in inflammatory events such as intestinal inflammation and colitis models made it clear about the importance of TRPM8 as an anti-inflammatory mediator. TRPM8 was shown a proven anti-inflammatory effect, including the colitis models. However, the role of TRPM8 in NSAIDs-induced intestinal injury has not been elucidated. Although the indomethacin model of ulceration is widely used in animal experiments, the functional role of TRPM8 receptors under indomethacin-induced enteropathy has not been directly studied. That is why in the current study, I investigated the effect of TRPM8 in intestinal lesions with the expected potential role of TRPM8 in NSAIDs-induced intestinal lesions, with investigating neuropeptides release patterns in response to indomethacin-induced intestinal injury in WT and TRPM8KO mice.

Chapter I: Role of TRPM8 in the pathogenesis of indomethacin-induced small intestinal injury

1. Background

In previous studies, I have reported that TRP channels are involved in GI tract events; neuronal TRPV1 and TRPA1 contribute to the progression of colonic inflammation [46]. Recent studies, including my own, showed that NSAID-induced GI ulcerogenic properties [86]. NSAIDs including indomethacin cause damage in the small intestine and the stomach in humans and experimental animals [87]. In terms of pathophysiological similarities, indomethacin-induced small intestinal injury in rodents may serve as a useful animal model for assessing NSAID-induced enteropathy as well as enteritis in Crohn's disease [88]. TRPM8 is expressed in the sensory neurons and afferent nerve terminals in somatic and visceral sensory nervous systems [46]. TRPM8 was expressed in mouse colonic afferent neurons and alleviated intestinal inflammation [89]. The expression of the TRP channel in extrinsic sensory neurons is considered a communication mechanism that can maintain homeostasis and modulate immune and inflammatory responses in the GI tract. Although the importance of the TRP channel in GI homeostasis and defense mechanism is well manifested, the role of TRPM8 in indomethacin-induced intestinal inflammation has not been elucidated. The first aim of my study is to investigate the exact role of TRPM8 in indomethacin-induced small intestinal injury using TRPM8-deficient mice and a specific TRPM8 agonist, WS-12.

2. Materials and Methods

2.1 Animals

In this study, 8–10-week-old male C57BL/6J mice weighing 22–27 g were purchased from Japan SLC (Shizuoka, Japan). TRPM8-deficient mice were established using C57BL/6J as described in a previous report [90]. All mice were housed in plastic cages with free access to food and water, and the temperature was maintained at 22°C ± 1°C under a 12-h light/dark cycle. All animal experimental procedures were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (Permission numbers: 18-004 and 19-004). Animal suffering was kept to a minimum whenever possible, and the smallest number of animals required for significant data interpretation was used.

2.2 Drugs and reagents

Indomethacin (Sigma-Aldrich, St. Louis, MO, the USA) was suspended in normal saline with a drop of Tween 80 (Wako Pure Chemical Industries Ltd., Osaka, Japan), and WS-12 (Tocris Bioscience, Bristol, the UK) was dissolved in 0.5% carboxymethylcellulose (CMC, Nacalai Tesque Inc., Kyoto, Japan) via sonication. These drugs were prepared immediately before use and were administered subcutaneously and intraperitoneally at a volume of 0.1 mL/10 g body weight.

2.3 Induction of intestinal injury

The animals were subjected to fasting for 18 h, provided with free access to food for 1 h, and then subcutaneously injected with indomethacin at a single dose of 8 mg/kg (Figure 5). The normal animals received vehicle only (saline with a drop of Tween 80). In some cases, WS-12 (3 and 10 mg/kg), a specific TRPM8 agonist, was administered intraperitoneally 30 min before and 8, 24, and 32 h following indomethacin administration. WS-12 was injected repeatedly as it was previously reported for WS-12 to exert TRPM8 activation with repeated or continuous administration [91]. So, in the current study, based on previous experiment and my current trial and error it was important to inject WS-12 repeatedly over that period of indomethacin treatment to get the required protective effect of WS-12. Control animals received 0.05% CMC alone. Then, they were sacrificed 48 h indomethacin administration, and each mouse was intravenously injected with 100 μL of 1% (w/v) Evans blue solution 30 min before sacrifice to examine for intestinal injury [92].

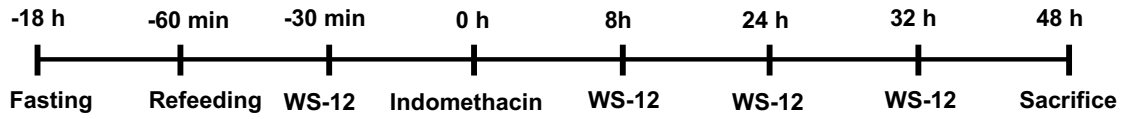

Figure 5. Induction of indomethacin-induced intestinal lesion

2.4 Macroscopic and histological observations

The small intestine was excised, washed with saline, fixed in 2% (v/v) formalin, opened longitudinally, and examined for injury under a dissecting microscope (S6D, Leica, Wetzlar, Germany) at a magnification of 10×. The number of lesions areas (mm^2) with visible injury were evaluated. The intestinal tissues were fixed in 10% (v/v) neutralized formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin. Histological injury was observed under a microscope (BX51, Olympus, Tokyo, Japan) at a magnification of 100×.

2.5 Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was assessed, as previously described but with some modifications [93]. The animals were sacrificed 48 h following indomethacin administration, and the small intestines were excised. Small intestinal tissues were homogenized in 50 mM phosphate buffer containing 0.5% (w/v) hexadecyltrimethylammonium bromide (pH: 6.0; Wako Pure Chemical Industries Ltd., Osaka, Japan). The homogenate samples were subjected to three freeze-thawing cycles and then centrifuged at 2,000 × g at 4°C for 10 min. The supernatant was added to 10 mM phosphate buffer (pH 6.0) with *o*-dianisidine HCl (Sigma-Aldrich Corp., St. Louis, MO, the USA) containing hydrogen peroxide. Changes in absorbance at a wavelength equal to 450 nm were recorded using MULTISKAN 60 (Thermo Fisher Scientific Inc., Waltham, MA, the USA). The MPO volumes were interpolated from a standard reaction curve for commercial MPO (Sigma-Aldrich Corp., St. Louis, MO, the USA). The Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, the USA) was used to measure protein content. The MPO activity was presented as micromoles H_2O_2/mg protein.

2.6 Determination of cytokine expression

The animals were sacrificed 48 h following indomethacin administration. Next, small intestinal tissues were removed, washed with cold phosphate-buffered saline (PBS), and stored in RNAlater (Ambion, Austin, TX, the USA) at 4°C until use. Tissue RNA was extracted using Sepasol RNA I Super G (Nacalai Tesque Inc., Kyoto, Japan), reverse transcribed with PrimeScript RT Master Mix (TaKaRa

Bio Inc., Kusatsu, Shiga, Japan), and analyzed quantitatively via polymerase chain reaction on the Thermal Cycler Dice Real-Time System (TaKaRa Bio Inc.) with the SYBR Premix ExTaq II (TaKaRa Bio Inc.). Predesigned primer sets for mouse interleukin (IL)-1β (MA025939) and tumor necrosis factor-alpha (TNF-α) (MA097070) were obtained from the Perfect Real-Time Supporting System (TaKaRa Bio Inc.). The mRNA expression level was standardized to that of TATA-binding protein (TBP).

2.7 Statistical analyses

Data were presented as means ± standard error of the mean. Statistical analyses were performed using the GraphPad Prism software version 6.0 h (GraphPad Software, La Jolla, CA, the USA). Multiple groups were compared using one- or two-way analysis of variance, followed by Holm–Sidak's multiple comparison test. Two group data were compared using the student's *t*-test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1 Effects of TRPM8 deficiency on indomethacin-induced intestinal injury

Indomethacin at a single dose caused injury along the small intestine, from the distal jejunum to the ileum, 48 h following indomethacin injection. Macroscopic hemorrhagic intestinal lesions were observed mostly in the ileum in both WT and TRPM8KO mice. However, the severity of the intestinal injury was apparently increased in TRPM8KO mice compared with WT mice. Indeed, the area of hemorrhagic injury visualized using Evans blue was significantly increased in TRPM8KO mice compared with the wild-type (WT) mice (Figure 6A and B). Two-way ANOVA revealed a significant main effect of indomethacin ($^{*}F\ (1, 24) = 36.03$, $P < 0.0001$), and a significant main effect of TRPM8KO ($^{\#}F\ (1, 24) = 233.0$ $P < 0.0001$). Based on the histological assessment, the area of the indomethacin-induced intestinal injury was larger in TRPM8KO mice than those in WT mice. That is, it had reached the muscularis mucosa in TRPM8KO mice (Figure 6C).

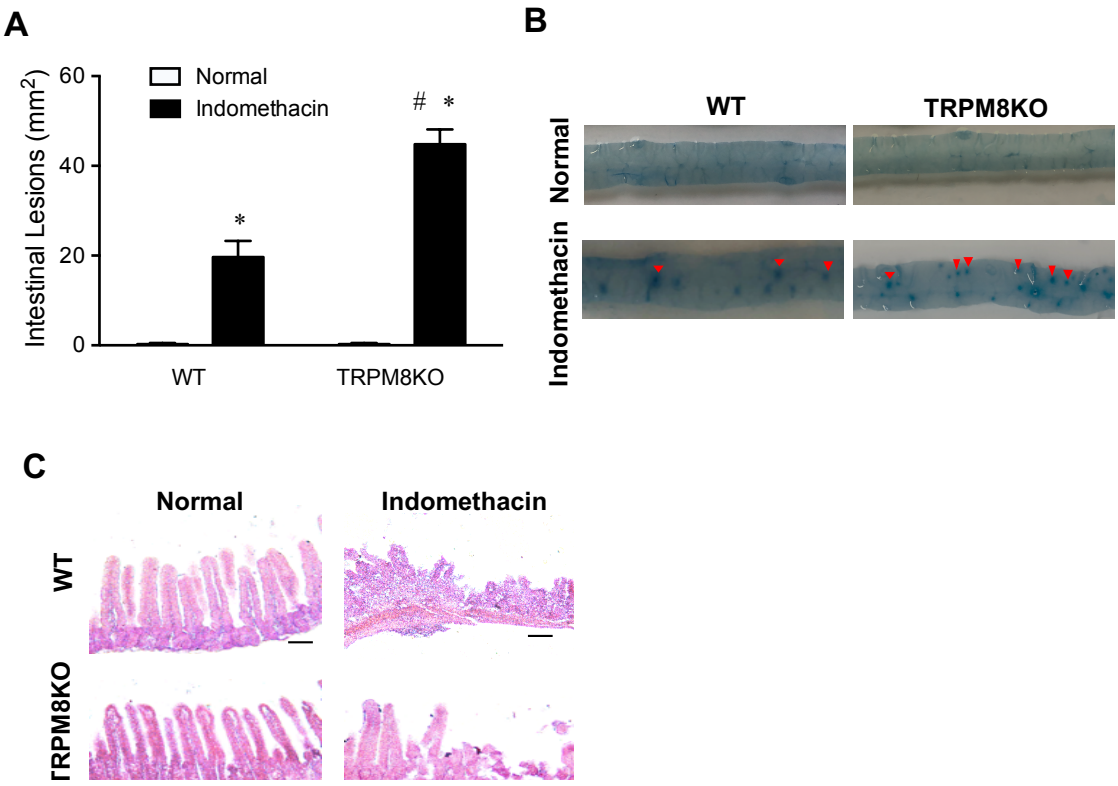

Figure 6. Indomethacin-induced intestinal injury in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice. Indomethacin (8 mg/kg) was administered subcutaneously, and the presence of intestinal injury was examined after 48 h following indomethacin injection (A) The area of intestinal injury and (B) typical macroscopic and (C) histological observations in normal (vehicle alone) and indomethacin-treated WT and TRPM8KO mice. Scale bars = 100 μm. Red arrows indicate intestinal lesion areas. Data were presented as means ± standard error of the mean (n = 6); $^{*}P < 0.05$ relative to the corresponding normal; $^{\#}P < 0.05$ relative to WT mice. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957. Figure 1(A-C).***

3.2 Effects of TRPM8 deficiency on an indomethacin-induced increase in MPO activity and inflammatory cytokine expressions in the intestinal mucosa

Indomethacin administration increased intestinal MPO activity, an index of neutrophil infiltration, and the inflammatory cytokines IL-1β and TNF-α mRNA expressions in WT mice. TRPM8KO mice showed further increases in the MPO activity (Figure 7A). Two-way ANOVA revealed a significant main effect of indomethacin ($^{*}F$ (1, 24) = 35.09 $P < 0.0001$), and a significant main effect of TRPM8KO ($^{\#}F$ (1, 23) = 4.559 P = 0.0436). In contrast, further enhancement of IL-1β and TNF-α mRNA expressions were not detected in TRPM8KO mice; the expression levels were similar in both mice (Figure 7B and C). and absence of significant effect of TRPM8KO ($^{*}F$ (1, 22) = 178.2 $P < 0.0001$), and absence of significant effect of TRPM8KO ($^{*}F$ (1, 26) = 43.40 $P < 0.0001$).

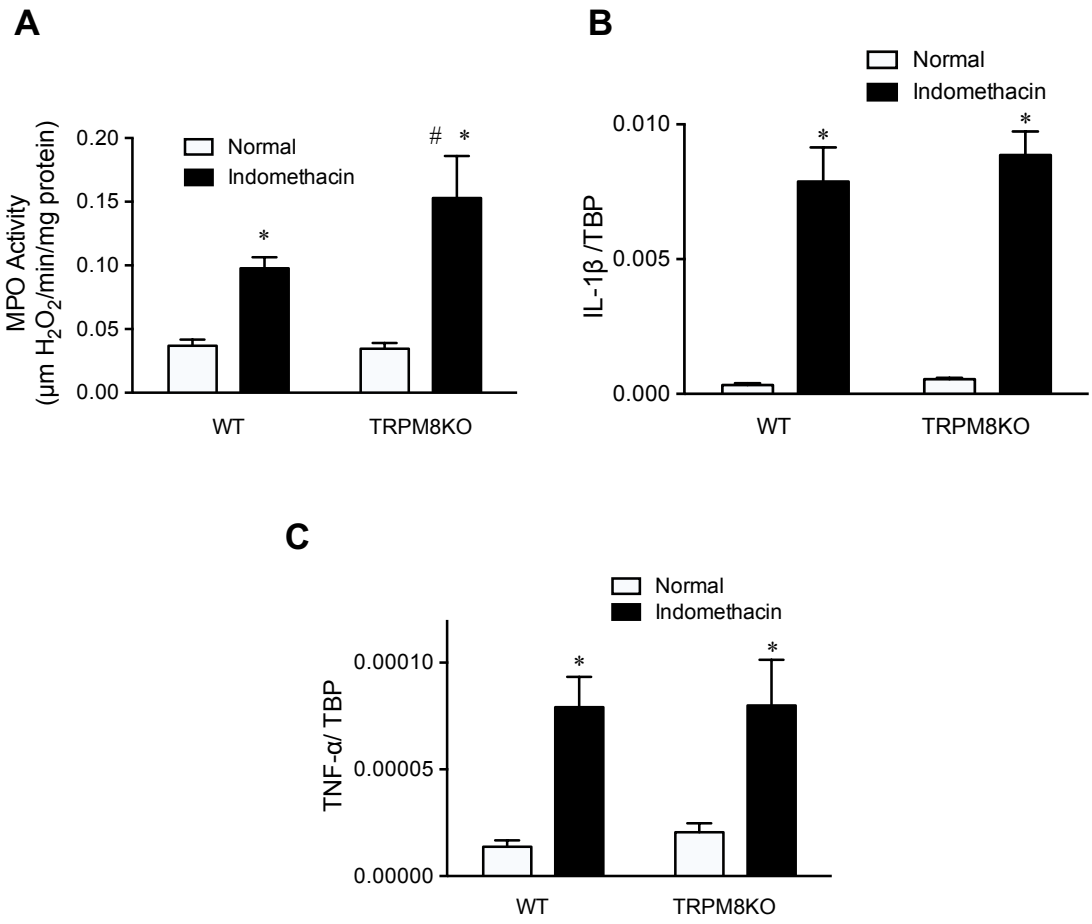

Figure 7. Changes in myeloperoxidase (MPO) activity and cytokine expression in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice after indomethacin administration. Indomethacin (8 mg/kg) was administered subcutaneously, and the MPO activity and cytokine expression in the small intestine were evaluated after 48 h following indomethacin injection. (A) The MPO activities were determined using the o-dianisidine method, and (B) interleukin-1β and (C) tumor necrosis factor-alpha mRNA expressions were determined using quantitative real-time polymerase chain reaction in normal (vehicle alone) and indomethacin-treated WT and TRPM8KO mice. The mRNA expression level was standardized to that of TATA-binding protein (TBP). Data were presented as means ± standard error of the mean (n = 5); *P < 0.05 relative to the corresponding normal; #P < 0.05 relative to WT mice. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957*****. Figure 2(A-C)**

3.3 Effects of TRPM8 agonist on indomethacin-induced intestinal injury

Then, I investigated the effect of TRPM8 activation using a specific TRPM8 agonist on indomethacin-induced intestinal injury to confirm the protective role of TRPM8. The repeated administration of WS-12 (3 and 10 mg/kg) dose-dependently reduced the severity of macroscopic intestinal injury in WT mice whereas no effects of WS-12 were found in TRPM8KO mice (Figure 8A and B). Using quantitative data, the lesion area was decreased by the administration of WS-12 partially at a dose of 3 mg/kg and significantly at a dose of 10 mg/kg. One-away ANOVA revealed a significant main effect of WS-12 in dose of 10 mg/kg ($t = 2.2$, $df = 8$, $P = 0.0566$) and significantly at a dose of 10 mg/kg ($^{*}F(2, 13) = 11.97$, $P = 0.0011$). However, this effect was totally abrogated in TRPM8KO mice. Student T-test revealed absence of significant effect of WS-12 in TRPM8KO mice ($t = 0.8035$, $df=13$, $P = 0.4361$).

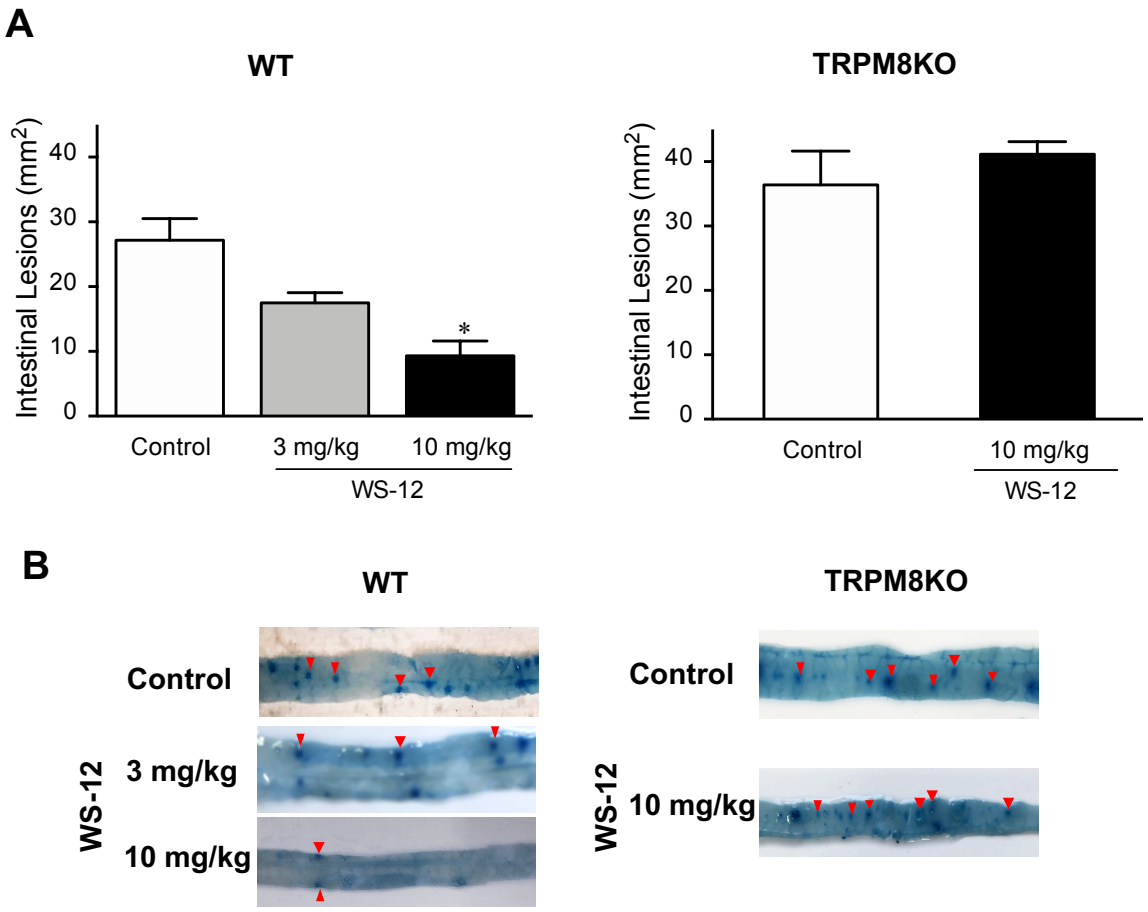

Figure 8. Effect of WS-12 on indomethacin-induced intestinal injury in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice. Indomethacin (8 mg/kg) was administered subcutaneously, and intestinal injury was examined after 48 h following indomethacin injection. WS-12 (3 and 10 mg/kg) was intraperitoneally administered 30 min before and 8, 24, and 32 h after indomethacin administration. (A) The area of intestinal injury, (B) typical macroscopic in controls (vehicle alone for WS-12) and WS-12-treated WT and TRPM8KO mice. Data were presented as means ± standard error of the mean (n = 6), in WT group receiving 3 mg/kg (n= 4); *P < 0.05 relative to control. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957*. Figure 3(A, B).**

3.4 The effects of TRPM8 agonist on histological observations and MPO on indomethacin-induced intestinal injury

Further, indomethacin-induced histological intestinal injury in WT mice was attenuated by WS-12 (10 mg/kg) whereas no effects of WS-12 were found in TRPM8KO mice (Figure 9A). Indomethacin-induced intestinal MPO activity upregulation was also attenuated by WS-12 (10 mg/kg). Two-way ANOVA revealed a significant main effect of WS-12 in WT mice ($^{*}F$ (1, 15) = 60.46, P < 0.0001) ($^{\#}F$ (1, 15) = 9.921, P = 0.0066). In contrast, the protective effects of WS-12 (10 mg/kg) against indomethacin-induced MPO upregulation were completely abrogated in TRPM8KO mice (Figure 9B). There was no significant difference in MPO activity between the control (CMC alone) and WS-12-treated groups in TRPM8KO mice. Two-way ANOVA revealed that no effects of WS-12 were found in TRPM8KO mice (F (1, 15) = 8.032, P = 0.0126).

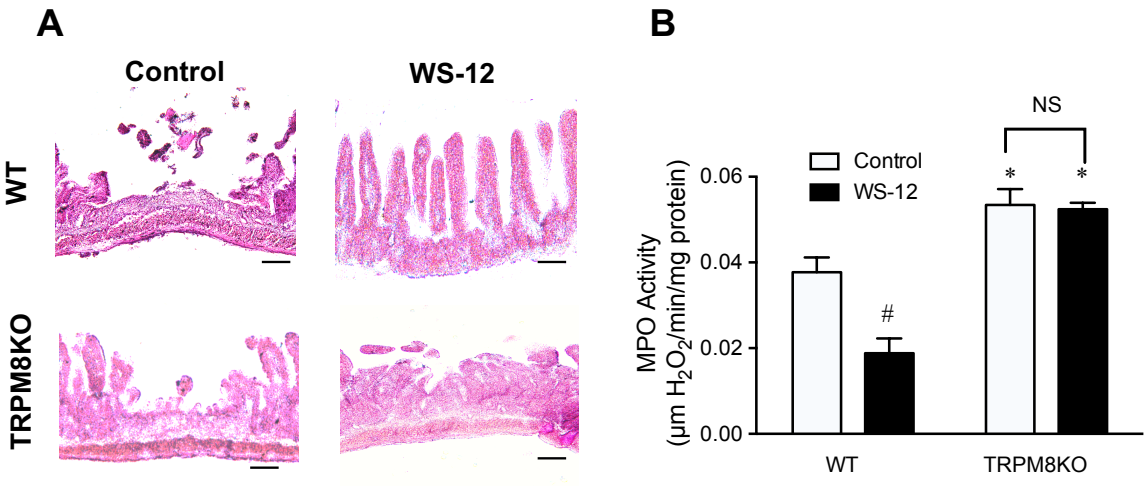

Figure 9. Effects of WS-12 on indomethacin-induced intestinal injury in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice. Indomethacin (8 mg/kg) was administered subcutaneously, and intestinal injury was examined after 48 h following indomethacin injection. WS-12 (10 mg/kg) was intraperitoneally administered 30 min before and 8, 24, and 32 h after indomethacin administration. (A) histological observations, and (B) myeloperoxidase (MPO) activity in controls (vehicle alone for WS-12) and WS-12-treated WT and TRPM8KO mice. Scale bars = 100 μm. Data were presented as means ± standard error of the mean (n = 5), in WT control group (n = 4); $^{\#}P < 0.05$ relative to control. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957*. Figure 3(C, D).**

4. Discussion

Several reports showed the roles of TRP channels and sensory afferent neurons in the regulation of intestinal inflammation [46, 94]. For instance, TRPV1 and TRPA1 contributed to the progression of colonic inflammation in DSS-induced colitis in mice [46]. Likewise, DSS-induced colitis was significantly attenuated in TRPV4KO as compared to WT mice [91]. Moreover, exogenous activation of TRPM8 by icilin shows an anti-inflammatory effect in mice with DSS-induced colitis model [25].

The current study found that indomethacin-induced small intestinal injury was significantly enhanced via the genetic deletion of TRPM8 whereas it was remarkably inhibited via the pharmacological activation of TRPM8 in mice. Therefore, I hypothesized that TRPM8 plays a protective role against indomethacin-induced small intestinal injury. NSAIDs cause injury in the stomach and the small intestine in humans and experimental animals [69, 95]. In terms of pathophysiological similarities, indomethacin-induced small intestinal injury in rodents may serve as a useful animal model for assessing NSAID-induced enteropathy as well as enteritis in Crohn's disease [88]. Several inflammatory responses, such as infiltration of neutrophils and upregulation of inflammatory cytokines, are involved in indomethacin-induced small intestinal injury [96]. TNF-α and IL-1β were considered essential mediators in IBD. Our previous studies suggested that the pathogenic mechanism of indomethacin-induced small intestinal lesions involves nitric oxide, neutrophils, and TNF-α, as well as enterobacteria. It may be considered that the translocation of enterobacteria in the mucosa is the first step required to activate various factors such as nitric oxide production and neutrophil activation. These factors together were resulting in the occurrence of intestinal lesions following administration of indomethacin [95]. In addition, neutrophils may exacerbate intestinal inflammatory diseases via the secretion of proteolytic enzymes and reactive oxygen and nitrogen intermediates [97]. In the current study, I observed that indomethacin-induced small intestinal injury was accompanied by an increase in mucosal MPO activity that was significantly enhanced in TRPM8KO mice compared with WT mice. As previously reported, upregulation of inflammatory cytokines such as TNF-α and IL-1β in the small intestine following indomethacin administration [93, 98]. However, in the current study, the cytokine expressions in TRPM8KO mice compared with WT mice were not further assessed. This notion should also be considered because the expression of these cytokines may be maximally upregulated to cause severe intestinal injury even in WT mice.

Similar to the current study results, Jong et al. [25] previously reported that TRPM8KO mice treated with DSS were more susceptible to experimentally induced colitis than WT mice. In contrast, Ramachandran et al. showed that the pharmacological activation of TRPM8 by icilin, a TRPM8 agonist, has anti-colitis effects in the DSS-colitis model [26]. However, TRPM8KO did not enhance the severity

of colitis in experimental colitis models induced by DSS and trinitrobenzene sulfonic acid (TNBS). To validate these differences, the current study examined the effect of WS-12, a significantly more selective agonist menthol derivative, on indomethacin-induced small intestinal injury [91].

TRPM8 is activated by several chemical agonists known to produce cool sensations, such as menthol, icilin, and eucalyptol [99]. Eucalyptol was found to attenuate inflammation and showed strong anti-inflammatory and analgesic effects that were absent in TRPM8KO mice [100]. In traditional medicine, menthol was used as an agent against abdominal discomfort and visceral pain [101]. Previous studies proved that treatment with the TRPM8 agonist menthol produced gastroprotective action through anti-apoptotic, immunomodulatory, and anti-inflammatory mechanisms that reduced TNF-α and IL-6 expression and increased the anti-inflammatory cytokine, IL-10 expression in the gastric mucosa [102]. In the current study, I used WS-12, a significantly more selective TRPM8 agonist, to investigate the role of TRPM8 activation on indomethacin-induced small intestinal injury [91]. The chemically related menthol analog, WS-12, had a half-maximal effective concentration value about 2,000 times less than that of menthol and is, consequently, the highest affinity TRPM8 ligand known to date [103].

Taken together, these findings in this chapter indicate the anti-inflammatory role of TRPM8 in indomethacin-induced small intestinal injury with attenuation of neutrophil infiltration and inflammatory cytokine expression.

Chapter II: TRPM8 expression profiles in the small intestine based on the role of CGRP and SP in the pathogenesis of indomethacin-induced intestinal injury.

1. Background

The expression of extrinsic sensory neurons by the TRP channel is considered a communication mechanism that modulates immune and inflammatory responses in the GI tract [37]. For instance, TRPV1 and TRPA1 play regulatory roles in intestinal inflammation via CGRP-mediated protective and SP-mediated deleterious mechanisms [104-106]. Furthermore, nerve growth factor (NGF) activates its receptor (tropomyosin receptor kinase A) TrkA, resulting in regulating ion channels, receptors, and signaling molecules with the involvement of CGRP [107]. These facts encouraged me in the current study to further investigate the protective role of TRPM8 based on neuropeptides release pathways.

My results and conclusions about TRPM8 deletion and activation following indomethacin injection in chapter I strongly suggest the anti-inflammatory role of TRPM8 in indomethacin-induced small intestinal injury. In the current chapter, the expression of TRPM8 in the small intestine based on the role of neuropeptides such as CGRP and SP in the pathogenesis of indomethacin-induced intestinal injury was immunohistochemically characterized. My findings suggest that TRPM8 contributes to the production of CGRP in injured intestine mucosa and may have a protective effect against indomethacin-induced small intestinal injury. In contrast, TRPM8 on the sensory afferent neurons may play a role in the progression of small intestinal injury in a SP-independent manner. In this chapter, I hypothesized that the involvement of neuropeptides, specially CGRP, with its well-known mucosal defense mechanisms, has an essential role in the protective effect of the TRPM8.

2. Materials and Methods

2.1 Animals

In this study, 8–10-week-old male C57BL/6J mice weighing 22–27 g were purchased from Japan SLC (Shizuoka, Japan). TRPM8-EGFP transgenic mice, which express EGFP under the direction of TRPM8 promoter, were established, as described previously [108]. All mice were housed in plastic cages with free access to food and water, and the temperature was maintained at 22°C ± 1°C under a 12-h light/dark cycle. All experimental animal procedures were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (Permission numbers: 18-004 and 19-004). Animal suffering was kept to a minimum whenever possible, and the smallest number of animals required for significant data interpretation was used.

2.2 Determination of CGRP Levels

The CGRP content in ileum homogenates was analyzed using enzyme immunoassay (EIA) (Bertin Bioreagent, France). Tissue preparation and analysis were performed based on the manufacturer's instructions. Data were presented as picogram per milligram (pg/mg) tissue weight.

2.3 Western blot analysis

Tissue preparation was performed, as described by Eijkelkamp et al. [109]. The proteins were separated using 10% -sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred into polyvinylidene fluoride or polyvinylidene difluoride membranes (Millipore, Bedford, MA, the USA) via electroblotting. The membranes were blocked with 5% (w/v) skim milk dissolved in Tween-PBS. The membranes were immunoreacted with rabbit anti-TRPM8 (1:500; Alomone Labs, Jerusalem.) and rabbit anti-β-actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, the USA) antibodies and were incubated overnight at 4°C. Then, they were stained with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) at room temperature for 2 h. Antibodies were diluted using tween tris-buffered saline (T-TBS). Immunoreactivity was detected via enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA, the USA), and the dominant band density was determined using the FUSION Solo 5 software (Vilber Lourmat, Marne-la-Vallée, France). The expression levels were normalized to that of β-actin.

2.4 Retrograde labeling

The origin of the primary afferent innervation to the mouse ileum was determined via retrograde tracing using fluorescent fluorogold dye (FG; Fluorochrome, Denver, Colorado, the USA). Approximately 5 µL of 4% fluorogold was injected circumferentially into the ileum at five sites under 2% isoflurane anesthesia. Tissue recovery occurred 4 days after fluorogold administration.

2.5 Immunohistochemistry

Immunohistochemical procedures were performed by Matsumoto et al., as previously described [110]. Small intestine DRG segments were collected, fixed for 2 h at 4°C via immersion in fresh 4% paraformaldehyde in 0.1 M phosphate buffer, and washed three times with PBS. The sections were then cryoprotected overnight in 0.1 M phosphate buffer containing 20% sucrose and were frozen in an optimal cutting temperature compound mounting medium (Sakura Finetek, Tokyo, Japan). Then, they were sectioned at a thickness of 30 µm for small intestinal samples and 10 µm for DRG samples on a cryostat (Leica Instruments) and were fixed into Super Frost Plus slides (Matsunami Glass, Osaka, Japan). First, the glass-mounted sections were incubated for 1 h each in 10% donkey serum containing 0.2% Triton X-100 and then washed three times with PBS for 10 min each. In the next steps, all the antibodies were diluted using 2% triton which was diluted using PBS with the addition of sodium azide. Subsequently, the sections were immunoreacted overnight at room temperature with sheep anti-CGRP antibodies. After washing in PBS, the sections were immunoreacted for 3 h at room temperature with Alexa Fluor 594 donkey anti-sheep IgG secondary antibodies. Then, they were immunoreacted overnight at room temperature with rabbit anti-PGP9.5 antibodies. After washing with PBS, the sections were immunoreacted for 3 h at room temperature with Alexa Fluor 488 donkey anti-rabbit IgG antibodies diluted using PBS.

In another experiment, the sections were immunoreacted overnight at room temperature with chicken anti-GFP antibodies. After washing with PBS, the sections were immunoreacted for 3 h at room temperature with Alexa Fluor 488 donkey anti-chicken IgG antibodies. They were immunoreacted overnight at room temperature with either sheep anti-CGRP or rabbit anti-SP antibodies, rabbit anti-PGP9.5 or goat anti-TrkA antibodies. After washing with PBS, the sections were immunoreacted for 3 h at room temperature with either Alexa Fluor 594 donkey anti-sheep IgG, Alexa Flour 594 donkey anti-rabbit IgG, or Alexa Flour 594 donkey anti-goat IgG. Optimized dilutions and the sources of the primary and secondary antibodies are listed in Tables 1 and 2, respectively.

Table 1. Primary antibodies used in this study.

Antigen	Host	Dilution	Source	Catalogue Number
CGRP	Sheep	1:2000	Enzo Life Sciences, Inc.	BML-CA1137
PGP 9.5	Rabbit	1:5000	Abcam, Cambridge, UK	Ab108986
GFP	Chicken	1:5000 (Ileum)	Cosmo Bio, LTD.	GFP-1010
GFP	Chicken	1:10000 (DRG)	Cosmo Bio, LTD.	GFP-1010
SP	Rabbit	1:1000	Immunostar, Hudson, USA	20064
TRKA	Goat	1:500	R&D Systems, USA	AF1056

Table 2. Secondary antibodies used in this study.

Secondary antibody	Conjugate probe	Dilution	Source	Catalogue Number
Donkey anti-sheep IgG	Alexa Fluor 594	1:800	Thermo Fisher Scientific. Inc. USA	A11016
Donkey anti-rabbit IgG	Alexa Fluor 594	1:800	Thermo Fisher Scientific. Inc. USA	A21207
Donkey anti-chicken IgG	Alexa Fluor 488	1:800 (Ileum)	Jackson ImmunoResearch. USA	703-546-155
Donkey anti-chicken IgG	Alexa Fluor 488	1:800 (DRG)	Jackson ImmunoResearch. USA	703-546-155
Donkey anti-goat IgG	Alexa Fluor 594	1:800	Thermo Fisher Scientific. Inc. USA	A11058
Donkey anti-rabbit IgG	Biotin-SP	1:400	Jackson ImmunoResearch. USA	711-066-152

The sections were evaluated using a confocal laser scanning microscope ($A1R^{+}$; Nikon, Tokyo, Japan) at a magnification of 200× or 100×. The Nikon NIS-Elements AR 4.20.00 software was used to capture images. The ImageJ software was utilized to quantify the positive signals for CGRP, SP, and TRPM8 expression. The positive areas of CGRP, SP, and TRPM8 were evaluated using adjusting threshold method and divided by the total intestinal villi areas. Next, the results were presented as a percentage of the total villi area.

2.6 Determination of NGF expression

The animals were sacrificed 48 h following indomethacin administration. Next, small intestinal tissues were removed, washed with cold PBS, and stored in RNAlater (Ambion) at 4°C until use. Tissue

RNA was extracted using Sepasol RNA I Super G (Nacalai Tesque Inc.), reverse transcribed with PrimeScript RT Master Mix (TaKaRa Bio Inc.) and analyzed quantitatively via polymerase chain reaction on the Thermal Cycler Dice Real-Time System (TaKaRa Bio Inc.) with the SYBR Premix ExTaq II (TaKaRa Bio Inc.). Predesigned primer sets for mouse NGF (RA066038) were obtained from the Perfect Real-Time Supporting System (TaKaRa Bio Inc.). The mRNA expression level was standardized to that of TBP.

2.7 Statistical analyses

Data were presented as means ± standard error of the mean. Statistical analyses were performed using the GraphPad Prism software version 6.0 h (GraphPad Software, La Jolla, CA, the USA). Multiple groups were compared using one- or two-way analysis of variance, followed by Holm–Sidak's multiple comparison test. Two group data were compared using the student's *t*-test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1 Changes in TRPM8 expression in indomethacin-induced intestinal injury

I investigated the expression of TRPM8 in the intestinal mucosa via immunohistochemical examination in TRPM8-EGFP transgenic mice and via Western blot analysis using an anti-TRPM8 antibody (Figure 10). I detected EGFP signals representing TRPM8 expression in the mucosa of control mice (Figure 10A). This expression appeared to increase in indomethacin-treated mice. Using quantitative analysis, I found that the area of TRPM8-positive neurons significantly increased 48 h following indomethacin administration (Figure 10B). Student t-test revealed a significant main effect of indomethacin ($t = 3.782$, $df = 12$, $P = 0.0026$). Similarly, Western blot analyses showed that the protein expression of TRPM8 was increased in the small intestine following indomethacin treatment, but was not statistically significant ($t = 1.288$, $df = 10$, $P = 0.2266$).

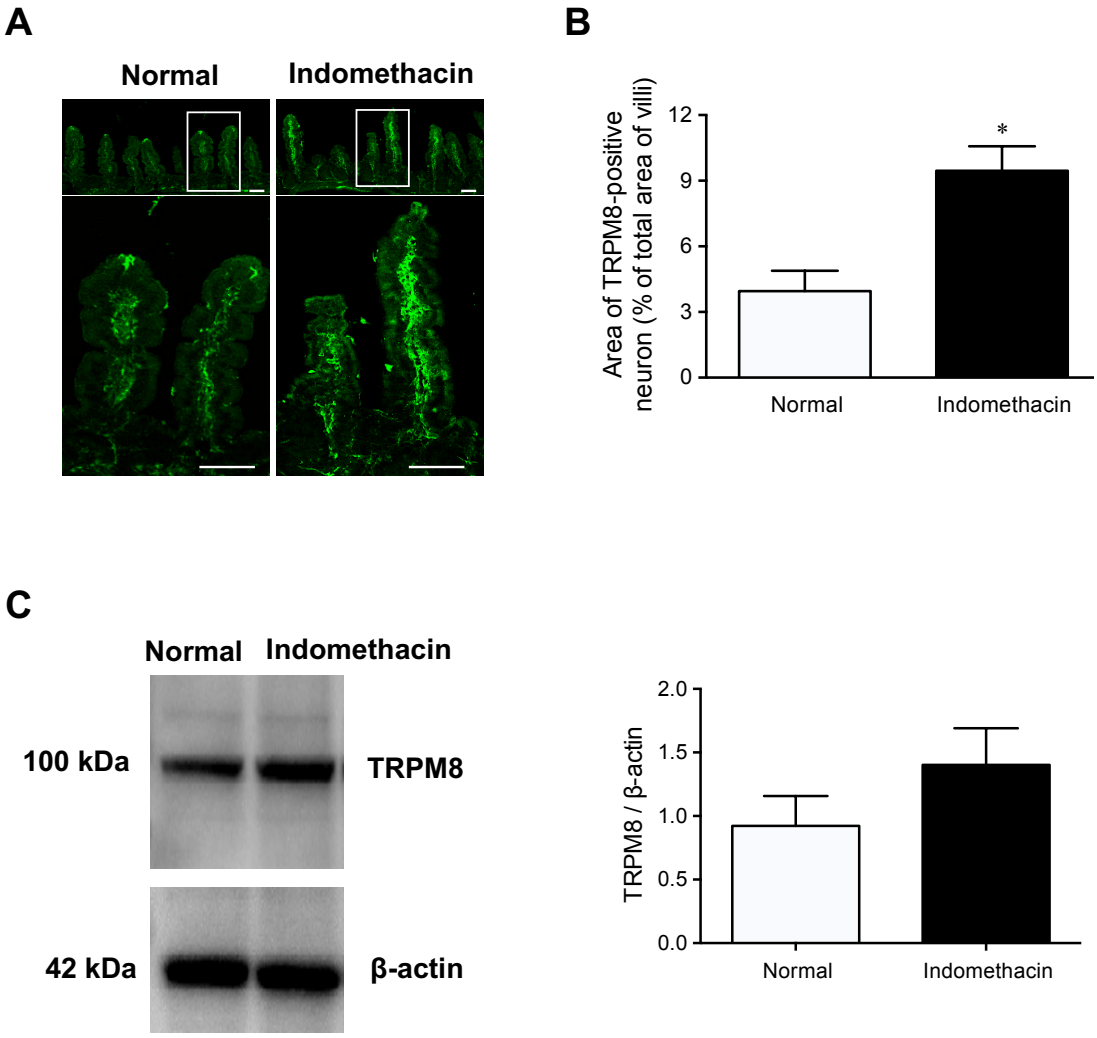

Figure 10. Changes in TRPM8 expression in the intestinal mucosa in mice with indomethacin-induced intestinal injury. The expression of TRPM8 was detected via immunohistochemistry (A, B) and Western blot analysis © in normal (vehicle alone) and indomethacin-treated wild-type (WT) mice. (A) Typical images and (B) quantified area of TRPM8-promoting enhanced green fluorescent protein (EGFP) signals presented as a percentage of total villi area in normal and indomethacin-treated mice. Scale bars = 100 μm. (C) The expression of TRPM8 and β-actin in the small intestine of normal and indomethacin-treated mice. Data were presented as means ± standard error of the mean (n = 6); $^{*}P <$ 0.05 relative to the normal group. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957*. Figure 4 (A-C).**

3.2 Expression profiles of TRPM8 immunoreactivities in the small intestine

Then I characterized the expression profiles of TRPM8 in the small intestine via immunohistochemistry staining using TRPM8-EGFP mice and several neural markers such as pan neural marker (PGP9.5), sensory neural markers (CGRP, and SP) (Figure 11). TRPM8-EGFP signals were colocalized with PGP9.5-immunopositive nerve fibers, indicating the presence of TRPM8-expressing neurons in the small intestine. Furthermore, TRPM8-EGFP signals were highly colocalized with CGRP-immunopositive neurons and partially colocalized with SP immunoreactivities in the small intestine. Thus, TRPM8-immunopositive neurons expressed mainly CGRP-positive neurons and partly SP-positive sensory neurons.

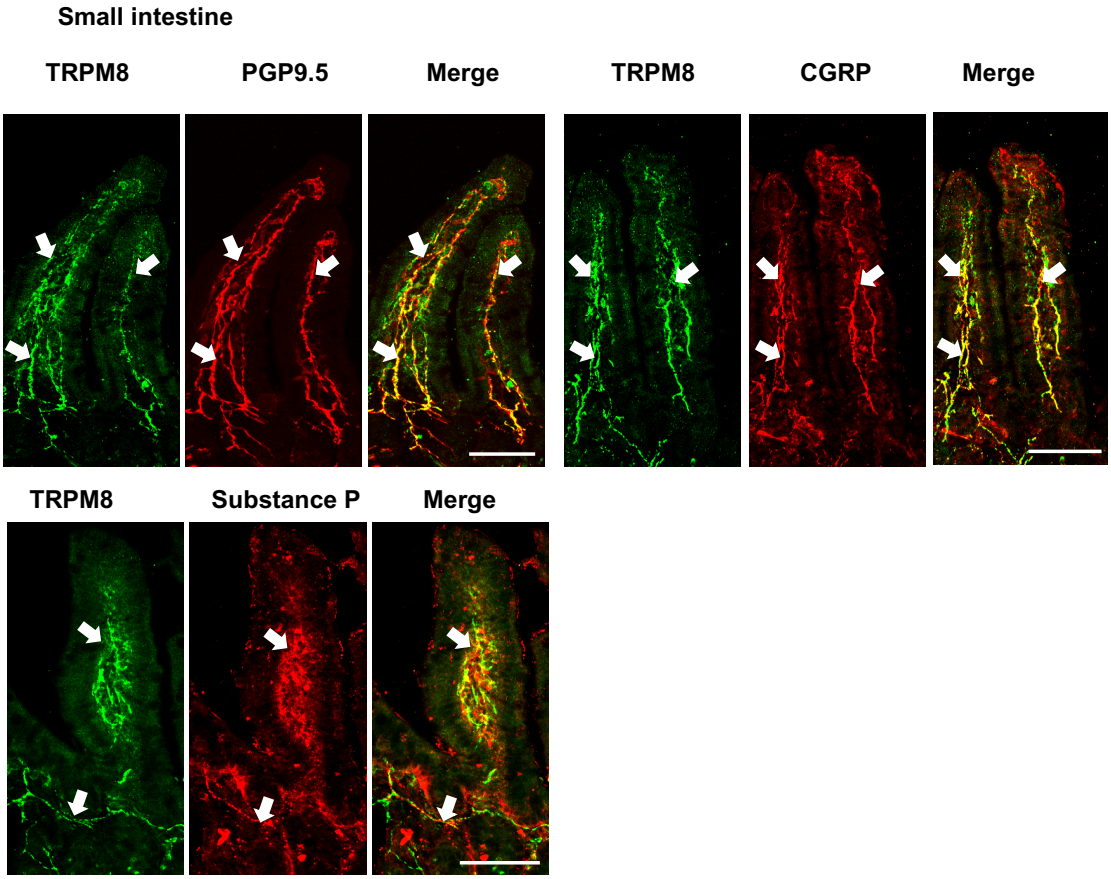

Figure 11. Expression profiles of TRPM8-immunoreactivities in the normal small intestine of TRPM8-enhanced green fluorescent protein (EGFP)-transgenic mice. Double labeling of TRPM8 (green) with PGP9.5 (red), calcitonin gene-related peptide (CGRP) (red), and substance P (SP) (red). Arrows indicate the colocalization of TRPM8-immunoreactivites with either PGP9.5, CGRP, or SP. Scale bars = 100 μm. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957.*** **Figure 5 (A).**

3.3 Characterization of TRPM8 immunoreactivities in dorsal root ganglia

Next, I investigated the origin of the primary afferent innervation of the small intestine using retrograde fluorescent tracer fluorogold dye (Figure 12). Most TRPM8-immunoreactive cell bodies were found in the DRG, and they were double-labeled with fluorogold retrogradely transported from the small intestine. I further observed the colocalization of TRPM8 with CGRP and SP in the DRG. TRPM8-EGFP signals were highly colocalized with CGRP-immunopositive neurons and partially colocalized with SP immunoreactivities. Taken together with the findings shown in Figure 11, TRPM8 was expressed in CGRP- and/or SP-positive primary afferent neurons originating from the DRG.

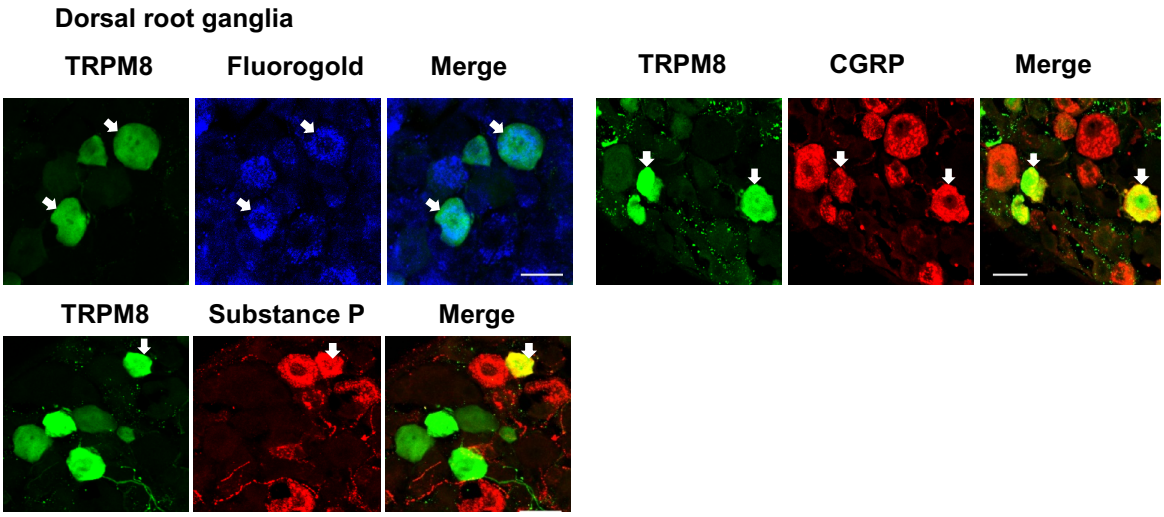

Figure 12. Expression profiles of TRPM8-immunoreactivities in dorsal root ganglia (DRG) of TRPM8-enhanced green fluorescent protein (EGFP)-transgenic mice. In DRG, double labeling of TRPM8 (green) with retrograde tracer fluorogold (blue), CGRP (red), and SP (red). Arrows indicate the colocalization of TRPM8 immunoreactivity with either fluorogold immunoreactive neurons, CGRP, and SP. Scale bars = 50 μm. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957.*** **Figure 5(B).**

3.4 Characterization of TrkA immunoreactivities in the small intestine

Then, I evaluated the immunoreactivity of neurotrophic TrkA, the receptor for NGF that are well known for having a relevant role in neuroimmune communication in the GI tract. I characterized localization of TrkA immunoreactivities in the small intestine via immunohistochemical double staining using TRPM8-EGFP mice and TrkA antibody (Figure 13). TRPM8-EGFP signals were colocalized with TrkA-immunopositive nerve fibers, indicating the presence of TrkA expressing neurons in the small intestine colocalized with TRPM8. Furthermore, TrkA signals were highly colocalized with CGRP-immunopositive neurons. Thus, TRPM8-immunopositive neurons expressed mainly TrkA-positive neurons, which also colocalize with CGRP.

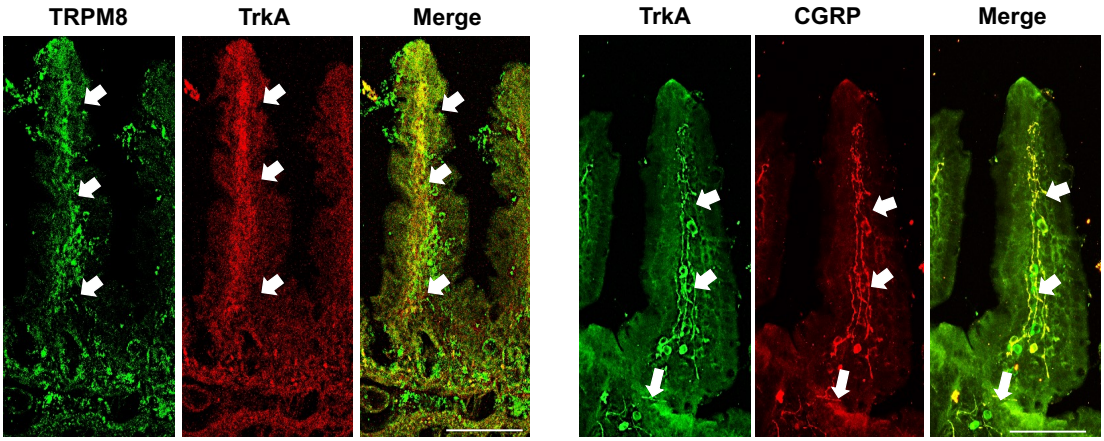

Figure 13. Expression profiles of TrkA-immunoreactivities in the small intestine of TRPM8-enhanced green fluorescent protein (EGFP)-transgenic mice. In the small intestine, double labeling of TRPM8 (green) with TrkA (Red), Arrows indicate the colocalization of TRPM8 immunoreactivity with TrkA immunoreactive neurons. Double labeling of TrkA (green) with CGRP (Red). Arrows indicate the colocalization of TrkA immunoreactivity with CGRP immunoreactive neurons. Scale bars = 50 μm.

3.5 Change in CGRP expressions in the mucosa in WT and TRPM8KO mice with intestinal injury

To better understand the role of CGRP in the pathogenesis of indomethacin-induced intestinal injury, I immunohistochemically investigated changes in CGRP expression in WT and TRPM8KO mice (Figure 14). The expression of CGRP in the intestinal mucosa was significantly upregulated following indomethacin administration in WT mice. However, this response was completely abrogated in TRPM8KO mice (Figure 14A and B). Two-way ANOVA revealed a significant main effect of indomethacin ($^{*}F$ (1, 27) = 5.856, P = 0.0225). To validate these results, the amount of CGRP in the intestinal tissues was assessed using EIA; indomethacin administration significantly increased the amount of CGRP in WT mice. However, this response was absent in TRPM8KO mice (Figure 14C). Two-way ANOVA revealed a significant main effect of indomethacin ($^{*}F$ (1, 18) = 4.776, P = 0.0423).

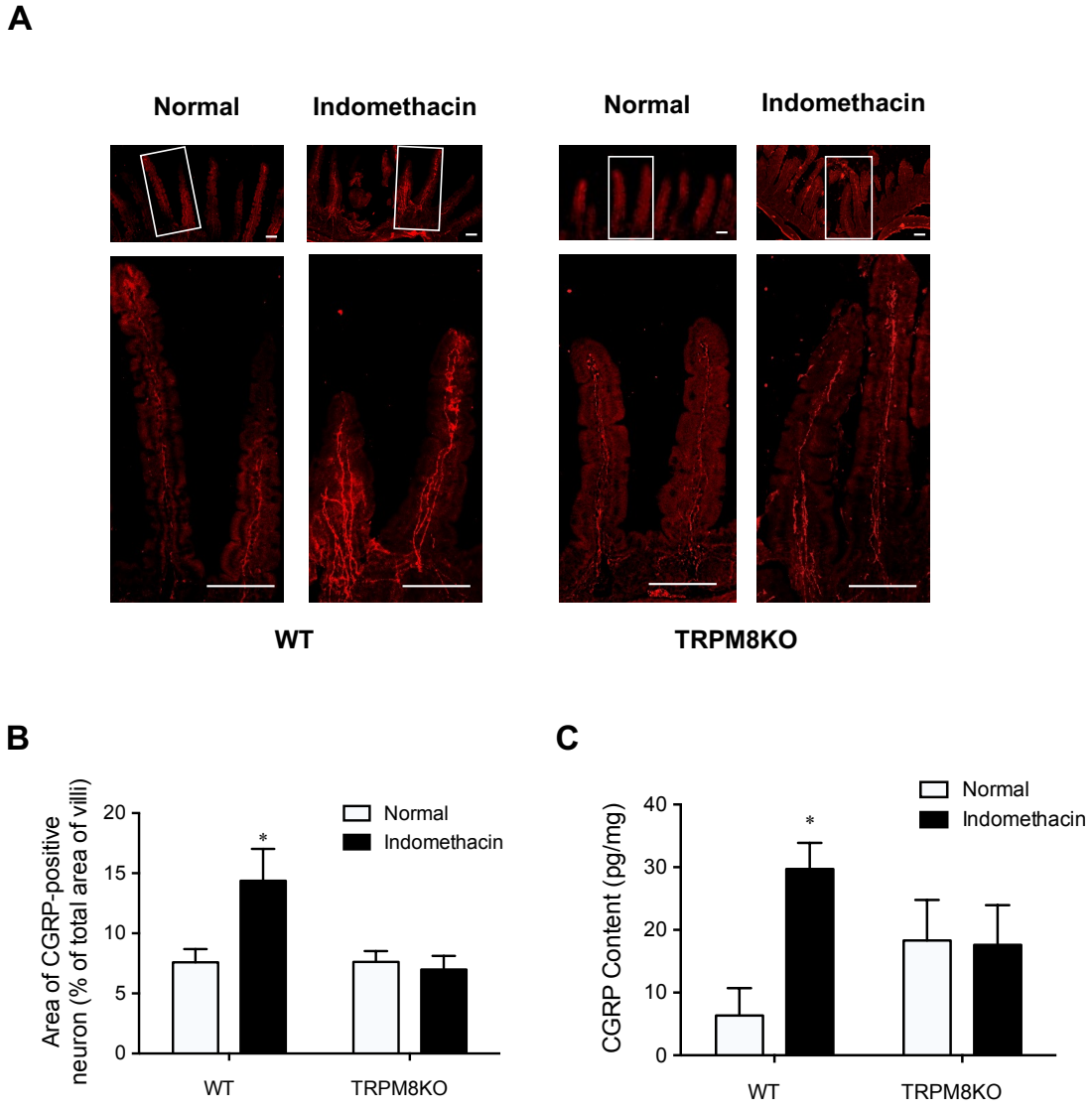

Figure 14. Changes in CGRP expression in the intestinal mucosa with indomethacin-induced intestinal injury in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice. **(**A) Typical images, (B) quantified area of CGRP-positive neurons presented as percentage of the total villi area, and (C) CGRP concentration (pg/mg in tissues) in intestinal homogenates determined using EIA in controls (vehicle alone) and indomethacin-injected WT and TRPM8KO mice. Scale bars = 100 μm. Data were presented as means ± standard errors of the mean (n = 5); *P < 0.05 relative to the WT normal. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957*****. Figure 6 (A-C).**

3.6 Change in SP expressions in the mucosa in WT and TRPM8KO mice with intestinal injury

To investigate the role of SP in the pathogenesis of indomethacin-induced intestinal injury, I immunohistochemically investigated changes in SP expression in WT and TRPM8KO mice (Figure 15). The expression of SP in the intestinal mucosa was significantly upregulated following indomethacin administration in both WT and TRPM8KO mice. The expression levels in WT and TRPM8KO mice were almost comparable (Figure 15A and B). Two-way ANOVA revealed a significant main effect of indomethacin ($^{*}F$ (1, 25) = 15.93, P = 0.0005). This result suggests that SP upregulation is brought about by the TRPM8-independent pathway.

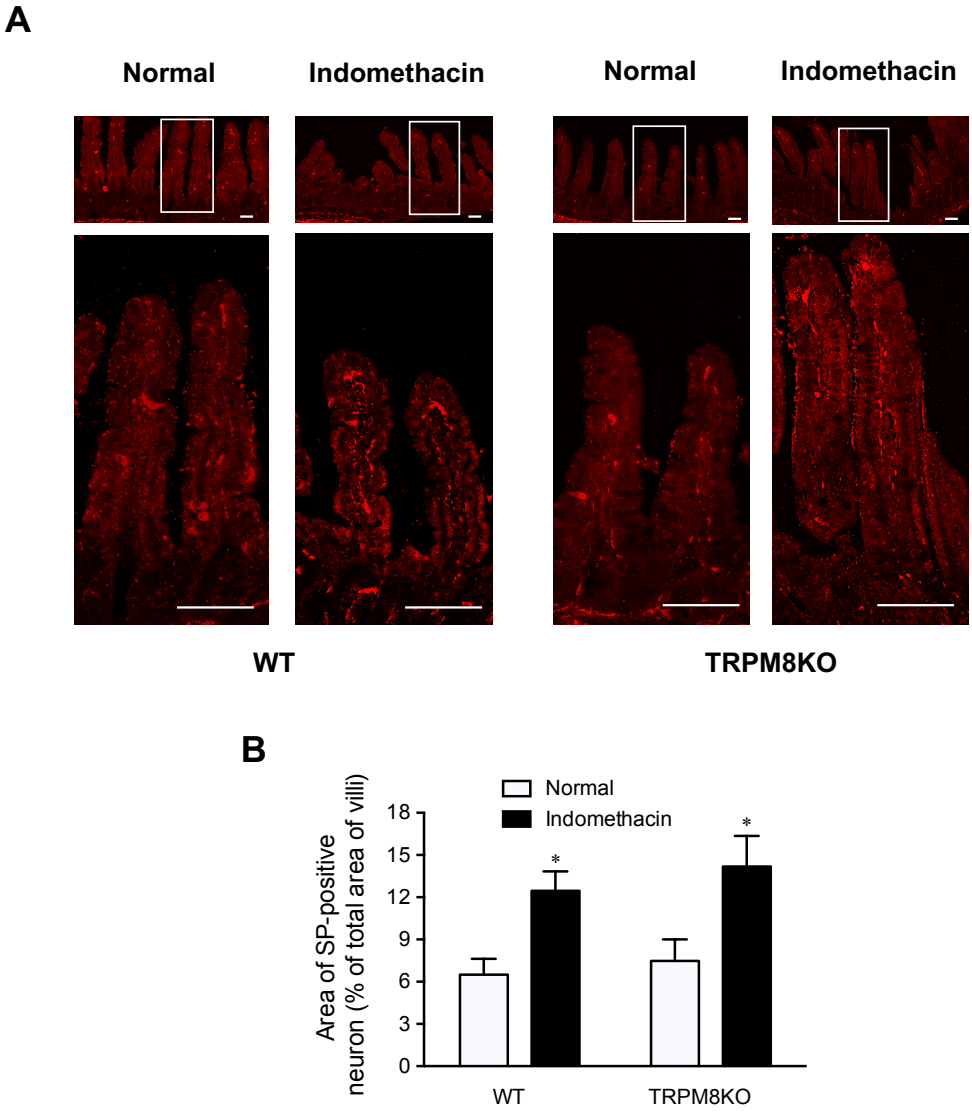

Figure 15. Changes in substance P (SP) expression in the intestinal mucosa with indomethacin-induced intestinal injury in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice. **(**A) Typical images and (B) quantified area of SP-positive neuron presented as percentage of the total villi area in normal (vehicle alone) and indomethacin-treated WT and TRPM8KO mice. Scale bars = 100 μm. Data were presented as means ± standard error of the mean (n = 5); *P < 0.05 relative to the corresponding normal. These figures are cited from ***Biol. Pharm. Bull. 2021, 44 (7), 947-957*****. Figure 7 (A, B).**

3.7 The effects of TRPM8 deficiency on NGF expressions in the intestinal mucosa

To better understand the relationship between NGF and CGRP pathways in the pathogenesis of indomethacin-induced intestinal injury, I investigated changes in NGF mRNA expression in WT and TRPM8KO mice (Figure 16). Besides having a well-described key role in inflammatory processes of the GI tract, NGF neurotrophin is relevant for promoting neuronal survival. The expression of NGF in the intestinal mucosa was significantly upregulated following indomethacin administration in WT mice. However, this response was completely abrogated in TRPM8KO mice. Two-way ANOVA revealed a significant main effect of indomethacin ($^{*}F$ (1, 17) = 4.838, P = 0.0420), and Two-way ANOVA revealed absence of significant effect of TRPM8KO ($^{\#}F$ (1, 17) = 6.136, P = 0.0240).

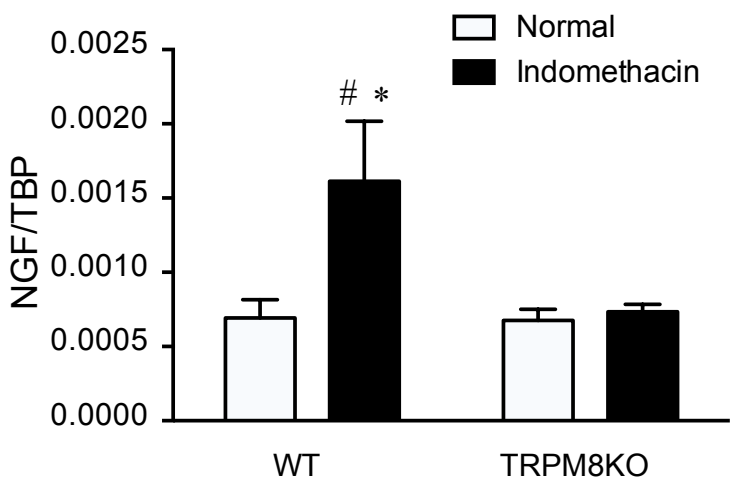

Figure 16. Changes in nerve growth factor (NGF) expression in wild-type (WT) and TRPM8-deficient (TRPM8KO) mice following indomethacin administration. Indomethacin (8 mg/kg) was administered subcutaneously, and the NGF expression in the small intestine was evaluated following 48 h after indomethacin injection. NGF mRNA expression was determined using quantitative real-time polymerase chain reaction in normal (vehicle alone) and indomethacin-treated WT and TRPM8KO mice. The mRNA expression level was standardized to that of TATA-binding protein (TBP). Data were presented as means ± standard error of the mean (n = 6); *P < 0.05 relative to the corresponding normal; #P < 0.05 relative to KO mice.

4. Discussion

Investigation of TRPM8 expression profile with related neuropeptides involved in its activation represents a crucial step to understand the protective effect of TRPM8 against NSAIDs-induced intestinal injury. In line with the experiments in chapter I, I characterized the expression of TRPM8 in the small intestine, DRG, and then examined the role of neuropeptides such as CGRP and SP in the pathogenesis of indomethacin-induced intestinal injury.

Since a specific anti-mouse TRPM8 antibody is not available for immunohistochemical analyses, I investigated the localization of TRPM8 in the small intestine using TRPM8-EGFP transgenic mice, which express EGFP under the direction of the TRPM8 promoter. The EGFP signals that might reflect the expression profiles of TRPM8 were commonly found in neural fibers and were significantly upregulated following indomethacin administration. The protein expression of TRPM8 determined via Western blot analyses showed the tendency to increase following indomethacin administration. A previous study revealed that TRPM8 was upregulated in the murine asthma model [111]. Thus, neuronal TRPM8 may be upregulated in intestinal mucosa inflammation treated with indomethacin.

Several studies showed that TRPM8 was expressed in mucosal sensory neurons for the regulation of several functions such as inflammation and visceral nociception [86, 112]. These functions are caused by the local release of neuropeptides such as CGRP and SP from sensory neurons [51]. Previous studies of patients with colitis and animal colitis models suggested that TRPV1 and TRPA1 were upregulated in primary sensory neurons [113]. In the DSS colitis model, TRPV1 and TRPA1 contribute to the progression of intestinal inflammation. TRP ion channel activation is followed by CGRP and SP release, which are anti- and pro-inflammatory, respectively [62]. The current study revealed that EGFP signals that might reflect the TRPM8 expression colocalized mainly with CGRP and partly with SP in the sensory neurons of the small intestine. My results indicate strong association between TRPM8 and CGRP expression profiles. At present, there is no direct evidence that upregulation of CGRP produces TRPM8-mediated intestinal protection. In our preliminarily experiment, available CGRP antagonist olcegepant failed to show clear abrogative effect against CGRP-mediated intestinal protective action. This is probably due to low bioavailability of this antagonist such as poor tissue distribution and persistence. Further studies are needed to confirm this point. However, along with the well proven protective effect of CGRP, upregulation of CGRP might be involved in TRPM8-mediated protective effect on indomethacin-induced small intestinal injury.

Further, retrograde tracing using fluorescent fluorogold dye showed that the extrinsic primary afferent innervation expressing CGRP and SP in the small intestine originated from the DRG. It has been shown

that TRPM8 is expressed in the group of C fibers and Aδ fibers [114]. On the other hand, CGRP and SP were released mainly by unmyelinated C fibers and very rarely by small myelinated Aδ fibers in the meninges [115]. In the current study, from the immunohistochemistry in the DRG staining, I found that the neuropeptide expression was mainly found in the small and medium-size fibers. Furthermore, previous experiments [29] and my current experiments showed high colocalization between TRPM8 and CGRP, suggesting the presence mainly in the C fibers. Nociceptive C-fibers can respond to stimuli such as mast cell-derived mediators, resulting in release of neuropeptides such as CGRP. The signals traveling through C-fibers lead to the release of neuropeptides [116].

In the GI tract, CGRP is well established as a protective neuropeptide released from the sensory afferent neurons via vasodilation and mucus secretion [117, 118]. The CGRP-mediated vasodilative hyperemic response and increased mucus secretion are well-known mucosal defense mechanisms against various noxious stimuli in the GI lumen. Further, CGRP has been reported to exert an anti-inflammatory effect via various effector functions of immune cells, including inhibiting inflammatory cytokines and upregulating anti-inflammatory cytokines [119]. In contrast, SP plays an essential role in the pathogenesis of inflammation by increasing vascular permeability, leukocyte activation, and mast cell degranulation [104]. To validate the association between these neuro-responses and the protective and deleterious effects of these neuropeptides, further studies should be conducted.

Several studies reported that the number of CGRP- and SP-positive sensory neurons increased in the inflamed mucosa in experimentally induced colitis [46]. The current study showed that both CGRP- and SP-positive sensory neurons were upregulated around the injured area in the small intestine treated with indomethacin. Interestingly, the upregulation of CGRP-positive sensory neurons was abrogated entirely in TRPM8KO mice. However, the upregulation of SP-positive sensory neurons was not affected in TRPM8KO mice. Thus, TRPM8 may contribute to the production of CGRP, but not SP, in the injured intestinal mucosa. CGRP has protective effects against experimentally induced colitis by DSS and TNBS [117,120].

In contrast, the deleterious effects of SP on the pathogenesis of colitis have been described [121, 122]. Thus, TRPM8-mediated CGRP release from the sensory afferent neurons may have a protective effect against indomethacin-induced small intestinal injury. In contrast, SP released from the sensory afferent neurons may play a role in the progression of small intestinal injury in a TRPM8-independent manner.

Kato's group previously reported the role of TRPV1 and TRPA1 in sensory afferent neurons in the progression of DSS-induced colitis in relation to CGRP and SP in contrast to TRPM8 [46]. The severity of colitis was attenuated in mice deficient of either TRPV1 or TRPA1. Further, TRPV1- and TRPA1-deficiencies prevented the upregulation of SP, but not CGRP, in DSS-induced inflamed colon. Thus,

the release of SP from the sensory afferent neurons expressing TRPV1 and TRPA1 may be involved in the progression of DSS-induced colitis. CGRP may have protective effects on colitis in TRPV1- and TRPA1-independent manner. I hypothesize that the different influences of TRP channels may be accounted for by the correlation of neuropeptides, such as CGRP and SP. TRP channels facilitate communication between the nervous and immune systems as TRP channels promote the regulation of inflammation through the release of neuropeptides [123]. Activating TRP channels enables crosstalk among neurons, immune cells, and epithelial cells to control inflammatory responses [65, 89]. TRP channels act either through a direct effect on the intracellular levels of cations or through indirect modulation of intracellular pathways to trigger pro- or anti-inflammatory mechanisms, depending on the inflammatory disease conditions [65]. For example, TRPA1, TRPV1, and TRPV4 channels activation resulting in intracellular calcium levels increasing and releasing neuropeptides such as CGRP and SP [65]. Based on these observations, CGRP may be upregulated by TRPM8 and SP by TRPV1 and TRPA1 in the sensory afferent neurons during inflammation. As previously mentioned, TRP channel actions contribute to neurogenic inflammation. Following TRPM8 activation, increased intracellular calcium levels might lead to the release of neuropeptides such as CGRP as action potentials generated following TRPM8 activation induces calcium influx and consequent CGRP release from trigeminal neurons [65]. CGRP is synthesized in trigeminal ganglia neuronal cell bodies and then stored in large, dense-core vesicles within the sensory nerve terminal to facilitate its secretion [124,125]. Following neuronal depolarization, excitation of trigeminal neurons caused subsequent CGRP release from the terminal via calcium-dependent exocytosis process that involves soluble N-ethyl amide-sensitive factor attachment protein receptor (SNARE) to promote vesicle docking and fusion to the membrane [125].

By contrast, TRPM8 was localized in high-threshold visceral afferent neurons and may also colocalize with TRPV1 and TRPA1 to restrain their downstream chemosensory and mechanosensory actions [89]. TRPM8 is present on colonic sensory neurons, which may couple with TRPV1 and TRPA1 to also inhibit their downstream chemosensory and mechanosensory actions [43]. Thus, it is possible that these channels that are colocalized in sensory neurons may inter-regulate the downstream neural and sensory responses. TRPM8 can reduce inflammation through crosstalk with other TRP channels. For example, TRPM8 activation has been found to suppresses TRPV1-mediated inflammatory neuropeptide release [25,26,89]. In addition, TRPM8 activation on sensory neurons using icilin caused desensitization of TRPA1, which plays a role in the release of inflammatory neuropeptides during inflammation, to its exogenous irritant and agonist allyl isothiocyanate (AITC), as confirmed in TRPA1KO mice [126,89] Consequently, TRPM8 performs an anti-inflammatory function to balance the pro-inflammatory responses of TRPV1 and TRPA1 via mediating chemosensory deactivation and inflammatory neuropeptide release [65].

TrkA receptor activation via NGF results in upregulating of TRP channels. The binding of NGF to TrkA was found to increase TRPM8 expression through phosphatidylinositol 3-kinase and p38 MAP kinase [127, 128]. NGF is a member of the neurotrophins family. In adults, NGF has a relevant role in neuroimmune communication in the GI tract [129]. Post-translational effects of NGF/TrkA binding include an increase in the concentration of neuropeptides SP and CGRP. In the current study, I found that mRNA expression of NGF in the intestinal mucosa was significantly upregulated following indomethacin administration in WT mice. However, this response was absent in TRPM8KO mice. This result is comparable to CGRP expression, making a strong relationship in TRPM8-mediated CGRP upregulation and NGF/TrkA pathway, resulting in intestinal protection only in WT mice with the absence of that response in TRPM8KO mice. In addition to its direct effects on immune-cell functions, NGF affect inflammatory responses by regulating the synthesis of CGRP, as it was found that neutralization of endogenous NGF using anti-NGF antibodies markedly reduces the synthesis of CGRP [130]. Likewise, in colitis model, it was found that anti-NGF treatment increased the severity of the experimental inflammation by reducing CGRP content in the gut [131]. Furthermore, in vitro studies using the DRG culture model have shown that cold and menthol sensitivity mediated by TRPM8 was upregulated by exogenous NGF [132]. Since it was proved that TRPM8 is co-expressed with receptors for pro-inflammatory mediators, TRPM8 is co-expressed with the high-affinity tyrosine kinase receptor for NGF, TrkA, a molecule with an essential role in pain signaling [18]. It has been further reported that chronic exposure of NGF increased CGRP contents via MEK signaling while acute exposure of NGF augmented capsaicin-evoked release of CGRP via protein kinase C and Src family kinase [133]. Thus, NGF/TrkA pathway may augment both the expression and release of CGRP in sensory neurons.

It has been described the importance of the gut microbiota in influencing the brain-gut axis that comprises bidirectional communication between the central and enteric nervous system, thereby linking emotional behavior, metabolism, and immune regulations [134]. Thermosensitive TRP channels, including TRPM8 and TRPA1, and microbiota have been suggested to involve thermogenesis and energy metabolism [135]. Moreover, we previously showed the involvement of intestinal microbiota in developing indomethacin-induced intestinal injury [95]. Thus, it is possible that alteration in intestinal microbiota via TRPM8 may be, in part, involved in the pathogenesis of intestinal injury.

Taken together, these findings in this chapter indicate that TRPM8, which expressed mainly in CGRP-positive sensory afferent neurons, was upregulated following indomethacin treatment. TRPM8-mediated upregulation of CGRP-positive neurons may be involved in suppression of indomethacin-induced intestinal injury. TRPM8-mediated protective mechanisms may involve TrkA/NGF pathway, because transcriptional upregulation of NGF was abrogated in the TRPM8KO mice and TrkA-immunopositive neurons were colocalized with TRPM8-EGFP signaling and CGRP-immunopositive

neurons in the small intestine. In contrast, SP in the sensory afferent neurons may play a role in the progression of small intestinal injury in a TRPM8-independent manner.

Conclusion

From Chapters I and II, I determined the roles of TRPM8 in indomethacin-induced intestinal injury in mice; the indomethacin-induced small intestinal injury was significantly exacerbated in TRPM8KO mice compared with WT mice. Besides, a TRPM8 agonist, WS-12, reduced the severity of intestinal injury in WT mice, but this response was abrogated in TRPM8KO mice, confirming the protective anti-inflammatory effect of TRPM8 against indomethacin-induced intestinal injury. Furthermore, the current study showed TRPM8 upregulation following indomethacin treatment. In addition, CGRP-positive sensory neurons were up-regulated around the injured area in the small intestine treated with indomethacin. In contrast, SP in the sensory afferent neurons may play a role in the progression of intestinal injury in a TRPM8-independent manner. Based on the findings obtained in this study, I hypothesized that TRPM8 mediated-CGRP upregulation in the sensory afferent neurons has a protective effect against indomethacin-induced small intestinal injury. CGRP protective mechanisms most probably involve the TrkA/NGF pathway. I found a strong relationship in TRPM8-mediated CGRP upregulation and NGF/TrkA pathway, resulting in intestinal protection only in WT mice with the absence of that response in TRPM8KO mice. Therefore, this study should contribute to developing novel therapeutic approaches for indomethacin-induced intestinal injury as TRPM8 is a potential target for the treatment and prevention of NSAIDs-induced enteropathy and IBD.

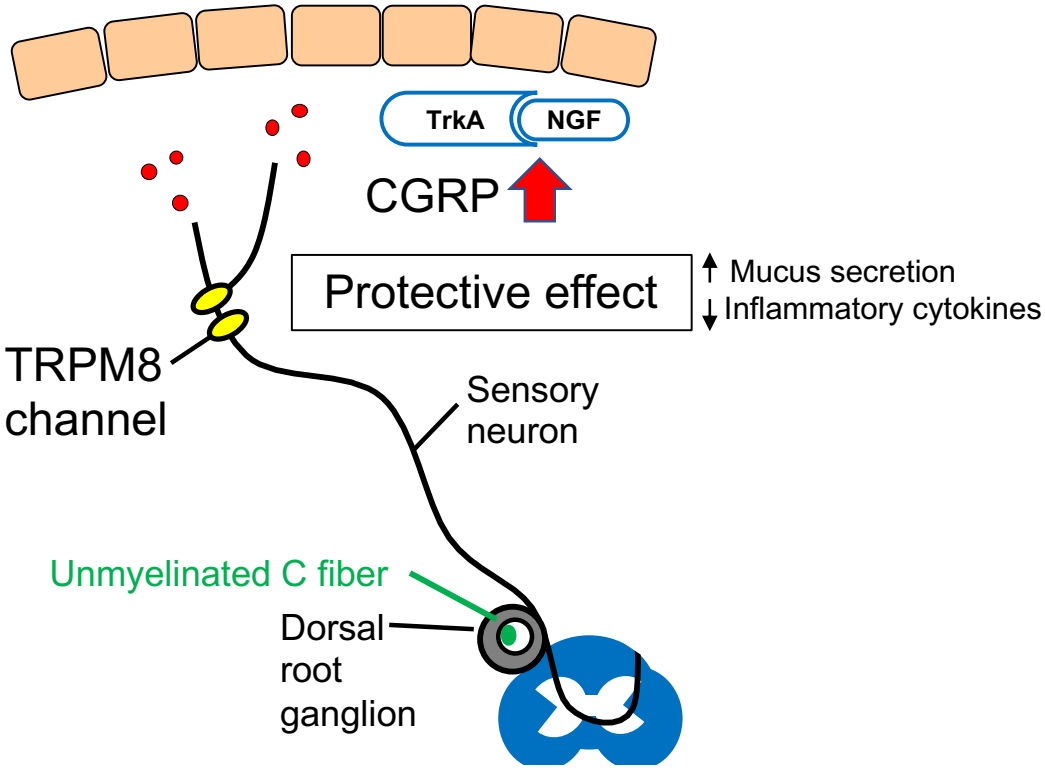

Figure 17. Schematic representation of TRPM8 protective effect against indomethacin-induced small intestinal injury. The protective effect of TRPM8 expressed in sensory afferent neurons is mediated by the upregulation of CGRP rather than SP.

References

1) Bagal, S. K.; Brown, A. D.; Cox, P. J.; Omoto, K.; Owen, R. M.; Pryde, D. C.; Sidders, B.; Skerratt, S. E.; Stevens, E. B.; Storer, R. I., Ion channels as therapeutic targets: a drug discovery perspective *J. Med. Chem.* **2013,** *56* (3), 593-624.
2) Smani, T.; Shapovalov, G.; Skryma, R.; Prevarskaya, N.; Rosado, J. A., Functional and physiopathological implications of TRP channels. *Biochim Biophys Acta. Mol. Cell Res.* **2015,** *1853* (8), 1772-1782.
3) Vriens, J.; Owsianik, G.; Voets, T.; Droogmans, G.; Nilius, B., Invertebrate TRP proteins as functional models for mammalian channels. *Pflügers Archiv.* **2004,** *449* (3), 213-226.
4) Julius, D., TRP channels and pain. *Annu. Rev. Cell Dev. Biol.* **2013,** *29*, 355-384.
5) Froghi, S.; Grant, C. R.; Tandon, R.; Quaglia, A.; Davidson, B.; Fuller, B., New Insights on the Role of TRP Channels in Calcium Signalling and Immunomodulation: Review of Pathways and Implications for Clinical Practice. *Clin. Rev. Allergy Immunol*. **2021**, 60 (2), 271-292.
6) Madej, M. G.; Ziegler, C. M., Dawning of a new era in TRP channel structural biology by cryo-electron microscopy. *Pflügers arch- eur. J. physiol.* **2018,** *470* (2), 213-225.
7) Owsianik, G.; Talavera, K.; Voets, T.; Nilius, B., Permeation and selectivity of TRP channels. *Annu. Rev. Physiol.* **2006,** *68*, 685-717.
8) Zhang, X.; Hu, M.; Yang, Y.; Xu, H., Organellar TRP channels. *Nat. Struct. Mol. Biol.* **2018,** *25* (11), 1009-1018.
9) Hardie, R. C.; Minke, B., The trp gene is essential for a light-activated Ca2+ channel in Drosophila photoreceptors. *Neuron.* **1992,** *8* (4), 643-51.
10) Bang, S. et al., Inhibition of sensory neuronal TRPs contributes to anti-nociception by butamben. *Neurosci Lett*, **2012**. 506(2): p. 297-302.
11) Zhang, X. et al., MCOLN1 is a ROS sensor in lysosomes that regulates autophagy. *Nat Commun.* **2016**. **7**: p. 12109.
12) Nilius, B.; Talavera, K.; Owsianik, G.; Prenen, J.; Droogmans, G.; Voets, T., Gating of TRP channels: a voltage connection? *J. Physiol.* **2005,** *567* (1), 35-44.
13) Karashima, Y.; Prenen, J.; Meseguer, V.; Owsianik, G.; Voets, T.; Nilius, B., Modulation of the transient receptor potential channel TRPA1 by phosphatidylinositol 4, 5-biphosphate manipulators. *Pflügers arch- eur. J. physiol*. **2008,** *457* (1), 77-89.
14) Taberner, F.J., et al., TRP channels interaction with lipids and its implications in disease. *Biochim. Biophys. Acta.* **2015**. 1848(9): p. 1818-27.
15) Bhave, G.; Hu, H.-J.; Glauner, K. S.; Zhu, W.; Wang, H.; Brasier, D.; Oxford, G. S.; Gereau, R. W., Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor

transient receptor potential vanilloid 1 (TRPV1). *Proceedings of the national academy of sciences.* **2003,** *100* (21), 12480-12485.

16) De Petrocellis, L.; Starowicz, K.; Moriello, A. S.; Vivese, M.; Orlando, P.; Di Marzo, V., Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB1 receptors and endovanilloids. *Exp. Cell Res.* **2007,** *313* (9), 1911-1920.

17) Caterina, M. J.; Schumacher, M. A.; Tominaga, M.; Rosen, T. A.; Levine, J. D.; Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* **1997,** *389* (6653), 816-824.

18) Peier, A. M.; Moqrich, A.; Hergarden, A. C.; Reeve, A. J.; Andersson, D. A.; Story, G. M.; Earley, T. J.; Dragoni, I.; McIntyre, P.; Bevan, S., A TRP channel that senses cold stimuli and menthol. *Cell* **2002,** *108* (5), 705-715.

19) Fleig, A.; Penner, R., The TRPM ion channel subfamily: molecular, biophysical and functional features. *Trends Pharmacol. Sci.* **2004,** *25* (12), 633-639.

20) Clapham, D. E., TRP channels as cellular sensors. *Nature* **2003,** *426* (6966), 517-524.

21) Huang, Y.; Fliegert, R.; Guse, A. H.; Lü, W.; Du, J., A structural overview of the ion channels of the TRPM family. *Cell Calcium* **2020,** *85*, 102111.

22) Simon, F.; Varela, D.; Cabello-Verrugio, C., Oxidative stress-modulated TRPM ion channels in cell dysfunction and pathological conditions in humans. *Cell. Signal.* **2013,** *25* (7), 1614-1624.

23) McNulty, S.; Fonfria, E., The role of TRPM channels in cell death. *Pflügers Archiv.* **2005,** *451* (1), 235-242.

24) Liu, Y.; Qin, N., TRPM8 in health and disease: cold sensing and beyond. *Adv. Exp. Med. Biol.* **2011**, 185-208.

25) de Jong, P. R.; Takahashi, N.; Peiris, M.; Bertin, S.; Lee, J.; Gareau, M. G.; Paniagua, A.; Harris, A. R.; Herdman, D. S.; Corr, M.; Blackshaw, L. A.; Raz, E., TRPM8 on mucosal sensory nerves regulates colitogenic responses by innate immune cells via CGRP. *Mucosal Immunol.* **2015,** *8* (3), 491-504.

26) Ramachandran, R.; Hyun, E.; Zhao, L.; Lapointe, T. K.; Chapman, K.; Hirota, C. L.; Ghosh, S.; McKemy, D. D.; Vergnolle, N.; Beck, P. L., TRPM8 activation attenuates inflammatory responses in mouse models of colitis. *Proceedings of the National Academy of Sciences* **2013,** *110* (18), 7476-7481.

27) Tsavaler, L.; Shapero, M. H.; Morkowski, S.; Laus, R., Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer Res.* **2001,** *61* (9), 3760-3769.

28) Yee, N. S.; Zhou, W.; Lee, M., Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma. *Cancer letters.* **2010,** *297* (1), 49-55.

29) Dhaka, A.; Earley, T. J.; Watson, J.; Patapoutian, A., Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. *J. Neurosci.* **2008,** *28* (3), 566-75.

30) Hayashi, T.; Kondo, T.; Ishimatsu, M.; Yamada, S.; Nakamura, K.; Matsuoka, K.; Akasu, T., Expression of the TRPM8-immunoreactivity in dorsal root ganglion neurons innervating the rat urinary bladder. *Neurosci. Res.* **2009,** *65* (3), 245-51.

31) Fisher, J. T., TRPM8 and dyspnea: from the frigid and fascinating past to the cool future? *Curr. Opin. Pharmacol.* **2011,** *11* (3), 218-23.

32) Walsh, D. A.; Mapp, P. I.; Kelly, S., Calcitonin gene-related peptide in the joint: contributions to pain and inflammation. *Br. J. Clin. Pharmacol.* **2015,** *80* (5), 965-978.

33) Liu, Z.; Wu, H.; Wei, Z.; Wang, X.; Shen, P.; Wang, S.; Wang, A.; Chen, W.; Lu, Y., TRPM8: a potential target for cancer treatment. *J. Cancer Res. Clin. Oncol.* **2016,** *142* (9), 1871-1881.

34) Thiel, G.; Backes, T. M.; Welck, J.; Steinhausen, S.; Fischer, A.-L.; Langfermann, D. S.; Ulrich, M.; Wissenbach, U.; Rössler, O. G., Pharmacological inhibition of TRPM8-induced gene transcription. *Biochem. Pharmacol.* **2019,** *170*, 113678.

35) Ordás, P., et al., Expression of the cold thermoreceptor TRPM8 in rodent brain thermoregulatory circuits. *J. Comp. Neurol*. **2021**. 529(1): p. 234-256.

36) Amato, A.; Terzo, S.; Lentini, L.; Marchesa, P.; Mulè, F., TRPM8 Channel Activation Reduces the Spontaneous Contractions in Human Distal Colon. *Int. J. Mol. Sci*. **2020**, 21 (15), 5403.

37) Lai, N. Y.; Mills, K.; Chiu, I. M., Sensory neuron regulation of gastrointestinal inflammation and bacterial host defence. *J. Intern. Med.* **2017,** *282* (1), 5-23.

38) Amato, A.; Liotta, R.; Mulè, F., Effects of menthol on circular smooth muscle of human colon: analysis of the mechanism of action. *Eur J Pharmacol*. **2014**, 740, 295-301.

39) Blackshaw, L. A., Transient receptor potential cation channels in visceral sensory pathways. *Br. J. Pharmacol.* **2014**, 171 (10), 2528-2536.

40) Camilleri, M., Physiological underpinnings of irritable bowel syndrome: neurohormonal mechanisms. *J. Physiol.* **2014,** *592* (14), 2967-2980.

41) Boesmans, W.; Owsianik, G.; Tack, J.; Voets, T.; Vanden Berghe, P., TRP channels in neurogastroenterology: opportunities for therapeutic intervention. *Br. J. Pharmacol.* **2011,** *162* (1), 18-37.

42) Khanna, R.; MacDonald, J. K.; Levesque, B. G., Peppermint oil for the treatment of irritable bowel syndrome: a systematic review and meta-analysis. *J. Clin. Gastroenterol.* **2014,** *48* (6), 505-512.

43) Hosoya, T.; Matsumoto, K.; Tashima, K.; Nakamura, H.; Fujino, H.; Murayama, T.; Horie, S., TRPM 8 has a key role in experimental colitis-induced visceral hyperalgesia in mice. *Neurogastroenterol. Motil.* **2014,** *26* (8), 1112-1121.

44) Winston, J.; Shenoy, M.; Medley, D.; Naniwadekar, A.; Pasricha, P. J., The vanilloid receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats. *Gastroenterology.* **2007,** *132* (2), 615-627.

45) Phillis, B. D.; Martin, C. M.; Kang, D.; Larsson, H.; Lindström, E. A.; Martinez, V.; Blackshaw, L. A., Role of TRPV1 in high-threshold rat colonic splanchnic afferents is revealed by inflammation. *Neurosci. Lett.* **2009,** *459* (2), 57-61.

46) Utsumi, D.; Matsumoto, K.; Tsukahara, T.; Amagase, K.; Tominaga, M.; Kato, S., Transient receptor potential vanilloid 1 and transient receptor potential ankyrin 1 contribute to the progression of colonic inflammation in dextran sulfate sodium-induced colitis in mice: Links to calcitonin gene-related peptide and substance P. *J Pharmacol Sci.* **2018,** *136* (3), 121-132.

47) Zielińska, A.; Sałaga, M.; Włodarczyk, M.; Fichna, J., Focus on current and future management possibilities in inflammatory bowel disease-related chronic pain. *Int. J. Colorectal Dis.* **2019,** *34* (2), 217-227.

48) Yarushkina, N.I., et al., Vulnerability of gastric and small intestinal mucosa to ulcerogenic action of indomethacin in C57/BL6/J mice and transient receptor potential channel vanilloid type 1 knockout mice. *J. Physiol. Pharmacol*. **2018**, 69(6).

49) Genton, L.; Kudsk, K. A., Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. *Am. J. Surg.* **2003,** *186* (3), 253-258.

50) Holzer, P., Transient receptor potential (TRP) channels as drug targets for diseases of the digestive system. *Pharmacol. Ther.* **2011,** *131* (1), 142-170.

51) Allais, L.; De Smet, R.; Verschuere, S.; Talavera, K.; Cuvelier, C. A.; Maes, T., Transient Receptor Potential Channels in Intestinal Inflammation: What Is the Impact of Cigarette Smoking? *Pathobiology.* **2017,** *84* (1), 1-15.

52) Holzer, P.; Farzi, A., Neuropeptides and the microbiota-gut-brain axis. *Adv. Exp. Med. Biol.* **2014**, 817, 195-219.

53) Benarroch, E. E., CGRP: sensory neuropeptide with multiple neurologic implications. *Neurology.* **2011**, 77 (3), 281-7.

54) Gerard, N. P.; Garraway, L. A.; Eddy Jr, R. L.; Shows, T. B.; Iijima, H.; Paquet, J. L.; Gerard, C., Human substance P receptor (NK-1): organization of the gene, chromosome localization and functional expression of cDNA clones. *Biochemistry.* **1991,** *30* (44), 10640-10646.

55) Datar, P.; Srivastava, S.; Coutinho, E.; Govil, G., Substance P: structure, function, and therapeutics. *Current topics in medicinal chemistry.* **2004,** *4* (1), 75-103.

56) Raddant, A. C.; Russo, A. F., Calcitonin gene-related peptide in migraine: intersection of peripheral inflammation and central modulation. *Expert. Rev. Mol. Med.* **2011,** *13*, e36-e36.

57) Russo, A.; Dickerson, I., CGRP: A multifunctional neuropeptide. In *Handbook of neurochemistry and molecular neurobiology*. **2006**, pp 391-427.

58) Ma, W. J.; Yin, Y. C.; Zhang, B. K.; Li, Y. J.; Li, W. Q., Calcitonin gene-related peptide-mediated pharmacological effects in cardiovascular and gastrointestinal diseases (Review). *Mol. Med. Rep.* **2021,** *23* (1), 1.

59) Kim, J.-H.; Yamamoto, T.; Lee, J.; Yashiro, T.; Hamada, T.; Hayashi, S.; Kadowaki, M., CGRP, a neurotransmitter of enteric sensory neurons, contributes to the development of food allergy due to the augmentation of microtubule reorganization in mucosal mast cells. *Biomed. Res.* **2014,** *35* (4), 285-293.

60) Poyner, D. R., Calcitonin gene-related peptide: Multiple actions, multiple receptors. *Pharmacol. Ther.* **1992,** *56* (1), 23-51.

61) Harrison, S. and P. Geppetti, Substance p. *Int J Biochem Cell Biol*, **2001**. 33(6): p. 555-76.

62) Csekő, K., et al., Role of TRPV1 and TRPA1 Ion Channels in Inflammatory Bowel Diseases: Potential Therapeutic Targets? *Pharmaceuticals (Basel),* **2019**. 12(2).

63) Brierley, S. M.; Hughes, P. A.; Page, A. J.; Kwan, K. Y.; Martin, C. M.; O'Donnell, T. A.; Cooper, N. J.; Harrington, A. M.; Adam, B.; Liebregts, T., The ion channel TRPA1 is required for normal mechanosensation and is modulated by algesic stimuli. *Gastroenterology.* **2009,** *137* (6), 2084-2095. e3.

64) Vriens, J.; Nilius, B.; Voets, T., Peripheral thermosensation in mammals. *Nature Reviews Neuroscience.* **2014**, 15 (9), 573-589.

65) Silverman, H. A.; Chen, A.; Kravatz, N. L.; Chavan, S. S.; Chang, E. H., Involvement of Neural Transient Receptor Potential Channels in Peripheral Inflammation. *Front. Immunol.* **2020,** *11*.

66) Peiris, M.; Weerts, Z.; Aktar, R.; Masclee, A. A. M.; Blackshaw, A.; Keszthelyi, D., A putative anti-inflammatory role for TRPM8 in irritable bowel syndrome-An exploratory study. *Neurogastroenterol. Motil.* **2021**, e14170.

67) Gupta, A.; Bah, M., NSAIDs in the treatment of postoperative pain. *Curr. Pain Headache Rep.* **2016,** *20* (11), 1-14.

68) Ramiro, S., I12. NSAIDS, conventional synthetic dmards and biologics IN spa: THEIR role and impact ON disease outcomes. *Rheumatology.* **2017,** *56* (suppl_2).

69) Bjarnason, I.; Hayllar, J., Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology.* **1993,** *104* (6), 1832-1847.

70) Yamada, T.; Deitch, E.; Specian, R. D.; Perry, M. A.; Sartor, R. B.; Grisham, M. B., Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. *Inflammation.* **1993,** *17* (6), 641-662.

71) Watanabe, T.; Fujiwara, Y.; Chan, F. K., Current knowledge on non-steroidal anti-inflammatory drug-induced small-bowel damage: a comprehensive review. *J. Gastroenterol.* **2020,** *55* (5), 481-495.

72) Matsumoto, T.; Kudo, T.; Esaki, M.; Yano, T.; Yamamoto, H.; Sakamoto, C.; Goto, H.; Nakase, H.; Tanaka, S.; Matsui, T.; Sugano, K.; Iida, M., Prevalence of non-steroidal anti-

inflammatory drug-induced enteropathy determined by double-balloon endoscopy: A Japanese multicenter study. *Scand. J. Gastroenterol.* **2008,** *43* (4), 490-496.

73) Kameda, N.; Higuchi, K.; Shiba, M.; Machida, H.; Okazaki, H.; Yamagami, H.; Tanigawa, T.; Watanabe, K.; Watanabe, T.; Tominaga, K., A prospective, single-blind trial comparing wireless capsule endoscopy and double-balloon enteroscopy in patients with obscure gastrointestinal bleeding. *J. Gastroenterol.* **2008,** *43* (6), 434-440.

74) Sunada, K.; Yamamoto, H., Double-balloon endoscopy: past, present, and future. *J. Gastroenterol.* **2009,** *44* (1), 1-12.

75) Higuchi, K.; Umegaki, E.; Watanabe, T.; Yoda, Y.; Morita, E.; Murano, M.; Tokioka, S.; Arakawa, T., Present status and strategy of NSAIDs-induced small bowel injury. *J. Gastroenterol.* **2009,** *44* (9), 879-888.

76) Goldstein, J. L.; Eisen, G. M.; Lewis, B.; Gralnek, I. M.; Zlotnick, S.; Fort, J. G., Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, and placebo. *Clin. Gastroenterol. Hepatol.* **2005,** *3* (2), 133-141.

77) Maiden, L.; Thjodleifsson, B.; Theodors, A.; Gonzalez, J.; Bjarnason, I., A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. *Gastroenterology.* **2005,** *128* (5), 1172-1178.

78) Ali Khan, M.; Howden, C. W., The Role of Proton Pump Inhibitors in the Management of Upper Gastrointestinal Disorders. *Gastroenterol. Hepatol.* **2018,** *14* (3), 169-175.

79) Otani, K.; Tanigawa, T.; Watanabe, T.; Shimada, S.; Nadatani, Y.; Nagami, Y.; Tanaka, F.; Kamata, N.; Yamagami, H.; Shiba, M., Microbiota plays a key role in non-steroidal anti-inflammatory drug-induced small intestinal damage. *Digestion.* **2017,** *95* (1), 22-28.

80) Washio, E.; Esaki, M.; Maehata, Y.; Miyazaki, M.; Kobayashi, H.; Ishikawa, H.; Kitazono, T.; Matsumoto, T., Proton pump inhibitors increase incidence of nonsteroidal anti-inflammatory drug–induced small bowel injury: a randomized, placebo-controlled trial. *Clin. Gastroenterol. Hepatol.* **2016,** *14* (6), 809-815. e1.

81) Endo, H.; Sakai, E.; Taniguchi, L.; Kessoku, T.; Komiya, Y.; Ezuka, A.; Kawamura, H.; Taguri, M.; Higurashi, T.; Ohkubo, H., Risk factors for small-bowel mucosal breaks in chronic low-dose aspirin users: data from a prospective multicenter capsule endoscopy registry. *Gastrointestinal Endoscopy.* **2014,** *80* (5), 826-834.

82) Dahan, A.; Amidon, G. L.; Zimmermann, E. M., Drug targeting strategies for the treatment of inflammatory bowel disease: a mechanistic update. *Expert. Rev. Clin. Immunol.* **2010,** *6* (4), 543-550.

83) Park, S. C.; Chun, H. J.; Kang, C. D.; Sul, D., Prevention and management of non-steroidal anti-inflammatory drugs-induced small intestinal injury. *World J. Gastroenterol.* **2011,** *17* (42), 4647.

84) Higashimura, Y.; Naito, Y.; Takagi, T.; Tanimura, Y.; Mizushima, K.; Harusato, A.; Fukui, A.; Yoriki, H.; Handa, O.; Ohnogi, H., Preventive effect of agaro-oligosaccharides on non-

steroidal anti-inflammatory drug-induced small intestinal injury in mice. *J. Gastroenterol. Hepatol.* **2014,** *29* (2), 310-317.

85) Jung, E. S.; Jang, H. J.; Hong, E. M.; Lim, H. L.; Lee, S. P.; Kae, S. H.; Lee, J., The Protective Effect of 5-Aminosalicylic Acid Against Non-Steroidal Anti-Inflammatory Drug-Induced Injury Through Free Radical Scavenging in Small Intestinal Epithelial Cells. *Medicina.* **2020,** *56* (10), 515.

86) Yu, X.; Yu, M.; Liu, Y.; Yu, S., TRP channel functions in the gastrointestinal tract. *Semin. Immunopathol.* **2016,** *38* (3), 385-96.

87) Konaka, A.; Nishijima, M.; Tanaka, A.; Kunikata, T.; Kato, S.; Takeuchi, K., Nitric oxide, superoxide radicals and mast cells in pathogenesis of indomethacin-induced small intestinal lesions in rats. *J. Physiol. Pharmacol.* **1999,** *50* (1).

88) Anthony, A.; Pounder, R. E.; Dhillon, A. P.; Wakefield, A. J., Similarities between ileal Crohn's disease and indomethacin experimental jejunal ulcers in the rat. *Aliment. Pharmacol. Ther.* **2000,** *14* (2), 241-5.

89) Harrington, A. M.; Hughes, P. A.; Martin, C. M.; Yang, J.; Castro, J.; Isaacs, N. J.; Blackshaw, A. L.; Brierley, S. M., A novel role for TRPM8 in visceral afferent function. *Pain.* **2011,** *152* (7), 1459-1468.

90) Dhaka, A.; Murray, A. N.; Mathur, J.; Earley, T. J.; Petrus, M. J.; Patapoutian, A., TRPM8 Is Required for Cold Sensation in Mice. *Neuron.* **2007,** *54* (3), 371-378.

91) Ma, S.; G, G.; Ak, V. E.; Jf, D.; H, H., Menthol derivative WS-12 selectively activates transient receptor potential melastatin-8 (TRPM8) ion channels. *Pak. J. Pharm. Sci.* **2008,** *21* (4), 370-8.

92) Kawahara, R.; Yasuda, M.; Hashimura, H.; Amagase, K.; Kato, S.; Takeuchi, K., Activation of alpha7 nicotinic acetylcholine receptors ameliorates indomethacin-induced small intestinal ulceration in mice. *European journal of pharmacology* **2011,** *650* (1), 411-7.

93) Kato, S.; Matsuda, N.; Matsumoto, K.; Wada, M.; Onimaru, N.; Yasuda, M.; Amagase, K.; Horie, S.; Takeuchi, K., Dual role of serotonin in the pathogenesis of indomethacin-induced small intestinal ulceration: pro-ulcerogenic action via 5-HT3 receptors and anti-ulcerogenic action via 5-HT4 receptors. *Pharmacol. Res.* **2012,** *66* (3), 226-34.

94) Balemans, D.; Boeckxstaens, G. E.; Talavera, K.; Wouters, M. M., Transient receptor potential ion channel function in sensory transduction and cellular signaling cascades underlying visceral hypersensitivity. *Am. J. Physiol. Gastrointest. Liver. Physiol.* **2017,** *312* (6), G635-g648.

95) Konaka, A.; Kato, S.; Tanaka, A.; Kunikata, T.; Korolkiewicz, R.; Takeuchi, K., Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. *Pharmacol. Res.* **1999,** *40* (6), 517-24.

96) Kato, S.; Nishio, H.; Ogura, M.; Takeuchi, K., Tacrolimus (FK506), an Immunosuppressive Agent, Prevents Indomethacin-Induced Small Intestinal Ulceration in the Rat: Inhibition of Inducible Nitric Oxide Synthase Expression. *J. Pharmacol. Sci.* **2007,** *103* (1), 40-47.

97) Fournier, B. M.; Parkos, C. A., The role of neutrophils during intestinal inflammation. *Mucosal Immunol*. **2012**, 5 (4), 354-366.

98) Ishida, T.; Miki, I.; Tanahashi, T.; Yagi, S.; Kondo, Y.; Inoue, J.; Kawauchi, S.; Nishiumi, S.; Yoshida, M.; Maeda, H.; Tode, C.; Takeuchi, A.; Nakayama, H.; Azuma, T.; Mizuno, S., Effect of 18beta-glycyrrhetinic acid and hydroxypropyl gammacyclodextrin complex on indomethacin-induced small intestinal injury in mice. *Eur. J. Pharmacol.* **2013,** *714* (1-3), 125-31.

99) Behrendt, H. J.; Germann, T.; Gillen, C.; Hatt, H.; Jostock, R., Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. *Br. J. Pharmacol.* **2004,** *141* (4), 737-745.

100) Caceres, A. I.; Liu, B.; Jabba, S. V.; Achanta, S.; Morris, J. B.; Jordt, S.-E., Transient Receptor Potential Cation Channel Subfamily M Member 8 channels mediate the anti-inflammatory effects of eucalyptol. *Br. J. Pharmacol.* **2017**, 174 (9), 867-879

101) Zielińska, M., et al., Role of transient receptor potential channels in intestinal inflammation and visceral pain: novel targets in inflammatory bowel diseases. *Inflamm. Bowel. Dis*. **2015**. 21(2): p. 419-27.

102) Rozza, A. L.; Meira de Faria, F.; Souza Brito, A. R.; Pellizzon, C. H., The gastroprotective effect of menthol: involvement of anti-apoptotic, antioxidant and anti-inflammatory activities. *PLoS One.* **2014**, 9 (1), e86686.

103) Beck, B.; Bidaux, G.; Bavencoffe, A.; Lemonnier, L.; Thebault, S.; Shuba, Y.; Barrit, G.; Skryma, R.; Prevarskaya, N., Prospects for prostate cancer imaging and therapy using high-affinity TRPM8 activators. *Cell Calcium.* **2007,** *41* (3), 285-294.

104) Engel, M. A.; Becker, C.; Reeh, P. W.; Neurath, M. F., Role of sensory neurons in colitis: increasing evidence for a neuroimmune link in the gut. *Inflamm. Bowel Dis.* **2011,** *17* (4), 1030-3.

105) Holzer, P., Vanilloid receptor TRPV1: hot on the tongue and inflaming the colon. *Neurogastroenterol. Motil.* **2004**, 16 (6), 697-9.

106) Engel, M. A.; Leffler, A.; Niedermirtl, F.; Babes, A.; Zimmermann, K.; Filipovic, M. R.; Izydorczyk, I.; Eberhardt, M.; Kichko, T. I.; Mueller-Tribbensee, S. M.; Khalil, M.; Siklosi, N.; Nau, C.; Ivanovic-Burmazovic, I.; Neuhuber, W. L.; Becker, C.; Neurath, M. F.; Reeh, P. W., TRPA1 and substance P mediate colitis in mice. *Gastroenterology.* **2011,** *141* (4), 1346-58.

107) Lippoldt, E. K.; Elmes, R. R.; McCoy, D. D.; Knowlton, W. M.; McKemy, D. D., Artemin, a Glial Cell Line-Derived Neurotrophic Factor Family Member, Induces TRPM8-Dependent Cold Pain. *J. Neurosci.* **2013,** *33* (30), 12543-12552.

108) Takashima, Y.; Daniels, R. L.; Knowlton, W.; Teng, J.; Liman, E. R.; McKemy, D. D., Diversity in the neural circuitry of cold sensing revealed by genetic axonal labeling of transient receptor potential melastatin 8 neurons. *J Neurosci.* **2007,** *27* (51), 14147-57.

109) Eijkelkamp, N.; Kavelaars, A.; Elsenbruch, S.; Schedlowski, M.; Holtmann, G.; Heijnen, C. J., Increased visceral sensitivity to capsaicin after DSS-induced colitis in mice: spinal cord c-Fos expression and behavior. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007,** *293* (4), G749-57.

110) Matsumoto, K.; Yamaba, R.; Inoue, K.; Utsumi, D.; Tsukahara, T.; Amagase, K.; Tominaga, M.; Kato, S., Transient receptor potential vanilloid 4 channel regulates vascular endothelial permeability during colonic inflammation in dextran sulphate sodium-induced murine colitis. *Br. J. Pharmacol.* **2018,** *175* (1), 84-99.

111) Liu, H.; Liu, Q.; Hua, L.; Pan, J., Inhibition of transient receptor potential melastatin 8 alleviates airway inflammation and remodeling in a murine model of asthma with cold air stimulus. *Acta. Biochim. Biophys. Sin. (Shanghai).* **2018,** *50* (5), 499-506.

112) Axelsson, H. E.; Minde, J. K.; Sonesson, A.; Toolanen, G.; Hogestatt, E. D.; Zygmunt, P. M., Transient receptor potential vanilloid 1, vanilloid 2 and melastatin 8 immunoreactive nerve fibers in human skin from individuals with and without Norrbottnian congenital insensitivity to pain. *Neuroscience.* **2009,** *162* (4), 1322-32.

113) Inoue, R., L.-H. Kurahara, and K. Hiraishi, TRP channels in cardiac and intestinal fibrosis. *Seminars in cell & developmental biology*. **2019**. 94: p. 40-49.

114) Kobayashi, K., et al., Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. *J. Comp. Neurol*. **2005**. 493(4): p. 596-606.

115) Bae, J.Y., et al., Quantitative analysis of afferents expressing substance P, calcitonin gene-related peptide, isolectin B4, neurofilament 200, and Peripherin in the sensory root of the rat trigeminal ganglion. *J. Comp. Neurol.* **2015**. 523(1): p. 126-38.

116) Siiskonen, H.; et al., Mast Cells and Sensory Nerves Contribute to Neurogenic Inflammation and Pruritus in Chronic Skin Inflammation. *Front. Cell. Neurosci*. **2019**, 13 (422).

117) Reinshagen, M.; Flämig, G.; Ernst, S.; Geerling, I.; Wong, H.; Walsh, J. H.; Eysselein, V. E.; Adler, G., Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J. Pharmacol. Exp. Ther.* **1998,** *286* (2), 657-61.

118) Holzer, P., Implications of tachykinins and calcitonin gene-related peptide in inflammatory bowel disease. *Digestion.* **1998,** *59* (4), 269-83.

119) Holzmann, B., Antiinflammatory activities of CGRP modulating innate immune responses in health and disease. *Curr. Protein Pept. Sci.* **2013,** *14* (4), 268-74.

120) Clifton, M. S.; Hoy, J. J.; Chang, J.; Idumalla, P. S.; Fakhruddin, H.; Grady, E. F.; Dada, S.; Corvera, C. U.; Bhargava, A., Role of calcitonin receptor-like receptor in colonic motility and inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007,** *293* (1), G36-44.

121) Evangelista, S.; Maggi, M.; Renzetti, A. R., Down-regulation of substance P receptors during colitis induced by trinitrobenzene sulfonic acid in rats. *Neuropeptides.* **1996,** *30* (5), 425-8.

122) Utsumi, D.; Matsumoto, K.; Amagase, K.; Horie, S.; Kato, S., 5-HT3 receptors promote colonic inflammation via activation of substance P/neurokinin-1 receptors in dextran sulphate sodium-induced murine colitis. *Br J Pharmacol* **2016,** *173* (11), 1835-49.

123) López-Requena, A.; Boonen, B.; Van Gerven, L.; Hellings, P. W.; Alpizar, Y. A.; Talavera, K., 15 Roles of Neuronal Trp. *Neurobiol TRP Channels.* **2017**, 277

124) Russell, F. A.; King, R.; Smillie, S. J.; Kodji, X.; Brain, S. D., Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev*. **2014**, 94 (4), 1099-1142.

125) Durham, P. L.; Masterson, C. G., Two mechanisms involved in trigeminal CGRP release: implications for migraine treatment. *Headache: The Journal of Head and Face Pain*. **2013**, 53 (1), 67-80.

126) Bautista, D. M.; Siemens, J.; Glazer, J. M.; Tsuruda, P. R.; Basbaum, A. I.; Stucky, C. L.; Jordt, S.-E.; Julius, D., The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature.* **2007**, 448 (7150), 204-208.

127) Van den Eynde, C.; Vriens, J.; De Clercq, K., Transient receptor potential channel regulation by growth factors. *BBA-Mol. Cell. Res.* **2021,** *1868* (4), 118950.

128) Kayama, Y.; Shibata, M.; Takizawa, T.; Ibata, K.; Nakahara, J.; Shimizu, T.; Toriumi, H.; Yuzaki, M.; Suzuki, N., Signaling Pathways Relevant to Nerve Growth Factor-induced Upregulation of Transient Receptor Potential M8 Expression. *Neuroscience.* **2017,** *367*, 178-188.

129) Huang, E.J. and L.F. Reichardt, Neurotrophins: roles in neuronal development and function. Annu. *Rev. Neurosci.* **2001**. 24: p. 677-736.

130) Bracci-Laudiero, L.; Aloe, L.; Caroleo, M. C.; Buanne, P.; Costa, N.; Starace, G.; Lundeberg, T., Endogenous NGF regulates CGRP expression in human monocytes, and affects HLA-DR and CD86 expression and IL-10 production. *Blood*. **2005**, 106 (10), 3507-3514.

131) Reinshagen M, Rohm H, Steinkamp M, et al. Protective role of neurotrophins in experimental inflammation of the rat gut. *Gastroenterology*. **2000**; 119:368-376.

132) Babes, A.; Cristian Ciobanu, A.; Neacsu, C.; Babes, R.-M., TRPM8, a sensor for mild cooling in mammalian sensory nerve endings. *Curr. Pharm. Biotechnol*. **2011**, 12 (1), 78-88.

133) Park, K. A.; Fehrenbacher, J. C.; Thompson, E. L.; Duarte, D. B.; Hingtgen, C. M.; Vasko, M. R., Signaling pathways that mediate nerve growth factor-induced increase in expression and release of calcitonin gene-related peptide from sensory neurons. *Neuroscience*. **2010**, 171 (3), 910-23.

134) Carabotti, M.; Scirocco, A.; Maselli, M. A.; Severi, C., The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann. Gastroenterol.* **2015,** *28* (2), 203-209.

135) Wen, J.; Bo, T.; Zhang, X.; Wang, Z.; Wang, D., Thermo-TRPs and gut microbiota are involved in thermogenesis and energy metabolism during low temperature exposure of obese mice. *J. Exp. Biol.* **2020,** *223* (Pt 11).

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