Experimental and computational evidence for an essential role of Na\textsubscript{\text{V}}1.6 in spike initiation at stretch-sensitive colorectal afferent endings

Bin Feng, Yi Zhu, Jun-Ho La, Zachary P. Wills\textsuperscript{1}, and G.F. Gebhart

Center for Pain Research, Department of Anesthesiology and \textsuperscript{1}Department of Neurobiology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA

Abbreviated title: Na\textsubscript{\text{V}}1.6 in spike initiation of colorectal afferents

Manuscript length: 49 pages, 9 figures, 4 tables

Words: 231 for Abstract, 489 for Introduction, 1899 for Discussion

Conflict of interest: The authors claim no conflict of interests.

Acknowledgements: supported by NIH grant R01 DK093525 (GFG). We thank Michael Burcham for assistance in preparation of figures and Dr. Brian Davis for his generous gift of transgenic mice that express YFP in sensory afferents (driven by SNS-Cre)

Correspondence: Bin Feng, Ph.D.

Center for Pain Research, University of Pittsburgh

W1402 BST, 200 Lothrop St., Pittsburgh, PA 15213, USA

E-mail: fengb@upmc.edu

Phone: 412-648-8123, fax: 412-383-5466


Copyright © 2015 by the American Physiological Society.
ABSTRACT

Stretch-sensitive afferents comprise ~33% of the pelvic nerve innervation of mouse colorectum, which are activated by colorectal distension and encode visceral nociception. Stretch-sensitive colorectal afferent endings respond tonically to stepped or ramped colorectal stretch whereas dissociated colorectal DRG neurons generally fail to spike repetitively upon stepped current stimulation. The present study investigated this difference in the neural encoding characteristics between the soma and afferent ending using pharmacological approaches in an in vitro mouse colon-nerve preparation and complementary computational simulations. Immunohistological staining and western blots revealed the presence of Nav1.6 and Nav1.7 channels at sensory neuronal endings in mouse colorectal tissue. Responses of stretch-sensitive colorectal afferent endings were significantly reduced by targeting Nav1.6 using selective antagonists (μ-conotoxin GIIla and μ-conotoxin PIIla) or TTX. In contrast, neither selective Nav1.8 (A803467) nor Nav1.7 (ProTX-II) antagonists attenuated afferent responses to stretch. Computational simulation of a colorectal afferent ending that incorporated independent Markov models for Nav1.6 and Nav1.7, respectively, recapitulated the experimental findings, suggesting a necessary role for Nav1.6 in encoding tonic spiking by stretch-sensitive afferents. In addition, computational simulation of a DRG soma showed that by adding a Nav1.6 conductance, a single-spiking neuron was converted into a tonic spiking one. These results suggest a mechanism/channel to explain the difference in neural encoding characteristics between afferent somata and sensory endings, likely caused by
differential expression of ion channels (e.g., Na\textsubscript{V}1.6) at different parts of the neuron.
INTRODUCTION

Irritable bowel syndrome (IBS) patients typically suffer from persistent pain and organ hypersensitivity, manifesting enhanced responses and reduced thresholds to mechanical distension of the distal colorectum (Naliboff et al. 1997). Understanding the afferent innervation of the colorectum is important as targeting colorectal afferents has proven to be effective in alleviating pain and hypersensitivity in IBS patients (e.g., intra-rectal instillation of local anesthetics (Verne et al. 2003; Verne et al. 2005) and oral intake of the guanylate cyclase-C agonist linaclotide (Busby et al. 2013; Chey et al. 2012; Rao et al. 2012).

The colorectum in the mouse is innervated by lumbar splanchnic and pelvic nerve pathways (Brierley et al. 2004; Feng and Gebhart 2011). Using an in vitro colorectum-nerve preparation and an unbiased electric search strategy, we categorized colorectal afferents into five mechanosensitive classes (serosal, mucosal, muscular, muscular-mucosal, mesenteric) and one mechanically-insensitive class (Feng et al. 2012a). Among these classes, only muscular and muscular-mucosal afferents tonically encode circumferential stretch of the colorectum (i.e., are stretch-sensitive) and subserve the encoding of nociceptive colorectal distension (Feng et al. 2010; Feng et al. 2013).

Patch clamp recordings reveal that colorectal dorsal root ganglion (c-DRG) somata have small diameters (<25-30μm), TTX-resistant inward sodium currents, a significant inflection/hump in the repolarization phase of the action potential (Beyak et al. 2004), and are immuno-positive for one or more neurochemical markers, including
TRPV1 (up to 85%), CGRP (up to 80%), and IB4 (up to 20%) (Christianson et al. 2006; La et al. 2011). Consistent with findings from c-DRG somata, almost all mechanosensitive colorectal afferents in mice are unmyelinated C-fibers with conduction velocities ≤1m/s (Feng and Gebhart 2011).

Our previous single-fiber studies suggest that a significant proportion (~33%) of colorectal afferent endings in the pelvic nerve (PN) innervation are stretch-sensitive and respond tonically to ramped circumferential colorectal stretch (0 to 170 mN in 34 sec) (Feng and Gebhart 2011; Feng et al. 2012b; Feng et al. 2012c) and could be categorized as class-1 tonic-spiking neurons according to Hodgkin’s scheme (Hodgkin 1948). In contrast, most if not all lumbosacral c-DRG somata (corresponding to the PN innervation) fail to spike repetitively (typically spiking only at the onset of stepped current stimulation (e.g., Fig. 2C in (Shinoda et al. 2010)), and can be categorized as class-3 single-spiking neurons. This discrepancy in spiking patterns between colorectal afferent somata and their sensory endings is likely contributed to by different compositions and/or densities of voltage-gated channels in their respective membranes. In small-diameter, presumptive nociceptive DRG neurons, most inward Na current is through Na\textsubscript{V}1.7 and Na\textsubscript{V}1.8 channels whereas a Na\textsubscript{V}1.6 current is less common and found mainly in larger-diameter DRG neurons (Cummins et al. 2005). However, Na\textsubscript{V}1.6 contributes to the tonic firing of neurons due to its low activation threshold, rapid repriming, and large persistent and resurgent current (Rush et al. 2005). We hypothesized that Na\textsubscript{V}1.6 contributes significantly to the tonic firing of stretch-sensitive colorectal afferent endings and
tested this hypothesis using combined single-fiber electrophysiology and computational simulation.
METHODS

Unless specified, experiments were conducted in male C57BL/6Tac mice, 6-8 weeks old, 20 – 30 g (Taconic, Germantown, NJ) and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunohistochemistry

As previously described (Feng et al. 2012c), mice that express YFP in sensory neurons driven by SNS-Cre (a gift from Dr. Brian M. Davis, University of Pittsburgh) were euthanized via CO₂ inhalation. The L6 DRG and distal colorectum were harvested and fixed with 4% paraformaldehyde in 0.16 M phosphate buffer containing 14% picric acid (Sigma-Aldrich). After cryoprotection in 20% sucrose, fixed tissue was embedded in OCT compound (Sakura Finetek, Tokyo, Japan), frozen, and sectioned at 20 μm for DRGs and at 70 μm for colorectum. Tissue sections were incubated with antibodies against either Naᵥ1.6 (1:1000, Alomone, ASC-009, Lot# AN1750) or Naᵥ1.7 (1:1000, Alomone, ACS-008, Lot# AN1125) and co-stained with goat antibodies against YFP (1:1000, Abcam, AB5450, Lot# GR136040-1, Cambridge, MA). The sections were further stained with Cy3-conjugated anti-rabbit IgG (1:200, Jackson Immunoresearch, 711-227-003, Lot# 95161) and Alexa Fluor® 488-conjugated anti-goat IgG (1:200, Molecular Probes, A11055, Lot# 989791 Eugene, OR). Confocal microscopy was carried out with a Nikon A1R point scanning microscope with either a 0.75NA 20X or 1.4NA 60X objective. Pinhole size was limited to 1 airy unit to maximize confocality of all images. 12-bit images were captured with limited laser and detector power to ensure fluorophore signals were not
3-D reconstructions were rendered using Nikon elements software (version AR 4.13.04).

To localize the two sodium channels in DRG neurons innervating the colorectum via pelvic nerves, a retrograde tracer Fast Blue (FB, 1% in sterile saline, EMS-Chemie GmbH, Groß-Umstadt, Germany) was injected into the distal colon wall as previously described (La et al. 2012). The L6 DRG were harvested 14 days later and processed for immunohistochemistry as above. Immunostained tissue sections were photographed using a microscope-mounted digital camera (DFC340FX; Leica).

**Western Blot**

As previously described (Zhu et al. 2012), tissue samples of L6 DRG, pelvic nerve, and colon were individually homogenized with a Teflon tube and mortar for less than 10 strokes in ice cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Sigma-Aldrich). Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo scientific, Rockford, IL, USA). The lysates were then mixed with Laemmli buffer (6×) and boiled for 5 min before loading. Samples (100 μg/lane) were then loaded and separated on a 5% SDS–PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk for 1 hour at room temperature and then incubated with primary antibody at 4°C overnight (Na\textsubscript{v}1.6 or Na\textsubscript{v}1.7 both at 1:200, Alomone), diluted with 5% milk/Tris-buffered saline with Tween 20 (TBST, Sigma–Aldrich). The blots were washed and then incubated with peroxidase-conjugated secondary antibody (1:2000 dilution).
in 5% milk/TBST, Jackson ImmunoResearch) for an hour at room temperature. An ECL kit (Amersham Biosciences, Piscataway, NJ, USA) was used for detection of immunoreactivity, and image of the blots were then taken with an LAS3000 imager (Fujifilm Inc., Japan).

**In Vitro Mouse Colon–pelvic Nerve Preparation**

As detailed previously (Feng et al. 2013), mice were euthanized via CO₂ inhalation followed by exsanguination after perforating the right atrium. The distal colorectum with attached PN was dissected and transferred to ice-cold Kreb’s solution bubbled with carbogen (95% O₂, 5% CO₂). The colorectum was opened longitudinally, pinned flat either mucosal or serosal side up depending on the experiment objective in a tissue chamber and the PN extended into an adjacent recording chamber filled with paraffin oil. The tissue chamber was superfused with a modified Krebs solution (in mM: 117.9 NaCl, 4.7 KCl, 25 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄∙7H₂O, 2.5 CaCl₂, 11.1 D-Glucose, 2 butyrate, and 20 acetate) at a temperature of ~30-32°C to which nifedipine (4μM) and indomethacin (3μM) was added. The PN was teased into fine bundles (~10μm thickness) for single-fiber recording.

As previously described (Feng and Gebhart 2011), mechanosensitive colorectal afferents were classified as serosal, muscular, mucosal or muscular-mucosal (M/M) based upon responses to probing with calibrated nylon monofilaments (0.4, 1, and 1.4g force), mucosal stroking (10mg force) and circumferential stretch. Muscular and M/M afferents both respond to stretch and their stimulus-response functions (SRFs)
to circumferential stretch were generated using a servo-controlled force actuator (Aurora Scientific, Aurora, Ontario, Canada). Custom-made claws (~1mm interval) were inserted along the antimesenteric edge of the colorectum to permit homogeneous, circumferential stretch by a slow ramped force (0 to 170mN at 5mN/sec) corresponding to intraluminal pressures of 0-45mmHg (Feng et al. 2010).

Application of Chemicals to Afferent Endings

The following compounds were applied directly to afferent endings in the colorectum as previously described (Feng and Gebhart 2011; Feng et al. 2013): tetrodotoxin (TTX), the NaV1.8 antagonist A803467 (A803), the NaV1.7 antagonist ProTx-II (PTX), and NaV1.6 antagonists μ-conotoxin GllIa (mCtxG) and μ-conotoxin PllIa (mCtxP). After establishing a baseline (control) SRF, the receptive ending was isolated (4X4 mm square X10 mm high tubing), the Krebs solution removed and replaced by 150μL of TTX, A803, PTX, mCtxG or mCtxP for 5 min. The tubing was then removed and a SRF acquired immediately afterwards (experiment). After re-exposing the ending to Krebs solution for 15 min, a third SRF (wash) was acquired to conclude the protocol. Generally, each fiber was exposed to 2-3 protocols using different drugs or concentrations with at least 15 min wash between protocols. Afferent responses after exposure to A803 did not recover after wash and those fibers were not tested with other drugs. To avoid direct application of drugs onto the pelvic nerve fibrils, afferents with receptive fields within 5mm of the nerve entry point into the colorectum were not studied pharmacologically when the colorectum was pinned serosal side up.
TTX was dissolved in Krebs solution at 3mM and prepared in aliquots of 3.3μL. A803 was dissolved in DMSO to 3mM and prepared in nitrogen-filled aliquots of 3.3μL (to avoid oxidation); DMSO (<0.3%) alone in Krebs solution has no significant effect on afferent responses to stretch (Feng et al. 2013; Kiyatkin et al. 2013). PTX was dissolved in 80% acetonitrile to 0.3mM and prepared in aliquots of 5μL; acetonitrile (0.8%) alone in Krebs solution has no significant effect on afferent responses to stretch (data not shown). mCtxG and mCtxP were dissolved in Krebs solution at 0.5mM and 0.1mM, respectively, and prepared in aliquots of 42μL. All aliquots were frozen, stored at -20°C and diluted on the day of an experiment to final concentrations in freshly oxygenated Krebs solution (TTX, PTX, mCtxG and mCtxP) or Krebs solution with pH adjusted to 7.4 by hydrochloric acid (A803, to avoid oxidation). TTX, PTX and A803 were purchased from Tocris (Bristol, UK), mCtxG from VWR (Radnor, PA) and mCtxP from Alomone. All other chemicals were purchased from Sigma-Aldrich.

**Circumferential Colorectal Stretch**

Circumferential colorectal stretch was quantified as a stretch ratio (λ) by measuring colorectal circumference during ramped stretch *in vitro*. Colorectal deformation was recorded through a stereo dissection microscope using a CCD camera; images were extracted every 2 sec during stretch and circumference was measured using ImageJ (v1.44p, NIH).

**Computational Simulation**

*Model geometry*
To facilitate comparisons, electrophysiological properties of colorectal afferent endings and DRG somata were simulated separately in the NEURON simulation environment (Carnevale and Hines 2005). The fundamental morphological and electrical features of a mouse colorectal afferent ending was represented by a multi-compartmental model as depicted in Fig. 1A. The nerve terminal model presented here is designed to emulate action potential encoding, assuming a transducer terminal region contiguous with a single spike-initiation zone similar to those developed to simulate the axon initial segment (AIS) of neurons in the central nervous systems (CNS) (e.g., (Hu et al. 2009)). The model consists of a transducer zone (trsd) where a generator potential is produced by a depolarizing current from mechanosensitive channels, a spike-initiation zone (siz) where action potential spikes are evoked by the generator potential, and a middle section (mid) in between in which Na\(^+\) and K\(^+\) channel densities gradually increase from the trsd side to siz side (to simulate the gradual change in ion channel densities); the passive compartment (pas) distal to siz provides space for axial diffusion of intracellular Na\(^+\) and K\(^+\) ions. All four compartments are cylinders 0.8 \(\mu\)m in diameter. To achieve spatial and temporal accuracy in simulation, the compartments were further divided into a total of 23 segments (10 in trsd, 5 each in siz and mid, and 3 in pas) so each segment length was less than 1/50th of the electrotonic length constant (570\(\mu\)m in the model)(Carnevale and Hines 2005). In contrast, dissociated DRG somata free of attached axons were simulated as a single-segment model depicted in Fig. 1B and assigned a diameter of the average colorectal DRG neuron (\(\Phi23\mu\)m) (Beyak et al.
The model incorporates four different Na\(^+\) conductances representing Na\(_{\text{V}1.6}\), Na\(_{\text{V}1.7}\), Na\(_{\text{V}1.8}\), and Na\(_{\text{V}1.9}\) and three K\(^+\) conductances, simulating the fast inactivating A-type current (K\(_A\)), slowly inactivating A-type current (K\(_D\)), and sustained current (K\(_S\)). Na\(_{\text{V}1.6}\) and Na\(_{\text{V}1.7}\) channels were represented by Markov models with multiple gating states to capture their unique and contrasting gating features (e.g., rapid vs. slow repriming and incomplete vs. complete inactivation). The other channels were modeled by Hodgkin-Huxley formulations. Na\(^+\),K\(^+\)-ATPase was simulated as a voltage- and [Na\(^+\)]-dependent outward current with a 3:2 transport ratio between Na\(^+\) and K\(^+\) ions. Intracellular Na\(^+\) and K\(^+\) concentrations are dynamically influenced by ion flow across the membrane via channels, pumps, and leak conductances as well as by passive axial diffusion, assuming a diffusion coefficient of 0.6 \(\mu\text{m}^2/\text{ms}\) (Fleidervish et al. 2010; Rugiero et al. 2010). Na\(^+\) and K\(^+\) reversal potentials were derived from the ion concentrations across the membrane.

**Na\(_{\text{V}1.6}\)** - Markov typed model (Fig. 6A) adopted from Khaliq et al. (Raman and Bean 2001). Na\(_{\text{V}1.6}\) current is determined by the open probability \(<O>\) and membrane potential \(V\): 

\[
I_{\text{Na6}} = g_{\text{Na6}} <O>(V-E_{\text{Na}})
\]

Rate coefficients (ms) that are membrane voltage-dependent (mV) are adopted from Khaliq et al., except that \(\zeta\) was adjusted to \(0.6 \exp(-V/25)\) to accommodate to the low firing rate of colorectal afferent neurons.

**Na\(_{\text{V}1.7}\)** - Markov typed model (Fig. 6A) adopted from Gurkiewicz et
NaV1.7 current is determined by the open probability $<O>$ and membrane potential $V$: $I_{Na^7} = \bar{g}_{Na^7} <O>(V-E_{Na})$. All model parameters were adopted from Gurkiewicz et al.

**NaV1.8** – Hodgkin-Huxley typed model adopted from Baker (Baker 2005)

$$I_{Na^8} = \bar{g}_{Na^8} m^3 h (V-E_{Na})$$

$$\dot{m} = \frac{m_n - m}{\tau_m}$$

$$\alpha_m = 3.83 \left[ 1 + \exp \left( \frac{-V + 2.58}{11.47} \right) \right]$$

$$\beta_m = 6.894 \left[ 1 + \exp \left( \frac{V + 61.2}{19.8} \right) \right]$$

$$m_n = \frac{\alpha_m}{\alpha_m + \beta_m}$$

$$\tau_m = \frac{1.0}{\alpha_m + \beta_m}$$

$$\alpha_h = 0.013536 \exp \left( \frac{-V + 105}{46.33} \right)$$

$$\beta_h = 0.61714 \left[ 1 + \exp \left( \frac{-V - 21.8}{11.998} \right) \right]$$

$$\dot{h} = \frac{h_n - h}{\tau_h}$$

$$h_n = \frac{\alpha_h}{\alpha_h + \beta_h}$$

$$\tau_h = \frac{1.0}{\alpha_h + \beta_h}$$

**NaV1.9** – Hodgkin-Huxley typed model adopted from Baker (Baker 2005)

$$I_{Na^9} = \bar{g}_{Na^9} m h (V-E_{Na})$$

$$\dot{m} = \frac{m_n - m}{\tau_m}$$

$$\alpha_m = 1.548 \left[ 1 + \exp \left( \frac{-V - 11.01}{14.871} \right) \right]$$

$$\beta_m = 8.685 \left[ 1 + \exp \left( \frac{V + 112.4}{22.9} \right) \right]$$

$$m_n = \frac{\alpha_m}{\alpha_m + \beta_m}$$

$$\tau_m = \frac{1.0}{\alpha_m + \beta_m}$$

$$\alpha_h = 0.2574 \left[ 1 + \exp \left( \frac{V + 63.264}{3.7193} \right) \right]$$

$$\beta_h = 0.53984 \left[ 1 + \exp \left( \frac{-V + 0.27853}{9.0933} \right) \right]$$

$$\dot{h} = \frac{h_n - h}{\tau_h}$$

$$h_n = \frac{\alpha_h}{\alpha_h + \beta_h}$$

$$\tau_h = \frac{1.0}{\alpha_h + \beta_h}$$
\[
h_h = \frac{\alpha_h}{\alpha_h + \beta_h} \quad \tau_h = \frac{1.0}{\alpha_h + \beta_h}
\]

**K_A** – Hodgkin-Huxley typed model adopted from Schild et al. (Schild et al. 1994)

with minor adjustments of parameters.

\[
I_{KA} = g_{KA} p^3 q (V - E_K)
\]

\[
\dot{p} = \frac{p_{in} - p}{\tau_p} \quad \dot{q} = \frac{q_{in} - q}{\tau_q}
\]

\[
p_{in} = 1.0 \left[ 1.0 + \exp \left( \frac{V + 15.79}{-10.0} \right) \right] \quad \tau_p = 5.0 \exp \left( -0.022^2 (V + 65)^2 \right) + 1.5
\]

\[
q_{in} = 1.0 \left[ 1.0 + \exp \left( \frac{V + 58}{7.0} \right) \right] \quad \tau_q = 45.0 \exp \left( -0.0035^2 (V + 10)^2 \right) + 10.5
\]

**K_D** – Hodgkin-Huxley typed model adopted from Schild et al. (Schild et al. 1994)

with minor adjustments of parameters.

\[
I_{KD} = g_{KD} x^4 y (V - E_K)
\]

\[
\dot{x} = \frac{x_{in} - x}{\tau_x} \quad \dot{y} = \frac{y_{in} - y}{\tau_y}
\]

\[
x_{in} = 1.0 \left[ 1.0 + \exp \left( \frac{V + 14.59}{-15.0} \right) \right] \quad \tau_x = 5.0 \exp \left( -0.022^2 (V + 65)^2 \right) + 3.5
\]

\[
y_{in} = 1.0 \left[ 1.0 + \exp \left( \frac{V + 48}{7.0} \right) \right] \quad \tau_y = 1800
\]

**K_S** - Hodgkin-Huxley typed model adopted from Schild et al. (Schild et al. 1994)

with minor adjustment of parameters.

\[
I_{KS} = g_{KS} n^4 (V - E_K)
\]

\[
\dot{n} = \frac{n_{in} - n}{\tau_n}
\]
\[ n_n = \frac{\alpha_n}{\alpha_n + \beta_n} \]
\[ \alpha_n = 0.1 \left( \frac{V + 52}{12.5} \right) \left[ 1 - \exp \left( -\frac{V + 52}{12.5} \right) \right] \]
\[ \beta_n = 0.125 \exp \left( -\frac{V + 60}{80} \right) \]
\[ \tau_n = \frac{1.0}{\alpha_n + \beta_n} \]

\[ \tau_\alpha = \frac{1}{\tau_n} \]

**Na\(^+\),K\(^+\)-ATPase** - adapted from Bondarenko et al. (Bondarenko et al. 2004) with parameters slightly adjusted.

\[ I_{\text{NaK}} = I_{\text{NaK,p}} + I_{\text{K,p}} \]
\[ I_{\text{NaK,p}} = 3I_{\text{NaK}} \]
\[ I_{\text{K,p}} = -2I_{\text{NaK}} \]

\[ f_{\text{NaK}} = \frac{1}{1 + 0.1245 \cdot \exp \left( -0.1 \frac{VF}{RT} \right) + 0.0365 \cdot \sigma \cdot \exp \left( -\frac{VF}{RT} \right)} \]

\[ \sigma = \frac{1}{7} \left[ \exp \left( \frac{[Na^+]_o}{67.3} \right) - 1 \right] \]

\[ K_{m,Na} = 13mM \quad K_{m,Ko} = 1.5mM \]

**Background current**

\[ I_B = \bar{g}_B (V - E_B) \]
\[ I_B = I_{\text{Na,B}} + I_{\text{K,B}} \]

\[ I_{\text{Na,B}} = \bar{g}_B \frac{E_B - E_K}{E_{Na} - E_K} (V - E_{Na}) \]
\[ I_{\text{K,B}} = \bar{g}_B \frac{E_{Na} - E_B}{E_{Na} - E_K} (V - E_K) \]

The summary of the ion channels and Na\(^+\),K\(^+\)-ATPase included in modeling is listed in table 1 below.

**Mechanosensitive (ms) ion channels**

To simulate the gating of a mechanosensitive (ms) ion channel, we used a two-state model that includes an open state (O) and a closed state (C), the rates of transition between which are \( \alpha \) and \( \beta \), two exponential functions of membrane tension.
\( \tau \) at the afferent ending:

\[ O \xrightarrow{\alpha, \beta} C \]

\[ \alpha = \frac{1}{A} \exp\left(-\frac{\tau - \tau_0}{2S}\right) \quad \beta = \frac{1}{A} \exp\left(\frac{\tau - \tau_0}{2S}\right) \]

Assume that the fraction of \( ms \) channels in the open state is denoted by \( p \), and we have:

\[ \dot{p} = \frac{p_o - p}{T_p} \]

\[ p_o, \] the open probability of the \( ms \) channel at steady-state, follows Boltzmann’s equation, consistent with previous theoretical and experimental studies on \( ms \) channels (Hao and Delmas 2010; Haselwandter and Phillips 2013; Wiggins and Phillips 2005).

\[ p_o = \frac{1}{1 + \exp\left(-\frac{\tau - \tau_0}{S}\right)} \]

To allow calculation of \( \tau \) from bulk colorectal deformation (circumferential stretch force \( F \), stretch ratio \( \lambda \), and their derivatives \( \dot{F} \) and \( \dot{\lambda} \) ), the passive mechanical properties of the colorectal wall tissue was simulated by a lumped parametric model consisting of 2 springs and 1 dashpot (Fig. 1C), which leads to the following equations:

\[
\begin{cases}
    x_2 = (\lambda - 1) \Delta L \\
    \tau \Delta w = k_2 (x_2 - x_1) \\
    \tau \Delta w = k_1 x_1 + c_1 \dot{x}_1
\end{cases}
\]

Assuming: \( T_1 = \frac{c_1}{k_1 + k_2} \Delta L \)

\[ m_1 = \frac{k_1 k_2 \Delta L}{(k_1 + k_2) \Delta w} \quad m_2 = \frac{k_2 c_1 \Delta L}{(k_1 + k_2) \Delta w} \]
Then: \[ \tau = \frac{m_1(\lambda - 1) + m_2\lambda - \tau}{T_\tau} \]

The \( ms \) channel conductance is divided into conductance for \( \text{Na}^+ \) and \( \text{K}^+ \) ions:

\[ g_{M} = g_{M,\text{Na}} + g_{M,\text{K}} = \frac{1}{R_{\text{Na}} + R_{\text{K}}} \]

Assuming the conductance for \( \text{Na}^+ \) and \( \text{K}^+ \) are proportional to their respective driving forces, i.e., \((E_{\text{Na}} - V)\) and \((V - E_{\text{K}})\), then we have:

\[ I_{\text{Na},M} = g_{M,\text{Na}} P \frac{E_{\text{Na}} - V}{E_{\text{Na}} - E_{\text{K}}} (V - E_{\text{Na}}) \quad \quad I_{\text{K},M} = g_{M,\text{K}} P \frac{V - E_{\text{K}}}{E_{\text{Na}} - E_{\text{K}}} (V - E_{\text{K}}) \]

\[ I_M = I_{\text{Na},M} + I_{\text{K},M} \]

Parameters for the \( ms \) channel in the study are listed below:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A )</td>
<td>10 ms</td>
</tr>
<tr>
<td>( S )</td>
<td>2.07 mN/m</td>
</tr>
<tr>
<td>( m_1 )</td>
<td>13.6 mN/m</td>
</tr>
<tr>
<td>( \tau_0 )</td>
<td>4.45 mN/m</td>
</tr>
<tr>
<td>( T_\tau )</td>
<td>1000 ms</td>
</tr>
<tr>
<td>( m_2 )</td>
<td>16900 (mN·ms)/m</td>
</tr>
</tbody>
</table>

The \( ms \) current evoked by a stepped colorectal stretch is plotted in Fig. 1C, which recapitulated features of a typical slowly adapting \( ms \) current observed experimentally in DRG neurons (Rugiero et al. 2010).

**Passive properties and initial conditions**

The passive electrical properties \( C_m, R_m \) and \( R_i \) in both models were set to 1 \( \mu \text{F/cm}^2 \), 10,000 \( \Omega \cdot \text{cm}^2 \), and 123 \( \Omega \cdot \text{cm} \), respectively. The initial ion concentrations for the soma model were set to be consistent with current-clamp recording conditions (i.e., 140mM [\( \text{Na}^+ \)]_o, 4.5mM [\( \text{Na}^+ \)]_i, 5mM [\( \text{K}^+ \)]_o, and 130mM [\( \text{K}^+ \)]_i), so was the resting membrane potential \( V_m \) of -64.3mV (Shinoda et al. 2010). The initial ion concentrations for the afferent ending model(145mM [\( \text{Na}^+ \)]_o, 4mM [\( \text{Na}^+ \)]_i, 6.3mM [\( \text{K}^+ \)]_o, and 155mM [\( \text{K}^+ \)]_i); were determined when the model reached equilibrium condition at
$V_m$ of -65mV, a potential well within the range of resting membrane potentials recorded from distal axons of small-diameter DRG neurons (-68 to -53mV) (Vasylyev and Waxman 2012). Simulations were run at 30°C to approximate the experimental conditions of *in vitro* single-fiber recordings. Rate constants of voltage-dependent channels and Na⁺,K⁺-ATPase were multiplied by a temperature factor (i.e., $Q_{10}^{(1-20)/10}$). The $Q_{10}$ values listed in Table 2 were adapted from Schild et al. (Schild et al. 1994). The numerical error tolerance in NEURON was set at $10^{-5}$.

**Data Recording and Analysis**

Action potentials (APs) were recorded extracellularly using a low-noise AC differential amplifier. Activity was monitored on-line, filtered (0.3 to 10 kHz), amplified (x10,000), digitized at 20kHz using a 1401 interface (CED, Cambridge, England) and stored on a PC. APs were discriminated off-line using Spike 2 software (CED). To avoid erroneous discrimination, no more than two clearly discriminable units in any record were studied. The stretch response threshold was defined as the force that evoked the first AP during ramped stretch. SRFs are presented as bins of evoked APs (0-57, 57-113, 113-170mN) by the ramped colorectal stretch. SRFs were normalized to the respective maximum binned spike number in control (baseline) tests. To facilitate comparisons, responses of stretch-sensitive afferents are also presented as total numbers of APs during ramped stretch. Data are presented throughout as mean ± SEM. One-way and two-way analyses of variance (ANOVA) or repeated-measures were performed as appropriate using SigmaPlot v11.0 (Systat software, Inc., San Jose, CA). Bonferroni post-hoc multiple comparisons were
performed when F values for main effects were significant. Differences were
considered significant when p<0.05 (denoted by *).
RESULTS

**The presence of NaV1.6 in colorectal afferents**

Portions of retrogradely labeled colorectal DRG neurons showed positive immunostaining for NaV1.6 and NaV1.7 (Fig. 2A). Co-localization of YFP (expressed in sensory neurons driven by SNS-Cre) with NaV1.6- and NaV1.7-immunoreactivity in the colorectum suggests the presence of NaV1.6 and NaV1.7 at distal colorectal afferent endings (Fig. 2B). The staining pattern of NaV1.6 is not homogenous along YFP-positive nerve fibers, but rather clusters at focal regions along the axons (see a 3-D reconstruction in the supplemental video), presumably regions of spike initiation, which is consistent with the sporadic NaV1.6 immunoreactivity in other sensory nerve terminals (Hossain et al. 2005). Both circular and longitudinal smooth muscle layers showed low-intensity staining of NaV1.6, suggesting the presence of some NaV1.6 protein in smooth muscle fibers. In some sections of colorectal tissue, we noted positive NaV1.6 staining that was YFP-negative, likely contributed to by NaV1.6 in efferent endings or enteric neurons (data not shown). Western blots confirmed the presence of NaV1.6 protein in both the pelvic nerve (PN) and L6 DRG, along with NaV1.7 (Fig. 2C). However, the two channels were barely detectable in protein extracts from colon because of relatively low content of neuronal proteins (stained by PGP9.5) in the tissue homogenates.

**Effect of TTX on stretch-sensitive colorectal afferents**

As illustrated in Fig. 3A, TTX was applied to the mucosal surface at the receptive
field (grey square surrounding “1” on the colorectal mucosal surface) and afferent responses to ramped stretch (0 to 170mN @ 5mmHg/sec) were assessed prior to and after TTX application, and again after washout. TTX at concentrations of 1μM and 3μM did not affect responses to stretch (Fig. 3B, $F_{2,8} = 0.12, p = 0.89$; Fig. 3C, $F_{2,10} = 2.2, p = 0.16$). In contrast, 10μM TTX inhibited virtually all afferent responses to stretch (Fig. 3D, $F_{2,24} = 28.3, p<0.001$; post-hoc comparison, $p<0.001$ for TTX vs. control). In additional experiments (n=7), a bile salt solution (0.25%) was applied locally to the receptive field for 5 min (to increase mucosal permeability) followed by 1μM TTX application. Application of bile salts significantly increased the response of afferents to stretch (Fig. 3E, $F_{3,36} = 14.9, p<0.001$; post-hoc comparison vs. control, $p = 0.023$), and subsequent application of 1μM TTX inhibited the response (post-hoc comparison vs. control, $p = 0.028$). The corresponding response thresholds to stretch were unaffected by 1μM or 3μM TTX (Fig. 3B inset, $F_{2,8} = 0.17, p=0.84$; Fig. 3C inset, $F_{2,10} = 0.84, p = 0.46$), but response threshold was significantly increased by 10μM TTX (Fig. 3D inset, $F_{2,24} = 24.9, p<0.001$) and 1μM TTX following bile salts (Fig. 3E inset, $F_{3,18} = 10.6, p<0.001$; post-hoc comparison, $p = 0.003$ for TTX vs. control). TTX was effectively removed after 15 min of wash ($p>0.05$, control vs wash, for all comparisons).

Responses to stretch at different concentrations of TTX are summarized in Fig. 3F (total spike numbers were normalized to control). When applied to the mucosal surface, the effect of TTX appears to be concentration-dependent, but was significant only at the greatest concentration tested (Fig. 3F, 10μM; $F_{4,47} = 15.6, p<0.001$,
post-hoc comparison, 10μM TTX vs. all others, p<0.02). Application of bile salts (0.25%) significantly increased the responses of afferents to stretch (Fig. 3F, F_{3,18} = 17.4, post-hoc comparison, bile salts vs. control, p = 0.009) and subsequent TTX application at a lower concentration (1μM) inhibited the response (post-hoc comparison, 1μM TTX vs. control, p = 0.006). There was no significant difference between control and washout (post-hoc comparison, p>0.3).

Effect of Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 antagonists on stretch-sensitive colorectal afferents

Both Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 channels are present in primary afferents and are blocked effectively by TTX with comparable EC\textsubscript{50}s (Table 3). Thus, we assessed the effects of Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 subtype-selective blockers on afferent responses to stretch.

As evidenced in Fig. 3, the colon mucosa impedes the diffusion of xenobiotics, including channel blocking molecules. To avoid concerns related to diffusion across the mucosa and interpretation of results, we conducted the following pharmacological studies with the serosal side of the colorectum facing up. When applied to the serosal surface, 1μM TTX effectively abolished afferent responses to stretch (Fig. 4A, F_{2,36} = 30.7, p<0.001, post-hoc comparison vs. control, p<0.001) and increased the response threshold (inset, F_{2,18} = 9.2, p = 0.002, post-hoc comparison vs. control, p = 0.003). The selective Nav1.7 antagonist PTX at the same concentration (1μM) did not affect either afferent responses (Fig. 4B, F_{2,32} = 0.07, p = 0.93) or response threshold
However, PTX at 3μM, a concentration greater than the EC50 for blocking NaV1.6, effectively attenuated afferent responses (Fig. 4C, F2,20 = 5.9, p = 0.02, post-hoc comparison vs. control, p = 0.04) and increased response threshold (inset, F2,10 = 9.6, p = 0.005, post-hoc comparison vs. control, p = 0.009).

Although blockage of NaV1.7 by PTX is almost irreversible (Johnson et al.), the response to stretch recovered to control completely after only 15min wash-out (post-hoc comparison vs. control, p = 0.95), a time at which NaV1.7 should remain in a blocked state. This strongly suggests that the attenuation of afferent responses to stretch by 3μM PTX arises through blocking NaV1.6, not NaV1.7. In addition, the selective NaV1.6 antagonist mCtxG (17μM) significantly reduced afferent responses to stretch (Fig. 4D, F2,36 = 15.2, p < 0.001, post-hoc comparison vs. control, p<0.001) and increased the response threshold (inset, F2,18 = 6.9, p = 0.006, post-hoc comparison vs. control, p = 0.008). Data are summarized (total spike number) in Fig. 4E. TTX at 1μM significantly reduced the response to stretch whereas the selective NaV1.7 blocker PTX at the same concentration (1μM) did not (Fig. 4E, F5,99 = 23.1, p<0.001; post-hoc comparison, TTX vs. control, p<0.001, 1μM PTX vs control, p>0.9). However, 3μM PTX effectively reduced the responses to stretch (post-hoc comparison, 3μM PTX vs control, p<0.001), likely from its non-specific blocking effect on NaV1.6. In addition, the selective NaV1.6 blocker mCtxG (17μM) significantly reduced the afferent responses to stretch (post-hoc comparison, mCtxG vs. control, p<0.001), suggesting an essential role of NaV1.6 in colorectal afferent encoding to stretch.
To verify the finding, the aforementioned selective Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 antagonists were applied to the mucosal surface of the colorectum (3 μM PTX and 50 μM mCtxG), as was another Na\textsubscript{v}1.6 antagonist from the μ-conotoxin family, mCtxP (10 μM). When applied from the mucosal side, 3μM PTX did not affect either afferent responses (Fig. 5A, $F_{2,14} = 2.76$, $p = 0.1$) or response threshold (inset, $F_{2,14} = 1.96$, $p = 0.18$) to stretch. In contrast, both mCtxG (50μM) and mCtxP (10 μM) significantly reduced afferent responses to stretch (Fig. 5B, $F_{2,12} = 28.1$, $p < 0.001$, post-hoc comparison vs. control, $p < 0.001$; Fig. 5C, $F_{2,14} = 19.4$, $p < 0.001$, post-hoc comparison vs. control, $p < 0.001$) and increased the response threshold (Fig. 5B inset, $F_{2,12} = 9.6$, $p = 0.003$; Fig. 5C inset, $F_{2,14} = 5.9$, $p = 0.014$). The summarized data in Fig. 5D are consistent with the findings presented in Figure 4 in which antagonists were applied from the serosal side: both Na\textsubscript{v}1.6 blockers (mCtxG and mCtxP) significantly reduced the response to stretch whereas the Na\textsubscript{v}1.7 blocker PTX did not ($F_{4,50} = 13.3$, $p < 0.001$, post-hoc comparison, mCtxG vs. control, $p < 0.001$, mCtxG vs. control, $p < 0.001$, PTX vs control, $p = 0.09$).

Effect of Na\textsubscript{v}1.8 antagonist A803 on stretch-sensitive colorectal afferents

When applied from the serosal side, the selective Na\textsubscript{v}1.8 antagonist A803 did not reduce afferent responses to stretch at either 1μM (Fig. 6A, $F_{2,20} = 1.37$, $p = 0.3$) or 10μM concentrations (Fig. 6C, $F_{2,24} = 2.97$, $p = 0.09$). Similarly, 3μM A803 applied from the mucosal side did not affect responses to stretch (Fig. 6B, $F_{2,10} = 4.1$, $p > 0.05$) whereas a greater concentration (10μM) slightly, but significantly attenuated
responses (Fig. 6D, $F_{2,20} = 6.0$, $p = 0.009$, post-hoc comparison vs. control, $p = 0.01$).

Response threshold was unaffected by A803 whether applied from the serosal (Fig. 6A inset, $F_{2,10} = 0.38$, $p = 0.69$; Fig. 6C inset, $F_{2,12} = 1.56$, $p = 0.25$) or mucosal side (Fig. 6B inset, $F_{2,10} = 0.39$, $p > 0.5$; Fig. 6D inset, $F_{2,20} = 0.74$, $p > 0.4$). Data from serosal and mucosal applications of A803 are summarized (total spike number) in Figs. 6E and 6F, respectively; there were no significant differences between control, A803 and wash (Fig. 6E, $F3,35 = 2.67$, $p = 0.06$; Fig. 6F, $F3,35 = 1.85$, $p = 0.16$).

Interestingly, 1μM A803 applied from the serosal side, a concentration well below the EC$_{50}$s for blocking NaV1.6 or NaV1.7 (Table 3), tended to increase the afferent responses to stretch.

Neuron membrane models for colorectal afferent endings and c-DRG somata

Details of the model structures and the mechanosensitive (ms) channel are illustrated in Fig. 1 and described in Methods. In addition, the simulations also include four voltage-gated Na$^+$ conductances, three voltage-gated K$^+$ conductances and the Na$^+$,K$^+$-ATPase pump; their maximum conductances and the pump current are presented in Table 4. The sodium channel conductance in the c-DRG soma model was estimated from the peak sodium current (~5nA) recorded from c-DRG neurons (Beyak et al. 2004). The density of NaV1.7 and NaV1.8 channels at the size in the afferent ending model were assigned to be 50 times the density at the soma, an estimation consistent with recent studies indicating 19-60 times sodium channel density at the AIS than at the soma (Baranauskas et al. 2013; Hu et al. 2009; Kole et al. 2008)
Plotted in Fig. 7A are Na\textsubscript{V} 1.6, 1.7, 1.8 and 1.9 currents calculated from voltage clamp simulations (-80 to 70mV). Different from other channels in the model that use Hodgkin-Huxley equations, Na\textsubscript{V} 1.6 and Na\textsubscript{V} 1.7 were simulated by Markov state models with corresponding diagrams plotted in Fig. 7B. The activation and inactivation channel conductances as functions of membrane voltage (Fig. 7C) were derived by simulating the corresponding single-electrode voltage clamp protocols in whole-cell configurations. Compared with Na\textsubscript{V} 1.7, the activation curve of Na\textsubscript{V} 1.6 is shifted in a hyperpolarized direction and the inactivation curve in a depolarized direction, suggesting greater open probability during action potential generation.

Na\textsubscript{V} 1.6 is critical for repetitive spiking

The simulated encoding response of a colorectal afferent ending to stepped circumferential stretch is presented in Fig. 8A. A stepped stretch of 50mN induced tonic action potential generation at the siz, which closely correlated with the transient increase in intracellular [Na\textsuperscript{+}] and transient decrease in intracellular [K\textsuperscript{+}]. Because experimental studies suggested the absence of Na\textsubscript{V} 1.6 currents in small-diameter DRG neurons (Cummins et al. 2005), the Na\textsubscript{V} 1.6 conductance in the c-DRG soma model was set to zero (Table 4). In contrast to the tonic firing in the afferent ending simulation (Fig. 8A), the c-DRG soma model under a stepped current clamp simulation (500ms) did not fire repetitively, even at a stimulation current three times rheobase (Fig. 8B). However, after adding a Na\textsubscript{V} 1.6 conductance (1/50th of Na\textsubscript{V} 1.6 conductance at siz) in the c-DRG soma model, the soma was able to fire tonically at a stepped current stimulation slightly greater than rheobase (x1.1) as shown in Fig. 8C.
In addition, after adding the \( \text{Na}_v1.6 \) conductance to the soma model, the rheobase decreased from 134.5pA in Fig. 8B to 22.9pA in Fig. 8C. In contrast, adding the same amount of \( \text{Na}_v1.7 \) or \( \text{Na}_v1.8 \) conductance to the soma model did not change the firing pattern (data not shown, but almost identical to Fig. 8B) other than the slight decrease of rheobase to 116.3pA and 129.5pA, respectively.

Simulation of afferent responses to ramped stretch after \( \text{Na}_v \) channel blockade

During the slow ramp protocol (0 to 170mN @ 5mN/sec), circumferential stretch deformed the colorectum homogeneously, which was recorded for post-hoc measurement of the circumferential stretch ratio as displayed in Fig. 9A. To mimic the ramped stretch protocol in single-fiber recordings (Figs. 3 to 6), the ramped force (0 to 170mN in 34 sec) and stretch ratio were used to drive action potential generation at the size in the afferent ending model with results displayed in Fig. 9B. To save computational time, the falling phase of the ramp, which mirrored the rising phase (34 sec) in the experimental protocol, was shortened in duration (6 sec) in the simulation. Pharmacological blockage of channels by TTX was mimicked by gradual reduction of maximum conductances of both \( \text{Na}_v1.6 \) and \( \text{Na}_v1.7 \) in the model (by 15%, 30% and 50%). In the simulation, the response to stretch is progressively reduced and completely inhibited by increasing blockage of both \( \text{Na}_v1.6 \) and \( \text{Na}_v1.7 \) conductances, simulating the effect of TTX (Fig. 9B). The pharmacological blockage of subtype-selective \( \text{Na}_v \) channels was simulated by reducing the corresponding maximal conductance by 50% (Fig. 9C). Computational simulations of the total...
number of spikes evoked by the ramped stretch stimulus are summarized in Fig. 9D, which favorably agrees with the findings from single-fiber studies (i.e., \( \text{Na}_V \text{1.6} \) is necessary for the encoding of colorectal afferent endings to stretch whereas blockage of \( \text{Na}_V \text{1.7} \) does not remarkably alter the firing pattern). Consistent with the experimental findings using 1\( \mu \text{M} \) A803 (Fig. 6E), blocking \( \text{Na}_V \text{1.8} \) in the model did not reduce afferent responses to stretch, but instead slightly increased firing.
DISCUSSION

The present study demonstrates that a TTX-sensitive current underlies the tonic encoding of ramped colorectal stretch by a group of unmyelinated sensory afferent endings innervating mouse colorectum. Prior studies on dissociated ‘nociceptive’ DRG neurons suggested that TTX-resistant currents dominate the inward sodium current that drives action potential generation (Blair and Bean 2002) because TTX typically did not inhibit action potential initiation in DRG somata (Choi and Waxman 2011). Afferents innervating the cornea have been shown to resist blockage by TTX and cooling (Brock et al. 1998; Carr et al. 2003), but a direct effect of TTX on visceral afferent endings has rarely been studied. Andresen et al. (Andresen et al. 1994) reported that intraluminal perfusion of 40nM TTX did not markedly affect the encoding function of A-type aortic baroreceptor endings to intraluminal pressure, which is unexpected because A-type baroreceptor somata generally exhibit a TTX-sensitive current (Li and Schild 2007). We found that mucosal application of TTX (10μM) reversibly inhibited the response of colorectal afferents to stretch whereas lower concentrations of TTX (1μM, 3μM) did not, suggesting that the 40nM concentration of TTX used by Andresen et al. was insufficient. An alternative interpretation is that the effect of TTX reported here resulted from spillover/leakage and blockage of the pelvic nerve. This is unlikely based upon 1) the large volume of bath solution (>200mL) relative to the volume of the TTX solution (150μL) and 2) the observation that responses to stretch of afferents in the same record with receptive fields outside the locus of TTX application were unaffected (e.g., fiber 2 in Fig. 3A).
The high TTX concentration required to block responses is likely due to epithelial tight-junctions within the colon mucosa that effectively prevent passive diffusion of large molecules (Camilleri et al. 2012). For example, when applied to the colonic mucosa, 300µM cyclic GMP was required to attenuate colorectal afferent responses to stretch (Feng et al. 2013). However, after pre-treating the colorectal mucosa with 0.25% bile salt solution, a detergent that increases gut permeability (Stenman et al. 2013), TTX completely inhibited afferent responses to stretch at 1µM, a concentration routinely used in patch-clamp studies on tissue slices to block TTX-sensitive currents (Gassner et al. 2009). In addition to increasing gut permeability, bile salts also have a direct effect on sensory afferents (Lieu et al. 2014), which could account for the significant increase in afferent responses to stretch after bile salts application. On the other hand, chemicals applied from the serosal surface appear to bypass the mucosal barrier without affecting the baseline response of the afferents. We documented that chemicals applied to the serosal surface can reach nerve endings at concentrations comparable to those used in patch-clamp studies (e.g., 1µM TTX effectively blocked afferent responses to stretch).

At the outset, we confirmed the presence of both Nav1.6 and Nav1.7 in colorectal afferent endings and examined the roles of Nav1.6, Nav1.7 and Nav1.8 using subtype-selective antagonists. We excluded study of Nav1.1 (no clear role in nociceptors), Nav1.3 (absent in adult DRG neurons), and Nav1.9 (lack of selective antagonists). We chose ProTX II (PTX) as the Nav1.7 antagonist due to its low EC50 (0.3 nM) compared with its EC50s for Nav1.6 (26nM) and Nav1.8 (146nM).
The two μ-Conotoxin NaV1.6 antagonists, mCtxG and mCtxP, have EC50s for NaV1.6 (0.68nM for mCtxG and 0.1nM for mCtxP) orders of magnitude less than for NaV1.7 or NaV1.8 (>100nM) (Wilson et al. 2011).

NaV1.7, which has been documented as important in pain sensation, was unexpectedly found not necessary for encoding tonic spiking of pelvic nerve afferent fibers to noxious colorectal stretch. Serosal application of 1μM PTX did not affect responses to stretch whereas the same concentration of TTX effectively blocked responses even though TTX has a much higher EC50 to block NaV1.7 than PTX (table 3). A greater concentration of PTX (3μM) slightly, but significantly attenuated responses to stretch, likely due to its nanomolar EC50 for NaV1.6. A prior study showed that PTX blocks both NaV1.6 and NaV1.7 at nanomolar concentrations, but the blockage of NaV1.6 by PTX has a significantly higher off-rate than blockage of NaV1.7, which accounts for the reversible blockage of NaV1.6 by PTX and almost irreversible blockage of NaV1.7 (Johnson et al.). The fact that the effect of 3μM PTX on the response to stretch was reversible (response recovered completely after only 15min wash-out, when NaV1.7 should have remained blocked) strongly suggests the involvement of NaV1.6 and not NaV1.7 in tonic spiking. Further, mCtxG at a concentration that selectively blocks NaV1.6 significantly reduced afferent responses to stretch, indicating a necessary role of NaV1.6 in encoding tonic spiking by stretch-sensitive colorectal afferent endings. An alternative interpretation of the blocking effect by Nav1.6 antagonist is the possible blockage of action potential propagation down the axon when applied from the serosal side. But blockage of
propagation by our pharmacological scheme seems unlikely because serosal application of selective NaV1.7 antagonist PTX did not seem to block the afferent response to stretch whereas a prior study clearly indicated that NaV1.7, not NaV1.6 is critical to action potential propagation in unmyelinated C-fibers (Schmalhofer et al. 2008). We repeated the experiments with PTX and mCtxG applied from the mucosal side at greater concentrations to overcome the mucosal barrier and also tested another selective NaV1.6 blocker mCtxP; results confirmed that NaV1.6, not NaV1.7 is necessary for tonic spiking by colorectal afferent endings.

The important role of NaV1.6 in tonic spiking is also supported by recordings from DRG somata. For example, siRNA knockdown of NaV1.6 effectively reduced the proportion of DRG somata that fire repetitively (Xie et al. 2013). NaV1.6 currents are restricted to medium to large diameter DRGs (Cummins et al. 2005), whereas small-diameter DRG somata lack NaV1.6 currents and usually do not fire tonically during stepped current stimulation (Hillsley et al. 2006; Huang et al. 2013; Shinoda et al. 2010). In addition, complementary modeling of a colorectal afferent ending and a soma recapitulated the experimental findings reported here and by others, collectively supporting a necessary role for NaV1.6 in tonic spiking at both afferent endings and somata. Interestingly, both Xie et al. (2013) and we noted positive NaV1.6 immunostaining in some small-diameter DRG somata, which are unlikely to contribute to the membrane current (Cummins et al. 2005)

Perhaps the different spiking properties arise from greater sodium channel densities in afferent endings (50 times in our simulation) than in their somata.
Nonetheless, the modeled soma responded tonically at a stimulus intensity slightly greater than rheobase after adding a small \( \text{Na}_v1.6 \) conductance (1/50 the conductance in siz). Thus, the lack of tonic firing in c-DRG somata is likely due to the absence of a \( \text{Na}_v1.6 \) conductance, not to the relative low density of other types of sodium channels (e.g., \( \text{Na}_v1.7 \) and \( \text{Na}_v1.8 \)) in somata. Different channel compositions at different regions of a same neuron have been documented in axon initial segments (AIS) in the CNS; \( \text{Na}_v1.6 \) channels are clustered at the distal AIS and absent in the soma membrane whereas \( \text{Na}_v1.2 \) is clustered at the proximal AIS (Baranauskas et al. 2013; Bender and Trussell 2012). The present findings suggest a mechanism/channel to explain differences in neural spiking characteristics between DRG somata and their sensory endings in end organs. Hence, excitability data recorded from dissociated DRG neurons likely do not reflect actual encoding at their sensory endings and thus need to be interpreted with caution.

The selective \( \text{Na}_v1.8 \) antagonist A803467 was also studied, but did not significantly affect afferent responses to stretch when applied either to the serosal or mucosal colorectal surface. Closer examination of the data revealed that colorectal afferents were differentially affected by A803467; responses to stretch in some afferents were attenuated whereas in others enhanced. In particular, serosal application of 1\( \mu \)M A803467, a concentration that selectively blocks \( \text{Na}_v1.8 \), tended to increase afferent responses to stretch (p = 0.06). Interestingly, model simulation also showed a slight increase in firing after blocking \( \text{Na}_v1.8 \). In fact, the role of \( \text{Na}_v1.8 \) in nociception and pain remains unclear (Knapp et al. 2012). Given the variability noted,
we focused on TTX-sensitive channels Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7, leaving Na\textsubscript{V}1.8 for subsequent study. Similarly, we included in our simulation model the gating formulas for an Na\textsubscript{V}1.9 conductance which appears to play a significant role during sustained noxious colon distension (Hockley et al. 2014) and will be the focus of a future study.

In previous sensory neuron models, Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 conductances were represented together as one TTX-sensitive conductance by a Hodgkin-Huxley styled formulation (Amir and Devor 2003; Baker 2005; Kovalsky et al. 2009; Schild et al. 1994; Tigerholm et al. 2013), which cannot simulate the unique gating features of Na\textsubscript{V}1.7 (i.e., complete inactivation and slow recovery from inactivation (Gurkiewicz et al. 2011)). The computational models employed here incorporated individual Markov models for Na\textsubscript{V}1.6 (Khaliq et al. 2003) and Na\textsubscript{V}1.7 (Gurkiewicz et al. 2011) previously verified with experimental data to capture their contrasting gating features (i.e., rapid vs. slow repriming, presence vs. absence of sustained current, and difference in inactivation voltage). The simulation revealed that the contrasting roles for Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 in tonic firing are likely caused by those aforementioned differences in channel gating properties, which will be investigated in future studies.

Our modeling also incorporated a novel mechanosensitive (ms) channel that drives action potential generation, which has not been reported previously. The rate constants between channel gating states were formulated as exponential functions of membrane tension, resulting in a Boltzmann-like steady-state channel open probability consistent with prior experimental and theoretical studies (Hao and Delmas 2010; Haselwandter and Phillips 2013; Wiggins and Phillips 2005).
Membrane tension at the afferent ending is linked to bulk circumferential colorectal stretch by a lumped parametric model that is routinely used to simulate passive mechanical properties of biological tissues (Feng and Gan 2004). The ms current evoked by a stepped stretch was consistent with a slowly-adapting ms current recorded in DRG neurons (Rugiero et al. 2010). The formulation also allows simulation of other types of ms (e.g., rapidly adapting) currents in future investigations when the scope of study extends beyond stretch-sensitive afferents.

The current computational model borrowed heavily from prior computational studies, including adoption of parameters for the voltage-gated channels and NaK-pump. The maximal conductances of sodium channels at the spike initiation zone was set to be 50 times the conductance at the soma; the conductance at the soma was determined from voltage-clamp data recorded on colorectal DRG neurons (Beyak et al. 2004). Parameters that were adjusted include maximal conductances of potassium channels necessary for the repolarization of the membrane depolarization, and gating parameters associated with mechanosensitive channels. We did not systematically adjust model parameters or conduct extensive sensitivity studies here, but instead focused on uncovering the differential roles of NaV1.6, NaV1.7 and NaV1.8 conductances in afferent encoding of ramped stretch, whose gating formulae were rigorously verified in previous studies (see Table 1 for references). Also, the geometry of the afferent ending model is intended to simulate the electrotonic character of free nerve endings, a morphological feature of the majority of the colorectal afferent terminals. However, some colorectal afferent endings appear to have lamina- and
array-like morphologies, which will not be adequately simulated by the current model geometry and need to be addressed in future studies.

In summary, we provide experimental and computational evidence for a necessary role of a Na\textsubscript{\text{V}}1.6 current in neural encoding of stretch-sensitive colorectal afferents. Immunohistochemistry and Western blotting revealed the presence of Na\textsubscript{\text{V}}1.6 and Na\textsubscript{\text{V}}1.7 at colorectal neuronal endings. Both TTX and selective Na\textsubscript{\text{V}}1.6 antagonists significantly attenuated afferent responses to stretch whereas a selective Na\textsubscript{\text{V}}1.7 antagonist only slightly reduced the response. Computational Markov type modeling recapitulated the pharmacological findings. A selective Na\textsubscript{\text{V}}1.8 antagonist did not significantly attenuate the responses to stretch and the exact role of Na\textsubscript{\text{V}}1.8 requires further study. These computational models provide a solid theoretical foundation for future studies of underlying mechanisms of neural encoding in different classes of colorectal afferents in both physiological and pathophysiological conditions.
FIGURE LEGENDS

Figure 1. Schematics for the computational simulation of action potential generation in colorectal afferent endings and dissociated DRG somata. (A) The colorectal afferent ending is simulated by a four-compartment cylinder consisting of a transducer (trsd), a spike initiation zone (siz), a transition zone in the middle (mid) and a passive conducting zone (pas) proximal to siz. (B) The soma is simulated as a one-segment membrane model. (C) The afferent ending model simulates encoding of mechanical stretch by inclusion of mechanosensitive (ms) channels in the transducer zone that are gated by the membrane tension $\tau$. A lumped parametric model is used to translate bulk colorectal deformation (stretch force $F$, stretch ratio $\lambda$, and their first derivatives) to the membrane tension $\tau$ at the afferent ending. Panel (C) also includes a representative ms current in response to a stepped colorectal stretch at 100mN.

Figure 2. Na$_{\text{V}}$1.6 and Na$_{\text{V}}$1.7 immunohistochemistry and Western blotting. (A) A proportion of mouse L6 DRG neurons retrogradely labeled with Fast Blue (FB) from the distal colon express Na$_{\text{V}}$1.6 and Na$_{\text{V}}$1.7 (arrows). Not all FB-labeled neurons expressed Na$_{\text{V}}$1.6 and Na$_{\text{V}}$1.7 (arrowheads). Scale bars = 50 $\mu$m. (B) Na$_{\text{V}}$1.6 and Na$_{\text{V}}$1.7 were co-localized with YFP (arrows) expressed in sensory neurons (driven by SNS-Cre). MP: myenteric plexus, CM: circular muscle, M: mucosa. Arrows indicate colocalization of YFP-expressing nerve fibers with either Na$_{\text{V}}$1.6- or Na$_{\text{V}}$1.7-immunoreactivity.. Many segments of YFP-expressing nerve fibers do not have Na$_{\text{V}}$1.6 or Na$_{\text{V}}$1.7 immunoreactivity (arrowheads). A Na$_{\text{V}}$1.7-immunoreactive MP neuron is indicated by an arrowhead. Scale bars = 10 $\mu$m. (C) Western-blot
detection of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 in protein extracts from pelvic nerve (PN), DRG and colon. The protein quantity of the two channels was below detection sensitivity in colon due to the low content of neuronal proteins in colon tissue homogenates (note the small quantity of neuron-specific protein PGP 9.5).

**Figure 3.** Localized mucosal application of tetrodotoxin (TTX) on a stretch-sensitive colorectal afferent ending. (A) Representative responses to ramped circumferential stretch (0-170mN @ 5mN/sec) of two stretch-sensitive colorectal afferents (ctrl) and their respective receptive fields (RF 1 and 2) on the flattened colorectal surface. RF 1 was isolated (illustrated by the grey square surrounding RF 1) and exposed to TTX (10\textmu M); the response was retested after 15 min wash (wash). TTX at 1 and 3 \textmu M modestly, but not significantly reduced afferent responses to stretch as shown in (B) and (C), respectively, whereas 10\textmu M TTX significantly attenuated most, if not all afferent responses to stretch (D). Application of 0.25\% bile salts significantly increased afferent responses to stretch and subsequent application of 1\textmu M TTX significantly attenuated afferent responses (E). TTX did not increase the response threshold to stretch at 1 or 3\textmu M (insets in B and C, in mN), but thresholds are significantly increased after 10\textmu M TTX (inset in D) as well as 1\textmu M TTX following bile salts application (inset in E). Responses to stretch were quantified as total number of action potentials during ramped stretch, normalized to control (=1), and are summarized in (F) (*, p<0.05).

**Figure 4.** Localized serosal application of TTX and selective Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 blockers on stretch-sensitive colorectal afferent endings. When applied from the
serosal side, 1μM TTX significantly attenuated most, if not all afferent responses to stretch (A) and increased response threshold (A inset). In contrast, the Na\textsubscript{v}1.7 blocker ProTX II (PTX, 1μM) did not affect afferent responses to stretch (B) or response threshold (B inset). PTX at 3μM, a concentration in excess of its EC\textsubscript{50} for Na\textsubscript{v}1.6, significantly attenuated the response to stretch (C) and increased response threshold (C inset). In Contrast, the selective Na\textsubscript{v}1.6 blocker \(\mu\text{-Conotoxin Gllla (mCtxG, 17}\mu\text{M})\) significantly reduced the response to stretch (D) and increased response threshold (D inset). (E) Normalized responses to stretch (total spike number) were significantly reduced by TTX, mCtxG and 3μM PTX, but not by 1μM PTX (*, p<0.05).

**Figure 5.** Localized mucosal application of selective Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 blockers on stretch-sensitive colorectal afferent endings. When applied from the mucosal side, the Na\textsubscript{v}1.7 blocker ProTX II (PTX, 3μM) did not affect afferent responses to stretch (A) or response threshold (A inset) whereas the selective Na\textsubscript{v}1.6 blocker \(\mu\text{-Conotoxin Gllla (mCtxG, 50}\mu\text{M})\) significantly reduced responses to stretch (B) and increased response threshold (B inset). Another selective Na\textsubscript{v}1.6 blocker, \(\mu\text{-Conotoxin PIIIa (mCtxP, 10}\mu\text{M})\), similarly reduced responses to stretch (C) and increased response threshold (C inset). (D) Normalized responses to stretch (total spike number) were significantly reduced by mCtxG and mCtxP, but not by PTX. The response to stretch after blocking Na\textsubscript{v}1.6 with mCtxG was significantly lower than after blocking Na\textsubscript{v}1.7 by PTX (*, p<0.05).

**Figure 6.** Effect of the selective Na\textsubscript{v}1.8 blocker A803467 (A803) on stretch-sensitive...
colorectal afferent endings. When applied from the serosal side, afferent responses to ramped stretch were unaffected by either 1μM (A) or 3μM A803 (C). Mucosal application of 3μM A803 did not affect responses to stretch (B), whereas 10μM A803 slightly, but significantly reduced responses ($F_{2,20} = 6.0$, $p = 0.009$, post-hoc comparison vs. control, $p = 0.01$). (D). Response thresholds (insets in A to D) were unaffected by A803. Normalized responses to stretch (total spike number) are plotted in (E) and (F) for A803 applied from the serosal and mucosal side, respectively; there were no significant differences between control, A803, and wash groups.

**Figure 7.** Simulation of voltage-gated sodium channel subtype (Na\textsubscript{\text{V}}1.6 to 1.9) in DRG somata. (A) Na\textsuperscript{+} currents (equal maximum membrane conductance of 10 pS/μm\textsuperscript{2}) evoked by a single-electrode voltage-clamp protocol (-100 to 70mV). Markov-type state models for Na\textsubscript{\text{V}}1.6 and Na\textsubscript{\text{V}}1.7 are illustrated in (B) to recapitulate their respective gating features (i.e., persistent and resurgent current [dark arrows] and complete inactivation [grey arrow] in A). The activation and inactivation functions of membrane voltage in (C) are from normalized peak conductance following simulations of activation and inactivation voltage-clamp protocols.

**Figure 8.** Modeled action potential firing patterns in the afferent ending (A) and DRG somata (B). The afferent ending model was stimulated by a stepped stretch protocol (50mN for 15 sec duration), which evoked repetitive action potential generation at the siz (A). The simulation recapitulates the profound changes of intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations at the siz that correlate with changes in membrane potential. In contrast, the DRG soma model, when stimulated by a stepped inward current, did not
fire repetitively (B). When a \( \text{Na}_V1.6 \) conductance was inserted into the model, the soma model started to fire repetitively at stimulus intensities above rheobase (C).

**Figure 9.** Simulation of afferent ending response to ramped colorectal stretch. Colorectal deformation during slow ramped stretch (0 to 170mN @ 5mN/sec) was measured experimentally, quantified as a circumferential stretch ratio \( \lambda \), and is plotted in (A). The experimentally measured mean stretch was used to drive the afferent ending model for action potential generation (B and C); total spike numbers are summarized in (D). The effect of TTX was simulated by reducing the maximum conductance of both \( \text{Na}_V1.6 \) and \( \text{Na}_V1.7 \) and effects of subtype-selective \( \text{Na}_V \) blockers were simulated by reducing the corresponding \( \text{Na}_V \) conductance. Selective blockage of \( \text{Na}_V1.7 \) or \( \text{Na}_V1.8 \) conductances did not markedly reduce afferent responses to stretch, whereas blockage of \( \text{Na}_V1.6 \) either selectively or by TTX completely inhibited the response.

**Supplemental video.** A 3-D reconstruction of the colorectal afferent endings (green, YFP driven by SNS-Cre) co-stained with Nav1.6 antibody (red), indicating the clustering of Nav1.6-like immuonstaining along the nerve terminals (yellow).
Table 1. Summary of voltage-gated ion channels and pump

<table>
<thead>
<tr>
<th>Channel / pump</th>
<th>Model type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaV1.6</td>
<td>13-state Markov</td>
<td>(Raman and Bean 2001)</td>
</tr>
<tr>
<td>NaV1.7</td>
<td>6-state Markov</td>
<td>(Gurkiewicz et al. 2011)</td>
</tr>
<tr>
<td>NaV1.8</td>
<td>Hodgkin-Huxley</td>
<td>(Baker 2005)</td>
</tr>
<tr>
<td>NaV1.9</td>
<td>Hodgkin-Huxley</td>
<td>(Baker 2005)</td>
</tr>
<tr>
<td>KA</td>
<td>Hodgkin-Huxley</td>
<td>(Schild et al. 1994)</td>
</tr>
<tr>
<td>KD</td>
<td>Hodgkin-Huxley</td>
<td>(Schild et al. 1994)</td>
</tr>
<tr>
<td>KS</td>
<td>Hodgkin-Huxley</td>
<td>(Schild et al. 1994)</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>[Na]ᵢ and V_m-dependent</td>
<td>(Bondarenko et al. 2004)</td>
</tr>
</tbody>
</table>

Table 2. Temperature factor $Q^{10}$ for voltage-gated channels and Na⁺,K⁺-ATPase

<table>
<thead>
<tr>
<th>Channel / pump</th>
<th>Rate Parameters</th>
<th>$Q^{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaV1.6</td>
<td>Forward &amp; reverse</td>
<td>1.5</td>
</tr>
<tr>
<td>NaV1.7</td>
<td>Forward</td>
<td>2.3</td>
</tr>
<tr>
<td>NaV1.7</td>
<td>Reverse</td>
<td>1.5</td>
</tr>
<tr>
<td>NaV1.8 and 1.9</td>
<td>m</td>
<td>2.3</td>
</tr>
<tr>
<td>NaV1.8 and 1.9</td>
<td>h</td>
<td>1.5</td>
</tr>
<tr>
<td>KA</td>
<td>p, q</td>
<td>1.93</td>
</tr>
<tr>
<td>KD</td>
<td>x, y</td>
<td>1.93</td>
</tr>
<tr>
<td>KS</td>
<td>n</td>
<td>1.4</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>I_max</td>
<td>1.14</td>
</tr>
</tbody>
</table>
### Table 3. EC$_{50}$s of subtype-selective blockers of voltage-gated sodium channels

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ (nM)</th>
<th>Na$_V$1.6</th>
<th>Na$_V$1.7</th>
<th>Na$_V$1.8</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTX</td>
<td>1 to 6</td>
<td>4</td>
<td></td>
<td>60,000,000</td>
<td>(Catterall et al. 2005)</td>
</tr>
<tr>
<td>PTX</td>
<td>26</td>
<td>0.3</td>
<td></td>
<td>146</td>
<td>(Schmalhofer et al. 2008)</td>
</tr>
<tr>
<td>mCtxG</td>
<td>680</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td></td>
<td>(Wilson et al. 2011)</td>
</tr>
<tr>
<td>mCtxP</td>
<td>100</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td></td>
<td>(Wilson et al. 2011)</td>
</tr>
<tr>
<td>A803</td>
<td>6740</td>
<td>6740</td>
<td>140</td>
<td></td>
<td>(Jarvis et al. 2007)</td>
</tr>
</tbody>
</table>

Abbreviations: TTX, tetrodotoxin; A803, A-803467; PTX, ProTx-II; mCtxG, $\mu$-conotoxin GIIIa; mCtxP, $\mu$-conotoxin PIIIa.

### Table 4. Maximum ion channel conductance (pS/µm$^2$) or pump current (pA/µm$^2$)

<table>
<thead>
<tr>
<th>G$<em>{max}$ or I$</em>{max}$</th>
<th>Afferent ending</th>
<th>Soma</th>
<th>Soma with Na$_V$1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trsd</td>
<td>mid</td>
<td>siz</td>
</tr>
<tr>
<td>ms</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$_V$1.6</td>
<td>1400</td>
<td>2800</td>
<td></td>
</tr>
<tr>
<td>Na$_V$1.7</td>
<td>2000</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>Na$_V$1.8</td>
<td>5000</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>Na$_V$1.9</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>$K_A$</td>
<td>450</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>$K_D$</td>
<td>400</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>$K_S$</td>
<td>40</td>
<td>210</td>
<td>380</td>
</tr>
<tr>
<td>Na$^+$,K$^+$-ATPase</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

- 44 -


Choi JS, and Waxman SG. Physiological interactions between Na(v)1.7 and Na(v)1.8 sodium channels: a computer simulation study. *J Neurophysiol* 106: 3173-3184, 2011.


Khaliq ZM, Gouwens NW, and Raman IM. The contribution of resurgent sodium


