Supporting Information

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SI Materials and Methods

Immunohistochemistry of Skin Biopsy and Intraepidermal Nerve Fiber Quantification. Skin biopsies from the distal leg (10 cm above the lateral malleolus), obtained with a disposable 3-mm punch after topical anesthesia with lidocaine, were processed as previously described (1, 2). Briefly, specimens were fixed [2% (wt/vol) paraformaldehyde–lysine–sodium periodate, 4 °C overnight], cryoprotected, and serially cut with a cryostat. Immunostaining was performed using polyclonal anti-protein gene product 9.5 antibodies (Ultraclone). Each 3-mm punch biopsy yielded ∼40 vertical 50-μm sections. To decrease variability of section length, quantification of intraepidermal nerve fiber (IENF) was performed on three nonconsecutive central sections (e.g., nos. 25, 27, 29) following previously published guidelines (3). All IENF crossing the dermal–epidermal junction, excluding fragments and secondary branching, were counted and their number divided by the length of the epidermis to obtain linear IENF density (IENF per millimeter). Findings were compared with sex- and age-adjusted normative values (3).

Genomic DNA Extraction. Genomic DNA was extracted from peripheral blood samples, collected in EDTA-containing tubes, according to standard procedures (4).

SCN10A Sequence Analysis and Exon Screening. *SCN10A* gene numbering is based on National Center for Biotechnology Information's RefSeq database, access numbers NG_031891.1 (genomic sequence) and CCDS33736.1 (coding sequence). Mutation screening was carried out for all 27 exons that constitute the *SCN10A* ORF. Genomic DNA was amplified by PCR with oligonucleotide primers complementary to flanking intronic sequences. Primers specific for *SCN10A* exons were designed using Primer3-input software (version 4.0). PCR amplification was carried out in a total volume of 25 μL containing 200 ng of genomic DNA, 1× DyNAzyme Buffer (Finnzymes, Celbio), 0.2 mM each GeneAmp dNTP (Applied Biosystems), 1 U of DyNAzyme DNA I DNA Polymerase (Finnzymes), and 0.5 μM of the specific primer pair. PCRs were performed on a GeneAmp PCR System 2700 (Applied Biosystems). Forward primer, 3′-TGGGCTCTAGATTTCCCAGA-5′, and reverse primer, 3′-GAGGCATTGGACAGGATGAT-5′, were used to amplify exon 11. Thermal cycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, followed by 7 min at 72 °C. PCR products (10uL) were confirmed by electrophoresis on a 2% (wt/vol) agarose gel, containing 0.0005% ethidium bromide. Bromophenol blue was used in the loading buffer (0.04%). PCR products were purified (2.5 μL of each) using ExoSAP-IT (GE Healthcare) and sequenced using Big Dye terminator, version 3.1, Cycle Sequencing Kit (Applied Biosystems) and ABI 3100 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using SeqScape, version 2.1.1, software (Applied Biosystems). DNA from 325 Caucasian control subjects (650 chromosomes) from the same geographical region as the index patient was analyzed for c.1661T > C substitution by PCR and high-resolution melting-curve analysis.

Plasmids. The wild-type (WT) construct (pcDNA5-SCN10A) that encodes human $Na_v1.8$ protein was purchased from Genionics. The L554P and A1304T mutations were introduced into constructs using QuikChange II XL site-directed mutagenesis (Stratagene) and referred to as L554P and A1304T hereinafter.

Primary Sensory Neuron Isolation and Transfection for Voltage-Clamp Studies. Animal studies followed a protocol approved by the Yale University and Veterans Administration West Haven Hospital Institutional Animal Care and Use Committees. For voltage-clamp recording, DRG neurons were isolated from homozygous $Na_v1.8$-cre mice (4-8 wk old) that lack endogenous $Na_v1.8$ and transfected by electroporation as previously reported (5). Briefly, DRGs were harvested from homozygous $Na_v1.8$-cre mice, incubated at 37 °C for 20 min in complete saline solution (CSS) [in mM: 137 NaCl, 5.3 KCl, 1 $MgCl_2$, 25 sorbitol, 3 $CaCl_2$, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), adjusted to pH 7.2 with NaOH] containing 0.5 U/mL Liberase TM (Roche Diagnostics) and 0.6 mM EDTA, followed by 15-min incubation at 37 °C in CSS containing 0.5 U/mL Liberase TL (Roche Applied Science), 0.6 mM EDTA, and 30 U/mL papain (Worthington Biochemical). DRGs were then centrifuged and triturated in 0.5 mL of DRG media containing 1.5 mg/mL BSA (low endotoxin) and 1.5 mg/mL trypsin inhibitor (Sigma). After trituration, 2 mL of DRG media was added to the cell suspension, which was filtered (40-μm nylon mesh cell strainer) (Becton Dickinson). The mesh was washed with 2 × 2 mL of DRG media. The cell suspension was evenly divided into two separate tubes, which were sequentially transfected with WT or mutant $Na_v1.8$ constructs using a Nucleofector IIS (Lonza) and Amaxa Basic Neuron SCN Nucleofector Kit (VSPI-1003) as described previously (5).

Briefly, the cell suspension was centrifuged (100 × *g* for 3 min), the supernatant was carefully removed, and the cell pellet was resuspended in 20 μL of Nucleofector solution, mixed with 2 μg of $hNa_v1.8$ WT or mutant construct plus 0.2 μg of EGFP, and transfected using Nucleofector IIS. After electroporation, 100 μL of calcium-free DMEM (37 °C) was added, and cells were incubated (37 °C) for 5 min to allow neurons to recover. The cell mixture was then diluted with 0.4 mL of DRG media containing 1.5 mg/mL BSA (low endotoxin) and 1.5 mg/mL trypsin inhibitor (Sigma) and seeded onto poly-D-lysine/laminin-coated coverslips (BD), and incubated at 37 °C in a 95% air/5% (vol/vol) CO_2 incubator to allow DRG neurons to attach to the coverslips. After 40 min, 1–1.5 mL of DRG media was added into each well, and the DRG neurons were maintained at 37 °C in a 95% air/5% (vol/vol) CO_2 incubator for 36 h before voltage-clamp recording. Transfection order of $hNa_v1.8$ WT and mutant constructs was alternated between cultures to minimize any effect of transfection order and viability of transfected neurons on channel expression.

Voltage-Clamp Electrophysiology. Voltage-clamp recordings were performed 36–54 h after transfection using an Axopatch 200B amplifier (Molecular Devices). Small DRG neurons (surface area, 250–490 μm^2) with robust green fluorescence and no apparent neurites were selected for voltage-clamp recording. Chamber temperature was maintained at 22 ± 1 °C using a temperature controller (HCC-100A; Dagan Corporation), a thermally conductive chamber (RCP-10T; Dagan Corporation), and a cooling module (model TCM-1; Warner Instruments). Pipette potential was adjusted to zero before seal formation, and liquid junction potential was not corrected. Capacity transients were canceled and voltage errors minimized with 85–90% series resistance compensation. Voltage-dependent currents were acquired with Clampex 9.2 at 5 min after establishing whole-cell configuration, sampled at 50 or 100 kHz, and filtered at 5 kHz. The pipette solution contained the following (in mM): 140 CsF, 10 NaCl, 1

EGTA, 10 dextrose, and 10 Hepes, pH 7.3 with CsOH (adjusted to 308 mOsmol/L with sucrose). The extracellular bath solution contained the following (in mM): 70 NaCl, 70 choline chloride, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 5 CsCl, 20 tetraethylammonium chloride (TEA·Cl), pH 7.32 with NaOH (327 mOsmol/L). Tetrodotoxin (1 μM), $CdCl_2$ (0.1 mM), and 4-aminopyridine (1 mM) were added in the bath solution to block endogenous voltage-gated sodium currents, calcium currents, and potassium currents, respectively.

For voltage-clamp recording, DRG neurons were held at −80 mV to inactivate $Na_v1.9$ channels (6). Current–voltage (I–V) relationships were measured using a series of 100-ms step depolarizations (−70 to +50 mV in 5-mV increments at 5-s intervals) from holding potential. Current density was calculated by normalizing maximal transient peak currents (I_{Trans}) with cell capacitance. Persistent currents were measured as mean amplitudes of currents between 93 and 98 ms after the onset of depolarization, and are presented as a percentage of the maximal transient peak current (I_{Trans}). The midpoint of activation ($V_{1/2,act}$), slope (k), maximal conductance (G_{max}), and reversal potentials (V_{rev}) were calculated by BoltzIV function using OriginPro8.5 (OriginLab Corporation) as follows:

$$I = G_{max}(V - V_{rev}) \Big/ \{1 + \exp[(V - V_{1/2,act})/k]\},$$

where I is the peak transient inward current. Activation curves were obtained by converting I to conductance (G) at each voltage (V) using the equation $G = I/(V - V_{rev})$.

Steady-state fast inactivation was assessed with a series of 500-ms prepulses (−90 to +10 mV in 5-mV increments); the remaining noninactivated channels were activated by a 40-ms step depolarization to 0 mV. Slow inactivation was assessed with 30-s prepulses at potentials ranging from −110 to +20 mV followed by a 30-ms hyperpolarization at −80 mV to allow $hNa_v1.8$ channels recover from fast inactivation; remaining available channels were activated by a 50-ms test pulse to 0 mV. Peak inward currents obtained from steady-state fast-inactivation and slow-inactivation protocols were normalized to maximal transient peak current (I_{max}) and fit with Boltzman function as follows:

$$I/I_{max} = A + (1 - A)/\{1 + \exp[(V - V_{1/2})/k]\},$$

where V represents the inactivating prepulse potential, and $V_{1/2}$ represents the inactivation midpoint. $V_{1/2,fast}$ and $V_{1/2,slow}$ represent the midpoints for steady-state fast inactivation and slow inactivation, respectively.

Recovery of $hNa_v1.8$ channels from fast inactivation (reprimming) was examined using a two-pulse protocol with interpulse intervals varying from 2 to 500 ms. Recovery rates were measured by normalizing peak current elicited by the test pulse (10-ms depolarization to 0 mV) to that of the prepulse (100 ms at 0 mV) after various recovery durations (2-500 ms) at different recovery potentials. Recovery time constants were calculated using monoexponential fits of the recovery fraction over recovery period.

Ramp currents were elicited with slow ramp depolarization from −80 to +40 mV over a 600-ms period at 0.2 mV/ms. The amplitude of ramp current was presented as a percentage of the maximal peak current (I_{Trans}).

Voltage-clamp data were analyzed using Clampfit 9.2 (Molecular Devices) and OriginPro 8.5 (OriginLab Corporation), and presented as means ± SEM. Statistical significance was examined using two-sample Student t test.

Current-Clamp Studies. Dorsal root ganglia (DRGs) from 4- to 8-wk-old Sprague Dawley rats were harvested and dissociated as described previously (7). Briefly, DRG neurons were dissociated with a 20-min incubation in Collagenase A (Roche) and 0.6 mM EDTA, followed by 20-min incubation in Collagenase D (Roche), 0.6 mM EDTA, and 30 U/mL papain; a suspension of DRG neurons was prepared as described above. Sodium channel and GFP constructs (channel/GFP ratio of 10:1) were electroporated into DRG neurons using Rat Neuron Nucleofector Solution (Amaxa) with wild-type $Na_v1.8$, and mutant derivative as described previously (5). Transfected DRG neurons were incubated at 37 °C in Ca^{2+}- and Mg^{2+}-free culture medium [DMEM plus 10% (vol/vol) FCS] for 5 min to increase cell viability. The cell suspension was then diluted in culture medium supplemented with nerve growth factor (50 ng/mL) and glial cell line-derived neurotrophic factor (50 ng/mL), plated on 12-mm circular coverslips coated with laminin and poly-ornithine and incubated at 37 °C in 5% (vol/vol) CO_2.

Current-clamp recordings were obtained from small (<30-μm diameter) GFP-labeled DRG neurons 40–48 h after transfection using EPC-10(HEKA) or MultiClamp 700B. Electrodes had a resistance of 1–3 MΩ when filled with the pipette solution, which contained the following (in mM): 140 KCl, 0.5 EGTA, 5 Hepes, and 3 Mg-ATP, pH 7.3 with KOH (adjusted to 315 mOsm with dextrose). The extracellular solution contained the following (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, pH 7.3 with NaOH (adjusted to 320 mOsm with dextrose). Whole-cell configuration was obtained in voltage-clamp mode before proceeding to the current-clamp recording mode. Cells with stable (<10% variation) resting membrane potentials more negative than −35 mV and action potential overshoot of >40 mV were used for data collection. Threshold was determined by the first action potential elicited by a series of depolarizing current injections that increased in 5-pA increments.

Voltage-clamp and current-clamp data were analyzed using Clampfit 9.2 (Molecular Devices), FitMaster (HEKA), and OriginPro 8.5 (OriginLab Corporation), and presented as means ± SEM. Statistical significance was examined using two-sample Student t test or two-portion z-test.

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