Dynamic-clamp analysis of wild-type human Na\textsubscript{v}1.7 and erythromelalgia mutant channel L858H

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Submitted 29 October 2013; accepted in final form 6 January 2014

Vasylyev DV, Han C, Zhao P, Dib-Hajj S, Waxman SG. Dynamic-clamp analysis of wild-type human Na\textsubscript{v}1.7 and erythromelalgia mutant channel L858H. J Neurophysiol 111: 1429–1443, 2014. First published January 8, 2014; doi:10.1152/jn.00763.2013.—The link between sodium channel Na\textsubscript{v}1.7 and pain has been strengthened by identification of gain-of-function mutations in patients with inherited erythromelalgia (IEM), a genetic model of neuropathic pain in humans. A firm mechanistic link to nociceptor dysfunction has been precluded because assessments of the effect of the mutations on nociceptor function have thus far depended on electrophysiological recordings from dorsal root ganglia (DRG) neurons transfected with wild-type (WT) or mutant Na\textsubscript{v}1.7 channels, which do not permit accurate calibration of the level of Na\textsubscript{v}1.7 channel expression. Here, we report an analysis of the function of WT Na\textsubscript{v}1.7 and IEM L858H mutation within small DRG neurons using dynamic-clamp. We describe the functional relationship between current threshold for action potential generation and the level of WT Na\textsubscript{v}1.7 conductance in primary nociceptive neurons and demonstrate the basis for hyperexcitability at physiologically relevant levels of L858H channel conductance. We demonstrate that the L858H mutation, when modeled using dynamic-clamp at physiological levels within DRG neurons, produces a dramatically enhanced persistent current, resulting in 27-fold amplification of net sodium influx during subthreshold depolarizations and even greater amplification during interspike intervals, which provide a mechanistic basis for reduced current threshold and enhanced action potential firing probability. These results show, for the first time, a linear correlation between the level of Na\textsubscript{v}1.7 conductance and current threshold in DRG neurons. Our observations demonstrate changes in sodium influx that provide a mechanistic link between the altered biophysical properties of a mutant Na\textsubscript{v}1.7 channel and nociceptor hyperexcitability underlying the pain phenotype in IEM.

pain; sodium channel; channelopathy; Hodgkin-Huxley equations; dynamic-clamp

MATERIALS AND METHODS

Voltage-clamp recordings of Na\textsubscript{v}1.7. Human Na\textsubscript{v}1.7 channels were stably expressed in HEK-293 cell line (Cummins et al. 1998). Pipettes...
were pulled from glass capillaries (cat. no. PG10165-4; World Precision Instruments, Sarasota, FL) and had resistance 1.5–2 MΩ when filled with the intracellular solution in mM: 140 CsCl, 10 NaCl, 0.5 EGTA, 10 HEPES, 3 MgATP, 10 glucose, pH 7.3 with CsOH. The extracellular solution was HBSS (cat. no. 14025; Invitrogen) in mM: 1.3 CaCl₂, 0.5 MgCl₂, 0.4 MgSO₄, 5.3 KCl, 0.4 KH₂PO₄, 4.2 NaHCO₃, 138 NaCl, 0.3 NaHPO₄, 5.6 glucose, supplemented with 15 mM NaCl (320 mosM). The liquid junction potential (+3.7 mV) between pipette and bath solutions was measured according to Neher (1992) and was not compensated. Whole cell voltage-clamp recordings were made using Axopatch 200B amplifier. Currents were low-pass filtered at 10 kHz and digitized/stored at 100 kHz by Digidata 1440A DAC using pCLAMP 10 software (Molecular Devices, Sunnyvale, CA). The series resistance was compensated by 80–85%. -P/4 protocol was used for current-voltage (I–V) and deactivation protocols to subtract uncompensated leak and capacitive currents. Recordings were made at room temperature (23–24°C). All data are presented as means ± SE. Data were analyzed using pCLAMP 10 and Origin 8.5 (OriginLab, Northampton, MA) software.

Assessment of Na⁺,1.7 contribution to TTX-S current in small DRG neurons. For assessment of the contribution of Na⁺,1.7 to the TTX-sensitive (TTX-S) current, DRG neurons were isolated from WT or Na⁺,1.7-knockout (KO) mice (11–16 wk old) and cultured as previously described (Dib-Hajj et al. 2009). Whole cell voltage-clamp recordings of small (20–25 μm) DRG neurons were obtained at room temperature (20–22°C) within 2–8 h in culture using an EPC 10 amplifier (HEKA Electronics) and fire-polished electrodes (1–2 MΩ) fabricated from 1.6-mm outer-diameter borosilicate glass micropettes (World Precision Instruments). The pipette potential was adjusted to zero before seal formation, and liquid junction potential was not corrected. Voltage errors were minimized with 80–90% series resistance compensation, and linear leak currents and capacitive artifacts were subtracted out using the P/6 method. Currents were acquired with PULSE software (HEKA Electronics) 5 min after establishing whole cell configuration, sampled at a rate of 50 kHz, and filtered at 2.9 kHz. The pipette solution contained in mM: 140 CsF, 10 NaCl, 1 EGTA, and 10 HEPES, pH 7.3 with CsOH (adjusted to 315 mosM). The extracellular bath solution contained in mM: 70 NaCl, 70 choline chloride, 3 KCl, 1 MgCl₂, 1 CaCl₂, 20 TEACl, 5 CsCl, 1.4 AP, 0.1 CaCl₂, and 10 HEPES, pH 7.31 with NaOH (326 mosM). The amplitude of TTX-S sodium current was estimated by two protocols as previously described (Rush et al. 2005). Cells were first held at −80 mV, and a 500-ms depolarizing prepulse to −50 mV was applied to inactivate the TTX-S sodium channels while leaving Na⁺,1.8 current intact, followed by a series of step depolarizations from −70 to +20 mV (in 5-mV increments). Cells exhibiting Na⁺,1.9 currents were excluded from data analysis. For the second protocol, cells were held at −80 mV, a 500-ms hyperpolarizing prepulse to −120 mV was applied to rescue the TTX-S sodium channels from inactivation, and total sodium currents were evoked by a series of depolarizing steps from −70 to +20 mV (in 5-mV increments). The TTX-S sodium current was obtained by subtraction of the currents obtained from the two protocols.

Dynamic-clamp recording. DRG neurons (soma diameter 21–26 μm; 24.4 ± 0.3, n = 25) obtained from postnatal day 0-5 Sprague-Dawley rats were grown in primary culture for 2–3 days (Ahn et al. 2013; Dib-Hajj et al. 2009). Small DRG neurons were dynamically clamped (Kemenes et al. 2011; Sauer et al. 2012; Sharp et al. 1993) in whole cell configuration using patch pipettes pulled from glass capillaries (cat. no. PG10165-4; World Precision Instruments). Pipette resistance was 1.5–2 MΩ when filled with the intracellular solution in mM: 140 KCl, 3 MgATP, 0.5 EGTA, 10 HEPES, 10 glucose, pH 7.3 with KOH (adjusted to 325 mosM with sucrose). The extracellular solution was HBSS (cat. no. 14025; Invitrogen) in mM: 1.3 CaCl₂, 0.5 MgCl₂, 0.4 MgSO₄, 5.3 KCl, 0.4 KH₂PO₄, 4.2 NaHCO₃, 138 NaCl, 0.3 NaHPO₄, 5.6 glucose (adjusted to 325 mosM with sucrose). Liquid junction potential (+3.8 mV) between pipette and bath solutions was not compensated. Membrane voltages and currents were recorded in dynamic-clamp using MultiClamp 700B amplifier (Molecular Devices) interfaced with CED Power1401 mk II DA1 and Signal software (Cambridge Electronic Design, Cambridge, United Kingdom), digitized by Digidata 1440A DAC, and stored on the hard disk using pCLAMP 10 software. Capacitance neutralization and bridge balance were rigorously employed to minimize the effect of electrode capacitance and series resistance on the dynamic-clamp recordings. I–V traces were filtered at 10 kHz and digitized at 50 kHz. Series resistance was compensated by 80–85%. Endogenous sodium current recorded in whole cell voltage-clamp was evoked from a holding potential of −50 mV by a test pulse to −10 mV with or without preceding 0.5-s prepulse to −100 mV to remove steady-state inactivation of TTX-S channels. TTX-S channels are mainly inactivated at −50 mV, whereas Na⁺,1.8 channels are still available for activation at this potential because steady-state inactivation of Na⁺,1.8 is shifted in a depolarized direction (Catterall et al. 2005). Thus, as described by Cummins and Waxman (1997) and Rush et al. (2005), as a measure of total TTX-S current, we used a conditioning/subtraction protocol and assessed the peak current on the trace obtained by digital subtraction of the current trace evoked by the test voltage without preceding prepulse from that with the prepulse, respectively.

We estimated the contribution of Na⁺,1.7 to the total TTX-S current in our assay based on measurements of the total TTX-S current amplitude in WT and Na⁺,1.7-KO DRG neurons (see above). To evaluate the effect of defined levels of L858H functional expression on excitability of small DRG neuron using dynamic-clamp recording, we first estimated the endogenous Na⁺,1.7 conductance and then substituted graded amounts of endogenous conductance by equal amounts of L858H channel conductance, thus achieving a specified substitution ratio (SR) via a dynamic-clamp operation, SR × gmax(L858H − WT), where gmax is maximal Na⁺,1.7 endogenous conductance.

Recordings were made at room temperature (23–24°C). All data are presented as means ± SE. Data were analyzed using pCLAMP 10 and Origin 8.5 software. Unless specified otherwise, the hypothesis that population means are significantly different was checked using Mann-Whitney nonparametric test (P < 0.05, P < 0.01, and P < 0.001).

Kinetic model of Na⁺,1.7 channel. We developed our model of Na⁺,1.7 channel using Hodgkin-Huxley equations d/(dV) = αm(h − m) − βm(m − h) and d/(dV) = αh(1 − h) − β(h, where m and h are channel activation and inactivation variable and α and β are forward (backward) rate constants, respectively. Channel states were independent with a first-order reaction between states. Thus channel activation and deactivation were considered as transitions between closed and open states, whereas channel inactivation and repriming were assumed to be transitions between primed and inactivated states, respectively. Na⁺,1.7 channel steady-state parameters and kinetics obtained based on electrophysiological recordings were converted into appropriate rate constants at various voltages using the equations α = m/τ, β = (1 − m)/τ. These reaction rate constants were fitted with Boltzmann equations of the form y = A2 + (A1 − A2)/(1 + exp(V − V1/2)/k)), where V is membrane voltage, V1/2 is voltage when reaction rate is half-maximal, and k is slope coefficient. Fits were converted into steady-state inactivation (activation) variables and inactivation (activation) time constants according to m = α/α + β and τ = 1/(α + β). The latter curves were overlaid on the experimental data to provide feedback to the rate constants fitting step. This cycle was manually repeated until the best possible fit of the experimental data was achieved. We obtained the following rate constants for the WT Na⁺,1.7 channel model:

\[ \alpha_m = 10.22 \pm 2.02 \times 10^{3} \exp(V - 7.19)/15.43 \], \[ \beta_m = 23.76 \times 10^{7} \exp(V - 20.73)/14.53 ] \]

\[ \alpha_h = 0.0744 \times 10^{2} \exp(V - 99.76)/11.07 ] \], \[ \beta_h = 2.54 \times 10^{-4} \exp(V - 7.8)/10.68 ] \]

We modeled the L858H IEM mutation because it has been well-studied at the voltage-clamp (Cummins et al. 2004) and current-clamp

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(Rush et al. 2006) levels. We focused on altered activation of the mutant channels because a hyperpolarizing shift in activation is common to all IEM mutant channels (Dib-Hajj et al. 2010). We did not model slow-inactivation in the mutant channel because we limited stimulation to 1-s trains at 10 Hz where the development of slow inactivation is not appreciable. The L858H Na\(_{1.7}\) channel model was described by the following equations:

\[
\alpha_n = 9.1 - 9.1/[1 + \exp(V + 11.52/22.49)], \quad \beta_n = 23.76/[1 + \exp(V + 87.6/14.53)];
\]

\[
\alpha_h = 0.0744/[1 + \exp(V + 99.76/11.07)], \quad \beta_h = 2.54 - 2.54/[1 + \exp(V - 7.8/10.68)].
\]

Sodium current was described by \(I_{sca} = g_{\text{max}} \times m^3 h \times (V_m - E_{Na})\), where \(V_m\) is membrane voltage potential and \(E_{Na} = 65\) mV is sodium reversal potential. Currents evoked by different voltage protocols were calculated in 0-μs precision using a custom program written in Origin 8.5 LabTalk.

Na\(_{1.7}\) current kinetics in response to square test pulses and the resulting \(I-V\) curve were identical either when calculated in LabTalk or obtained from dynamic-clamp recordings on a vendors-supplied physical cell model. All data are presented as means ± SE. Data were analyzed using pCLAMP 10 and Origin 8.5 software.

**RESULTS**

**Kinetic model of WT Na\(_{1.7}\) based on Hodgkin-Huxley equations.** Channel kinetics were described based on a Hodgkin-Huxley model of sodium channel with several independent states and a first-order reaction between the states. Channel activation and deactivation were considered as transitions between closed and open states, whereas channel inactivation and repriming were assumed to be transitions between primed (open or closed) and inactivated states (Hille 1978; Hodgkin and Huxley 1952).

Na\(_{1.7}\) currents were recorded in whole cell voltage-clamp to obtain channel steady-state and kinetics parameters. Steady-state inactivation \((h_s)\) was calculated as the ratio of peak current amplitude to the maximal peak current amplitude elicited by a 0-mV test voltage following a 1-s prepulse at different voltages ranging from −100 to −40 mV in 5-mV increments from a holding potential of −100 mV (Fig. 1A). Steady-state activation \((m_s)\) was defined as the ratio of Na\(_{1.7}\) \(g = I_{\text{peak}}(V_m - E_{Na})\) determined at the respective membrane potentials to the Na\(_{1.7}\) \(g_{\text{max}}\) (Fig. 1A). Steady-state relationships of recombinant Na\(_{1.7}\) \((n = 8)\) cells were best fit using a Boltzmann equation with the following parameters: \(V_{1/2} = -73.9\) mV, \(k = 6.2\) mV and \(V_{1/2} = -20.4\) mV, \(k = 7.2\) mV for channel steady-state inactivation \((n = 8)\) and steady-state activation \((n = 17)\), respectively. Our model accurately described Na\(_{1.7}\) channel steady-state properties as can be seen from the respective Boltzmann fits of the modeled steady-state curves, \(V_{1/2} = -72.6\) mV, \(k = 5.7\) mV and \(V_{1/2} = -20.4\) mV, \(k = 7.1\) mV (Fig. 1A).

Na\(_{1.7}\) current activated in a sigmoidal manner with an apparent delay (Fig. 1, E and F), which suggests that the channel undergoes multiple closed-state transitions before activation. Channel activation kinetics were best described by a single exponential raised to the third power (Fig. 1E). Channel activation (Fig. 1B; \(n = 8\)) was determined from current traces elicited by test voltages ranging from −55 to 60 mV in 5- to 10-mV increments applied from −100-mV holding potential. Deactivation time constant (Fig. 1B; \(n = 14\)) was determined from a single-exponential fit of the respective portions of current traces (“tail currents”) measured at different test voltages ranging from −100 to −20 mV following a 0.5-ms voltage step to −10 mV from a holding potential of −100 mV.

The falling phase of the current (90 to 10% amplitude) was fitted with a single exponential to obtain the inactivation time constant (Fig. 1C; \(n = 7\)). The following protocol was used to evaluate channel repriming kinetics [for details, see Cummins et al. (1998)]. First, a 20-ms test voltage step to 0 mV was applied from a holding potential of −100 mV to inactivate the channel, and then repriming voltage steps of different durations (from 2 to 1,000 ms) were applied at a given voltage followed by the second 20-ms test voltage to 0 mV. The ratios of peak current amplitudes measured at the first and second test voltages were plotted as a function of the repriming step duration; this function was fitted with a single exponential to obtain a time constant of channel removal from inactivation (Fig. 1C; \(n = 14\)).

Our model effectively described channel kinetics as can be seen from the close match between the modeled and experimentally determined activation-deactivation (Fig. 1B) and inactivation-repriming (Fig. 1C) time constants at a wide range of physiological membrane voltages. The obtained Hodgkin-Huxley variables \(m^2\) and \(h\) were both highly voltage-dependent with submillisecond activation kinetics resulting in a transient channel open probability in response to a series of voltage steps (Fig. 1D). Our model accurately followed the kinetics of Na\(_{1.7}\) current evoked by a series of depolarization steps (Fig. 1, F and G). The resulting \(I-V\) relationships also provided a match between modeled and experimentally determined data at a wide range of physiological membrane voltages (Fig. 1H).

Thereafter, we utilized this model (WT) and its modification for the L858H mutant channel to study mechanisms of functional contribution of Na\(_{1.7}\) channel to neuronal excitability in normal and pathological conditions.

Na\(_{1.7}\) conductance regulates current threshold for AP generation in a linear manner. We performed a quantitative analysis of the effect of graded additions or subtractions of sodium conductance resulting from Na\(_{1.7}\) channel function on small DRG neuron excitability using dynamic-clamp. Although we had the capability to transfect DRG neurons with Na\(_{1.7}\) channels to study the effect of the channel on neuronal excitability (Dib-Hajj et al. 2009; Rush et al. 2006), transfection does not permit accurate calibration of the level of Na\(_{1.7}\) channel that is being expressed. Thus we carried out dynamic-clamp recording in DRG neurons based on experimentally determined Na\(_{1.7}\) gating properties and an assumption that Na\(_{1.7}\) contributes on average 70% of the TTX-S current in small DRG neurons. We based our estimate of the Na\(_{1.7}\) contribution of 70% of the TTX-S current in small DRG neurons on our measurements of total TTX-S currents in mice that are deficient in Na\(_{1.7}\) (Fig. 2). Figure 2A shows representative family traces of TTX-S sodium currents recorded in DRG neurons from WT and Na\(_{1.7}\)-KO mice, respectively. Peak current densities were \(143 ± 17\) pA/pF \((n = 16)\) for TTX-S sodium channels recorded from WT DRG neurons. However, DRG neurons from Na\(_{1.7}\)-KO mice produced significantly smaller TTX-S sodium currents with current densities \(-32\% (46 ± 9\) pA/pF, \(n = 16)\) of that in WT DRG neurons (Fig. 2B).

Using dynamic-clamp with the Na\(_{1.7}\) channel model, we studied how increases in the level of Na\(_{1.7}\) conductance affect small DRG neuron excitability. Dynamic-clamp allowed us to

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*J Neurophysiol* • doi:10.1152/jn.00763.2013 • www.jn.org
add graded increments of Nav1.7 current to the cell, matching the injected conductances to the predicted contribution of Nav1.7 channels to the TTX-S current (70% of total TTX-S current in each cell is assumed to be produced by Nav1.7; see above). We found that the current threshold (defined when the 2nd differential of AP changes its sign) inversely correlated in a linear fashion ($r^2 = 0.98$, incremental threshold change) with the addition of Nav1.7 conductance (Fig. 3, A and B). Current threshold decreased from its original value (426 ± 82 pA, n = 9) to a value of 310 ± 65 pA (n = 9) when Nav1.7 was doubled by dynamic-clamp. Since current threshold variability was large (current threshold in 9 control cells was 350, 230, 470, 525, 340, 240, 90, 840, and 750 pA) in small DRG neurons, we normalized the effect of the Nav1.7 channel addition on current threshold and expressed it in the form $100\% \times \frac{\text{CT}}{\text{CT}_0}$, where $\text{CT}$ is current threshold change at the respective level of Nav1.7 addition and $\text{CT}_0$ is native current threshold. The normalized current threshold change significantly decreased (Mann-Whitney, $P < 0.001$) from 5.6 ± 0.8% (addition of 12.5% Nav1.7) to -28 ± 3.5% (addition of 100% Nav1.7). Importantly, stimulation with a current equivalent to the current threshold when
Nav1.7 conductance was doubled did not elicit an AP in a DRG neuron with native levels of Nav1.7 (Fig. 3, A and B). Electronic addition of Nav1.7 conductance also resulted in an enhancement of AP firing probability (Fig. 3C). A 10-Hz train of 10 current pulses 10-ms duration each applied at the threshold level evoked 1.7 ± 0.3 APs in control cells vs. 4.3 ± 0.5 (n = 8; P < 0.01), 6.1 ± 0.7 (n = 8; P < 0.01), 8.4 ± 0.7 (n = 8; P < 0.001), and 9.4 ± 0.5 (n = 8; P < 0.001) after electronic addition of 12.5, 25, 50, and 100% Nav1.7 conductance, respectively (Fig. 3D).

It is reasonable to suggest that electronic subtraction of Nav1.7 conductance will reduce neuronal excitability. Indeed, incremental addition of graded levels of negative Nav1.7 conductance resulted in an increase of current threshold in a linear fashion (r² = 0.99) and in incremental (r² = 0.98) reduction of AP firing probability (Fig. 4, A and B). A 10-Hz train of 10 current pulses applied at 1.5× threshold level evoked 9.9 ± 0.1 APs (n = 15) in control cells vs. 9.4 ± 0.4 (n = 15), 7.8 ± 0.6 (n = 15; P < 0.001), 5.3 ± 1.7 (n = 15; P < 0.001), and 2.0 ± 1.0 (n = 15; P < 0.001) after dynamic-clamp subtraction of 12.5, 25, 50, and 100% Nav1.7 conductance, respectively (Fig. 4, A and B). In the same cells, current threshold increment significantly increased from 3.9 ± 0.7 to 9.4 ± 1.6% (n = 13; P < 0.001), 17.1 ± 2.3% (n = 13, P < 0.001), and 32.8 ± 3.4% (n = 13; P < 0.001) in response to 12.5, 25, 50, and 100% reduction of Nav1.7 conductance. Since the Nav1.7 channel begins to activate at −55 to −50 mV (Fig. 1A), it is possible that subtraction of Nav1.7 conductance results in a reduced sodium current at subthreshold voltages, lowering the net current influx so that, to achieve AP threshold, the stimulus intensity would need to be increased to compensate for the lower sodium charge. Our measurements of Nav1.7 channel activity in a dynamic-clamped DRG neuron support this hypothesis. Nav1.7 began to activate at about −53 mV, reached 62% of its peak value at −32 mV threshold voltage, and reached peak amplitude at −19 mV (Fig. 4C). In the example shown, Na1.7 sodium influx at subthreshold voltages comprises ~21% of the total sodium charge due to Na1.7 channel activity occurring in the time interval between stimulus onset and AP undershoot (Fig. 4C). Subsequently, the channel inactivates quickly, and the resulting current is not present during the interpulse interval provided membrane potential is not ramping back to the threshold level (Fig. 4D, but see Fig. 5C). The relatively slow channel repriming kinetics at membrane voltages close to resting membrane potential (RMP; Fig. 1C) still allow the channel to recover from inactivation during 10-Hz stimulation cycle without significant accumulation of inactivation (Fig. 4D, bottom). These data suggest that Na1.7 channel activity at subthreshold membrane voltages drives the set point of current threshold for AP generation, thus regulating AP firing probability.

Model of Na1.7 L858H mutant predicts an enhanced level of channel activity during repetitive firing of DRG neuron. The L858H mutation in human Na1.7 has been shown to be associated with a neuropathic pain syndrome, IEM (Yang et al. 2004). The mutation produces a hyperpolarizing shift in channel activation, slows channel deactivation, and causes an increase in amplitude of the Na1.7 current in response to slow, small depolarizations (Cummins et al. 2004). When expressed in the native cell environment, transfection with mutant channels renders small DRG neurons hyperexcitable and is associated with depolarized RMP and reduced current threshold (Rush et al. 2006). To study the mechanism of Na1.7 L858H-induced neuronal hyperexcitability in more detail, we performed quantitative evaluations of the effect of graded dynamic-clamp substitutions of L858H mutant conductance for WT Na1.7 conductance on the electrical excitability of small DRG neurons. In the absence of empirical data, we assumed that the level of functional WT and L858H channels in the DRG neuron plasma membrane was the same.

First, we constructed a Hodgkin-Huxley (Hodgkin and Huxley 1952) model of the L858H channel based on the previously reported data (Cummins et al. 2004) by appropriately modifying our kinetic model of WT Na1.7 channel. We adjusted activation rate constants to account for the −14 mV shift of L858H channel steady-state activation and for the altered mutant deactivation (Fig. 5B). The resulting kinetic model of L858H channel had a significantly enhanced window current (window current is defined as m³ × h at steady-state; Fig. 5B) and an apparent leftward shift of I–V curve in a manner consistent with data reported by Cummins et al. (2004) (Fig. 5, A and B). Maximal steady-state channel open probability increased 17-fold from 9.5 × 10⁻³ at −32.3 mV to 1.6 × 10⁻² at −51.5 mV (Fig. 5B); when measured at −63-mV RMP, it increased 255-fold from 3.8 × 10⁻⁶ to 9.7 × 10⁻⁴ (Fig. 5B). Here and thereafter, both WT and L858H models were calculated in a 28-pF equipotential sphere of 1 μm² × cm⁻¹ capacitance; conductance density was set to 0.029 S/cm². We further studied differences of WT and L858H channel behavior in response to a voltage command shaped in the form of membrane potential previously recorded from spontaneously active small DRG neuron (Fig. 5C, top). Both WT and L858H channels were active at subthreshold levels during ramplike membrane depolarizations between APs (Fig. 5C, left) as well as during APs (Fig. 5C, right). L858H model channel was already
activated at a $-62$-mV initiation point producing $-115$-pA inward current, whereas WT channel produced only $-0.5$-pA current at this voltage. During the second cycle of AP firing, L858H channel began to activate at $-70$-mV undershoot ($-2$ pA) and reached $-209$ pA at $-54$ mV, whereas WT channel just began to activate ($-2$ pA) at $-54$ mV. At the $-33.5$-mV AP voltage threshold, WT current was $-115$ pA, whereas L858H current was $-657$ pA. L858H current peaked ($-659$ pA) at $-32$ mV, and WT current peaked ($-320$ pA) at $-2.9$ mV. Within the postpeak $0.5$ ms (WT) and $1$ ms (L858H), current declined sharply to about $-20$ pA at the $56.8$-mV AP overshoot. This pattern of L858H channel activity resulted in a substantial enhancement of the sodium influx at subthreshold membrane voltages (WT, $4.1 \times 10^{-13}$ C and L858H, $1.1 \times 10^{-11}$ C) as well as during AP (WT, $2.8 \times 10^{-13}$ C and L858H, $7.7 \times 10^{-13}$ C). The differences in the pattern of WT and L858H channel activity remain unchanged throughout the repetitive cycle of AP firing (Fig. 5, C and D). Steady-state inactivation and inactivation kinetics were not affected by L858H mutation. Hence, both WT and L858H channels showed a similar level of use-dependent inactivation (Fig. 5, C and D).

The model predicts a substantial enhancement of $\text{Nav}_1.7$ L858H mutant activity over a wide range of physiological membrane voltages during interspike intervals, at subthreshold levels of depolarization, and in the course of AP, thus increasing the sodium charge inflow.

Our kinetic model of the L858H mutant channel, consistent with the data published previously (Rush et al. 2006), predicted that expression of L858H mutant should enhance neuronal excitability. To test this hypothesis, we evaluated the effect of defined levels of L858H functional expression on electrical excitability of small DRG neurons using dynamic-clamp recording. A single-allele mutation of $\text{SCN9A}$, assuming equal efficiency of expression of WT and mutant channels, most probably results in 1:1 ratio of WT and L858H expressions in
sensory neurons of the affected individual; however, the exact stoichiometry of WT-to-L858H expression is not known. We therefore used dynamic-clamp to assess the effect of substitutions of graded amounts of L858H current for WT current. In designing our experiments on rat DRG neurons, we first estimated the endogenous Nav1.7 conductance (see above for details) and then dynamically substituted graded amounts of endogenous conductance by equal amounts of L858H channel conductance. Essentially, we performed dynamic-clamp operation $SR \times g_{\text{max}}(\text{L858H} - \text{WT})$.

We first tested the effect of WT-to-L858H substitution on current threshold. An AP was first evoked in a control DRG neuron without any current substitution by a 10-ms current pulse of threshold intensity (Fig. 6A). WT-to-L858H substitution was then implemented and resulted in a substantial inward current that developed at subthreshold voltages during stimulation and an apparent shortening of the delay for AP generation. The amplitude of the AP was not affected and was (from overshoot to undershoot) 113.9 mV in control vs. 114.8 (112.6) mV at a 25% (50%) SR [i.e., at 25% (50%) L858H]. At the same time, the maximal rate of AP rise became progressively smaller, being 114.2, 109.5, and 96.1 mV/ms in control and 25 and 50% SR, respectively (Fig. 6, C and D). We suggest that this deceleration of AP rate of rise could be the result of

![Figure 4](http://jn.physiology.org/)

**Fig. 4.** Removal of Na\(_{\text{v}1.7}\) conductance raises current threshold for AP generation and reduces AP firing probability. *A:* APs were evoked by 10-ms-long current pulses applied at 1.5x threshold amplitude in control (Ctrl; black) and after electronic subtraction of the incremental values of Na\(_{\text{v}1.7}\) conductance. Dynamic-clamp subtraction of Na\(_{\text{v}1.7}\) conductance (expressed as the incremental decrease over the endogenous Na\(_{\text{v}1.7}\) conductance) is noted on the y-axis. Scale bar, 200 ms. *B,* top: averages (n = 13) of current threshold change in response to the subtraction of respective proportion of endogenous Na\(_{\text{v}1.7}\) conductance. The solid line is a linear regression fit ($r^2 = 0.99$) of the data. Statistical analysis on the top was performed between threshold increments obtained at 12.5% and at the respective percentage of conductance increment. *B,* bottom: averages (n = 15) of the number of APs evoked by the protocol described in A and plotted as a function of dynamically subtracted Na\(_{\text{v}1.7}\) conductance; the solid line is a linear regression fit ($r^2 = 0.98$) of the data. **$P < 0.01$ and ***$P < 0.001$.

*C:* AP (top) evoked by a 10-ms-long current stimulus of 1.5x threshold intensity in control (black) and after dynamic-clamp subtraction of 12.5% of Na\(_{\text{v}1.7}\) conductance (blue) and the respective Na\(_{\text{v}1.7}\) model current (bottom). *D:* APs (top) evoked by the protocol described above in control (black) and after dynamic-clamp subtraction (middle) of 12.5% of endogenous Na\(_{\text{v}1.7}\) conductance. The $I$–$V$ phase plot of the model Na\(_{\text{v}1.7}\) conductance dynamically subtracted during neuronal repetitive firing is shown on the bottom. Note that the positive-going (outward) dynamic-clamp current shown in C and D is flipped over 0 line to facilitate comparison of Na\(_{\text{v}1.7}\) current across the manuscript.
additional inactivation of endogenous channels due to the depolarization of RMP described below; however, we cannot exclude the possibility that the reduction of net (L858H − WT) current seen at the respective voltage of AP maximal rise rate (20 mV; 527 pA at 25% SR and 385 pA at 50% SR) is a causative factor (Fig. 6B, right). Substitution of WT-to-L858H conductance in incremental amounts resulted in the reduction of AP current threshold in a linear manner ($r^2 = 0.96$, Fig. 6C, top; $r^2 = 0.97$, Fig. 6C, bottom). Current threshold was 668 ± 142 pA (control, $n = 5$) and was reduced to 598 ± 188, 457 ± 92, 328 ± 53, and 133 ± 20 pA ($n = 5; P < 0.05$) after 12.5, 25, 50, and 100% WT-to-L858H substitution, respectively (Fig. 6C). The reduction of current threshold was accompanied by enhancement of AP firing probability (Fig. 6D). A 10-Hz train of 10 current pulses applied at the threshold level evoked on average 1.3 ± 0.2 APs in control and 8.9 ± 0.5, 9.3 ± 0.6, 8.9 ± 1.0, and 8.4 ± 1.0 APs ($n = 5; P < 0.01$) after 12.5, 25, 50, and 100% WT-to-L858H substitution, respectively (Fig. 6E).

The present findings confirm earlier results (Rush et al. 2006) showing that the reduction of current threshold and the enhancement of AP firing probability are accompanied by RMP depolarization (Fig. 7C). At a mechanistic level, the present results add to the previous findings by showing that RMP depolarization is driven by the persistent window activity of L858H channel at membrane voltages close to RMP (Fig. 5B, middle). The effect of WT conductance subtraction in (L858H − WT) model on DRG neuron RMP was negligible since addition or subtraction of up to 100% of WT Na,1.7 conductance, performed in a set of additional experiments, did not significantly affect RMP of DRG neurons in the −65- to −62-mV range of recorded RMP (Fig. 7A and B). The average RMP of DRG neurons was (mean ± SE, $n = 6$; $P > 0.05$): −64 ± 0.6 mV in control and −63.5 ± 0.8, −63.8 ± 0.7, −64.0 ± 0.5, −63.6 ± 0.6, −63.6 ± 0.6, −63.7 ± 0.7, −63.9 ± 0.8, and −64.0 ± 1.0 mV after addition of −100, −50, −25, −12.5, +12.5, +25, +50, and +100% WT conductance, respectively (Fig. 7A and B). In contrast, WT-to-L858H substitution significantly depolarized RMP from −63.4 ± 0.6 mV in control to −62.3 ± 0.5 mV ($n = 6; P > 0.05$), −60.7 ± 0.8 mV ($n = 6; P < 0.05$), −58.4 ± 0.9 mV ($n = 6; P < 0.01$), and −56.6 ± 1.3 mV ($n = 6; P < 0.01$) after
12.5, 25, 50, and 100% WT-to-L858H conductance exchange, respectively (Fig. 7D).

L858H mutant enhances DRG neuron excitability by increasing sodium influx during subthreshold membrane depolarizations and interspike intervals. Our model of the L858H mutant predicts an enhanced level of channel activity at subthreshold and suprathreshold voltages during repetitive firing of DRG neuron (see Fig. 5 and text above). The additional sodium charge inflow predicted by our results would be expected to be proexcitatory and should lead to enhanced neuronal excitability. We tested this hypothesis in dynamically clamped DRG neurons by assessing the response to a 10-Hz
train of 10-ms depolarizing stimuli of two models: WT and (L858H/WT). We wanted to compare numerically the effect of these two models of channel activity on neuronal excitability side by side and first present data at 12.5% WT channel conductance addition and the respective 12.5% WT-to-L858H conductance exchange, since 12.5% SR is the minimal substitution studied for which AP firing is affected.

We found that L858H channels were already active at 63.4 mV RMP, producing current responsible for a 1-mV depolarizing shift of the RMP (63.4 ± 0.6 mV in control and 62.3 ± 0.5 mV after 12.5% SR, n = 6, P > 0.05, but with a clear depolarizing trend of RMP), whereas WT current was not detectable above the noise, thus having no effect on RMP (64 ± 0.6 mV in control and 63.6 ± 0.6 mV after addition of 12.5% WT conductance; Fig. 8, A–D). WT current began to activate (−3 pA) at −50.9 mV and reached its −216-pA maximum amplitude at 12.4 mV, a value of membrane voltages well above −40-mV AP threshold. In contrast, (L858H − WT) net current was already active (−21 pA) at −63-mV RMP and reached its maximum (−452 pA) at −30.6 mV. During interspike intervals, the net current resulting from (L858H − WT) substitution gradually increased from −1 pA at −69.5 mV to −20 pA at −64 mV, contributing to the development of slow, ramplike depolarization of the membrane potential (Fig. 8, D and E), whereas current produced by WT model was not detectable above the noise (Fig. 8, B and E). Significantly more net charge was carried by the (L858H − WT) compared with WT (in pA*ms/nA, we normalized current charge to the amplitude of native Nav1.7 current to account for cell-to-cell variability in the level of endogenous Nav1.7 current): 6.6 ± 1.2 (WT, n = 8) vs. 48 ± 10 (L858H, n = 5; P < 0.01) during subthreshold depolarization; and −0.2 ± 0.32 (WT, n = 8) compared with 58.2 ± 10.4 (L858H, n = 5; P < 0.01) during interspike intervals; whereas the charge during AP actually was smaller for mutant cells, 12.6 ± 6.3 (L858H, n = 5), compared with WT, 22 ± 3.5 (n = 8). These data indicate that the substantial increase of L858H channel activity at subthreshold membrane voltages results in a significant amplification of net sodium influx, subsequent depolarization of the membrane potential, reduction of current threshold, and ensuing enhancement of AP firing probability. These observations reveal enhancement of small DRG neuron excitability by substitution of as little as 12.5% of the Nav1.7 channels of the cell with L858H channel (i.e., with expression of the mutant channel at a density much lower than expected for a cell with 1 mutant allele) and demonstrate the powerful effect of the mutant channels on nociceptor excitability.
Finally, as a model of nociceptors in patients carrying the L858H mutation, where single-allele mutation of SCN9A probably results in a ratio close to 1:1 of WT and L858H, we assessed the effect of a 50% SR of L858H channels. The model produced $-37 \pm 2 \text{ pA (n = 6)}$ persistent current at rest (Fig. 9, A and B), which depolarized RMP on average by 5 mV from $-63.4 \pm 0.6 \text{ mV in control to } -58.4 \pm 0.9 \text{ mV (n = 6; P < 0.01)}$ after 50% WT-to-L858H conductance exchange (Fig. 7D and Fig. 9, A and B). This current activated further at subthreshold membrane potentials in response to depolarizing current injection, reached $-432 \pm 112 \text{ (n = 6)}$ peak at 2.7 ± 0.4 ms poststimulus before AP threshold, and then promptly declined to essentially zero level within the next 3.6 ± 0.7 ms (Fig. 9A). The net charge inflow due to the (L858H – WT) model activity during subthreshold depolarization and during AP was $0.8 \pm 0.3 \text{ pC and } 0.3 \pm 0.2 \text{ pC (n = 6)}$, respectively (Fig. 9C). During first interspike intervals, the net current resulting from 50% (L858H – WT) SR produced $2.9 \pm 0.5 \text{ pC charge inflow, contributing to the development of slow, ramp-like depolarization of the membrane potential (Fig. 9, B and C).}

These observations of increased sodium influx underlying enhancement of small DRG neuron excitability following L858H substitution provide a mechanistic link between altered function of mutant channels and nociceptor hyperexcitability underlying the pain phenotype in patients carrying the Na$_{1.7}$ L858H mutation.

**DISCUSSION**

Our dynamic-clamp recording in native rat DRG neurons shows that increasing the Na$_{1.7}$ conductance density lowers threshold for a single AP and increases the number of APs fired in response to a train of depolarizing stimuli. Our data show a linear inverse relationship between functional Na$_{1.7}$ conductance and current threshold (the minimal stimulus capable of eliciting an AP). Consistent with the latter, we found a direct correlation between Na$_{1.7}$ conductance and AP firing probability. The relationship be-
between Na\textsubscript{v1.7} conductance and AP firing probability was also linear in the 0–25% range of additional Na\textsubscript{v1.7} conductances. Saturation at 50–100% level was at least in part due to reaching maximal possible number of APs that could be evoked by our stimulation protocol.

It is generally accepted that TTX-S channels, including Na\textsubscript{v1.7} channel, can function as a subthreshold sodium channel that amplifies membrane response to small depolarizing stimuli at subthreshold membrane voltages both in DRG neuron soma (Blair and Bean 2002; Choi and Waxman 2011; Kovalsky et al. 2009; Rush et al. 2007) and at the axon endings of primary sensory neurons (De Col et al. 2008; Pinto et al. 2008; Vasylyev and Waxman 2012). The functional impact of Na\textsubscript{v1.7} channel activity on neuronal excitability and pain signal processing has been generally studied using an “all-or-none” paradigm using genetic knockout (Nassar et al. 2004) of Na\textsubscript{v1.7} channel function. Functional contributions of Na\textsubscript{v1.7} to DRG neuron excitability, including current and voltage thresholds for AP generation and AP repetitive firing, have also been studied using computer simulations of DRG neurons. (Choi and Waxman 2011; Herzog et al. 2001; Kouranova et al. 2008; Kovalsky et al. 2009; Sheets et al. 2007). However, dynamic-clamp recording is superior to computer simulation for quantitative study of Na\textsubscript{v1.7} channel function because it records the response of native neurons without making assumptions regarding which conductances to include in the computer model (Kemenes et al. 2011; Samu et al. 2012; Sharp et al. 1993). Our immediate and quite unexpected observation was that Na\textsubscript{v1.7}, when studied in the native DRG neuron environment, regulated the set point for AP current threshold in a remarkably linear manner: increasing or reducing Na\textsubscript{v1.7} conductance by as little as 12.5% or as much as 100% produced a graded effect with a high degree of linearity over a 200% range of Na\textsubscript{v1.7} conductances. We found that substitution of as little as 12.5% of channels with L858H produces hyperexcitability in DRG neurons. This observation predicts that expression of L858H produces hyperexcitability of DRG neurons even if the 50% reduction of current density seen after expression of L858H in HEK-293 cells (Cummins et al. 2004) applies to nociceptors.

Within the domain permitted by our stimulation protocol, Na\textsubscript{v1.7} also regulated AP firing probability in a linear manner. This observation may be relevant to the pathophysiology of acquired pain since abnormal accumulations of Na\textsubscript{v1.7} have been demonstrated at nerve endings within painful neuromas in rats (Persson et al. 2011) and humans (Black et al. 2008), and Na\textsubscript{v1.7} levels and TTX-S current density have been shown to increase in DRG neurons in response to inflammation (Black et al. 2004) and in diabetic rats (Chattopadhyay et al. 2008, 2011). We would note, however, that although our previous patch-clamp analysis of small-diameter DRG neuron axons in vitro demonstrated a resting potential similar to that in DRG neuron somata, and sequential activation during AP clamp of TTX-S and TTX-resistant currents with characteristics attributed to Na\textsubscript{v1.7} and Na\textsubscript{v1.8} (Vasylyev and Waxman 2012) similar to that Fig. 9. A single-allele SCN9A mutation: L858H functional evaluation in small DRG neuron. A: AP evoked by a current pulse of threshold intensity in control (black) and after dynamic-clamp 50% exchange of WT-to-L858H conductance (red). APs are shown on the top (stimulation protocol is shown on top of APs), and the respective Na\textsubscript{v1.7} current differential is presented in the bottom. B: AP repetitive firing (top) evoked by a 10-Hz train of current pulses (same as in A) at threshold intensity in control (black) and after 50% WT-to-L858H SR (blue); dynamic-clamp recording of the 50% (L858H – WT) current is shown on the bottom. C: dynamic-clamp (L858H – WT) model currents at 50% SR of endogenous Na\textsubscript{v1.7} conductance were integrated over 3 different time intervals: 1) from stimulus onset to AP threshold (arrow 1 to 2 in A); 2) from threshold to undershoot (arrows 2 and 3 in A); and 3) from undershoot to the next stimulus onset. Sodium charge (pA*ms) was normalized to the peak value of the native Na\textsubscript{v1.7} sodium current (n = 6). Kruskal-Wallis ANOVA nonparametric test for 3 populations was used to determine whether the samples come from different populations (P < 0.01).
seen in DRG neuron somata (Blair and Bean 2002), we cannot exclude the possibility that the properties and functional role of Na$_{1.7}$ are not identical in DRG neuron somata vs. sensory axons and their terminals.

Dynamic-clamp requires an input of a kinetic model of the channel under study. Several kinetic models of TTX-S sodium channels, including Na$_{1.7}$ channel, have been recently proposed (Gurkiewicz et al. 2011; Herzog et al. 2001; Kovalsky et al. 2009; Sheets et al. 2007). These models rely on experimental data for TTX-S channels steady-state and kinetic properties with some degree of variability since these were obtained in different studies. This variability may be attributed, at least in part, to differences in recording solutions and in voltage protocols. TTX-S channel recordings were often performed using fluoride-based intracellular solution, which shifts the voltage dependence of Na$_{1.7}$ channel steady-state and kinetic parameters (Coste et al. 2004; Meadows et al. 2002; Saab et al. 2003; Sheets et al. 2007). At the same time, current-clamp recordings of AP firing in DRG neurons are commonly performed in a physiologically relevant chloride-based intracellular solution (Dib-Hajj et al. 2009; Estacion et al. 2011; Rush et al. 2006). It is reasonable to develop the kinetic model of Na$_{1.7}$ channel based on voltage-clamp data obtained under conditions similar to those where this model is utilized. Thus we developed a kinetic model of recombinant Na$_{1.7}$ channel based on voltage-clamp recordings obtained using intracellular solution essentially similar to that used for dynamic-clamp; the single difference between voltage-clamp and dynamic-clamp pipette solutions was that potassium chloride was replaced by cesium chloride on 1:1 basis (see MATERIALS AND METHODS). We also used the same HBSS bath solution for voltage-clamp and dynamic-clamp recordings. Additionally, we performed a detailed analysis of Na$_{1.7}$ channel activation kinetics. Although an $m^3$ model is generally accepted for sodium channel gating in squid giant axon (Hodgkin and Huxley 1952), mammalian skeletal muscles (Chanda and Bezanilla 2002), and mammalian sensory neurons (Herzog et al. 2001; Kostyuk et al. 1981; Ogata and Tatebayashi 1993; Sheets et al. 2007), an $m^2$ model of TTX-S sodium channel activation has been suggested in mammalian central neurons (Baranauskas and Martina 2006). Consistent with previous studies of TTX-S sodium current (Herzog et al. 2001; Kostyuk et al. 1981; McCormick et al. 2007; Ogata and Tatebayashi 1993; Sheets et al. 2007), we found that recombinant Na$_{1.7}$ channel activated with an apparent delay, suggesting multiple transitions between closed states, in a sigmoidal manner with activation kinetics best described by $m^3$ model.

The Na$_{1.7}$ L858H mutation was identified in a patient with hereditary primary erythromelalgia (Yang et al. 2004). Subsequent studies showed that the L858H mutation produces a $-13.5$-mV hyperpolarizing shift of Na$_{1.7}$ channel activation, slows channel deactivation, and enhances Na$_{1.7}$ current amplitude in response to slow, small depolarizations in a manner consistent with its erythromelalgia phenotype (Cummins et al. 2004). We modeled kinetics of the L858H mutant by appropriately adjusting WT channel activation rate constants to obtain the respective alterations in kinetics and in $I-V$ relationships similar to those reported for recombinant WT and L858H channels (Cummins et al. 2004). Our L858H model predicted enhancement of Na$_{1.7}$ channel activity at subthreshold membrane voltages. In response to a voltage command shaped in the form of membrane voltage of spontaneously firing DRG neuron, substitution of only 12.5% of the channels of the cell with the L858H model, compared with WT, resulted in a 27-fold increase of sodium influx at subthreshold membrane voltages and in a 3-fold sodium influx increase during the AP. When we evoked APs in dynamic-clamp using a 10-ms depolarizing stimulus, persistent L858H channel activity at subthreshold voltages produced $>600\%$ additional sodium influx during small depolarizations and resulted in the appearance of a significant sodium influx during interspike intervals ($-0.2 \pm 0.32$, WT; compared with $58.2 \pm 10.4$, L858H). Such a substantial increase of the sodium influx is proexcitatory, and we were not surprised to observe a hyperexcitable neuronal phenotype due to L858H introduced by dynamic-clamp. Substitution of 50% WT-to-L858H conductance in our dynamic-clamp experiments produced persistent current that depolarized RMP on average by 5 mV and resulted in a reduction of current threshold on average by 51% without changing AP overshoot. This persistent current activated further at subthreshold membrane potentials in response to depolarizing current injection, reached its peak before AP threshold, and then promptly declined to essentially zero level within the next few milliseconds. The net charge inflow due to (L858H – WT) model was larger during subthreshold depolarization than during the AP and produced a significant charge inflow during interspike intervals driving a slow, ramplike depolarization of the membrane potential. On the basis of the present results, we attribute the L858H-induced RMP depolarization described by Rush et al. (2006) and seen in this study to the persistent current due to window channel activity. Steady-state open channel probability at $-63$-mV RMP increased 255-fold from $3.8 \times 10^{-6}$ (WT) to $9.7 \times 10^{-4}$ (L858H). Maximal conductance of endogenous Na$_{1.7}$ current (see MATERIALS AND METHODS) was $317 \pm 68$ nS ($n = 6$, WT experiments) and $354 \pm 64$ nS ($n = 6$, L858H experiments). Addition of 50% of the respective conductance resulted, assuming the calculated steady-state open probability, in 0.08-pA (WT) and 22-pA (L858H) persistent current at $-63$-mV RMP for dynamic-clamped neuron.

Our results obtained via dynamic-clamp at physiological levels of WT and mutant Na$_{1.7}$ conductance show, for the first time, that current threshold of small DRG neurons is regulated by Na$_{1.7}$ in a linear manner. Our observations also demonstrate that persistent activity of the L858H mutant channel in small DRG neurons amplifies sodium influx at subthreshold membrane voltages, so as to depolarize RMP and reduce current threshold for AP generation, thus producing hyperexcitability in nociceptive DRG neurons. Taken together, these findings establish a quantitative mechanistic link between the altered biophysical properties of a mutant Na$_{1.7}$ channel and nociceptor hyperexcitability underlying the pain phenotype in IEM.

**GRANTS**

This work was supported in part by grant from the Rehabilitation Research & Development Service and Medical Research Service, Department of Veterans Affairs to S. G. Waxman. The Center for Neuroscience and Regeneration
MECHANISTIC ROLE OF NaV,1.7 IN NEURONAL HYPEREXCITABILITY

Research is a collaboration of the Paralyzed Veterans of America with Yale University.

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

AUTHOR CONTRIBUTIONS

REFERENCES


