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A gain-of-function voltage-gated sodium channel 1.8 mutation drives intense hyperexcitability of A- and C-fiber neurons

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Abstract

Therapeutic use of general sodium channel blockers, such as lidocaine, can substantially reduce the enhanced activity in sensory neurons that accompanies chronic pain after nerve or tissue injury. However, because these general blockers have significant side effects, there is great interest in developing inhibitors that specifically target subtypes of sodium channels. Moreover, some idiopathic small-fiber neuropathies are driven by gain-of-function mutations in specific sodium channel subtypes. In the current study we focus on one subtype, the voltage-gated sodium channel 1.8 (Na_v1.8). Na_v1.8 is preferentially expressed in nociceptors and gain-of-function mutations in Na_v1.8 result in painful mechanical hypersensitivity in humans. Here, we used the recently developed gain-of-function Na_v1.8 transgenic mouse strain, *Possum*, to investigate Na_v1.8-mediated peripheral afferent hyperexcitability. This gain-of-function mutation resulted in increased mechanically-evoked action potential firing in subclasses of A β , A δ and C-fibers. Moreover, mechanical stimuli initiated bursts of action potential firing in specific subpopulations that continued for minutes after removal of the force and were not susceptible to conduction failure. Surprisingly, despite the intense afferent firing, the behavioral effects of the Na_v1.8 mutation were quite modest as only frankly noxious stimuli elicited enhanced pain behavior. These data demonstrate that a Na_v1.8 gain-of-function point mutation contributes to intense hyperexcitability along the afferent axon within distinct sensory neuron subtypes.

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Gain-of-function mutation in Na_v1.8 channel produces its most marked effect along the axon, resulting in intense spontaneous bursting and increased mechanically-evoked action potentials in specific afferent subpopulations.

1. Introduction

Voltage-gated sodium channels are fundamental to neuronal excitation. This family consists of channels with nine identified α -subunits that are expressed throughout the mammalian nervous system. One subunit, the sodium channel 1.8 ($\text{Na}_v1.8$ or *Scn10a*), is tetrodotoxin (TTX)-resistant and preferentially expressed in dorsal root ganglion (DRG) sensory neurons. More specifically, $\text{Na}_v1.8$ expression is localized to a large percentage of putative nociceptors [2], and its role in generating ectopic spontaneous activity of nociceptors during chronic inflammation, neuropathy and tissue injury continues to be of keen interest [37].

Human patients with idiopathic small-fiber neuropathy were found to have gain-of-function $\text{Na}_v1.8$ mutations that produce peripheral mechanical hypersensitivity and DRG neuron hyperexcitability [19]. The mutations in these patients were diverse in location along the $\text{Na}_v1.8$ peptide sequence, but all led to increased voltage-gated sodium currents in small-diameter DRG neurons, many of which are nociceptors [7; 19]. Recently, a mouse with a hypermorphic mutation to the *Scn10a* allele (*Scn10a^{Psm/Psm}*) was developed [7]. These mice have elevated sodium conductance and a peculiar phenotype termed “*Possum*”, which is characterized by full body immobility for approximately 30 seconds in response to scruffing of the neck [7]. Additionally, these mice display a cold hypersensitivity, electroencephalographic changes and sinus bradycardia.

Here, we investigated the functional consequence of a gain-of-function $\text{Na}_v1.8$ mutation in primary afferent fiber activity along the peripheral axon and terminals. We show that cutaneous afferent fibers in *Possum* mice have a profoundly dysfunctional phenotype in that they exhibit highly elevated action potential firing rates in response to sustained force applied to the peripheral terminals. Moreover, a subset of these fibers exhibit extended after-firing following mechanical activation. These observations confirm identification of specific afferent populations in which $\text{Na}_v1.8$ is functionally expressed and demonstrate how a gain-of-function $\text{Na}_v1.8$ mutation markedly enhances the excitability of both C- and A-type peripheral afferent fibers. This transgenic mouse may serve as a useful model for studying a variety of painful conditions where $\text{Na}_v1.8$ channels are implicated.

2. Methods

2.1 Animals

Adult male *Scn10a* mice of at least 7 weeks of age were used, which were either wild-type (*Scn10a^{WT}*) or hypermorphic (*Scn10a^{Psm/Psm}*) with a point mutation in the *Scn10a* gene on a C57Bl/6J background as previously described [6]. Mice were anesthetized by isoflurane and killed by either cervical dislocation for skin-nerve experiments or decapitation for patch-clamp and calcium imaging experiments. All animals were maintained by the Medical College of Wisconsin and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. Experimenters were blinded to mouse genotype throughout the data collection for behavioral experiments.

2.2 Behavior

Each behavioral test was conducted independently, separated by at least 2 days. Mechanical threshold was assessed on the plantar side of the hindpaw by measuring the 50% paw withdrawal threshold with a series of calibrated von Frey filaments (0.38 to 37 mN) using the Up-Down method [9; 16]. The frequency of withdrawal to suprathreshold mechanical stimuli was evaluated as a measure of mechanical responsiveness to punctate stimuli within the purported noxious range [23; 48]. To perform this test we applied a 3.31 mN von Frey monofilament to the plantar surface of each paw ten times, alternating between paws with at least 5 seconds between applications. In addition, a frankly noxious mechanical test was performed by applying a spinal needle to the plantar hindpaw and alternating between paws with at least 10 seconds between applications. To test dynamic light touch, we used a 7 mm paintbrush to stroke each hindpaw posterior to anterior.

To assess responsiveness to external pungent chemical stimuli, a mustard oil (MO, allyl isothiocyanate, Sigma-Aldrich, St. Louis, MO) assay was performed by applying 10 μ l of either 10% MO diluted in mineral oil or a mineral oil vehicle control to the glabrous hindpaw skin. The number of paw lifts and licks were recorded, and combined. In an additional experiment, we systemically blocked activity of Na_v channels prior to MO application using an intraperitoneal (i.p.) injection of the antagonist, A-803467 (Tocris Bioscience, Bristol, UK), which has been reported to block $\text{Na}_v1.8$ at a 30 mg/kg concentration diluted in 5% dimethyl sulfoxide / 95% polyethylene glycol [27]. Mice were euthanized following the MO experiments.

2.3 Teased fiber skin-nerve recordings

The *ex vivo* hairy skin-saphenous nerve preparation was utilized to determine mechanical response properties of cutaneous primary afferent fibers in $\text{Scn10a}^{\text{WT}}$ and $\text{Scn10a}^{\text{Psm/Psm}}$ mice following established protocols [34; 42]. Briefly, *ex vivo* skin-nerve preparations were dissected and immediately placed into the recording chamber superfused with oxygenated synthetic interstitial fluid at $32 \pm 0.5^\circ\text{C}$ [32]. The skin was placed in the chamber corium side up. The saphenous nerve was desheathed and fascicles teased apart until functionally single fibers could be distinguished through the use of a mechanical search stimulus. Due to the $\text{Scn10a}^{\text{Psm/Psm}}$ phenotype where mechanically-evoked bursts (MEB) were elicited by mechanical stimuli, we had to use a combination of a blunt glass rod or 4 mN von Frey filament followed by an electrical search stimulus to identify single units. The blunt glass rod allowed for the rapid identification of active afferent fibers. In some cases, the glass rod evoked the MEB response before the fiber could be characterized, and therefore we also used a 4 mN von Frey filament to mechanically identify active fibers. To confirm that a single, identifiable unit would be recorded, we then applied a small electrical stimulus that would trigger a response from any other fibers that may present on the electrode. Mechanically-insensitive units were identified in both $\text{Scn10a}^{\text{WT}}$ and $\text{Scn10a}^{\text{Psm/Psm}}$ mice, but were not included in the results of this study. Fibers were characterized by conduction velocity and mechanical threshold using calibrated von Frey filaments (range 0.044–147.0 mN). Conduction velocity was measured by inserting a Teflon-coated steel needle into the most mechanically-sensitive area of the receptive field and applying square-wave pulses (500 μ s), and the action potential latency and the distance between electrodes were

quantified. We classified units by their conduction velocity and adaptation properties as previously described [34]. Fibers conducting > 10 m/sec were A β fibers, those conducting 1.2–10 m/sec were A δ fibers and those conducting < 1.2 m/sec were C-fibers. The A β -fibers were further classified as rapidly adapting (RA) or slowly adapting (SA) based on adaptive properties to force. SA fibers responded throughout a sustained mechanical force and adapted slowly to the force, whereas RA fibers responded primarily at the onset and offset of force. The SA-A β fibers were further subdivided into low-threshold, which began responding to forces below 4 mN with an initial burst of action potentials followed by a decay, and high-threshold, which only began to respond to forces at 4 mN or higher with a regular firing rate during sustained force [34; 38]. The A δ -fibers were classified as either slowly adapting A-mechanoreceptors (AM) or rapidly adapting Down-hair (D-hair) receptors. A feedback-controlled probe (0.8 mm ceramic cylinder) was used to apply sustained increasing forces (10, 20, 40, 70, 100, and 150 mN) for 10 seconds to the most mechanically-sensitive area of the receptive field, with at least 1 min between applications. Action potential waveforms were visualized on an oscilloscope and audibly monitored using an audio amplifier and speaker. Action potentials were recorded, discriminated and analyzed using the data acquisition software LabChart 6 (ADInstruments, Colorado Springs, CO) on a PC for offline analysis.

2.4 Dorsal root ganglion cell culture

Lumbar dorsal root ganglia (DRG) 1–6 were isolated bilaterally and placed into 1 ml Hank's Balanced Salt Solution (Thermo Fischer Scientific, Waltham, MA). Neurons were cultured as previously described [3]. Ganglia were dissociated into single somata via enzymatic digestion and trituration through a P200 pipette tip. The neurons were plated onto laminin-coated glass coverslips and incubated for 2 hours to allow adherence. The neurons were then provided with complete cell medium consisting of DMEM and Hams-F12, supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine, 0.8% D-glucose, 100 units penicillin and 100 μ g/ml streptomycin. No growth factors were added. Calcium imaging was performed 18–24 hours after the neurons were plated and whole-cell patch clamp recordings were performed 20–35 hours after plating. Only small-diameter somata (< 27 μ m) were used for calcium imaging (Scn10a^{WT} $n=3$ animals; Scn10a^{Psm/Psm} $n=3$ animals) and whole-cell patch clamp experiments (Scn10a^{WT} $n=5$ animals; Scn10a^{Psm/Psm} $n=5$ animals) to enrich for C-fiber type neurons.

2.5 Whole-cell patch clamp recordings

Whole-cell patch clamp recordings were performed on cultured sensory neurons in a chamber superfused with extracellular buffer containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose; pH of 7.4 ± 0.03 and osmolality of 310 ± 3 mOsm. Patch pipettes had resistances of 2.4–5.5 M Ω and were backfilled with a solution containing 135 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 0.2 mM NaGTP, 2.5 mM ATPNa₂, and 10 mM HEPES. This intracellular buffer had a pH of 7.20 ± 0.03 and an osmolality of 290 ± 3 mOsm.

Neurons were stained with Isolectin B4 (IB4) (10 μ g/mL, Sigma-Aldrich) prior to commencement of recordings. Once a stable whole-cell patch was obtained and cell

capacitance was compensated for, spontaneous action potential activity was determined through 1-minute recordings where current was clamped to 0 pA. Rheobase measurements were obtained by injecting square pulse currents of 10 ms duration of increasing magnitude. Neurons were then returned to voltage clamp mode and focal mechanical stimulation of the soma was performed using a closed glass pipette (tip diameter = 1–2 μm) attached to a piezo stack actuator (PA25, Piezosystem, Jena) moving at 3.5 $\mu\text{m}/\text{ms}$. Neurons were mechanically stimulated in 1.8 μm increments up to 10.9 μm or until the patch seal was lost, whichever came first. Each mechanical step was 200 ms in duration and mechanical steps were separated by at least 25 seconds to avoid sensitization or desensitization. Recordings where the seal could not be maintained for at least 3.6 μm of stimulating pipette displacement were discarded, and recordings were only performed if the leak current was ≤ 200 pA. Series resistances were kept to <10 M Ω and compensated at 60%.

Recordings utilized an EPC9 amplifier (HEKA, Germany) and Pulse software (HEKA, Germany). Off-line analysis was completed using PulseFit and FitMaster software (HEKA, Germany). Baseline to trough noise averaged 9.27 ± 0.44 pA; thus, only currents with magnitudes ≥ 20 pA were considered responses, and current kinetics were determined by fitting the current obtained at the largest mechanical step with a monoexponential decay equation. The time constant of inactivation (τ_1) was then used to categorize neurons as rapidly adapting (RA; $\tau_1 \leq 10$ ms), intermediately adapting (IA; $10 < \tau_1 \leq 30$ ms), slowly adapting (SA; $\tau_1 > 30$ ms), or mechanically insensitive (MI).

2.6 Calcium imaging

Calcium imaging was performed using the dual-wavelength fluorescence indicator FURA-2AM (Thermo Fisher Scientific) as described previously [3]. Briefly, isolated DRG neurons were loaded with 2.5 $\mu\text{L}/\text{ml}$ FURA-2AM in extracellular buffer containing 2% BSA for 45 min at room temperature, followed by 30 min wash in extracellular buffer for de-esterification. Neurons were superfused at 6 ml/min using an AutoMate pressurized perfusion system. Experiments were conducted at room temperature ($23 \pm 1^\circ\text{C}$). Fluorescence images were captured with a cooled CCD camera (CoolSNAP FX; Photometrics, Tucson, AZ). MetaFluor imaging software was utilized in order to detect and analyze intracellular calcium changes throughout the experiment (Molecular Devices, Sunnyvale, CA). Baseline measurements were recorded during superfusion with extracellular buffer. Next, either 30 or 100 μM MO was applied for 1 min. A $\geq 20\%$ increase in intracellular calcium from baseline was considered a response. At the end of each protocol, 50 mM KCl solution was applied to depolarize neurons, thereby allowing for identification of viable neurons.

2.7 Data analysis

Single fiber data was compared between Scn10a^{Psm/Psm} mice and Scn10a^{WT} controls. For each fiber type, mechanical threshold was compared using Mann-Whitney *U* test for non-parametric data, conduction velocity was compared using student's *t*-test, and the number of mechanically-evoked action potentials across the force range was compared using two-way ANOVA for parametric data using Prism 5 software (GraphPad, La Jolla, CA). For behavioral tests, responsiveness in the needle test was compared using a Fisher's Exact Test.

Mechanical threshold was analyzed with a Mann-Whitney U test for non-parametric data when using a series of discrete von Frey filaments for comparison. The frequency test, dynamic light touch assay and number of hyperalgesic responses during the needle test were compared using student's t -tests. For MO experiments, responses between genotypes and treatment groups were compared using a one-way ANOVA with Tukey's post-hoc analysis. For patch-clamp experiments, mechanical thresholds between subtypes and genotypes were compared using a one-way ANOVA. Current densities in response to mechanical steps from 1.8 – 10.9 μm were compared between groups using a 2-way ANOVA. Current kinetics were examined between groups using a Chi-Square test followed by Fisher's exact test for specific group comparisons. Spontaneous action potential firing was compared between $\text{Scn10a}^{\text{Psm/Psm}}$ and $\text{Scn10a}^{\text{WT}}$ using a student's t -test. For calcium imaging, the percent responders to MO were compared between $\text{Scn10a}^{\text{Psm/Psm}}$ and $\text{Scn10a}^{\text{WT}}$ neurons using the Fisher's Exact test. The amplitude of responses to MO was compared using an unpaired t -test.

3. Results

3.1 Marked increase in mechanically-evoked action potentials in nociceptors from $\text{Na}_v1.8$ mutant mice

Since the physiological consequences of gain-of-function $\text{Na}_v1.8$ mutations at the cellular level have not been well explored, we first asked how the mutation might modulate action potential firing rates in cutaneous afferent neurons. We recorded mechanically-evoked action potentials from the peripheral axons of saphenous nerve fibers, which include almost entirely cutaneous afferents innervating the hairy skin of the dorsal hindpaw. Because $\text{Na}_v1.8$ is expressed in both A- and C-fibers in rat and mice [17; 22; 44], we expected to find measurable differences in afferent sensitivity across several afferent fiber subtypes in *Possum* mice. We first determined whether A-mechanoreceptor (AM) and C-fiber nociceptors were affected by the $\text{Na}_v1.8$ hypermorphism. We recorded from single afferent fibers and found that the mechanically-evoked action potential firing rate more than doubled in these nociceptors. C-fibers from $\text{Scn10a}^{\text{Psm/Psm}}$ mice fired 2.5-fold more action potentials when averaged across all forces tested (10 to 150 mN; 10 sec each) (Fig. 1A) than $\text{Scn10a}^{\text{WT}}$ controls (Fig. 1A). AM fibers displayed a 2-fold increase in overall action potential firing rate over wild-type controls (Fig. 1B). We found a slight increase in conduction velocity in AM fibers, but not C-fibers, from $\text{Scn10a}^{\text{Psm/Psm}}$ mice compared to controls. There were no differences in von Frey mechanical thresholds for either fiber type (Table 1). Collectively, these data suggest that the $\text{Na}_v1.8$ mutation ($\text{Scn10a}^{\text{Psm/Psm}}$) results in hyperexcitability in the form of enhanced suprathreshold firing of both myelinated and unmyelinated primary afferent fibers to force.

3.2 Intense after-stimulus action potential firing in nociceptors from $\text{Na}_v1.8$ mutants

During recordings of mechanical response properties, we noted that many fibers continued firing after cessation of mechanical stimulation. We then quantified the after-stimulus firing, or mechanically-evoked bursts (MEBs). We found a large portion of hyper-responsive fibers among both AM and C-fibers from $\text{Scn10a}^{\text{Psm/Psm}}$ mice. A total of 65% of C-fibers and 90% of AM fibers exhibited MEBs of action potentials (Fig. 1C). These MEB trains lasted as

long as 40 minutes, and averaged 544 sec in C-fibers and 220 sec in AM fibers (Fig. 1D). Examples of selected MEB trains are shown in Figures 1E and 1H. The MEB trains were generally not observed at low forces (10 and 20 mN) in either C-fibers or AM fibers, and most trains occurred at forces $\geq$ 40 mN (Fig. 1F, 1I).

To determine whether action potential firing rates during MEB trains differed from the firing rates that occurred during mechanical stimulation, we compared the peak action potential firing rates during each of these segments. No differences were found between action potential firing rates during or post stimulation in either C-fibers or AM fibers (Fig. 1G, 1J).

Because of the pronounced after-firing in response to mechanical stimulation, we wondered if another sensory modality could elicit this post-stimulation bursting phenotype. We applied a 20-sec, 32–2°C cold ramp to C-fiber receptive fields and were able to elicit a cold-evoked burst of action potentials post-stimulation that was similar to the MEBs (Supplementary Fig. 1). Since $\text{Na}_v1.8$ is neither a direct mechanical or cold transducer, these results suggest that noxious stimuli, independent of modality, applied to peripheral terminals of $\text{Na}_v1.8$ -expressing nociceptors are capable of evoking post-stimulation action potential discharge.

3.3 Marked increase in mechanically-evoked action potentials in fast-conducting A β -fibers

Though initially thought to be nociceptor specific, $\text{Na}_v1.8$ has been shown to be expressed widely in DRG neurons, including in somata of A β fibers [10; 44]. Therefore we also measured mechanically-evoked action potentials in A β -fibers that are generally considered to be non-nociceptive. No overall differences in mechanically-evoked action potentials were observed in slowly-adapting A β (SA-A β) fibers between $\text{Scn10a}^{\text{Psm/Psm}}$ and controls (Fig. 2A). Interestingly, within the SA-A β fibers, we found a hyperexcitable subpopulation based on mechanical threshold. Low-threshold SA-A β fibers, characterized by mechanical thresholds less than 4 mN, displayed no difference in mechanically-evoked action potential firing (Fig. 2B). However, high-threshold SA-A β fibers, characterized by mechanical thresholds greater than 4 mN, exhibited a nearly 2-fold increase in mechanically-evoked action potentials in $\text{Scn10a}^{\text{Psm/Psm}}$ mice compared to controls (Fig. 2C). Surprisingly, we also found increased mechanically-evoked action potentials in rapidly-adapting A β (RA-A β) fibers, which fired 2.5-fold more action potentials over all forces in $\text{Scn10a}^{\text{Psm/Psm}}$ compared to controls (Fig. 2D). No differences were observed in rapidly adapting A δ (D-hair) fibers (Fig. 2E). The von Frey mechanical thresholds and conduction velocities did not differ between genotypes in any of the A β or D-hair populations (Table 1). In sum, the changes in excitability in light-touch A β -fibers were restricted to specific subpopulations.

3.4 Specific A β -fiber subpopulations are hyperexcitable

Much like what was observed in the C- and AM fiber populations, we found that half of the high-threshold SA-A β fibers of $\text{Scn10a}^{\text{Psm/Psm}}$ mice exhibited MEBs (Fig. 2F). These mechanically-evoked bursts averaged 189 seconds in duration (Fig. 2G). A typical example is shown in Figure 2H. The MEB trains were primarily observed beginning at 40 mN with the most substantial trains occurring at 100 mN (Fig. 2I). No differences were observed between action potential firing rates during the mechanical stimulus and post-stimulus MEBs in $\text{Scn10a}^{\text{Psm/Psm}}$ SA-A β fibers (Fig. 2J).

3.5 No differences in response to focal mechanical stimulation of the soma membrane in patch-clamp recordings

We next used whole-cell patch-clamp recordings of isolated lumbar dorsal root ganglia neurons to determine whether hyperexcitability occurs at the level of the soma of putative nociceptors. We focused on small-diameter neurons, which, in general, correspond to C-fiber neurons *in vivo* [15]. Neurons were further subtyped by Isolecetin B4 (IB4)-binding, which is a broad marker for non-peptidergic neurons [3; 39]. We hypothesized that IB4 may be a marker of the hyperexcitable C-fiber subpopulation (65%) since a similar percentage of Na_v1.8-positive DRG neurons in rat (60%) and mouse (40%) co-stain with IB4 [21; 44]. On the other hand, the Na_v1.8 current reportedly contributes to the generation of multiple, non-decremental action potentials in IB4-negative small-diameter DRG neurons [10].

To test hyperexcitability at the soma, we first examined responses to focal mechanical stimulation of Scn10a^{Psm/Psm} and Scn10a^{WT} small-diameter neurons. We found no overall differences in peak mechanical current densities between Scn10a^{Psm/Psm} and control neurons (data not shown). Additionally, there were no differences between IB4-positive and IB4-negative subgroups of the two genotypes for mechanical thresholds (Fig. 3A) or current densities over a range of 1.8 to 10.95 μ m mechanical stimulation steps (Fig. 3B). We investigated this further by evaluating specific subpopulations identified by the decay kinetics of the mechanically-evoked currents, which included rapid, intermediate and slow current inactivation (Fig. 3C). Again, no differences were observed between Scn10a^{Psm/Psm} and Scn10a^{WT} neurons for any category of mechanical current in either IB4 positive or negative small neurons (Fig. 3D).

To determine if the MEB activity we observed at the fiber level could also be observed in patch-clamp recordings of the DRG somata, we recorded action potential firing following mechanical stimulation in neurons under current clamp mode. Surprisingly, we rarely observed continued action potential firing after cessation of the mechanical stimulus in either Scn10a^{Psm/Psm} (21%) or Scn10a^{WT} (17%) neurons. Because Na_v channelopathies can include spontaneous activity in nociceptors, we also recorded non-evoked (ongoing) action potential firing in the soma. Interestingly, we found increased spontaneous firing rates in Scn10a^{Psm/Psm} small-diameter neurons, compared to wild-type neurons (Fig. 3E). In sum, these data demonstrate that the MEB activity observed at the afferent level is likely due to hyperexcitability along the peripheral axon toward the central terminal, but is not due to sensitization of mechanically sensitive ion channels.

3.6 Scn10a^{Psm/Psm} mice exhibit hyperalgesic responses only to frankly noxious mechanical stimuli

A fascinating aspect of the *Possum* phenotype is that the extreme hyper-responsiveness at the cutaneous afferent level did not translate into a measureable behavioral response using paw withdrawal threshold, a traditional assay of mechanical sensitivity [7]. In agreement, early reports using the Na_v1.8-deficient mice also revealed no differences in paw withdrawal threshold but did show nearly a total loss in response to noxious mechanical stimulation of the tail [1]. With such a marked phenotype in the cutaneous afferent fibers, we expanded the mechanical tests to include a wider range of mechanical assays would reveal differences

with the $Scn10a^{Psm/Psm}$ mice. Therefore, we used repeated application of a 3.31 mN von Frey monofilament to determine if enhanced behavioral response requires extended suprathreshold stimulation, a dynamic light touch stroke using a 7 mm paintbrush to examine responses to light mechanical sensations, and the Up-Down method to confirm paw withdrawal thresholds [9; 16] (Fig. 4 A–C). Despite using this array of tactile stimuli, we still found no difference in behavioral responses between $Scn10a^{Psm/Psm}$ and wild-type mice.

We therefore hypothesized that traditional behavioral assays for mechanical sensitivity may not reach the “noxious” range that elicits the afferent fiber hyper-responsiveness observed in the skin-nerve preparation. We reasoned that a spinal needle behavioral assay would deliver an overtly noxious punctate mechanical stimulus that has a greater potential to reach the hyperexcitable range without causing injury [24]. Using the needle assay, we found response rates of 100% in $Scn10a^{Psm/Psm}$ mice and 97% in wild-type controls (Fig. 4D). To better identify the severity of the response, we also recorded specific response types elicited by the needle, which ranged from a fast paw withdrawal (flinch) to a hyperalgesic-type response (fluttering, licking, and/or extended elevation of hindpaw in the air) [24]. The $Scn10a^{Psm/Psm}$ mice exhibited nearly 3-fold more hyperalgesic responses to the needle (Fig. 4E). Together, these data suggest that intense mechanical stimuli in the overtly noxious range is required to elicit the hyperexcitable afferent response at the receptive terminal and subsequently evoke a hyperalgesic behavioral response. This also may explain why these animals appear to function normally in their living environment, without obvious signs of discomfort.

3.7 $Scn10a^{Psm/Psm}$ mice have increased behavioral responses to noxious chemical stimuli

We also asked whether the *Possum* phenotype could only be elicited by scruffing the neck. Blasius *et al.* suggested that the behavior may be part of the flight response [7], though it is not clear if the *Possum* mice are simply displaying the classic prey response or if they become immobilized with severe pain from the mechanically-stimulated hyperexcitable afferents. Therefore, we applied mustard oil (MO; allyl isothiocyanate; 10%) to the glabrous hindpaw skin to chemically stimulate nociceptor subpopulations in the similar region used for skin-nerve experiments. MO is a potent and noxious agonist of the Transient Receptor Potential Ankyrin 1 (TRPA1) channel [3; 29], a channel that is involved in the detection of a diverse array of stimuli, including mechanical force [8; 34]. Following application of MO to the hindpaw, we found that the $Scn10a^{Psm/Psm}$ mice entered into a *Possum*- or torpor-like state, remaining relatively motionless for much of the 5-minute experimental period. As a result, the number of responses (lift and licks) to MO averaged 2.0 ± 0.7 in $Scn10a^{Psm/Psm}$ mice compared to 10.4 ± 0.7 in $Scn10a^{WT}$ mice (Fig. 5A). These data suggest that the over-excitation of afferent terminals, likely those that express TRPA1, is capable of driving the *Possum* behavior. To determine if $Na_v1.8$ -expressing fibers contribute to this phenotype, we performed an i.p. injection of the Na_v channel blocker, A-803467 (30 mg/kg) and repeated the MO experiments in both $Scn10a^{Psm/Psm}$ and $Scn10a^{WT}$ mice. A-803467 has been reported to be selective and useful tool for identifying $Na_v1.8$ contribution to afferent excitation [27; 45], however recent reports have shown evidence that it may also interact with other Na_v channels and voltage-gated calcium

channels [5; 46]. We found that $Scn10a^{Psm/Psm}$ mice no longer entered a *Possum*- or torpor-like state phenotype and exhibited an average of 5.3 ± 0.4 responses to MO following administration of A-803467 (Fig. 5A). The response was similar to that in $Scn10a^{WT}$ mice, which exhibited a partially inhibited MO response, with an average of 6.0 ± 1.4 responses (Fig. 5A).

Therefore, we further investigated the relationship between TRPA1 and the hyper-responsive afferent subpopulation. We conducted functional calcium imaging on isolated small-diameter lumbar DRG neurons using both modest concentration (30 μ M) and a near maximal concentration (100 μ M) of MO. We found that the percentage of neurons that responded to MO between genotypes did not differ at either concentration (Fig. 5B). Of the neurons that responded to MO, we found that the response amplitude was similar between genotypes (Fig 5C). Together with the patch-clamp data, these findings indicate that differences in excitability do not likely occur at the level of the soma. Thus, the gain-of-function mutation appears to primarily drive hyperexcitability along the peripheral axon.

4. Discussion

Sodium channel polymorphisms have been implicated in a diverse array of neuropathies, though the mechanisms that underlie the associated pain remain enigmatic. Thus, determining how specific gain-of-function mutations of voltage-gated sodium channels lead to the hyperexcitability of sensory neurons along the axon and at the terminals is important as more effective pharmacological therapeutics are developed. Here we show that cutaneous primary afferent neurons of *Possum* mice that have a $Na_v1.8$ hypermorphism display a striking phenotype: subpopulations of both A- and C-fibers exhibit markedly increased mechanically-evoked action potential firing rates throughout a wide range of forces and the firing often extends well beyond the stimulus into mechanically-evoked bursting. Interestingly, these animals exhibited hyperalgesia only to an overtly noxious mechanical stimulus, but not to other types of lower-intensity tactile stimuli. Additionally, we found that chemical activation of TRPA1 in the skin *in vivo* induced a *Possum*-like behavioral phenotype where mice became immobilized.

4.1 Functional $Na_v1.8$ is broadly expressed across primary afferent subpopulations

The $Na_v1.8$ channel is preferentially expressed in DRG neurons; however, there has been debate regarding the specific neuronal subpopulations that express it [17; 21; 41; 44] and growing evidence suggests that its expression may not be nociceptor specific. Therefore, it is important to identify the populations of sensory neurons where $Na_v1.8$ plays a functional role in transmission of nociceptive information to the spinal cord. In this study, we used the hypermorphic $Na_v1.8$ mutant combined with functional recordings of identified sensory neurons in order to determine how a gain-of-function $Na_v1.8$ mutation alters signaling in specific cutaneous afferent fiber populations.

Here we report that *Possum* mice exhibited increased action potential firing in subtypes of cutaneous A β , A δ and C-fibers, which is consistent with data showing that $Na_v1.8$ is expressed by A- and C-fiber subpopulations [44]. However, it remains unclear which of these fiber subpopulations subserve a nociceptive role. While SA-A β fibers are typically

categorized as low threshold mechanoreceptors that detect pressure (SAI) or stretch (SAII) [28], approximately 20% of SA-A β fibers have been postulated to be nociceptive [18; 49; 50]. A subset of the SA-A β fibers (25%) also exhibited enhanced firing in the Na $_v$ 1.8 mutant. These SA-A β fibers exhibited higher von Frey thresholds ($\sim$ 4 mN), steadily firing throughout the duration of each sustained force with increased overall firing rates, which resemble the properties of AM nociceptors. We also found enhanced firing in RA-A β neurons, which innervate hair follicles in hairy skin [35; 40] and Meissner's corpuscles in glabrous skin [26]. We cannot rule out that Na $_v$ 1.8-positive, RA-A β fibers might contribute to nociception; however, it is generally agreed that these afferents are not nociceptors.

Of the A δ fibers, only the AM fibers exhibited a hyperexcitable phenotype. AM fibers are largely considered to be nociceptive, and this is consistent with our data showing that approximately 90% of AM fibers were hyperexcitable. The other category of A δ fibers, D-hair afferents, were unaffected. This is consistent with data showing that D-hair afferents encode movement of zigzag and aucho hair follicles [35], suggesting that they likely contribute to gentle touch. Thus, although Na $_v$ 1.8 is functionally expressed across many fibers, it is likely that subpopulations of AM fibers, C-fibers and possibly high-threshold SA-A β fibers may contribute to mechanical hyperalgesia during ongoing pain.

4.2 How might Na $_v$ 1.8 kinetics contribute to MEBs in Possum neurons?

Genetic mutation of sodium channels expressed in small-diameter DRG neurons can result in hyperexcitability and other changes in channel activation kinetics. These functional properties have been reported to result in increased pain perception [19; 25]. The increased pain perception may arise, in part, from heightened spontaneous action potential firing [36; 47]. Indeed, we observed increased spontaneous action potential firing in Scn10a^{Psm/Psm} small-diameter neurons in the current study. Additionally, the tetrodotoxin-resistant channel Na $_v$ 1.8 and the tetrodotoxin-sensitive channel Na $_v$ 1.7 (Scn9a) have been shown to lower action potential thresholds [11; 33; 51], thereby increasing the probability that these neurons will fire action potentials spontaneously. Consequently, a single point mutation within a highly conserved region within Na $_v$ 1.7 has been reported to result in decreased in voltage activation threshold, increased action potential firing and slower inactivation rates leading to increased pain [11; 14; 20].

DRG recordings from *Possum* small-diameter neurons revealed decreased rheobase, or the amount of depolarizing current required to elicit an action potential, and a slowing of the slowly inactivating current [7]. Because the slowly inactivating Na $_v$ 1.8 current contributes to repeated action potential generation [6; 43] it is possible that the slowed current in *Possum* gain-of-function neurons facilitates the extended action potential firing observed in the current study. Collectively, these changes may increase excitability of these gain-of-function Na $_v$ 1.8-expressing neurons, resulting in the enhanced action potential firing rate and MEBs. This type of shift may be responsible for the pain associated with gain-of-function sodium channel mutations in humans.

4.3 Relatively normal behavior despite intense afferent fiber firing in Possum mice

A surprising aspect of the study was that the gain-of-function $\text{Na}_v1.8$ mutation did not result in marked changes in behavior. Studies have shown upregulation of $\text{Na}_v1.8$ following inflammation in animal models [4]. Increased $\text{Na}_v1.8$ expression levels have been shown to contribute to nocifensive behaviors that include mechanical and thermal hypersensitivity [27; 30]. Further, a recent study showed that optogenetic activation of $\text{Na}_v1.8$ -expressing afferents evoked prominent nocifensive behaviors including enhanced paw withdrawal, licking, jumping and vocalization [12]. Given the ongoing spontaneous activity, intense mechanically-evoked firing, and prolonged mechanically-evoked bursts post-stimuli that we observed in the afferent fibers of *Possum* mice, we expected to find increased nocifensive responses across all of the behavioral assays used in the current study. However, this did not occur. Our behavioral data showed enhanced responsiveness only to frankly noxious mechanical stimuli (needle test) and chemical activation via mustard oil.

One possible explanation of the disconnection between behavior and afferent firing is that *Possum* mice develop enhanced engagement of descending inhibitory pathways, which tonically suppress the intense afferent input [13]. For instance, the lack of ongoing pain behavior *despite* the hyperexcitable afferent input may be a result of continuous release of antinociceptive compounds, including endogenous cannabinoids that may decrease activity at the spinal cord level or higher [31]. Thus, the ongoing afferent input in *Possum* mice may be attenuated by descending neural control, enabling normal behavioral responses to innocuous to moderate mechanical forces and only frankly noxious input can override the tonic descending inhibition to evoke pain behavior. Collectively, our data demonstrate that a gain-of-function $\text{Na}_v1.8$ point mutation drives hyperexcitability along the axon of distinct sensory neuron subtypes, and opens the door for future studies to investigate how descending inhibitory pathway can tonically suppress nociceptive afferent input.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Scn10a^{Psm/Psm} nociceptors exhibited increased and extended mechanically-evoked action potential firing. Hairy skin-saphenous nerve recordings revealed that action potential firing rate was increased during sustained force (10 s) in both (A) C-fibers and (B) AM fibers from *Scn10a*^{Psm/Psm} mice. (C) Mechanically-evoked bursts (MEBs) of action potentials in response to mechanical stimulation were present in 65% of C-fibers and 90% of AM fibers of *Scn10a*^{Psm/Psm} mice. (D) The duration of these MEBs averaged 544 sec in C-fibers and 220 sec in AM fibers. (E) Examples of action potential firing to a 40 mN sustained force in *Scn10a*^{Psm/Psm} (Psm/Psm) and *Scn10a*^{WT} (WT) C-fibers. Note that only *Scn10a*^{Psm/Psm} preparations exhibited MEBs. (F) MEBs were observed at all forces tested (10, 20, 40, 70, 100 and 150 mN), but were minimal until forces reached 40 mN and higher. (G) No differences in C-fiber action potential firing rates were observed between those fired during the sustained force at 70 mN and at the maximal post-stimulation burst. (H) Examples of action potential firing to a sustained 100 mN force in *Scn10a*^{Psm/Psm} and *Scn10a*^{WT} AM fibers. Note that only *Scn10a*^{Psm/Psm} fibers exhibited MEBs. (I) MEBs were observed at all forces tested, but the greatest numbers of post-stimulation action potentials were fired at 40 mN and higher. (J) No differences in AM fiber action potential firing rates were observed between those fired during the sustained force at 70 mN and at the maximal post-stimulation burst. *** $P < 0.001$ and † $P < 0.05$. Data reported as mean $\pm$ s.e.m.

Figure 2.

Scn10a^{Psm/Psm} A-fiber subpopulations exhibit increased extended mechanically-evoked bursts of action potentials following stimulation. Action potential firing rate in response to sustained force (10 s) was similar between *Scn10a*^{Psm/Psm} and controls in (A) slowly-adapting A β fibers (SA-A β) and (B) specifically within the low-threshold SA-A β fiber subpopulation. (C) The *Scn10a*^{Psm/Psm} high-threshold SA-A β fiber subpopulation exhibited increased firing rates compared to controls; this was also seen in (D) rapidly-adapting A β fibers. (E) No differences between genotypes were observed between rapidly-adapting down-hair A δ fibers (D-hair). (F) Mechanically-evoked bursts (MEBs) were present in 5% of low-threshold SA-A β and 50% of high-threshold SA-A β fibers from *Scn10a*^{Psm/Psm} (G) that lasted nearly 4 min in high-threshold SA-A β fibers. (H) Examples of action potential firing to a 100 mN sustained force in *Scn10a*^{Psm/Psm} and *Scn10a*^{WT} high-threshold SA-A β fibers. Note that only *Scn10a*^{Psm/Psm} exhibited MEBs in all recordings. (I) MEBs were observed at all forces tested, but the greatest numbers of post-stimulation action potentials were fired at 40 mN and higher. (J) No differences in action potential firing rates were observed between those fired during the sustained force at 70 mN and at the maximal post-stimulation burst. ** $P < 0.01$. Data reported as mean $\pm$ s.e.m.

Figure 3.

Whole-cell patch-clamp recording revealed no difference in response to focal mechanical stimulation. Small-diameter lumbar dorsal root ganglion cells were stimulated with a punctate mechanical force for 200 ms in 1.8 μ m increments up to 10.9 μ m. Cells were subtyped by Isolectin B4 (IB4)-binding, a marker for non-peptidergic neurons. We found no differences in (A) mechanical thresholds between Scn10a^{Psm/Psm} and Scn10a^{WT} cells in IB4-positive and IB4-negative cells. (B) Peak current density did not differ between genotypes and IB4 binding subtypes across mechanical steps. (C) Examples of slowly-adapting, intermediately-adapting and rapidly-adapting recordings. (D) No differences were observed in the percentages of cells that had slowly, intermediately or rapidly adapting responses, or that were mechanically insensitive between Scn10a^{Psm/Psm} and Scn10a^{WT} cells in their respective IB4-positive and IB4-negative groups. (E) The percentage of non-evoked, spontaneous firing rates was higher in Scn10a^{Psm/Psm} small-diameter neurons, compared to controls. (A), (B) and (E) data reported as mean $\pm$ s.e.m.

Figure 4.

Scn10a^{Psm/Psm} mice exhibited increased sensitivity to overtly noxious stimuli. All behavioral tests were administered by placing enclosed mice on an elevated mesh screen and applying force to the glabrous skin of the hindpaw. For mechanical stimulation, left and right hindpaw responses were counted and averaged to calculate the percent response for paw withdrawal threshold. The experimenter was blinded to genotype. Mechanical sensitivity to (A) blunt punctate force using repeated application of a 3.31 von Frey filament (*Scn10a*^{WT} *n*=10; *Scn10a*^{Psm/Psm} *n*=12) or (B) light touch using a 7 mm paintbrush revealed no differences between *Scn10a*^{Psm/Psm} and controls (*Scn10a*^{WT} *n*=10; *Scn10a*^{Psm/Psm} *n*=11). (C) Paw withdrawal threshold using the Up and Down method also revealed no differences between genotypes (*Scn10a*^{WT} *n*=10; *Scn10a*^{Psm/Psm} *n*=12). (D) No differences in response frequency to spinal needle prick were observed; however, (E) *Scn10a*^{Psm/Psm} mice exhibited an increase in hyperalgesic responses to the needle (*n*=14 per genotype). * *P*<0.05 and *** *P*<0.001. Data reported as mean ± s.e.m.

Figure 5. *Scn10a*^{Psm/Psm} and *Scn10a*^{WT} mice responded similarly to the noxious TRPA1 agonist, mustard oil. (A) Application of mustard oil (MO) to the glabrous hindpaw skin resulted in a *Possum*-like behavior in *Scn10a*^{Psm/Psm} mice. As a result they responded less (lifts and licks) than wild-type controls. Injection of A-803467 resulted in a shift in responses by both genotypes. *Scn10a*^{Psm/Psm} mice exhibited increased responses without the *Possum*-like behavior, and decreased responses in *Scn10a*^{WT} mice. No differences were observed in vehicle controls. There were no differences in the (B) percentage or (C) amplitude of response of cultured small-diameter neurons to 30 and 100 μ M mustard oil.

Table 1Summary of fiber properties in Scn10a^{WT} and Scn10a^{Psm/Psm} mice.

Fiber type	Genotype	n	Median von Frey Threshold (mN)	Lower Quartile	Upper Quartile	Mean Conduction Velocity (m/s)	± SEM
RA-Aβ	Scn10a ^{WT}	9	0.66	0.47	1.63	17	3.0
	Scn10a ^{Psm/Psm}	14	0.66	0.27	0.90	14.1	1.4
Low-Threshold SA-Aβ	Scn10a ^{WT}	11	1.63	0.66	1.63	14.5	1.0
	Scn10a ^{Psm/Psm}	17	0.66	0.27	1.63	12.5	0.6
High-Threshold SA-Aβ	Scn10a ^{WT}	4	4.00	4.00	4.00	16.2	3.8
	Scn10a ^{Psm/Psm}	6	4.00	4.00	6.11	12.5	0.9
D-hair	Scn10a ^{WT}	9	0.27	0.27	0.66	6.4	1.3
	Scn10a ^{Psm/Psm}	11	0.27	0.66	1.63	5.6	0.9
AM	Scn10a ^{WT}	14	4.00	4.00	4.00	3.9	0.6
	Scn10a ^{Psm/Psm}	22	4.00	3.41	6.82	5.9*	0.6
C	Scn10a ^{WT}	11	4.00	4.00	11.00	0.5	0.1
	Scn10a ^{Psm/Psm}	12	4.00	4.00	11.70	0.7	0.1