



## A novel role for TRPM8 in visceral afferent function

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### ABSTRACT

Transient receptor potential ion channel melastatin subtype 8 (TRPM8) is activated by cold temperatures and cooling agents, such as menthol and icilin. Compounds containing peppermint are reported to reduce symptoms of bowel hypersensitivity; however, the underlying mechanisms of action are unclear. Here we determined the role of TRPM8 in colonic sensory pathways. Laser capture microdissection, quantitative reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence, and retrograde tracing were used to localise TRPM8 to colonic primary afferent neurons. *In vitro* extracellular single-fibre afferent recordings were used to determine the effect of TRPM8 channel activation on the chemosensory and mechanosensory function of colonic high-threshold afferent fibres. TRPM8 mRNA was present in colonic DRG neurons, whereas TRPM8 protein was present on nerve fibres throughout the wall of the colon. A subpopulation (24%,  $n = 58$ ) of splanchnic serosal and mesenteric afferents tested responded directly to icilin (5  $\mu\text{mol/L}$ ). Subsequently, icilin significantly desensitised afferents to mechanical stimulation ( $P < .0001$ ;  $n = 37$ ). Of the splanchnic afferents responding to icilin, 21 (33%) also responded directly to the TRPV1 agonist capsaicin (3  $\mu\text{mol/L}$ ), and icilin reduced the direct chemosensory response to capsaicin. Icilin also prevented mechanosensory desensitization and sensitization induced by capsaicin and the TRPA1 agonist AITC (40  $\mu\text{mol/L}$ ), respectively. TRPM8 is present on a select population of colonic high threshold sensory neurons, which may also co-express TRPV1. TRPM8 couples to TRPV1 and TRPA1 to inhibit their downstream chemosensory and mechanosensory actions.

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### 1. Introduction

The transient receptor potential (TRP) channel melastatin 8 (TRPM8) is a nonselective cation channel activated by innocuous and noxious cool temperatures [5,21,39,46]. TRPM8 is also activated by compounds evoking cooling sensations such as (–)-menthol, menthylamide, and the supercooling agent icilin [5,44,49]. In addition, TRPM8 has a role in injury-evoked cold and mechanical allodynia [12,49].

Sensory neurons expressing TRPM8 have a diverse neurochemical profile [56,57]. Early studies show the majority of TRPM8 sensory neurons are nonpeptidergic, are of small diameter, and lack co-expression of classic nociceptive markers such as TRP vanilloid 1 (TRPV1) [46,55]. A role for TRPM8 in nociceptive pathways has since been described, specifically in the development of injury-evoked cold and mechanical allodynia, in conjunction with TRP ankyrin 1 (TRPA1) [12,49]. Furthermore, capsaicin-sensitivity has

been shown in menthol- and cold-sensitive sensory neurons, suggesting co-expression with TRPV1 [40,45,60]. TRPM8 has therefore emerged as a potential target for pain modulation [33,37,57]. The mechanisms by which TRPM8 may mediate nociceptive signalling are only partially understood at best. Studies in the tongue and skin indicate a peripheral mechanism whereby TRPM8 activation evokes antinociception attributed to the desensitization of TRPV1-mediated responses [26,33].

Recent studies by our group and others have demonstrated the importance of various TRP channels to determine the sensory properties of primary afferent fibres supplying the colon in health and disease, namely, TRPV1, TRPV4, and TRPA1 [3,6,9,11,15,52]. Nociceptive signalling from the colon is carried by lumbar splanchnic and sacral pelvic nerves. Splanchnic nerves supply the mid to distal colon, whereas pelvic nerves supply the colorectum [8,10]. Splanchnic and pelvic afferent fibres have peripheral endings in specific layers of the colon wall and neuronal cell bodies in the thoracolumbar (TL; T10-L1) and lumbosacral (LS; L6-S1) dorsal root ganglia (DRG), respectively [51]. In the mouse colorectum, there are 5 primary afferent fibre subtypes based on mechanosensory properties and the location of receptive fields [8,10]. Colonic

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afferent subtypes signal chemical and mechanical events. Fibres within the splanchnic pathway have higher thresholds to mechanical stimuli than pelvic afferents, and primarily constitute a nociceptive pathway [10].

Recent studies show TRPM8 is expressed in visceral organs and the sensory pathways supplying them, particularly the urinary tract [29,54,61,62]. In animal models of post-inflammatory colonic hypersensitivity, preparations containing peppermint and caraway oil significantly attenuate heightened sensitivity to mechanical stimuli [2]. Clinical trials using herbal remedies containing peppermint attenuate symptoms of colonic hypersensitivity, which manifests clinically in patients with irritable bowel syndrome (IBS) as abdominal pain [14,24,27,34,41]. Of particular importance is the observation that the severity of abdominal pain is significantly reduced after peppermint administration [14,41]. Little is understood of the mechanisms underlying these clinical findings. We aimed to determine whether TRPM8 is expressed by colonic afferent nerve fibres. Furthermore, we aimed to identify a role for TRPM8 in modulating the signalling of nociceptive visceral events and investigate interactions with TRPV1 and TRPA1 channels.

## 2. Materials and methods

All experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia. Male C57/BL6 (12–16 weeks of age) were used for all experiments unless stated otherwise.

### 2.1. Retrograde labelling

Cholera toxin subunit B conjugated to AlexaFluor 488 (CTB-488) or 555 (CTB-555) (Invitrogen, Carlsbad, CA) was injected at 3 sites subserosally within the wall of the distal colon [32]. After 3 to 4 days, animals were either transcardially perfused with ice-cold fixative (4% paraformaldehyde in 0.1 mol/L phosphate buffer (Sigma-Aldrich, St. Louis, MO) for immunohistochemical studies, as previously described [9] or humanely killed by placing in a carbon dioxide-filled chamber for dissociated ganglia cell culture and mRNA expression studies.

### 2.2. Immunohistochemistry

After transcardial perfusion of retrogradely traced or nontraced mice, the distal colon, TL (T10–L1) and LS (L6–S1) DRG were removed and postfixed for 2 hours at room temperature. Distal colon and DRG were then used for transverse sections. Flat-sheet, whole-mount preparations of colonic layers (serosa, mesentery, longitudinal muscle, myenteric ganglia, and circular muscle) were also used. After fixation, tissue for sectioning was cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich, St. Louis, MO) overnight at 4°C and frozen in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Alphen aan den Rijn, Netherlands). Serial frozen sections (10–12 µm) were cut and processed for immunohistochemistry. Colon for whole-mount preparations underwent phosphate buffer saline (PBS; Sigma-Aldrich) washes to remove fixative and graded ethanol washes to remove fat in the mesentery before being processed for immunohistochemistry [28].

Nonspecific binding of secondary antibodies was blocked with 5% normal donkey or chicken serum diluted in 0.2% Triton-TX 200 (Sigma-Aldrich) in PBS. Tissue sections and colonic whole-mounts were incubated with primary antisera, diluted in 0.2% Triton-TX/PBS, overnight at 4°C. Two rabbit anti-TRPM8 antisera were used: ACC-049 at 1:200 (Alomone Laboratories, Jerusalem, Israel) and ab74845 at 1:2000 (Abcam, Cambridge, MA). A goat anti-calcitonin gene-related peptide (CGRP 1:200; Abcam) and

goat-anti TRPV1 (1:200; GT15129 Neuromics Inc., Edina, MN) were also used. Secondary antibodies raised in either donkey or chicken coupled to AlexaFluor488, AlexaFluor568, and AlexaFluor647 (Molecular Probes, Invitrogen) were used at 1:200 for 1 hour at room temperature for visualization. Negative controls were prepared as above with the primary antibody omitted. Images were obtained using an epifluorescent microscope (BX51, Olympus, Hamburg, Germany) and confocal scanning microscope (SP5, Leica Microsystems, Wetzlar, Germany). Only neuronal cells with intact nuclei were included in this study and the number of labeled neurons was expressed as a percentage of neurons in the whole DRG from 6 to 8 DRG sections (30-µm apart) per mouse and averaged across 3 mice. The optimal working dilution of each antibody was determined empirically. Omission of the primary or secondary antibody resulted in the absence of labelling. The protocol used for double-labelling immunohistochemistry did not affect the distribution or the intensity of each label compared with the corresponding single-labelling pattern. In addition, the secondary antibodies used did not cross-react with unmatched primaries, showing that the secondary antibodies bind specifically to the targeted primary antibodies. Preabsorption controls were used for the Alomone TRPM8 antisera. The TRPM8 antisera was incubated overnight at 4°C with immunising peptide as per manufacturer's instructions and applied to sections as described above.

### 2.3. Western blot

Snap-frozen tissue was partially thawed and weighed. A 30-mg quantity (30 mg wet weight) of coarsely chopped tissue was placed in lysis buffer (RIPA buffer; Sigma) and protease inhibitor mix (Roche Applied Science, Australia) and homogenised using a pestle and mortar on ice. Homogenate was centrifuged at 4°C for 15 minutes and the supernatant collected. Samples (10 µg/µL) were denatured by heating at 70°C for 10 minutes in loading buffer (Invitrogen) and resolved using standard SDS-PAGE on Tris-acetate pre-cast gels (Invitrogen) and then transferred onto nitrocellulose membranes using dry iBlot system (Invitrogen). Pre-stained protein standards (Invitrogen) labelled with Western MagicMark (Invitrogen) were run alongside samples to estimate the molecular weight of resultant chemiluminescent bands. Non-specific binding was blocked by incubation with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T; Zymed Laboratories, Invitrogen) and 10% fat-free skim milk for 60 minutes at room temperature. Membranes were incubated with anti-TRPM8 antisera (ACC-049; 1:400) diluted in 4% skim milk in 0.1%TBS-T overnight at 4°C. Signal was detected with a horseradish peroxidase-conjugated goat anti-rabbit-IgG secondary antibody (1:10,000; 31460, Pierce Protein Research Products, Thermo Scientific, Rockford, IL) incubated for 1 hour at room temperature in TBS-T containing 4% fat-free skim milk. Signals were detected with SuperSignal ECL kit reagents (RPN2132, Amersham Biosciences, GE Healthcare Biosciences, Uppsala, Sweden) as per the manufacturer's instructions, and exposed to ultraviolet light for 30 seconds to 5 minutes. Controls for Western blots included lanes with no protein added (lysis buffer in loading buffer only) and lanes without primary antibodies added. β-Actin (1:1500; 4967; Cell Signalling Technology, Danvers, MA) was used a positive marker.

### 2.4. Dissociated ganglion cell culture and laser capture microdissection

TL and LS DRG (n = 4) were removed 4 days after injections of fluorescent dye into the colon (CTB-AF555) and digested with 4 mg/mL collagenase II (GIBCO, Invitrogen) and 4 mg/mL dispase (GIBCO) for 30 minutes at 37°C, followed by 4 mg/mL collagenase II for 10 minutes at 37°C. Neurons were mechanically dissociated into a single-cell suspension via trituration through fire-polished

Pasteur pipettes. Neurons were resuspended in Hanks' buffered salt solution (HBSS; GIBCO) and spot-plated onto 50-mm Zeiss duplex dishes (Carl Zeiss, Oberkochen, Germany), then maintained at 37°C in 5% CO<sub>2</sub> for 2 hours, allowing optimal cell adhesion. Retrogradely labeled neurons were isolated using a PALM Microlaser Technologies microdissection system (Carl Zeiss) and catapulted directly into a lysis/stabilization buffer containing carrier RNA (4 ng/μL) (Qiagen, Valencia, CA).

### 2.5. Quantitative RT-PCR

RNA was isolated from whole, snap-frozen DRG (n = 3) using RNeasy-Mini extraction kit (Qiagen) and from isolated LCM cells using RNeasy Micro-Kit (Qiagen) following the manufacturer's instructions. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on whole DRG and LCM neurons was performed using a Chromo4 (MJ Research, Waltham, MA) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and Opticon Monitor software (MJ Research). Qiagen QuantiTect SYBR Green RT-PCR 1-step kits were used according to the manufacturer's specifications, with the following primers TRPM8 primers: forward: 5'-GCT GTG GCC TCG TAT CAT TT-3'; reverse: 5'-GAG CAG CAC ATA GGC AAA CA-3'; TRPV1 primers: forward: 5'-CGTC CAAACCTGCGAATGAAGTTC-3'; reverse: 5'-CCTCCATCTCTGTGT CACTGG-3'; β-tubulin primers: forward: 5'-CCAAGTCTGGGAGGTC ATC-3'; reverse: 5'-TGAGAGGAGGCCTCATTGTAG-3' (GeneWorks, Adelaide, Australia). Reaction conditions used were as follows: reverse transcription: 50°C (30 minutes); initial PCR activation: 95°C (15 minutes); Annealing, denaturing, and extension cycles: 94°C (15 seconds), 55°C (30 seconds), and 72°C (30 seconds) repeated for 50 cycles. Size of amplified products was confirmed by gel electrophoresis. Each assay was run in at least triplicate in separate experiments. Control PCRs were performed with RNase-free water (Qiagen). The comparative cycle threshold method was used to quantify the abundance of target transcripts in whole ganglia and isolated neurons. Quantitative data are expressed as mean ± SD, and significant differences in transcript expression determined by a Mann–Whitney test at a significance level of *P* < .05. The expression of TRPM8 and TRPV1 in laser-captured, nonlabeled (general population) neurons was similar to that in whole TL DRG, suggesting that the method of neuronal dissociation and culturing for 2 hours before isolation did not affect the expression levels of TRPM8 and TRPV1.

### 2.6. In vitro electrophysiology and pharmacology

The colon was opened and pinned flat mucosal side up in a specialised organ bath. The colonic compartment was superfused with a modified Krebs solution of the following composition (mmol/L): 117.9 NaCl, 4.7 KCl, 25 NaHCO<sub>3</sub>, 1.3 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>(H<sub>2</sub>O)<sub>7</sub>, 2.5 CaCl<sub>2</sub>, and 11.1 D-glucose, bubbled with carbogen (95% O<sub>2</sub>/5% CO<sub>2</sub>) at 34°C. In all preparations the L-type calcium channel antagonist nifedipine (1 μmol/L) was added to suppress smooth muscle activity and the prostaglandin synthesis inhibitor indomethacin (3 μmol/L) added to suppress potential inhibitory actions of endogenous prostaglandins. Splanchnic nerve bundles were extended into a paraffin-filled recording compartment, in which, after fine dissection strands, were laid onto a glass plate and single fibres placed onto recording electrodes. Receptive fields were identified as described previously [10]. Once identified, receptive fields were tested with three distinct mechanical stimuli to enable classification: static probing with calibrated von Frey hairs (2 g force applied 3 times for a 2–3 seconds), mucosal stroking with calibrated von Frey hairs (10 mg force applied 10 times), and circular stretch (1–5 g, in 1-g increments; each weight applied for 1 minute, with a 1-minute interval between applications).

Stretch across receptive fields was applied using a claw and cantilever system and was initiated by the addition of weights. Once individual afferent fibres were classified and the stimulus-response was established, a small metal ring was placed around the receptive field and icilin (500 nmol/L to 10 μmol/L; Sigma-Aldrich) was applied for 2 minutes. Mechanosensitivity to a limited range of stimuli was re-tested after application. Experiments were performed in wild-type C57/BL6 mice and mice with disruption to the TRPA1 gene, as previously described in detail [35]. Capsaicin (3 μmol/L) or allylthiocyanate (AITC; 40 μmol/L) was applied for 2 minutes after icilin. The mechanosensitivity of the receptive field was reassessed after drug removal. In all cases, stimulus-response functions are expressed as spikes per second meaned over the full period of stimulus. Effects of drugs were investigated at a submaximal mechanical stimulus so that responses were not saturated, allowing observation of increases or decreases in mechanosensitivity. Differences before and after the addition of a single drug dose were compared using a paired Student's *t* test. Data are expressed as mean ± standard error of mean; n = number of observations.

## 3. Results

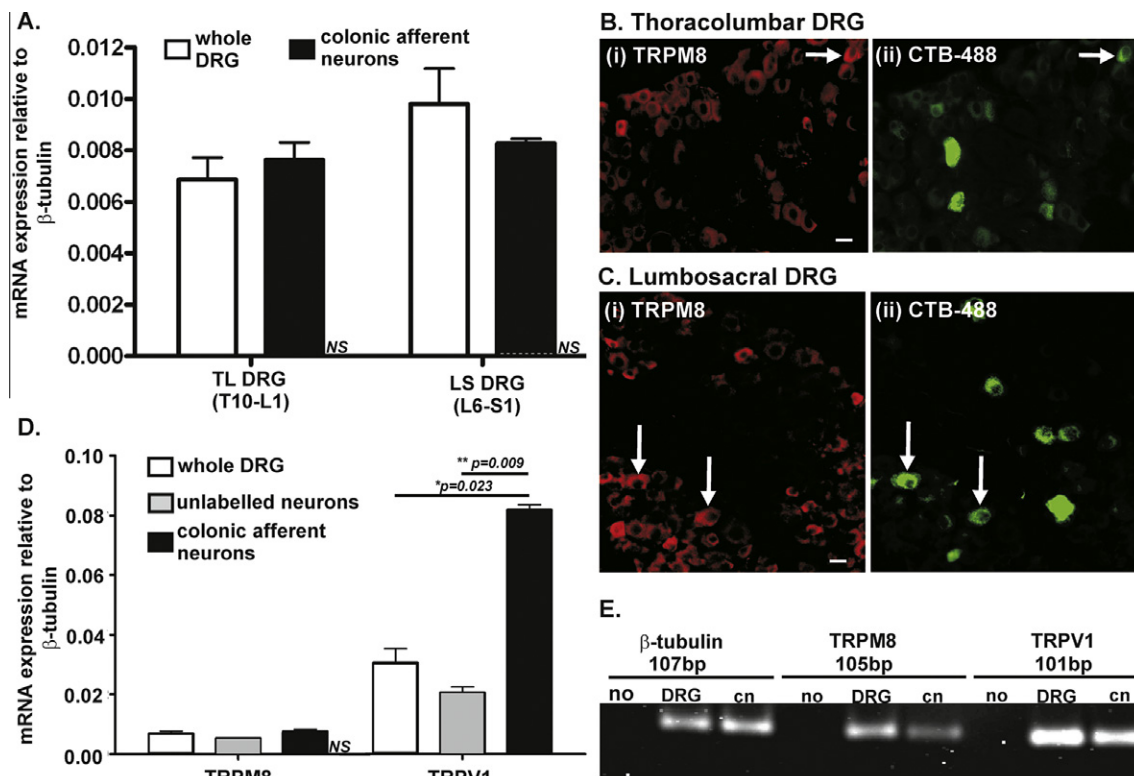
### 3.1. Expression of TRPM8 in colonic afferent pathways

We investigated the expression of TRPM8 in colonic and noncolonic sensory neurons using a combination of techniques. Quantitative RT-PCR of mRNA content indicated messenger RNA for TRPM8 was present but did not significantly differ between whole TL and LS DRG (Fig. 1A). Laser-capture microdissection combined with quantitative RT-PCR indicated that TRPM8 transcripts were detected in similar abundance in retrogradely labelled TL and LS colonic neurons (Fig. 1A). Quantification of mRNA levels also indicated that colonic afferent neurons express similar levels of TRPM8 to the general population of DRG neurons (Fig. 1A).

Consistent with previous studies in mice [20], fluorescence immunohistochemistry revealed TRPM8 protein in small diameter neurons in both ganglia, with 5.4 ± 0.3% of neurons in the TL DRG (Fig. 1B i) and 7% ± 1.2% of neurons in the LS DRG (Fig. 1C i) immunoreactive (IR) for TRPM8. The small percentage of retrogradely labeled colonic afferent DRG neurons in the TL (Fig. 1B ii) and LS (Fig. 1C ii) was consistent with previous studies [11,32]. TRPM8 immunoreactivity was localised to 12.96 ± 3% of retrogradely labeled colonic neurons in the TL DRG (Fig. 1B) and 21.68 ± 1.7% in the LS DRG (Fig. 1C). The retrogradely labeled neurons accounted for a small population (7–9%) of all TRPM8-IR neurons across both sensory ganglia, indicating that colonic neurons are not more likely to express TRPM8 than the general population of DRG neurons.

The expression of TRPM8 in colonic afferent neurons was compared with expression of TRPV1 in the same neurons (Fig. 1D). TRPV1 expression is enriched in TL colonic afferent neurons (Fig. 1D) when compared with the general population of neurons. Previous studies report that approximately 80% of TL colonic afferent neurons display TRPV1-like immunoreactivity [8]. We observed RT-PCR products corresponding to the predicted nucleotide length of TRPM8 and TRPV1 in whole DRG and in laser-captured retrograde labeled colonic afferent neurons (Fig. 1E).

In the distal colon, TRPM8 immunoreactivity was present in a small population of nerve fibres in the outermost layers of the colonic wall, where they were associated with mesenteric blood vessels (Fig. 2A). Some TRPM8-IR fibres were also present in the myenteric ganglia (Fig. 2B) and in the circular muscle (Fig. 2C). TRPM8-IR fibres were scarce in the mucosa, but were present in the muscularis mucosae at the base of the mucosa (Fig. 2C). In whole-mount preparations of mouse colon, TRPM8 immunoreactivity



**Fig. 1.** Expression and protein localisation of TRPM8 in colonic afferent neurons. (A) Comparative TRPM8 mRNA expression in whole dorsal root ganglia (DRG) and in isolated colonic afferent neurons from thoracolumbar (TL) and lumbosacral (LS) DRG, respectively. Colonic afferent neurons were retrograde labelled from the colon and isolated by laser capture microdissection. mRNA levels were determined after analysis of real-time polymerase chain reaction cycle thresholds relative to  $\beta$ -tubulin. TRPM8 immunoreactivity (red) in (B) thoracolumbar DRG and (C) lumbosacral DRG, combined with retrograde labelling from the colon with CTB-488 (green) to identify colonic afferent neurons. Arrows indicate neurons showing retrograde labelling and TRPM8 immunoreactivity. Scale bars = 20  $\mu$ m. (D) Relative mRNA expression of TRPM8 and TRPV1 in whole thoracolumbar DRG, isolated unlabelled sensory DRG neurons, and isolated retrograde labelled colonic afferent DRG neurons. TRPV1 mRNA expression is enriched in colonic afferent neurons, whereas TRPM8 mRNA expression is similar to that observed in noncolonic populations. E: Agarose gel electrophoresis of amplified reverse transcription and polymerase chain reaction products from whole thoracolumbar DRG and labelled colonic afferent neurons (cn) using primers specific for  $\beta$ -tubulin, TRPM8, and TRPV1; no denotes no RNA template added.

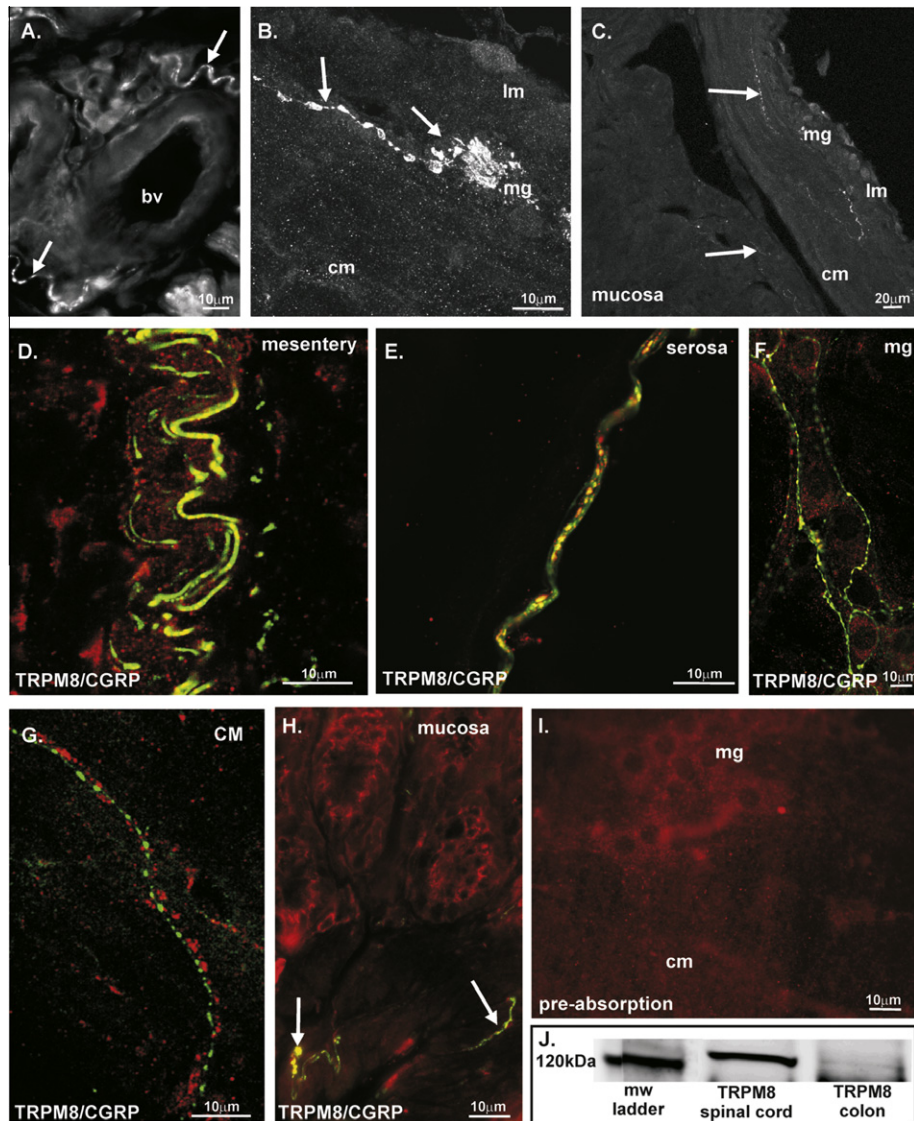
co-localised with CGRP-immunoreactivity in nerve fibres alongside blood vessels (Fig. 2D), in the serosa (Fig. 2E), and in the myenteric ganglia (Fig. 2F). Intramuscular TRPM8-IR nerve fibres did not contain CGRP but were closely apposed to CGRP-IR fibres (Fig. 2G). TRPM8-IR nerves at the base of the mucosa were CGRP-IR (Fig. 2H). TRPM8-IR was abolished by preabsorbing the antibody with the immunising peptide (Fig. 2I). Western blotting of spinal cord homogenate, where TRPM8 protein is abundantly present [20,59], and colon homogenate showed that the antisera used bound to a band at approximately 120 kDa (Fig. 2J) and is present, albeit very scarce, in the colon. The expected size of TRPM8 is 128 kDa (SwissProt. #Q8R4D5).

### 3.2. Effect of icilin on colonic afferent fibres

Single-fibre recordings of splanchnic serosal and mesenteric afferents demonstrated a population of fibres that responded directly to application of icilin (5  $\mu$ mol/L) (Fig. 3A). Of the 58 splanchnic afferents tested, 14 (24%) responded directly to icilin (Fig. 3B). Upon retesting of mechanosensitivity after icilin responses, afferent receptive fields had significantly reduced responses to mechanical stimulation (Fig. 3C and D). Furthermore, afferents that did not respond directly to icilin application were also significantly desensitised to mechanical stimuli (Fig. 3E). There was no statistical association between the size of the chemosensory response and the level of mechanical desensitisation. However, the level of mechanical desensitisation after icilin was dose dependent (Fig. 3F).

Co-immunoreactivity for TRPM8 and TRPV1 was present in  $27.4 \pm 5\%$  of retrogradely traced neurons in the TL DRG (eg, Fig. 4A). In the colonic wall, nerve fibers around blood vessels showed TRPM8/TRPV1-immunoreactivity (Fig. 4B). We found that 7 of 21 (33%) splanchnic afferent fibres tested responded directly to both icilin and the TRPV1 agonist capsaicin (3  $\mu$ mol/L) (Fig. 4C). Prior incubation of the TRPM8 agonist icilin subsequently affected TRPV1 function. Specifically, we found that the direct chemosensory responses of splanchnic serosal afferent fibres to capsaicin (3  $\mu$ mol/L) were significantly reduced by icilin pretreatment, but only in those afferents that responded directly to icilin (Fig. 4D). Capsaicin-responsive splanchnic afferent nerves are reproducibly and significantly mechanically desensitised after responding to capsaicin (Fig. 4Ei) [8]. However, this pronounced capsaicin-induced mechanical desensitisation did not occur in afferents pretreated with icilin (Fig. 4Eii). This effect occurred in fibers responding directly to icilin (Fig. 4Eii) and in those that did not (Fig. 4Eiii). These data show that preincubation with icilin inhibits direct chemosensory responses and mechanical desensitisation mediated by TRPV1.

Like TRPM8, the TRPA1 channel is also activated by noxious cold [23]. In addition, we have found that TRPA1 plays an important role in mechanosensory function in colonic afferents [9]. TRPA1 agonists evoke mechanical hypersensitivity and activation of TRPV1-induced mechanical desensitization requires TRPA1 [9]. Based on these observations, we hypothesized that activation of TRPM8 could also modulate TRPA1-mediated mechanosensation. Here we found that the TRPA1 agonist AITC (mustard oil; 40  $\mu$ mol/L) increases serosal afferent responses to mechanical



**Fig. 2.** Localisation of TRPM8 in peripheral endings in the mouse colon. (A, B and H) Cryostat sections (10 μm) and (D–G and I) flat-sheet, whole-mount preparations of mouse colon showing (A–C) TRPM8 immunoreactivity and (D–H) TRPM8 (red) and CGRP (green) immunoreactivity. TRPM8 immunoreactivity was localised to nerve fibres (A) around mesenteric blood vessels (bv), (B) in the myenteric ganglia (mg), (C) in the circular muscle (cm), and at the base of the mucosa (arrows). TRPM8-IR fibres containing CGRP-immunoreactivity were present in the (D) mesentery, (E) serosa, and (F) myenteric ganglia. G: TRPM8-IR fibres in the muscle did not co-label for CGRP-immunoreactivity but were closely associated with CGRP-IR fibres. H: TRPM8-IR fibres at base of mucosa partially overlapped with CGRP-IR. I: Preabsorbing the TRPM8 antisera with the immunising peptide abolished TRPM8-immunoreactivity. J: Western blot of mouse spinal cord and colon homogenate labelled with TRPM8 antisera. Antisera bound to abundant levels of protein in the spinal cord and a sparse level of protein in the colon at approximately 120 kDa.

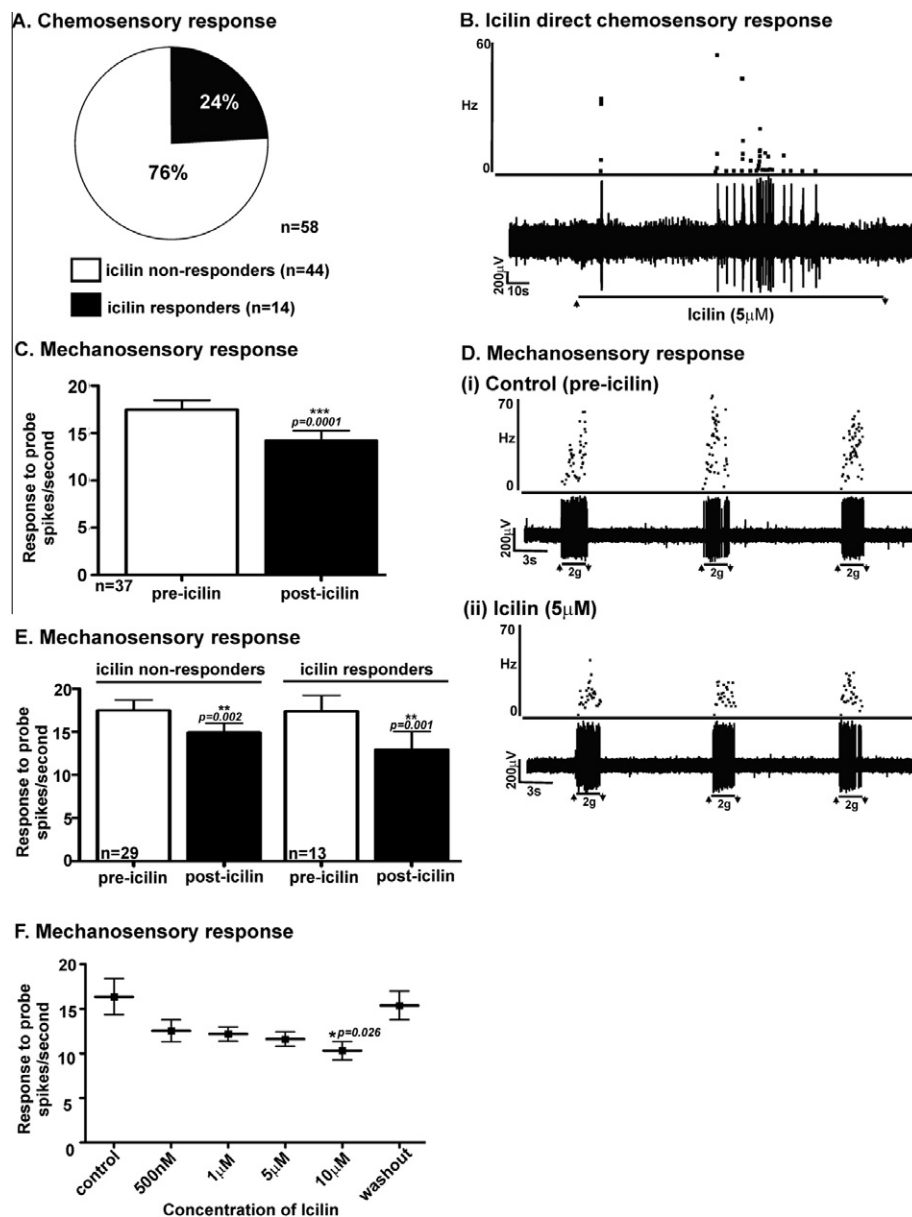
stimuli (Fig. 5Ai), as previously described [9]. However, after treatment with icilin, this AITC-induced mechanical hypersensitivity did not occur (Fig. 5Aii). Furthermore, in TRPA1<sup>-/-</sup> mice, the icilin-induced mechanical desensitisation of serosal afferent fibres was absent (Fig. 5B). We also confirmed our previous findings demonstrating that the capsaicin-mediated desensitisation was also absent in TRPA1<sup>-/-</sup> mice (Fig. 5B) [9]. It could be argued that icilin is acting directly on TRPA1 [50] but the icilin (5 μmol/L) induced excitation of splanchnic afferent fibres (5 of 12 fibers tested, Fig. 5C) was similar in TRPA1<sup>-/-</sup> and wild-type fibres (Fig. 5D). Overall, these findings suggest that both TRPM8 and TRPV1 interfere with TRPA1-mediated mechanosensory function, and that TRPM8 also interacts with TRPV1.

#### 4. Discussion

This study provides anatomical and molecular evidence for the expression of TRPM8 in a subpopulation of colonic afferent

neurons. The functional studies also provide evidence for direct activation of high-threshold colonic afferent fibres through TRPM8 and an interaction of TRPM8 with both TRPV1 and TRPA1, which alters the properties of mechanoreceptive colonic afferent endings. Our major findings are firstly that pro- and anti-nociceptive roles may exist for TRPM8 in high-threshold colonic afferents, as it produces initial activation followed by mechanical desensitisation. Second, we show TRPM8 activation subsequently effects TRPV1 and TRPA1 sensitivity, resulting in diminished agonist-evoked responses.

We found TRPM8 mRNA expression in retrogradely labeled colonic DRG afferent neurons. Correspondingly, in the periphery, TRPM8 protein was localized to nerve endings within various layers of the colonic wall. TRPM8 mRNA expression was similar in colonic afferent neurons compared with other DRG neurons in both the TL and LS DRG, indicating a lack of specialization according to peripheral targets. Although this is the first report of TRPM8 expression in colonic afferents, it has been localised to



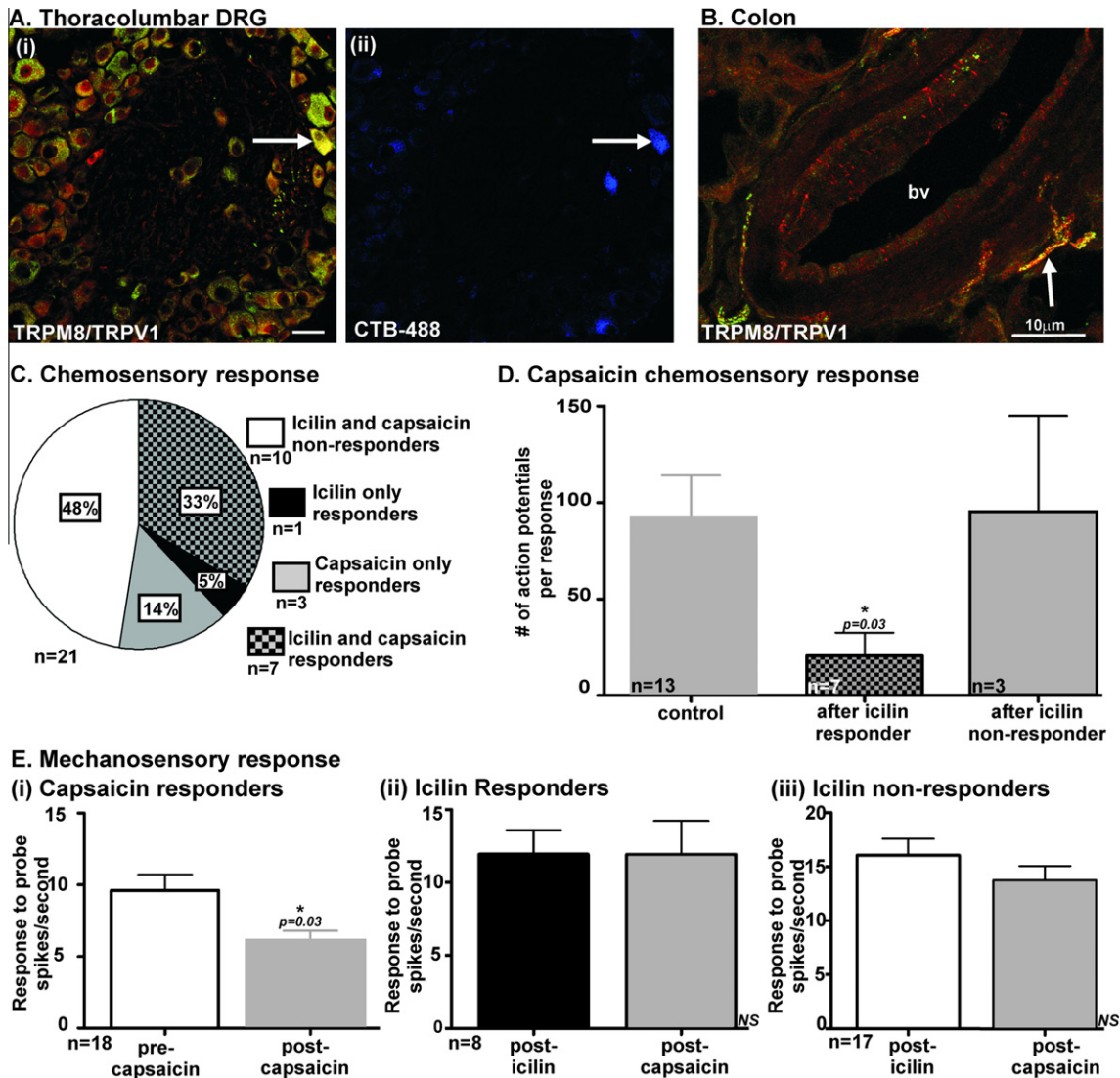
**Fig. 3.** Effect of icilin on colonic afferent sensory fibres. (A) Of the splanchnic serosal and mesenteric fibre mechanosensitive receptive fields tested, 30% had a direct chemosensory response to application of icilin (5  $\mu$ M). (B) Original recording showing effect of icilin on splanchnic serosal afferent fibre activity. Upper trace shows instantaneous frequency plots; lower trace shows original recording of neural activity during application of icilin. (C) After incubation with icilin for 2 minutes, mechanosensitivity of splanchnic serosal and mesenteric afferents was significantly reduced. (D) Original recording showing reduced mechanical responsiveness of a splanchnic serosal afferent fibre mechanosensitive response to probing with 2 g von Frey hair after icilin 5- $\mu$ mol/L application. Upper trace shows instantaneous frequency plots; lower trace shows original recording of neural activity before (i) and after (ii) incubation with icilin. (E) Splanchnic afferent fibres not directly responding to icilin were significantly desensitized to mechanical stimuli; however the level of desensitisation was greater in fibres that responded directly to icilin. (F) Desensitisation of splanchnic serosal afferents to mechanical stimuli was dose dependent, with significant desensitisation reached at 5  $\mu$ mol/L. After removal and washout of icilin, mechanosensitivity of splanchnic serosal afferent fibres returned to control levels. NS, not significant ( $P > .05$ ).

vagal afferent neurons innervating the upper GI tract [61,62] and recently to sensory neurons innervating the bladder [29]. Within the bladder, TRPM8 has been implicated in overactive bladder and painful bladder syndrome [36]. As visceral organs are not subject to temperature changes as experienced by skin, it could be suggested that a physiological role of TRPM8 is more important in skin. However, a range of endogenous agents have been shown to act on TRPM8, such as lysophospholipids, endovanilloids, and endocannabinoids [4,18,19,58].

We observed direct excitation of a population of high-threshold colonic afferent nerve endings by the TRPM8 agonist, icilin. Pharmacological studies demonstrate that icilin, at low concentrations, specifically activates TRPM8 [7]. In somatosensory neurons, icilin

activates TRPM8 with extremely variable latency followed by extensive desensitization, provided that calcium is present [16]. It has also been shown that, at high concentrations, icilin can activate TRPA1, in addition to TRPM8 [38,50]. However, in the current study, we found that icilin evoked similar afferent responses in both wild-type and TRPA1 knock-out mice, suggesting that icilin acted directly on TRPM8 in colonic afferent fibres. Correspondingly, cold hypersensitivity induced by icilin is retained in TRPA1<sup>-/-</sup> mice but is absent in TRPM8<sup>-/-</sup> mice [25]. Previous studies have also shown that icilin has excitatory effects in TRPA1 knock-out mice and in sensory pathways that do not contain TRPA1 [13].

An important finding, potentially explaining the antinociception mediated by TRPM8 at peripheral sensory endings, was the

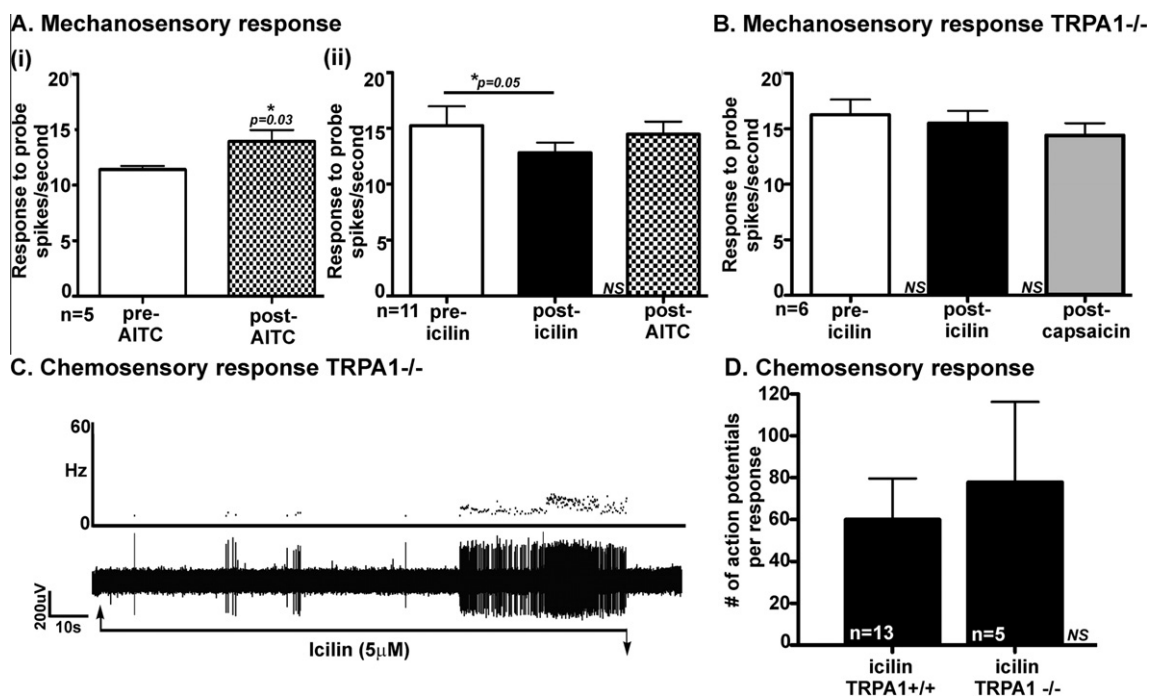


**Fig. 4.** TRPM8 and TRPV1 co-localisation in colonic afferent neurons. (A) In thoracolumbar DRG, (i) co-expression (yellow) of TRPM8 (green), and TRPV1 (red) immunoreactivity was localised to (ii) colonic afferent neurons (blue) identified with retrograde labeling with CTB-488. (B) In colonic wall, TRPM8 (green) immunoreactivity was present in some TRPV1-IR (red) nerve fibers around blood vessels (co-localisation in yellow). (C) Of the 24 serosal afferent fibres tested 9 (37.5%) responded directly to icilin and also 3  $\mu\text{mol/L}$  capsaicin. Equal proportions did not respond to either icilin or capsaicin. (D) Afferent fibers responding to icilin had reduced neural activity induced by capsaicin. (E) (i) Capsaicin caused significant mechanical desensitisation in splanchnic afferent fibers, which was abolished by preincubation with icilin (ii) in fibres responding directly to icilin but not (iii) in fibres that did not respond directly to icilin. Scale bars = 20  $\mu\text{m}$ . NS, not significant ( $P > 0.05$ ).

way in which TRPM8 activation affected the function of other TRP channels. A population of mechanoreceptive nerve endings that responded directly to icilin also responded to the TRPV1 agonist capsaicin, suggesting co-expression of these channels in colonic afferents. Direct chemosensory responses to capsaicin were reduced after incubation with icilin, which also prevented capsaicin-induced mechanical desensitisation, suggesting not only co-expression but also direct interaction. Consistent with these results, we found TRPV1/TRPM8 co-localisation in a population of colonic afferent neurons and peripheral nerve fibres around blood vessels in the colon wall. The ability of TRPM8 to cross-desensitize TRPV1 suggests that it is able to temporarily suppress activity of TRPV1 and hence to have analgesic actions. With regard to the mechanism of this interaction, TRPM8 and TRPV1 are both regulated by protein kinase C (PKC)-mediated phosphorylation [47], and functional interactions between the 2 channels could occur either directly or indirectly via recruitment of common second messengers. PKC activation by mediators such as bradykinin has been shown to blunt TRPM8-mediated and facilitate

TRPV1-mediated afferent activation [47], suggesting that the TRPM8–TRPV1 interaction may be subject to external modulation. Furthermore, bradykinin receptor 2 activation sensitizes TRPV1 but inhibits TRPM8 function, the latter via a reduction in intracellular PIP2 levels [17,22,48].

TRPM8/TRPV1 co-localisation within the neural circuitry of sensory pathways appears to be dependent on the type of sensory neuron assessed and its target organ. Neurochemical studies show TRPM8 sensory neurons co-express TRPV1 in varying degrees [1,29,43,56,57,62]. In the trigeminal ganglion, very few neurons co-express TRPM8 and TRPV1 [1], whereas in lumbosacral DRG neurons supplying the bladder, one-third of TRPM8-IR neurons co-express TRPV1-IR [29]. We found TRPM8/TRPV1-immunoreactivity in  $27.4 \pm 5\%$  of colonic afferent neurons. TRPM8 has also been shown to modulate central glutamatergic nociceptive inputs into the spinal cord dorsal horn [20,49,59], a population of which also express TRPV1 targeting the same types of dorsal horn neurons [63]. Cross-sensitisation of TRPV1 has previously been described at peripheral endings of sensory neurons, induced by the TRPM8



**Fig. 5.** Contribution of TRPA1 to icilin-induced mechanical desensitisation. (A) (i) TRPA1 agonist AITC (40  $\mu\text{mol/L}$ ) significantly increased fibre responses to mechanical stimuli. (ii) This increase was not significant in fibres after incubation with icilin. (B) In TRPA1<sup>-/-</sup> mice, icilin and capsaicin did not cause mechanical desensitisation. (C) Original recording showing direct chemosensory response of icilin on a splanchnic serosal afferent fibre in a TRPA1 knock-out mouse (TRPA1<sup>-/-</sup>). Upper trace shows instantaneous frequency plots; lower trace shows original recording of neural activity during application of icilin. (D) Direct affect of icilin on fiber activity was unchanged in TRPA1<sup>-/-</sup> mice. NS, not significant ( $P > 0.05$ ).

agonist menthol [26]. However, menthol also acts on TRPA1, as cold hypersensitivity evoked by menthol is present in TRPM8<sup>-/-</sup> mice and is lost in TRPA1<sup>-/-</sup> mice [25]. An increase in TRPM8 expression within TRPV1-expressing sensory neurons has been shown in neuropathic and inflammatory pain models [20].

Icilin reduced afferent mechanosensory responses after evoking direct excitation, but it also caused mechanical desensitisation in fibres that showed no direct response. Moreover, icilin pretreatment reduced capsaicin desensitisation without necessarily evoking a direct response. This suggests either that the desensitisation may occur via actions elsewhere from TRPM8, which is unlikely as discussed above, or that only low levels of TRPM8 expression are required for coupling to the pathway of desensitisation, whereas high levels are required for coupling to generation of action potentials. This may be reflected in the sparsity of colonic neurons clearly immunoreactive for TRPM8, which contrasts with the large number of fibres desensitized by icilin. We have shown this type of phenomenon previously [9] whereby very few afferents showed direct chemosensory responses to TRPA1 activation with AITC and yet a large population became mechanically hypersensitive after AITC.

Normal mechanosensory responses of colonic high-threshold afferents require expression of TRPA1, and activation of TRPA1 augments colonic afferent responses to mechanical stimuli [9]. Therefore TRPM8 may cause desensitization via some sort of interaction with TRPA1. We provide evidence for this by showing that icilin prevented mechanical hypersensitivity induced by TRPA1 activation with AITC. Correspondingly, the effect of icilin on mechanical desensitisation was absent in TRPA1 knock-out mice. However, the direct chemosensory responses to icilin were intact in TRPA1 knockouts. This suggests the mechanical desensitisation induced by icilin depends on interaction of TRPM8 with TRPA1. Importantly, it also shows that TRPM8 does not desensitize afferent endings nonselectively.

There are bidirectional interactions between TRPA1 and TRPV1 in transduction of sensory stimuli in both somatic and viscerosensory pathways [9,13,42,53]. Therefore the ability of TRPM8 to reduce mechanosensory function may involve TRPV1 and TRPA1 in a type of cascade. For example, icilin activation of TRPM8 may reduce TRPV1 function, which in turn affects TRPA1-mediated mechanosensation. It is also possible that TRPM8 interacts with TRPV1 and TRPA1 independently.

Our data provide a possible mechanism for the visceral antinociceptive properties of peppermint in animal models [2] and human studies [14,24,27,34,41] via exogenous activation of TRPM8 on peripheral sensory endings. This may be important in the clinical setting, because these classes of afferent become mechanically hypersensitive after recovery from colonic inflammation [30,31]. Therefore, reducing their mechanosensitivity via TRPM8 activation could have significant therapeutic benefit. This study provides a mechanism by which existing herbal remedies or future therapeutic drugs containing TRPM8 agonists may act to reduce abdominal pain, particularly of colonic origin. This potentially occurs via activation of TRPM8 on high-threshold colonic afferent sensory endings that subsequently cross-desensitizes TRPV1 activation. Furthermore, activation of TRPM8 also reduces the activity of TRPA1 channels, effectively dampening mechanosensitivity.

#### Conflict of interest

The authors have no conflict of interests to declare.

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