A monoclonal antibody that targets a $Na_V1.7$ voltage sensor for pain and itch relief

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Supplementary Figure 1. ELISA Responses of SVmab1 and CTmab using Intact $Na_V1.7$ DII VSD, Related to Figure 1. Data are shown as means ± S.E.M (n=3).

Supplementary Figure 2. The Loop Region (between S3 and S4) Sequence Alignment of Na_V Subtypes from Human (**a**) and from Species Used for the Electrophysiological Recordings in HEK293 cells (**b**), Related to Figure 1B. The red-colored region in $hNa_V1.7$ was chosen for raising SVmab1 and the amino acid residues that are colored blue denote species-specific differences in sequence. Note that the sequence difference among the Na_V subtypes used for electrophysiological recording is as significant as that of human Na_V subtypes.

Supplementary Figure 3. Intraplantar (i.pl.) Injection of SVmab1 Reduces Formalin-Induced Inflammatory Pain in the 1st and 2nd Phase, Related to Figure 5. *P<0.05, vs. control antibody (CTmab), n = 6 mice/group. Antibody was injected 30 min prior to the formalin injection.

Supplementary Figure 4. SVmab1 Suppresses Action Potentials and Transient and Persistent Sodium Currents in Small-Sized DRG Neurons, Related to Figure 6.

(**A**) SVmab1 dose-dependently suppresses action potential in dissociated DRG neurons. Left, traces of single action potential. Right, action potential amplitude. *P<0.05, n=25-30 neurons/group. n.s., no significance. (**B**) SVmab1 inhibits persistent sodium currents ($I_{Na}Ps$) in dissociated neurons. Left, traces of persistent sodium currents ($I_{Na}Ps$) before treatment (control) and after treatment with CTmab (300 nM) and SVmab1 (300 nM). Right, amplitudes of $I_{Na}Ps$ in dissociated neurons. *P<0.05, compared to control; #P<0.05, compared with CTmab (300 nM);

n=10-15 neurons/group. (**C**) SVmab1 (300 nM) inhibits action potentials in small-sized neurons of whole mount DRGs from naïve mice. Top, traces of action potentials. Bottom, number of spikes, **P*<0.05, n=5-10 neurons/group. **(D)** SVmab1 (300 nM) suppresses transient sodium currents (I_{Na}s, density) in small-sized neurons of whole mount DRGs. n=5-10 neurons/group. All the data are shown as means ± S.E.M.

Supplementary Figure 5. SVmab1 Delays the Conduction of C-fiber Stimulation-Induced Synaptic Responses in Lamina II Neurons in Spinal Cord Slices, Related to Figure 6.
(A) Photo of a mouse spinal cord slice with the dorsal root attached. Note the distal end of the dorsal root is inserted into a suction electrode. **(B)** Schematic showing the patch clamp recording in the superficial dorsal horn of a spinal cord slice. **(C)** Traces of evoked EPSCs (eEPSCs) following treatment of SVmab1 (300nM) and CTmab (300 nM). Note that the eEPSC is delayed by SVmab1. **(D)** Ratio of eEPSC delay. **P*<0.05, n=5 neurons/group. The data are shown as means ± S.E.M

Supplementary Figure 6. Schematic of Peripheral and Central Actions of the $Na_V1.7$ Monoclonal Antibody (SVmab1) on Pain and Itch, Related to Figures 6 and 7. $Na_V1.7$ is expressed by pain and itch conducting unmyelinated C-fiber primary sensory neurons in DRGs. The peripheral terminals of these nociceptive/pruriceptive neurons innervated skin, muscle, and joint, and the central terminals of these neurons project to the spinal cord superficial dorsal horn (lamina I and lamina II). $Na_V1.7$, synthesized in cell bodies of C-fiber DRG neurons, is transported to spinal cord central terminals which form synapses to itch- and pain-selective projection neurons in the lamina I (green and red ovals in the dotted box). These $Na_V1.7$-

expressing C type afferent terminals also form synapses with excitatory interneurons (blue oval) in the lamina IIo, where patch-clamp recordings were performed. Furthermore, these lamina II interneurons synapse to pain- and itch-selective projection neurons and are essential for both pain and itch transmission. Systemic injection of SVmab1 produces peripheral actions by suppressing $Na_V1.7$-mediated neuronal excitability in DRG neuronal somata and conduction of action potentials in peripheral and central axons. Intrathecal injection of SVmab1 has central actions by suppressing $Na_V1.7$-mediated glutamatergic synaptic transmission in lamina IIo interneurons. As a result of peripheral and central modulation of SVmab1, pain and itch in both acute and chronic conditions are suppressed. * indicates the action sites of the antibody.

Extended Experimental Procedures

Whole-cell patch-clamp recordings in HEK293 cells

HEK293 cells were transfected with plasmids containing Na_V channel cDNAs mixed with the plasmid containing GFP using lipofectamine 2000 (Invitrogen) at 1 µg of DNA per well of a 6-well plate. Approximately, 24 hr after transfection, whole-cell recordings were performed on single isolated green cell identified under a fluorescence microscope at RT. Glass pipettes (Sutter instrument Co.) were prepared (2–3 MΩ) using a vertical puller (Sutter instrument Co.). Data were acquired with an Axopatch 200B amplifier controlled by Clampex 10 via a Digidata 1440A data acquisition system (Axon Instruments). Currents were sampled at a rate of 10 kHz and filtered at 3 kHz. The pipette solution contained (in mM): 10 NaCl, 110 CsCl, 20 TEA, 2.5 $MgCl_2$, 5 EGTA, 3 ATP, 5 HEPES, pH 7.0 (adjusted with CsOH), and the osmolarity was adjusted to 300 mOsmol/L with glucose. The extracellular bath solution contained (in mM): 100 NaCl, 5 CsCl, 30 TEA, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.1 $CdCl_2$, 5 HEPES, 25 Glucose, 5 4-aminopyridine, pH 7.4 (adjusted with CsOH), and the osmolarity was adjusted to 300 mOsmol/L with glucose.

To record current-voltage relationships, after establishing the whole cell configuration, cells were held at −120 mV and current traces were elicited using 30 ms voltage steps between −80 and +60 mV with 10 mV increments. I-V curves were generated by plotting normalized peak currents (I/I_{max}) as a function of depolarization potential.

The voltage-dependence of channel activation was calculated by measuring the peak currents at test potentials ranging from −90 mV to +10 mV evoked in 5 mV increments from a holding potential of −120 mV. The conductance (G_{Na}) was calculated according to the equation $G_{Na} = I_{Na}/(V_g - V_r)$, where I_{Na} is the peak amplitude of the Na^+ current, V_g is the test potential, and V_r is the reversal potential for Na^+. The conductance-voltage curves were drawn according to the equation $G_{Na}/_{max}G_{Na} = 1/\{1+ \exp [(V_{g0.5} - V_g)/k_g]\}$, where $_{max}G_{Na}$ is the maximum value for G_{Na}, $V_{g0.5}$ is the potential at which G_{Na} is $0.5_{max}G_{Na}$, and kg is the slope factor (potential required for an e-fold change). The voltage-dependence of steady-state inactivation was determined using 500 ms conditioning pre-pulses ranging from −110 mV to −30 mV from a holding potential of –120 mV in 5 mV increments, followed by a test pulse to –10 mV for 30 ms. The peak I_{Na} was normalized to its respective maximum value ($_{max}I_{Na}$) and plotted as a function of the pre-pulse

potential. The steady-state inactivation curves were drawn according to the equation $I_{Na}/_{max}I_{Na} = 1/\{1 + \exp[(V_h - V_{h0.5})/k_h]\}$ where V_h is the pre-pulse potential, $V_{h0.5}$ is the potential at which I_{Na} is 0.5 $_{max}I_{Na}$, and k_h is the slope factor. Data analysis and curve fitting were performed with OrignPro (OriginLab Corp).

Pain models and behavioral testing of pain

To produce inflammatory pain, diluted formalin (5%, 20 μl) was injected into the plantar surface of a hindpaw. Neuropathic pain was produced by chronic constriction injury (CCI) of the sciatic nerve. Mice were anesthetized with isoflurane, and three ligatures with 7-0 prolene were placed around the nerve proximal to the trifurcation (1 mm between ligatures). The ligatures were loosely tied until a short flick of the ipsilateral hind limb was observed.

Animals were habituated to the environment for at least 2 days before the testing. All the behaviors were tested blindly. We assessed formalin-evoked spontaneous inflammatory pain by measuring the time (seconds) mice spent on licking or flinching the affected paw every 5 min for 45 min. For testing mechanical sensitivity after nerve injury, we confined mice in boxes placed on an elevated metal mesh floor and stimulated their hindpaws with a series of von Frey hairs with logarithmically increasing stiffness (0.02-2.56g, Stoelting), presented perpendicularly to the central plantar surface. We determined the 50% paw withdrawal threshold by Dixon's up-down method (Dixon, 1980). For testing motor function, a rota-rod system was used. Mice were tested for three trails separated by 10 min intervals and during the tests, the speed of rotation was accelerated from 2 to 20 r.p.m. in 3 min, and the falling latency was recorded (Liu et al., 2012). The behavioral tests of pain were performed blindly.

Itch models and behavioral testing of itch

We purchased compound 48/80 and chloroquine from Sigma-Aldrich. Mice were habituated to the testing environment daily for at least two days before analysis. Mice were shaved at the back of the neck the day before injection. Mice were left in small plastic chambers (14 × 18 × 12 cm) on an elevated metal mesh floor and allowed 30 min for habituation before examination. To elicit acute itch, we injected 50 μl of pruritic agent compound 48/80 (100 μg) or chloroquine (CQ, 200 μg) intradermally in the nape of the neck, or GRP (1 nmol) intrathecally (Sun and Chen, 2007) and counted the number of scratches every 5 min for 30 min after the injection. A

scratch was counted when a mouse lifted its hindpaw to scratch the shaved region and returned the paw to the floor or to the mouth for licking (Liu, 2012).

To induce chronic itch, we painted the neck skin with acetone and diethyether (1:1) following by water (AEW) twice a day for 4 days, and examined spontaneous itch by counting the number of scratches for 60 min on day 5 (Liu et al., 2012). We also generated the allergic contact dermatitis (ACD) model of chronic itch by applying the hapten 1-fluoro-2, 4-dinitrobenzene (DNFB) onto the back skin (Zhao et al., 2013). DNFB was dissolved in a mixture of acetone:olive oil (4:1) for sensitization and challenge. One day before sensitization, the surface of abdomen and the nape of neck was shaved. Mice were sensitized with 0.5% DNFB solution (50 μl) by topical application to a ~2 cm^2 area of shaved abdomen skin. Five days later, mice were challenged with 0.2% DNFB solution (30 μl) by painting the shaved neck area, then every other day for one week. Spontaneous scratching behaviors were videoed for 1 hour, at 24 hours after each challenge. The behavioral tests of itch were performed blindly.

Spinal cord drug delivery

For spinal intrathecal injection, spinal cord puncture was made with a 30G needle between the L5 and L6 level to deliver reagents (10 μl) to the cerebral spinal fluid (Hylden and Wilcox, 1980).

Whole-cell patch clamp recordings in dissociated DRG neurons and whole mount DRG

The dissociated DRGs were removed aseptically from mice (4-6 weeks) and incubated with collagenase (1.25mg/ml, Roche)/dispase-II (2.4 units/ml, Roche) at 37°C for 90 min, then digested with 0.25% trypsin for 8 min at 37°C, followed by 0.25% trypsin inhibitor. Cells were mechanically dissociated with a flame polished Pasteur pipette in the presence of 0.05% DNAse I (Sigma). DRG cells were plated on glass cover slips and grown in a neurobasal defined medium (with 2% B27 supplement, Invitrogen) with 5 μM AraC and 5% carbon dioxide at 36.5°C. DRG neurons were grown for 24 hours before use.

The L4-L5 whole mount DRGs were carefully removed from the vertebral column and placed in cold oxygenated ACSF. The connective tissue was gently removed under a microscope and the ganglia were digested with a mixture of 1.0 mg/ml protease and 1.6 mg/ml collagenase (Sigma) for 30 min at 37°C. The ganglion was transferred into a holding chamber containing

normal Mg^{2+}-free ACSF with CNQX (2 μM) bubbled with 95% O_2 and 5% CO_2 at room temperature.

Whole-cell voltage and current clamp recordings were performed at room temperature (28°C) to measure transient and persistent sodium currents and action potentials, respectively, with Axopatch-200B amplifier (Axon Instruments) and Digidata 1440A data acquisition system (Axon Instruments). The patch pipettes were pulled from borosilicate capillaries (Chase Scientific Glass Inc.). When filled with the pipette solution, the resistance of the pipettes was 4-5 MΩ. The recording chamber (300 μl) was continuously superfused (3-4 ml/min). Series resistance was compensated for (> 80%), and leak subtraction was performed. Data were low-pass-filtered at 2 KHz, sampled at 10 KHz. The pClamp10 (Axon Instruments) software was used during experiments and analysis.

For sodium current recording, pipette solution contained (in mM): CsCl 100, sodium L-glutamic acid 5, TEACl 30, $CaCl_2$ 0.1, $MgCl_2$ 2, EGTA 11, HEPES 10, adjusted to pH 7.4 with CsOH. The external solution was composed of (in mM): NaCl 90, choline chloride 30, TEACl 20, $CaCl_2$ 0.1, $MgCl_2$ 5, $CoCl_2$ 5, HEPES 10, glucose 10 adjusted to pH 7.4 with NaOH. In voltage-clamp experiments, the transient sodium current (I_{Na}) was evoked by a test pulse to +0 mV from the holding potential, -70 mV. The persistent sodium current ($I_{Na}P$) was recorded by applying a 3 s depolarization ramp current from -80 to -10 mV at a holding potential of -60 mV (Xie et al., 2011). The plot was fitted using the Origin software (Origin, Northampton, MA, USA). The pipette solution for current-clamp experiments was composed of (in mM): K-gluconate 145, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 10, HEPES 5, K_2ATP 5, adjusted to pH 7.4 with KOH. The external solution contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, adjusted to pH 7.4 with NaOH. In current-clamp experiments, action potentials were recorded under current clamp (-60 mV), with 1 second depolarizing current pulses with 200 pA amplitude.

Spinal cord slice preparation and patch clamp recordings

A portion of the lumbar spinal cord (L4-L5) was removed from mice (4-6 weeks old) under urethane anesthesia (1.5 - 2.0 g/kg, i.p.) and kept in pre-oxygenated ice-cold Krebs solution. Transverse slices (400-600 μm) were cut on a vibrating microslicer. The slices were perfused with Kreb's solution (8-10 ml/min) that was saturated with 95% O_2 and 5% CO_2 at 36±1^oC for at least 1-

3 h prior to experiment. The Kreb's solution contains (in mM): NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11. The whole cell patch-clamp recordings were made from lamina IIo neurons in voltage clamp mode. Patch pipettes were fabricated from thin-walled, borosilicate, glass-capillary tubing (1.5 mm o.d., World Precision Instruments). After establishing the whole-cell configuration, neurons were held at the potential of -70 mV to record sEPSCs (Park et al., 2011). The resistance of a typical patch pipette is 5-10 MΩ. The internal solution contains (in mM): potassium gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, and ATP-Mg 5. Membrane currents were amplified with an Axopatch 200B amplifier (Axon Instruments) in voltage-clamp mode. Signals were filtered at 2 kHz and digitized at 5 kHz. Data were stored with a personal computer using pCLAMP 10 software and analyzed with Mini Analysis (Synaptosoft Inc.).

To measure the nerve conduction in the dorsal root, transverse spinal cord slice (350–450 μm thick) with dorsal root (4-6 mm) attached and the evoked EPSCs were measured from lamina IIo neurons following dorsal root stimulation. The dorsal root was electrically stimulated (3 mA, 100 μs) through a suction electrode with an isolated current stimulator and latency from initial point of stimulus trace to initial point of the eEPSC was measured in the presence of the antibodies.

Supplemental References

Dixon,W.J. (1980). Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. *20*, 441-462.

Liu,T., Berta,T., Xu,Z.Z., Park,C.K., Zhang,L., Lu,N., Liu,Q., Liu,Y., Gao,Y.J., Liu,Y.C., Ma,Q., Dong,X., and Ji,R.R. (2012). TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. J Clin. Invest *122*, 2195-2207.

Park,C.K., Lu,N., Xu,Z.Z., Liu,T., Serhan,C.N., and Ji,R.R. (2011). Resolving TRPV1- and TNF-a-mediated spinal cord synaptic plasticity and inflammatory pain with neuroprotectin D1. J Neurosci *31*, 15072-15085.

Sun,Y.G. and Chen,Z.F. (2007). A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature *448*, 700-703.

Xie,R.G., Zheng,D.W., Xing,J.L., Zhang,X.J., Song,Y., Xie,Y.B., Kuang,F., Dong,H., You,S.W., Xu,H., and Hu,S.J. (2011). Blockade of persistent sodium currents contributes to the riluzole-induced inhibition of spontaneous activity and oscillations in injured DRG neurons. PLoS. ONE. *6*, e18681.

Zhao,Z.Q., Huo,F.Q., Jeffry,J., Hampton,L., Demehri,S., Kim,S., Liu,X.Y., Barry,D.M., Wan,L., Liu,Z.C., Li,H., Turkoz,A., Ma,K., Cornelius,L.A., Kopan,R., Battey,J.F., Jr., Zhong,J., and Chen,Z.F. (2013). Chronic itch development in sensory neurons requires BRAF signaling pathways. J Clin. Invest. *123*, 4769-4780.