Supplementary Information

SI Methods

Patients

The study was approved by the institutional review board of Fundación para el Estudio de las Enfermedades Neurometabólicas and informed consent was obtained from the patients prior to the initiation of the study. The index case (case IV. 2, **Figure 1**) is an adult female who presented with a history of lifelong intense pain episodes localized primarily in the distal extremities, especially in the joints of fingers, wrists and ankles. DNA was isolated from peripheral blood using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according the manufacturers manual. We performed polymerase chain reaction amplification using specific primer sets for all exons that constitute the open reading frame of the *SCN9A* and *SCN11A* genes, and intronic flanking regions of these exons [1]. Amplicons were gel purified using the QIAEXII Gel Extraction Kit (Qiagen, Hilden, Germany), and their nucleotide sequences were determined by fluorescence sequencing with the BigDye terminator kit and ABI3730 Genetic Analyzers (Applied Biosystems, Boston, MA, USA).

Plasmids

The GFP-2A-tagged wild-type construct (pcDNA3-$Na_v1.9$, WT) was previously described [2]. The p.Arg222His mutation (referred to as R222H within figure labels) was introduced into the construct using Quickchange ® II XL site-directed mutagenesis (Stratagene) and confirmed by Sanger sequencing of the insert.

Superior Cervical Ganglion Neuron Isolation and Transfection

All protocols for care and use of animals were approved by the IACUC at the Veterans Administration Connecticut Healthcare System, West Haven. Superior cervical ganglion (SCG) neurons do not produce endogenous tetrodotoxin-resistant currents $Na_v1.8$ and $Na_v1.9$ [3] and have been previously shown to support the expression of $Na_v1.9$ currents following transfection [4], thus producing a platform for expression and functional analysis of Nav1.9. As described previously [4], SCG were isolated from neonatal (birth to 5 days old, gender was not determined) Sprague–Dawley rats (7 preparations for both WT and p.Arg222His mutant, 7 neonatal rats were used in each preparation) , incubated at 37 °C for 20 min in oxygenated complete saline solution (CSS) (in mM: 137 NaCl, 5.3 KCl, 1 $MgCl_2$, 25 sorbitol, 3 $CaCl_2$, and 10 HEPES, adjusted to pH 7.2 with NaOH) containing 1.5 mg/ml Collagenase A (Roche Diagnostics) and 0.6 mM EDTA and then exchanged with an oxygenated, 37 °C CSS solution containing 1.5 mg/ml Collagenase D (Roche Diagnostics), 0.6 mM EDTA, and 30 U/ml papain (Worthington Biochemicals) and incubated for another 20 min. SCG were then centrifuged and triturated in DRG media: DMEM/F12 (1:1) with 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen), and 10% fetal bovine serum (Hyclone), which contained 1.5 mg/ml bovine serum albumin (Sigma-Aldrich) and 1.5 mg/ml trypsin inhibitor (Sigma-Aldrich). After trituration, the cell suspension was transfected with 2.5 µg of GFP-2A-tagged WT or p.Arg222His mutant constructs with a Nucleofector IIS device (Lonza), using the Amaxa® Basic Neuron SCN Nucleofector®™ Kit (VSPI-1003) and SCN Basic Neuro Program 6. Transfected neurons were allowed to recover for 5 min at 37 °C in calcium-free DMEM media. The cell suspension was then diluted with DRG media containing 1.5 mg/ml bovine serum albumin and 1.5 mg/ml trypsin inhibitor, seeded onto poly-D-lysine/laminin precoated coverslips (BD Biosciences), and incubated at 37 °C for 45 min

to allow SCG neurons to attach to the coverslips. DRG media containing 50 ng/ml each of mNGF (Alomone Labs, Jerusalem, Israel) and GDNF (Peprotec, Rocky Hill, N.J.) was then added to each well to a final volume of 1.0 ml, and the cells were maintained at 37 °C in a 95% air/5% CO_2 (vol/vol) incubator for 40–48 h before voltage clamp recording.

Voltage-Clamp Recording

Voltage-clamp recordings were obtained at 22 ± 1 °C, within 40–48 h after transfection using an EPC-10 amplifier (HEKA Electronics). Small SCG neurons (<25 μm) with robust green fluorescence and no apparent neurites were selected for voltage-clamp recording. 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 ~90% series resistance compensation. Voltage-dependent currents were acquired with Patchmaster at 5 min after establishing whole cell configuration, sampled at 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 mOsmol/l with dextrose). The extracellular bath solution contained (in mM) 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 5 CsCl, 20 tetraethylammonium chloride (TEA·Cl), pH 7.3 with NaOH (327 mOsmol/l). TTX (500 nM), $CdCl_2$ (0.1 mM), and 4-aminopyridine (1 mM) were added in the bath solution to block endogenous TTX-sensitive voltage-gated sodium currents, calcium currents, and potassium currents, respectively.

To generate activation curves, cells were held at -120 mV and stepped to potentials of -100 to +20 mV in 5 mV increments (with an interval of 5 s) for 100 ms. Peak inward currents obtained from activation protocols were converted to conductance values using the equation,

$G=I/(V_m - E_{Na})$, where G is the conductance, I is the peak inward current, V_m is the membrane potential step used to elicit the response, and E_{Na} is the reversal potential for sodium channel. Conductance data were normalized and fit with a Boltzmann equation of the form $G=G_{max}/(1+exp[(V_{1/2,act} - V_m)/k)]$, where $V_{1/2},act$ is the midpoint of activation, and k is a slope factor. To generate steady-state fast inactivation curves, cells were held at -120 mV and stepped to inactivating potentials of -140 to +20 mV in 10 mV increments (with an interval of 10 s) for 500 ms followed by a 50 ms step to -40 mV, the voltage where peak current occurs. For slow-inactivation, cells were held at -120 mV, and the protocol consisted of a 30-s step to potentials varying from -120 to -30 mV in 10 mV increments (with an interval of 10 s), followed by a 100-ms step to -100 mV to remove fast-inactivation and a 50-ms step to -40 mV to elicit a test response. Peak inward currents obtained from steady-state fast-inactivation and slow-inactivation protocols were normalized by the maximum current amplitude and fit with a Boltzmann equation of the form $I = A + (1 - A)/ (1+exp[(V_m - V_{1/2,inact})/k)]$, where V_m represents the inactivating prepulse membrane potential, and $V_{1/2,inact}$ represents the midpoint of inactivation. Deactivation was estimated from current decay, using a 25-ms short depolarizing pulse to -40 mV followed by a 150-ms repolarizing pulse to potentials ranging from -110 to -60 mV with 5-mV increments (with an interval of 10 s), and the holding potential for deactivation was -120 mV. Deactivation kinetics were calculated by fitting the decaying currents with a single exponential function. The response to a ramp stimulus was evaluated by a small slow ramp depolarization protocol, which starts from the holding potential of -120 mV and steadily increases to 0 mV over 600 ms for rate of 0.2 mV/ms.

DRG Neuron Isolation and Transfection

DRG from 4- to 8-week-old male Sprague–Dawley rats (WT: 6 preparations; p.Arg222His mutant: 16 preparations, one rat used in each preparation) were harvested, and the neurons dissociated as described previously [4, 5]. Briefly, DRG were harvested, incubated at 37 °C for 20 min in oxygenated CSS containing 1.5 mg/ml Collagenase A (Roche Diagnostics) and 0.6 mM EDTA, and then exchanged with an oxygenated, 37 °C CSS solution containing 1.5 mg/ml Collagenase D (Roche Diagnostics), 0.6 mM EDTA, and 30 U/ml papain (Worthington Biochemicals) and incubated for another 20 min. A suspension of DRG neurons was then prepared by trituration as described above, 2 ml of DRG media was added to the suspension, and the suspension was filtered using a 70-µm nylon cell strainer (Becton Dickinson). The cell strainer was washed with 292 ml of DRG media, the washes were combined with the suspension and then centrifuged at 100 g for 3 min, supernatants were removed, and cells were transfected as described above using Amaxa® Basic Neuron SCN Nucleofector®™ Kit (VSPI-1003) and SCN Basic Neuro Program 6. After transfection, cells were allowed to recover in calcium-free DMEM, seeded, and incubated for 45 min as described above. DRG media containing 50 ng/ml each of mNGF (Alomone Labs, Jerusalem, Israel) and GDNF (Peprotec, Rocky Hill, N.J.) was then added to each well to a final volume of 1.0 ml, and the cells were maintained at 37°C in a 95% air/5% CO_2 (vol/vol) incubator for 40–48 h before current-clamp recording.

Current-Clamp Recordings

Current-clamp recordings were obtained at 22 ± 1°C as previously described [6] from small (<30 µm) green fluorescent protein-labeled DRG neurons 40–48 h after transfection using EPC-10 amplifier (HEKA). The pipette solution contained (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 (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 current-clamp recording mode. Current threshold was determined by the first action potential elicited by a series of 200-ms depolarizing current injections that increased in 5-pA increments. Voltage threshold was determined by assessing the first-order derivative *dV/dt* of membrane potential. Cells with stable (<10% variation) resting membrane potentials more negative than -40 mV and overshooting action potentials (>80 mV resting membrane potential to peak) were used for additional data collection. Action potential frequency was determined by quantifying the number of action potentials elicited in response to depolarizing current injections (500-ms).

Data Analysis

Electrophysiological data were analyzed using Fitmaster (HEKA Electronics) and Origin 8.5.1 (Microcal) and presented as mean ± standard error (SE). Statistical significance was determined by Student's *t* tests (current-clamp except firing frequency and spontaneous activity), Mann–Whitney test (firing frequency), or two proportion *z* test (comparison of proportion of cells producing spontaneous activity).

Multistate structural modeling

Structural modeling of the $Na_v1.9$ channel was performed as previously described [7, 8]. Briefly, sequence alignment of voltage-gated sodium channels was obtained using ClustalW2 and was manually refined to improve the alignment [9, 10]. Modeling of voltage-sensing domain (VSD)

of $Na_v1.9$ domain I was based on templates of voltage-gated channels in multiple states as determined by molecular dynamic simulations [11, 12]. Modeling was carried out using SWISS-MODEL [13, 14] and refined as described previously [15, 16]. Models were energy minimized, analyzed, and visualized in Molecular Operating Environment (MOE; Chemical Computing Group). Virtual mutagenesis and ligand-receptor interaction analysis were performed using MOE.

References

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