Sodium channel NaV1.9 mutations associated with insensitivity to pain dampen neuronal excitability

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Voltage-gated sodium channel (NaV) mutations cause genetic pain disorders that range from severe paroxysmal pain to a congenital inability to sense pain. Previous studies on NaV1.7 and NaV1.8 established clear relationships between perturbations in channel function and divergent clinical phenotypes. By contrast, studies of NaV1.9 mutations have not revealed a clear relationship of channel dysfunction with the associated and contrasting clinical phenotypes. Here, we have elucidated the functional consequences of a NaV1.9 mutation (L1302F) that is associated with insensitivity to pain. We investigated the effects of L1302F and a previously reported mutation (L811P) on neuronal excitability. In transfected heterologous cells, the L1302F mutation caused a large hyperpolarizing shift in the voltage-dependence of activation, leading to substantially enhanced overlap between activation and steady-state inactivation relationships. In transfected small rat dorsal root ganglion neurons, expression of L1302F and L811P evoked large depolarizations of the resting membrane potential and impaired action potential generation. Therefore, our findings implicate a cellular loss of function as the basis for impaired pain sensation. We further demonstrated that a U-shaped relationship between the resting potential and the neuronal action potential threshold explains why NaV1.9 mutations that evoke small degrees of membrane depolarization cause hypexcitability and familial episodic pain disorder or painful neuropathy, while mutations evoking larger membrane depolarizations cause hypoexcitability and insensitivity to pain.

Introduction

Chronic pain is a prevalent and pernicious medical problem that represents an enormous public health burden. Understanding the molecular mechanisms and identifying critically important proteins involved in nociception may reveal novel therapeutic targets.

Strong evidence supports the contribution of 3 distinct voltage-gated sodium (NaV) channels (NaV1.7, NaV1.8, and NaV1.9), expressed predominantly in peripheral neurons including pain-signaling neurons, to normal and pathological pain perception (1–6). Whereas NaV1.7 is a fast-gating and tetrodotoxin-insensitive (TTX-S) neuronal NaV channel, NaV1.8 and NaV1.9 (also known as SN1 and NaN, respectively) exhibit TTX resistance (TTX-R) (7–12). NaV1.9 contributes to a persistent sodium current in small-diameter, nociceptive sensory neurons in dorsal root ganglia (DRGs) (13–15) and has been implicated in neuronal pain signaling triggered by inflammation (16–19). These physiological contributions of NaV1.9 may stem from its unique biophysical properties. Specifically, NaV1.9 exhibits voltage dependence of activation and inactivation that overlap near the resting membrane potential (RMP), slow inactivation gating kinetics, and a very large, persistent current (11, 20–24). These properties have been hypothesized to enable NaV1.9 to regulate the threshold for excitability of peripheral nociceptive sensory neurons by modulating both the RMP and responses to sub-threshold stimuli (14, 15, 17, 23).

Voltage-gated sodium channels have been implicated in genetic pain disorders by the discovery of mutations in the genes encoding NaV1.7, NaV1.8, and NaV1.9 (1–3, 5, 25, 26). Mutations in NaV1.7 and NaV1.8 have been extensively analyzed, and a clear genotype-phenotype correlation has emerged (2, 27, 28). For NaV1.7, mutations producing gain-of-function biophysical changes at the channel level are associated with disorders of paroxysmal pain (inherited erythromelalgia, paroxysmal extreme pain disorder), whereas those with loss-of-function properties are associated with congenital insensitivity to pain (CIP). Likewise, for NaV1.8, gain-of-function mutations have been identified in subjects with painful peripheral neuropathy. Further, gain-of-function NaV1.7 mutations promote hyperexcitability of DRG neurons in vitro, whereas global knockout of this gene in mice produces the opposite phenotype of insensitivity to pain, with reduced action potential firing in DRG neurons (29), consistent with the increase in threshold of DRG neurons produced by blockade of NaV1.7 (30). This collective work on NaV1.7 and NaV1.8 has established a clear relationship between gain of function at the channel level and severe pain, and channel loss of function and insensitivity to pain, for these 2 channel subtypes.

By contrast, no clear relationship between channel dysfunction and clinical phenotype has yet emerged from early work on NaV1.9 mutations. Mutations of NaV1.9 have recently been associated with...
and were unable to distinguish a sharp from a blunt object when blinded. Light touch, temperature, and vibration sensations were normal for both affected individuals. Motor nerve conduction velocities were normal for both patients, and nerve fibers were deemed normal upon examination by light and electron microscopy. Motor milestones as well as cognitive and neurological development were normal. The proband reported a normal sense of smell, unlike CIP patients with Na\(^{+}\)V1.7 mutations, who have anosmia (39, 40). The proband reported severe pruritus since childhood, resulting in painless excoriating lesions of the thorax, ears, and nose from scratching her wounds. She had surgery at age 13 for nasal septum prostheses due to repeated nasal trauma and subsequent deformity. Although the original report on this subject (38) did not document autonomic disturbances, the proband subsequently reported a history of persistent diarrhea of unknown cause as well as episodic abdominal pain beginning in childhood and persisting into adulthood. Sweating was reported as normal.

Targeted sequence analysis of Na\(^{+}\)V1.9, Na\(^{+}\)V1.8, and Na\(^{+}\)V1.9 in the proband identified a heterozygous Na\(^{+}\)V1.9 coding variant (c.3904C>T, p.Leu1302Phe [L1302F]) (Figure 1B), which was recently reported in an unrelated family with insensitivity to pain (32). The variant affects a highly conserved residue in the S6 segment of domain III and is absent in the Genome Aggregation Database (http://gnomad.broadinstitute.org/). Other family members, including the unaffected maternal grandmother and 2 unaffected maternal aunts, did not harbor the L1302F variant. We

either loss of pain perception (26, 31, 32) or the opposite phenotypes of familial episodic pain and painful peripheral neuropathy (33–37). All mutant Na\(^{+}\)V1.9 channels for which biophysical data are available show hyperpolarizing shifts in channel activation, which is consistent with a gain of function at the channel level, despite the contrasting nature of the associated clinical phenotypes. The physiological basis for this paradox has not been resolved.

In this study, we elucidated the functional consequences of the second Na\(^{+}\)V1.9 mutation (L1302F) associated with insensitivity to pain and investigated the impact of the 2 known Na\(^{+}\)V1.9 mutations associated with this condition on the excitability of nociceptive neurons. Our observations demonstrate how these Na\(^{+}\)V1.9 mutations, which produce a severe gain of function at the channel level, can cause reduced excitability of dorsal root ganglion neurons, consistent with a loss of pain sensibility at the clinical level.

Results
Phenotype and genetics of a proband with insensitivity to pain. We studied a previously described French woman with insensitivity to pain (38). Briefly, both the proband and her mother (Figure 1A) suffered multiple painless orthopedic injuries during childhood including several painless fractures of the lower extremities. Between the ages of 4 and 13 years, the proband suffered a total of 11 fractures, including several painless fractures of the right and left tibiae. There was radiographic evidence of advanced destruction of the calcaneum and talus. The affected subjects did not perceive a noxious stimulus such as pin-prick as painful and were unable to distinguish a sharp from a blunt object when blinded. Light touch, temperature, and vibration sensations were normal for both affected individuals. Motor nerve conduction velocities were normal for both patients, and nerve fibers were deemed normal upon examination by light and electron microscopy. Motor milestones as well as cognitive and neurological development were normal. The proband reported a normal sense of smell, unlike CIP patients with Na\(^{+}\)V1.7 mutations, who have anosmia (39, 40). The proband reported severe pruritus since childhood, resulting in painless excoriating lesions of the thorax, ears, and nose from scratching her wounds. She had surgery at age 13 for nasal septum prostheses due to repeated nasal trauma and subsequent deformity. Although the original report on this subject (38) did not document autonomic disturbances, the proband subsequently reported a history of persistent diarrhea of unknown cause as well as episodic abdominal pain beginning in childhood and persisting into adulthood. Sweating was reported as normal.

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Causative variants were discovered in this or other genes with bio-
ified the L1302F variant in the proband, but no other potentially
resolve the heritability question. Whole-exome sequencing veri-
(deceased), although DNA from the parents was not available to
presumed that the variant was inherited from the affected mother
logical plausibility for a genetic pain disorder.
expressing WT (∼13) NaV1.9 channels. The
mean values, and error bars indicate the SEM.
and D were normalized to cell capacitance. Data points in C represent
WT (black circles) or L1302F (solid orange circles). Current values
expressing WT (black circles) or L1302F (solid orange circles) channels. Figure 2, A and B, illustrates the average TTX-R whole-
ction of fast inactivation time constants determined from cells
nels occurred at more hyperpolarized test potentials (Figure 2D).
ics were similar (Figure 2C), but peak activation of mutant chan-
tures peaked at approximately –65 mV. The peak current density
threshold currents of 40 pA, 55 pA, and 110 pA, respectively.
spontaneously or produced overshooting action potentials with
cells regained excitability when held at –60 mV and either fired
voltage dependence of activation observed for L1302F, creates an
window current of L1302F would potentiate this effect. Consistent
expression of L1302F evoked a marked depolarization of the average RMP by 11.5 mV as compared with WT channel expression in small DRG neurons
(Figure 4A and Table 2). All neurons expressing WT NaV1.9 fired
action potentials, with an average current threshold of 290 ± 38 pA (Figure 4B and Table 2). A representative action potential from
firing WT NaV1.9 exhibited a whole-cell current that
peaked at approximately –40 mV, whereas L1302F current den-
sities peaked at approximately –65 mV. The peak current density
(solid orange circle indicated by the arrow in Figure 4A) failed
as large as 500 pA (Figure 4A and Table 2). A representative action potential from
neurons transfected with L811P, which was
RMP, a smaller effect than was observed for
neurons exhibiting an 8.2-mV depolarized RMP values (Figure 4A, solid orange circles).
Because we expected that the depolarized RMP would contrib-
ute to the effect of the mutant channel on DRG neuron excitability,
emonstrated a relatively normal membrane potential in cells
in these mutant channels by injecting a hyperpolarizing
to achieve a holding potential of –60 mV. All 4 of these
cells regained excitability when held at –60 mV and either fired
ously produced overshooting action potentials with
threshold currents of 40 pA, 55 pA, and 110 pA, respectively.
As an example, a cell recorded at its native RMP of –26.6 mV
action potential with stimuli as large as 500 pA
(Figure 4C), whereas the cell fired an action potential at a threshold current as low as 40 pA when held at –60 mV (Figure 4D).
We observed similar phenomena in
neurons transfected with L811P, which was
previously associated with insensitivity to
pain (26). Small DRG neurons expressing
L811P exhibited an 8.2-mV depolarized RMP, a smaller effect than was observed for

Table 1. Functional properties of WT and mutant human NaV1.9 channels

<table>
<thead>
<tr>
<th></th>
<th>Peak current density (pA/pF)</th>
<th>Voltage dependence of activation</th>
<th>Voltage dependence of inactivation</th>
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<tbody>
<tr>
<td></td>
<td>V1/2 (mV)</td>
<td>k</td>
<td>V1/2 (mV)</td>
</tr>
<tr>
<td>WT</td>
<td>23.4 ± 2.2 (n = 17)</td>
<td>54.2 ± 1.3 (n = 17)</td>
<td>55.3 ± 2.3 (n = 8)</td>
</tr>
<tr>
<td>L1302F</td>
<td>19.1 ± 1.7 (n = 13)</td>
<td>81.2 ± 1.3 (n = 13)</td>
<td>51.1 ± 3.6 (n = 7)</td>
</tr>
</tbody>
</table>

*P < 0.05 and P < 0.001, by t test compared with WT values.
channels, including NaV1.8, which contributes substantially to the inactivation of L1302F or L811P caused inactivation of voltage-gated sodium currents. In both A and B, the solid lines represent Boltzmann fits to averaged data. C) Superimposed steady-state inactivation and activation curves calculated for WT (gray lines) and L1302F (orange lines) sodium currents. The steady-state inactivation and activation curves for WT intersect near ~50 mV, while those for L1302F intersect at approximately ~70 mV. Shaded areas indicate the window current between ~100 and ~10 mV, with the solid orange-shaded area representing the window current for L1302F channels and the hatched area indicating the window current for WT channels. Data points in A and B represent mean values, and error bars indicate the SEM.

L1302F (Table 2 and Figure 4E). As indicated by the solid purple diamonds in Figure 4E, 8.7% of neurons (4 of 46 cells) expressing L811P were nonexcitable at their native RMP. When held at –60 mV, however, all 4 cells fired spontaneously. Figure 4F illustrates representative spontaneous firing in the cell (indicated by the arrow in Figure 4E) recorded continuously for 30 seconds, with no external stimuli.

We posited that RMP depolarizations evoked by expression of L1302F or L811P caused inactivation of voltage-gated sodium channels, including NaV1.8, which contributes substantially to the action potential upstroke (42). This effect is predicted to attenuate action potential amplitude in neurons that do fire and to completely impair action potential generation in the most severely depolarized neurons expressing mutant NaV1.9 channels. A comparison of spontaneous action potential waveforms among cells expressing WT or mutant channels is shown in Figure 5A, which depicts a marked depolarization of the RMP and an attenuated overshoot in cells expressing either L1302F or L811P.

We previously demonstrated that the action potential amplitude in small adult mouse DRG neurons decreases as the membrane potential is depolarized from ~90 mV to ~30 mV and that this amplitude–membrane potential relationship depends on the activity of both TTX-S and TTX-R (e.g., NaV1.8) sodium channels (43). Consistent with the hypothesis that a lower action potential amplitude is due to inactivated Na channels, we observed that the action potential amplitude was attenuated significantly in cells expressing L1302F and L811P at their native resting potentials (Table 2) but was rescued by holding cells at ~60 mV (Table 3). The magnitude of the reduction in action potential amplitude by L1302F (20%) was greater than that for L811P (8.5%), paralleling the larger RMP depolarization in cells expressing L1302F (11.5 mV for L1302F versus 8.2 mV for L811P; Table 2).

Because it is difficult to measure the responses to injected current in spontaneously firing neurons, we excluded them from the analysis of RMP, input resistance, and amplitude of action potential. We did not observe a difference in the percentage of cells expressing L1302F or L811P that fired spontaneously at the native RMP as compared with cells expressing WT channels (Table 2). However, cells expressing L1302F or L811P showed a significantly higher proportion of spontaneously firing cells than did WT cells when using a holding potential of ~60 mV (Table 3, and Figure 5B). These observations are consistent with the conclusion that, at the channel level, mutations confer a gain of function, which evokes neuronal hyperexcitability at a physiological RMP.

Input resistance was significantly smaller in DRG neurons expressing L1302F than in cells expressing WT channels at both the native RMP and when held at ~60 mV (Tables 2 and 3). Neur...
tions expressing L811P also exhibited a significantly lower input resistance at −60 mV, but input resistance measured at the native RMP was not significantly different (Table 3). Lower input resistance, also consistent with a gain of function at the channel level, can be attributed to the large conductance of L1302F and L811P channels at negative potentials owing to the hyperpolarized voltage dependence of activation.

Whereas all cells expressing WT NaV1.9 channels fired action potentials when assessed at the native RMP, 13% of cells expressing L1302F and 8.7% of cells expressing L811P did not fire at the native RMP (Table 2). In an analysis in which we excluded spontaneously firing cells, the current threshold was significantly smaller in L1302F-expressing neurons when held at −60 mV (Table 3), presumably due to the relief of resting sodium channel inactivation, while the current threshold was not significantly different in cells expressing L811P (Table 3). However, if spontaneously firing cells were included in this analysis, then the current threshold determined for both L1302F and L811P was significantly smaller than the threshold for WT-expressing cells (Table 3).

**Impact of RMP depolarization on current threshold and action potential amplitude.** Expression of either L1302F- or L811P-mutant channels produced large RMP depolarizations in small DRG neurons. To model the effect of this depolarization on neuron excitability, we examined nontransfected adult rat DRG neurons within 8 hours of isolation using whole-cell current-clamp recording. Inward currents were injected to clamp the membrane potential at a series of voltages from −60 mV to −30 mV in 2.5-mV increments. This experiment demonstrated that the current threshold for action potential generation falls in direct relationship to the extent of membrane depolarization until a critical point, at which further depolarization requires larger stimuli to initiate a response, is reached (Figure 6A). This U-shaped curve is similar to the relationship between current threshold and resting potential predicted from studies in which the membrane potential was modulated by injections of hyperpolarizing and depolarizing current more centrally along the somatosensory pathway in rat dorsal column axons (44).

This relationship provides an explanation for why large depolarizations caused by expression of L1302F (11.5 mV) or L811P (8.2 mV) are associated with hypoexcitability, whereas lesser degrees of membrane depolarization, which are evoked by other NaV1.9 mutations associated with familial episodic pain disorder or pain-
ful neuropathy, cause neuronal hyperexcitability. The representative action potential traces recorded from nontransfected DRG neurons at different clamping voltages (–60 mV, –52.5 mV, –42.5 mV, and –40 mV) shown in the bottom panel of Figure 6A further validate this conclusion. The relationship between action potential amplitude and the extent of RMP depolarization is best fit by a Boltzmann function having a midpoint voltage (–38.7 ± 2.3 mV; Figure 6B) that is close to the midpoint voltage for fast inactivation of NaV1.8, which contributes a major component of the inward current underlying the action potential upstroke, and is consistent with the midpoint voltage (–37 mV) determined previously for TTX-R current in DRG neurons (43).

Discussion

Syndromes of insensitivity to pain have been associated with mutations of 2 voltage-gated sodium channels, NaV1.7 and NaV1.9. In the case of NaV1.7, a sodium channel that sets the gain on nociceptive DRG neurons (30, 45, 46), the congenital inability to sense pain is associated with recessive mutations that produce loss of function at the channel level (39, 47, 48). By contrast, dominant NaV1.7 mutations that produce gain-of-function changes, including a hyperpolarizing shift in the voltage dependence of activation at the channel level, are associated with genetic disorders featuring increased pain sensation (49–52). Unlike the distinct relationships between channel defects and clinical phenotypes for NaV1.7, dominant mutations that produce gain of function in NaV1.9 at the channel level have been associated with syndromes characterized by both insensitivity to pain (26, 31) and severe pain (6, 33, 34, 36). The mechanistic link between NaV1.9 gain of function and insensitivity to pain has been elusive.

The results of our functional profiling provide a mechanistic explanation for a loss of pain sensibility in these patients. Specifically, our voltage-clamp studies revealed a large hyperpolarizing shift in the voltage dependence of activation for L1302F (–26.9 mV) that was qualitatively similar to the results reported for the L811P mutation (26). This biophysical effect causes a larger window of overlap between the voltage dependence of activation and steady-state inactivation (Figure 3C), and this phenomenon is expected to promote large depolarizing current near the RMP in DRG neurons carrying these mutations. Consistent with this prediction, we observed large depolarizations in the average resting potential (11.5 mV for L1302P, and 8.2 mV for L811P) in DRG neurons expressing either mutation, with resulting impairments in action potential generation.

To examine the effect of this depolarization on excitability, we assessed the effect of holding potential on current threshold in non-

Table 3. Electrophysiological properties of small DRG neurons expressing WT or mutant NaV1.9 (holding potential of –60 mV)

<table>
<thead>
<tr>
<th></th>
<th>Spont. firing cells (%)</th>
<th>Input resistance (MΩ)</th>
<th>Current threshold excl. spont. firing cells (pA)</th>
<th>Current threshold incl. spont. firing cells (pA)</th>
<th>AP amplitude (mV)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>0/24 (0%)</td>
<td>519 ± 55 (n = 19)</td>
<td>284 ± 44 (n = 24)</td>
<td>284 ± 44 (n = 24)</td>
<td>116 ± 1.9 (n = 24)</td>
</tr>
<tr>
<td>L1302F</td>
<td>7/30 (23%)</td>
<td>374 ± 30 (n = 21)</td>
<td>139 ± 31 (n = 23)</td>
<td>106 ± 26 (n = 30)</td>
<td>108 ± 2.7 (n = 23)</td>
</tr>
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</table>

Comparison of WT and L1302F

WT: 12/60 (20%) 654 ± 44 (n = 54) 226 ± 24 (n = 54) 218 ± 24 (n = 56) 116 ± 0.90 (n = 54)

L811P: 30/60 (50%) 417 ± 31 (n = 30) 239 ± 37 (n = 30) 120 ± 24 (n = 60) 113 ± 1.7 (n = 30)

*Current threshold for spontaneously firing cells was assigned a value of 0 pA; \( p < 0.05 \) and \( p < 0.001 \), by \( z \) test compared with WT values; \( p < 0.05 \), \( p < 0.01 \), and \( p < 0.001 \), by \( t \) test compared with WT values. incl., including; excl., excluding; Spont., spontaneously.

Figure 5. Effects of mutant channels on action potential properties.

(A) Representative recordings demonstrating that expression of L1302F (orange trace) and L811P (purple trace) was associated with a depolarized RMP and an attenuated action potential overshoot as compared with cells expressing WT channels (blue trace). (B) Proportions of spontaneously firing cells. Colors represent the percentage of spontaneously firing cells for WT (blue), L1302F (orange), and L811P (purple) when studied at the native RMP or when using a holding potential of –60 mV. The plotted data sets for the native RMP were L1302F: 18 of 50 cells (36%) versus WT: 12 of 44 cells (27%), \( P = 0.35 \), by \( z \) test, and L811P: 18 of 64 cells (28%) versus WT: 9 of 60 cells (15%), \( P = 0.079 \), by \( z \) test. When cells were held at –60 mV, 23% of cells expressing L1302F (7 of 30) as opposed to 0% of cells expressing WT fired spontaneously (\( *P = 0.012 \), by \( z \) test), whereas 50% of cells expressing L811P (30 of 60) fired spontaneously as compared with 3.6% (2 of 56) of cells expressing WT (\( ***P < 0.0001 \), by \( z \) test).
transfected adult rat DRG neurons. This experiment demonstrated that the current threshold for action potential generation decreases gradually with membrane depolarization until a critical point, at which further depolarization requires larger stimuli to initiate a response, is reached. Notably, when we injected current into nontransfected cells to produce membrane depolarizations similar to those caused by expression of L1302F (+11.5 mV) or L811P (+8.2 mV), we observed a reduced ability to fire action potentials. These findings are in contrast to our previous observations that a 4- to 6-mV depolarization in the resting potential of DRG neurons associated with multiple NaV1.9 mutations results in neuronal hyperexcitability and paroxysmal pain disorders (27, 34, 53–55). These divergent observations regarding the effects of mutations on neuronal excitability are explained by the U-shaped relationship between resting potential and current threshold (Figure 6A), which demonstrates hyperexcitability of neurons with a moderately depolarized RMP and hypoexcitability of neurons with a severely depolarized RMP.

An explanation at the channel level for how depolarization induced by L1302F or L811P expression leads to hypexcitability is provided by our observation that a reduction in action potential amplitude is correlated with the degree of RMP depolarization, with a relationship suggesting involvement of NaV1.8 channels, which produce most of the inward current underlying action potentials (43, 56). Although both human and mouse mutations evoke a similar hypopolarized activation voltage dependence, there were notable differences in the time course of inactivation, sodium current density, and voltage dependence of inactivation (26), all of which confound direct comparisons and diminish the reliability of extrapolating across species.

Some parallels can be drawn between the divergent cellular effects of NaV1.9 gain-of-function mutations and those of the skeletal muscle channel NaV1.4, mutations, which are associated with genetic disorders of muscle contraction (57, 58). Gain-of-function NaV1.4 mutations predominantly cause myotonia or periodic paralysis, symptoms that represent either enhanced or diminished sarcolemmal excitability, respectively. This spectrum of pathophysiological effects has been attributed to specific mechanisms of channel dysfunction and the manner by which dysfunctional channels affect the resting potential. Mutations that impair fast inactivation typical of myotonic disorders promote hyperexcitability by increasing sodium channel availability and by prolonging the muscle action potential, which promotes greater t-tubular potassium accumulation and a greater probability of after-depolarizations. By contrast, an enhanced persistent
sodium current (59, 60) or an anomalous gating pore current (61, 62) typical of mutations associated with either hyperkalemic or hypokalemic periodic paralysis, respectively, causes sustained depolarization of the resting potential and inexcitability owing to inactivation of WT sodium channels. The cellular effects of Na₉.1.9 mutations associated with insensitivity to pain are most reminiscent of the behavior of Na₉.1.4 in hypokalemic periodic paralysis, in that abnormal sodium conductance renders the membrane depolarized and inexcitable.

In conclusion, we determined that large hyperpolarizing shifts in the voltage dependence of activation in mutant Na₉.1.9 channels associated with insensitivity to pain evoke a degree of membrane depolarization that renders DRG neurons hypoexcitable, probably because of inactivation of other peripheral nerve sodium channels (including Na₉.1.8). Thus, our observations provide a mechanistic explanation for loss of pain sensibility associated with Na₉.1.9 mutations that exhibit severe gain of function at the channel level.

Methods

Study subject and molecular genetics. A French proband with insensitivity to pain and her family were recruited as part of a larger study to investigate the molecular basis of loss of sensitivity to pain. At age 33, the proband was enrolled in our study, at which time an updated medical history was collected from the patient’s self-reports. Genetic studies included Sanger sequencing of SCN9A (NaV1.7), SCN10A (NaV1.8), and SCN11A (NaV1.9). Subsequently, whole-exome sequencing was performed using the SureSelect V4 Capture Reagent (Agilent Technologies), followed by 80× on-target sequencing using a HiSeq 2000 Sequencer (Illumina).

Plasmids and cell transfection. The L811P and L1302F mutations were introduced into full-length human Na₉.1.9 (GenBank accession number NP_0554858.2) cDNA including a C-terminal triple FLAG epitope as previously described (22). All recombinant cDNAs were sequenced in their entirety to confirm the presence of the intended modifications and the absence of unwanted mutations.

Heterologous expression experiments were conducted using ND7/23 cells (Sigma-Aldrich) grown at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS (Atlanta Biologicals), 2 mM L-glutamine, and penicillin/streptomycin (50 units/ml and 50 μg/ml, respectively). Unless otherwise stated, all tissue culture media were obtained from Life Technologies (Thermo Fisher Scientific).

Stable expression of mutant Na₉.1.9 channels in ND7/23 cells (Sigma-Aldrich) was achieved using the piggyBac transposon system as previously described (63, 64) using a transposon vector containing a puromycin resistance gene (pB-Na₉.1.9_mut-5xFLAG-PuroR). ND7/23 cells were cotransfected with a NaV1.9 plasmid and pCMV-hyPBase encoding a hyperactive version of the piggyBac transposase (65) using FUGENE-6 (Roche Applied Science). ND7/23 cells were transfected using Nucleofector IIS and protocol GFP using a Nucleofector IIS (Lonza) and an Amaxa Basic Neuron SCN Bridge containing bath solution served as reference electrodes. Whole-cell conductance was calculated from a holding potential of –120 mV. The peak current was normalized between –100 and +40 mV (10 s at the holding potential between pulses). The normalized G-V curves with a Boltzmann function. The voltage dependence of activation was calculated by fitting the normalized G-V curves with a Boltzmann function. The voltage dependence of channel availability was assessed following a 300-msec prepulse to various potentials and normalizing to the current measured following a pulse to –40 mV (membrane held for 20 s at the holding potential between pulses). The normalized G-V curves were fit with the Boltzmann function: G = 1/(1 + exp[V – V₁/₂]/k) to determine the V₁/₂ and slope factor (k).

Isolation and transfection of primary sensory neurons. DRGs from 4- to 6-week-old female and male Sprague-Dawley rats were harvested and dissociated as described previously (67). Briefly, DRG neurons were dissociated with a 20-minute incubation in 1.5 mg/ml collagenase A (Roche) and 0.6 mM EDTA, followed by an 18-minute incubation in 1.5 mg/ml collagenase D (Roche). 0.6 mM EDTA, and 30 U/ml papain (Worthington Biochemical Corp.). DRGs were then centrifuged and triturated in 0.5 ml of media containing 1.5 mg/ml BSA (low endotoxin) and 1.5 mg/ml trypsin inhibitor (Sigma-Aldrich). After trituration, 2 ml of DRG media were added to the cell suspension, which was filtered through a 70-μm nylon mesh cell strainer (BD Technologies). The mesh was washed twice with 2 ml of DRG media. The cells were then cotransfected with WT or mutant human Na₉.1.9 plasmids and a plasmid encoding GFP using a Nucleofector IIS (Lonza) and an Amaxa Basic Neuron SCN Nucleofector Kit (VSP1-1003). Briefly, the cell suspension was centrifuged (100 × g for 3 min), and the cell pellet was resuspended in 20 μl Nucleofector solution, mixed with 2 μg WT, L1302F, or L811P Na₉.1.9 plasmid plus 0.2 μg GFP plasmid, and transfected using Nucleofector IIS and protocol SCN-BNP 6. After transfection, cells were allowed to recover in calcium-free DMEM, fed with DRG media supplemented with nerve growth factor solution for approximately 2 hours (1 h at 37°C, and then at 28°C) before electrophysiology experiments.
factor (50 ng/ml) and glial cell line-derived neurotrophic factor (50 ng/ml), and maintained at 37°C with 5% CO₂ for 40 to 55 hours before current-clamp recording. Recordings were obtained from nontransfected adult rat DRG neurons within 8 hours of isolation.

**Current-clamp electrophysiology.** The pipette solution contained the following: 140 mM KCl, 0.5 mM EGTA, 5 mM HEPES, 3 mM Mg-ATP, and 10 mM dextrose, pH 7.30, with KOH (adjusted to 310 mOsm with sucrose). The bath solution contained the following: 140 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 10 mM dextrose, pH 7.30, with NaOH (adjusted to 320 mOsm with sucrose). A whole-cell configuration was obtained in voltage-clamp mode before proceeding to the current-clamp recording mode. The electrophysiologist was blinded with respect to exogenous sodium channel expression in DRG neurons (either WT or mutant Naᵥ1.9) until after data analysis. Small DRG neurons with a diameter of less than 30 μm and green fluorescence were selected for recording. A stable (<10% variation) RMP for 30 seconds and the presence of endogenous Naᵥ1.8 currents (>1 nA) evaluated by holding neurons at −50 mV were used as additional criteria for inclusion. Input resistance was determined by the slope of a linear fit to hyperpolarizing responses to current steps from −5 pA to −40 pA in 5-pA increments. The minimum current injection required to achieve firing of a single action potential (designated as the current threshold) was determined at the first action potential elicited by a series of depolarizing current injections (200 ms) applied in 5-pA increments. Action potential amplitude was measured by the spike height from the peak to the RMP. Spontaneously firing cells were excluded from the analysis of RMP, input resistance, and action potential amplitude.

**Statistics.** Voltage-clamp data were analyzed and plotted using a combination of Clampfit 9.2 (Molecular Devices), SigmaPlot 12.5 (Systat Software), and Origin 7.0 (OriginLab). Statistical analyses were carried out using the statistical analysis option in SigmaPlot. Current-clamp data were analyzed using Fitmaster (HEKA Elektronik) and Origin (Microcal Software) software. Percentages of nonfiring cells and spontaneously firing cells were compared using the t test. Unless otherwise noted, statistical significance was determined using an independent 2-tailed t test. All electrophysiology data are presented as the mean ± SEM, and error bars in the figures represent the SEM. The number of cells (n) used for each experimental condition is indicated in the figures, figures legends, or tables. A P value of less than 0.05 was considered significant.

**Study approval.** The human study was approved by an independent ethics review board (protocol CIP-SEQ-001; Quorum Review, Inc., Seattle, WA, USA), and the provand provided written informed consent for participation and publication of the findings. Animal use was approved by the IACUC of the Veterans Administration West Haven Hospital.

**Author contributions**

JH, CGV, AC, and YPG performed experiments, collected and analyzed data, and contributed to the writing of the manuscript; SDDH, CJC, SGW, and ALG designed the study, evaluated results, and wrote the manuscript.

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