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

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The colorectum in the mouse is innervated by lumbar splanchnic and pelvic nerve (PN) pathways (Brierley et al. 2004; Feng and Gebhart 2011). Using an in vitro colorectal-nerve preparation and an unbiased electric search strategy, we categorized colorectal afferents into five mechanosensitive classes [serosal, mucosal, muscular, muscular-mucosal (M/M), mesenteric] and one mechanically-insensitive class (Feng et al. 2012a). Among these classes, only muscular and M/M 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, 2013).

Patch-clamp recordings reveal that colorectal dorsal root ganglion (c-DRG) somata have small diameters (<25–30 μm), tetrodotoxin (TTX)-resistant inward sodium currents, a significant inflection/hump in the repolarization phase of the action potential (AP) (Beyak et al. 2004), and are immuno-positive for one or more neurochemical markers, including transient receptor potential vanilloid 1 (up to 85%), calcitonin gene-related peptide (up to 80%), and isoleucin B4 (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 ≤1 m/s (Feng and Gebhart 2011).

Our laboratory’s previous single-fiber studies suggest that a significant proportion (~33%) of colorectal afferent endings in the PN innervation are stretch-sensitive and respond tonically to ramped circumferential colorectal stretch (0 to 170 mN in 34 s) (Feng and Gebhart 2011; Feng et al. 2012b, 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 Chen et al. 2010), and can be categorized as class-3 single-spiking neurons. This discrepancy in spiking patterns between colorectal 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.

The colorectum typically innervates by lumbar splanchnic and pelvic nerve (PN) pathways (Brierley et al. 2004; Feng and Gebhart 2011). Using an in vitro colorectal-nerve preparation and an unbiased electric search strategy, we categorized colorectal afferents into five mechanosensitive classes [serosal, mucosal, muscular, muscular-mucosal (M/M), mesenteric] and one mechanically-insensitive class (Feng et al. 2012a). Among these classes, only muscular and M/M 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, 2013).
METHODS

Unless specified, experiments were conducted in male C57BL/6Tac mice, 6–8 wk 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 yellow fluorescent protein (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 (1:1,000, Alomone, ASC-009, lot no. AN1750) or NaV1.7 (1:1,000, Alomone, ACS-008, lot no. AN1125) and co-stained with goat antibodies against YFP (1:1,000, Abcam, Cambridge, MA, AB5450, lot no. GR136040-1). The sections were further stained with Cy3-conjugated anti-rabbit IgG (1:200, Jackson Immunoresearch, 711-227-003, lot no. 95161) and Alexa Fluor 488-conjugated anti-goat IgG (1:200, Molecular Probes, Eugene, OR, A11055, lot no. 989791). Confocal microscopy was carried out with a Nikon A1R point scanning microscope with either a 0.75 numerical aperture 60 objective. Pinhole size was limited to 1 airy unit to maximize confocality of all images. Twelve-bit images were captured with limited laser and detector power to ensure fluorophore signals were not saturated. Z-stack images were captured in 1 μm (×20) or 0.5 μm (×60) increments. Three-dimensional 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 PNs, a retrograde tracer Fast Blue (1% in sterile saline, EMS-Chemie, 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, PN, and colon were individually homogenized with a Teflon tube and mortar for less than 10 strokes in ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors (Sigma-Aldrich). Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). 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 h at room temperature, then incubated with primary antibody at 4°C overnight (NaV1.6 or NaV1.7 both at 1:200, Alomone), and 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 (1:2,000 in 5% milk/TBST, Sigma Immunoresearch) for 1 h at room temperature. An ECL kit (Amersham Biosciences, Piscataway, NJ) was used for detection of immunoreactivity, and image of the blots was then taken with an LAS3000 imager (Fujifilm, Japan).

In Vitro Mouse Colon-PN Preparation

As detailed previously (Feng et al. 2013), mice were euthanized via CO₂ inhalation followed by exsanguination after the right atrium was perforated. 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 (4 μM) and indomethacin (3 μM) were added. The PN was teased into fine bundles (~10 μm thickness) for single-fiber recording.

As previously described (Feng and Gebehart 2011), mechanosensitive colorectal afferents were classified as serosal, muscular, mucosal or M/M based upon responses to probing with calibrated nylon monofilaments (0.4, 1, and 1.4 g force), mucosal stroking (10 mg 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 (~1-mm interval) were inserted along the anti-mesenteric edge of the colorectum to permit homogeneous, circumferential stretch by a slow ramped force (0 to 170 mN at 5 mN/s) corresponding to intraluminal pressures of 0–45 mmHg (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 Gebehart 2011; Feng et al. 2013): TTX, the Naₐ,1.8 antagonist A803467 (A803), the Naₐ,1.7 antagonist ProTx-II (PTX), and Naₐ,1.6 antagonists μ-conotoxin GIIIA (mCtxG) and μ-conotoxin GIIb (mCtxP). After establishing a baseline (control) SRF, the receptive ending was isolated (4 × 4 mm² × 10 mm high tubing) and the Krebs solution removed and replaced by 150 μl of TTX, A803, PTX, mCtxG or mCtxP for 5 min. The tubing was then removed, and a SRF acquired immediately afterwards (experiment). After reexposing the ending to Krebs solution for 15 min, a third SRF (wash) was acquired to conclude the protocol. Generally, each fiber was exposed to two to three 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 PN fibrils, afferents with receptive fields within 5 mm 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 3 mM and prepared in aliquots of 3.3 μl. A803 was dissolved in DMSO to 3 mM and prepared in nitrogen-filled aliquots of 3.3 μl (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 0.3 mM and prepared in aliquots of 5 μl; 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 0.5 mM and 0.1 mM, respectively, and prepared in aliquots of 42 μl. 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 charge-coupled device camera; images were extracted every 2 s during stretch, and circumference was measured using ImageJ (v.1.44p, National Institutes of Health).

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 multicompartmental model, as depicted in Fig. 1A. The nerve terminal model presented here is designed to emulate AP encoding, assuming a transducer terminal region contiguous with a single spike-initiation zone (siz) similar to those developed to simulate the axon initial segment (AIS) of neurons in the central nervous systems (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 (ms channels), a siz where AP 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 for axial diffusion of intracellular Na+ and K+ ions. All four compartments are cylinders 0.8 μm 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 μm 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 (Φ 23 μm) (Beyak et al. 2004).

Voltage-gated ion channels and pump. The model incorporates four different Na+ conductances representing NaV1.6, NaV1.7, NaV1.8, and NaV1.9 and three K+ conductances, simulating the fast inactivating A-type current (K_A), slowly inactivating A-type current (K_S), and sustained current (K_S). NaV1.6 and NaV1.7 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. NaV1.6-K+-ATPase was simulated as a voltage- and intracellular Na+ concentration ([Na+]_i)-dependent outward current with a 3:2 transport ratio between Na+ and K+ ions. [Na+]_i and intracellular K+ concentration ([K+]_i) 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 μm²/s (Fleidervish et al. 2010; Rugiero et al. 2010). Na+ and K+ reversal potentials were derived from the ion concentrations across the membrane.

NaV1.6, NaV1.6 is simulated by a Markov-type model (see Fig. 6A) adopted from Khaliq et al. (2003) (see also Raman and Bean 2001). NaV1.6 current is determined by the open probability <O> and membrane potential V: I_{Na6} = \tilde{g}_{Na6} <O> (V - E_{Na6}), where \tilde{g}_{Na6} is maximum NaV1.6 conductance, and E_{Na6} is sodium reversal potential. Rate coefficients (ms) that are membrane voltage-dependent (mV) are adopted from Khaliq et al. (2003), except that \xi was adjusted to 0.6 \exp(-V/25) to accommodate to the low firing rate of colorectal afferent neurons.

NaV1.7, NaV1.7 is simulated by a Markov-type model (see Fig. 6A) adopted from Gurkiewicz et al. (2011). NaV1.7 current is determined by the open probability <O> and membrane potential V: I_{Na7} = \tilde{g}_{Na7} <O> (V - E_{Na7}), where \tilde{g}_{Na7} is maximum NaV1.7 conductance. All model parameters were adopted from Gurkiewicz et al. (2011).

NaV1.8, NaV1.8 is simulated by a Hodgkin-Huxley-type model adopted from Baker (2005).

I_{Na6} = \tilde{g}_{Na6}n^4h(V - E_{Na6})
where $I_{Na9}$ is $Na_N$ current; $\bar{g}_{Na9}$ is maximum Na\textsubscript{v}1.9 conductance.

$K_M. K_M$ is simulated by a Hodgkin-Huxley-type model adopted from Schild et al. (1994) with minor adjustments of parameters.

$I_{KA} = \bar{g}_{KA} g (V - E_K)$

$\dot{p} = \frac{p_n - p}{\tau_p}$

$\dot{q} = \frac{q_n - q}{\tau_q}$

$p_n = 1.0 \left[ 1 + \exp \left( \frac{V + 15.79}{-10.0} \right) \right]$

$\tau_p = 5.0 \exp \left[ -0.0222 (V + 65)^2 \right] + 1.5$

$q_n = 1.0 \left[ 1 + \exp \left( \frac{V + 58}{7.0} \right) \right]$

$\tau_q = 45.0 \exp \left[ -0.0035 (V + 10)^2 \right] + 10.5$

where $I_{KA}$ is $K_M$ voltage-gated potassium current; $\bar{g}_{KA}$ is maximum $K_M$ conductance; $p$ and $q$ are Hodgkin-Huxley typed voltage-gating parameters for $K_M$ current; $\dot{p}$ and $\dot{q}$ are first derivative of $p$ and $q$, respectively; and $E_K$ is potassium reversal potential.

$K_D, K_D$ is a Hodgkin-Huxley-type model adopted from Schild et al. (1994) with minor adjustments of parameters.

$I_{KD} = \bar{g}_{KD} g (V - E_K)\dot{x} = \frac{x_n - x}{\tau_x}$

$y = \frac{y_n - y}{\tau_y}$

$x_n = 1.0 \left[ 1 + \exp \left( \frac{V + 14.59}{-15.0} \right) \right]$

$\tau_x = 5.0 \exp \left[ -0.0222 (V + 65)^2 \right] + 3.5$

$y_n = 1.0 \left[ 1 + \exp \left( \frac{V + 48}{7.0} \right) \right]$

$\tau_y = 1,800$

where $I_{KD}$ is $K_D$ voltage-gated potassium current; $\bar{g}_{KD}$ is maximum $K_D$ conductance; $x$ and $y$ are Hodgkin-Huxley typed voltage-gating parameters for $K_D$ current; and $\dot{x}$ and $\dot{y}$ are first derivative of $x$ and $y$, respectively.

$K_N, K_N$ is a Hodgkin-Huxley-type model adopted from Schild et al. (1994) with minor adjustment of parameters.

$I_{KS} = \bar{g}_{KS} g (V - E_K)$

$n = \frac{n_n - n}{\tau_n}$

$n_n = \frac{\alpha_n}{\alpha_n + \beta_n}$

$\tau_n = 1.0 \left[ \frac{V + 52}{12.5} \right] \left[ 1 - \exp \left( \frac{V + 52}{-12.5} \right) \right]$

$\alpha_n = 0.1 \left[ \frac{V + 52}{12.5} \right] \left[ 1 - \exp \left( \frac{V + 52}{-12.5} \right) \right]$

$\beta_n = 0.125 \exp \left( -\frac{V + 60}{80} \right)$

where $I_{KS}$ is $K_N$ voltage-gated potassium current; $\bar{g}_{KS}$ is maximum $K_N$ conductance; $n$ is Hodgkin-Huxley typed voltage-gating parameters for $K_N$ current; and $\dot{n}$ is first derivative of $n$.

$Na^+/K^+/ATPase, Na^+/K^+/ATPase$ is adapted from Bondarenko et al. (2004) with parameters slightly adjusted.

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$I_{NaK} = \tilde{I}_{NaK} \cdot f_{NaK}$

$$I_{NaK} = I_{Na,p} + I_{K,p}$$

$I_{Na,p} = 3I_{NaK}$

$I_{K,p} = -2I_{NaK}$

$$f_{NaK} = \frac{1}{1 + 0.1245 \cdot \exp \left(-\frac{V F}{RT}\right) + 0.0365 \cdot \sigma \cdot \exp \left(-\frac{V F}{RT}\right)}$$

where $I_{NaK}$ is total current from Na$^+$-K$^+$-ATPase activities; $\tilde{I}_{NaK}$ is maximum $I_{NaK}$; $f_{NaK}$, $K_{Na,n}$, and $K_{Na,o}$ are parameters for the Na$^+$-K$^+$-ATPase current; $\tilde{K}^+$ is extracellular K$^+$ concentration; $I_{Na,p}$ is sodium current from Na$^+$-K$^+$-ATPase activities; $I_{K,p}$ is potassium current from Na$^+$-K$^+$-ATPase activities; $V$ is membrane voltage; $F$ is Faraday constant; $R$ is universal gas constant; $T$ is absolute temperature; $[Na]_o$ is extracellular Na concentration; and $\sigma$ is a function of $[Na]_o$.

**BACKGROUND CURRENT.**

$$I_B = \tilde{I}_B (V - E_B)$$

$I_{Na,B} = I_{Na,B} + I_{K,B}$

$I_{Na,B} = \frac{E_{Na} - E_K}{E_{Na} - E_K} (V - E_{Na})$

$I_{K,B} = \frac{E_{Na} - E_K}{E_{Na} - E_K} (V - E_K)$

where $I_B$ is background current; $\tilde{I}_B$ is maximum conductance of background; $I_{Na,B}$ is background current contributed by sodium; $I_{K,B}$ is background current contributed by potassium; and $E_B$ is background current reversal potential.

The summary of the ion channels and Na$^+$-K$^+$-ATPase included in the model is listed in Table 1.

**Mechanosensitive ion channels.** To simulate the gating of a 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:

$$O \xrightarrow{\alpha} C$$

$$\alpha = \frac{1}{A} \exp \left(-\frac{\tau - \tau_0}{2S}\right)$$

where $S$ is a parameter determining $\alpha$ and $\beta$.

**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>Na$^+$,1.6</td>
<td>13-state Markov</td>
<td>Raman and Bean 2001</td>
</tr>
<tr>
<td>Na$^+$,1.7</td>
<td>6-state Markov</td>
<td>Gürkiewicz et al. 2011</td>
</tr>
<tr>
<td>Na$^+$,1.8</td>
<td>Hodgkin-Huxley</td>
<td>Baker 2005</td>
</tr>
<tr>
<td>Na$^+$,1.9</td>
<td>Hodgkin-Huxley</td>
<td>Schilt et al. 1994</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Hodgkin-Huxley</td>
<td>Schilt et al. 1994</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Hodgkin-Huxley</td>
<td>Schilt et al. 1994</td>
</tr>
<tr>
<td>Na$^+$-K$^+$-ATPase</td>
<td>[Na$^+$], and $V_m$ dependent</td>
<td>Bondarenko et al. 2004</td>
</tr>
</tbody>
</table>

$Na$, voltage-gated sodium channel; $K_p$, fast inactivating A-type current; $K_{iso}$, slowly inactivating A-type current; $K_{sc}$, sustained current; [Na$^+$], intracellular Na$^+$ concentration; $V_m$, membrane potential.

$$\beta = \frac{1}{A} \exp \left(\frac{\tau - \tau_0}{2S}\right)$$

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

$$\tilde{p} = p - \frac{p}{T_p}$$

in which,

$$p_{\alpha} = \frac{\alpha}{\alpha + \beta}$$

and

$$T_p = \frac{1}{\alpha + \beta}$$

where $\rho$ is first derivative of $p$, and $T_p$ is exponential decay time constant for $p$.

$p_{\alpha}$, 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).

$$p_{\alpha} = \frac{1}{1 + \exp \left(-\frac{\tau - \tau_0}{S}\right)}$$

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 two springs and one dashpot (Fig. 1C), which leads to the following equations:

$$\frac{\partial}{\partial t} + \frac{\Delta L}{\Delta w} k_1 x_1 + c_1 \dot{x}_1$$

$$\frac{\partial}{\partial t} + \frac{\Delta L}{\Delta w} k_2 x_2 + c_2 \dot{x}_2$$

where $x$ is Hodgkin-Huxley type voltage-gating parameter for $K_{iso}$ current; $\dot{x}$ is first derivative of $x$; $\Delta L$ is unit length of the simulated neural membrane patch; $\Delta w$ is unit width of the simulated neural membrane patch; $k_1$ and $k_2$ are two linear spring components in the lumped parametric model that transfers bulk colorectal deformation into neural membrane tension; and $c_1$ is one linear dashpot component in the lumped parametric model.

Assuming:

$$T_{\tau} = \frac{c_1}{k_1 + k_2}$$

$$m_1 = \frac{k_1 k_2 \Delta L}{(k_1 + k_2) \Delta w}$$

$$m_2 = \frac{k_2 c_1 \Delta L}{(k_1 + k_2) \Delta w}$$

Then:

$$\dot{\tau} = \frac{m_1 (\lambda - 1) + m_2 \dot{\lambda} - \tau}{T_{\tau}}$$

where $T_{\tau}$ is the exponential decay time constant for $\tau$, and $\dot{\tau}$ is first derivative of $\tau$.

The ms channel conductance ($g_M$) is divided into conductance for Na$^+$ ($g_{Na,m}$) and K$^+$ ions ($g_{K,m}$):

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

\[
\begin{align*}
I_{Na,M} &= g_{Na,K} \frac{E_{Na} - V}{E_{Na} - E_K} (V - E_{Na}) \\
I_{K,M} &= g_{K,M} \frac{V - E_K}{E_{Na} - E_K} (V - E_K) \\
I_M &= I_{Na,M} + I_{K,M}
\end{align*}
\]

where \(g_{Na,K}\) is maximum conductance of m current; \(I_{Na,M}\) is m current contributed by sodium; and \(I_{K,M}\) is m current contributed by potassium.

Parameters for the m channel in the study are as follows: parameter determining \(\alpha\) and \(\beta\) (\(A\)), 10 ms; parameter determining \(\alpha\) and \(\beta\) (\(T\)), 4.45 mM \(Na^+\); parameter determining \(\tau\) and \(\beta\) (\(S\)), 2.07 mM \(Na^+\); exponential decay time constant for \(\tau\) (\(T\)), 1.000 ms; parameter determining \(\tau\) (\(m\)), 13.6 mM \(Na^+\); parameter determining \(\tau\) (\(h\)), 16,900 (mM ms)/m.

The m 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, \(C_m\) (specific membrane capacitance of neural endings and somata), \(R_m\) (membrane resistivity of neural endings and somata), and \(R_i\) (axial resistivity of neural endings) in both models were set to 1 \(\mu\)F/cm\(^2\), 10,000 \(\Omega\)/cm\(^2\), and 123 \(\Omega\)/cm, respectively. The initial ion concentrations for the soma model were set to be consistent with current-clamp recording conditions (i.e., 140 mM \([Na^+]_o\), 4.5 mM \([Na^+]_i\), 5 mM \([K^+]_o\), and 130 mM \([K^+]_i\) so was the resting membrane potential \(V_m\) of −64.3 mV (Shinoda et al. 2010). The initial ion concentrations for the afferent ending model (145 mM \([Na^+]_o\), 4 mM \([Na^+]_i\), 6.3 mM \([K^+]_o\), and 155mM \([K^+]_i\)) were determined when the model reached equilibrium condition at \(V_m\) of −65 mV, a potential well within the range of \(V_m\) values recorded from distal axons of small-diameter DRG neurons (−68 to −53 mV) (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 \([i.e., Q_{10} = 2 - 24(10)]\). The \(Q_{10}\) values listed in Table 2 were adapted from Schild et al. (1994). The numerical error tolerance in NEURON was set at 10\(^{-5}\).

### Data Recording and Analysis

APs were recorded extracellularly using a low-noise AC differential amplifier. Activity was monitored on-line, filtered (0.3 to 10 kHz).

### Table 2. Temperature factor \(Q_{10}\) for voltage-gated channels and \(Na^+\)-\(K^+\)-ATPase

<table>
<thead>
<tr>
<th>Rate Parameters</th>
<th>(Q_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Na_{1,6}) forward and reverse</td>
<td>1.5</td>
</tr>
<tr>
<td>(Na_{1,7}) forward</td>
<td>2.3</td>
</tr>
<tr>
<td>(Na_{1,7}) reverse</td>
<td>1.5</td>
</tr>
<tr>
<td>(Na_{1,8 and 1.9})</td>
<td>2.3</td>
</tr>
<tr>
<td>(Na_{1,8 and 1.9})</td>
<td>1.5</td>
</tr>
<tr>
<td>(K_h)</td>
<td>1.93</td>
</tr>
<tr>
<td>(K_q)</td>
<td>1.93</td>
</tr>
<tr>
<td>(K_n)</td>
<td>1.4</td>
</tr>
<tr>
<td>(Na^+)-(K^+)-ATPase</td>
<td>1.14</td>
</tr>
</tbody>
</table>

\(I_{max}\), maximum current; \(m\) and \(h\), Hodgkin-Huxley typed voltage-gating parameters for sodium channels; \(p\) and \(q\), Hodgkin-Huxley typed voltage-gating parameters for \(K_h\) current; \(x\) and \(y\), Hodgkin-Huxley typed voltage-gating parameters for \(K_q\) current; \(n\), Hodgkin-Huxley typed voltage-gating parameters for \(K_n\) current.

### RESULTS

#### The Presence of \(Na_{1,6}\) in Colorectal Afferents

Portions of retrogradely labeled colorectal DRG neurons showed positive immunostaining for \(Na_{1,6}\) and \(Na_{1,7}\) (Fig. 2A). Colocalization of YFP (expressed in sensory neurons driven by SNS-Cre) with \(Na_{1,6}\) and \(Na_{1,7}\)-immunoreactivity in the colorectum suggests the presence of \(Na_{1,6}\) and \(Na_{1,7}\) at distal colorectal afferent endings (Fig. 2B). The staining pattern of \(Na_{1,6}\) is not homogenous along YFP-positive nerve fibers, but rather clusters at focal regions along the axons (see a three-dimensional reconstruction in Supplementary Video S1; the online version of this article contains supplemental data), presumably regions of spike initiation, which is consistent with the sporadic \(Na_{1,6}\) immunoreactivity in other sensory nerve terminals (Hossain et al. 2005). Both circular and longitudinal smooth muscle layers showed low-intensity staining of \(Na_{1,6}\), suggesting the presence of some \(Na_{1,6}\) protein in smooth muscle fibers. In some sections of colorectal tissue, we noted positive \(Na_{1,6}\) staining that was YFP-negative, likely contributed by \(Na_{1,6}\) in effenter endings or enteric neurons (data not shown). Western blots confirmed the presence of \(Na_{1,6}\) protein in both the PN and L6 DRG, along with \(Na_{1,7}\) (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 (gray square surrounding “1” on the colorectal mucosal surface), and afferent responses to ramped stretch (0 to 170 mN @ 5 mmHg/s) were assessed before and after TTX application and again after washout. TTX at concentrations of 1 \(\mu\)M and 3 \(\mu\)M 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, 10 \(\mu\)M 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 1 \(\mu\)M TTX application. Application of bile salts significantly increased the response of
afferents to stretch (Fig. 3E, F_{3,46} = 14.9, P < 0.001; post hoc comparison vs. control, P = 0.023), and subsequent application of 1 μM TTX inhibited the response (post hoc comparison vs. control, P = 0.028). The corresponding response thresholds to stretch were unaffected by 1 μM 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 10 μM TTX (Fig. 3D, inset, F_{2,24} = 24.9, P < 0.001) and 1 μM TTX following bile salts (Fig. 3E, inset, F_{3,18} = 10.0, 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. washout, 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, 10 μM; F_{4,47} = 15.6, P < 0.001, post hoc comparison, 10 μM 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 (1 μM) inhibited the response (post hoc comparison, 1 μM 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} values (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, 1 μM 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 (1 μM) did not affect either afferent responses (Fig. 4B, $F_{2,32} = 0.07, P = 0.93$) or response threshold (inset, $F_{2,16} = 0.08, P = 0.93$). However, PTX at 3 μM, a concentration greater than the EC₅₀ for blocking NaV1.6, effectively attenuated afferent responses (Fig. 4C, $F_{2,20} = 5.9, P = 0.02$, post hoc comparison vs. control, $P = 0.04$) and increased response threshold (inset, $F_{2,10} = 9.6, P = 0.005$, post hoc comparison vs. control, $P = 0.009$). Although blockage of NaV1.7 by PTX is almost irreversible (Johnson et al. 2007), the response to stretch recovered to control completely after only 15 min wash-out (post hoc

*$P < 0.05.$
reduced afferent responses to stretch (Fig. 4, inset).

Fig. 4. Localized serosal application of TTX and selective Na_v.1.6 and Na_v.1.7 blockers on stretch-sensitive colorectal afferent endings. A: when applied from the serosal side, 1 μM TTX significantly attenuated most, if not all, afferent responses to stretch and increased response threshold (inset). B: in contrast, the Na_v.1.7 blocker ProTX II (PTX; 1 μM) did not affect afferent responses to stretch or response threshold (inset). C: PTX at 3 μM, a concentration in excess of its EC_{50} for Na_v.1.6, significantly attenuated the response to stretch and increased response threshold (inset). D: in contrast, the selective Na_v.1.6 blocker µ-conotoxin GIIa (mCtxG; 17 μM) significantly reduced the response to stretch and increased response threshold (inset). E: normalized responses to stretch (total spike number) were significantly reduced by TTX, mCtxG and 3 μM PTX, but not by 1 μM PTX. *P < 0.05.

Table 3. EC_{50} values of subtype-selective blockers of voltage-gated sodium channels

<table>
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<tr>
<th></th>
<th>EC_{50}</th>
<th>Na_v.1.6</th>
<th>Na_v.1.7</th>
<th>Na_v.1.8</th>
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<tr>
<td>PTX</td>
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<tr>
<td>mCtxG</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>Wilson et al. 2011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mCtxP</td>
<td>100</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>Wilson et al. 2011</td>
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</tr>
<tr>
<td>A803</td>
<td>6,740</td>
<td>6,740</td>
<td>140</td>
<td>Jarvis et al. 2007</td>
<td></td>
</tr>
</tbody>
</table>

Values are in nM. TTX, tetrodotoxin; PTX, ProTx-II; mCtxG, µ-conotoxin GIIa; mCtxP, µ-conotoxin PIIia; A803, A803467.

comparison vs. control, P = 0.95), a time at which Na_v.1.7 should remain in a blocked state. This strongly suggests that the attenuation of afferent responses to stretch by 3 μM PTX arises through blocking Na_v.1.6, not Na_v.1.7. In addition, the selective Na_v.1.6 antagonist mCtxG (17 μM) significantly reduced afferent responses to stretch (post hoc comparison, 3 μM PTX vs. control, P < 0.001), likely from its nonspecific blocking effect on Na_v.1.6. In addition, the selective Na_v.1.6 blocker mCtxG (17 μM) significantly reduced the afferent responses to stretch (post hoc comparison, mCtxG vs. control, P < 0.001), suggesting an essential role of Na_v.1.6 in colorectal afferent encoding to stretch.

To verify the finding, the aforementioned selective Na_v.1.6 and Na_v.1.7 antagonists were applied to the mucosal surface of the colorectum (3 μM PTX and 50 μM mCtxG), as was another Na_v.1.6 antagonist from the µ-conotoxin family, mCtxP (10 μM). When applied from the mucosal side, 3 μM 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 (50 μM) 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.
significant differences between control, A803 and wash (Fig. 4). 

When applied from the serosal side, the selective NaV1.8 antagonists were applied from the serosal side: both NaV1.6 

5. Localized mucosal application of selective NaV1.6 and NaV1.7 blockers on stretch-sensitive colorectal afferent endings. A and B: when applied from the mucosal side, the NaV1.7 blocker PTX (3 µM) did not affect afferent responses to stretch (A) or response threshold (A, inset), whereas the selective NaV1.6 blocker mCtxG (50 µM) significantly reduced responses to stretch (B) and increased response threshold (B, inset). C: another selective NaV1.6 blocker, μ-conotoxin PIIIa (mCtxP; 10 µM), similarly reduced responses to stretch and increased response threshold (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 NaV1.6 with mCtxG was significantly lower than after blocking NaV1.7 by PTX. *P < 0.05.

Effect of NaV1.8 Antagonist A803 on Stretch-Sensitive Colorectal Afferents

When applied from the serosal side, the selective NaV1.8 antagonist A803 did not reduce afferent responses to stretch at either 1 µM (Fig. 6A, F_{2,20} = 1.37, P = 0.3) or 10 µM concentrations (Fig. 6C, F_{2,24} = 2.97, P = 0.09). Similarly, 3 µM 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 (10 µM) 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 Fig. 6, E and F, respectively; there were no significant differences between control, A803 and wash (Fig. 6E, F_{3,35} = 2.67, P = 0.06; Fig. 6F, F_{3,35} = 1.85, P = 0.16). Interestingly, 1 µM A803 applied from the serosal side, a concentration well below the EC_{50} values 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 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 (~5 nA) recorded from c-DRG neurons (Beyak et al. 2004). The density of NaV1.7 and NaV1.8 channels at the site 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 NaV1.6, 1.7, 1.8 and 1.9 currents calculated from voltage clamp simulations (~80 to 70 mV). Different from other channels in the model that use Hodgkin-Huxley equations, NaV1.6 and NaV1.7 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 NaV1.7, the activation curve of NaV1.6 is shifted in a hyperpolarized direction, and the inactivation curve in a depolarized direction, suggesting greater open probability during AP generation.

NaV1.6 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 50 mN induced tonic AP generation at the site, which closely correlated with the transient increase in [Na^+]_i and transient decrease in [K^+]_i. 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 (500 ms) 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 (×1.1) as shown in Fig. 8C. In addition, after adding the Na\textsubscript{v}1.6 conductance to the soma model, the rheobase decreased from 134.5 pA in Fig. 8B to 22.9 pA in Fig. 8C. In contrast, adding the same amount of Na\textsubscript{v}1.7 or Na\textsubscript{v}1.8 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 116.3 pA and 129.5 pA, respectively.

### Simulation of Afferent Responses to Ramped Stretch after Na\textsubscript{v} Channel Blockade

During the slow ramp protocol (0 to 170 mN @ 5 mN/s), circumferential stretch deformed the colorectum homoge-

![A - F](https://www.jn.org/download/figshare/figshare.10.220.33.5/figshare.10.220.33.5/jn_2017-06-28-17628)

Table 4. Maximum ion channel conductance or pump current

<table>
<thead>
<tr>
<th>G\textsubscript{max} or I\textsubscript{max}</th>
<th>Afferent Ending</th>
<th>Soma</th>
<th>Soma with Na\textsubscript{v}1.6</th>
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</thead>
<tbody>
<tr>
<td>ms, pA/μm\textsuperscript{2}</td>
<td>trsd</td>
<td>mid</td>
<td>siz</td>
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<tr>
<td>Na\textsubscript{v}1.6, pS/μm\textsuperscript{2}</td>
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<td>80</td>
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</tr>
</tbody>
</table>

G\textsubscript{max}, maximum conductance; ms, mechanosensitive channel; trsd, transducer zone; mid, middle section; siz, spike-initiation zone; pas, passive compartment.
neously, which was recorded for post hoc measurement of the circumferential \( \lambda \) as displayed in Fig. 9A. To mimic the ramped stretch protocol in single-fiber recordings (Figs. 3–6), the ramped force (0 to 170 mN in 34 s) and \( \lambda \) were used to drive AP generation at the siz 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 s) in the experimental protocol, was shortened in duration (6 s) in the simulation. Pharmacological blockage of channels by TTX was mimicked by gradual reduction of maximum conductances of both Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 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 Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 conductances, simulating the effect of TTX (Fig. 9B). The pharmacological blockage of subtype-selective Na\textsubscript{V} channels was simulated by reducing the

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

**Fig. 8.** Modeled action potential firing patterns in the afferent ending (A) and DRG somata (B). A: the afferent ending model was stimulated by a stepped stretch protocol (50 mN for 15-s duration), which evoked repetitive action potential generation at the siz. The simulation recapitulates the profound changes of intracellular Na\textsuperscript{+} ([Na\textsuperscript{+}]) and K\textsuperscript{+} concentrations ([K\textsuperscript{+}]) at the siz that correlate with changes in membrane potential (V). B: in contrast, the DRG soma model, when stimulated by a stepped inward current, did not fire repetitively. C: when a Na\textsubscript{V}1.6 conductance was inserted into the model, the soma model started to fire repetitively at stimulus intensities above rheobase.

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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., NaV1.6 is necessary for the encoding of colorectal afferent endings to stretch, whereas blockage of NaV1.7 does not remarkably alter the firing pattern). Consistent with the experimental findings using 1 μM A803 (Fig. 6E), blocking NaV1.8 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 AP generation (Blair and Bean 2002) because TTX typically did not inhibit AP 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. (1994) reported that intraluminal perfusion of 40 nM 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 (10 μM) reversibly inhibited the response of colorectal afferents to stretch, whereas lower concentrations of TTX (1 μM, 3 μM) did not, suggesting that the 40 nM 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 PN. This is unlikely based upon

1) the large volume of bath solution (>200 ml) relative to the volume of the TTX solution (150 μl); 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, 300 μM cyclic GMP was required to attenuate colorectal afferent responses to stretch (Feng et al. 2013). However, after pretreating 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 1 μM, 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., 1 μM TTX effectively blocked afferent responses to stretch).

At the outset, we confirmed the presence of both Na\(_\text{v}1.6\) and Na\(_\text{v}1.7\) in colorectal afferent endings and examined the roles of Na\(_\text{v}1.6\), Na\(_\text{v}1.7\) and Na\(_\text{v}1.8\) using subtype-selective antagonists. We excluded study of Na\(_\text{v}1.1\) (no clear role in nociceptors), Na\(_\text{v}1.3\) (absent in adult DRG neurons), and Na\(_\text{v}1.9\) (lack of selective antagonists). We chose PTX as the Na\(_\text{v}1.7\) antagonist due to its low EC\(_50\) (0.3 nM) compared with its EC\(_50\)s for Na\(_\text{v}1.6\) (26 nM) and Na\(_\text{v}1.8\) (146 nM) (Schmalhofer et al. 2008). The two μ-conotoxin Na\(_\text{v}1.6\) antagonists, mCtxG and mCtxP, have EC\(_50\) values for Na\(_\text{v}1.6\) (0.68 mM for mCtxG and 0.1 nM for mCtxP) orders of magnitude less than for Na\(_\text{v}1.7\) or Na\(_\text{v}1.8\) (>100 nM) (Wilson et al. 2011).

Na\(_\text{v}1.7\), which has been documented as important in pain sensation, was unexpectedly found not necessary for encoding tonic spiking of PN afferent fibers to noxious colorectal stretch. Serosal application of 1 μM PTX did not affect responses to stretch, whereas the same concentration of TTX effectively blocked responses, even though TTX has a much higher EC\(_50\) to block Na\(_\text{v}1.7\) than PTX (Table 3). A greater concentration of PTX (3 μM) slightly, but significantly, attenuated responses to stretch, likely due to its nanomolar EC\(_50\) for Na\(_\text{v}1.6\). A prior study showed that PTX blocks both Na\(_\text{v}1.6\) and Na\(_\text{v}1.7\) at nanomolar concentrations, but the blockage of Na\(_\text{v}1.6\) by PTX has a significantly higher off-rate than blockage of Na\(_\text{v}1.7\), which accounts for the reversible blockage of Na\(_\text{v}1.6\) by PTX and almost irreversible blockage of Na\(_\text{v}1.7\) (Johnson et al. 2007). The fact that the effect of 3 μM PTX on the response to stretch was reversible (response recovered completely after only 15 min wash-out, when Na\(_\text{v}1.7\) should have remained blocked) strongly suggests the involvement of Na\(_\text{v}1.6\) and not Na\(_\text{v}1.7\) in tonic spiking. Further, mCtxG at a concentration that selectively blocks Na\(_\text{v}1.6\) significantly reduced afferent responses to stretch, indicating a necessary role of Na\(_\text{v}1.6\) in encoding tonic spiking by colorectal afferent endings. An alternative interpretation of the blocking effect by Na\(_\text{v}1.6\) antagonist is the possible blockage of AP 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 Na\(_\text{v}1.7\) antagonist PTX did not seem to block the afferent response to stretch, whereas a prior study clearly indicated that Na\(_\text{v}1.7\), not Na\(_\text{v}1.6\), is critical to AP 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 Na\(_\text{v}1.6\) blocker, mCtxP; results confirmed that Na\(_\text{v}1.6\), not Na\(_\text{v}1.7\), is necessary for tonic spiking by colorectal afferent endings.

The important role of Na\(_\text{v}1.6\) in tonic spiking is also supported by recordings from DRG somata. For example, small interfering RNA knockdown of Na\(_\text{v}1.6\) effectively reduced the proportion of DRG somata that fire repetitively (Xie et al. 2013). Na\(_\text{v}1.6\) currents are restricted to medium-to-large-diameter DRGs (Cummins et al. 2005), whereas small-diameter DRG somata lack Na\(_\text{v}1.6\) 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 Na\(_\text{v}1.6\) in tonic spiking at both afferent endings and somata. Interestingly, both Xie et al. (2013) and we noted positive Na\(_\text{v}1.6\) 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 Na\(_\text{v}1.6\) conductance (1/50th the conductance in siz). Thus the lack of tonic firing in c-DRG somata is likely due to the absence of a Na\(_\text{v}1.6\) conductance, not to the relative low density of other types of sodium channels (e.g., Na\(_\text{v}1.7\) and Na\(_\text{v}1.8\)) in somata. Different channel compositions at different regions of a same neuron have been documented in AIS in the central nervous system; Na\(_\text{v}1.6\) channels are clustered at the distal AIS and absent in the soma membrane, whereas Na\(_\text{v}1.2\) is clustered at the proximal AIS (Baranaukauskas 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 Na\(_\text{v}1.8\) antagonist A803 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 A803; responses to stretch in some afferents were attenuated, whereas in others enhanced. In particular, serosal application of 1 μM A803, a concentration that selectively blocks Na\(_\text{v}1.8\), tended to increase afferent responses to stretch (P = 0.06). Interestingly, model simulation also showed a slight increase in firing after blocking Na\(_\text{v}1.8\). In fact, the role of Na\(_\text{v}1.8\) in nociception and pain remains unclear (Knapp et al. 2012). Given the variability noted, we focused on TTX-sensitive channels Na\(_\text{v}1.6\) and Na\(_\text{v}1.7\), leaving Na\(_\text{v}1.8\) for subsequent study. Similarly, we included in our simulation model the gating formulas for an Na\(_\text{v}1.9\) conductance, which appears to play a significant role during repeated noxious colon distension (Hockley et al. 2014) and is beyond the focus of the current study.

In previous sensory neuron models, Na\(_\text{v}1.6\) and Na\(_\text{v}1.7\) 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. 2014), which cannot simulate the unique gating features of Na\(_\text{v}1.7\) [i.e., complete inactivation and slow recovery from inactivation (Gurkiewicz et al. 2011)]. The computational models employed here incorporated individual Markov models for Na\(_\text{v}1.6\) (Khaliq et al. 2003) and Na\(_\text{v}1.7\) (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\(_\text{v}1.6\) and Na\(_\text{v}1.7\) in tonic firing are likely caused by those aforementioned differences in channel gating properties.
Our modeling also incorporated a novel ms channel that drives AP 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 Na-K pump. The maximal conductances of sodium channels at the soma 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 ms channels. We did not systematically adjust model parameters or conduct extensive sensitivity studies here, but instead focused on uncovering the differential roles of Na\textsubscript{V}1.6, Na\textsubscript{V}1.7 and Na\textsubscript{V}1.8 conductances in afferent encoding of ramped stretch, whose gating formulas 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{V}1.6 current in neural encoding of stretch-sensitive colorectal afferents. Immunohistochemistry and Western blotting revealed the presence of Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 at colorectal neuronal endings. Both TTX and selective Na\textsubscript{V}1.6 antagonists significantly attenuated afferent responses to stretch, whereas a selective Na\textsubscript{V}1.7 antagonist only slightly reduced the response. Computational Markov type modeling recapitulated the pharmacological findings. A selective Na\textsubscript{V}1.8 antagonist did not significantly attenuate the responses to stretch, and the exact role of Na\textsubscript{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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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