Differential Inhibition of Nav1.7 and Neuropathic Pain by Hybridoma-Produced and Recombinant Monoclonal Antibodies that Target Nav1.7

Differential activities of Nav1.7-targeting monoclonal antibodies

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Abstract The voltage-gated Na⁺ channel subtype Nav1.7 is important for pain and itch in rodents and humans. We previously showed that a Nav1.7-targeting monoclonal antibody (SVmab) reduces Na⁺ currents and pain and itch responses in mice. Here, we investigated whether recombinant SVmab (rSVmab) binds to and blocks Nav1.7 similar to SVmab. ELISA tests revealed that SVmab was capable of binding to Nav1.7-expressing HEK293 cells, mouse DRG neurons, human nerve tissue, and the voltage-sensor domain II of Nav1.7. In contrast, rSVmab showed

no or weak binding to Nav1.7 in these tests. Patch-clamp recordings showed that SVmab, but not rSVmab, markedly inhibited Na⁺ currents in Nav1.7-expressing HEK293 cells. Notably, electrical field stimulation increased the blocking activity of SVmab and rSVmab in Nav1.7-expressing HEK293 cells. SVmab was more effective than rSVmab in inhibiting paclitaxel-induced mechanical allodynia. SVmab also bound to human DRG neurons and inhibited their Na⁺ currents. Finally, potential reasons for the differential efficacy of SVmab and rSVmab and future directions are discussed.

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Introduction

Voltage-gated Na⁺ (Nav) channels are essential for the initiation and propagation of action potentials in excitable cells. Humans and rodents possess nine highly homologous Nav channel subtypes (Nav1.1–Nav1.9), and each subtype plays a distinct role in regulating physiological processes [1, 2]. Dysregulation of Na⁺ channels causes diseases such as cardiac arrhythmia, epilepsy, ataxia, periodic paralysis, and chronic pain [3–11]. Especially, human genetic studies have revealed a critical role of Nav1.7 in pain and itch sensation. Loss-of-function mutations in the human Nav1.7 gene (*SCN9A*) result in congenital insensitivity to pain and anosmia without affecting other sensations such as touch and temperature [12, 13]. In contrast, gain-of-function mutations lead to episodic pain such as primary erythromelalgia and

paroxysmal extreme pain disorder [14, 15]. Nav1.7 is mainly expressed in primary sensory neurons such as dorsal root ganglion (DRG) neurons [16, 17]. Although Nav1.7 is required for normal heat pain and neuropathic pain in mice [18–20], there is a striking species difference in its expression between mouse and human DRGs. PCR analysis of seven DRG-expressed Nav subtypes has revealed that the human DRG has higher expression of Nav1.7 (~50% of total Nav expression) and lower expression of Nav1.8 (~12%), whereas the mouse DRG has higher expression of Nav1.8 (~45%) and lower expression of Nav1.7 (~18%) [21]. Furthermore, Nav1.7 expression and function are upregulated in mouse and human DRG neurons in chronic pain conditions such as painful diabetic neuropathy [22] and paclitaxel-induced neuropathy [21]. Thus, subtype-specific Nav1.7 inhibitors are in high demand for pain management.

Nav channels consist of an α subunit that forms the ion conduction pore and one or two β subunits that modulate the function of the α subunit and thus affect Nav-related neuropathic pain [9]. The α subunit is composed of a single polypeptide with four repeat domains (DI–DIV), each repeat containing six transmembrane helical segments (S1–S6). The first four segments (S1–S4) comprise the voltage-sensor domain (VSD) and the last two (S5–S6) form the pore domain. S4 in the VSD contains the gating charge arginine residues that sense membrane potential changes and, together with the C-terminal half of S3 (S3b), form a helix-loop-helical unit termed the voltage-sensor paddle [23], which contributes to the voltage-dependent gating of Nav channels [24–26]. This paddle is the receptor site for voltage-sensor toxins. By binding to the paddle, voltage-sensor toxins modulate the voltage-dependence of Nav and Kv channels [27–29]. Based on this knowledge, we developed a monoclonal antibody, SVmab, which targets a region of the paddle (S3–S4 loop) of both human and mouse Nav1.7. SVmab binds to the VSD II and inhibits the activity of Nav1.7 in HEK293 cells and mouse DRG neurons, with an effective concentration range of 100 nmol/L–300 nmol/L [19]. We also raised another monoclonal antibody that targets the S1–S2 loop of human and mouse Nav1.7. Because this antibody does not inhibit the activity of Nav1.7, we used it as a control antibody and named it CTmab [19]. Notably, SVmab but not CTmab has pronounced analgesic and anti-pruritic effects in mouse models of inflammatory pain, neuropathic pain, and acute and chronic itch [19].

Recently, Liu *et al.* produced and purified a recombinant SVmab protein (termed arSVmab) based on the published sequence [30]. They evaluated the activity of arSVmab in a battery of binding and functional assays and surprisingly found that binding of arSVmab to either the peptide immunogen or the full-length Nav1.7 channel expressed in

HEK293 cells was below detectable level [30]. Although this antibody showed some inhibition of Na^+ currents in Nav1.7-expressing HEK293 cells in certain voltage protocols, its blocking effect on Nav1.7 currents was not significantly different from their control antibody [30]. Notably, *in vivo* tests in animal models of pain were not conducted in their studies.

To address the discrepancy between the hybridoma-derived and recombinant SVmab antibodies, we generated our own recombinant monoclonal antibody (named rSVmab) and investigated its properties with regard to Nav1.7 binding and inhibition as well as its effects on pain through a side-by-side comparison with SVmab.

Materials and Methods

Reagents

The hollow fiber bioreactor was from Fiber Cell Systems Inc. (MD); Dulbecco's modified Eagle's medium (DMEM) was from Gibco or Sigma; penicillin-streptomycin was from Gibco; rSVmab was produced by Genscript Co., according to the published sequence of SVmab and using the isotype of IgG1; control mouse IgG was from Santa Cruz Biochem; the human Nav1.7 ion channel cell line was from SB Drug Discovery (Glasgow, UK); Alexa-fluor 488 was from Thermo Scientific. The hNav1.5-pcDNA 3.1 plasmid was provided by Dr. Geoffrey Pitt (Cornell School of Medicine). We also obtained an anti-Nav1.7 antibody that targets its intracellular loop (<http://www.alomone.com/p/anti-nav1.7/asc-008/44>) from Alamone (Jerusalem, Israel). The control (DYKDDDDK) and blocking (VEL-FLADVEG) peptides were synthesized by Genscript. SFX-Insect cell medium was from Hyclone Laboratories (UT); n-dodecyl- β -D-maltopyranoside (DDM) for the human Nav1.7 VSDII purification was from Anatrace; carbonate-bicarbonate buffer was from Sigma and Tween-20 for ELISA was from Bio-Rad; ELISA microwell plates were from Nunc; anti-FLAG was from Sigma and HRP-conjugated secondary antibody was from Thermo Scientific; tetramethylbenzidine (TMB)-based substrate solution for an ELISA reaction was from KPL Inc. (MA); PVDF membranes and secondary antibody for western blotting of SVmab and rSVmab were from Bio-Rad and Li-Cor (IRDye® 800CW) (NE); paclitaxel was from Sigma.

Animals

Adult CD1 mice (8–10 weeks old, male) were obtained from Charles River Laboratories and used for PCR analysis. All the animal procedures were approved by the Animal Care Committee of Duke University Medical

Centre. To induce chemotherapy-associated neuropathic pain, paclitaxel (2 mg/kg, i.p.) was injected on days 0, 2, 4, and 6 [31]. Some electrophysiological experiments were carried out independently at the University of Cincinnati in accordance with the Animal Care and Use Committee of the University of Cincinnati, and under the guidelines of the National Institutes of Health. Male and female C57BL/6J mice aged 7 to 8 weeks (Jackson Laboratories) were used for DRG collection. Mice were housed 2 to 3 per cage, had free access to food and water, and were exposed to a 14/10-h light/dark cycle. The ambient environment was controlled at constant temperature ($22 \pm 1^\circ\text{C}$) and humidity (60%–70%).

Human DRGs

Non-diseased human DRGs were obtained through the National Disease Research Interchange (Philadelphia, PA) with permission from the Duke University Institutional Review Board. Postmortem L3–L5 DRGs were dissected from donors and delivered in ice-cold culture medium to the laboratory at Duke University within 24 h–72 h of the donor's death.

Antibody Preparation and Purification

Regarding the recombinant antibody production, recombinant plasmids encoding the heavy and light chains of the antibody were transiently co-transfected into suspension Expi293F cell cultures (Life Technologies, Carlsbad, CA). The culture supernatants collected on day 6 were used for purification using a HiTrapTM protein G column (GE, PA). SVMab and CTmab were produced from hybridoma cells in the Duke Cell Culture Facility using a hollow fiber bioreactor (FiberCell Systems Inc. MD) with DMEM (Gibco or Sigma), 5% CDM-HD (FiberCell Systems Inc.), and 1% penicillin-streptomycin (Gibco). The hybridoma cells were grown in RPMI, 20% fetal bovine serum (FBS), 1% glutamine (ThermoFisher), and 1% penicillin-streptomycin (Gibco) prior to expansion in the bioreactor. SVMab and CTmab were purified on a HiTrapTM protein G column (GE). The first batch of SVMab was provided by Abmart (www.ab-mart.com). Buffer exchange for all antibodies was done on a PD-10 column (GE).

Human Nav1.7 Voltage Sensor Domain II (VSD II) Expression and Purification

The VSD II of human Nav1.7 (hVSD II, amino-acids 716–851) fused to the N-terminal segment of the gene (amino-acids 1–18) was cloned into the pFastBac vector (Invitrogen, NY) in-frame with a C-terminal 10×His affinity tag. Baculovirus was produced according to the

manufacturer's protocol (Invitrogen, Bac-to-Bac Baculovirus expression system). For protein expression, *Spodoptera frugiperda* 9 (Sf9) insect cells were infected with baculovirus at 1.2×10^6 cells/mL and grown at 27°C for 72 h in an orbital shaker. The cell pellets were resuspended in buffer A (50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin, 1 $\mu\text{g/mL}$ aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and DNase I) and lysed by sonication (5×30 pulses). For protein extraction, 40 mmol/L n-dodecyl- β -D-maltopyranoside (DDM) was added to the lysate and stirred at 4°C for 2 h. Insoluble material was removed by centrifugation ($8000 \times g$, 30 min), and TALON resin (Clontech) was added to the supernatant and stirred with 10 mmol/L imidazole for 30 min at 4°C . The resin was then washed with ten column volumes of buffer B (20 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 1 mmol/L DDM, 10 mmol/L imidazole) and the protein was eluted with 5 column volumes of buffer C (20 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 1 mmol/L DDM, 200 mmol/L imidazole). The fractions containing hVSDII were further purified by size-exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare (PA) in 20 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 1 mmol/L DDM, 2 mmol/L dithiothreitol. The protein peak was collected and analyzed by SDS-PAGE. Purified hVSDII was never frozen and thawed before the ELISA assay.

Enzyme-Linked Immunosorbent Assays (ELISA) for the VSD II of Nav1.7

Before coating, hVSD II was diluted into carbonate buffer (0.05 mol/L carbonate-bicarbonate buffer, pH 9.6, Sigma) at 10 $\mu\text{g/mL}$. ELISA microwell plates (Nunc) were coated by adding 100 μL of diluted hVSD II protein. The plates were incubated for 2 h at room temperature (RT) and then overnight at 4°C . After washing the plates 3 times with PBST (PBS containing 0.05% Tween-20, Bio-Rad), they were blocked with 100 μL 5% skim milk in PBST (Bio-Rad) for 1 h at RT. The plates were then washed 3 times with PBST buffer. Primary antibodies (SVMab, rSVMab, CTmab, and anti-FLAG) were diluted in PBST buffer to the desired concentration and 100 μL of each antibody was added to each well. The plates were then incubated at RT for 1 h. After 3 washes with PBST, the HRP-conjugated secondary antibody (diluted 1:1000 in PBST buffer, Thermo Scientific) was added and incubated for 1 h at RT. The plates were then washed 3 times with PBST buffer and 100 μL tetramethylbenzidine (TMB)-based substrate (KPL Inc.) was added. After 20 min incubation at RT, 100 μL of 1 mol/L HCl for 10 min at RT was used to stop the reaction. The resulting color reaction was measured at

450 nm with an ELISA reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA).

Western Blot

Nav1.7-targeting antibodies were loaded onto an SDS-PAGE gel (4%–15%) without reducing reagent and transferred onto a PVDF membrane (Bio-Rad). The membrane was washed 3 times with TBST (TBS + 0.05% Tween-20, Bio-Rad) and blocked with 5% skim milk (Bio-Rad) in TBST for 1 h at RT. After washing 3 times with TBST buffer, the membrane was incubated for 1 h at RT with secondary antibody (IRDye-800CW, Li-Cor, 1:10,000 in TBST buffer). The membrane was analyzed on an Odyssey CLx reader (Li-Cor).

Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells were obtained from the ATCC and maintained at the Duke cell culture facility. Cells were cultured in high-glucose (4.5 g/L) DMEM containing 10% (v/v) FBS (Gibco) and streptomycin/penicillin. For Nav1.7-expressing HEK293 cells, an additional antibiotic, blasticidin, was included. Transfection was performed by Lonza electroporation (Lonza, Portsmouth, NH) at 70% confluence using 2 µg cDNA. The transfected cells were cultured in the same growth medium for 48 h before biochemical or electrophysiological studies.

Antibody Labeling with Biotin, Alexa-Fluor 488, and HRP

For ELISA analysis to detect antibody-binding to Nav1.7, biotin was conjugated to CTmab, SVMab, rSVMab, and mouse IgG using an EZ-LinkTM Sulfo-NHS-biotinylation kit (Thermo Scientific) at 0.1 mg biotin per mg antibody, according to the manufacturer's instructions. The mixture was incubated overnight at 4°C. To visualize the antibody-binding to Nav1.7-expressing cells, CTmab, SVMab, and rSVMab (1 mg) were conjugated with Alexa-fluor 488 (10 µg) in 0.1 mol/L sodium bicarbonate buffer overnight at 4°C, according to the manufacturer's instructions (Thermo Scientific). We also conjugated the Nav1.7 detection antibody (50 µg) with horseradish peroxidase (HRP, 50 µg) overnight at 4°C using a Sure-LINKTM HRP conjugation kit (KPL, Inc., Gaithersburg, MD), according to the manufacturer's manual. Non-binding HRP, biotin, or Alexa-fluor 488 was removed by dialysis (4°C, 24 h) for salt exchange using a membrane with a 10 kDa cut-off (Sigma).

Binding Assay and ELISA for Full-Length Nav1.7 and Nav1.5

Human spinal nerve tissue (20 mg) or HEK-293T cells expressing Nav1.5 or Nav1.7 (1×10^5) were incubated with biotinylated antibodies (SVMab, rSVMab, CTmab, mouse IgG, 1, 100, 300, and 1000 nmol/L) in PBS for 15 min at RT and centrifuged at 1,000 g for 10 min to remove non-binding antibodies. The tissues or cells were lysed in a low-salt buffer (10 mmol/L HEPES, 50 mmol/L NaCl, 5 mmol/L EDTA, with protease inhibitor cocktail (Sigma), 1% Triton X-100) at 4°C for 1 h. The tissues and cells were homogenized and then centrifuged at 15,000 g for 10 min to remove non-solubilized membrane components. The samples were incubated in a streptavidin-coated plate (96-well, Thermo Scientific) at RT for 2 h. The plate was washed twice with low-salt harvest buffer containing 1% Triton X-100. The samples were then incubated on the plate for 2 h at RT with the HRP-conjugated detection antibody against Nav1.7 (1 µg/mL). The wells were washed 5 times in PBST (containing 1% Tween-20) and incubated with the TMB-based substrate for 15 min. The substrate reaction was then stopped and the absorbance was read using a spectrophotometer (Bio-Rad) at 450 nm. To assess frequency-dependent binding, field stimulation (60 V/cm at 0, 1, and 10 Hz, 4 min) was applied to the 96-well plate during antibody incubation.

Immunohistochemistry

Human DRGs were fixed in 4% paraformaldehyde overnight, then sections (12 µm) were cut in a cryostat. The sections were first blocked with 2% BSA for 1 h at room temperature, incubated with SVMab (2 µg/µL) primary antibody overnight at 4°C, and then they were incubated for 2 h at RT with cyanine 3 (Cy3)-conjugated secondary antibodies (1:400; Jackson ImmunoResearch, Cambridgeshire, UK). The sections were then stained with DAPI (1:1000, Sigma, 5 min) and examined under a Nikon fluorescence microscope (Japan). Images were captured with a CCD Spot camera and analyzed with NIH Image software or Adobe PhotoShop.

Primary Cultures of Mouse DRG Neurons

DRGs were removed aseptically from mice (5–7 weeks old) and incubated with collagenase (1.25 mg/mL, Roche)/dispase-II (2.4 units/mL, Roche, IN) 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), then plated on glass coverslips and grown in a Neurobasal defined

medium (with 2% B27 supplement, Invitrogen) with 5 $\mu\text{mol/L}$ AraC under 5% CO_2 at 37°C. The neurons were grown for 24 h before use. To determine antibody-binding to Nav1.7, the neurons were incubated with Alexa-fluor 488-labeled antibodies (300 nmol/L, 30 min) at RT. In experiments conducted at the University of Cincinnati, mice were deeply anesthetized with isoflurane (5% for induction and 2% for maintenance) for bilateral DRG collection under a dissecting microscope. The sheath of the L4 or L5 DRG was carefully removed in ice-cold solution (Hank's balanced salt solution, HBSS). DRGs were then transferred to papain solution (3 mL, 30 U/mL) for 20 min followed by collagenase solution (3 mL, 1.5 mg/mL) for another 20 min in a 37°C incubator. Dissociated neurons were seeded onto 6–8 poly-D-lysine-coated glass coverslips with Neurobasal medium (Life Technologies, Grand Island, NY) containing 1% glutamine, 2% B-27, and 1% penicillin-streptomycin. Cells were cultured overnight before recording. For live cell labeling to check Nav1.7 antibody binding to DRG neurons, cultured DRG neurons were washed twice with 40°C pre-cold PBS and incubated with FITC-conjugated Nav1.7 antibody (300 nmol/L) in extracellular solution for 30 min at 4°C, then washed with PBS 3 times, fixed with 4% paraformaldehyde for 15 min before imaging.

Whole-Cell Recordings in HEK293 Cells and Mouse DRG Neurons

Whole-cell recordings were performed at room temperature using an Axopatch-200B amplifier with a Digidata 1440A (Axon Instruments, Sunnyvale, CA). The patch pipettes were pulled from borosilicate capillaries (World Precision Instruments, Inc., Sarasota, FL). Pipette resistance was 4 $\text{M}\Omega$ –6 $\text{M}\Omega$. To record Na^+ currents, the internal solution contained (in mmol/L): 140 CsCl, 10 EGTA, 10 HEPES, and 2 Mg-ATP, adjusted to pH 7.3 with CsOH. The extracellular solution for HEK293 cell recording contained (in mmol/L) 140 NaCl, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 10 glucose, adjusted to pH 7.3 with NaOH. The extracellular solution for mouse DRG neuron recordings contained (in mmol/L): 131 NaCl, 10 TEACl, 10 CsCl, 1 CaCl_2 , 2 MgCl_2 , 0.3 CdCl_2 , 3 4-aminopyridine, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH. In voltage-clamp mode, transient Na^+ currents (I_{Na}) were evoked by a test pulse to 0 mV from the holding potential (−70 mV). Tetrodotoxin (TTX)-resistant Na^+ currents were recorded in the presence of 1 $\mu\text{mol/L}$ TTX. To assess frequency-dependent inhibition, field stimulation (60 V/cm at 0, 1, and 10 Hz) was applied to the recording chamber before the activation of Na^+ channels [32]. Currents were filtered at 2 kHz and sampled at 10 kHz. pClamp10

software (Axon Instruments, Molecular Devices) was used during experiments and for analysis.

Whole-cell patch-clamp was also used to record Na^+ currents in mouse DRG neurons at the University of Cincinnati. Cultured DRG cells were transferred to the recording chamber and continuously perfused at RT in oxygenated solution containing (in mmol/L) 145 NaCl, 3 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 , 7 glucose, and 10 HEPES (pH 7.3, titrated with NaOH). Small- to medium-sized neurons (<40 μm in diameter), visualized under an inverted microscope (IX71; Olympus America Inc., Center Valley, PA), were selected for recording using an AxoPatch-200B patch-clamp amplifier (Molecular Devices Corp., Sunnyvale, CA). Patch pipettes (3 $\text{M}\Omega$ –5 $\text{M}\Omega$) were fabricated from borosilicate glass with a Sutter P97 micropipette puller (Sutter Instrument, CA) and filled with internal solution containing (in mmol/L) 130 CsF, 10 HEPES, 10 NaCl, 10 EGTA, 10 TEACl, 10 glucose, and 1 CaCl_2 (pH 7.3, titrated with CsOH). The capacitance transient was cancelled and series resistance was compensated >80%. Recorded neurons were deemed acceptable for further testing only if the resting potential was more negative than −45 mV and the measured access resistance was <10 $\text{M}\Omega$. The bath solution was then switched to Na^+ current recording solution containing (in mmol/L): 10 NaOH, 130 TMACl, 3 KCl, 1 CaCl_2 , 1 MgCl_2 , 0.1 CdCl_3 , 1 4-AP, 20 TEACl, and 10 HEPES (pH 7.3, titrated with HCl). The total Na^+ current (TTX-sensitive plus TTX-resistant) and the slow inactivating TTX-resistant Na^+ current were evoked by a test pulse to −20 mV from a holding potential of −80 mV, or −50 mV, respectively, in the absence of TTX [33]. Recordings were made during drug application for 6 min at 40-s intervals. Patch-clamp data were acquired and analyzed using pClamp 10 (Molecular Devices). The experimenter who performed the patch-clamp recordings was blinded to the antibodies tested until the completion of all experiments.

Electrophysiology in HEK293 Cells Expressing Nav1.1, Nav1.2, Nav1.6, and Nav1.8

The effects of SVMab and rSVMab on these Nav subtypes were tested in HEK293 cells using SB Drug Discovery (www.sbdrugdiscovery.com). The experimenter who performed the patch-clamp recording was blinded to the antibody reagents tested. In QPatch voltage-clamp recordings from HEK293 cells expressing Nav1.1, Nav1.2, and Nav1.6, a 50-ms voltage pulse from −120 mV to 0 mV from a holding potential of −120 mV was applied every 15 s to monitor the time-dependent effects of drugs against the Na^+ channel. The maximum inward current values during the peak recordings at 0 mV were used to analyze and plot all graphs. The testing protocol for

voltage-gated Na^+ channels consisted of first adding extracellular physiological solution twice for a minimum of 4 min (control period), a testing concentration of the SVmab and rSVmab (300 nmol/L) for at least 4 min followed by a 10-min wash and a saturating inhibitory concentration of propafenone hydrochloride (300 $\mu\text{mol/L}$) and two washout periods. Manual patch-clamp recordings from HEK293 cells expressing Nav1.8 were carried out at room temperature. On the day of the experiment, the Nav1.8 cells were cultured according to the standard operating procedures of SB Drug Discovery. Briefly, cells were harvested using TrypLETM Express, re-suspended in their medium, and kept in a shaker for up to 6 h. Internal and external physiological solutions were identical to those for the QPatch assay. The patch pipettes were pulled from borosilicate glass and had tip resistances of 2 $\text{M}\Omega$ –5 $\text{M}\Omega$ when filled with the internal solution. Manual patch-clamp experiments were carried out using an Axon 200B amplifier and a Digidata 1440A acquisition system (Axon Instruments, Molecular Devices). The pClamp software (version 10) from Axon Instruments was used to stimulate and record electrical activity. Capacitative transients were compensated electronically, but the voltage-drop across the series resistance and the liquid junction potential were not compensated. The series resistance was generally <10 $\text{M}\Omega$.

Human DRG Neuron Cultures

Upon delivery, DRGs were rapidly dissected from the nerve roots and minced in Ca^{2+} -free HBSS (Gibco). Human DRG cultures were prepared as previously reported [34]. They were digested at 37°C in a humidified O_2 incubator for 120 min with collagenase Type II (Worthington, NJ, 290 units/mg, 12 mg/mL final concentration) and dispase II (Roche, 1 unit/mg, 20 mg/mL) in PBS with 10 mmol/L HEPES, pH adjusted to 7.4 with NaOH. They were mechanically dissociated using fire-polished pipettes, filtered through 100- μm nylon mesh and centrifuged for 5 min (500 $\times$ g). The hDRG cell pellet was re-suspended and plated on 0.5 mg/mL poly-D-lysine-coated glass coverslips. Cultures were grown in Neurobasal medium supplemented with 10% FBS, 2% B-27, and 1% penicillin/streptomycin.

Whole-Cell Patch-Clamp Recordings from Dissociated Human DRG Neurons

Whole-cell patch-clamp recordings from small-diameter (<50 μm) hDRG neurons were conducted at RT. We used patch pipettes to measure transient Na^+ currents and action potentials with an EPC10 amplifier (HEKA) and an Axopatch-200B amplifier with a Digidata 1440A (Axon Instruments, Molecular Devices). The patch pipettes were pulled from borosilicate capillaries (World Precision

Instruments, Inc.). The resistance was 3 $\text{M}\Omega$ –4 $\text{M}\Omega$ when filled with the pipette solution. The recording chamber (300 μL) was continuously superfused at 3 mL/min–4 mL/min. Series resistance was compensated (>80%) and leak subtraction was performed. Data were low-pass-filtered at 2 KHz and sampled at 10 KHz. Patchmaster (HEKA) and pClamp10 (Axon Instruments) softwares were used during experiments and analysis. When recording Na^+ currents, the pipette solution contained (in mmol/L): CsCl 130, NaCl 9, MgCl_2 1, EGTA 10, HEPES 10, adjusted to pH 7.3 with CsOH. The external solution for recording transient Na^+ currents contained: NaCl 131, TEACl 10, CsCl 10, CaCl_2 1, MgCl_2 2, CdCl_2 0.3, 4-aminopyridine 3, HEPES 10, glucose 10, adjusted to pH 7.4 with NaOH. In voltage-clamp experiments, transient Na^+ currents (I_{Na}) were evoked by a test pulse to 0 mV from the holding potential (−70 mV).

Behavioral Tests

Mice were habituated to the environment for at least 2 days before experiments. To assess mechanical allodynia, the plantar surface of the left hind-paw was stimulated using a series of von Frey fibers with logarithmically increasing stiffness (0.02 g–2.56 g, Stoelting, Wood Dale, IL) presented perpendicularly to the central plantar surface. The paw withdrawal threshold was determined following Dixon's up-down method. The frequency response was measured by stimulating the hind-paw with a 0.4 gram von Frey hair 10 times and the percentage withdrawal response was calculated as the frequency [35].

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Biochemical and electrophysiological data were tested using one-way or two-way ANOVA followed by the *post-hoc* Bonferroni test or unpaired two-tailed Student's *t*-test. Statistical analyses were performed with Prism 6.0 (GraphPad) and the criterion for statistical significance was $P < 0.05$. For the electrophysiological data generated at the University of Cincinnati (Fig. 8), linear regression was used to calculate and compare the slopes of the time-vs-peak current plots among different antibodies.

Results

SVmab and rSVmab have Different Binding Affinities for the Nav1.7 Voltage-Sensor Domain II

We previously showed that SVmab binds to the VSD II isolated from Nav1.7 [19]. To test if SVmab and rSVmab

bind to this VSD II with comparable affinity, we performed ELISA experiments. Recombinant Nav1.7 VSD II fused with a C-terminal hexahistidine tag (VSDII-His₆) was prepared and coated onto the ELISA plate. Increasing concentrations (0 nmol/L, 66 nmol/L, and 133 nmol/L) of SVmab and rSVmab were delivered, after which a secondary antibody conjugated to horseradish peroxidase (HRP) was added for detection. Consistent with our previous report [19], SVmab showed robust ELISA signals to Nav1.7 VSD II in a concentration-dependent manner (Fig. 1A). Notably, rSVmab showed weaker ELISA signals than SVmab by ~ 5-fold, although they still showed a concentration-dependent increase, ~ 3-fold above the level of the negative control anti-FLAG antibody at 266 nmol/L (Fig. 1A). We also noted that the first batch of SVmab that was used for most of the previous *in vitro*

experiments exhibited a stronger ELISA response to the Nav1.7 VSD II than a recent batch of antibody (Fig. 1B). Because it is possible that the apparent discrepancy in ELISA responses of the antibodies was due to their differential sensitivity to the secondary antibody, rather than to the Nav1.7 VSD II, we ran both antibodies on SDS-PAGE (4%–15% gradient gel) under non-reducing conditions and performed western-blots using a secondary antibody. The results showed no appreciable difference in western blot signals between SVmab and rSVmab, strongly suggesting that the observed difference in ELISA signals is due to the different affinities of the antibodies for the Nav1.7 VSD II (Fig. 1C). We also ran both antibodies on SDS-PAGE under a reducing condition and found that the heavy chains of SVmab and rSVmab migrated slightly differently (Fig. 1D), possibly due to differences in their

Fig. 1 Biochemical properties of SVmab and rSVmab. **A** Binding activity of SVmab and rSVmab in ELISA. hVSD II (1 μ g) was coated on the microwell plate in 0.05 mmol/L carbonate-bicarbonate buffer, pH 9.6. Anti-FLAG antibody was used as a negative control. All ELISA measurements were performed in triplicate. Data are mean $\pm$ SEM. **B** Antibody binding in ELISA. SVmab (66 nmol/L), SVmab (first batch, 66 nmol/L), and CTmab (66 nmol/L) were used.

Anti-FLAG antibody (266 nmol/L) was added as a positive control. All ELISA measurements were performed in triplicate. Data are mean $\pm$ SEM. **C** SDS-PAGE (left) and western blots (right) of SVmab (2 μ g) and rSVmab (2 μ g) under a non-reducing condition. The gel (left) was stained with Coomassie blue. **D** SDS-PAGE of SVmab (1 μ g) and rSVmab (1 μ g) under a reducing condition. The gel was stained with Coomassie blue.

post-translational modification such as glycosylation or chemical modification.

SVmab and rSVmab have Different Binding Affinities for Nav1.7 in HEK293 Cells, Human Nerve Tissue, and Mouse DRG Neurons

We next asked whether SVmab and rSVmab bind to the full-length Nav1.7 channel. In the first step, we developed an ELISA assay using SVmab and rSVmab as the capture antibodies (Fig. 2A). We also included two control antibodies, mouse IgG antibody, and CTmab that targets the S1–S2 loop of the Nav1.7 VSD II [19]. We used CTmab as a control antibody because it does not affect either Na⁺ currents or pain and itch, despite its binding to the Nav1.7

VSD II [19]. We conjugated a commercial Nav1.7 antibody with HRP as the detection antibody. Notably, the detection antibody recognizes an intracellular loop between domains I and II of Nav1.7, whereas the capture antibodies recognize the extracellular loops (S1–S2 or S3–S4 loops of the VSD II). The ELISA plates were coated with streptavidin, which binds biotin-conjugated capture antibodies (Fig. 2A). Nav1.7-expressing HEK293 cells were treated with the capture antibody for 15 min to bind human Nav1.7 proteins. Then the cells were solubilized with Triton and incubated on streptavidin-coated plates for 2 h, after which the detection antibody was added for visualization.

As shown in Fig. 2B, IgG at 1, 100, 300, and 1000 nmol/L had ELISA signals below the detection limit,

Fig. 2 SVmab, rSVmab, and CTmab differentially bind to Nav1.7 in Nav1.7-expressing HEK293 cells and human spinal nerve tissue. **A** Schematic of ELISA analysis. **B–D** Differential binding of SVmab, rSVmab, and CTmab to HEK cells expressing Nav1.7 (**B**) and Nav1.5

(**C**) as well as Nav1.7-expressing human nerve tissue (**D**) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to IgG control; $n = 5$ (with cell lysate) and $n = 3$ (no cell lysate), one-way ANOVA followed by *post-hoc* comparison.

and served as background and baseline. SVmab exhibited a concentration-dependent increase in ELISA responses to the Nav1.7 channels extracted from HEK cells ($P < 0.05$ vs IgG control at 300 nmol/L). In contrast, rSVmab showed a significant ELISA response to Nav1.7 compared to IgG only at the highest concentration (1000 nmol/L, $P < 0.05$). CTmab displayed the highest ELISA responses to Nav1.7 among all the antibodies. Given that CTmab does not inhibit Na^+ currents [19], this result suggests that binding to Nav1.7 is not sufficient for the blocking activity of a Nav1.7-targeting antibody. In the absence of a Nav1.7-containing cell lysate, all the antibodies showed the background level of ELISA signals (Fig. 2B). For comparison, CTmab, SVmab, and rSVmab showed no appreciable ELISA responses to Nav1.5-expressing HEK cells, which were transfected with Nav1.5 cDNA (Fig. 2C), suggesting that the interactions of both SVmab and CTmab with Nav1.7 are specific. We also performed the ELISA experiments with the four antibodies to Nav1.7 in human

spinal nerve tissue lysates. Unlike the mouse DRG, Nav1.7 is the major Na^+ channel in the human DRG [21]. SVmab and CTmab but not rSVmab had significant ELISA responses to human Nav1.7 in naïve tissue ($P < 0.05$ vs IgG; Fig. 2D).

Since SVmab inhibits Na^+ currents in mouse DRG neurons [19], we next compared the binding activity of CTmab, SVmab, and rSVmab in these neurons. To visualize the binding, we labeled the antibodies with the green fluorescent dye Alexa-fluor 488. Incubation of mouse DRG neuron cultures with the labeled antibodies (300 nmol/L, 30 min) resulted in binding of CTmab and SVmab to $\sim 10\%$ of the neurons (Fig. 3A, B). However, rSVmab only labeled 2% of mouse DRG neurons; this percentage was lower than that of SVmab and CTmab ($P < 0.05$, $n = 4$ cultures, Fig. 3A, B). Taken together, these results show that (i) SVmab and CTmab bind to Nav1.7 in HEK cells, human nerve tissue, and mouse DRG

Fig. 3 Alexa-fluor 488-labeled SVmab, rSVmab, and CTmab differentially bind to Nav1.7 in mouse DRG neurons. **A** Images of labeled DRG neurons binding to SVmab and CTmab but not rSVmab. Right,

contrast images of the left panel. Scale bar, 100 μm . **B** Percentage of DRG neurons binding each antibody (* $P < 0.05$, $n = 3$ cultures, one-way ANOVA followed by *post-hoc* comparison).

Fig. 4 SVmab, rSVmab, and CTmab differentially regulate Na⁺ currents in Nav1.7 cells and do not affect Na⁺ currents in HEK293 cells expressing Nav1.1, Nav1.2, Nav1.5, Nav1.6, and Nav1.8. **A–B** Effects of SVmab, rSVmab, and CTmab on Nav1.7-expressing cells. **A** Representative traces of Na⁺ currents. **B** Time-course of Na⁺ currents before and after 4-min perfusion with CTmab (300 nmol/L, *n* = 5), rSVmab (300 nmol/L, *n* = 6), SVmab (100 nmol/L, *n* = 3), and SVmab (300 nmol/L, *n* = 10). Note continuing inhibition of currents after the perfusion (***P* < 0.01, ****P* < 0.001, ns, not significant, two-way ANOVA). **C–D** SVmab, rSVmab, and CTmab (300 nmol/L) do not affect Nav1.5 currents in Nav1.5-expressing cells. **C** Representative traces of Na⁺ currents after exposure to CTmab, SVmab, and rSVmab (300 nmol/L). **D** Normalized Na⁺ currents before and after exposure to antibodies (*n* = 5 cells/group). HEK cells were transfected with Nav1.5 cDNA and β-1/3 subunit DNA, and the recordings were made 48 h later. **E** Effects of SVmab and rSVmab (300 nmol/L) on Na⁺ currents in HEK293 cells expressing Nav1.1, Nav1.2, and Nav1.6 (QPatch) and Nav1.8 (manual patch). Neither SVmab nor rSVmab affected the currents. As a positive control, propafenone (300 μmol/L), as indicated by white bars, substantially reduced the currents.

neurons, and (ii) rSVmab has a much lower binding affinity for Nav1.7 in these cell and tissue systems.

SVmab and rSVmab Differentially Regulate Na⁺ Currents in Nav1.7-Expressing HEK293 Cells

To compare the blocking activity of SVmab, rSVmab, and CTmab, we measured their effects on inhibiting Na⁺ currents through human Nav1.7 expressed in HEK293 cells using patch-clamp recording. To facilitate this process, we chose a stable cell line expressing both α (Nav1.7) and β subunits. Because we previously used cells transiently transfected with Nav1.7 only, the effects of SVmab on Nav1.7 may differ from our previous measurements in terms of efficacy and kinetics [36–38]. We only analyzed cells with large Na⁺ currents (>500 nA). The cells were perfused with the antibodies for 4 min, followed by washout for >10 min. As we previously showed [19], CTmab (300 nmol/L, 4 min) had no effect on Na⁺ currents over a period of 15 min (Fig. 4A, B). However, SVmab perfusion (4 min) elicited a marked and concentration-dependent inhibition of Na⁺ currents at 100 and 300 nmol/L (30% and 60%; *P* < 0.05; Fig. 4B). SVmab showed slow kinetics of inhibition of the Na⁺ currents, and this inhibition was also sustained; after washout, there was no sign of recovery (Fig. 4B). However, rSVmab perfusion (4 min, 300 nmol/L) only resulted in a mild reduction of Na⁺ current (10%, *P* > 0.05 vs baseline, *n* = 5 cells; Fig. 4A, B). Collectively, SVmab and rSVmab differ not only in their binding affinity for Nav1.7, but also in their ability to block it.

SVmab and rSVmab do not Regulate Na⁺ Currents in HEK293 Cells Expressing Nav1.1, Nav1.2, Nav1.5, Nav1.6, and Nav1.8

To assess the specific inhibitory effects of SVmab and rSVmab on Nav1.7, we also tested their effects on Nav1.5-mediated Na⁺ currents using manual patch clamp recordings. Transfection of HEK293 cells with hNav1.5 cDNA resulted in large Na⁺ currents, which were not affected by any of the antibodies (SVmab, CTmab, and rSVmab at 300 nmol/L, Fig. 4C, D). QPatch analysis showed that SVmab and rSVmab (300 nmol/L, 4 min) had no appreciable effects on Na⁺ currents generated by cells expressing Nav1.1, Nav1.2, and Nav1.6 (Fig. 4E). Manual patch-clamp recordings also revealed no effects of SVmab and rSVmab (~ 300 nmol/L, 4 min) on Na⁺ currents generated by cells expressing Nav1.8 (Fig. 4E). As a positive control, 300 μmol/L propafenone profoundly inhibited the currents through Nav1.1, Nav1.2, Nav1.6, and Nav1.8 (Fig. 4E).

Frequency-Dependent Inhibition of and Binding to Nav1.7 by SVmab and rSVmab in HEK293 Cells

We had previously shown that SVmab inhibits Na⁺ currents in Nav1.7-expressing HEK293 cells in a frequency (use)-dependent manner [19]. To further address activity/frequency-dependent inhibition of Nav1.7 by SVmab and rSVmab, we applied field stimulation (60 V/cm, at 0, 1, and 10 Hz) to the recording chamber just before activation of the Na⁺ channels (Fig. 5A). Following electrical stimulation, low concentration of SVmab (100 nmol/L) induced more reduction of Na⁺ currents (70%–80%) than SVmab (100 nmol/L) without field stimulation (*P* < 0.001, Fig. 5B, C). However, CTmab (100 nmol/L) did not reduce the Na⁺ currents following stimulation (Fig. 5C). rSVmab showed mild inhibition of the currents at 10 Hz (Fig. 5D, E). To examine whether the field stimulation indeed increases Nav1.7 activity, we tested the stimulation-dependent effects of carbamazepine, which is known to inhibit the function of Nav channels in a use or activity-dependent manner [32]. We found that carbamazepine (100 μmol/L) induced much greater reduction of the Na⁺ currents after stimulation at 10 Hz (Fig. 5F, G). Taken together, these data suggest that SVmab inhibition of Nav1.7 is largely activity/frequency-dependent, while rSVmab shows mild activity-dependence. We next asked whether the increased reduction of Nav1.7 currents by SVmab in the presence of field stimulation is due to increased binding of SVmab to Nav1.7. To address this question, we performed live-cell ELISA on Nav1.7-expressing HEK293 cells with and without field stimulation in a setting similar to the patch clamp recording (Fig. 5H).

Fig. 5 Electrical field stimulation enhances the functional inhibition of Nav1.7 by SVmab, rSVmab, and carbamazepine in HEK293 cells expressing Nav1.7. **A** Schematic of electrical field stimulation followed by the induction of Na⁺ currents. **B–C** Effects of SVmab (100 nmol/L, 10 Hz), SVmab (100 nmol/L, 1 Hz), SVmab (100 nmol/L, no stimulation), and CTmab (100 nmol/L, 10 Hz) on Na⁺ currents in Nav1.7-expressing cells. **B** Representative traces of Na⁺ currents. **C** Time-dependent inhibition of Nav1.7 currents by SVmab (***P* < 0.001; two-way ANOVA, *n* = 4–5 cells). **D–E** Effects of rSVmab (100 nmol/L, 10 Hz, 1 Hz, or no stimulation) on Na⁺ currents in Nav1.7-expressing cells. **D** Representative traces of Na⁺ currents. **E** Time-dependent inhibition of Nav1.7 currents by rSVmab (**P* < 0.05; two-way ANOVA, *n* = 5 cells). **F–G** Representative of carbamazepine (100 μmol/L, 10 Hz, 1 Hz, or no stimulation) on Na⁺ currents in Nav1.7-expressing cells. **F** Representative traces of Na⁺ currents. **G** Time-dependent inhibition of Nav1.7 currents by carbamazepine (***P* < 0.001; two-way ANOVA, *n* = 6–7 cells). **H** Electrical field stimulation increased the binding of SVmab and rSVmab to Nav1.7 expressed in HEK293 cells (**P* < 0.05, one-way ANOVA, *n* = 6 cultures).

Notably, we found that the binding of both SVmab and rSVmab to the cells expressing Nav1.7 increased upon field stimulation (Fig. 5H), while control IgG showed no increase (Fig. 5H). This result suggests that the binding of SVmab and rSVmab to Nav1.7 is activity/frequency-dependent.

Different Effects of SVmab and rSVmab on TTX-Sensitive and TTX-Resistant Na⁺ Currents in Mouse DRG Neurons

Mouse DRG neurons express functional Nav1.7 [16, 19, 39]. We further assessed the effects of SVmab on transient Na⁺ currents induced in dissociated mouse DRG neurons with small diameters (<25 μm). SVmab induced a slow but substantial inhibition (~50%) of transient Na⁺ currents (Fig. 6A, B). Notably, 10 nmol/L protoxin II, a specific inhibitor of Nav1.7 at low concentrations (~10 nmol/L) [29, 40], elicited a level of inhibition similar to SVmab (Fig. 6A, B), suggesting that SVmab suppresses the Na⁺ currents generated by Nav1.7. To test whether SVmab also inhibits the Na⁺ currents generated by Nav1.8, the major Nav channel in mouse DRG neurons [21], we recorded the TTX-resistant Na⁺ currents generated by Nav1.8 and Nav1.9, and found that SVmab did not inhibit these currents, suggesting that SVmab primarily inhibits Nav1.7 currents in mouse DRG neurons (Fig. 6C, D).

Next, we tested whether the inhibitory effect of SVmab on Nav1.7 is abrogated by a blocking peptide used to raise SVmab in mouse DRG neurons, as shown in HEK293 cells expressing Nav1.7 [19]. Pre-incubation of the blocking peptide (10 μmol/L) with SVmab impaired the inhibitory effect of SVmab in these neurons, while the control peptide had no such effect (Fig. 7A, B). SVmab also failed to suppress Na⁺ currents in stable HEK293 cells expressing

Nav1.7 and the β subunit in the presence of the blocking peptide (10 μmol/L, Fig. 7C), consistent with our previous study [19], suggesting that the extracellular S3b–S4 region is important for the functional inhibition of Nav1.7 by SVmab. Taken together, these data suggest that the SVmab blocks Na⁺ currents that is mediated by Nav1.7 in mouse DRG neurons.

In a separate study conducted at the University of Cincinnati, we also tested the effects of CTmab, SVmab, and rSVmab on Na⁺ currents in small and medium-sized mouse DRG neurons (<40 μm in diameter). Each recorded neuron was only tested for one antibody at a single dose of 300 nmol/L. Recording began at the time of antibody application for 6 min at 40-s intervals. Cells that did not complete the 6-min recording were omitted from final analysis.

Representative recordings and effects of the three antibodies are shown in Fig. 8A–D. CTmab had no effect on the currents, while SVmab induced a progressive inhibition. rSVmab did not show inhibitory effects within the 6 min but seemed to show a small inhibitory effect at 5 min (*P* = 0.054 for the rSVmab group, linear regression, probability of nonzero slope). The average responses to all three antibodies are presented in Fig. 8E. Acute bath application of SVmab reduced total Na⁺ currents compared to rSVmab or CTmab (*P* < 0.0001 for the SVmab group only, linear regression, probability of slope being nonzero). SVmab at 300 nmol/L also inhibited the slow-inactivating Na⁺ currents (Fig. 8F; averaged data, linear regression, *P* < 0.001 probability of nonzero slope). Although TTX was not included in this study, the slow inactivating Na⁺ currents (held at −50 mV) could have been partially TTX-resistant currents [33].

SVmab and rSVmab Differentially Inhibit Neuropathic Pain After Chemotherapy

We have shown that intrathecal injection of SVmab effectively reduces the neuropathic pain symptom mechanical allodynia after chronic constriction injury of the sciatic nerve [19]. To test whether SVmab also elicits relief in other neuropathic pain conditions, we used a chemotherapy model. Paclitaxel-induced neuropathic pain is resistant to anti-inflammatory treatments [41]. We assessed mechanical allodynia, a cardinal feature of neuropathic pain, *via* both paw withdrawal threshold and frequency. Paclitaxel induced a rapid and remarkable mechanical allodynia, as indicated by reduction in withdrawal threshold and increase in withdrawal frequency on day 3 (Fig. 9A, B). Spinal injection of SVmab (50 μg) *via* the intrathecal route increased the withdrawal threshold (*P* < 0.001 *vs* CTmab, Fig. 9A). Given the high molecular weight of SVmab (~150 kDa), the dose administered was very low

Fig. 6 SVmab blocks TTX-sensitive Na^+ currents in mouse DRG neurons. **A–B** Perfusion with SVmab (300 nmol/L) and protoxin II (10 nmol/L) but not CTmab (300 nmol/L) suppressed transient Na^+ currents in small DRG neurons. **A** Representative current traces.

B Time-course of currents before and 4 min after drug perfusion. Note continuing inhibition by SVmab during washout. Also note that SVmab and protoxin II induced similar inhibition ($*P < 0.05$, two-way ANOVA). **C–D** SVmab and CTmab did not alter TTX-resistant Na^+ currents. **C** Representative traces of TTX-resistant Na^+ currents. **D** Time course of TTX-resistant Na^+ currents before and 4 min after antibody perfusion. *n.s.* not significant.

(0.3 nmol). In agreement with our previous report [19], intrathecal CTmab (50 μg) had no effect on mechanical sensitivity (Fig. 9A). A second intrathecal injection of SVmab 24 h later also suppressed the paclitaxel-induced increase in withdrawal frequency. Intrathecal injection of rSVmab (50 μg) also increased the withdrawal threshold

($P < 0.001$ vs CTmab, Fig. 9A). However, intrathecal rSVmab did not reduce the withdrawal frequency after the second injection (Fig. 9B). Thus, SVmab is more effective than rSVmab in suppressing chemotherapy-evoked neuropathic pain, but rSVmab also has an appreciable *in vivo* effect.

Fig. 7 Inhibition of Na^+ currents by SVmab is abrogated by a blocking peptide in mouse DRG neurons and Nav1.7-expressing HEK293 cells. **A–B** Effects of the control peptide DYKDDDDK (10 μ mol/L, $n = 7$) with SVmab, the blocking peptide VELFLAD-VEG (10 μ mol/L, $n = 10$) with SVmab, or SVmab alone (300 nmol/L, $n = 6$). **A** Representative current traces. **B** Time course of currents

before and 4 min after drug perfusion ($*P < 0.05$, *n.s.*, not significant, two-way ANOVA). **C** Effects of the blocking peptide (10 μ mol/L) with SVmab (300 nmol/L) on currents in Nav1.7-expressing cells ($n = 8$ cells). Note no inhibition of Nav1.7 currents by SVmab in the presence of the blocking peptide.

SVmab Binds to and Inhibits Nav1.7 in Human DRG Neurons

Finally, we tested whether SVmab also binds to and inhibits Nav1.7 in human DRG neurons. Immunohistochemistry revealed that SVmab was capable of binding neurons and axons of human DRGs (Fig. 10A). Notably, SVmab binding primarily occurred on the cell surface

(Fig. 10B), supporting specific binding to the extracellular site of human Nav1.7. Patch-clamp recordings from small-diameter human DRG neurons ($<50 \mu\text{m}$) showed that SVmab (300 nmol/L) elicited $\sim 20\%$ – 30% inhibition of Na^+ currents ($P < 0.05$ compared to CTmab, Fig. 10C). Thus, SVmab is also active in human tissue. In contrast, CTmab (300 nmol/L, 4 min) had no effect on Na^+ currents in these neurons (Fig. 10C).

Fig. 8 SVmab, rSVmab, and CTmab differentially regulate total and slow-inactivating Na^+ currents in mouse DRG neurons. **A–C** Representative traces of whole-cell voltage-clamp recordings from acutely cultured neurons exposed to monoclonal antibodies. The currents were evoked by a test pulse every 40 s to -20 mV from a holding potential of -80 mV followed by -50 mV. **D** Peak currents plotted against duration of antibody application. **a**: CTmab, **b**: SVmab, **c**: rSVmab (300 nmol/L). **E–F** Responses of total currents (**E**) and slow-inactivating currents (**F**) to application of Nav1.7 antibodies. Percentage change of peak currents averaged from all tested neurons plotted against time of antibody application ($P < 0.0001$, linear regression, probability of nonzero slope for SVmab).

Discussion

Our data showed that SVmab and rSVmab exhibit differential but specific blocking effects on Na^+ currents elicited in Nav1.7-expressing HEK293 cells, in mouse DRG cells, and in human DRG cells. Furthermore, these two antibodies showed differential pain-relief effects on a model of chemotherapy-induced neuropathic pain. SVmab had significantly higher activity than rSVmab in all experiments.

The mild effect of rSVmab on HEK293 cells is in agreement with the results of Liu *et al.* [30]. Consistent with the differences, SVmab had a significantly higher binding affinity for Nav1.7 in all systems tested (HEK293 cells expressing Nav1.7, mouse DRG neurons, human nerve tissue, and the Nav1.7 VSD II) than rSVmab. Despite its low affinity, the effects of rSVmab on Nav1.7 were specific because it bound to Nav1.7-expressing HEK293 cells at a high concentration compared to the control

Fig. 9 SVmab, rSVmab, and CTmab differentially alter neuropathic pain after chemotherapy. **A** Spinal administration of SVmab and rSVmab, but not CTmab, reduced paclitaxel-induced mechanical allodynia, evaluated by withdrawal threshold. Antibodies were injected intrathecally 3 days after a single paclitaxel injection (6 mg/kg) in male CD1 mice ($***P < 0.001$ vs CTmab group, two-way ANOVA with Bonferroni *post-hoc* comparison, $n = 5$ mice per group). **B** Spinal administration of SVmab, but not CTmab or rSVmab, decreased withdrawal frequency. Antibodies were injected intrathecally 4 days after paclitaxel injection ($***P < 0.001$ vs CTmab group, two-way ANOVA with Bonferroni *post-hoc* comparison, $n = 5$ mice per group).

antibody IgG1 and also had an effect on neuropathic pain *in vivo*. In contrast, CTmab, despite its higher-affinity binding, had no effect on Nav1.7 function in any of the systems, underlining the functional specificity of SVmab and rSVmab.

The study of Liu *et al.* showed that the binding of arSVmab to the peptide corresponding to the loop between S3 and S4 was below the detection limit [30]. Here, we showed that the peptide abrogated the blocking effect of SVmab on Na^+ currents in mouse DRGs and in HEK293 cells expressing Nav1.7, consistent with our previous study [19]. We previously showed that SVmab and CTmab bind to Nav1.7 VSD II [19], which was confirmed in the present study (Fig. 1A). Importantly, we found that SVmab and CTmab bound to the full-length Nav1.7 channels expressed in HEK293 cells and human nerve tissue to which rSVmab

Fig. 10 SVmab binds human DRG neurons and inhibits their Na^+ currents. **A–B** Immunohistochemistry showing the binding of SVmab to human DRG neurons visualized by Cy3-conjugated secondary antibody. The box in **A** is enlarged in **B**. Arrows in **A** indicate labeled axons; arrows in **B** indicate surface labeling of DRG neurons (scale bars, 50 μ m). **C** Normalized Na^+ currents before and after perfusion with CTmab (300 nmol/L, $n = 5$ neurons) and SVmab (300 nmol/L, $n = 8$ neurons; $*P < 0.01$, two-way ANOVA).

showed significantly weaker binding. There are several possible explanations for this apparent discrepancy. First, it

is possible that the weaker affinity of rSVmab leads to peptide binding below the detection level. Second, SVmab may have additional and serendipitous interactions with the rest of Nav1.7 (e.g. the S1–S2 loop or the pore region) besides the S3–S4 loop, which could have increased the affinity of SVmab for either the Nav1.7 VSD II or the full-length channel. Third, SVmab may recognize the S3–S4 loop in a conformation-dependent manner. In the voltage-gated Na⁺ channel, the S3b helix is followed by a structured S3–S4 loop that is connected to the S4 helix [42]. Consistent with this possibility, the S3–S4 loop sequence that we used for raising SVmab includes part of the S3b helix and overlaps significantly with the binding sites of the voltage-sensor toxins HwTx and ProTxII [29, 40, 43]. It is believed that these toxins bind to and stabilize the VSD in a specific conformation, thereby affecting the gating of Nav1.7 [29, 43].

In a validation study of mouse DRG neurons at the University of Cincinnati, we also saw significant inhibition of Na⁺ currents by SVmab, whereas CTmab had no effect and rSVmab showed very mild inhibition (Fig. 8). It was unexpected that SVmab also inhibited the slow-inactivating Na⁺ currents, which could be TTX-resistant Na⁺ currents when holding the membrane potential at −50 mV before depolarizing to −20 mV [33]. In contrast, our data also showed that SVmab at the same concentration (300 nmol/L) did not inhibit TTX-resistant Na⁺ currents in mouse DRG neurons (Fig. 6C, D) or Nav1.8-mediated Na⁺ currents in HEK293 cells (Fig. 4E). This discrepancy may be due to different recording conditions in different labs (e.g., different holding and depolarization potentials). Because TTX was not included during Na⁺ channel recording to completely suppress TTX-sensitive currents for the experiments illustrated in Figure 8, part of the inhibitory effects shown in Figure 6B may have been the result of either inhibition of the TTX-resistant Na⁺ currents mediated by Nav1.8/Nav1.9 or inhibition of the remaining TTX-sensitive currents. Given the greater complexity of native DRG neurons than HEK293 cells, we cannot exclude the possibility that SVmab interacts with different Na⁺ channel subtypes in native settings. Thus, more experiments are needed to determine the selectivity of the antibody in native and pathological conditions, including possible effects on Nav1.6 [7, 44] and Nav1.8/Nav1.9. Given a critical role of Nav1.8 and Nav1.9 in neuropathic pain and their predominant expression in sensory neurons [16, 45, 46], additional inhibition of TTX-resistant Na⁺ currents may increase the therapeutic efficacy of the antibody.

What are the possible reasons for the different functional efficacy and binding properties of SVmab and rSVmab? First, we reason that different post-translational modifications of the antibody (i.e. glycosylation or chemical

modification) produced in two different systems (hybridoma vs HEK293 cells) could explain the discrepancy. Post-translational modifications, including glycosylation and chemical modification, are known to have significant effects on the structure and function of recombinant antibodies [47–56]. Consistent with this possibility, the apparent molecular weights of the heavy chains of SVmab and rSVmab differed on SDS-PAGE (Fig. 1D). If this is the case, it would be interesting to investigate how post-translational modifications regulate the different efficacies of SVmab and rSVmab. Second, we cannot exclude the possibility that the hybridoma clone that produced SVmab of high activity was gradually dominated by another clone that produces a less active antibody during hybridoma culture and selection. We recently realized that the first batch of the antibody that was used for most of the previous *in vitro* experiments exhibited a stronger ELISA response to the Nav1.7 VSD II than a recent batch of SVmab (Fig. 1B) and there is batch-to-batch variation in binding and activity of the antibody, which could cause difference in its blocking activity. Third, although it is a remote possibility, we cannot completely rule out an error in antibody sequencing. Nevertheless, we performed hybridoma sequencing from two different companies and obtained a consensus sequence of the variable region.

We observed that although CTmab shows stronger binding to Nav1.7 than SVmab, it is unable to block Nav1.7 activity. On the other hand, although rSVmab shows weaker binding to Nav1.7 than CTmab and SVmab, it still has some *in vivo* activity in our behavioral tests (Fig. 9A). It is noteworthy that the control antibody used by Liu *et al.* showed some blocking activity of the Nav1.7 currents, but the source of this control antibody has not been fully described [30].

These findings might have implications for the development of ion channel-targeting antibodies. Because ion channels undergo multiple conformational changes associated with gating at a fast time scale (milliseconds or less), antibody binding to a conformation that affects gating is less likely to be a high-affinity event. Therefore, screening high-affinity antibody binders for initial selection may not be an ideal strategy for developing antibodies that block ion channel activity. One potential approach is to engineer bispecific antibodies to have one Fab portion with blocking activity and another portion with a high affinity for the target ion channel.

In agreement with the previous report [19], our results showed that the efficacy of SVmab is activity/frequency dependent. Following electrical field stimulation at 10 Hz, a low concentration of SVmab (100 nmol/L) induced a pronounced inhibition of the Nav1.7 current (Fig. 4C, D). Thus, SVmab could be more effective in neuropathic pain conditions where hyperexcitability of sensory neurons

develops [9, 57, 58]. It is interesting that rSVmab showed some *in vivo* activity in the mechanical allodynia threshold test despite its apparently low potency against Nav1.7, suggesting that Nav1.7 contributes differently to the withdrawal threshold and frequency in the paclitaxel-induced mechanical allodynia model. We cannot completely rule out the possibility of an additional target of rSVmab and SVmab for their *in vivo* effects on the mechanical allodynia threshold.

Our data also suggest that post-translational modification might be an important factor in the development of therapeutic antibodies targeting surface proteins such as ion channels, especially under *in vivo* or *ex vivo* conditions. Because antibodies are large molecules, it is critical to have unhindered access to their targets in neurons within tissues. To this end, it might be advantageous to use antibodies with less glycosylation or smaller fragments (e.g. Fab or nanobody). Notably, in a pilot study we observed that the Fab fragments of SVmab effectively inhibit Na^+ currents in mouse and human DRG neurons and suppress inflammatory and neuropathic pain [59]. Further characterization of the *in vitro*, *ex vivo*, and *in vivo* efficacy of the Fab fragments is under way. It is also noteworthy that a complete inhibition of Nav1.7 function can be detrimental, given the important role of this channel in normal pain perception and other physiological functions. Partial inhibition of Nav1.7 function, especially preferential inhibition of Nav1.7 hyper-function in pathological pain conditions may offer better control of clinical pain. It is also worth mentioning that patients and mice lacking Nav1.7 have increased opioid tone and the insensitivity to pain can be reversed by naloxone [60]. Thus, Nav1.7 inhibitors such as SVmab may also alleviate pathological pain by enhancing the function of the endogenous opioid system.

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