Novel SCN9A Mutations Underlying Extreme Pain Phenotypes: Unexpected Electrophysiological and Clinical Phenotype Correlations

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The importance of Na⁺,1.7 (encoded by SCN9A) in the regulation of pain sensing is exemplified by the heterogeneity of clinical phenotypes associated with its mutation. Gain-of-function mutations are typically pain-causing and have been associated with inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD). IEM is usually caused by enhanced Na⁺,1.7 channel activation, whereas mutations that alter steady-state fast inactivation often lead to PEPD. In contrast, nonfunctional mutations in SCN9A are known to underlie congenital insensitivity to pain (CIP). Although well documented, the correlation between SCN9A genotypes and clinical phenotypes is still unclear. Here we report three families with novel SCN9A mutations. In a multiaffected dominant family with IEM, we found the heterozygous change L245 V. Electrophysiological characterization showed that this mutation did not affect channel activation but instead resulted in incomplete fast inactivation and a small hyperpolarizing shift in steady-state slow inactivation, characteristics more commonly associated with PEPD. In two compound heterozygous CIP patients, we found mutations that still retained functionality of the channels, with two C-terminal mutations (W1775R and L1831X) exhibiting a depolarizing shift in channel activation. Two mutations (A1236E and L1831X) resulted in a hyperpolarizing shift in steady-state fast inactivation. To our knowledge, these are the first descriptions of mutations with some retained channel function causing CIP. This study emphasizes the complex genotype–phenotype correlations that exist for SCN9A and highlights the C-terminal cytoplasmic region of Na⁺,1.7 as a critical region for channel function, potentially facilitating analgesic drug development studies.

Key words: congenital insensitivity to pain; inherited erythromelalgia; Nav1.7; pain; paroxysmal extreme pain disorder; SCN9A

Introduction

Naturally occurring mutations in SCN9A have been linked with both hyposensitive and hypersensitive pain states, emphasizing the important role of the encoded ion channel Na⁺,1.7 in the regulation of nociceptive neuron excitability (Dib-Hajj et al., 2013). Gain-of-function mutations in SCN9A are linked with debilitating, chronic pain syndromes, typified by inherited erythromelalgia (IEM), paroxysmal extreme pain disorder (PEPD), and small-fiber neuropathy (SFN). IEM and PEPD are rare but clinically distinct conditions; IEM is characterized by an episodic burning sensation in the hands and feet that is often coupled with redness and swelling, whereas PEPD is associated with extreme bouts of pain within proximal regions of the body, typically rectal and ocular. Patients with idiopathic SFN present with more widespread axonal degeneration resulting in pain and other sensory impairments, and a recent report found SCN9A mutations in 8 of 28 affected individuals (Faber et al., 2012). Loss-of-function mutations, by contrast, have been associated with a complete absence of pain in affected individuals, a condition known as congenital insensitivity to pain (CIP).

Electrophysiological characterization has revealed that, like other voltage-gated sodium channels, Na⁺,1.7 shows voltage-dependent activation and inactivation, with the latter having fast and slow components. Known SCN9A-associated CIP mutations are incapable of supporting functional Na⁺,1.7 expression, suggesting a crucial role of this current for nociception (Cox et al.,...
2006, 2010) and the essential dependence of nociception on NaV1.7 was recently confirmed in mice (Gingras et al., 2014). SFN mutations appear to impair the slow inactivation of NaV1.7 currents, and it has been suggested that the resulting overtexcitability results in an increased sensitivity of small-diameter DRG neurons to otherwise benign insults (Faber et al., 2012). Overexcitability is also observed when mutations found in patients with IEM or PEPD are heterologously expressed in DRG neurons (Dib-Hajj et al., 2008; Estacion et al., 2008; Eberhardt et al., 2014); however, the underlying electrophysiological characteristics of the mutated NaV1.7 are thought to differ from each other. IEM mutations generally cause a hyperpolarizing shift in channel activation, whereas PEPD is typically associated with a depolarizing shift in fast inactivation (albeit often incomplete), resulting in persistent and/or resurgent currents. Why, or how, subtle changes in channel gating result in different phenotypes is currently unclear, but rare mutations found in patients with mixed IEM and PEPD symptoms have been shown to simultaneously display altered activation and inactivation properties (Estacion et al., 2008).

To further understand the effects of discrete molecular and functional changes in both hyposensitive and hypersensitive pain conditions, we electrophysiologically characterized four novel mutations in NaV1.7 identified in two individual cases of CIP (W1775R, A1236E, L1831X) and one case of IEM (L245V). Surprisingly, all three CIP-related mutations gave rise to functional NaV1.7 channels, exhibiting changes in activation and/or fast inactivation gating properties, coupled with a significant reduction in total current. In contrast, the single IEM-related mutation showed no change in channel activation properties but did show significant changes in inactivation properties. Our results challenge a simple correlation between electrophysiological properties observed in vitro and patient phenotype.

Materials and Methods

Identification of novel SCN9A mutations. The cases reported here were one of the human genes we routinely sequenced. Genomic DNA was isolated from peripheral blood by standard methods. All coding exons and flanking splice sites of SCN9A were bidirectionally sequenced using previously reported primers (Cox et al., 2006).

Construction of expression plasmids. The wild-type SCN9A-polyo IRES-DsRed2 construct (FLRED) and the SCN1B-encephalomyocardiitis virus-SCN2B-polyo IRES-EGFP (JCS) construct were generated as previously described (Cox et al., 2006). These bear either SCN9A and DsRed2 (FLRED) or SCN1B, SCN2B, and EGFP (JCS) on the same vector expressed from the same promoter, and the inclusion of DsRed2 and EGFP in these constructs helped in the identification of positively cotransfected cells when patch clamping. The NaV1.7-L245V, NaV1.7-A1236E, NaV1.7-A1632E, NaV1.7-W1775R, and NaV1.7-L1831X mutations were introduced into FLRED using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) or by recombinant PCR.

Cell culture and transfection. HEK293 cells were used for all experiments. Briefly, HEK293 cells were maintained in a 75 cm2 flask with DMEM (4500 mg/L glucose) supplemented with 5% FBS, 1-glutamine, and penicillin/streptomycin (culture medium) at 37°C, 5% CO2. Cells were split every third day of culture. For transfection, HEK293 cells were plated directly onto 35 mm plastic dishes (Falcon) and incubated for 24 h at 37°C, 5% CO2. After 24 h, the culture medium was replaced and the cells were incubated for 30 min. After 30 min, 500 μL of OptiMEM (In- vitrogen) containing 1 μg of SCN9A cDNA (mutant or wild-type), 42.5 ng of β1/β2 cDNA, and 10 μL of lipofectamine 2000 (Invitrogen) was added to each dish of cells, which were then incubated for 24 h at 37°C, 5% CO2. For the negative control, β1/β2 cDNA (42.5 ng) was transfected alone. After 24 h, the cells were split and diluted 1:20 in culture medium and plated directly onto 35 mm dishes and incubated for 24 h. All cells were recorded from 24 to 48 h after transfection.

Electrophysiological analysis. All electrophysiological recordings were performed using an AxoPatch 200B amplifier and a Digidata 1440A digitizer (Axon Instruments), controlled by Clampex software (version 10, Molecular Devices). Filamented borosilicate microelectrodes (GC150TF-15, Harvard Apparatus) were coated with beeswax and fire polished using a microforge (Narishige) to give resistances of 2–3 MΩ. For voltage-clamp experiments, the following solutions were used. Extracellular solution (values in mM) as follows: 137 NaCl, 3 KCl, 2 CaCl2, 10 HEPES, 1 glucose, pH 7.4 with NaOH. Intracellular solution (values in mM) as follows: 137 CsF, 10 EGTA, 10 TEA-Cl, 2 MgCl2, 10 HEPES, 10 NaCl, 1 CaCl2, pH 7.2 with CsOH. Unless otherwise stated, standard whole-cell currents were acquired at 25 kHz and filtered at 10 kHz (low-pass Bessel filter) at 22°-24°C. After achieving whole-cell configuration, a holding potential of −100 mV was applied and series resistance was compensated by 70%. All currents were leak subtracted using a p/4 protocol. Liquid junction potentials were calculated using Clampex software and corrected for before analysis. For resurgent current recordings, the β4 peptide (KLLTFLIKKTKREK, Invitro- gen) was included in the intracellular pipette solution at 100 μM. The peptide was added on the day of experiment from a 10 mM stock (ddH2O). Voltage-dependent activation was fitted to a Boltzmann equation

\[
y(x) = \frac{A2 + (A1 - A2)(1 + \exp(-(V - Vh)/k2))}{1 + \exp(-(V - Vh)/k1)} - \frac{A1}{1 + \exp(-(V - V0)/k1)}
\]

where A1 is the maximal amplitude, Vh is the potential of half-maximal activation, x is the clamped membrane potential, V0 is the reversal potential, and k is a constant. Voltage dependence of inactivation was fitted to a single Boltzmann equation:

\[
y(x) = \frac{A1 - A2}{1 + \exp(-(V - Vh)/k2)} + A2
\]

or a double Boltzmann equation:

\[
y(x) = \frac{A1}{1 + \exp(-(x - V0)/k1)} + \frac{A2}{1 + \exp(-(x - x02)/k2)}
\]

where k1 and k2 were constrained to 4.5. All Boltzmann equations were fitted using Origin software.Voltage dependence of channel deactivation was fitted to a double exponential of the form: f(t) = Af exp(−(t/τf)) + As exp(−(t/τs)) + C, using Clampfit software.

Data analysis. All electrophysiological data were extracted using Clampfit (version 10, Molecular Devices) and analyzed using Origin 9.0 (OriginLab). Significance was determined at p < 0.05. Individual p values are given for each comparison made.

Results

Identification of novel SCN9A mutations and patient phenotype

A large family with an inherited erythromelalgia phenotype and two unrelated individuals with apparent congenital insensitivity to pain were referred to us for genetic testing (for location of novel mutations in NaV1.7, see Fig. 1).

Family 1. The index case referred to us suffered from severe episodic pain in his hands and feet associated with excess heat perception. Three other members of the family were similarly affected and another five cases suspected according to family history. Episodes usually became distressing in the second decade of life but retrospectively started approximately a decade earlier. Episodes were precipitated by exercise, hot weather, and viral illness, and soothed by cold. Attacks usually started with pain restricted to the surface of the affected body part, but later during an episode pain was also perceived deeper within the tissue; during an attack, the affected part of the body exhibited allodynia and erythema, but this was not usually accompanied by swelling. The episodes tended to get more frequent and of greater severity with age; however, family members have developed a number of strategies to minimize the number and severity of the attacks, such as exercising in bare feet and wearing open sandals. Attacks could reliably be aborted by cooling within the first 5 min, but not thereafter. The pain phenotype was inherited as a dominant trait,
with both males and females similarly affected and passage from mother to son and father to daughter. Other possible causes of erythromelalgia had previously been excluded; and importantly, none of the affected family members reported pain on defecating or in facial (ocular) areas. We found the novel heterozygous SCN9A change c.733C>G (causing the NaV1.7 change p.L245V) in the referred index case. We thence confirmed that three other clinically affected individuals bore the mutation, whereas one clinically unaffected individual who requested testing did not.

**Family 2.** The index case was a 5-year-old girl referred to us as having no evidence of feeling pain. She had not shown any signs of distress during early life’s immunizations or teething but lacked any injuries to her lips, tongue, or terminal digits often seen in other individuals with congenitally inherited insensitivity to pain. However, the parents became concerned that she failed to respond appropriately to repeated falls and minor injuries, culminating with her developing a limp when 3 years of age and being found to have a fractured femur. She complained of no pain during this episode, and once placed in a plaster cast the bone healed normally. As speech developed, she was able to articulate that she did not feel discomfort or pain after injuries or falls but realized that other people found these events unpleasant. Her development and general health were normal. We tested the patient by applying pressure with a rigid 0.8 cm plastic tube onto the proximal region of the nail bed of the index finger while the patient’s hand lay flat on a hard unyielding surface. This procedure did not result in lasting damage to the nail or bruising, but control individuals report touch sensation at gentle pressures applied, which disappears immediately upon removal of the weight and pain lasting ~10 s when the applied weight exceeds ~1 kg. The patient, by contrast, could perceive pressure but did not report any unpleasant sensation at maximal applied weights exceeding ~10 kg. The patient was also anosmic. Screening of SCN9A identified two mutations, which were inherited from her father by applying pressure with a rigid 0.8 cm plastic tube onto the proximal region of the nail bed of the index finger (see above). Screening of SCN9A revealed two mutations, which were inherited from her father. The mutations identified are a mutation in the extracellular C-terminal tail. L245V (IEM) is located within the intracellular loop between domains 4 and 5 of the first region. Channel pore (green). The proposed location for each mutation is shown. Y163X (CIP) is located within the second TM domain of the first condition, the steepness of the voltage dependence, A1236E exhibited no significant shift (1.3 mV), whereas no significant difference was observed with WT (~25.2 ± 1.6 mV), whereas A1236E exhibited no significant shift (~21.5 ± 1.8 mV). In addition, the steepness of the voltage dependence, k, was significantly shallower for W1775R (~7.5 ± 0.2), A1236E (~7.3 ± 0.4), and L1831X (~7.6 ± 0.2) compared with WT (~3.7 ± 0.3). No change in reversal potential (Vrev) was observed between WT, mutant, or β1/β2 negative control currents, consistent with Na+ selective currents. Analysis of fast inactivation properties showed that A1236E and L1831X exhibited a hyperpolarizing shift in V1/2 values (~80.7 ± 1.3 and ~82.2 ± 1.3 mV, respectively) compared with WT (~71.1 ± 1.3 mV), whereas no significant difference was observed with W1775R (~73.4 ± 2.2 mV) (Fig. 2B,D,F). In addition, there was a significant difference in the slope k of channel inactivation for W1775R (11.6 ± 1.1), K932R, which was located in the intracellular loop between domains 5 and 6 (Fig. 1B). The third region contains the intracellular C-terminal tail. L245V (IEM) is located within the intracellular loop between domains 4 and 5 of the first region. Channel pore (green). The proposed location for each mutation is shown. 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A1236E (9.1 ± 0.8), and L1831X (7.9 ± 0.6), compared with WT (6.1 ± 0.4).

**Characterization of a novel IEM-associated mutation**

Electrophysiological analysis was performed on the IEM-associated Na<sub>v</sub>1.7 mutation, L245V. Individual parameter comparisons are summarized in Table 1. Analysis of channel activation properties showed that L245V gave rise to functional channels and exhibited peak activation currents that were not significantly different to WT (−398.5 ± 44.7 and −361.2 ± 39.5 pA/pF, respectively; Fig. 3A). Furthermore, L245V and WT activation currents exhibited almost identical subthreshold persistent current remaining even 500 ms following activation, compared with WT (−25.2 ± 1.3 mA, respectively, which would ultimately result in peak current following activation, compared with WT. In contrast to WT, L245V fast inactivating currents exhibited incomplete fast inactivation, leaving a substantial persistent current remaining even 500 ms following initial channel activation at several test pulses (Fig. 3B, D), and it is likely that the inability of L245V channels to fully undergo fast inactivation allows a secondary slower inactivation process with rightward shifted voltage dependence to become apparent. In addition to incomplete fast inactivation, L245V also exhibited a significant hyperpolarizing shift in the V<sub>50</sub> of slow inactivation (L245V: −41.4 ± 0.9 mV; WT: −31.4 ± 1.8 mV), a slowing of deactivation kinetics (L245V: 0.20 ± 0.02 ms; WT: 0.09 ± 0.1 ms at −60 mV), as well as an increased ramp current in response to a depolarizing ramp stimulus (Fig. 3E–G). We next investigated whether the changes in channel inactivation associated with L245V give rise to the emergence of resurgent currents by including the β4 peptide (KKLTIFILKTREK; 100 μM) in the intracellular pipette solution. We were, however, unable to detect clear resurgent currents beyond the observed tail currents in the absence of the β4 peptide for the wild-type, the L245V mutation, or the previously described A1632E mutation (Fig 3C).

**Discussion**

Given the fundamental role of Na<sub>v</sub>1.7 ion channels in pain, understanding how molecular and functional changes affect sensory nerve function could facilitate the development of novel analgesics, as well as improve current treatment strategies for related chronic pain syndromes. In this study, we identified and electrophysiologically characterized four naturally occurring novel mutations in Na<sub>v</sub>1.7 that give rise to either hyposensitive (CIP) or hypersensitive (IEM) pain conditions.

**The link between Na<sub>v</sub>1.7 functional change and pathogenesis**

Previous studies have concluded that pain-insensitive mutations are due to a total loss of Na<sub>v</sub>1.7 channel function (Cox et al., 2006, 2010; Ahmad et al., 2007), which, given the fundamental role of Na<sub>v</sub>1.7 in regulating electrical activity in sensory neurons, intuitively explains the painless phenotype. Here, however, we present three novel CIP-associated mutations in Na<sub>v</sub>1.7 that all retain some functionality. Electrophysiological characterization of the three mutations showed that each had a significant reduction in peak current following activation, compared with WT. In addition, each mutation also exhibited changes in activation and/or inactivation properties. Both W1775R and L1831X exhibited an ~10 mV depolarizing shift in channel activation, whereas A1236E and L1831X exhibited a similar hyperpolarizing shift in channel fast inactivation. Such changes in channel activation/inactivation properties would have the effect of reducing the number of channels activated, as well as reducing the number of channels available for activation, respectively, which would ultimately result in peak current following activation, compared with WT. In contrast to WT, L245V fast inactivating currents exhibited incomplete fast inactivation, leaving a substantial persistent current remaining even 500 ms following initial channel activation at several test pulses (Fig. 3B, D), and it is likely that the inability of L245V channels to fully undergo fast inactivation allows a secondary slower inactivation process with rightward shifted voltage dependence to become apparent. In addition to incomplete fast inactivation, L245V also exhibited a significant hyperpolarizing shift in the V<sub>50</sub> of slow inactivation (L245V: −41.4 ± 0.9 mV; WT: −31.4 ± 1.8 mV), a slowing of deactivation kinetics (L245V: 0.20 ± 0.02 ms; WT: 0.09 ± 0.1 ms at −60 mV), as well as an increased ramp current in response to a depolarizing ramp stimulus (Fig. 3E–G). We next investigated whether the changes in channel inactivation associated with L245V give rise to the emergence of resurgent currents by including the β4 peptide (KKLTIFILKTREK; 100 μM) in the intracellular pipette solution. We were, however, unable to detect clear resurgent currents beyond the observed tail currents in the absence of the β4 peptide for the wild-type, the L245V mutation, or the previously described A1632E mutation (Fig 3C).
Naturally contribute towards a loss of Na\(_V\), function. Nonetheless, our findings suggest that substantial, yet incomplete, block of Na\(_V\), and modulation of channel activation/inactivation properties might be sufficient for pain therapy. Interestingly, a patient has previously been reported with “partial congenital insensitivity to pain” who is a compound heterozygote for a 1 bp splice donor deletion midway through the SCN9A gene and a C1719R missense mutation (which maps upstream of the C-terminal intracellular region) (Staud et al., 2011). Although no biophysical analysis was performed, it was speculated by the authors that the incomplete pain insensitivity seen in the patient may be consistent with hypomorphic effects of one or both mutations. In contrast, the two families we describe appear to have complete pain insensitivity (although one patient lacked any injuries to her lips, tongue, or terminal digits often seen in other individuals with congenitally inherited insensitivity to pain) but carried mutations with at least some residual activity.

The C-terminal region of the voltage-gated sodium channel family is well conserved, with an EF-hand and downstream IQ motif annotated and verified in several Na\(_V\) channels (Wingo et al., 2004; Kubota et al., 2009; Miloushev et al., 2009). The Na\(_V\), W1775 residue, which is mutated in CIP Family 2, is completely conserved across all human Na\(_V\) family members, and the analogous amino acid in the cardiac sodium channel, Na\(_V\), (W1798) is known to be located within the first of 2 EF-hands. EF-hands are helix-loop-helix structural domains found in a large family of calcium binding proteins. The EF-hand of voltage-gated sodium channels is known to bind and stabilize the fast inactivation gate and also binds with beta1 and beta3 subunits (Spampanato et al., 2004; Cusdin et al., 2010). Mutations within C-terminal EF-hands of human Na\(_V\) channels have previously been reported to cause diseases, such as potassium-aggravated myonia and Long QT syndrome (Kubota et al., 2009; Gläser et al., 2012). Downstream of the EF-hand is a calmodulin-binding IQ motif, which can modulate Na\(_V\) currents (Mori et al., 2003; Staud et al., 2011).

Figure 2. Electrophysiological characterization of W1775R, A1236E, and L1831X in HEK293 cells. For each trace, averaged WT (black dash) and \(\beta_1/\beta_2\) only (blue dash) traces are overlaid.

Comparisons to WT values are shown in Table 1. A, Left, Representative raw trace of W1775R channel currents in response to the channel pulse protocol shown (activation). Right, Current–voltage relationship of the peak activation currents normalized to cell size (pA/pF) for W1775R (red). All traces were fitted with a Boltzmann equation (see Materials and Methods), where \(V_{0.5} = -12.4 \pm 1.3\) mV, \(k = -7.5 \pm 0.2\), \(V_{\text{max}} = 71.4 \pm 4.9\) mV, and \(I_{\text{max}} = -115.9 \pm 15.0\) pA/pF (\(n = 7\)). B, Left, Representative raw trace of W1775R currents in response to a test pulse to -10 mV following a series of prepulses (protocol shown in inset; fast inactivation). Right, Peak fast inactivation currents for W1775R (red) were normalized to the maximum current and plotted against the prepulse potential used. Data were fitted with a Boltzmann equation (see Materials and Methods), where \(V_{0.5} = -3.8 \pm 0.6\) mV, \(k = 7.6 \pm 0.5\), \(V_{\text{max}} = 7.9 \pm 0.6\) mV, and \(I_{\text{max}} = 103.3 \pm 14.0\) pA/pF (\(n = 9\)). C, top, Left, Representative raw trace of A1236E channel currents in response to the channel pulse protocol. Right, Current–voltage relationship of the peak activation currents normalized to cell size (pA/pF) for A1236E (red). All traces were fitted with a Boltzmann equation, where \(V_{0.5} = 21.5 \pm 1.8\) mV, \(k = -7.3 \pm 0.4\), \(V_{\text{max}} = 67.5 \pm 3.8\) mV, and \(I_{\text{max}} = -41.0 \pm 11.9\) pA/pF (\(n = 8\)). D, Left, Representative raw trace of A1236E currents in response to a test pulse to -10 mV following a series of prepulses as in B. Right, Peak fast inactivation currents for A1236E (red) were normalized to the maximum current and plotted against the prepulse potential used. Data were fitted with a Boltzmann equation, where \(V_{0.5} = -80.7 \pm 1.3\) mV and \(k = 9.1 \pm 0.8\) (\(n = 8\)). E, top, Left, Representative raw trace of L1831X channel currents in response to the channel pulse protocol. Right, Current–voltage relationship of the peak activation currents normalized to cell size (pA/pF) for L1831X (red). All traces were fitted with a Boltzmann equation, where \(V_{0.5} = -15.4 \pm 1.8\) mV, \(k = -7.6 \pm 0.2\), \(V_{\text{max}} = 72.5 \pm 5.3\) mV, and \(I_{\text{max}} = -103.3 \pm 14.0\) pA/pF (\(n = 9\)). F, top, Left, Representative raw trace of L1831X currents in response to a test pulse to -10 mV following a series of prepulses. Right, Peak fast inactivation currents for L1831X (red) were normalized to the maximum current and plotted against the prepulse potential used. Data were fitted with a Boltzmann equation, where \(V_{0.5} = -82.2 \pm 1.3\) mV and \(k = 7.9 \pm 0.6\) (\(n = 9\)).
Reddy Chichili et al., 2013). In CIP Family 3, there is a truncating mutation (L1831X) that causes deletion of this IQ motif from the C terminus of the mutant NaV1.7 channel. The mutations reported here highlight the importance of this region of the channel for normal function.

In contrast to loss-of-function mutations, we also characterized the novel IEM-associated mutation, L245V. Until recently, mutations in NaV1.7 that give rise to the hypersensitive pain conditions IEM or PEPD have been functionally distinguished by exhibiting either a hyperpolarizing shift in channel activation or a...
depolarizing shift in channel inactivation, respectively (Dib-Hajj et al., 2013). Here, however, we showed that, unlike typical IEM-associated mutations, L245V exhibited no shift in the voltage dependence of channel activation. This is consistent with other recently identified IEM-associated mutations. Eberhardt et al. (2014) recently presented an IEM mutation (A1632T) that had no change in channel activation but instead displayed a depolarizing shift in the voltage dependence of fast inactivation. The apparent lack of a shift in channel activation was also evident following the characterization of another IEM-associated mutation, G616R (Choi et al., 2010). These findings therefore question the validity of using a single marker to determine whether a mutation is IEM or PEPD causing.

Despite there being no change in channel activation, L245V did have changes in other gating characteristics, exhibiting incomplete fast inactivation, a hyperpolarizing shift in slow inactivation, a slowing of channel deactivation, the emergence of persistent and associated tail currents, as well as increased ramp currents. Understanding the relevance these changes have in determining an IEM or PEPD phenotype is difficult, as there is a significant overlap between changes in Na\textsubscript{v}1.7 function and pathogenesis; a slowing of channel deactivation, the presence of persistent currents, as well as an increase in ramp current density have all been previously associated with both IEM and PEPD phenotypes (Lampert et al., 2006; Cheng et al., 2008, 2011; Choi et al., 2011; Eberhardt et al., 2014). More recently, it has been suggested that, in the absence of a shift in channel activation, the presence of persistent and resurgent currents is likely a determinant of whether a mutation is IEM or PEPD causing (Jarecki et al., 2010; Theile et al., 2011; Eberhardt et al., 2014). This, however, is not supported by the significant persistent and associated tail current presented here by L245V. Other electrophysiological characteristic indicators that have been proposed to distinguish IEM from PEPD include the effects associated mutations have on neuronal physiology. It has been suggested that, although both IEM and PEPD mutations lower the action potential threshold and enhance action potential firing from sensory neurons when overexpressed in DRG cultures, IEM-associated, but not PEPD-associated, mutations typically cause a depolarization of the resting membrane potential (Dib-Hajj et al., 2010). Although not directly studied here for L245V, Eberhardt et al. (2014) showed that the IEM-associated A1632T mutation did enhance excitability but had no effect on the resting membrane potential of sensory nerves. They concluded that the presence of persistent and resurgent currents is a characteristic hallmark for PEPD-associated mutations; however, in contrast to this, we clearly observed persistent currents for the IEM-associated L245V mutation, and a recent publication showed that application of the sea-anemone toxin ATX-II elicited resurgent currents predominantly in A\textsubscript{\delta} fibers, associated with a pricking/tingling pain rather than the burning pains reported in IEM and PEPD, which are thought to be mostly C-fiber mediated (Klinger et al., 2012).

**Phenotype prediction arising from mutations in SC9N9A**

Understanding the etiology of SC9N9A-associated hyposensitive and hypersensitive pain conditions is central to the development of therapies aimed at treating these debilitating disorders. Within recent years, much has been done to try and further understand the role Na\textsubscript{v}1.7 channels have in the regulation of neuronal excitability and pain; however, the current mismatch between functional change and clinical phenotype suggests that phenotype-prediction arising from mutations in SC9N9A is complex and unlikely to be determined by any single change in channel function. As presented here, both hyposensitive and hypersensitive pain conditions arising from mutations in SC9N9A are likely to be determined by the overall functionality of Na\textsubscript{v}1.7 channels as described by all gating parameters in addition to the size of the current passed.

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