Increased Resurgent Sodium Currents in Nav1.8 Contribute to Nociceptive Sensory Neuron Hyperexcitability Associated with Peripheral Neuropathies

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Neuropathic pain is a significant public health challenge, yet the underlying mechanisms remain poorly understood. Painful small fiber neuropathy (SFN) may be caused by gain-of-function mutations in Nav1.8, a sodium channel subtype predominantly expressed in peripheral nociceptive neurons. However, it is not clear how Nav1.8 disease mutations induce sensory neuron hyperexcitability. Here we studied two mutations in Nav1.8 associated with hypersensitive sensory neurons: G1662S reported in painful SFN; and T790A, which underlies increased pain behaviors in the *Possum* transgenic mouse strain. We show that, in male DRG neurons, these mutations, which impair inactivation, significantly increase TTX-resistant resurgent sodium currents mediated by Nav1.8. The G1662S mutation doubled resurgent currents, and the T790A mutation increased them fourfold. These unusual currents are typically evoked during the repolarization phase of action potentials. We show that the T790A mutation greatly enhances DRG neuron excitability by reducing current threshold and increasing firing frequency. Interestingly, the mutation endows DRG neurons with multiple early afterdepolarizations and leads to substantial prolongation of action potential duration. In DRG neurons, siRNA knockdown of sodium channel β_4 subunits fails to significantly alter T790A current density but reduces TTX-resistant resurgent currents by 56%. Furthermore, DRG neurons expressing T790A channels exhibited reduced excitability with fewer early afterdepolarizations and narrower action potentials after β_4 knockdown. Together, our data demonstrate that open-channel block of TTX-resistant currents, enhanced by gain-of-function mutations in Nav1.8, can make major contributions to the hyperexcitability of nociceptive neurons, likely leading to altered sensory phenotypes including neuropathic pain in SFN.

Key words: beta subunit; hyperexcitability; pain; resurgent; sodium channel

Significance Statement

This work demonstrates that two disease mutations in the voltage-gated sodium channel Nav1.8 that induce nociceptor hyperexcitability increase resurgent currents. Nav1.8 is crucial for pain sensations. Because resurgent currents are evoked during action potential repolarization, they can be crucial regulators of action potential activity. Our data indicate that increased Nav1.8 resurgent currents in DRG neurons greatly prolong action potential duration and enhance repetitive firing. We propose that Nav1.8 open-channel block is a major factor in Nav1.8-associated pain mechanisms and that targeting the molecular mechanism underlying these unique resurgent currents represents a novel therapeutic target for the treatment of aberrant pain sensations.

Introduction

Hyperactivity of small-diameter sensory neurons is frequently associated with neuropathic pain. Indeed, painful small fiber neuropathy (SFN) may be caused by abnormal hyperexcitability

of nociceptive sensory neurons. However, the underlying mechanisms remain poorly understood, contributing to an unmet medical need. Recent studies on families with SFN indicate that, in ~35% of patients, hereditary mutations in two genes *SCN9A*

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(Nav1.7) and *SCN10A* (Nav1.8) are involved (Faber et al., 2012a; Hoeijmakers et al., 2012; Themistocleous et al., 2014).

Nav1.7 and Nav1.8 are preferentially expressed in nociceptive sensory neurons and are crucial contributors to neuropathic pain (Akopian et al., 1999; Cummins et al., 2007; Dib-Hajj et al., 2007). Although they share >75% sequence similarity, they exhibit several unique biophysical properties. For example, Nav1.7 undergoes slower recovery from inactivation and slower closed-state inactivation (Cummins et al., 1998). Nav1.8 exhibits depolarized voltage dependencies and slower open-channel kinetics (Akopian et al., 1996). These unique biophysical properties help determine the distinct roles they play in action potential generation. Whereas Nav1.7 is essential for setting voltage threshold, Nav1.8 is a major contributor to the rising phase (Cummins et al., 2007). Mutations identified in patients with painful SFN typically endow sodium channels with a hyperpolarized voltage dependence of activation and/or impaired inactivation kinetics, therefore facilitating the generation and repetitive firing of action potentials (Cummins et al., 2004; Fertleman et al., 2006; Faber et al., 2012b; Huang et al., 2013).

Resurgent currents mediated by sodium channels represent another important factor that influences neuronal excitability. In contrast to classic sodium currents elicited by depolarization, resurgent currents are unusual currents typically evoked during the repolarization phase of action potentials (Raman and Bean, 1997) by an open-channel blocker (Bant and Raman, 2010). Resurgent currents have been observed in multiple neuronal populations (Afshari et al., 2004; Cummins et al., 2005; Enomoto et al., 2006; Kim et al., 2010), and can promote generation of high-frequency action potential firing (Raman and Bean, 1997; Xie et al., 2016). Nav1.6 is the major carrier of resurgent current in DRG neurons, but other TTX-sensitive (TTX-S) sodium channel subtypes (e.g., Nav1.7) also exhibit an intrinsic ability to generate resurgent currents (Grieco and Raman, 2004; Cummins et al., 2005; Jarecki et al., 2010; Patel et al., 2015). Intriguingly, this ability is augmented under conditions that slow the rate of fast inactivation (Grieco and Raman, 2004; Jarecki et al., 2010). Our recent studies demonstrate that small-sized DRG neurons produce TTX-resistant (TTX-R) resurgent currents, which might be mediated by Nav1.8-like channels (Tan et al., 2014). Compared with Nav1.7-mediated resurgent currents, the TTX-R resurgent currents display much slower kinetics and are produced at more positive potentials. Computer simulations indicate that increased Nav1.7-mediated resurgent currents are important for inducing high-frequency action potential firing in nociceptive neurons in paroxysmal extreme pain disorder. By contrast, the influences of abnormal Nav1.8-mediated resurgent currents largely remain unknown. Given the distinct roles that specific sodium channel isoforms play in DRG action potential generation, it is of special interest to uncover whether abnormal Nav1.8-mediated resurgent currents might influence nociceptive neuron excitability in painful SFN and other painful conditions.

T790A and G1662S are Nav1.8 mutations identified in the *Possum* transgenic mouse strain and humans with painful SFN, respectively (Blasius et al., 2011; Han et al., 2014). Previous studies have shown that both of these mutations impair fast inactivation. We hypothesized that this impaired fast inactivation would lead to enhanced resurgent currents that increase action potential

firing in sensory neurons. In this study, we found that these mutations did indeed significantly increase Nav1.8-mediated resurgent currents when expressed in rat small-diameter DRG neurons. We show that increased Nav1.8-mediated resurgent currents substantially broaden action potentials, induce multiple early afterdepolarizations (EADs), and increase firing frequency, therefore greatly enhancing excitability of nociceptive neurons. We propose that increased resurgent currents induced by gain-of-function mutations in Nav1.8 contribute to enhanced hyperexcitability of nociceptive neurons that underlies altered sensory phenotypes, including neuropathic pain in SFN.

Materials and Methods

Sodium channel constructs and mutagenesis. The cDNA constructs encoding the mouse Nav1.8 (mNav1.8) and human Nav1.8 (hNav1.8) were synthesized as codon-optimized cDNA by GenScript and subcloned into a pcDNA3.1(+) expression vector. Two mNav1.8 mutations, T790A and G1663S, the second equal to G1662S in hNav1.8, as well as the G1662S mutation in hNav1.8 were constructed using the QuikChange XL (Stratagene) mutagenesis kit following the manufacturer's instructions (Stratagene). Mutations were confirmed by sequencing.

Cell culture and transfection. Rat DRG neurons were acutely dissociated and cultured according to the procedure described previously (Cummins et al., 2000). Briefly, young adult male Sprague Dawley rats, in adherence with animal procedures approved by the Indiana University School of Medicine and the School of Science Institutional Animal Care and Use Committees, were killed by carbon dioxide overexposure followed by decapitation. All DRGs or just lumbar DRGs (L4–L5) were removed quickly from the spinal cord and then incubated in DMEM containing collagenase (1 mg/ml) and protease (1 mg/ml). After the ganglia were triturated in DMEM supplemented with 10% FBS, cells were plated on glass coverslips coated with poly-D-lysine and laminin. Cultures were maintained at 37°C in a 5% CO₂ incubator. The Helios Gene Gun (Bio-Rad) was used to transiently cotransfect rat DRG neurons, as described previously (Herzog et al., 2003; Dib-Hajj et al., 2009; Jarecki et al., 2010). Cells were cotransfected with a plasmid encoding the recombinant VGSC and an internal ribosome entry site-EGFP (IRES-EGFP) vector plasmid that also encoded a Nav1.8 shRNA targeting the rat Nav1.8 (Mikami and Yang, 2005) but not the codon optimized mNav1.8 or hNav1.8 sequences. Under control conditions, the endogenous Nav1.8-type currents have an average current density of 947 ± 72 pA/pF ($n = 70$) and the Nav1.8 shRNA reduces endogenous Nav1.8-type current amplitudes in DRG neurons by 98% (Jarecki et al., 2010).

Electrophysiological recordings. DRG recordings were obtained from cells 2 d after transfection. The Nav1.8 current density was not significantly different from endogenous Nav1.8-type current density. Transfected cells were selected for recordings based on their expression of EGFP. Whole-cell patch-clamp recordings were performed at room temperature (~21°C) and 34°C using an EPC-10 amplifier and the Pulse program (HEKA Electronics). The temperature was increased to 34°C using a bipolar temperature controller (CL-100, Harvard Apparatus), a quick exchange heated/cooled platform for 35 mm Petri dishes (QE-IHC, Warner Instruments) equipped with a thermal cooling module (TCM-1, Warner Instruments). Final temperatures were confirmed using a digital thermometer (Thermo Fisher Scientific).

For voltage-clamp recordings, fire-polished electrodes (1.0–2.0 M Ω) were fabricated from 1.7 mm capillary glass using a P-97 or P-1000 puller (Sutter Instruments), and the tips were coated with sticky wax (KerrLab) to minimize capacitive artifacts and enhance series resistance compensation. The pipette solution contained the following (in mM): 140 CsF, 1.1 EGTA, 10 NaCl, and 10 HEPES, pH 7.3. The bathing solution was as follows (in mM): 130 NaCl, 30 TEA chloride, 1 MgCl₂, 3 KCl, 1 CaCl₂, 0.05 CdCl₂, 10 HEPES, and 10 D-glucose, pH 7.3 (adjusted with NaOH). TTX (500 nM) was added to the bath solution to block endogenous TTX-S currents in DRG neurons. The liquid junction potential for these solutions was <8 mV; data were not corrected to account for this offset. The pipette potential was zeroed before contacting the cell. After estab-

lishing the whole-cell recording configuration, the resting potential was held at -80 mV for 5 min to allow adequate equilibration between the micropipette solution and the cell interior. Linear leak subtraction, based on resistance estimates from 4–5 hyperpolarizing pulses applied before a depolarizing test potential, was used for all voltage-clamp recordings. Membrane currents were usually filtered at 5 kHz and sampled at 20 kHz. Voltage errors were minimized using 80%–93% series resistance compensation, and the capacitance artifact was canceled using the computer-controlled circuitry of the patch-clamp amplifier. To avoid contamination from window currents, resurgent current was obtained by subtracting persistent current from the current measured after 30 ms into the repolarizing pulse. Peak resurgent current at each test potential was normalized to the transient current with maximal amplitude (obtained from the I – V protocol). Normalized resurgent current amplitude was plotted as a function of voltage.

For current-clamp recordings, fire-polished electrodes (4.0 – 5.0 M Ω) were fabricated from 1.2 mm capillary glass using a P-97 or P-1000 puller (Sutter Instruments). The pipette solution contained the following (in mM): 140 KCl, 5 MgCl₂, 5 EGTA, 2.5 CaCl₂, 4 ATP, 0.3 GTP, and 10 HEPES, pH 7.3 (adjusted with KOH). The bathing solution contained the following (in mM): 140 NaCl, 1 MgCl₂, 5 KCl, 2 CaCl₂, 10 HEPES, and 10 glucose, pH 7.3 (adjusted with NaOH). Neurons were allowed to stabilize for 3 min in the current-clamp mode before initiating current injections to measure action potential activity.

Procedure for in vivo injection of siRNA into the DRG. Male Sprague Dawley rats under isoflurane anesthesia were used for these procedures. siRNAs were prepared using cationic linear polyethylenimine-based transfection reagents (*in vivo* jetPEI, Polyplus Transfection, distributed by VWR Scientific). The siRNA reagents were “smartpools” consisting of four different siRNA constructs combined into 1 and were purchased from Dharmacon. The four siRNA sequences directed against rat Nav β 4 (catalog #M-101002-01) were as follows: Construct 1, GGAUCGUGAAGAAUGAUA; Construct 2, UCCAAGUGGUUGAUAAAUU; Construct 3, GCAAUACUCAGGCGAGAUG; and Construct 4, AAACAACUCUGCUACGAUC. The nontargeting control was directed against firefly luciferase gene (catalog #D-001210-02). This luciferase sequence (UAAGGCUAUGAAGAGAUAC) is unrelated to the shRNA sequence recently reported to have extensive off-target effects in hippocampal neurons (Hasegawa et al., 2017). Aliquots of 3 μ l of containing siRNA/jetPEI mixture (80 pmol of siRNA) were injected into each L4 and L5 DRG on one side, through a small glass needle inserted close to the DRG as previously described (Xie et al., 2013; Barbosa et al., 2015). Three days after injection, ipsilateral L4 and L5 DRGs were harvested. Dissociated DRG neurons were examined by immunocytochemistry to verify knockdown, and another fraction was used for transfection and whole-cell patch clamp. A previous study using the same methodology showed that two separate individual Nav β 4 siRNA constructs from the smartpool reduced immunostaining and had similar behavioral effects as the smartpool (Xie et al., 2016), providing evidence against sequence-dependent off-target effects of the siRNA construct used (Jackson and Linsley, 2010). Indiana University School of Medicine Institutional Animal Care and Use Committee approved the animal protocols described.

Immunocytochemistry. To verify knockdown of Nav β 4 protein, L4 and L5 ipsilateral DRGs harvested and cultured from rats injected with nontargeting control and β 4-siRNA were examined 5 d after dissociation in parallel with whole-cell patch-clamp experiments. DRG neurons were fixed with 4% PFA (0.1 M phosphate buffer, pH 7.4) for 20 min and washed in PBS. Cells were then permeabilized in 1% Triton X-100 in PBS for 20 min at room temperature ($\sim 21^\circ\text{C}$), washed in PBS, blocked for 2 h (10% normal goat serum, 0.1% Triton X-100 in PBS) at room temperature, and washed with PBS. Cells were then incubated with polyclonal anti-Nav β 4 antibody (1:500, #Ab80539, Abcam) diluted in blocking solution overnight at 4°C . After additional PBS washes, cells were incubated with secondary antibody AlexaFluor-488 Goat Anti-Rabbit IgG (Molecular Probes, Invitrogen) in blocking solution at 1:1000 concentration for 2 h at room temperature. Coverslips were mounted in Prolong Gold Antifade (Invitrogen) and DRG neurons imaged using Axio Observer Z1 Widefield Microscope with a 20 $\times$ objective (Carl Zeiss). Images were analyzed with NIS Elements Advance Research (Nikon) software, and

corrected total cell fluorescence was calculated in Excel (Microsoft) by applying measurements obtained from image analysis using the following equations adapted from Barbosa et al. (2015):

$$CTCF = \frac{\text{Integrated Density}}{\text{Area of selected cell}} \times \text{mean fluorescence of background readings}$$

where

$$\text{Integrated Density} = (\text{mean intensity} \times \text{area of the cell})$$

Experimental design and statistical analysis. The acquisition of control and experimental data was randomized. Data were analyzed using the software programs PulseFit (HEKA) and Prism 5.0 (GraphPad Software). All data are shown as mean $\pm$ SE. The number of separate experimental cells is presented as n . Channel conductance was calculated using the equation $G(\text{Nav}) = I/(V - V_{\text{rev}})$ in which I , V , and V_{rev} represent inward current value, membrane potential, and reversal potential, respectively. Statistical analysis was performed by Student's t test and χ^2 analysis, and $p < 0.05$ indicated a significant difference.

Results

The *Possum* mutation in Nav1.8 that slows fast inactivation increases TTX-R resurgent currents

We first asked whether alteration of gating properties by the T790A mutation, originally characterized in the *Possum* mouse line, could be replicated in rat DRG neurons. Endogenous rat Nav1.8 currents are knocked down by $>98\%$ under our experimental conditions (Jarecki et al., 2010) by the rat Nav1.8 targeting shRNA (Mikami and Yang, 2005). As shown in Figure 1A, when transiently expressed in rat DRG neurons, the *Possum* mutation substantially slowed down inactivation of mNav1.8 channels at room temperature (21°C). Nav1.8 inactivation was well fit by two exponentials (Fig. 1B). At 10 mV, the fast-component time constant was 2.42 ± 0.17 ms and 3.47 ± 0.22 ms ($p < 0.001$) for WT and T790A mNav1.8 channels, respectively, whereas the slow-component value was 11.19 ± 1.07 ms and 17.78 ± 1.62 ms ($p < 0.005$), respectively. The *Possum* mutation did not affect the voltage dependence of steady-state inactivation (Fig. 1C). These major observations are fully consistent with those characterized for *Possum* mouse DRG neurons. However, we also observed that the *Possum* mutation shifted the voltage dependence of mNav1.8 activation by -11.9 mV in rat DRG neurons at room temperature (21°C) (WT, -12.3 ± 0.3 mV; T790A, -24.2 ± 0.4 mV; Fig. 1C). There was not a statistical difference in current density associated with mNav1.8 and T790A mutant channels; the average peak current density was -1054 ± 234 pA/pF and -1230 ± 249 pA/pF ($p > 0.5$), respectively. It is important to note that these peak current densities are remarkably similar to that observed for rat Nav1.8 currents under control conditions (-1063 ± 128 pA/pF, $n = 20$).

We then examined the change in gating properties of mNav1.8 by the T790A mutation at 34°C , a temperature close to physiological body temperature. As reported previously (Zimmermann et al., 2007), the increase in temperature did not evidently shift the voltage dependence of either steady-state mNav1.8 activation or inactivation (Fig. 1C) but accelerated the fast component of mNav1.8 inactivation kinetics (Fig. 1A,B; Table 1). At 34°C , the *possum* mutation also significantly slowed down mNav1.8 inactivation (compared with WT mNav1.8) by increasing the fast-component time constant from 1.47 ± 0.14 ms to 2.26 ± 0.23 ms ($p < 0.01$) and the slow-component value from 9.38 ± 0.68 ms to 13.45 ± 0.94 ms ($p < 0.005$), respectively. Moreover, as observed at 21°C , the *possum* mutation did not

significantly affect steady-state mNav1.8 inactivation at 34°C compared with WT but shifted the voltage dependence of mNav1.8 activation by -14.6 mV (WT, -8.6 ± 0.5 mV; T790A, -23.2 ± 0.5 mV; Fig. 1C). It is important to note that, although some ion channels exhibit pronounced temperature sensitivity, our data support the conclusion of Zimmerman et al. (2007) that Nav1.8 inactivation properties are relatively resistant to temperature-induced changes.

Recently, we identified a slow TTX-R resurgent current in small-diameter DRG neurons that is predicted to be generated by Nav1.8 and that is predicted to modulate nociceptor excitability (Tan et al., 2014). The resurgent current mechanism may compete with channel classic fast inactivation (Cannon and Bean, 2010) and human disease mutations that impair fast inactivation of TTX-S channels substantially increase resurgent currents (Jarecki et al., 2010). We thus tested whether mNav1.8 has intrinsic ability to generate TTX-R resurgent currents and whether the *Possum* mutation would alter Nav1.8-mediated resurgent current generation. It is important to note that, when Nav1.8 channels are expressed in the ND7/23 heterologous cell line and interrogated with a resurgent current protocol (Fig. 2A), classic tail currents are elicited but not resurgent currents. However, mNav1.8 channels transfected into DRG neurons generate robust resurgent currents that are nearly identical to endogenous TTX-R slow resurgent currents previously described (Fig. 2C). We used a 20 ms initial depolarization in the resurgent current protocol. Although Nav1.8 inactivation is often incomplete with 20 ms depolarizations, we used this duration because it allows direct comparisons to previous studies on TTX-S resurgent currents, grossly mimics nociceptor action potential durations, and minimizes the confounding influences of slow inactivation (Blair and Bean, 2003). As illustrated by the superimposed currents in Figure 2B, the classic Nav1.8 tail currents observed in ND7/23 cells arise nearly instantaneously and decay rapidly, whereas the slow resurgent currents observed with DRG mNav1.8 transfection have much slower onset and decay kinetics. These slow resurgent currents are observed with mNav1.8 transfection but were not observed when Nav1.4, Nav1.5, Nav1.6, or Nav1.7 channels are transfected into Nav1.8-shRNA treated DRG neurons (Jarecki et al., 2010). We find that, in the majority of mNav1.8-transfected rat DRG neurons (7 of 8 cells at 21°C; 16 of 20 cells at 34°C), TTX-R resurgent currents were inducible when the membrane was repolarized to voltages ranging from 5 to -40 mV. The currents peaked at -10 to -15 mV (Fig. 1C). The time to peak and the decay time constant for the WT currents elicited at -15 mV were 45.1 ± 8.6 ms and 618.3 ± 81.2 ms at 21°C (12.0 ± 2.3 ms and 532.8 ± 51.9 ms at 34°C), respectively. These data indicate that, while resurgent current decay shows little temperature dependence, the onset of resurgent currents is substantially faster at warmer temperatures ($p < 0.005$). These mNav1.8 resurgent currents exhibited a more depolarized voltage dependence of activation compared with the fast resurgent currents mediated by other Nav subtypes (including Nav1.6 and Nav1.7), which peak at ~ -40 mV (Jarecki et al.,

Figure 1. The *Possum* T790A mutation alters gating properties of mNav1.8 in rat DRG neurons at 21°C and 34°C. **A**, The *Possum* mutation significantly slowed fast inactivation. Left, Right, Typical current traces were elicited by a 50 ms depolarizing potential of 10 mV. Currents were normalized to the maximal amplitudes. **B**, Summary of time constants of the fast and slow components of inactivation. Time constants were obtained by fitting double exponential functions. **C**, The *Possum* mutation shifted voltage-dependent activation, but not steady-state inactivation, to more negative potentials. Steady-state inactivation was estimated using a standard double pulse protocol in which currents were induced by a 20 ms depolarizing potential of 0 mV following a 500 ms prepulse at potentials that ranged from -100 to 10 mV with a 10 mV increment. Data points for both activation and inactivation kinetics were well fitted with the Boltzmann equation. At 21°C (circle symbols), $V_{1/2}$ for activation: mNav1.8, -12.3 ± 0.3 mV ($n = 13$); T790A, -24.2 ± 0.4 mV ($n = 16$). $V_{1/2}$ for inactivation: mNav1.8, -32.7 ± 0.5 mV ($n = 9$); T790A, -36.5 ± 0.7 mV ($n = 13$). At 34°C (square symbols), $V_{1/2}$ for activation: mNav1.8, -8.6 ± 0.5 mV ($n = 16$); T790A, -23.2 ± 0.5 mV ($n = 16$). $V_{1/2}$ for inactivation: mNav1.8, -27.7 ± 1.6 mV ($n = 14$); T790A, -33.1 ± 0.9 mV ($n = 14$). $**p < 0.01$, $***p < 0.001$.

Table 1. Inactivation time constants of mNav1.8 and T790A^a

Temperature (°C)	mNav1.8		n	T790A		n
	τ_{fast} (ms)	τ_{slow} (ms)		τ_{fast} (ms)	τ_{slow} (ms)	
21	2.42 ± 0.17	11.19 ± 1.07	15	3.47 ± 0.22	17.78 ± 1.62	19
34	1.47 ± 0.14	9.38 ± 0.68	21	2.26 ± 0.23	13.45 ± 0.94	19
p	<0.001	>0.05		<0.001	<0.001	

^aSodium currents were elicited by a 50 ms depolarizing potential of 10 mV from a holding potential of -80 mV (see Fig. 1A). Data are mean $\pm$ SEM.

2010). WT mNav1.8-mediated resurgent current amplitude (measured as indicated in Fig. 2B, double-headed arrow) was $2.1 \pm 0.4\%$ of the peak transient current at 21°C and $2.2 \pm 0.3\%$ at 34°C (Fig. 2F,H). TTX-R resurgent currents could be elicited in almost all T790A-transfected rat DRG neurons (9 of 9 cells at 21°C ; 13 of 15 cells at 34°C). The largest resurgent current was attained at -15 mV to -20 mV (Fig. 2C,D). Interestingly, although the *Possum* mutation did not significantly change the voltage at which TTX-R resurgent currents peaked, the plot of normalized resurgent currents shows that there is substantially increased current at voltages between -30 and -45 mV (Fig. 2E,G). The *Possum* mutation was found to induce a greater than fourfold increase in relative resurgent current amplitude. The peak resurgent current amplitude was $10.2 \pm 0.8\%$ of the peak transient current at 21°C and $9.0 \pm 0.9\%$ at 34°C (Fig. 2F,H). As is illustrated in Figure 2B, resurgent current amplitude is measured from the peak of the slow onset phase to the residual current at the end of the 1 s repolarization step. Accurate measurement of resurgent current amplitude is complicated by the slow kinetics of Nav1.8 currents and by the relatively depolarized voltage dependence of Nav1.8 resurgent currents.

Figure 2. The *Possum* T790A mutation enhances mNav1.8 resurgent currents. **A**, Representative tail currents induced by a resurgent current protocol (top left) recorded from ND7/23 cells expressing Nav1.8 channels. The resurgent current protocol is a two-step protocol, in which the cell membrane is initially depolarized to 30 mV for 20 ms, followed by 1 s hyperpolarizing steps to potentials ranging from 5 to -85 mV. **B**, Comparison of Nav1.8 tail current (blue trace) with a mNav1.8 resurgent current recorded a DRG neuron (orange trace) elicited with a -15 mV hyperpolarizing voltage step. Dashed line and double headed arrow indicate how resurgent current amplitude was estimated. **C, D**, Representative resurgent currents recorded from DRG neurons expressing mNav1.8 (left) and T790A (right) channels at 21°C (**C**) and 34°C (**D**). **E**, Comparison of normalized resurgent current–voltage relationships of mNav1.8-mediated ($n = 7$) and T790A-mediated ($n = 9$) resurgent currents at 21°C . **F**, Summary of ratio resurgent currents at 21°C . Filled and open symbols represent mNav1.8 and T790A mutant channels, respectively. **G**, Comparison of normalized resurgent current–voltage relationships of mNav1.8-mediated ($n = 15$) and T790A-mediated ($n = 13$) resurgent currents at 34°C . **H**, Summary of ratio resurgent currents at 34°C . In **A–H**, Cells were held at -80 mV, and all recordings were performed in the presence of 500 nM TTX. **E, G**, Filled and open symbols represent mNav1.8 and T790A mutant channels, respectively. Data points are mean $\pm$ SE. * $p < 0.05$, *** $p < 0.001$.

The *Possum* mutation in Nav1.8 substantially increases sensory neuron excitability

Raman et al. (1997) reported that in cerebellar Purkinje neurons, resurgent currents are required for generating high-frequency action potentials. Therefore, we next asked how increasing Nav1.8-mediated resurgent currents might modulate excitability of nociceptive neurons at room temperature and physiological body temperature. In this study, 20 small-sized neurons with mNav1.8 channels and 24 with T790A mutant channels were patched and recorded at 21°C in the whole-cell current-clamp mode, and 22 with mNav1.8 channels and 22 with T790A mutant channels were studied at 34°C . Our recordings indicated that the *Possum* mutation did not change resting membrane potential (mNav1.8, -57.2 ± 0.8 mV vs T790A, -58.3 ± 1.6 mV, $p >$

0.05) but increased the number of neurons that fired spontaneously. At 21°C , spontaneous firing was observed in $\sim 13\%$ of mNav1.8-transfected neurons (3 of 23 cells), which is close to the percentage (14%) of neurons transfected with rat Nav1.8 channels that exhibit spontaneous activity (Han et al., 2014). In contrast, the expression of T790A mutant channels was associated with an almost threefold increase in the proportion of spontaneous firing neurons, with 57 percentage of DRG neurons (13 of 23 cells) exhibiting spontaneous activity (Fig. 3A,B). Increasing temperature may induce a higher proportion of spontaneous firing DRG neurons. At 34°C , the proportion was 27.3% (6 of 22 cells) for mNav1.8 and 63.6% (14 of 22 cells) for T790A, respectively. The difference in spontaneous activity was significant at both 21°C and 34°C (χ^2 analysis; $p < 0.02$).

The *Possum* mutation substantially broadened the duration of single action potentials elicited by a short (1 ms) current injection. Although small resurgent currents were inducible in the majority of WT mNav1.8-transfected DRG neurons, all of the action potentials elicited in these neurons were comparatively narrow at both temperatures examined (Fig. 3C,D). As described for nociceptive neurons by other studies (Djouhri et al., 1998; López de Armentia et al., 2000; Ritter et al., 2015), these WT action potentials exhibit a prominent shoulder during the repolarization phase. WT mNav1.8-transfected DRG neurons generated action potentials with an average duration (measured at the base) of 16.0 ± 0.7 ms at room temperature and 11.0 ± 1.0 ms at 34°C (Fig. 2D). The duration ranged from 10.1 to 23.1 ms at 21°C and from 4.8 to 24.7 ms at 34°C . The durations measured at 34°C are generally consistent with those measured at the base previously in rat C-nociceptors *in vivo* (Fang et al., 2005). By contrast, T790A-transfected neurons produced strikingly prolonged action potentials with an average duration of 163.5 ± 44.8 ms at 21°C and 575.4 ± 134.0 ms at 34°C . The duration ranged widely from 16.1 to 702.4 ms at 21°C ; and, surprisingly, the duration varied from 7.9 to 1737 ms at 34°C . This suggests that the *Possum* mutation substantially broadened action potentials in nociceptive neurons by >10 -fold. It is worth noting that multiple EADs were present during the plateau phase of action potentials in most T790A-transfected neurons (Fig. 3C). A similar phenomenon has also been observed with long-QT3 syndrome mutations, which impair fast inactivation of cardiac sodium channels and cause fatal arrhythmias (Nuyens et al., 2001; Song et al., 2012). EADs were not observed in any WT mNav1.8-transfected DRG neuron. Compared with WT mNav1.8-transfected DRG neurons, T790A-transfected DRG neurons exhibited a lower current threshold for action potential initiation (Fig. 3E). The *Possum* mutation greatly increased firing frequency elicited by a long (2 s) current injection. As shown in Figure 3F, G, 100 pA current injection failed to induce action potentials in the majority of WT mNav1.8-transfected DRG neurons (18 of 22 cells at 21°C ; 15 of 21 cells at 34°C). Even when injected with 200 pA current, firing frequency in most WT-transfected DRG neurons (17 of 22 cells at 21°C ; 20 of 21 cells at 34°C) was not >0.5 Hz (Fig. 3H). In contrast, the mean firing frequency of T790A-transfected DRG neurons by the same current injections was 3.3 ± 0.8 Hz and 4.7 ± 1.1 Hz at 21°C (2.1 ± 0.6 Hz and 5.1 ± 1.6 Hz at 34°C), respectively. Although the increase in action potential number induced by 200 pA current injection is not statistically significant at 34°C , the action potentials had a much longer duration for T790A-transfected neurons than for mNav1.8-transfected neurons (Fig. 3F). Among the 21 T790A-transfected neurons, five only generated one action potential, and the action potential had

Figure 3. The *Possum* mutation upregulated excitability of small-sized DRG neurons at 21°C and 34°C. **A**, Typical spontaneous firing recorded from mNav1.8- or T790A-transfected DRG neurons. **B**, Bar graph represents the percentage of DRG neurons with (black) or without (white) spontaneous firing. +SA, Spontaneous activity; −SA, no spontaneous activity. **C**, Representative single action potentials elicited by a 1 ms current injection in DRG neurons transfected with mNav1.8 or T790A mutant channels. Injected currents ranged from 0 to 3200 pA. **i, ii**, Two typical types of single action potentials on T790A-transfected neurons. Gray voltage traces represent resting membrane potential. EAD was indicated by arrows during the shoulder of the repolarization elicited in T790A-transfected neurons (right). **D, E**, Summary of action potential durations (APD₉₀) and current thresholds. At 21°C, the average APD₉₀s were 16.0 ± 0.7 ms (mNav1.8, $n = 21$) and 163.5 ± 44.8 ms (T790A, $n = 23$), respectively. The average current thresholds were 900.0 ± 59.6 pA (mNav1.8) and 454.5 ± 60.9 pA (T790A), respectively. At 34°C, the average APD₉₀s were 11.0 ± 1.0 ms (mNav1.8, $n = 22$) and 575.4 ± 134.0 ms (T790A, $n = 18$), respectively. The average current thresholds were 1047.0 ± 111.2 pA (mNav1.8) and 735.7 ± 90.6 pA (T790A), respectively. **F**, Representative trains of action potentials evoked by a 2 s injection of 100 or 200 pA currents. **G, H**, Summary of the number of action potentials evoked during the 2 s injection. Filled and open circles represent mNav1.8 and T790A mutant channels, respectively. **A, C, F**, Dotted line indicates zero voltage level. * $p < 0.05$, *** $p < 0.001$.

a 2 s long plateau phase at ~ 0 mV due to the firing of multiple EADs.

Knockdown of Navβ4 reduced sensory neuron excitability by decreasing Nav1.8-mediated resurgent currents

Navβ4 subunit is highly expressed in DRG neurons (Yu et al., 2003). There is evidence to support that its cytoplasmic tail functions as an open-channel blocker, which underlies the generation of resurgent currents (Grieco et al., 2005; Bant and Raman, 2010; Theile and Cummins, 2011; Barbosa et al., 2017). To confirm the roles of increased Nav1.8-mediated resurgent currents in nociceptive neuron hyperexcitability, we next asked whether resurgent current reduction by Navβ4 knockdown would decrease the excitability of T790A-transfected DRG neurons. The following experiments were performed only at room temperature because the

T790A mutation similarly increased Nav1.8-mediated resurgent currents and upregulated DRG neuron excitability at 21°C and 34°C (Figs. 2, 3).

In adult rat L4 and L5 DRG neurons, Navβ4 siRNA treatment was found to depress expression of Navβ4 proteins by 90% (Fig. 4A,B). However, there was no statistical difference in T790A mutant channel current density (control, -0.98 ± 0.19 nA/pF, $n = 8$; Navβ4 siRNA, -0.78 ± 0.17 nA/pF, $n = 13$; $p > 0.05$). Navβ4 knockdown did not significantly affect inactivation kinetics, the voltage dependence of activation, or steady-state inactivation of T790A mutant channels (Fig. 4C,D). Navβ4 knockdown did not significantly alter the fraction of transfected DRG neurons that generated TTX-R resurgent currents (control, 100%, 8 of 8 cells; Navβ4 siRNA, 77%, 10 of 13 cells) but substantially reduced the relative T790A resurgent current ampli-

Figure 4. Knockdown of Navβ4 reduced the ability of T790A mutant channels to generate resurgent currents in L4–L5 DRG neurons. **A**, Left, Diagram represents L4–L5 DRGs injected by Navβ4 siRNA. Right, Immunofluorescent reactions showed Navβ4 subunit expression levels in DRG neurons, in which T790A mutant channels were not transfected. Scale bars, 50 μm. **B**, Summary of fluorescence in L4–L5 DRG neurons with or without siRNA treatment. **C**, Knockdown of Navβ4 did not significantly change either the fast component time constant or the slow component of inactivation kinetics. For τ_{fast}: control, 3.9 ± 0.4 ms (*n* = 8); β4siRNA, 3.5 ± 0.3 ms (*n* = 12), *p* > 0.1. For τ_{slow}: control, 21.1 ± 2.7 ms; β4siRNA, 21.0 ± 2.7 ms, *p* > 0.1. **D**, Knockdown of Navβ4 did not alter steady-state activation or inactivation of T790A mutant channels. V_{1/2} for activation: control, −21.8 ± 0.7 mV (*n* = 7); β4siRNA, −26.2 ± 0.5 mV (*n* = 9). V_{1/2} for inactivation: control, −33.0 ± 1.1 mV (*n* = 7); β4siRNA, −37.4 ± 0.6 mV (*n* = 9). **E**, Representative resurgent current traces for control and β4siRNA elicited by a two-step protocol as described in Figure 2A. Neurons were transfected with T790A channels. **F**, Normalized current–voltage relationships of T790A-mediated resurgent currents. **G**, Summary of ratio resurgent current. **C–G**, All DRG neurons were held at −80 mV and pretreated with 500 nM TTX. Filled and open circles represent control and β4siRNA treatment, respectively. **p* < 0.05.

tude from 12.1 ± 2.0% to 5.3 ± 0.9% (*p* < 0.05) of the peak transient current (Fig. 4E, G). Navβ4 knockdown did not alter the current–voltage relationship of resurgent currents (Fig. 4F).

As DRG neurons also express several TTX-S sodium channel subtypes (e.g., Nav1.6, Nav1.7) that are able to produce TTX-S resurgent currents, and because those resurgent currents are altered by changing Navβ4 subunit expression level to influence neuronal excitability (Barbosa et al., 2015), we added 500 nM TTX into the bath solution to block all TTX-S resurgent currents in the following current-clamp recordings, allowing us to focus on TTX-R-dependent action potential activity. Navβ4 knockdown did not change resting membrane potential or current threshold (data not shown) and did not significantly reduce the proportion of T790A-transfected neurons with spontaneous firing. Under control conditions, 60.0% of L4–L5 DRG neurons with T790A mutant channels (12 of 20 cells) spontaneously generated action potentials, which is consistent with the observation in neurons isolated from all DRGs (Fig. 2B). After treatment with Navβ4 siRNA, 9 of 20 transfected neurons (45.0%) displayed spontaneous activity (Fig. 5A, B). This difference was not significant. However, as can be seen in Figure 5C, single evoked action potentials were substantially narrower after Navβ4 siRNA treatment. The average durations measured under these two conditions were 253.8 ± 93.4 ms and 38.1 ± 8.2 ms (*p* < 0.05), respectively (Fig. 5D, left). Under control conditions, 50% of transfected neurons (10 of 20 cells) generated single action potential with duration of >50 ms, but the percentage is only 10% (2 of 20 cells) after treatment with Navβ4 siRNA (Fig. 5D, right; *p* < 0.01). The reduction indicated that an increase in Nav1.8-mediated resurgent currents can play a crucial role in broadening nociceptive neuron action potentials. With 2 s injected currents of 100 pA, T790A-transfected neurons treated with Navβ4 siRNA displayed fewer action potentials than control T790A-transfected neurons

(Fig. 5E–G). The decrease in action potential number elicited was not significant at 200 pA. Overall, our data indicate that Navβ4 is not only important for generation of prolonged action potentials but also can impact firing frequency in nociceptive neurons transfected with T790A mutant channels.

An SFN mutation in Nav1.8 that impairs inactivation increases resurgent current

Next, we asked whether a human mutation, G1662S, identified in patients with painful SFN, endowed Nav1.8 with enhanced resurgent current generation. Han et al. (2014) reported that the SFN mutation impaired inactivation of human Nav1.8 expressed in mouse DRG neurons. Here we transfected rat DRG neurons with hNav1.8 WT and G1662S channel constructs. As shown in Figure 6A, B, the SFN mutation significantly slows fast inactivation of Nav1.8 current elicited by a 50 ms depolarizing potential of 10 mV. At 10 mV, the fast-component time constant was 1.89 ± 0.18 ms and 2.41 ± 0.25 ms (*p* > 0.05) for WT and G1662S hNav1.8 channels, respectively, whereas the slow-component value was 9.83 ± 0.78 ms and 13.95 ± 1.49 ms (*p* < 0.05), respectively. The SFN mutation did not shift the voltage dependence of activation but induced a slight (6.6 mV) positive shift in the voltage dependence of inactivation (Fig. 6D). Accordingly, the shift induced an increased overlap of the activation and inactivation curves, which is predicted to result in increased window currents (Fig. 6E). TTX-R resurgent currents were elicited using the same protocol as described for mNav1.8 and T790A mutant channels (Fig. 6F). Larger TTX-R resurgent currents were induced with G1662S mutant channels, compared with WT hNav1.8 (Fig. 6G). The average relative amplitude was 5.44 ± 0.80% of peak transient currents, significantly larger than WT hNav1.8-mediated resurgent currents (3.3 ± 0.61%; *p* < 0.05). The fraction of cells exhibiting detectable resurgent currents was

Figure 5. Knockdown of Navβ4 subunit reduced excitability in L4–L5 DRG neurons transfected with T790A mutant channels. **A**, Typical spontaneous firing recorded from T790A-transfected DRG neurons with or without Navβ4 siRNA treatment. **B**, Bar graph represents the percentage of DRG neurons with (white) or without (black) spontaneous firing. +SA, Spontaneous activity; –SA, no spontaneous activity. **C**, Typical single action potentials elicited by a 1 ms current injection. Arrows indicate EADs during the shoulder of the repolarization. **D**, Summary of action potential duration (APD90). Left, Scatter plot of APD90. The average durations were 253.8 ± 93.4 ms (control, $n = 20$) and 38.1 ± 8.2 ms (β4 siRNA, $n = 20$). Right, Histogram showing the percentage of cells generating action potentials with the duration of 0–25, 25–50, 50–200, and >200 ms. **E**, Typical action potential trains elicited by a 2 s injection of 100 (left) or 200 (right) pA current. **F**, **G**, Summary of the number of action potentials elicited by a 2 s injection of 100 or 200 pA current, respectively. **A**, **C**, **E**, Dotted line indicates zero voltage level. * $p < 0.05$.

also greater with G1662S channels ($p < 0.05$; Fig. 6H). The resurgent currents hNav1.8 and G1662S resurgent currents peaked at -15 mV and showed a slow onset and decay similar to those mediated by mNav1.8 and T790A mutant channels (Fig. 6F). The SFN mutation did not alter the voltage dependence of activation of TTX-R resurgent currents (Fig. 6I).

For comparison, we also introduced the human disease mutation G1662S into the mouse Nav1.8 ortholog; G1662 is equivalent to the Gly residue at position 1663 in mNav1.8. The G1663S mutation did not evidently affect the time constants of mNav1.8 inactivation (τ_{fast} : mNav1.8, 2.4 ± 0.2 ms, $n = 15$ vs G1663S, 2.6 ± 0.2 ms, $n = 8$, $p > 0.05$; τ_{slow} : mNav1.8, 11.2 ± 1.1 ms vs

Figure 6. Effects of the G1662S mutation on hNav1.8 gating properties and hNav1.8-mediated resurgent currents at 21°C. **A**, The G1662S mutation slows the rate of fast inactivation. Currents were evoked by a 50 ms depolarizing potential of 10 mV. **B**, Summary of time constants of the fast and slow components of inactivation. Time constants were obtained by fitting with double exponential functions. G1662S significantly changed the slow time constant of fast inactivation at 10 mV. **C**, The G1662S mutation did not affect the current density. The averaged current density of WT hNav1.8 is 0.4997 ± 0.07 nA/pF, $n = 23$; and the current density of hNav1.8 channel with G1662S mutation is 0.4477 ± 0.09 nA/pF, $n = 16$. **D**, The G1662S mutation did not affect the voltage–conductance relationship but shifted steady-state inactivation of hNav1.8 to more positive potentials. Families of currents for I – V curves and steady-state inactivation were induced using the same protocols as described in the Figure 1C legend. Data points are mean $\pm$ SE. $V_{1/2}$ for activation: hNav1.8, -20.2 ± 0.56 mV, $n = 17$; G1662S, -23.96 ± 0.58 mV, $n = 10$. $V_{1/2}$ for inactivation: hNav1.8, -47.64 ± 1.06 mV, $n = 16$; G1662S, -41.06 ± 0.60 mV, $n = 12$. **E**, Comparison of the overlap of steady-state activation and inactivation curves between hNav1.8 (black curves) and G1662S (gray curves). **F**, The G1662S mutation increased hNav1.8-mediated resurgent currents. Resurgent currents were induced with the protocol shown and described in the Figure 2 legend. **G**, Summary of resurgent/peak current ratio. Cells were held at -80 mV and pretreated in 500 nM TTX. **H**, The G1662S mutation increased the cell fraction that generated resurgent currents (hNav1.8, $n = 23$; G1662S, $n = 16$). **I**, Comparison of normalized current–voltage relationships of hNav1.8- and G1662S-mediated resurgent currents. $*p < 0.05$.

Figure 7. An SFN mutation of hNav1.8 alters mNav1.8 channel inactivation and resurgent currents at 21°C. **A**, The G1663S mutation did not significantly affect the rate of fast inactivation for mNav1.8. **B**, The G1663S mutation did not affect the voltage dependence of activation but induced a positive shift of steady-state inactivation of mNav1.8. $V_{1/2}$ for activation: mNav1.8, -12.3 ± 0.3 mV, $n = 13$; G1663S, -15.3 ± 0.7 mV, $n = 8$. $V_{1/2}$ for inactivation: mNav1.8, -32.7 ± 0.5 mV, $n = 13$; G1663S, -29.2 ± 0.4 mV, $n = 8$. **C**, G1663S mutation enhanced window current of mNav1.8. **D**, Representative mNav1.8 and G1663S resurgent current traces. The G1663S mutation increased mNav1.8-mediated resurgent current amplitudes. **E**, Comparison of normalized current–voltage relationships of mNav1.8- and G1663S-mediated resurgent currents. **F**, Comparison of resurgent current ratios from mNav1.8 and G1663S channels. $*p < 0.05$.

G1663S, 12.5 ± 2.7 ms, $p > 0.05$; Fig. 7A). The G1663S mutation did not evidently alter the voltage dependence of mNav1.8 activation either but slightly impaired the voltage dependence of inactivation by 3.5 mV ($V_{1/2}$ for inactivation: mNav1.8, -32.7 ± 0.5 mV, $n = 13$; G1663S, -29.2 ± 0.4 mV, $n = 8$; $p < 0.05$; Fig. 7B,C). Our results show that the G1663S mutation also enhanced resurgent currents of mNav1.8 (Fig. 7D–F). The peak G1663S resurgent currents in absolute terms (1.04 ± 0.20 nA, $n = 9$) were twice as large as WT mNav1.8-mediated resurgent currents (0.49 ± 0.12 nA, $n = 7$; $p < 0.05$). The average relative amplitude was $3.61 \pm 0.42\%$ of peak transient currents, also significantly larger than WT mNav1.8-mediated resurgent currents ($2.08 \pm 0.40\%$; $p < 0.05$; Fig. 7F). These results are consistent with the observations in the human Nav1.8 construct (Fig. 6).

Discussion

Our data demonstrate, for the first time, that (1) human Nav1.8 channels can gen-

erate resurgent sodium currents, (2) pathophysiological Nav1.8 mutations can significantly increase Nav1.8 resurgent currents, and (3) increased Nav1.8 open-channel block is likely to be an important contributor to nociceptive neuron hyperexcitability. In this study, we showed that TTX-R resurgent currents were increased by a gain-of-function Nav1.8 mutation in painful SFN and a mutation identified in the *Possum* transgenic mouse line that is associated with enhanced pain sensitivities. We demonstrate that increased Nav1.8-mediated resurgent currents not only enabled nociceptive neurons to generate high-frequency bursts of action potentials but also substantially broadened action potentials and generated multiple EADs. We also found that Nav β 4 is a major determinant for resurgent current generation of Nav1.8. Nociceptor hyperexcitability is a crucial factor in altered pain phenotypes, and Nav1.8 resurgent currents are likely to contribute to neuropathic pain in SFN as well as inflammatory pain conditions.

Our data provide compelling evidence that Nav1.8 has the intrinsic ability to produce resurgent currents and accounts for the generation of endogenous TTX-R resurgent currents in DRG neurons (Tan et al., 2014). The endogenous rat TTX-R resurgent currents and those mediated by recombinant mNav1.8 and hNav1.8 are all resistant to TTX, display similar voltage dependence of activation, and, importantly, show a very slow onset and decay. These characteristics are quite different from resurgent currents mediated by other sodium channel subtypes (Jarecki et al., 2010; Barbosa et al., 2015). There is considerable evidence accumulating to suggest that open-channel block induced by Nav β 4 is a major factor for TTX-S resurgent current generation. Our siRNA-mediated knockdown experiment indicates that Nav β 4 is also important for TTX-R resurgent current generation. The Nav β 4 cytoplasmic tail likely functions as an open-channel blocker, which directly competes with channel inactivation to dock transiently within (or nearby) the channel pore (Grieco et al., 2005). We predict that the large TTX-R resurgent currents detected in our expression system are also evoked in human DRG neurons. There is one residue that is different between the cytoplasmic tails of rat and human Nav β 4 (Yu et al., 2003), but this residue at position 188 (a Thr in rat and an Ile in human) is suggested to have little effect on Nav β 4-induced resurgent currents (Lewis and Raman, 2011). However, whereas we achieved close to 90% knockdown of Nav β 4 protein, Nav1.8 resurgent currents were only reduced by 56%. This raises the possibility that other open-channel blockers, in addition to Nav β 4, interact with Nav1.8 channels and contribute to Nav1.8 resurgent currents.

The *Possum* T790A mutation impairs inactivation but also shifts activation by -11.9 mV in rat DRG neurons. This observation is different from those in a previous report, which indicated that the mutation does not modify the voltage dependence of Nav1.8 activation in the *Possum* transgenic mouse DRG neurons (Blasius et al., 2011). The activation shift identified in our study is unlikely due to different cell backgrounds. Such a shift was also observed when T790A channels were expressed in the ND7/23 cell line (Fig. 8). The hyperpolarized shift in activation is unlikely to contribute to enhanced resurgent currents but can also impact excitability. Interestingly, T790 is equivalent in position to T704 in human Nav1.4 and T698 in rat Nav1.4 sequences.

Figure 8. Gating properties of mNav1.8 and T790A mutant channels expressed in ND7/23 cells at 21°C. Normalized I – V curves (**A**), channel conductance (**B**), and steady-state inactivation (**C**) were assayed as described in the Figure 1 legend. $V_{1/2}$ for activation: mNav1.8, 4.2 ± 0.8 mV, $n = 5$; T790A, -12.1 ± 0.6 mV, $n = 6$. $V_{1/2}$ for inactivation: mNav1.8, -41.9 ± 1.3 mV, $n = 5$; T790A, -41.9 ± 1.5 mV, $n = 6$.

A mutation at this position in Nav1.4 (T704M) is associated with hyperkalemic periodic paralysis. Cummins et al. (1993) and Bendahhou et al. (1999) found that hNav1.4-T704M and rNav1.4-T698M induce a hyperpolarizing shift in voltage dependence of Nav1.4 activation but do not impact inactivation. Our data demonstrate that this threonine residue, which is conserved across nine sodium channel isoforms, can influence both activation and inactivation gating of Nav1.8. This could represent an important difference in Nav1.8 gating compared with other sodium channel isoforms.

It is widely accepted that, whereas Nav1.7 helps set voltage threshold for action potential firing, Nav1.8 contributes mainly to the rising phase (Cummins et al., 2007). Many studies have shown that nociceptive sensory neurons generate action potentials with a prominent shoulder during the falling phase (Djoughri et al., 1998; López de Armentia et al., 2000). Blair and Bean (2002) demonstrated that TTX-R, not TTX-S, sodium current is active during this shoulder phase. Our present data demonstrate that an increase in TTX-R resurgent currents can substantially broaden the shoulder of the action potential. The broadening was greater at 34°C. This likely reflects the resistance of overall Nav1.8 gating to temperature-induced changes (Zimmermann et al., 2007) coupled with the faster onset of Nav1.8 resurgent currents at warmer temperatures. Although the *Possum* mutation slows down fast inactivation and increases window currents, which presumably contributes to increased excitability, our Nav β 4 knockdown experiments clearly indicated that the decrease in Nav1.8-mediated resurgent currents due to Nav β 4 knockdown results in shortened action potential durations, decreased EADs, and reduced firing frequency. Interestingly, Nav1.7 paroxysmal extreme pain disorder mutations slow fast inactivation and increase Nav1.7-mediated TTX-S resurgent currents, but paroxysmal extreme pain disorder mutations do not significantly broaden action potential duration (Dib-Hajj et al., 2008; Jarecki et al., 2010; Theile et al., 2011). We propose that, whereas Nav1.7-mediated resurgent currents are mainly responsible for enhancing repetitive firing in nociceptive neurons, Nav1.8-mediated resurgent currents contribute to broadening of action potentials and to repetitive firing.

We also observed a substantial reduction in the rheobase (current threshold) for action potential firing with the T790A mutation. Bant and Raman (2010) demonstrated that Nav β 4-induced open-channel block of TTX-sensitive resurgent currents in cerebellar granule cells not only contributes to enhanced resurgent currents and increased repetitive firing, but also to increased persistent currents, a depolarized voltage dependence of inactivation.

tion, and a lower rheobase. In our study, Nav β 4 knockdown did not significantly impact rheobase in T790A-transfected cells. This could reflect the possible interaction of Nav1.8 an additional open-channel blocker. The change in rheobase with T790A is also likely, at least in part, to result from the mutation induced alterations in other biophysical properties, such as enhanced activation and impaired inactivation.

Our data indicate that hNav1.8 generates TTX-R resurgent currents almost twice as large as the mouse ortholog in rat DRG neurons. We did not observe a major difference in the inactivation properties of mouse and human Nav1.8 channels transfected in rat DRG neurons, so it is unclear precisely why hNav1.8 generated larger resurgent currents. The SFN mutation G1662S is the first human disease-causing mutation shown to enhance generation of TTX-R resurgent currents. This mutation increased Nav1.8-mediated resurgent currents by 65%. In addition, whereas only 30% of neurons transfected with WT hNav1.8 generated resurgent currents, 63% of neurons transfected with G1662S did. It is likely that these changes in resurgent current amplitude and frequency are sufficient to increase nociceptive neuron excitability.

We propose that a fourfold increase in resurgent current amplitude, such as that observed with the *Possum* mutation, underlies the extreme neuronal hyperexcitability, aberrant pain sensitivity, and possibly even the freezing response to pinch in *Possum* mice (Blasius et al., 2011; Garrison et al., 2014). The SFN mutation had a smaller impact on resurgent current amplitude than the *Possum* mutation. A more moderate increase in resurgent currents is consistent with the more moderate increases in excitability reported for the SFN G1662S mutation (Han et al., 2014). In hNav1.8, the G1662S SFN mutation induced a significant slowing of inactivation. Although the change in fast inactivation time constant is similar for both the T790A and the G1662S mutations, the T790A mutation has a greater impact on late currents, and this difference likely contributes to the much larger resurgent currents with the *Possum* mutation. Lewis and Raman (2013) showed that open-channel blockers might have higher affinity in sodium channels with DIVS4 deployed than with DIVS4 in the resting or partially deployed configuration. We assume that T790A mutant channels exhibit a lower affinity for the open-channel blocker than WT Nav1.8 channels at voltages more negative than -20 mV, and this coupled with augmented window currents results in increased current amplitudes at voltages between -25 and -45 mV. Our data indicate that either increasing window currents or slowing fast inactivation is likely to enhance Nav1.8-mediated resurgent current generation.

In conclusion, our findings provide novel mechanistic insight into how disease mutations alter generation of Nav1.8-mediated resurgent currents and how aberrant TTX-R resurgent currents regulate hyperexcitability of nociceptive sensory neurons. Our data demonstrate, for the first time, that painful Nav1.8 mutations induce aberrant TTX-R resurgent currents, and these are likely to serve as substantial contributors to the pathological mechanisms underlying nociceptive neuron hyperexcitability associated with neuropathic pain. We have previously shown that inflammatory mediators increase TTX-R resurgent currents (Tan et al., 2014). Thus, inhibition of TTX-R resurgent currents by pharmacological agents or reduction of Nav1.8 open-channel block by knocking down Nav β 4 expression may present an effective strategy to treat neuropathic pain in SFN and other painful conditions.

References

- Afshari FS, Ptak K, Khaliq ZM, Grieco TM, Slater NT, McCrimmon DR, Raman IM (2004) Resurgent Na currents in four classes of neurons of the cerebellum. *J Neurophysiol* 92:2831–2843. [CrossRef Medline](#)
- Akopian AN, Sivilotti L, Wood JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* 379:257–262. [CrossRef Medline](#)
- Akopian AN, Souslova V, England S, Okuse K, Ogata N, Ure J, Smith A, Kerr BJ, McMahon SB, Boyce S, Hill R, Stanfa LC, Dickenson AH, Wood JN (1999) The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. *Nat Neurosci* 2:541–548. [CrossRef Medline](#)
- Bant JS, Raman IM (2010) Control of transient, resurgent, and persistent current by open-channel block by Na channel β 4 in cultured cerebellar granule neurons. *Proc Natl Acad Sci U S A* 107:12357–12362. [CrossRef Medline](#)
- Barbosa C, Tan ZY, Wang R, Xie W, Strong JA, Patel RR, Vasko MR, Zhang JM, Cummins TR (2015) Nav β 4 regulates fast resurgent sodium currents and excitability in sensory neurons. *Mol Pain* 11:60. [CrossRef Medline](#)
- Barbosa C, Xiao Y, Johnson AJ, Xie W, Strong JA, Zhang JM, Cummins TR (2017) FHF2 isoforms differentially regulate Nav1.6-mediated resurgent sodium currents in dorsal root ganglion neurons. *Pflugers Arch* 469:195–212. [CrossRef Medline](#)
- Bendahhou S, Cummins TR, Tawil R, Waxman SG, Ptáček LJ (1999) Activation and inactivation of the voltage-gated sodium channel: role of segment S5 revealed by a novel hyperkalemic periodic paralysis mutation. *J Neurosci* 19:4762–4771. [CrossRef Medline](#)
- Blair NT, Bean BP (2002) Roles of tetrodotoxin (TTX)-sensitive Na^+ current, TTX-resistant Na^+ current, and Ca^{2+} current in the action potentials of nociceptive sensory neurons. *J Neurosci* 22:10277–10290. [CrossRef Medline](#)
- Blair NT, Bean BP (2003) Role of tetrodotoxin-resistant Na^+ current slow inactivation in adaptation of action potential firing in small-diameter dorsal root ganglion neurons. *J Neurosci* 23:10338–10350. [CrossRef Medline](#)
- Blasius AL, Dubin AE, Petrus MJ, Lim BK, Narezkina A, Criado JR, Wills DN, Xia Y, Moresco EM, Ehlers C, Knowlton KU, Patapoutian A, Beutler B (2011) Homomorphic mutation of the voltage-gated sodium channel encoding gene *Scn10a* causes a dramatic stimulus-dependent neurobehavioral phenotype. *Proc Natl Acad Sci U S A* 108:19413–19418. [CrossRef Medline](#)
- Cannon SC, Bean BP (2010) Sodium channels gone wild: resurgent current from neuronal and muscle channelopathies. *J Clin Invest* 120:80–83. [CrossRef Medline](#)
- Cummins TR, Zhou J, Sigworth FJ, Ukomadu C, Stephan M, Ptáček LJ, Agnew WS (1993) Functional consequences of a Na^+ channel mutation causing hyperkalemic periodic paralysis. *Neuron* 10:667–678. [CrossRef Medline](#)
- Cummins TR, Howe JR, Waxman SG (1998) Slow closed-state inactivation: a novel mechanism underlying ramp currents in cells expressing the hNE/PN1 sodium channel. *J Neurosci* 18:9607–9619. [CrossRef Medline](#)
- Cummins TR, Black JA, Dib-Hajj SD, Waxman SG (2000) Glial-derived neurotrophic factor upregulates expression of functional SNS and NaN sodium channels and their currents in axotomized dorsal root ganglion neurons. *J Neurosci* 20:8754–8761. [CrossRef Medline](#)
- Cummins TR, Dib-Hajj SD, Waxman SG (2004) Electrophysiological properties of mutant Nav1.7 sodium channels in a painful inherited neuropathy. *J Neurosci* 24:8232–8236. [CrossRef Medline](#)
- Cummins TR, Dib-Hajj SD, Herzog RI, Waxman SG (2005) Nav1.6 channels generate resurgent sodium currents in spinal sensory neurons. *FEBS Lett* 579:2166–2170. [CrossRef Medline](#)
- Cummins TR, Sheets PL, Waxman SG (2007) The roles of sodium channels in nociception: implications for mechanisms of pain. *Pain* 131:243–257. [CrossRef Medline](#)
- Dib-Hajj SD, Cummins TR, Black JA, Waxman SG (2007) From genes to pain: Na v 1.7 and human pain disorders. *Trends Neurosci* 30:555–563. [CrossRef Medline](#)
- Dib-Hajj SD, Estacion M, Jarecki BW, Tyrrell L, Fischer TZ, Lawden M, Cummins TR, Waxman SG (2008) Paroxysmal extreme pain disorder M1627K mutation in human Nav1.7 renders DRG neurons hyperexcitable. *Mol Pain* 4:37. [CrossRef Medline](#)
- Dib-Hajj SD, Choi JS, Macala LJ, Tyrrell L, Black JA, Cummins TR, Waxman

- SG (2009) Transfection of rat or mouse neurons by biolistics or electroporation. *Nat Protoc* 4:1118–1126. [CrossRef Medline](#)
- Djouhri L, Bleazard L, Lawson SN (1998) Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. *J Physiol* 513:857–872. [CrossRef Medline](#)
- Enomoto A, Han JM, Hsiao CF, Wu N, Chandler SH (2006) Participation of sodium currents in burst generation and control of membrane excitability in mesencephalic trigeminal neurons. *J Neurosci* 26:3412–3422. [CrossRef Medline](#)
- Faber CG, Hoeijmakers JG, Ahn HS, Cheng X, Han C, Choi JS, Estacion M, Lauria G, Vanhoutte EK, Gerrits MM, Dib-Hajj S, Drenth JP, Waxman SG, Merkies IS (2012a) Gain of function Nav1.7 mutations in idiopathic small fiber neuropathy. *Ann Neurol* 71:26–39. [CrossRef Medline](#)
- Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, Persson AK, Hoeijmakers JG, Gerrits MM, Pierro T, Lombardi R, Kapetis D, Dib-Hajj SD, Waxman SG (2012b) Gain-of-function Nav1.8 mutations in painful neuropathy. *Proc Natl Acad Sci U S A* 109:19444–19449. [CrossRef Medline](#)
- Fang X, McMullan S, Lawson SN, Djouhri L (2005) Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. *J Physiol* 565:927–943. [CrossRef Medline](#)
- Fertleman CR, Baker MD, Parker KA, Moffatt S, Elmslie FV, Abrahamson B, Ostman J, Klugbauer N, Wood JN, Gardiner RM, Rees M (2006) SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. *Neuron* 52:767–774. [CrossRef Medline](#)
- Garrison SR, Weyer AD, Barabas ME, Beutler BA, Stucky CL (2014) A gain-of-function voltage-gated sodium channel 1.8 mutation drives intense hyperexcitability of A- and C-fiber neurons. *Pain* 155:896–905. [CrossRef Medline](#)
- Grieco TM, Raman IM (2004) Production of resurgent current in Nav1.6-null Purkinje neurons by slowing sodium channel inactivation with betapompilidotoxin. *J Neurosci* 24:35–42. [CrossRef Medline](#)
- Grieco TM, Malhotra JD, Chen C, Isom LL, Raman IM (2005) Open-channel block by the cytoplasmic tail of sodium channel beta4 as a mechanism for resurgent sodium current. *Neuron* 45:233–244. [CrossRef Medline](#)
- Han C, Vasylyev D, Macala LJ, Gerrits MM, Hoeijmakers JG, Bekelaar KJ, Dib-Hajj SD, Faber CG, Merkies IS, Waxman SG (2014) The G1662S Nav1.8 mutation in small fibre neuropathy: impaired inactivation underlying DRG neuron hyperexcitability. *J Neurol Neurosurg Psychiatry* 85:499–505. [CrossRef Medline](#)
- Hasegawa Y, Mao W, Saha S, Gunner G, Kolpakova J, Martin GE, Futai K (2017) Luciferase shRNA presents off-target effects on voltage-gated ion channels in mouse hippocampal pyramidal neurons. *eNeuro* 4:ENEURO.0186–17.2017. [CrossRef Medline](#)
- Herzog RI, Cummins TR, Ghassemi F, Dib-Hajj SD, Waxman SG (2003) Distinct repriming and closed state inactivation kinetics of Nav1.6 and Nav1.7 sodium channels in mouse spinal sensory neurons. *J Physiol* 551:741–750. [CrossRef Medline](#)
- Hoeijmakers JG, Merkies IS, Gerrits MM, Waxman SG, Faber CG (2012) Genetic aspects of sodium channelopathy in small fiber neuropathy. *Clin Genet* 82:351–358. [CrossRef Medline](#)
- Huang J, Yang Y, Zhao P, Gerrits MM, Hoeijmakers JG, Bekelaar K, Merkies IS, Faber CG, Dib-Hajj SD, Waxman SG (2013) Small-fiber neuropathy Nav1.8 mutation shifts activation to hyperpolarized potentials and increases excitability of dorsal root ganglion neurons. *J Neurosci* 33:14087–14097. [CrossRef Medline](#)
- Jackson AL, Linsley PS (2010) Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov* 9:57–67. [CrossRef Medline](#)
- Jarecki BW, Piekarczyk AD, Jackson JO 2nd, Cummins TR (2010) Human voltage-gated sodium channel mutations that cause inherited neuronal and muscle channelopathies increase resurgent sodium currents. *J Clin Invest* 120:369–378. [CrossRef Medline](#)
- Kim JH, Kushmerick C, von Gersdorff H (2010) Presynaptic resurgent Na⁺ currents sculpt the action potential waveform and increase firing reliability at a CNS nerve terminal. *J Neurosci* 30:15479–15490. [CrossRef Medline](#)
- Lewis AH, Raman IM (2011) Cross-species conservation of open-channel block by Na channel $\beta 4$ peptides reveals structural features required for resurgent Na current. *J Neurosci* 31:11527–11536. [CrossRef Medline](#)
- Lewis AH, Raman IM (2013) Interactions among DIV voltage-sensor movement, fast inactivation, and resurgent Na current induced by the Nav $\beta 4$ open-channel blocking peptide. *J Gen Physiol* 142:191–206. [CrossRef Medline](#)
- López de Armentia M, Cabanes C, Belmonte C (2000) Electrophysiological properties of identified trigeminal ganglion neurons innervating the cornea of the mouse. *Neuroscience* 101:1109–1115. [CrossRef Medline](#)
- Mikami M, Yang J (2005) Short hairpin RNA-mediated selective knockdown of Nav1.8 tetrodotoxin-resistant voltage-gated sodium channel in dorsal root ganglion neurons. *Anesthesiology* 103:828–836. [CrossRef Medline](#)
- Nuyens D, Stengl M, Dugarmaa S, Rossenbacker T, Compennolle V, Rudy Y, Smits JF, Flameng W, Clancy CE, Moons L, Vos MA, Dewerchin M, Benndorf K, Collen D, Carmeliet E, Carmeliet P (2001) Abrupt rate accelerations or premature beats cause life-threatening arrhythmias in mice with long-QT3 syndrome. *Nat Med* 7:1021–1027. [CrossRef Medline](#)
- Patel RR, Barbosa C, Xiao Y, Cummins TR (2015) Human Nav1.6 channels generate larger resurgent currents than human Nav1.1 channels, but the Nav $\beta 4$ peptide does not protect either isoform from use-dependent reduction. *PLoS One* 10:e0133485. [CrossRef Medline](#)
- Raman IM, Bean BP (1997) Resurgent sodium current and action potential formation in dissociated cerebellar Purkinje neurons. *J Neurosci* 17:4517–4526. [CrossRef Medline](#)
- Raman IM, Sprunger LK, Meisler MH, Bean BP (1997) Altered subthreshold sodium currents and disrupted firing patterns in Purkinje neurons of Scn8a mutant mice. *Neuron* 19:881–891. [CrossRef Medline](#)
- Ritter DM, Zemel BM, Lepore AC, Covarrubias M (2015) Kv3.4 channel function and dysfunction in nociceptors. *Channels (Austin)* 9:209–217. [CrossRef Medline](#)
- Song W, Xiao Y, Chen H, Ashpole NM, Piekarczyk AD, Ma P, Hudmon A, Cummins TR, Shou W (2012) The human Nav1.5 F1486 deletion associated with long QT syndrome leads to impaired sodium channel inactivation and reduced lidocaine sensitivity. *J Physiol* 590:5123–5139. [CrossRef Medline](#)
- Tan ZY, Piekarczyk AD, Priest BT, Knopp KL, Krajewski JL, McDermott JS, Nisenbaum ES, Cummins TR (2014) Tetrodotoxin-resistant sodium channels in sensory neurons generate slow resurgent currents that are enhanced by inflammatory mediators. *J Neurosci* 34:7190–7197. [CrossRef Medline](#)
- Theile JW, Cummins TR (2011) Recent developments regarding voltage-gated sodium channel blockers for the treatment of inherited and acquired neuropathic pain syndromes. *Front Pharmacol* 2:54. [CrossRef Medline](#)
- Theile JW, Jarecki BW, Piekarczyk AD, Cummins TR (2011) Nav1.7 mutations associated with paroxysmal extreme pain disorder, but not erythromelalgia, enhance Nav $\beta 4$ peptide-mediated resurgent sodium currents. *J Physiol* 589:597–608. [CrossRef Medline](#)
- Themistocleous AC, Ramirez JD, Serra J, Bennett DL (2014) The clinical approach to small fibre neuropathy and painful channelopathy. *Pract Neurol* 14:368–379. [CrossRef Medline](#)
- Xie W, Strong JA, Ye L, Mao JX, Zhang JM (2013) Knockdown of sodium channel Nav1.6 blocks mechanical pain and abnormal bursting activity of afferent neurons in inflamed sensory ganglia. *Pain* 154:1170–1180. [CrossRef Medline](#)
- Xie W, Tan ZY, Barbosa C, Strong JA, Cummins TR, Zhang JM (2016) Upregulation of the sodium channel Nav $\beta 4$ subunit and its contributions to mechanical hypersensitivity and neuronal hyperexcitability in a rat model of radicular pain induced by local DRG inflammation. *Pain* 157:879–891. [CrossRef Medline](#)
- Yu FH, Westenbroek RE, Silos-Santiago I, McCormick KA, Lawson D, Ge P, Ferreira H, Lilly J, DiStefano PS, Catterall WA, Scheuer T, Curtis R (2003) Sodium channel $\beta 4$, a new disulfide-linked auxiliary subunit with similarity to $\beta 2$. *J Neurosci* 23:7577–7585. [CrossRef Medline](#)
- Zimmermann K, Leffler A, Babes A, Cendan CM, Carr RW, Kobayashi J, Nau C, Wood JN, Reeh PW (2007) Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures. *Nature* 447:855–858. [CrossRef Medline](#)