A Sodium Channel Gene *SCN9A* Polymorphism That Increases Nociceptor Excitability

Mark Estacion, PhD,[1,2,3] T. Patrick Harty, PhD,[1,2,3] Jin-Sung Choi, PhD,[1,2,3] Lynda Tyrrell, MA,[1,2,3] Sulayman D. Dib-Hajj, PhD,[1,2,3] and Stephen G. Waxman, MD, PhD[1,2,3]

Sodium channel $Na_V1.7$, encoded by the *SCN9A* gene, is preferentially expressed in nociceptive primary sensory neurons, where it amplifies small depolarizations. In studies on a family with inherited erythromelalgia associated with $Na_V1.7$ gain-of-function mutation A863P, we identified a nonsynonymous single-nucleotide polymorphism within *SCN9A* in the affected proband and several unaffected family members; this polymorphism (c. 3448C&T, Single Nucleotide Polymorphisms database rs6746030, which produces the amino acid substitution R1150W in human $Na_V1.7$ [$hNa_V1.7$]) is present in 1.1 to 12.7% of control chromosomes, depending on ethnicity. In this study, we examined the effect of the R1150W substitution on function of the $hNa_V1.7$ channel, and on the firing of dorsal root ganglion (DRG) neurons in which this channel is normally expressed. We show that this polymorphism depolarizes activation (7.9–11mV in different assays). Current-clamp analysis shows that the 1150W allele depolarizes (6mV) resting membrane potential and increases (~2-fold) the firing frequency in response to depolarization in DRG neurons in which it is present. Our results suggest that polymorphisms in the $Na_V1.7$ channel may influence susceptibility to pain.

Ann Neurol 2009;66:862–866

It is now clear that, in the human nervous system, 9 different isoforms of voltage-gated sodium channels function in different ways, collaborating to produce electrical activity within nerve cells. The $Na_V1.7$ sodium channel (encoded by the gene *SCN9A*), in particular, is preferentially expressed in pain-signaling dorsal root ganglion (DRG) neurons (nociceptors)[1,2] and has been shown to play a critically important role in these cells, amplifying small depolarizations so as to increase the gain in pain signaling.[3] Gain-of-function mutations of $Na_V1.7$ have been shown to cause the painful disorders inherited erythromelalgia (IEM)[4,5] and paroxysmal extreme pain disorder,[6] whereas loss-of-function mutations cause congenital insensitivity to pain,[7] underscoring the important role of $hNa_V1.7$ in human pain signaling.

In studies on a family with IEM associated with $Na_V1.7$ gain-of-function mutation A863P,[8] we identified a nonsynonymous single-nucleotide (c. 3448C>T) polymorphism within *SCN9A* (Single Nucleotide Polymorphisms database [dbSNP] rs6746030) in the affected proband, and in several unaffected family members (father and brother of proband), and reported that this polymorphism is present in 14% of ethnically matched, Caucasian control chromosomes.[8] This polymorphism, in exon 18 of *SCN9A*, substitutes a nonpolar tryptophan (W) for a positively charged arginine (R) at residue 1150 in the reference $Na_V1.7$ sequence[9] and is located within the C-terminus of L2, the loop that joins domains II and III of the channel. R1150 occurs within a highly conserved sequence of sodium channels and is invariant in all mammalian $Na_V1.7$ channels isolated thus far. Moreover, almost all other sodium channels possess a polar or positively charged residue at this site (arginine in $Na_V1.2$, $Na_V1.5$, $Na_V1.7$, and $Na_V1.8$, lysine in $Na_V1.3$ and $Na_V1.4$, glutamine in $Na_V1.1$ and $Na_V1.6$, and cysteine/histidine in $Na_V1.9$ from different species). Given the conservation of this residue in $Na_V1.7$ channels from different species and the presence of a positively charged residue in most other sodium channels, the substitution by a tryptophan residue suggests a functional effect of the polymorphism on the biophysical properties of the $Na_V1.7$ channel, which is known to play a central role in human pain signaling. We therefore studied the effect of the R1150W substitution on the function of the $Na_V1.7$ sodium channel, and on the firing of pain-signaling DRG neurons in which this channel is normally expressed. Here we show that expression of this polymorphism has important functional implications.

Subjects and Methods

Exon 18 of *SCN9A*, which carries the c. 3448C>T polymorphism, was amplified from a Caucasian control sample of 91 individuals (182 chromosomes), as previously described.[8] The amplicons were sequenced, and frequency of the C and T alleles, which encode the 1150R and 1150W versions of $hNa_V1.7$, was determined.

The functional properties of $hNa_V1.7_{1150R}$ and $hNa_V1.7_{1150W}$ isoforms heterologously expressed in HEK293 cells were assessed using patch-clamp recordings.[8,10] A tryptophan was substituted for arginine at residue 1150 within

From the [1]Department of Neurology and [2]Center for Neuroscience and Regeneration Research, Yale University School of Medicine, New Haven CT; and [3]Rehabilitation Research Center, Veterans Affairs Connecticut Health Care System, West Haven, CT.

Address correspondence to Dr Waxman, Neuroscience Research Center, Bldg 34, VA Connecticut Healthcare System (127A), 950 Campbell Avenue, West Haven, CT 06516. E-mail: Stephen.Waxman@yale.edu

The authors declare that they have no conflict of interest.

Received Jul 2, 2009, and in revised form Sep 22. Accepted for publication Oct 2, 2009. Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21895

the tetrodotoxin-resistant version of $hNa_V1.7$ plasmid using QuikChange XL site-directed mutagenesis (Stratagene, La Jolla, CA).[10] Recordings were performed in voltage-clamp mode in both transiently transfected HEK293 cells (including human β_1 and β_2 subunits)[8] and stably expressing clonal HEK293 cell lines (without β subunits).[11] The effect of the $hNa_V1.7_{1150W}$ allele on excitability was assessed using current-clamp recording from small (22–28μm)-diameter rat DRG neurons, which are largely nociceptors, following transfection with either the $hNa_V1.7_{1150R}$ or the $hNa_V1.7_{1150W}$ channel, together with green fluorescent protein (GFP), using Rat Neuron Nucleofector Solution (Lonza, Walkersville, MD).[8]

Results

Previously, we reported the c. 3448C>T polymorphism within *SCN9A* (dbSNP rs6746030) in 14% of ethnically matched Caucasian control chromosomes, with a sample size of 100 chromosomes.[8] We have now determined the allele frequency in a larger sample of 91 Caucasian control subjects (Coriell Institute, Camden, NJ) and found the following distribution: 70.32% (64/91 individuals) homozygous for the C allele, 28.57% (26/91 individuals) heterozygous for the C and T alleles, and 3.29% (3/91 individuals) homozygous for the T allele. Thus, the frequency of the T allele within this Caucasian control population is 17.58% (32/182 chromosomes).

We analyzed the effects of the R1150W polymorphism by transiently expressing $hNa_V1.7_{1150R}$ and $hNa_V1.7_{1150W}$ alleles, together with sodium channel β_1 and β_2 subunits and GFP, within HEK293 cells. This analysis, using patch-clamp voltage-clamp methods,[8,11] demonstrated a 7.9mV depolarizing shift in the $V_{1/2}$ (midpoint of voltage-dependence) for activation ($hNa_V1.7_{1150R}$ $V_{1/2} = -15.9 \pm 0.8$mV, n = 33; $hNa_V1.7_{1150W} = -8.0 \pm 1.1$mV, n = 17; $p < 0.001$), as shown in Figure 1. The $V_{1/2}$ for fast inactivation (Fig 2A) was unchanged by the polymorphism ($hNa_V1.7_{1150R}$ $V_{1/2} = -76.9 \pm 0.8$mV, n = 33; $hNa_V1.7_{1150W}$ $V_{1/2} = -77.9 \pm 7.5$mV, n = 17). Although the slope factor was shallower for the $hNa_V1.7_{1150W}$ allele compared with the $hNa_V1.7_{1150R}$ allele ($hNa_V1.7_{1150R}$, $k = 6.9 \pm 0.1$; $hNa_V1.7_{1150W}$, $k = 7.5 \pm 0.3$; $p < 0.05$), availability for the 2 alleles was not significantly different at potentials from −130 to −10mV, suggesting that this does not contribute to differences in excitability. The parameters of slow inactivation (Fig 2B) were not significantly altered by the $hNa_V1.7_{1150W}$ allele compared with the $hNa_V1.7_{1150R}$ allele ($hNa_V1.7_{1150R}$ $V_{1/2} = -80.0 \pm 1.4$mV, $k = 15.8 \pm 0.6$, fit minimum $= 0.14 \pm 0.02$ [n = 33]; $hNa_V1.7_{1150W}$ $V_{1/2} = -76.8 \pm 4.8$mV, $k = 14.3 \pm 0.7$, fit minimum $= 0.15 \pm 0.03$ [n = 17]).

We confirmed the depolarizing effect of the R1150 polymorphism on the voltage dependence of activation in a second set of recordings on stably expressing HEK293 cell lines that revealed an 11mV depolarizing shift of activation voltage dependence ($hNa_V1.7_{1150R}$ $V_{1/2} = -29.6 \pm 2.0$mV, n = 7; $hNa_V1.7_{1150W}$ $V_{1/2} = -19.5 \pm 2.0$mV, n = 6; $p < 0.01$). This analysis of stably transfected cells confirmed the absence of a change in fast inactivation.

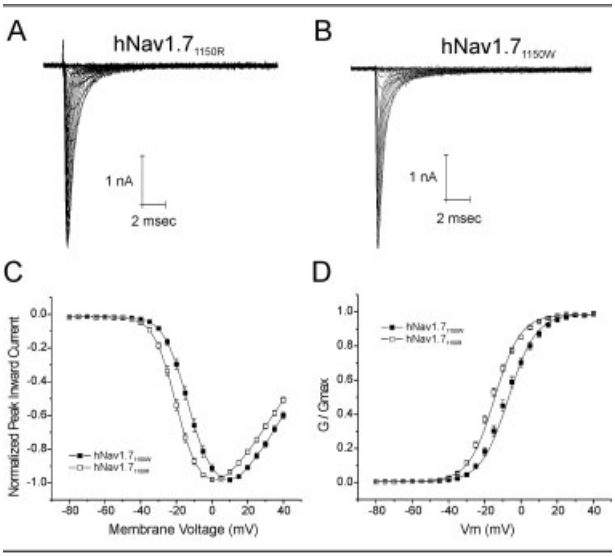

Fig 1. Activation voltage dependence was assessed in HEK293 cells transiently transfected with NaV β1 and β2 subunits and either (A, C) the $hNaV1.7_{1150R}$ or (B, D) the $hNaV1.7_{1150W}$ sodium channel. Typical data traces are shown in panels A and B. The peak inward currents recorded from either isoform averaged around 3–4 nA. There was no significant difference in current density (0.25 ± 0.03 nA/pF for $hNa_v1.7_{1150R}$, 0.22 ± 0.04 nA/pF for $hNa_v1.7_{1150W}$). (C) The peak current-voltage curves, normalized to Imax for each cell, derived from the traces recorded during the activation protocol, are plotted as a function of test potential with the $hNa_v1.7_{1150R}$ (n = 33) responses shown by open squares and the $hNa_v1.7_{1150W}$ (n = 17) responses shown by closed squares. Error bars are ±SEM. (D) The conductance-voltage curves derived from the I-V data normalized to the value Gmax derived from the Boltzman fit are plotted with $hNa_v1.7_{1150R}$ (open squares) *and $hNa_v1.7_{1150W}$* (closed squares). *The midpoint of activation voltage-dependence for $hNa_v1.7_{1150W}$ is shifted 7.9 mV depolarized compared to $hNa_v1.7_{1150R}$. Error bars are ±SEM.*

To assess the effect of the 1150W allele on excitability, we carried out current-clamp recordings[12,13] on small rat DRG neurons, which are largely nociceptors, after transfection with $hNa_V1.7_{1150W}$ or $hNa_V1.7_{1150R}$ and GFP, recording from cells that displayed GFP signal indicating successful transfection. This analysis revealed a statistically significant 6mV depolarizing shift in resting potential in $hNa_V1.7_{1150W}$-transfected neurons compared with $hNa_V1.7_{1150R}$-transfected neurons ($hNa_V1.7_{1150R}$: −57.4 ± 1.1mV, n = 48; $hNa_V1.7_{1150W}$: −51.5 ± 1.3mV, n = 30; $p < 0.005$). Current threshold showed a trend toward a reduction

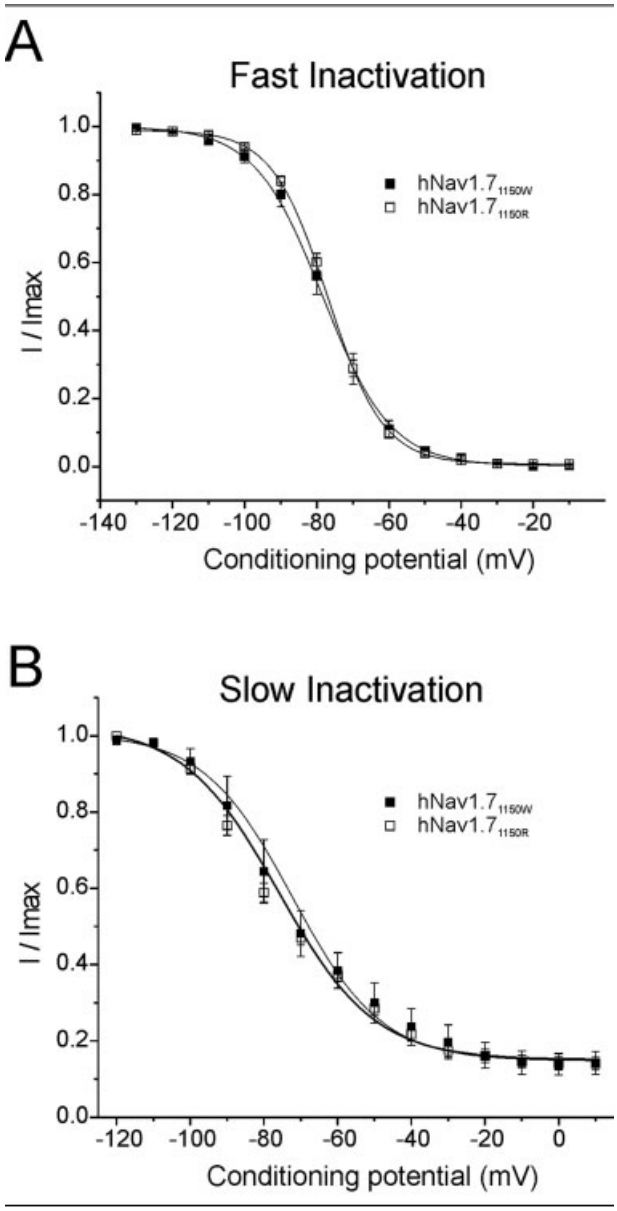

Fig 2. Inactivation voltage dependence. (A) Fast-inactivation was assessed after expression of the $hNa_v1.7_{1150R}$ or the $hNa_v1.7_{1150W}$ sodium channels in HEK293 cells using a protocol consisting of a 500 msec conditioning pulse followed by a 40 msec test pulse to 0 mV to assess the fraction of available channels. For each conditioning pulse potential, the peak current recorded is normalized to the maximum peak recorded during the trial and is plotted for $hNa_v1.7_{1150R}$ (open squares) *and $hNa_v1.7_{1150W}$* (closed squares). *Error bars are ±SEM. (B) Slow-inactivation was assessed using a protocol consisting of a 30-second conditioning pulse followed by a 100 msec pulse to −120 mV to restore the fast-inactivation state and then pulsed to 0 mV to assess the fraction of available channels. For each conditioning pulse potential, the peak current recorded is normalized to the maximum peak recorded during the trial and is plotted with $hNa_v1.7_{1150R}$ shown by open squares and $hNa_v1.7_{1150W}$ shown by closed squares. Error bars are ±SEM.*

in cells expressing $hNa_V1.7_{1150W}$ (215 ± 32pA, n = 20) compared with $hNa_V1.7_{1150W}$ (238 ± 25pA, n = 48), although this was not statistically significant (p = 0.57) . This analysis also showed that, in response to depolarizing current stimulation, DRG neurons expressing the 1150W allele fired more action potentials compared with similar cells expressing the 1150R allele, with cells expressing $hNa_V1.7_{1150W}$ generating about twice as many action potentials at stimulus intensities ranging from 50 to 500pA ($p < 0.05$ at all stimulus intensities >100 pA) (Fig 3).

Discussion

The $Na_v1.7$ sodium channel, which is preferentially expressed in nociceptive DRG neurons,[1,2] plays an important role in electrogenesis in these cells, where it amplifies small depolarizations so as to increase the gain in pain signaling.[3] In our sample of 91 Caucasian control subjects, the single nucleotide polymorphism c. 3448C>T (dbSNP rs6746030) is present within 17.58% of ethnically matched control chromosomes.[8] Examination of the dbSNP shows that the frequency of the rs6746030 polymorphism in control samples is ethnicity dependent: American Caucasians of European descent, 12.7% (sample size: 118 chromosomes); American Chinese of Han descent, 5.6% (sample size: 90 chromosomes); Americans of Japanese descent, 1.1% (sample size: 90 chromosomes); sub-Saharan African, 13.3% (sample size: 120 chromosomes).

The present observations demonstrate that the 1150W allele of $Na_V1.7$ shifts activation voltage dependence 7.9 to 11mV in a depolarizing direction after expression with (7.9mV) or without (11mV) β_1 and β_2 subunits in a heterologous expression system, and has a strong effect on the function of DRG neurons, depolarizing their resting potential and increasing their firing rate. The mechanism by which the 1150W $Na_V1.7$ allele increases the firing rate of DRG neurons is not yet fully understood, and may involve interactions of the $Na_V1.7$ channel protein with factors that are specifically expressed within DRG neurons. Prior studies indicate that a depolarizing shift in $Na_V1.7$ activation voltage dependence can contribute to a decrease in excitability[13,14] if electrogenic pumps are not considered, but this shift will also reduce the window current in nociceptors housing the $hNa_V1.7_{1150W}$ allele. This latter change would be expected to attenuate the standing influx of Na^+ ions required for maintenance of Na^+/K^+ adenosine triphosphatase activity in DRG neurons in which $Na_V1.7$ is a dominant source of window current, thereby depolarizing these nociceptors as a result of diminishing hyperpolarizing pump current and/or a reduction of ion gradients due to insufficient pump rate.[15,16] The present results are consistent with earlier findings, demonstrating that depolarization of nociceptive DRG neurons can make these

cells hyperexcitable as a result of the presence within these cells of $Na_V1.8$ sodium channels, which support repetitive firing and are relatively resistant to inactivation by depolarization.[13] In nociceptive DRG neurons, depolarization by 6mV, that is, of the same magnitude as produced by the $hNa_V1.7_{1150W}$ allele, has been shown to increase excitability.[8]

Irrespective of the underlying mechanism, our results demonstrate that expression of the 1150W polymorphism in $Na_V1.7$ doubles the firing frequency in small DRG neurons. The firing frequencies we observed in DRG neurons expressing $hNa_V1.7_{1150W}$ are similar, in fact, to those seen in DRG neurons expressing the Q10R $Na_V1.7$ mutation, found in a patient with IEM with onset of pain at 14 years, much older than in most patients with IEM.[17] Why this patient, who presumably harbored this $Na_V1.7$ mutation throughout life, did not experience pain earlier, and why most humans carrying the 1150W $Na_V1.7$ polymorphism do not develop a chronic pain syndrome such as IEM, is not yet understood. Nonetheless, even in the absence of detailed understanding of biophysical mechanisms, increased firing frequencies in nociceptive DRG neurons would be expected to produce pain or lower pain threshold,[18] a prediction that could be tested by correlating pain phenotype versus genotype in a population of human subjects. Interestingly, although the 1150W $Na_V1.7$ polymorphism is found in ~13.3 to 17.5% of Caucasian and sub-Saharan African control chromosomes (and is present at lower frequencies, 1.1–5.6%, in Asian control chromosomes), Drenth et al[19] described its presence in a patient carrying the diagnosis of sporadic primary erythromelalgia, suggesting that it may cause a pain syndrome with low penetrance (in contrast to most IEM mutations, which exhibit nearly 100% penetrance[4]), or that its phenotypic expression may be modulated by disease-modifier genes, as has been shown for another sodium channel, $Na_V1.6$.[20] Whether the 1150W polymorphism influences the severity of IEM in a patient with an IEM mutation and 1150W is not known, because in that pedigree[19] there were no other subjects with the IEM mutation.

Gain-of-function mutations of $Na_v1.7$ have previously been demonstrated to produce clinical syndromes characterized by severe pain, whereas loss-of-function

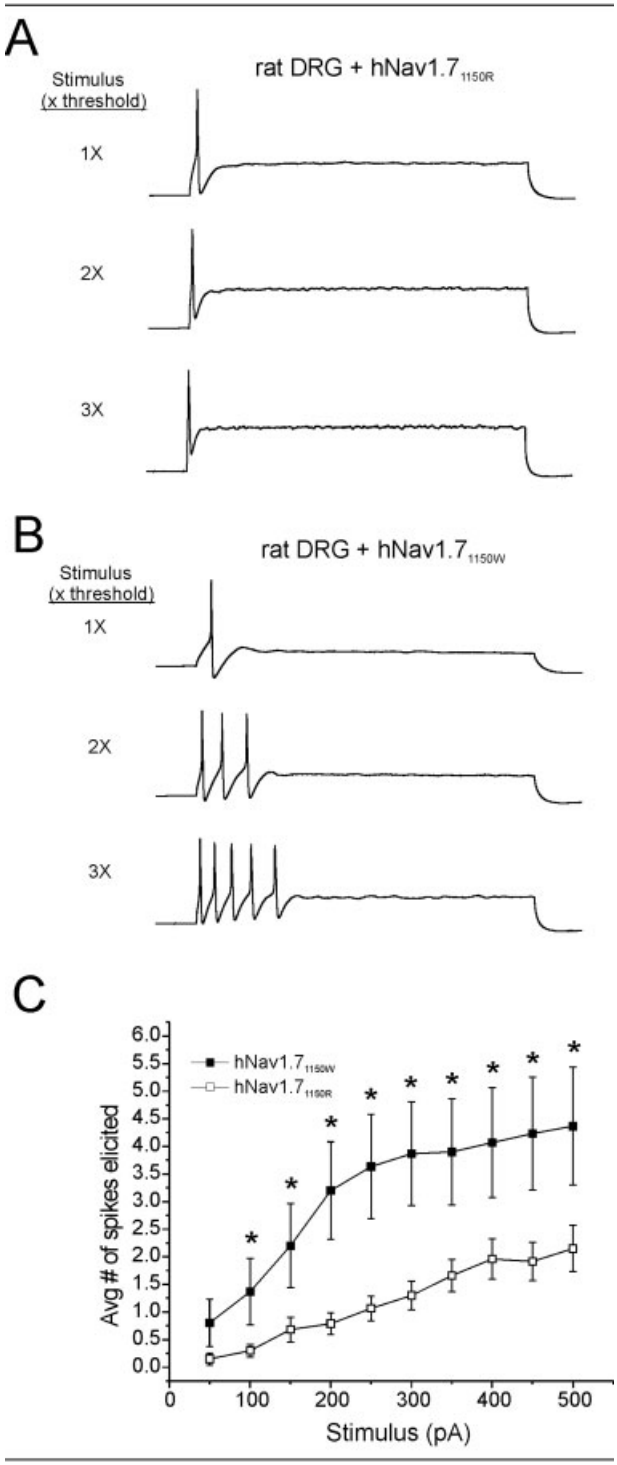

Fig 3. Enhanced firing of rat dorsal root ganglion (DRG) neurons after expression of $hNaV1.7_{1150W}$. Avg = average. (A) Example traces showing action potentials elicited from neurons transfected with $hNa_v1.7_{1150R}$ construct. The three traces shown illustrate the response to 1x, 2x and 3x threshold current injections (250pA, 500pA, 750pA). The stimulus duration is 1 second. (B) Example traces of the action potentials elicited from neurons transfected with $hNa_v1.7_{1150W}$ construct. The three traces shown illustrate the response to 1x, 2x and 3x threshold current injections (150pA, 300pA, 450pA). (C) The mean total number of action potentials (defined as spikes overshooting 0 mV) for current injection pulses of 1 second duration is plotted as a function of stimulus current intensity. The mean response of neurons expressing $hNa_v1.7_{1150W}$ channels (filled squares, n = 30) *was significantly elevated, compared to the mean response of neurons expressing $hNa_v1.7_{1150R}$ channels* (open squares, n = 48) *for stimulus current injections exceeding 100 pA ($p < 0.05$). Error bars are ±SEM.*

mutations of $Na_V1.7$ produce inability to experience pain. The results reported here indicate that a polymorphism in *SCN9*A, the gene encoding the human $Na_V1.7$ sodium channel, can influence the excitability of nociceptive DRG neurons. These observations suggest the possibility that polymorphisms of the $Na_V1.7$ sodium channel may contribute to alterations in pain sensitivity or susceptibility to chronic pain, and underscore the potential importance of $Na_V1.7$ as a molecular target for treatment of pain.

This work was supported by grants from the Rehabilitation Research Service and Medical Research Service, Department of Veterans Affairs (SGW), and from the Erythromelalgia Association (SGW).

We thank Emmanuella Eastman and Larry Macala for excellent technical assistance.

The Center for Neuroscience and Regeneration Research is a Collaboration of the Paralyzed Veterans of America and Yale University.

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