PROTOCOL
(R21NS137014-01)

Cell culture

Cell lines. HEK293 cell line stably expressing human wide-type $Na_V1.7$ (HEK1.7) is provided by Dr. Theodore Cummins. HEK293 cell lines stably expressing human wide-type $Na_V1.6$ (HEK1.6), $Na_V1.3$ (HEK1.3), $Na_V1.1$ (HEK1.1), $Na_V1.5$ (HEK1.5), and CHO cells stable expression of human $Na_V1.8$ (CHO1.8) will be obtained from Charles River. Neuronal NG108-15 (NG108) neuronal-like cells and F11 cells (hybrid cells of mouse neuroblastoma cells with embryonic rat DRG neurons) will be purchased from ATCC (Manassas, VA). ND7/23 neuronal cells will be obtained from Sigma-Aldrich. Rat DRG-neuronal 50B11 cells (50B11) will be used as reported previously. These cells will be cultured and transfected (PEI 40) using standard techniques.

Electrophysiology (EP)

EP recordings will be performed, as we described previously with minor modifications at room temperature (22~25°C) [4, 10], in a blind manner where the electrophysiologist will be not aware of the treatment. Patch pipettes 0.9-2.5MΩ resistance will be formed from borosilicate glass (King Precision Glass Co., Claremont, CA) and fire polished. Recordings will be made with an Axopatch 700B amplifier (Molecular Devices, Downingtown, PA). Signals will be filtered at 5 kHz and sampled at 20 and 50 kHz (adopted from literature) [1-3, 5-9, 12, 13] with a Digidata 1440A digitizer and pClamp10 software (Molecular Devices, San Jose, CA). Series resistance (3–5MΩ) will be monitored before and after the recordings, and data will be discarded if the resistance changed by 20%. After achieving the whole-cell recording, capacitance (Cm) and series resistance (Rs) will be compensated accordingly.

Whole-cell voltage-clamp recording on dissociated DRG neurons [11]. To record voltage-activated I_{Ca} in DRG dissociated neuron, the internal pipette solution contained (in mM): 110 Cs-methylsulfate, 10 TEA-Cl, 1 CaCl2, 1 $MgCl_2$, 10 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Li_2-GTP, at pH of 7.2 with CsOH and osmolarity of 296 to 300 mOsm. Small- to medium-size PSNs (≤40 μm soma diameter) were chosen to record T-type low-voltage activated (LVA) I_{Ca} because these are polymodal nociceptors and the majority of acutely dissociated small- and medium-sized PSNs express T-currents under both basal and pathological conditions. To selectively record low-voltage activated (LVA) I_{Ca}, the neurons were preincubated in a Tyrode's solution with 0.2 μM ω-conotoxin GVIA, 0.2 μM nisoldipine and 0.2 μM ω-conotoxin MVIIC for at least 30 min. ω-conotoxin GVIA irreversibly blocks N-type I_{Ca}, and ω-conotoxin MVIIC irreversibly blocks P-/Q-type I_{Ca}. The concentrations used were saturating in preliminary experiments. Any residual high-voltage activated (HVA) I_{Ca} following incubation of HVA calcium channel blockers was eliminated by using fluoride in the internal pipette solution. The fluoride (F^-)-based internal solution, which was used in all experiments examining LVA I_{Ca}, contained (in mM): 135 tetra-methyl ammonium hydroxide (TMA-OH), 10 EGTA, 40 HEPES, and 2 $MgCl_2$, adjusted to pH 7.2 with hydrofluoric acid (HF). A selective and reversible T-type Ca^{2+} channel blocker, TTA-P2 (3, 5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide, Alomone Labs, Jerusalem, Israel) was used to confirm the T-type $I_{Ca.}$ Leak currents were digitally subtracted using a P/4 leak subtraction protocol. The peak T-current was measured after subtracted from the

current at the end of the depolarizing test potential to avoid contamination with residual HVA currents. Voltage protocols consisted of 100-ms depolarizing steps from a holding potential of −60 mV for HVA, or 400-ms depolarizing steps from a holding potential of −90mV for LVA to +60 mV, in 10mV increments with 5s intervals between steps.

Sodium channel current (I_{Na}) recording in cultured cell lines. Whole-cell voltage-clamp to recording I_{Na} will be performed in HEK1.7, HEK1.1, HEK1.3, HEK1.6, HEK1.5, HEK1.8, CHO1.8, NG108-15 cells, and F11cells in current-density (I-V) and fast-inactivation voltage protocols. External solution consists of the following (in mM): 110 NaCl, 20 tetraethylammonium-Cl, 0.01 $CaCl_2$, 0.1 $CaCl_2$, 5 $MgCl_2$, 10 HEPES and 5.56 mM glucose (pH 7.4, 310–315 mosM/L). The internal pipette solution consisted of (in mM): 10 NaCl, 130 CsCl, 5 $MgCl_2$, 5 EGTA, 2.5 Na^{2+}ATP and 10 HEPES (pH 7.2). After formation of a tight seal (maximal leak amplitude <150 pA), membrane resistance and capacitance will be determined. The voltage dependence of activation will be assessed from holding potential using 50 ms pulses (test-pulse) to a range of test potentials from −100 mV to +50 mV in 5 mV or 10 mV incremental steps with an interval of 5 s. Current density will be calculated by normalizing maximal peak currents with cell capacitance. The voltage dependence of steady-state fast inactivation will be measured using a two-step protocol. A 500 ms pre-pulse with various potentials ranging from V_{hold} to +10 mV in 5 or 10 mV incremental steps will be used to inactivate the channels. This pre-pulse will be immediately followed by a 40 ms test-pulse at 0 mV to determine the remaining fraction of available channels. Inward current measured during the test-pulse to 0 mV will be normalized to the cell's maximum test-pulse inward current. To determine the conductance-voltage (I–V) relationships of voltage-dependent activation, the peak current densities during each voltage command step will be fitted to a smooth curve with a Boltzmann equation: $I = G_{max}(V - E_{rev})/(1 + \exp([V - V_{50}]/k))$, which provided the maximum conductance (G_{max}). Normalized activation curves will be fitted with a Boltzmann equation $G/G_{max}=1/(1+\exp([V-V_{50}]/k))$, where G will be calculated as follows: $G=I/(V-E_{rev})$. The steady-state inactivation curves will be fitted with $I/I_{max} = 1/(1 + \exp([V - V_{50}]/k))$. In all the equations, V_{50} denotes the half-activation and half inactivation potentials, Vm is the membrane potential, Erev is the reversal potential, k is the slope factor, G is the conductance, and I is the current at a given Vm; G_{max} and I_{max} are the maximum conductance and current, respectively. Current density will be obtained by dividing the maximum peak current (pA) by the cell capacitance (pF). Voltage errors defined by Rs x Imax will be minimized by using 80-85% series resistance compensation, and the compensation will be readjusted before each voltage-clamp protocol.

TTXs and TTXr I_{Na} recording in DRG dissociated neurons. Isolated I_{Na} will be recorded from single small/medium DRG neurons (≤35 mm in diameter, 4wk after AAV-DRG injection in naïve rats) and differentiated hiPSC-SNs in bath solution that contained the following (in mM): 80 NaCl, 50 choline-Cl, 30 TEA-Cl, 2 CaCl2, 0.2 CdCl2, 10 HEPES, and 5 glucose, pH 7.3 with NaOH. Internal solution containing the following (in mM): 70 CsCl, 30 NaCl, 30 TEA-Cl, 10 EGTA, 1 CaCl2, 2 MgCl2, 2 Na2ATP, 0.05 GTP, 10 HEPES, and 5 glucose, pH 7.3 with CsOH. A voltage protocol will be adopted to separate TTXr I_{Na} and TTXs I_{Na}. In brief, a 500 ms prepulse to -120 or -50 mV will be applied before a 50 ms test pulse from -100 to 40 mV with steps of 5 or 10 mV by test pulses from -50 to 0 mV. Both TTXs and TTXr I_{Na} will be apparent after the -120 mV prepulse; only TTX-R I_{Na} will be obtained after the -50 mV prepulse, and the TTXs component will be obtained by digitally subtracting the TTXr I_{Na} from the total I_{Na}. EP recording will be performed

in differentiated hiPSC-SNs with extensive neurite growth at DIV21-28. To isolate somatic I_{Na}, a brief prepulse to voltage (-40mV) near spikes inactivating hiPSC-SN axonal spikes but not somatic spikes will be performed, as described previously. Total, TTXs and TTXr I_{Na} in dissociated PSNs and differentiated hiPSC-SNs will be also recorded with 1mM of TTX in the bath solution.

Voltage-gated potassium channel current (I_{Kv}) in NG108-15 cells [11]**.** Electrophysiological recordings of I_{Kv} were conducted in non-differentiated NG108-15 cells, bathed at room temperature (25°C) in Modified Tyrode's solution consists of the following (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 10 HEPES at pH of 7.4 with NaOH and an osmolarity of 300 mOsm. The recording pipette solution in all the experiments contain (in mM): 95 K-Gluconate, 2 KCl, 2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP, 0.2 EGTA, 10 HEPES at pH of 7.2 and 290 mOsm. Voltage protocols consists of 300-ms square-wave commands from a holding potential of −80 mV for I_{Kv} channels to +120 mV, in 10-mV increments with 5-s intervals between steps.

Whole-cell current-clamp recording on dissociated DRG neurons. Whole-cell current-clamp recording of dissociated DRG neurons will be performed, as described previously. Dissociated small- and medium-sized DRG neurons (≤40□m in diameter) from sham-operated animals, rats with TNI only, and dissociated DRG neurons with clear GFP expression from TNI rats injected with AAV6-GFPNP or AAV6-NaViPA1 at 8-week after TNI and 6-week after vector injection will be used for recording (n=5 rats per group). The membrane input resistance will be calculated by dividing the ending amplitude of steady-state hyperpolarizing voltage deflection by the injected current. APs will be generated by injection of a series of current pulses (180 to 280 pA in steps of 20 pA, 250 ms). The baseline potentials will be recorded for 20 ms before the stimulus pulses will be injected into the neurons. Resting membrane potential (RMP) will be defined as the mean value of the 20 ms pre-stimulus potential in the first trial and the AP rheobase as the minimum current required to evoke the first AP. The neurons with stable resting membrane potentials (RMP) more negative than -40 mV and overshooting APs (>80 mV RMP to peak) will be used for additional data collection. AP frequency will be determined by quantifying the number of APs elicited in response to depolarizing current injections (250 ms).

Generation of lentivector expressing 1.7/1.8iPAs and NP for hiPSC-SNs transduction
Lentiviral (LV) expression plasmid pWPT-GFP will be used to express designed dual 1.7/1.8iPAs and NP (control). LV will be packaged using pWPT-GFP1.7/1.8iPAs and pWPT-GFPNP with packaging plasmid pCMVdR8.74 and envelop plasmid pVSV-g, concentrated, and products titrated in the range of $1x10^8$ to $2x10^8$ transduction unit/mL. Cultured hiPSC-SNs will be infected by LV-GFP, GFP1.7/1.8iPAs or LV-GFPNP in the presence of 8mg of polybrene (Sigma-Aldrich, St. Louis, MO) per mL at an optimized multiplicity of infection ≈5.

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