Visceral and Somatic Pain Modalities Reveal Na_V1.7-Independent Visceral Nociceptive Pathways

Short title: Role of Na_V1.7 in Visceral Nociception

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Author Contributions

Study concept and design (JRFH, RGC, SM, MATG, WJW, DCB, CMC, GM); funding and supervision (JMB, WJW, DCB, CK, CMC, GM); acquisition and analysis of data (JRFH, RGC, SM, MATG, CM, AT, ALW, VCG, FRN, TP). JNW provided reagents without which the studies would not have been possible. All authors contributed to the interpretation of data and writing the manuscript. CM is funded by the Dr Hadwen Trust and did not participate in experiments involving animals, or cells or tissues from animals or from human embryos. All authors approved the final version of the manuscript.

Keywords

Visceral pain; visceral nociception; voltage gated sodium channel; Nav1.7; colorectal; heat pain.

Key Points Summary

- Voltage-gated sodium channels play a fundamental role in determining neuronal excitability
- Specifically, voltage-gated sodium channel subtype $Na_V1.7$ is required for sensing acute and inflammatory somatic pain in mice and humans but its significance in pain originating from the viscera is unknown.
- Using comparative behavioural models evoking somatic and visceral pain pathways, we identify the requirement for $Na_V1.7$ in regulating somatic (noxious heat pain threshold) but not in visceral pain signalling.
- These results enable us to better understand the mechanisms underlying the transduction of noxious stimuli from the viscera, suggest that the investigation of pain pathways should be undertaken in a modality-specific manner and help to direct drug discovery efforts towards novel visceral analgesics.

Abstract

Voltage-gated sodium channel NaV1.7 is required for acute and inflammatory pain in mice and humans but its significance for visceral pain is unknown. Here we examine the role of NaV1.7 in visceral pain processing and the development of referred hyperalgesia using a conditional nociceptor-specific NaV1.7 knockout mouse (NaV1.7$^{Nav1.8}$) and selective small-molecule NaV1.7 antagonist PF-5198007. NaV1.7$^{Nav1.8}$ mice showed normal nociceptive behaviors to intracolonic application of either capsaicin or mustard oil, stimuli known to evoke sustained nociceptor activity and sensitization following tissue damage, respectively. Normal responses following induction of cystitis by cyclophosphamide were also observed in both NaV1.7$^{Nav1.8}$ and littermate controls. Loss, or blockade, of NaV1.7 did not affect afferent responses to noxious mechanical and chemical stimuli in nerve-gut preparations in mouse, or following antagonism of NaV1.7 in resected human appendix stimulated by noxious distending pressures. However, expression analysis of voltage-gated sodium channel α subunits revealed NaV1.7 mRNA transcripts in nearly all retrogradely-labelled colonic neurons suggesting redundancy in function. By contrast, using comparative somatic behavioral models we identify that genetic deletion of NaV1.7 (in NaV1.8-expressing neurons) regulates noxious heat pain threshold and that this can be recapitulated by the selective NaV1.7 antagonist PF-5198007. Our data demonstrates that NaV1.7 (in NaV1.8-expressing neurons) contributes to defined pain pathways in a modality-dependent manner, modulating somatic noxious heat pain but is not required for visceral pain processing, and advocates that pharmacological block of NaV1.7 alone in the viscera may be insufficient in targeting chronic visceral pain.

Abbreviations

BSA	Bovine serum albumin
CIP	Congenital insensitivity to pain
CT	Quantification cycles
DRG	Dorsal root ganglia
FB	Fast Blue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IC/BPS	Interstitial cystitis/bladder pain syndrome
LS	Lumbosacral
Na_V	Voltage-gated sodium channel
PEPD	Paroxysmal extreme pain disorder
PO	*Per os*
QST	Quantitative standardized testing
TL	Thoracolumbar
TRPV1	Transient receptor potential cation channel V1
TTX-R	Tetrodotoxin-resistant
TTX-S	Tetrodotoxin-sensitive

Introduction

Chronic pain originating from internal organs affects significant proportions of the population with analgesics restricted by dose-limiting side-effects. Persistent pain and visceral hypersensitivity manifests as reduced thresholds for mechanical distension of visceral organs and are strongly associated with inflammation. The targeting of peripheral sensory input, either by peripheral nerve block (Cherry *et al.*, 1985; Brown, 1989; Eisenberg *et al.*, 1995) or local anaesthetics (Verne *et al.*, 2003; Verne *et al.*, 2005) has proven effective in treating visceral pain. However, our understanding of key sensory afferent transduction mechanisms responsible for visceral nociception is limited. Here, we investigate voltage-gated sodium channel $Na_V1.7$ in both visceral and somatic pain behaviors and show that peripheral pain pathways of the viscera are functionally distinct from classical nociceptors, providing evidence supporting functional diversity of nociception and confirmation that novel analgesic development must be applied in a mechanism-specific manner.

Rare human genetic conditions link $Na_V1.7$ to pain perception, with loss-of-function mutations causing congenital insensitivity to pain (CIP) (Cox *et al.*, 2006; Goldberg *et al.*, 2007). Recapitulation of the human painless phenotype using knockout mice genetically engineered to globally lack $Na_V1.7$ results in complete loss of responses to acute, inflammatory and neuropathic pain (Gingras *et al.*, 2014). Using tissue-specific $Na_V1.7$ knockout mice (including nociceptor-specific $Na_V1.7^{Nav1.8}$ mice (Nassar *et al.*, 2004), pan-sensory neuron $Na_V1.7^{Advill}$ mice (Minett *et al.*, 2012) and pan-sensory and sympathetic neuron $Na_V1.7^{Wnt1}$ mice (Minett *et al.*, 2012)) modality-specific pain pathways associated with acute heat and mechanical detection, hyperalgesia and allodynia have been linked with differing Na_V repertoires.

Intriguingly, CIP patients feel no visceral pain with reports of both painless childbirth and rupture of appendix (Melzack & Wall, 1988; Zimmermann *et al.*, 1988; Wheeler, 2015), suggesting that $Na_V1.7$ may be required for visceral nociception. Rectal pain is a symptom of paroxysmal extreme pain disorder (PEPD), another condition associated with rare $Na_V1.7$ mutations (Fertleman *et al.*, 2006), with defecation capable of triggering pain attacks implicating a link to anorectal distension. In patients with interstitial cystitis/bladder pain syndrome (IC/BPS), pain perception associates with $Na_V1.7$ mutations (Reeder *et al.*, 2013). Like other chronic pain conditions, a hallmark of IC/BPS is ongoing pain in the absence of obvious pathophysiology (Dimitrakov & Guthrie, 2009). Therefore $Na_V1.7$ could be involved in maintaining spontaneous pain, such as peripheral or central sensitization, in addition to evoked pain attributed to mechanical stimulation. Surprisingly, whilst broad-spectrum sodium channel blockers are effective in treating chronic visceral pain, selective $Na_V1.7$ antagonists (ProTx-II) and monoclonal blocking antibodies targeting $Na_V1.7$ have been unable to fully recapitulate loss of $Na_V1.7$ mutant phenotypes to other chronic pain models (Schmalhofer *et al.*, 2008; Lee *et al.*, 2014). Indeed, selective antagonism of $Na_V1.7$ with ProTx-II also failed to block afferent responses to stretch of the colorectum (Feng *et al.*, 2015), suggesting the contribution of $Na_V1.7$ to visceral pain processing is still unclear. In light of recent findings that $Na_V1.7$ is essential for some (acute heat and mechanical pain, inflammatory hyperalgesia and neuropathic allodynia), but not all (acute cold pain, cancer-induced bone pain and oxaliplatin-evoked allodynia) pain modalities, we investigated visceral pain and referred hyperalgesia using a conditional nociceptor-specific $Na_V1.7$ knockout mouse ($Na_V1.7^{Nav1.8}$) and selective $Na_V1.7$ antagonist PF-5198007. Thus, using comparative behavioral models evoking somatic and visceral pain pathways we identify specific mechanisms regulating noxious heat pain threshold and show that Na$_V$1.7 in Na$_V$1.8-expressing neurons is not required for visceral pain signalling.

Materials and Methods

Experiments were performed in adult mice weighing 20 – 35 g. Conditional nociceptor-specific NaV1.7 knockout mice (NaV1.7$^{Nav1.8}$) and their littermate controls were generated as described previously (Nassar *et al.*, 2004). Observers performing behavioral and *ex vivo* electrophysiological experiments were blind to the genotypes of the animals. Animals were acclimatized for at least one week before behavioral testing in temperature and light-controlled (12hr light/dark cycle) rooms. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986 or with the EU Directive 2010/63/EU for animal experimentation, with approval of the University of Granada Research Ethics Committee (Granada, Spain). Human tissues were collected and utilised with approval of the East London and The City HA Local Research Ethics Committee (London, UK; NREC 10/H0703/71) in accordance with the Declaration of Helsinki and following full written informed consent.

Behavioral experiments

Experiments were performed on both male and female knockout and wild-type littermate control mice. Visceral pain and referred hyperalgesia was assessed using previously described methods, with small modifications (Olivar & Laird, 1999; Laird *et al.*, 2001; Gonzalez-Cano *et al.*, 2013). Briefly, mice were acclimatized for 40 minutes to test chambers (consisting of a transparent box on an elevated wire mesh floor) after which 50μl of capsaicin (0.1 or 1%), mustard oil (0.01 or 0.1%) or vehicle was instilled intrarectally *via* a thin cannula inserted into the anus and the animal returned to the chamber. The number of spontaneous pain behaviors (including licking of abdomen, stretching of abdomen and abdominal retractions) were recorded for the subsequent 20 minutes. In a separate set of experiments, visceral pain behaviors caused by cyclophosphamide-induced cystitis were examined following a previously described protocol (Olivar & Laird, 1999). Again after a 40 min habituation, animals were removed from the test chamber and cyclophosphamide (100 or 200mg/kg) or vehicle injected intraperitoneally. The animals were returned to the chamber and pain behaviors recorded according to the following scale: 0 = normal, 1 = piloerection, 2 = strong piloerection, 3 = labored breathing, 4 = licking of the abdomen and 5 = stretching and contraction of the abdomen. If more than one of these behaviors was noted during a single observation period, then only the type and not quantity of each different pain behavior was scored (i.e. if two stretching and contractions (5 points) and one abdominal licking (4 points) was observed, then a score of 9 was assigned).

After the evaluation of spontaneous pain behaviors (primary behavioral endpoint), the presence of referred hyperalgesia was determined by measuring the withdrawal response to a punctate mechanical stimulation (von Frey hair filaments 0.02 – 2 g (0.19-19.6 mN), Touch-Test Sensory Evaluators, North Coast Medical Inc., USA) of the abdomen using the up-down paradigm 20 minutes after algogen administration (Chaplan *et al.*, 1994). Avoiding the perianal and external genitalia, the mid-range 0.4 g von Frey hair filament was applied (three times for 2-3 sec at 5 sec intervals) to the lower and mid abdomen. If a positive response (consisting of immediate licking/scratching of the application site, sharp retraction of the abdomen or jumping) was observed, then probing was repeated in consecutive tests with a weaker von Frey filament. By contrast if there was no response to probing then a stronger von Frey filament was used. Once the withdrawal threshold (secondary behavioral endpoint) was ascertained, mice were humanely killed by concussion of the brain and cervical dislocation of the neck.

Electrophysiological recordings of visceral afferent activity

Nerves innervating murine and human gastrointestinal tissues were isolated and electrophysiological activity recorded using previously described methods (Peiris *et al.*, 2011; Hockley *et al.*, 2014). Mice were humanely killed by concussion of the brain and cervical dislocation of the neck. The distal colon with associated lumbar splanchnic nerves was removed and transferred to a recording chamber superfused (7 ml/min; 32-34 ^{o}C) with carbogenated Krebs buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4.7H_2O$, 11.1 glucose, and 25 $NaHCO_3$) supplemented with nifedipine (10 µM), atropine (10µM) and indomethacin (3 µM). The same supplemented Krebs buffer was used to luminally perfuse (100 µl/min) the colon after cannulation.

To translate murine experimental recordings into human tissue, we recorded extrinsic nerve activity from resected human appendices. We have previously shown that the appendix represents a valid human *ex vivo* model of visceral afferent activity amenable to the testing of mechanical and chemical stimuli (Peiris *et al.*, 2011). Specifically, the extrinsic nerves of the appendix are a branch of those innervating the right colon along the ileocolic artery and represent a readily available tissue in normal non-inflamed (e.g. from colon cancer resections) states. Resected appendices were obtained from 5 patients undergoing elective surgery at Barts Health NHS Trust, London after full written consent was attained. Appendices were removed from patients undergoing right hemicolectomies as part of their normal surgical treatment for bowel cancer or slow transit constipation (see Table 1 for details) with the permission of the histopathologist and were returned to the morbid anatomy department after completion of the studies. Once removed, appendix specimens were immediately placed in cold Krebs buffer and handled in a comparable manner to mouse distal colon tissues. Removal of the tip and cannulation enabled intraluminal perfusion, in addition to superfusion with Krebs buffer (7 mL/min; 32-34 ^{o}C) supplemented with 10 µM nifedipine and 10 μM atropine. Under a dissection microscope, mesenteric neurovascular bundles were blunt dissected and associated nerves identified and cleared of connective tissue. Using borosilicate glass suction electrodes, multi-unit activity from whole lumbar splanchnic nerves (rostral to the inferior mesenteric ganglia) of mouse, or from mesenteric nerves of human bowel tissues, was recorded. Signals were amplified and band pass filtered (gain 5 K; 100-1300 Hz; Neurolog, Digitimer Ltd, UK) and digitized at 20 kHz (micro1401; Cambridge Electronic Design, UK) before display on a PC using Spike 2 software. The signal was digitally filtered for 50 Hz noise (Humbug, Quest Scientific, Canada) and a threshold of twice the background noise (typically 100 μV) was used to determine action potential firing counts.

Electrophysiological protocols

Following a stabilizing period of 30 minutes, noxious intraluminal distending pressures were applied by blocking the luminal perfusion out-flow of the cannulated mouse distal colon or resected human appendix. The noxious pressures reached evoke pain behaviors *in vivo* and are above threshold for all known visceral afferent mechanoreceptors (Ness & Gebhart, 1988; Hughes *et al.*, 2009). In murine experiments, a combined sequential protocol was used to initially assess multiple aspects of visceral afferent mechanosensitivity and chemosensitivity. Specifically, a set of 6 rapid phasic distensions (0-80 mm Hg, 60 s at 9 min intervals) followed by slow ramp distension (0-145 mmHg, ~5-6 min) were implemented prior to bath superfusion of separate 20 ml volumes of 1 μM bradykinin and 1mM ATP at 40 min intervals. In separate experiments, the effect of pharmacological inhibition of Na_V1.7 on visceral afferent sensitivity to mechanical distension or noxious stimulation by capsaicin, mustard oil or bradykinin was tested. A set of 9 rapid phasic distensions (0-80 mm Hg, 60 s at 9 min intervals) followed by a 30 min stabilization period and bath superfusion of 1 μM bradykinin in a

20 ml volume were performed. Prior to the 7th phasic distension, bath superfusion of the selective Nav1.7 antagonist PF-5198007 (100 nM; 500 mL; (Alexandrou *et al.*, 2016)) or vehicle (0.1 % DMSO) was initiated and maintained for the duration of the remaining three distensions and bradykinin application. In some experiments, after a wash-out period, repeat phasic distensions were performed during which 250 ml tetrodotoxin (TTX; 100 nM) was superfused. In separate experiments, a ramp distension (0-145mmHg) was performed followed by bath superfusion of capsaicin (500nM) and mustard oil (250 μM) at 1 hour interval. Five minutes prior to application of capsaicin, either 100nM PF-5198007 or vehicle (0.1% DMSO) was applied for the duration of the subsequent stimulations. Human appendix specimens were stimulated in a comparable manner by repeat ramp distension (0-60 mm Hg, ~30 s at 9 min intervals). Baseline responses were established for three distensions prior to the superfusion of PF-5198007 (100 nM or 1 μM) for 50 min during subsequent distensions.

Retrograde labelling of gut-specific sensory neurons and single-cell qRT-PCR

Distal colon-specific sensory neurons were retrogradely labelled, picked and the expression of mRNA transcripts of interest determined by qRT-PCR. A mid-line 1.5cm laparotomy was performed on male mice after induction of anaesthesia with 1.5% isoflurane. Multiple injections of Fast Blue (FB: 0.2 µl per site, 2% in saline, Polysciences Gmbh, Germany) were made using a fine pulled-glass needle and microinfusion pump (0.4 µl/min) into the wall of the distal colon. Prior to suturing of the peritoneal muscle layer and securing the skin with Michel clips, the abdominal cavity was flushed with saline to remove any excess FB. Post-operative care (monitoring body weight and soft diet) and analgesia (buprenorphine 0.05-0.1 mg/kg daily) was provided for the duration of the protocol. Three to five days after surgery, mice were humanely killed by concussion of the brain and cervical dislocation of the neck, and thoracolumbar (TL: T10-L1) and lumbosacral (LS: L5-S2) dorsal root ganglia (DRGs) were harvested and cultured separately for gene expression experiments. Dissected ganglia were incubated at 37°C (in 5% CO_2) in Lebovitz L-15 Glutamax (GIBCO, UK) media containing 1mg/ml collagenase type 1A (Sigma) and 6mg/ml bovine serum albumin (BSA; Sigma, UK) for 15 min, followed by L-15 media containing 1mg/ml trypsin (Sigma, UK) and 6mg/ml BSA for 30 min. Ganglia were gently triturated and collected by brief centrifugation at 500 *g*. The supernatant (containing dissociated cells) was collected and the cycle of gentle trituration and centrifugation repeated. Cells from TL and LS DRG were plated separately onto poly-D-lysine–coated coverslips (BD Biosciences, UK) and incubated in Lebovitz L-15 Glutamax media containing 2 % penicillin/streptomycin, 24 mM $NaHCO_3$, 38mM glucose and 10 % fetal bovine serum. Fast Blue positive colonic sensory neurons were individually harvested from cultures of retrogradely labelled DRG (either TL: T10-L1 or LS: L5-S2) by pulled glass pipette. By breaking the pipette tip (containing the cell) into a tube containing preamplification mastermix (2.5µl 0.2x primer/probe mix, 5µl CellDirect 2x reaction buffer (Invitrogen), 0.1 µl SUPERase-in (Ambion, TX, USA), 1.2 µl TE buffer (Applichem, Germany) and 0.2 µl Superscript III Reverse Transcriptase/Platinum Taq mix (Invitrogen)) and freezing immediately, mRNA transcripts were preserved. Only those individual Fast Blue positive neurons free from debris and other non-neuronal cells (e.g. satellite glia) were collected. An image of each harvested neuron was also captured using a camera (DCC1545M, ThorLabs Inc, NJ, USA) attached to the inverted microscope enabling an estimation of cell size to be ascertained. In the absence of cells, samples of the bath solution were collected for no-template control experiments. Using the following thermal cycling protocol, preamplification of cDNA was achieved: 50°C for 30 minutes, 95°C for 2 minutes, then 21 cycles of (95°C for 15 seconds, 60°C for 4 minutes). After dilution (1:5 TE buffer), Taqman qPCR assays were run for each gene of interest (Taqman Assay ID: Na$_V$1.1, Mm00450580_m1; Na$_V$1.2, Mm01270359_m1; Na$_V$1.3, Mm00658167_m1; Na$_V$1.4, Mm00500103_m1; Na$_V$1.5, Mm01342518_m1; Na$_V$1.6, Mm00488110_m1; Na$_V$1.7, Mm00450762_s1; Na$_V$1.8, Mm00501467_m1; Na$_V$1.9, Mm00449367_m1; GAPDH, Mm99999915_g1; Applied Biosystems) using the following cycling protocol: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of (95°C for 15 seconds, 60°C for 1 minutes). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as an internal positive control, with all single-cell RT-PCR products expressing GAPDH and bath control samples were negative for all Taqman reactions. Relative expression of Na$_V$s was normalized to GAPDH quantification cycles (CT) using $2^{-\Delta CT}$ formula. Quantitative assessment of gene expression was determined by quantification cycle values less than the threshold of 35 being considered as positive.

Ramping hotplate pain behaviors

Behavioral phenotyping experiments were performed using both male and female mice, and pharmacology experiments were carried out in male mice. Acute heat pain was assessed using a ramping hotplate comparable to that used in human standardised quantitative testing (QST) protocols (Rolke *et al.*, 2006). Mice were acclimatized in a chamber for 6 minutes daily for the 3 days preceding dosing. After which, following a 30 second acclimatization, the chamber floor was slowly heated from 31°C at a rate of 3.4°C/min and the temperature and time taken until observing a pain behavior was recorded (behavioral endpoint; the occurrence of either licking or shaking of the hind paw and/or rapid shifting of weight (stomping) from one foot to the other). After baseline measurements were made, mice were dosed *via* oral administration (P.O.) with either vehicle or PF-5198007 at 1 or 3mg/ml with a dose volume of 10ml/kg and 1hr later, the ramping hotplate repeated. Mice were humanely killed by concussion of the brain and cervical dislocation of the neck immediately after final assessment of thermal pain threshold.

Skin-nerve preparation

Multi-unit extracellular afferent recordings were made from the tibial nerve innervating the glaborous skin of the hind paw as previously described (Milenkovic *et al.*, 2008) but with some modifications. Briefly, mice were humanely killed by concussion of the brain and cervical dislocation of the neck, the hind limbs were then shaved, removed and the tibial nerve and associated glaborous skin dissected free. The preparation was mounted glaborous skin downwards in a recording chamber superfused (10ml/min; 36±1˚C) with carbogenated (95% O_2, 5% CO_2) Krebs buffer (in mM: NaCl 107, KCl 3.48, $NaHCO_3$ 26.2, $MgSO_4(.7H_2O)$ 0.69, NaH_2PO_4 1.67, Na-gluconate 9.64, sucrose 7.6, glucose 5.5, CaCl 1.53). The epiperineurium was removed from the distal end of the tibial nerve and suction electrode recordings comparable to those of visceral afferent activity were made. Following a 60 minute stabilisation period, a heat stimulus (Krebs perfused onto the skin at a focal point equivalent to the heel portion of the paw) lasting 50 seconds was applied, this increased in temperature from 36˚C to 52˚C at a rate of 0.4˚C/second to mimic the noxious heat ramp used *in vivo*. In total, a series of 10 heat stimulations were performed at 15 minutes intervals. The first 4 heat stimulation formed the baseline reading with bath superfusion of PF-5198007 (30nM) or vehicle (0.1% DMSO) initiated and maintained for the duration of the next 2 stimulations (30 minutes), PF-5198007 (100nM) or vehicle (0.1% DMSO) for the following 2 heat stimulations (30 minutes) and heat stimulations 9 and 10 carried out during the superfusion (15 minutes) of TTX (100nM or 300nM) and lidocaine (1mM), respectively. In separate experiments, the effect of genotype and selective sodium channel antagonists were assessed in response to a cold stimulus (36 to 6˚C at a rate of 0.4˚C/second) delivered in the same manner as the heat stimulus, with comparable stimulation and protocols as above.

Data analysis

Pain behaviors and mechanical thresholds were compared across experimental groups with 2-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test, using either SigmaPlot 12.0 (Systat Software Inc., CA, USA) or Prism 6 (GraphPad Inc., USA). Referred hyperalgesia, expressed as the mechanical threshold producing 50% of responses, was calculated using: 50% mechanical threshold (g) = $[(10\ (X_f + \kappa\delta))\ /10]$, where X_f = value (in logarithmic units) of the final von Frey filament used; κ = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli (Dixon, 1980). Peak changes or total sum firing of electrophysiological nerve activity in multi-unit experiments were determined by subtracting baseline firing (2 minutes before distension or drug application) from increases in nerve activity following distension or noxious chemical superfusion. Estimation of cell size from single-cell images was achieved by averaging the height and width of each cell (ImageJ 1.49V analysis software, NIH, USA). Total sum firing of electrophysiological nerve activity in response to each hot or cold stimulation was obtained by subtracting any signal evoked by heat/cold stimulation in the presence of lidocaine (1mM). Expression data was visualized using R and the ggplot2 graphics package (Wickham, 2009). Statistical significance was set at $P < 0.05$. Data are displayed as mean ± SEM.

Drugs

Stock concentrations of capsaicin (1%; 10% ethanol, 10% tween, 80% saline), mustard oil (1%; 70% ethanol, 30% saline), cyclophosphamide (saline), bradykinin (10mM; water), lidocaine (1M; water) and ATP (300mM; water) were purchased from Sigma-Aldrich and prepared as described. Tetrodotoxin (15μg/ml stock) was purchased from Nanning Leaf Pharmaceuticals (Canada) and diluted in saline. PF-5198007 was manufactured in-house by Pfizer and solubilized in DMSO at a 10mM stock. For *in vitro* experiments, PF-5198007 was applied at a concentration of 100nM (ensuring almost 100% inhibition of mouse Na$_V$1.7 (IC$_{50}$ 5.2nM) and selectivity over Na$_V$1.1 and Na$_V$1.6 (IC$_{50}$ 149nM and 174nM, respectively)(Alexandrou *et al.*, 2016)). For *in vivo* studies PF-5198007, 1mg/ml or 3mg/ml, was suspended in 0.5% methylcellulose + 0.1% Tween-80 in distilled water. Doses of PF-5198007 were selected to achieve a free plasma concentration of ~100nM (littermate: 1mg/kg, 58 ± 10 nM, N = 5; 3mg/kg, 842 ± 91 nM, N = 10; Na$_V$1.7$^{Nav1.8}$: 1mg/kg, 68 ± 12 nM, N = 5; 3mg/kg, 634 ± 69 nM, N = 9). Vehicle was dosed as a 10ml/kg solution of 0.5% methylcellulose + 0.1% Tween-80 in distilled water. All other compounds were diluted in appropriate experimental buffer to working concentrations on the day of experimentation, unless otherwise stated.

<u>Results</u>

Visceral pain behaviors to colorectal sensitizing noxious stimuli were unaffected by deletion of Na$_V$1.7

We used a conditional Na$_V$1.7 knockout mouse strain, where *floxed* (*SCN9A*) Na$_V$1.7 mice were crossed with mice in which Cre expression is driven by the Na$_V$1.8 promotor (Na$_V$1.7$^{Nav1.8}$) resulting in tissue-specific ablation of Na$_V$1.7 in sensory neurons expressing nociceptive markers (Nassar *et al.*, 2004; Shields *et al.*, 2012). Capsaicin acts at TRPV1 and will activate the vast majority of visceral afferent terminals (>85% (Christianson *et al.*, 2006; Malin *et al.*, 2009)) leading to neurogenic inflammation and prolonged ongoing afferent activity due to sensitization (Laird *et al.*, 2001; Laird *et al.*, 2002). Intracolonic instillation of capsaicin in littermate control mice led to dose-dependent increases in observed pain behaviors consisting of abdominal contractions and licking (Fig. 1A). The deletion of Na$_V$1.7 from Na$_V$1.8-positive neurons, however, did not attenuate pain behaviors at either dose of capsaicin tested (P = 0.72, N = 6-8, 2-way ANOVA). In separate experiments, the potent algogen mustard oil was instilled intracolonically leading to the activation and sensitization of afferents and induction of localized tissue damage as previously described (Laird *et al.*, 2002). Substantial pain behaviors were observed in both Na$_V$1.7$^{Nav1.8}$ and littermate controls (Fig. 1B), which were not significantly different in terms of the magnitude of their response (P = 0.79, N = 6-8, 2-way ANOVA). The time course of pain behaviours induced by capsaicin and mustard oil did not differ between littermate controls and Na$_V$1.7$^{Nav1.8}$ mice. These findings show that Na$_V$1.7 expressed in Na$_V$1.8-positive neurons is not required for the development of visceral pain or for sustained spontaneous nociceptor activity as a result of sensitization.

Referred hyperalgesia is a common characteristic of visceral pain, with the sensitization of somatic structures in the same metameric field to the affected organ driven in part by spinal convergence of somatic and visceral afferents inputs (Cervero, 1983; Mertz *et al.*, 1995). Whilst primary inflammatory hyperalgesia has been shown to be dependent on Na_V1.7 in Na_V1.8-expressing neurons, whether Na_V1.7 contributes to the development of secondary hyperalgesia remains unstudied. The development of mechanical sensitivity of the abdomen in response to intracolonic instillation of either capsaicin (0.1%) or mustard oil (0.01%) was independent of ablation of Na_V1.7 from Na_V1.8-expressing neurons, with 50% withdrawal thresholds significantly reduced 20 minutes after treatment irrespective of genotype (capsaicin; $P < 0.01$, $N = 6\text{-}8$, 2-way ANOVA; mustard oil, $P < 0.01$, $N = 6\text{-}8$, 2-way ANOVA).

Pain responses to cyclophosphamide-induced cystitis are unaffected by deletion of Na_V1.7

To model bladder pain/cystitis in $Na_V1.7^{Nav1.8}$ mice, cyclophosphamide was administered leading to the progressive development of visceral pain behaviors for the duration of the 4 hour observation window. Cyclophosphamide treatment produces mucosal erosion and haemorrhage of the bladder in addition to edema (Fraiser *et al.*, 1991). The development and time course of pain behaviors observed did not differ between littermate controls and $Na_V1.7^{Nav1.8}$ mice to either dose of cyclophosphamide tested (Fig. 2A, $P = 0.93$, $N = 6\text{-}8$, 2-way ANOVA). Indeed both $Na_V1.7^{Nav1.8}$ mice and littermate controls also showed marked referred hyperalgesia when tested 4 hours after cyclophosphamide treatment (Fig. 2B). The referred hyperalgesia did not differ dependent on genotype suggesting that persistent activation of nociceptors by a developing noxious chemical stimuli is not driven by a requirement for Na_V1.7 to be present.

Visceral afferent mechanosensitivity is blocked by TTX but is unaffected by deletion of Na_V1.7 or blockade with a selective small-molecule Na_V1.7 antagonist

In order to distinguish between the multiple roles that Na_V1.7 makes to nociceptive processing, we investigated the contribution of Na_V1.7 to mechanosensitivity and chemosensitivity at the peripheral terminals of sensory neurons innervating the gastrointestinal tract. To do this multi-unit e*x vivo* extracellular electrophysiological recordings of lumbar splanchnic nerve activity were made from the distal colon of mice. Tissues were dissected free and cannulated to enable mechanical and chemical stimuli to be applied by luminal distension or bath superfusion. Phasic distension of the colon to noxious pressures (0-80 mm Hg) was used to model mechanical stimulation of the bowel and evoke increased afferent firing for the duration (60 second) of the distension. Consistent with previous reports, adaptation in the response to repeat stimulation (at 9 minute intervals) was observed during subsequent distensions with the response stabilizing by the fourth to sixth distension (see Fig. 3A & C) (Hockley *et al.*, 2014). In $Na_V1.7^{Nav1.8}$ mice, there was no significant difference in either the initial peak distension response or in the degree of tachyphylaxis observed during repeat distensions compared to littermate controls (Fig. 3C, P = 0.62, N = 13-14, 2-way repeated-measures (RM) ANOVA). Previous studies have suggested that not only the magnitude, but also the dynamic quality, of the distension paradigm used may be important for delineating gut motor events, specifically noxious stimuli (Sengupta & Gebhart, 1994; Booth *et al.*, 2008). Given the proposed role of Na_V1.7 as a threshold channel contributing to the amplification of depolarizing stimuli in sensory neurons (Dib-Hajj *et al.*, 2013), we used a slow ramp distension protocol to supramaximal distension pressures (0-145 mm Hg) in order to investigate the impact of loss of Na_V1.7 on responses across a range of innocuous and noxious distending pressures. In littermate controls, afferent firing increased proportionally to intraluminal pressure with a peak firing rate of 37.5 ± 5.7 spikes/s at 145 mm Hg. Significantly less firing was observed in NaV1.7$^{Nav1.8}$ mice to equivalent distending pressures (at 145 mm Hg, 25.7 ± 4.2 spikes/s; *P* < 0.0001, *N* = 19, 2-way ANOVA). However, firing rates in NaV1.7$^{Nav1.8}$ mice to ramp distension were unchanged within the physiologically-relevant 0-80 mm Hg range compared to controls (*P* > 0.05, Bonferroni's post-hoc analysis). Within the supramaximal range (80 -145 mm Hg), there was a reduction in firing, suggesting NaV1.7 may be involved in transducing non-physiological extremes of pressure in the colon but not innocuous or even noxious mechanical stimuli.

Given that NaV1.7 is ablated only in NaV1.8-positive neurons, it is possible that visceral afferents that are both sensitive to noxious mechanical stimuli and are negative for NaV1.8 may be contributory to the responses observed. In order to test this hypothesis, in a further set of experiments, repeat phasic distensions were continued and the effect of the selective small-molecule NaV1.7 antagonist PF-5198007 (100nM) was assessed on responses in both NaV1.7$^{Nav1.8}$ and littermate control mice. Responses in littermate control mice to repeat phasic distensions were unchanged following pre-incubation with, and in the presence of, 100nM PF-5198007 compared to vehicle (Fig. 3E, *P* = 0.86, *N* = 7, 2-way RM ANOVA). Further, the afferent response following application of 100nM PF-5198007 in NaV1.7$^{Nav1.8}$ mice also did not significantly differ from that observed in wild-type animals (*P* = 0.87, *N* = 6-7, 2-way RM ANOVA). However, irrespective of genotype, application of 100nM TTX to preparations did fully block afferent firing to noxious phasic distension (Fig. 3E). Together this shows that mechanosensitivity in visceral afferents is dependent on TTX-sensitive voltage-gated sodium channels but not NaV1.7.

Loss, or antagonism, of NaV1.7 does not alter visceral afferent responses to acute inflammatory and algogenic mediators

To investigate the involvement of NaV1.7 in modulating visceral afferent sensitivity to inflammatory and algogenic mediators used in our *in vivo* studies, capsaicin and mustard oil were applied to distal colon preparations and visceral afferent responses recorded from both littermate and NaV1.7$^{\text{Nav1.8}}$ mice, and in the presence or absence of 100nM PF-5198007. In separate experiments, bradykinin and ATP, as inflammatory mediators typically present during injury or infection, and that may be evoked by mustard oil/cyclophosphamide treatment contributing to ongoing nociceptor sensitization were also tested.

Responses to application of 500nM capsaicin did not differ between NaV1.7$^{\text{Nav1.8}}$ mice and littermate mice in vehicle control experiments (0.1% DMSO; NaV1.7$^{\text{Nav1.8}}$ vs. littermate; P = 0.50, N = 6 both groups, unpaired t-test, Fig. 4A). In addition, superfusion of 100nM PF-5198007 during, and 5 minutes prior to, capsaicin (500nM) application did not significantly change the evoked afferent discharge in either genotype (NaV1.7$^{\text{Nav1.8}}$: 100nM PF-5198007 vs. 0.1% DMSO, P = 0.82, N = 6, unpaired t-test; littermate: 100nM PF-5198007 vs. 0.1% DMSO, P = 0.59, N = 6, unpaired t-test, Fig. 4A). Afferent firing evoked by mustard oil was also unchanged in both NaV1.7$^{\text{Nav1.8}}$ mice and littermate controls (0.1% DMSO: NaV1.7$^{\text{Nav1.8}}$ vs. littermate, P = 0.46, N = 6, unpaired t-test, Fig. 4B), irrespective of the presence of NaV1.7 antagonist (NaV1.7$^{\text{Nav1.8}}$: 100nM PF-5198007 vs. 0.1% DMSO, P = 0.44, N = 6, unpaired t-test; littermate: 100nM PF-5198007 vs. 0.1% DMSO, P = 0.93, N = 6, unpaired t-test, Fig. 4B).

Bath superfusion of 1mM ATP in littermate mice resulted in significant afferent discharge with a peak change in firing of 1.39 ± 0.50 spikes/s. In NaV1.7$^{\text{Nav1.8}}$ mice, the response was comparable to littermate controls (2.33 ± 0.80 spikes/s, P = 0.32, N = 7-8, unpaired t-test). Responses to application of 1μM bradykinin were greater than that observed for ATP, however did not differ dependent on genotype (littermate, 9.11 ± 3.32 vs. Na$_V$1.7$^{Nav1.8}$, 8.56 ± 3.04 spikes/s, P = 0.90, N = 7-8, unpaired t-test). Further, in distal colon preparations from littermate controls pre-incubated with 100nM PF-5198007, peak firing response to 1μM bradykinin was unchanged (vehicle (0.1% DMSO) 5.16 ± 2.00 versus 100nM PF-5198007 4.31 ± 0.63 spikes/s, P = 0.70, N = 7, unpaired t-test); this was also true of tissues from Na$_V$1.7$^{Nav1.8}$ mice pre-incubated with the Na$_V$1.7 antagonist (100nM PF-5198007; P = 0.17, N = 6-7, unpaired t-test). Collectively, these data suggest that Na$_V$1.7 within the peripheral terminal of colonic sensory neurons is not required in order to transduce both noxious mechanical and chemical algogenic stimuli, in agreement with behavioral experiments.

Localization of Na$_V$ expression in colonic sensory neurons

We next investigated the expression of voltage-gated sodium channel α subunits present in colonic sensory neurons. Specifically, using single-cell qRT-PCR we examined the expression of mRNA transcripts for Na$_V$1.1, Na$_V$1.2, Na$_V$1.3, Na$_V$1.4, Na$_V$1.5, Na$_V$1.6, Na$_V$1.7, Na$_V$1.8 and Na$_V$1.9 in gut-projecting sensory neurons. Both lumbar splanchnic and pelvic innervation have been shown to contribute to the transmission of noxious stimuli from the distal colon (Brierley *et al.*, 2004). As such, the expression of these channels was determined in colonic sensory neurons in dorsal root ganglia (DRG) T10 to L1 levels (thoracolumbar: TL) that are known to possess the greatest number of sensory neurons projecting *via* the lumbar splanchnic nerve, and separately in DRG L5 to S2 levels (lumbosacral: LS); the afferents from which have been shown to project predominantly *via* the pelvic nerve. Of the 30 cells collected per mouse (N = 3), the average size of colonic sensory neurons harvested was 32.0 ± 0.2 μm for TL (N = 3) and 30.7 ± 1.0 μm for LS (N = 3). In the Na$_V$1.7$^{Nav1.8}$ mice used in the studies described here, Na$_V$1.7 was selectively ablated from all Na$_V$1.8-positive sensory neurons. To confirm the proportion of colonic sensory neurons affected by this gene ablation, the expression of Na$_V$1.7 and Na$_V$1.8 was first examined. Na$_V$1.7 was present in 100% of thoracolumbar and 95.6 ± 2.22% of lumbosacral colonic sensory neurons. High expression of Na$_V$1.8 was also observed in both thoracolumbar (95.6 ± 2.22 %) and lumbosacral (91.1 ± 4.44 %) colonic sensory neuron populations. Importantly, significant co-expression of both these sodium channels in individual colonic sensory neurons was found, with 95.4% of Na$_V$1.7-positive neurons also expressing Na$_V$1.8, suggesting that the vast majority of colonic sensory neurons in Na$_V$1.7$^{Nav1.8}$ mice would be affected by the genetic deletion. The expression of the remaining tetrodotoxin-sensitive (TTX-S: Na$_V$1.1, Na$_V$1.2, Na$_V$1.3, Na$_V$1.4 and Na$_V$1.6) and TTX-resistant voltage-gated sodium channels (TTX-R: Na$_V$1.5 and Na$_V$1.9) was also determined (Catterall *et al.*, 2005). Of the TTX-S sodium channels, Na$_V$1.6 was present in the greatest frequency (86.7%; Fig. 5A) of thoracolumbar colonic sensory neurons after Na$_V$1.7. Significant proportions of thoracolumbar colonic sensory neurons also expressed either Na$_V$1.1 (44.4 ± 5.88 %), Na$_V$1.2 (68.9 ± 8.89 %) or Na$_V$1.3 (53.3 ± 10.2 %), although co-expression was not always observed (see Fig. 5C). As expected, both the skeletal myocyte voltage-gated sodium channel Na$_V$1.4 and the cardiac myocyte Na$_V$1.5 channel were expressed by low proportions of thoracolumbar colonic sensory neurons (6.67 ± 6.67 % and 17.8 ± 5.88 %, respectively). In agreement with previous studies, mRNA transcripts for TTX-R Na$_V$1.9 were observed in 84.4 ± 44.4 % of thoracolumbar neurons (Hockley *et al.*, 2016). By comparison, the expression of Na$_V$1.1, Na$_V$1.2, Na$_V$1.3, Na$_V$1.4, Na$_V$1.7 and Na$_V$1.8 did not significantly differ between populations of lumbosacral compared to thoracolumbar colonic sensory neurons (Fig. 5A, all $P > 0.05$, TL *vs.* LS, unpaired t-test). Interestingly, significant differences were observed between the frequency of expression of Na$_V$1.5 (TL *vs.* LS, $P < 0.05$, unpaired t-test) and NaV1.6 (TL *vs.* LS, $P < 0.01$, unpaired t-test) in lumbosacral compared to thoracolumbar colonic sensory neurons. Indeed, transcripts for both NaV1.5 and NaV1.6 were observed in approximately half of lumbosacral colonic sensory neurons (48.9 ± 8.01 % and 51.1 ± 5.88 %, respectively). The expression of NaV1.9 (which has been shown previously to contribute to afferent sensitivity of the lumbar splanchnic nerve(Hockley *et al.*, 2014)) in lumbosacral colonic sensory neurons were consistent with the frequency of expression observed in the thoracolumbar populations ($P = 0.42$, $N = 3$, unpaired t-test). Taken together, these data not only support the expression of NaV1.7 by a majority of colonic sensory neurons innervating the distal colon, but also highlight an as yet unexplored complexity in the molecular patterning of voltage-gated sodium channels present in these neurons.

Deletion of NaV1.7 impairs somatic noxious thermal thresholds, which can be recapitulated by NaV1.7 antagonism

Given that no differences in acute visceral pain or referred hyperalgesia could be observed in mice lacking NaV1.7 in NaV1.8-positive neurons or to block of NaV1.7 by the selective inhibitor PF-5198007, we next sought to investigate the role of NaV1.7 in somatic acute pain behaviors. In order to investigate the contribution of NaV1.7 in NaV1.8-positive sensory neurons to the modulation of thermal thresholds, we utilized a ramping hotplate behavioral paradigm. In littermate controls, this latency was 261 ± 5 seconds ($N = 38$) corresponding to a temperature rise of ~13.6°C (baseline floor temperature 31°C ramping to 44.6 ± 0.2°C; Fig. 6A). This increase in temperature required to evoke a behavioral response was equivalent to a previous study using a modified ramping Hargreaves' test (Minett *et al.*, 2014a). NaV1.7$^{Nav1.8}$ mice showed an attenuated response to ramping hotplate with an augmented latency (274 ± 5 s) and significantly increased thermal threshold (46.1 ± 0.3°C, $N = 36$, $P < 0.0001$, unpaired t-test; Fig. 6A) in agreement with previous observations (Minett *et al.*, 2014a). The attenuation of complex behaviors associated with the ramping hotplate test suggests involvement of Na$_V$1.7 in pain signalling to noxious thermal stimulation of the skin under certain conditions.

Using the ramping hotplate, we went on to confirm the ability for the selective Na$_V$1.7 inhibitor PF-5198007 to modulate thermal pain behaviors (see Fig. 6B). In littermate mice, application of PF-5198007 (1mg/kg P.O.) significantly increased the thermal threshold for observing pain behaviors with a concomitant increase in the latency to response when compared to vehicle controls ($P < 0.01$, $N = 10$, 2-way ANOVA with Bonferroni's post-hoc *vs.* vehicle; Fig. 6B). In both vehicle and PF-5198007 treatment groups, the thermal threshold of Na$_V$1.7$^{Nav1.8}$ mice remained significantly greater than littermate controls but did not differ between groups. Application of a higher dose of PF-5198007 (3mg/kg P.O.) also led to an increase in thermal threshold during hotplate ramp, which was comparable to thresholds observed in Na$_V$1.7$^{Nav1.8}$ mice and significantly different from vehicle groups ($P < 0.05$, $N = 10$, 2-way ANOVA with Bonferroni's post-hoc *vs.* vehicle). These data suggest that whilst pain behaviors can be evoked in the absence, or antagonism, of Na$_V$1.7, the expression of Na$_V$1.7 in sensory neurons modulates heat pain thresholds to noxious thermal stimuli.

Na$_V$1.7 also contributes to cutaneous afferent firing to both noxious hot, but not cold, thermal stimuli

To investigate whether Na$_V$1.7 was necessary for sensory transduction at the peripheral terminal of somatic afferents, *ex vivo* multi-unit electrophysiological recordings of the tibial nerve from skin-nerve preparations of Na$_V$1.7$^{Nav1.8}$ mice and littermate controls were made (Fig. 6C & D). In support of hotplate experiments, a ramping thermal stimuli (focal water jet from 36°C to 52°C (at ~0.4°C/sec)) was applied to the corium side of the skin and the evoked nerve activity recorded. Total firing during the heat-evoked stimuli was significantly attenuated in Na$_V$1.7$^{Nav1.8}$ mice compared to littermate controls (Fig. 6E, $P < 0.0001$, $N = 26$-29, 2-way ANOVA with Bonferroni's post-hoc). Bath superfusion of 100nM TTX led to significant inhibition of firing regardless of genotype compared to vehicle controls (Fig. 6E, $P < 0.05$, $N = 9$-11 and $P < 0.0001$, $N = 10$-11, Na$_V$1.7$^{Nav1.8}$ and littermate controls, respectively, 2-way ANOVA with Bonferroni's post-hoc), suggesting that the transduction of noxious thermal stimuli at the peripheral terminal of sensory afferents is enhanced by the presence of Na$_V$1.7 in Na$_V$1.8-positive neurons, but is dependent on other TTX-S Na$_V$s that might be present. Application of 100nM PF-5198007 in littermate controls was able to recapitulate the attenuated response observed in Na$_V$1.7$^{Nav1.8}$ mice (Fig. 6F, $P < 0.05$, $N = 9$-10, 2-way ANOVA with Bonferroni's post-hoc vs. vehicle (0.1% DMSO)). In addition, PF-5198007 in Na$_V$1.7$^{Nav1.8}$ mice further reduced afferent responses to heat ramp suggesting that afferent firing at the peripheral terminal is dependent predominantly on expression of Na$_V$1.7 in Na$_V$1.8-positive sensory neurons. However, this does not discount contributions of Na$_V$1.7 to other sensory populations spinally or supra-spinally involved in the nociceptive processing of thermal stimuli.

In addition, we investigated cutaneous afferent firing to evoked cold stimuli by localized perfusion of a cooling perfusate over the receptive field from 36°C to ~6°C (at ~0.4°C/sec). In previous studies, Na$_V$1.7 has been shown to be involved in acetone-induced cooling, but not noxious cold sensation (Minett *et al.*, 2012). Responses evoked by cold stimulation of the skin did not differ between Na$_V$1.7$^{Nav1.8}$ mice and littermate controls (Fig. 6G, $P > 0.05$, $N = 18$, 2-way ANOVA with Bonferroni's post-hoc), however application of 100nM TTX completely abolished cold-evoked responses compared to vehicle ($P < 0.01$, $N = 6$ and $P < 0.0001$, $N = 5$-6, littermate and Na$_V$1.7$^{Nav1.8}$ mice, respectively, 2-way ANOVA with Bonferroni's post-hoc). Finally incubation with the selective $Na_V1.7$ antagonist PF-5198007 (100nM) did not significantly attenuate cold evoked afferent firing (Fig. 6H), supporting the posit that $Na_V1.7$ does not contribute to the transduction or amplification of cold-evoked depolarizations at the peripheral terminal.

Mesenteric nerve responses to phasic distension in human appendix are unaffected by inhibition of $Na_V1.7$

Finally, in order to understand whether our findings in murine visceral afferents translate to human we used *ex vivo* extracellular recordings of surgically resected appendices to investigate $Na_V1.7$ function in response to mechanical stimuli. The human appendix has been used previously as a pre-clinical model of visceral nociception (Peiris *et al.*, 2011). The appendix was cannulated and stimulated by repeat noxious ramp distension (0-60 mm Hg) and mesenteric nerve firing recorded. Ramp distension evoked a concomitant increase in human visceral afferent firing with a peak change in firing of 10.1 ± 1.5 spikes/s (N = 5), with reproducible responses observed to subsequent distensions. Application of PF-5198007 did not significantly impair visceral afferent firing to ramp distension at either low or high distending pressures (Fig. 7B, P = 0.26, N = 5, 2-way RM ANOVA). This confirms our mouse data highlighting that $Na_V1.7$ appears not to significantly impact visceral afferent sensitivity to acute mechanosensation. As such, $Na_V1.7$ imparts functionality on sensory neurons in a modality-specific manner and therefore the analgesic assessment of $Na_V1.7$ antagonists should be determined in a mechanism-dependent fashion.

Discussion

Nociceptive processing in somatic and visceral pain has common underlying pathways, including convergence in neuroanatomy, overlap in psychological representation and commonality in cellular transductions. However, important differences exist in the manifestation, perception and psychology of these pain modalities. Traditionally, visceral afferents are characterized based on mechanical sensitivity and activation by chemical mediators (including bradykinin and ATP (Su & Gebhart, 1998; Brierley *et al.*, 2004; Grundy, 2004)), with functional assessment required to define nociceptive properties. Compared to somatic counterparts, visceral sensory neurons almost exclusively possess characteristics attributed to nociceptors (unmyelinated C-fibres (Sengupta & Gebhart, 1994), peptidergic (Robinson *et al.*, 2004) and high expression of Na$_V$1.8/TTX-R sodium currents (Beyak *et al.*, 2004)), yet collectively transduce innocuous unconscious and conscious sensations in addition to pain. As such, visceral sensory neurons do not fit well with classical views of nociceptors and established schema for nociceptive transduction pathways.

Here, we add to this by showing that visceral pain signalling *in vivo* to acute and sensitizing noxious stimuli is independent of Na$_V$1.7. We confirm by way of *ex vivo* electrophysiological recordings of mouse visceral afferent fibres that deletion of, or selective small-molecule antagonism of Na$_V$1.7, does not attenuate responses to persistent noxious mechanical (including repeat phasic and sustained ramp distension) and chemical stimuli (including capsaicin, mustard oil, bradykinin and ATP). This lack of efficacy in Na$_V$1.7 antagonism in blocking visceral afferent activation extends to recordings from resected human appendix tissues when applying noxious distending pressures. Surprisingly, mouse visceral sensory neurons almost always express Na$_V$1.7 suggesting that, whilst present, Na$_V$1.7 appears not to contribute to the modulation of afferent excitability to depolarizing stimuli, or the propagation of action potentials. Furthermore, the lack of phenotype observed in $Na_V1.7^{Nav1.8}$ mice suggests $Na_V1.7$ is not necessary for transducing noxious visceral input centrally by $Na_V1.8$-expressing neurons. By contrast somatically, deletion of $Na_V1.7$ does modulate acute heat pain thresholds, which can be replicated using selective $Na_V1.7$ antagonism. Strikingly, loss of $Na_V1.7$ from $Na_V1.8$-expressing neurons, or small-molecule antagonism, are able to attenuate afferent firing evoked by ramping heat stimuli applied to skin-nerve preparations. This implicates $Na_V1.7$ in modulating thermal transduction sensitivity in somatic afferents. This was not true of cold stimuli, where $Na_V1.7$ does not have a role in afferent responses. Our data demonstrates that whilst $Na_V1.7$ does modulate defined somatic pain pathways, it is not required for those visceral pain modalities investigated here and advocates that selective pharmacological block of $Na_V1.7$ in the viscera may prove ineffective in targeting chronic visceral pain caused by spontaneous nociceptor activity, sensitizing inflammatory mediators or evoked mechanical distension: principal clinical drivers of visceral pain.

Voltage-gated sodium channels are vital for the transmission of painful stimuli in primary afferents. Importantly, the relative significance of individual sodium channels is dependent on the pain modality considered, with $Na_V1.7$ essential in transducing somatic acute thermal and mechanical pain, in conjunction to inflammatory hyperalgesia and neuropathic allodynia (Minett *et al.*, 2014b). Similarly, $Na_V1.8$ is critical for extreme cold pain (Abrahamsen *et al.*, 2008), with chemotherapy-induced allodynia dependent on $Na_V1.6$ (Sittl *et al.*, 2012; Deuis *et al.*, 2013). Normal visceral nociceptor activity, by contrast, is dependent on both $Na_V1.8$ (Laird *et al.*, 2002) and $Na_V1.9$ (Hockley *et al.*, 2014). Surprisingly, the role of $Na_V1.7$ in visceral pain processing is poorly understood in spite of human genetic data linking $Na_V1.7$ to pain signalling.

Substantive evidence for the involvement of Na_V1.7 in visceral pain processing comes from human genetic studies. Patients with congenital insensitivity to pain linked to mutations in Na_V1.7 do not feel pain, including pain originating from internal structures (broken bones (Cox *et al.*, 2006; Goldberg *et al.*, 2007)) and hollow organs (e.g. during appendicitis or child-birth (Melzack & Wall, 1988; Zimmermann *et al.*, 1988)). Mutations in *SCN9A* gene encoding Na_V1.7 are also causal in paroxysmal extreme pain disorder (PEPD) where severe burning pain may occur in rectal, ocular and mandibular regions. Intriguingly, defecation and micturition can both trigger such rectal pain attacks (Fertleman *et al.*, 2006; Meglic *et al.*, 2014), implicating hypersensitivity of visceral mechanoreceptors in initiating pain attacks. Whilst Na_V1.7 is linked with multiple aspects of the pain pathway, this is the first report detailing the contribution of Na_V1.7 to visceral pain processing. Using single-cell qRT-PCR of gut-specific sensory neurons we show that mRNA transcripts for Na_V1.7 are expressed by the vast majority of colonic sensory neurons, consistent with Na_V1.7 immunoreactivity in extrinsic afferent terminals of the distal colorectum (Feng *et al.*, 2015). Co-expression of Na_V1.7 in Na_V1.8-positive neurons was substantial in gut-projecting populations, suggesting that nearly all visceral sensory neurons would be affected by Na_V1.8-specific knockout of Na_V1.7 (Nassar *et al.*, 2004). However, it is possible that some Na_V1.7-positive Na_V1.8-negative colonic neurones remain, which may be sufficient to maintain pain behaviours. Visceral afferent firing to mechanical and chemical activation were unaffected following loss of, or antagonism of, Na_V1.7, but could be blocked by TTX as shown previously (Campaniello *et al.*, 2016). As such, TTX-S Na_Vs other than Na_V1.7 are involved in transducing noxious visceral stimuli. Established roles for TTX-R Na_V1.8 and Na_V1.9 correlate well with their extensive expression shown here; however little is known about the expression of TTX-S Na_Vs within a viscerally-projecting population. Na_V1.6 is essential in pelvic afferent endings for spike initiation and repetitive firing (Feng *et al.*, 2015), a concept that would fit with the extensive presence of Na$_V$1.6 mRNA transcripts observed here. Further, using toxin antagonists of Na$_V$1.7 (ProTx-II) and Na$_V$1.6 (μ-conotoxin GIIIa and μ-conotoxin PIIIa), a requirement on Na$_V$1.6, but not Na$_V$1.7, was observed for the encoding of stretch-sensitive pelvic afferents (Feng *et al.*, 2015). Taken together, these observations present compelling evidence that Na$_V$1.7 is redundant in visceral afferent nociception to spontaneous or evoked noxious stimuli.

Clearly whilst not necessary for normal sensation in the gut, the high relative expression of Na$_V$1.7 suggests that aberrant Na$_V$1.7 function, such as that present in some monogenic pain disorders, could significantly impact visceral sensation. Intriguingly, the propensity for mutations in Na$_V$1.7 to evoke regional pain phenotypes in PEPD patients (i.e. rectal and not 'true visceral' pain) could be driven by differences we observe here in the expression of some sodium channels (Na$_V$1.5(Renganathan *et al.*, 2002) and Na$_V$1.6(Cummins *et al.*, 2005)) located in thoracolumbar, versus lumbosacral, visceral sensory neurons. Precedent for background neuronal phenotype contributing to the manifestation of functional effects already exists with the same mutation in Na$_V$1.7 causing hypo- and hyper-excitability when expressed in either sympathetic or sensory neurons (Rush *et al.*, 2006). The extensive expression of Na$_V$1.7 suggests that mutations subverting its endogenous function may significantly alter phenotype even if not required for that pain modality normally. As such it is possible that non-canonical roles of Na$_V$1.7 may help explain the contradiction of how CIP patients associated with loss of Na$_V$1.7 do not feel visceral pain. For example, recent evidence of Na$_V$1.7 deletion upregulating endogenous opioid expression suggests a complex transcriptional modulatory, as well as electrogenic, contribution by Na$_V$1.7, however this did not alter the expression of other Na$_V$ subtypes present in DRG (Minett *et al.*, 2015). Importantly, the use of a selective small-molecule antagonist of Na$_V$1.7 enables us to discount developmental differences in gene deletion studies in the phenotypes observed here. Comparison with somatic pain behaviors enables confirmation of a modality-specific action for Na$_V$1.7 expression and confirms the ability of the antagonist PF-5198007 in replicating gene deletion studies. Na$_V$1.7 is required for modulating heat pain thresholds after burn injury (Shields *et al.*, 2012) and for acute noxious heat sensing in a population of Na$_V$1.8-negative neurons (Minett *et al.*, 2012). Surprisingly, we found using an adapted ramping hotplate test that loss of Na$_V$1.7 from Na$_V$1.8-positive neurons could also alter acute heat pain thresholds and this could be recapitulated using PF-5198007. In all cases, mice remained sensitive to noxious heat, suggesting that Na$_V$1.7 is not required in Na$_V$1.8-expressing neurons but can modulate the thermal threshold sensitivity. Notably, we observed a desensitization of the heat pain threshold from ~44°C by 2-3°C following antagonism of Na$_V$1.7, as such fixed temperature hotplate tests typically used to measure withdrawal latencies at 50°C or 55°C would be above threshold in either case masking potential phenotypic differences. A similar non-redundant role for Na$_V$1.7 in Na$_V$1.8-expressing neurons was observed to an adapted Hargreaves' test (Minett *et al.*, 2014a). This further highlights the involvement of multiple sub-populations of neurons on stimulus-intensity specific responses underpinning noxious thermal detection.

In summary, using a combination of gene deletion knockout mice and pharmacological tool molecule we demonstrate that Na$_V$1.7, although expressed extensively by gut-projecting sensory neurons, contributes minimally to visceral pain pathways associated with algogenic sensitizing chemicals and evoked activation of visceral afferents by noxious stimuli. The patterning of sodium channel expression shown here reveals a previously unstudied molecular complexity to visceral sensory neurons. Combined with a detailed study of somatic thermal sensitivity, we show that assessment of candidate analgesic targets to pain mechanisms must be considered in a modality-specific manner. As such, $Na_V1.7$ antagonism of peripheral visceral afferents may not represent a viable therapeutic rationale for the treatment of chronic visceral pain associated with evoked distension or inflammation of the viscera.

Figure Legends

Table 1

Patients details from which resected appendix specimens were used. Appendix specimens from 5 patients were collected and used in electrophysiological nerve recordings.

Figure 1

Spontaneous visceral-pain related behaviors in $Na_V1.7^{Nav1.8}$ and littermate mice following intracolonic administration of capsaicin (A and C) or mustard oil (B and D). Number of acute pain related behaviors (licking of abdomen, stretching, abdominal retractions) induced by capsaicin (A) or mustard oil (B) during a 20 min period. Referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured 20 min after the administration of capsaicin (C) or mustard oil (D). Mean ± SEM of values obtained in 6-10 animals. *$P < 0.05$ and **$P < 0.01$ vs. vehicle.

Figure 2

Visceral pain related behaviors evoked by cyclophosphamide-induced cystitis in $Na_V1.7^{Nav1.8}$ and littermate mice. (A) Behavioral pain responses were recorded at 30 minute intervals during the 240 min observation period after cyclophosphamide injection. (B) Referred mechanical hyperalgesia was evaluated by stimulation of the abdomen with von Frey filament 4h after cyclophosphamide administration. Mean ± SEM of values obtained in 6-10 animals. *$P < 0.05$ and **$P < 0.01$, vs. vehicle.

Figure 3

Visceral afferent responses to noxious distension of the distal colon in $Na_V1.7^{Nav1.8}$ mice and following small-molecule $Na_V1.7$ antagonism. Example rate histogram of colonic splanchnic nerve activity and intraluminal pressure trace to repeat phasic distension (0-80 mm Hg; 60 s; 9 min intervals) in $Na_V1.7^{Nav1.8}$ (B) and littermate (A) mice. (C) Peak change in firing rate during phasic distensions in both genotypes (*P = 0.46*, 2-way repeated-measures ANOVA). (D) Average firing rates to ramp distension (0-145 mm Hg) at 5 mm Hg increments in littermate and $Na_V1.7^{Nav1.8}$ mice. (E) Effect of 100nM PF-5198007, vehicle (0.1% DMSO) or 100nM TTX on total firing evoked during repeat 0-80 mm Hg phasic distensions in littermate and $Na_V1.7^{Nav1.8}$ mice.

<u>Figure 4</u>

Effect of capsaicin and mustard oil on visceral afferent responses. Change in peak firing rate to application of 500nM capsaicin (A) and 250μM mustard oil (B) in littermate and $Na_V1.7^{Nav1.8}$ mice, both in the absence and presence of 100nM PF-5198007.

<u>Figure 5</u>

Expression of voltage-gated sodium channel mRNA transcripts in mouse colonic sensory neurons by single-cell qRT-PCR. (A) Proportions of thoracolumbar and lumbosacral colonic sensory neurons expressing transcripts for $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.5$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$. (B) Relative expression of Na_V transcripts in thoracolumbar and lumbosacral colonic sensory neurones (C) Co-expression analysis of voltage-gated sodium channels in both thoracolumbar and lumbosacral colonic sensory neuronal populations. Each segment in the wheel-diagrams is representative of a single cell with a coloured segment signifying positive expression.

<u>Figure 6</u>

Somatic pain behaviors and tibial nerve activity to noxious thermal stimulation in $Na_V1.7^{Nav1.8}$ and littermate mice. (A) Thermal pain thresholds in $Na_V1.7^{Nav1.8}$ mice are significantly increased following ramping hotplate behavioral testing. (B) Average thermal pain thresholds following the application of selective $Na_V1.7$ antagonist PF-

5198007 (1 or 3mg/kg) or vehicle in $Na_V1.7^{Nav1.8}$ and littermate mice. Example raw traces, rate histogram and temperature recordings of tibial nerve activity in littermate (C) and $Na_V1.7^{Nav1.8}$ mice (D). (E) Sum firing of tibial nerve activity during focal heat stimulation in skin-nerve preparations of $Na_V1.7^{Nav1.8}$ and littermate mice in the presence of TTX (100nM) or vehicle (0.1% distilled H_2O). $^{\#\#\#\#}P < 0.0001$, $Na_V1.7^{Nav1.8}$ baseline vs. littermate baseline. (F) Effect of PF-5198007 on evoked tibial nerve firing by heat stimulation in $Na_V1.7^{Nav1.8}$ and littermate mice. (G) Sum firing of tibial nerve activity during focal cold stimulation in skin-nerve preparations of $Na_V1.7^{Nav1.8}$ and littermate mice in the presence of TTX (100nM) or vehicle (0.1% distilled H_2O). (H) Effect of PF-5198007 on evoked tibial nerve firing by cold stimulation in $Na_V1.7^{Nav1.8}$ and littermate mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

Figure 7

Effect of selective small-molecule antagonism of $Na_V1.7$ in resected human appendices following repeat noxious distension. (A) Example rate histogram of appendix mesenteric nerve activity and intraluminal pressure trace following repeat ramp distension (0-60 mm Hg; 10 min interval). Application of PF-5198007 was initiated at the start of the black bar and maintained for 50 min during which distensions were continued. (B) Average firing rates to repeat ramp distension (0-60 mm Hg; $N = 5$) of human appendix prior to, and after, addition of PF-5198007; neither low-threshold or high-threshold afferent firing is affected by antagonism of $Na_V1.7$. Both change in peak firing rate (C) and total afferent firing (D; Area Under Curve) were unchanged by bath superfusion with PF-5198007 ($N = 5$).

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Table 1

#	Disease	Operation	Tissue	Age	Sex
1	Cancer	Right hemicolectomy	Appendix	83	F
2	Cancer	Right hemicolectomy	Appendix	42	F
3	Cancer	Right hemicolectomy	Appendix	72	F
4	Slow Transit Constipation	Subtotal Colectomy	Appendix	69	M
5	Cancer	Right hemicolectomy	Appendix	70	M
			Mean age / M:F ratio	*67*	*1:1.5*

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