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A selective role for TRPV4 ion channels in visceral sensory pathways

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Abstract

Background & Aims—Although there are many candidates as molecular mechanotransducers, so far there has been no evidence for molecular specialization of visceral afferents. Here we show that colonic afferents express a specific molecular transducer that underlies their specialized mechanosensory function: the transient receptor potential channel TRPV4.

Methods & Results—We found TRPV4 mRNA is highly enriched in colonic sensory neurons compared with other visceral and somatic sensory neurons. TRPV4 protein was found in colonic nerve fibers from patients with inflammatory bowel disease, and it colocalized in a subset of fibers with the sensory neuropeptide CGRP in mice. We characterized the responses of eight subtypes of vagal, splanchnic and pelvic mechanoreceptors. Mechanosensory responses of colonic serosal and mesenteric afferents were enhanced by a TRPV4 agonist and dramatically reduced by targeted deletion of TRPV4 or by a TRP antagonist. Other subtypes of vagal and pelvic afferents, by contrast, were unaffected by these interventions. The behavioral responses to noxious colonic distension were also substantially reduced in mice lacking TRPV4.

Conclusions—These data indicate that TRPV4 contributes to mechanically-evoked visceral pain, with relevance to human disease. In view of its distribution in favor of specific populations of visceral afferents, we propose that TRPV4 may present a selective novel target for the reduction of visceral pain, which is an important opportunity in the absence of current treatments.

Introduction

Chronic visceral pain represents a major unmet need for treatment. A hallmark of chronic pain is heightened sensitivity to mechanical events; in the gastrointestinal tract these are mainly

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smooth muscle contraction and distension of hollow organs^{1, 2}. In irritable bowel syndrome, peripheral cellular changes are becoming increasingly evident³ suggesting that peripheral sensory mechanisms assume a key role in generating pain. Opportunities for improved treatment most likely will arise from targeting specific mechanisms of activation of nociceptive neurons.

Mechanosensory information from the viscera follows three major pathways to the central nervous system: the vagal, splanchnic (thoraco-lumbar spinal) and pelvic (lumbo-sacral spinal) nerves. We have recently shown there are 8 main functional subtypes of mechanosensitive afferents to the mouse gastrointestinal tract according to the location of their mechanoreceptive fields in mucosa, muscle, serosa, and mesentery^{4, 5}. Each pathway contains different functional classes of afferents. Some classes are common between pathways, for example serosal afferents are found in both splanchnic and pelvic pathways, whilst others are unique to a particular pathway, such as mesenteric afferents, which are exclusively splanchnic. Between them, these afferent subtypes signal the full range of qualities of the mechanical environment within and outside the gastrointestinal lumen. Overall, the vagal pathway contains afferents from more proximal regions tuned to detect non-noxious stimuli involved in homeostasis and feeding behavior; the pelvic pathway contains both nociceptive and non-nociceptive afferents from distal organs, whilst splanchnic afferents have generally higher mechanical thresholds than pelvic afferents, and constitute primarily a pain sensing pathway from throughout the abdominal and pelvic viscera⁶.

The Transient Receptor Potential (TRP) family of ion channels are emerging candidates as molecular sensory transducers, and as such form part of the front line of sensory function⁷. The TRP family comprises 5 main members (TRPA, TRPC, TRPM, TRPP, TRPV) in mammals, which share several key properties: they are mostly non-selective cation channels and have six transmembrane domains. TRPs are proposed to be primary transducers of thermal, mechanical and chemical stimuli. Specifically TRPV4 is implicated in mechanosensation⁸ and pain^{9, 10}. TRPV4 is the mammalian homologue of the *C. elegans* gene Osm-9, which is critical in osmotic and mechanical avoidance suggesting that it functions to detect noxious osmotic and mechanical stimuli. TRPV4^{-/-} mice show abnormal systemic osmotic regulation and decreased cutaneous pain, implicating TRPV4 in mechanotransduction. TRPV4 is expressed in sensory neurons^{10, 11}, and is regulated endogenously by the arachidonic acid metabolite, 5,6-EET¹², which is produced in response to a variety of stimuli.

Our aim in this study was to determine the role of TRPV4 in visceral sensation. We accomplished this first by investigating its localization and expression along visceral afferent pathways, second by assessing the functional effects of TRPV4 ligands and genetic mutation on a range of visceral mechanosensitive afferents, third by translating the findings to a conscious animal model; finally we determine the translation of TRPV4 localization to human tissue.

Materials and Methods

All experiments were approved by the Animal Ethics Committees of the IMVS and the University of Adelaide, and the human ethics committee of the Royal Adelaide Hospital.

Retrograde labeling

As described previously^{13, 14}, the fluorescent retrograde neuronal tracer cholera toxin subunit B conjugated to fluorescein isothiocyanate (CTB-FITC) was injected at several sites within the wall of the descending colon or proximal stomach. After three days dorsal root or nodose ganglia were removed either for *in situ* hybridization or laser capture microdissection.

In situ hybridization

Using a method developed in our laboratory^{13, 14}, animals were transcardially perfused with 4% paraformaldehyde and thoracolumbar (T10-L1) and lumbosacral (L6-S1) DRG were removed, cryoprotected and frozen in embedding medium. Serial frozen sections (12µm) were cut and post fixed, then underwent a standard protocol for preparation prior to hybridization. Under RNase free conditions a digoxigenin (DIG) labeled oligonucleotide probe TGTGCTGGTGGTTGTCTCTGCGGCGCTCTACCTGGCTGGGATCGAGGC (1750–1797) was used to target TRPV4, with a complementary sense probe used as a negative control, which revealed minimal labeling above background. The probe was designed to target conserved areas across the splice variants. Detection of the DIG label was performed using CARD amplification (Perkin-Elmer, USA) combined with streptavidin conjugated AlexaFluor 546 (SAF546) (Invitrogen, USA). Images were obtained using an epifluorescent microscope (Olympus BX51) using filters appropriate for FITC and SAF546 labeling.

Only cells with intact nuclei were included. Number of labeled neurons was expressed as percentage of neurons in the whole section. This measure was determined from 4 to 8 ganglion sections/mouse and averaged across 5–6 mice/target. The number of neurons labeled by each antisense probe was compared with several negative control experiments including use of complementary sense probe, omission of probe from the hybridization buffer and competition with excess unlabeled probe. A negligible level of autofluorescence was observed in control experiments when compared to the antisense probe. A neuron was counted as TRPV4 positive if the majority of the cytoplasm was fluorescent at a level clearly above the maximum attributable to autofluorescence. No labelling was seen in non-neuronal structures such as glia. Proportions of positive neurons for each target in whole DRG or nodose ganglia were the same regardless of whether or not ganglia were retrogradely labeled, indicating that expression was not induced by interventions during tracing. Cross sectional diameter was averaged over a representative 96 neurons per label (N=6). Similar values were obtained using fluorescence or light (Nomarsky) microscopy. One-way ANOVAs or unpaired t-tests were used to determine differences in TRPV4 expression between populations.

Laser capture microdissection and quantitative RT-PCR

DRG (T10-L1 and L6-S1) and nodose ganglia were removed from five male or female mice (20–30g). RNA was isolated from whole DRG using methods described elsewhere^{13, 14} and used for quantitative RT-PCR (QRT-PCR).

Nodose and DRG were also harvested then sectioned after retrograde labeling with CTB-FITC as described above. Retrogradely labeled neurons were microdissected on a PALM Microlaser Technologies microdissection system and catapulted directly into a lysis and stabilization buffer and RNA was isolated. QRT-PCR were performed using a Chromo4 real-time instrument and Opticon Monitor software (MJ Research). We used Qiagen QuantiTect® SYBR® Green RT-PCR one-step kits according to the manufacturer's specifications, with the following primers and conditions: TRPV4: Forward 5'-3'

CGTCCAAACCTGCGAATGAAGTTC, Reverse 5'-3'

CCTCCATCTCTTGTGTCACCTGG; Reverse Transcription: 50°C (30mins); Initial PCR activation: 95°C (15 mins); PCR cycles: 94°C (15sec), 47°C (30sec) and 72°C (30sec) repeated for 44 cycles. β -tubulin was assessed using a Qiagen Quantitect® Primer Assay. Size of amplified products was confirmed by gel electrophoresis. Each assay was run at least 3x in separate experiments. Control PCRs were performed with distilled RNase-free water. The comparative cycle threshold method was used to quantify the abundance of target transcripts in whole ganglia and LCM neurons, as described^{13, 14}. Quantitative data are expressed as mean $\pm$ SD, and significant differences in transcript expression determined by a Mann-Whitney test.

Immunohistochemistry

Distal colon was removed after transcardial perfusion with 4% paraformaldehyde. Either transverse sections (20 μ m) or wholemounts (mucosa-stripped) were examined. A goat anti-calcitonin gene-related peptide (CGRP 1/200; Calbiochem, catalogue #PC205L) was used overnight at 4°C for sections or 37°C for wholemounts¹⁵. A rabbit anti-TRPV4 (1/1000; Alomone, catalogue no. ACC- 034) was used under the same conditions¹⁰. Secondary antibodies coupled to AlexaFluor®488 and AlexaFluor®555 were used for visualization. Negative controls were prepared as above with the primary antibody omitted or in tissue from TRPV4^{-/-} mice.

Targeted deletion of TRPV4

Mice with disrupted TRPV4 genes were generated by homologous recombination in embryonic stem cells as previously described¹⁶. Individual colonies of homozygous wild-type and TRPV4^{-/-} mice were generated from heterozygote breeding pairs. Homozygous TRPV4^{+/+} or ^{-/-} littermates were blindly allocated for experiments and their genotype confirmed by PCR of tail DNA. Results were encoded until statistical analysis. TRPV4^{+/+} or ^{-/-} mice were otherwise indistinguishable by body weight, general behavior or gross anatomical features of the gut or elsewhere.

In vitro electrophysiology and pharmacology

Similar to preparations we have recently published⁵, the distal colon plus attached lumbar splanchnic or pelvic nerves were removed from TRPV4^{+/+} and ^{-/-} mutant mice. The colon was opened and pinned mucosal side up in a specialized organ bath. The colonic compartment was superfused with modified Krebs solution composed of (mM): 117.9NaCl, 4.7KCl, 25NaHCO₃, 1.3NaH₂PO₄, 1.2MgSO₄(H₂O)₇, 2.5CaCl₂, 11.1D-Glucose, 2NaButyrate, and 20NaAcetate bubbled with 95% O₂/5% CO₂ at 34°C. The L-type calcium channel antagonist nifedipine (1 μ M) was added to suppress smooth muscle activity and the cyclo-oxygenase inhibitor indomethacin (3 μ M) to suppress potential actions of endogenous prostaglandins. The nerve bundle was extended into the paraffin filled recording compartment whereafter finely dissected strands were laid onto a mirror and single fibers recorded.

Receptive fields were identified as described previously⁵, then tested with three distinct mechanical stimuli to enable classification: focal compression with calibrated von Frey hairs (70,160,400,1000mg, 2g & 4g force; each force applied for 3 $\times$ 3sec), mucosal stroking with calibrated von Frey hairs (10,200,500, and 1000mg; each applied 10 times) and circular stretch (1–5g, in 1g increments; each applied for 1 min, with 1 min interval between applications). Stretch was applied via a claw attached to the tissue near the receptive field connected to a cantilever system. Conduction velocity and electrical threshold were determined by stimulation via a concentric electrode placed over the receptive field. In a separate group of experiments, the colon was initially left intact and a balloon (details below) inserted. This was distended isobarically with air under the control of a precision manometer, and the response of fibers to distension measured. Mechanoreceptive fields were subsequently examined and classified serosal or mesenteric after opening the colon as described above.

Gastroesophageal mechanoreceptors were recorded using a similar technique, as described in detail previously⁴. They were classified into mucosal and tension receptors according to their responses to calibrated von Frey hairs (10–1000 mg) stroked over the mucosa and circumferential stretch applied across the receptive field using a claw and pulley/cantilever system (0.5–5 g).

Once the individual afferent fiber had been classified and its stimulus-response relationship established reproducibly, increasing concentrations of 5,6-EET or ruthenium red (RR) were

applied in separate experiments into a small metal ring placed around the receptive field. Each concentration was allowed to equilibrate (2min for 5,6-EET and 10min for RR) then the ring was removed and mechanosensitivity to a limited range of stimuli was quickly re-tested. In each pharmacological experiment usually only one afferent fiber was studied.

Stimulus response functions are expressed as spikes/second or spikes/stroke (for mucosal responses). Effects of drugs were investigated at a sub-maximal mechanical stimulus, allowing observation of increases or decreases in mechanosensitivity. Differences between stimulus-response functions were assessed using two-way analysis of variance (ANOVA). Differences between corresponding points on separate stimulus-response curves were assessed by a Bonferroni post-hoc test. Differences between adjacent points on the same stimulus-response curve were assessed by one-way ANOVA for repeated measures with a Dunnett's post-hoc test. Data are expressed as mean $\pm$ SEM. N=number of animals and n=number of observations.

Colorectal distension and visceromotor response

To record the visceromotor response (VMR) to colorectal distension (CRD), electromyographic (EMG) electrodes were surgically implanted in the abdominal musculature during brief anesthesia. The CRD balloon consisted of a polyethylene plastic cylinder (length 1.5cm; diameter 0.9cm) secured to polyethylene tubing. The balloon was inserted transanally until 0.5cm clear of the anal verge. The electrode wires and tubing were taped to the tail. The animal was allowed to recover fully from anesthesia, then EMG activity was recorded during phasic balloon inflation (80mmHg) achieved by connecting the balloon to a compressed air cylinder. Inflation pressures were adjusted via the tank regulator, and gas flow through the system was controlled by a custom-made distension control device to enable computer triggering of balloon inflation. Each distension was tested five times for 10sec each, with 30sec separating each distension, which gave rise to reproducible EMG responses. No sensitization or desensitization of the VMR was observed over the timecourse of this procedure greater than 10% change from the mean response. Animals were humanely killed by anesthetic overdose immediately following the protocol, at which point the colon was examined and showed no evidence of macroscopic damage due to distension.

Results

Expression of TRPV4 in visceral afferent pathways

Using a combination of techniques that we have recently developed for assessing molecular expression in identified neuronal populations^{13, 14}, we investigated expression of TRPV4 in visceral and non-visceral sensory neurons. A small proportion of neurons in sensory ganglia were retrogradely labeled from stomach or colon: 5.0 $\pm$ 0.7% of nodose ganglion neurons from stomach; 2.9 $\pm$ 0.1% in thoraco-lumbar and 2.3 $\pm$ 0.2% in lumbo-sacral dorsal root ganglia (DRG) from colon. Fluorescence *in-situ* hybridization (FISH) combined with retrograde tracing revealed TRPV4 mRNA in three times as many colon-innervating sensory neurons than other neurons of similar size in thoraco-lumbar DRG (Fig 1B&D), indicating TRPV4 expression is more prevalent in colonic sensory neurons. In lumbo-sacral DRG and nodose ganglia the proportion of identified visceral afferents expressing TRPV4 was also larger than other ganglion neurons, but not to the extent seen in thoraco-lumbar ganglia (Fig 1A&C).

Retrogradely labeled visceral afferent neurons were also investigated using laser-capture and quantitative RT-PCR of mRNA content. RT-PCR products corresponding to the predicted nucleotide length of TRPV4 were detected in nodose ganglia, in thoraco-lumbar and lumbo-sacral DRG, and in retrogradely labeled visceral afferents in each of these ganglia (Fig 1E(i)). Quantification of mRNA levels indicated that identified visceral afferents expressed TRPV4 more abundantly than the general population of neurons in their respective ganglia (Fig 1E

(iii)). Within the thoraco-lumbar DRG TRPV4 was 20-fold more abundant in identified colonic neurons than in whole ganglia. Differences between pathways were also evident, with colonic thoraco-lumbar neurons expressing 8-fold the levels of TRPV4 in identified gastric sensory neurons in nodose ganglia (Fig 1E(iii)). This was despite the fact that relative TRPV4 levels in whole thoraco-lumbar DRG were actually less than those in the other ganglia (Fig 1E(ii)).

TRPV4 protein co-localized almost exclusively with the sensory neuropeptide CGRP in colonic nerve fibers in TRPV4^{+/+} mice. TRPV4 labeling was lost in ^{-/-} mice, whereas patterns of CGRP labeling were unchanged (data not shown). Labeling was confined to the outermost (serosal and mesenteric) layers, frequently associated with blood vessels (Fig 2A), and scarce in intramuscular or mucosal endings (Fig 2B).

Role of TRPV4 in visceral afferent pathways

In view of the enrichment of TRPV4 in visceral sensory pathways apparent from molecular and anatomical studies, we investigated its functional role in visceral mechanosensation. In order to gain a complete picture of mechanosensory function, we investigated all 8 major subtypes of mechanoreceptors in different regions of gut using standardized *in vitro* single fiber recording techniques^{4, 5}. Colonic mechanoreceptors with receptive fields on serosa and mesentery responded to high intensities of mechanical stimulation with von Frey hairs (>50% above 400mg). This was correlated with a high threshold to distension of the intact colon by balloon (45 ± 3.2 mmHg, n=4) or circumferential stretch of the preparation as a flat sheet (10.6 ± 0.3 g, n=4; see Supplementary Information for methods). Mechanosensory responses were dramatically reduced in mice with targeted disruption of TRPV4, and their mechanosensory thresholds were increased (Fig 3A,B). These changes could not be attributed to altered excitability of afferents, because their electrical thresholds and conduction velocities were identical in TRPV4^{+/+} and ^{-/-} mice (Fig 4), and are therefore more attributable to a direct role of TRPV4 in mechanosensation. Changes in mechanosensitivity were specific to splanchnic and pelvic afferents with receptive fields in the serosa and mesentery at similar locations to fibers showing CGRP and TRPV4 labeling in our anatomical studies. In contrast, afferents sensitive to mucosal stroking and muscular tension showed no deficits in TRPV4^{-/-} mice. This was the case in both vagal and pelvic pathways (Fig 3B,C). Thus, changes in function upon deletion of TRPV4 are in keeping with its location in fibers innervating the outer layers of organs and enrichment of its expression in a particular neuronal population that innervates viscera.

The endogenous selective TRPV4 agonist 5,6-EET caused sensitization of the mechanosensory response of splanchnic and pelvic colonic mesenteric and serosal afferents of wild-type mice, but had no effect on those of TRPV4 null-mutants (Fig 5A and Fig 6A, B). The non-selective TRP channel blocker ruthenium red (RR) reduced mechanosensitivity in these afferents in wild-type mice, but had no effect on those from TRPV4 null-mutants (Fig 5B and Fig 6A, B). Representative examples of experiments showing these effects are shown in figure 5. In contrast with serosal and mesenteric afferents, vagal and pelvic mucosal, muscular and muscular mucosal fibers were relatively insensitive to both of these TRPV4 ligands (Fig 6C, and data not shown). From these data it is clear that alterations in the pharmacology of visceral afferents parallel the changes in mechanosensitivity in TRPV4 null-mutants.

Studies of TRPV4 in intact animals and humans

Colorectal balloon distension evokes voluntary contractions of the external abdominal muscles indicative of pain or discomfort in both conscious animals and humans^{1, 17}, and replicates symptoms of chronic inflammatory or functional visceral pain in patients with inflammatory bowel disease or irritable bowel syndrome^{18, 19}. This therefore represents a well-established behavioral response to noxious visceral activation and is relevant to clinical observations. We

measured the abdominal EMG responses to noxious colorectal distension (Fig 7A), which were reduced by approximately half in mice lacking TRPV4, indicating that changes at the cellular level described above translate to alterations in sensory function in intact animals. We observed no other signs of changes in behavior or conscious sensory capacity in TRPV4 null-mutants.

An important step was to determine if TRPV4 is distributed similarly across species, so we investigated TRPV4 expression in tissue from humans. Tissue was obtained from surgical resections in patients with active colitis (Crohn's disease and ulcerative colitis), and from non-inflamed, uninvolved tissue taken from patients undergoing surgery for bowel cancer. We observed punctate TRPV4 immunoreactivity in fine fibers innervating mainly the submucosa and serosa (Fig 7B), where it was observed usually in association with blood vessels (within 20µm). Vascular smooth muscle and endothelium were also weakly positive for TRPV4 as previously reported²⁰. The circular and longitudinal smooth muscle layers of the colon were largely negative (Fig 7C), as were neurons of the myenteric ganglia. In tissue obtained from patients with active colitis we observed that serosal blood vessels were more densely innervated, often being completely wrapped by TRPV4 positive fibers within the section (Fig 7D), whereas vessels in uninflamed colon were only partly encircled (not shown). In one of the Crohn's disease patients, TRPV4-positive lamellar endings were found within longitudinal muscle close to the serosal border (Fig 7C); these were not seen in any other specimens. Thus human colonic resections showed TRPV4 immunolabeling in similar structures to those observed in mouse, being concentrated in the outer layers of the organ. We also observed altered localization of TRPV4 expression during active inflammation of the organ.

Discussion

This study provides anatomical, molecular and functional evidence for a specialized role of TRPV4 in a sub-population of colonic mechanoreceptors; it extends functional evidence into behavior of the whole animal and anatomical evidence into observations of human tissue. These findings indicate a molecular specialization of sensory neurons from the viscera.

Distinct populations of colonic mechanoreceptors exist which have high thresholds to mechanical stimulation that project centrally in the splanchnic and pelvic nerves^{6, 21}; some studies have shown these to have receptive fields on serosal and mesenteric blood vessels^{5, 22}. Our preliminary observations indicate these afferents respond to distension only at intraluminal pressures that evoke pain in other species *in vivo*^{1, 23, 24}. Therefore we speculate that the properties of colonic serosal/mesenteric afferents in the current study are consistent with a role as visceral nociceptors. The mechanosensory responses of these afferents were dramatically reduced in TRPV4 null-mutant mice. In contrast, vagal and pelvic mucosal and muscular afferents showed no deficits in TRPV4^{-/-} mice. TRPV4 therefore plays a selective role in afferents we believe to be visceral nociceptors. The pharmacology of visceral afferents was closely correlated with the changes in mechanosensitivity in TRPV4 null-mutants; thus only serosal and mesenteric colonic fibers showed increases in mechanosensitivity in response to the selective endogenous TRPV4 agonist 5,6-EET, and decreases in response to the TRP antagonist ruthenium red (RR), and these effects were lost in TRPV4^{-/-} mice. RR is recognized as a non-selective TRP channel blocker, yet its effect on mechanosensitivity was lost totally upon deletion of TRPV4. This may indicate that serosal/mesenteric afferents utilise TRPV4 for mechanosensation to the exclusion of other TRPs, otherwise we should have seen a residual effect of RR in TRPV4 null-mutants. Other populations of afferents were relatively unaffected by 5,6-EET and RR, and no changes in this situation occurred in fibers from TRPV4^{-/-} mice, paralleling the lack of changes in mechanosensitivity of these afferents in TRPV4^{-/-}. Our observation of mechanical sensitization of afferents by low concentrations of the TRPV4 agonist 5,6-EET suggests that when it is produced endogenously it may mediate

ongoing activation of the channel. This would provide a delicate fine-tuning mechanism for mechanosensitivity.

Our functional evidence was correlated with intense enrichment of TRPV4 expression in splanchnic and pelvic colonic afferents traced to their cell bodies in the thoraco-lumbar and lumbo-sacral DRG. This was evident both in terms of the proportions of neurons expressing TRPV4 mRNA and the relative quantities of TRPV4 mRNA in the colonic population of DRG neurons. Although previous studies have shown TRPV4 is localized in a sub-population of DRG neurons¹⁰, it has not until now been associated with those innervating a particular target. Our findings indicate a particularly important role for TRPV4 in visceral afferents, but do not preclude its role also in somatic afferents. Correspondingly, TRPV4 null-mutants show deficits in somatic pain^{9, 10, 25}. Interestingly, the role of TRPV4 is often not manifested in somatic pain until a sensitizing stimulus is administered, whereas its role in the colon is evident in normal conditions. There may be additional roles for TRPV4 in visceral afferents, such as one in detecting changes in osmolality, as has been demonstrated for this channel in other systems^{8, 16}. There may also be alternative roles for visceral afferents expressing TRPV4, such as in mediating local vasodilatation, which is suggested by their expression of the vasodilator CGRP and their proximity to blood vessels.

Studies of other candidate mechanotransduction molecules, including members of the acid sensing ion channel (ASIC) and TRP families, have shown that each channel may contribute differently to mechanosensory function in individual subtypes of visceral afferents innervating each visceral organ^{26–28}. Whilst mutation of other channels had effects on several populations of afferents, none of these were exclusively involved in serosal/mesenteric afferent function, in contrast to the highly specific role we have revealed here for TRPV4. The loss of function we observed in TRPV4 null-mutants is also substantially larger than any previously reported in mice lacking other channels. From this and other investigations it is emerging that each class of sensory neuron has a “signature” of expression of a range of channels, which most likely confers its unique mechanosensory properties. We consider that TRPV4 is a major contributor to the “signature” of visceral nociceptors.

We found that observations at the cellular level translate to alterations in sensory function in intact animals. Mice lacking TRPV4 showed dramatic reduction of abdominal EMG responses to noxious colorectal distension. This appeared to be specifically related to perception of the experimental stimulus, because we observed no other signs of changes in behavior or conscious sensory capacity in TRPV4 null-mutants, as others have shown^{16, 25, 29}. Our anatomical findings in animal experiments were translated to studies in human tissue, showing TRPV4 expression in human colon. Thus human colonic resections showed TRPV4 immunolabeling in similar structures to those observed in mouse. TRPV4 was concentrated in the outer layers of the organ and in association with blood vessels, in particular during active inflammation of the colon associated with Crohn’s disease and ulcerative colitis. Both of these conditions are characterized by recurrent abdominal pain¹⁹, therefore the distribution of TRPV4 we observed may provide a basis for these symptoms, along with other pathways associated with inflammation.

In conclusion, our *in vivo* data support TRPV4 as a strong candidate for mediating symptoms of visceral pain. In view of its distribution in favor of visceral afferents we suggest to be nociceptive, we propose that TRPV4 may present a selective novel target for the reduction of visceral pain. This is an important opportunity, because the chronicity of irritable bowel syndrome implicates that it demands continual inhibition without the impairment of other aspects of sensory function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TRPV4	transient receptor potential channel – vanilloid 4
DRG	dorsal root ganglia
TL	thoracolumbar
LS	lumbosacral
FISH	fluorescence in situ hybridization

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Figure 1. Expression of TRPV4 in visceral afferents

A–C. Fluorescence *in situ* hybridization of TRPV4 mRNA expression (red) in nodose ganglia and dorsal root ganglia (DRG), combined with retrograde labelling of visceral sensory neurons from stomach (**A**), and colon (**B and C**) with CTB-FITC (green). Arrows indicate neurons showing retrograde labeling from the gut: blue arrows indicate those positive for TRPV4, and yellow arrows denote TRPV4-negative neurons. Graphs show proportion of neurons expressing TRPV4 in either whole DRG or in retrogradely labelled cells ($p < 0.001$ vs general population, *t*-test). **D.** *In situ* hybridization showed the majority TRPV4 positive neurons were 15–20 μ m in size (i), similar to the distribution of retrogradely labeled visceral afferents (CTB-FITC) in each ganglion (ii). These data indicate that differences in mRNA levels are not related to cell sizes. Most of these are in the C-fiber range, which was confirmed by conduction velocity (Fig 4). **E.** (i) Agarose gel electrophoresis of amplified RT-PCR products from three different ganglia from wild-type (+/+) and TRPV4 null-mutant (–/–) mice using primers specific for TRPV4 and β -actin; no = no RNA template added. (ii) Relative mRNA levels in each sensory ganglion following analysis of real-time PCR cycle thresholds. Thoracolumbar (TL) DRG had lowest levels of TRPV4 expression, which was significant compared with nodose ganglia and lumbosacral DRG (* $p < 0.05$, ** $p < 0.01$ Mann-Whitney U test). (iii) In contrast colonic neurons within TL DRG showed the highest levels of relative expression – approximately 20-fold that in the whole TL DRG ($p < 0.001$).

Figure 2. Colocalization of CGRP and TRPV4 in peripheral endings in mouse colon

A. i & ii: Fibers colocalizing CGRP and TRPV4 are restricted to the outer layers (arrows, ser=serosa), with the mucosa (muc) showing little selective label. v & vi: Section showing mesenteric blood vessels (bv) are flanked by fibers colocalizing CGRP and TRPV4 (arrows). iii & iv, vii & viii: Wholemounts of mesenteric blood vessels also showing colocalization of CGRP and TRPV4 in nerve fibers (arrows). Scale bars on sections=25µm, on wholemounts=250µm. **B.** i & ii: CGRP was present in dense networks of fibers within mucosal villi in a colonic section. TRPV4 does not co-localize with CGRP in mucosa. v & vi: CGRP is abundant in submucosa and external muscle layers of mouse colon, whereas TRPV4 is absent. iii & iv: CGRP+ fibers around myenteric ganglia in mouse colon wholemount do not colocalize TRPV4. Scale bars in B=250µm.

Figure 3. Selective deficits in mechanosensory function in TRPV4 null

A. Most splanchnic colonic afferents belonged to two classes based on location of receptive field - mesenteric and serosal afferents. Upper graphs: Both showed dramatically reduced stimulus-response functions to a static von Frey hair (vfh) in TRPV4^{-/-} mice compared with +/+ (p<0.0001, 2-way ANOVA). Significance at individual stimulus intensities is shown by asterisks (* p<0.05, ** p<0.01, ***p<0.001, Bonferroni test). Lower graphs: activation thresholds to von Frey hairs were significantly increased for a greater percentage of both afferent subtypes. **B.** Four major classes of colonic pelvic afferents were recorded-serosal, mucosal, muscular and muscular/mucosal based on the location and responsiveness of their receptive fields. Pelvic serosal afferents showed similar deficits in TRPV4^{-/-} to their

splanchnic equivalents (i), whereas all other pelvic afferent subtypes were unchanged regardless of their adequate stimulus (ii–v). **C.** Two classes of gastroesophageal vagal afferents were observed-mucosal and tension receptors. Neither showed any significant change (NS=not significant) in mechanosensitivity in TRPV4^{-/-} mice.

A Conduction velocity

i) Mesenteric

ii) Serosal

B Electrical activation threshold

i) Mesenteric

ii) Serosal

Figure 4. TRPV4 null-mutants have normal afferent biophysical properties

A. Splanchnic colonic mesenteric (i) and serosal (ii) afferents show similar conduction velocities. **B.** Both populations also had similar activation thresholds to electrical stimuli delivered by a concentric electrode placed on their receptive fields.

Figure 5. Original records showing effects of TRPV4 ligands on colonic afferent mechanosensitivity Upper traces show instantaneous frequency plots, and lower traces show original recordings of neural activity before (i) and after (ii) incubation with ligands. **A.** The TRPV4 agonist 5,6-EET increased mechanical sensitivity of a splanchnic mesenteric afferent fiber to a 2g probe. **B.** The non-selective TRP channel antagonist Ruthenium red caused inhibition of mechanosensitivity to this stimulus in a different fiber.

A Splanchnic colonic **B Pelvic colonic** **C Vagal gastro esophageal** **Figure 6. TRPV4 ligands selectively modulate mechanosensory function**

A. Splanchnic colonic mesenteric afferents show increased responses to static application of a 2g von Frey hair in the presence of the TRPV4 agonist 5,6-EET (i), which is lost in the TRPV4 null-mutant (ii). Conversely, the non-selective TRP blocker ruthenium red concentration-dependently inhibits mechanosensory responses (iii)— an effect that is also lost in the TRPV4 null-mutants (iv). Data not shown indicate similar effects on mesenteric endings. **B.** Pelvic colonic serosal afferents show a similar pattern of potentiation by 5,6-EET and inhibition by ruthenium red, which was also lost in the TRPV4 null-mutant. **C.** No significant effect of TRPV4 ligands was seen in vagal tension receptors. Data not shown indicate similar lack of effect of these compounds on pelvic mucosal, muscular, and muscular/mucosal endings.

* $P < 0.01$, ** $P < 0.001$ vs control, repeated measures ANOVA with Dunnett's post-hoc test.

Figure 7. Translational studies of TRPV4 function and expression

A. Abdominal EMG responses of conscious mice to colorectal balloon distensions (5x80mmHg) were reduced by 55% in TRPV4 null-mutants (N=7, $p<0.05$). **B.** 14µm section of colon from a patient with Crohn's disease (CD) showing serosal blood vessel encircled by TRPV4-immunopositive fiber. Punctate TRPV4 labelling is also evident in other areas of the serosa. **C.** Varicose TRPV4-immunopositive ending in longitudinal muscle within 100µm of serosal border in Crohn's disease. This was seen in only one of 5 CD patients. **D.** Section of serosa/mesentery of a CD patient approximately 2mm from colon, showing 2 blood vessels surrounded by TRPV4-positive fibers. **E.** Circular and longitudinal muscle layers were TRPV4-negative.

ser=serosa, lm=longitudinal muscle, cm=circular muscle, bv=serosal blood vessel. Scale bars
B-D=40 μ m, E=500 μ m.