Cellular/Molecular

The Voltage-Gated Sodium Channel $Na_v1.9$ Is an Effector of Peripheral Inflammatory Pain Hypersensitivity

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We used a mouse with deletion of exons 4, 5, and 6 of the SCN11A (sodium channel, voltage-gated, type XI, α) gene that encodes the voltage-gated sodium channel $Na_v1.9$ to assess its contribution to pain. $Na_v1.9$ is present in nociceptor sensory neurons that express TRPV1, bradykinin B_2, and purinergic $P2X_3$ receptors. In $Na_v1.9^{-/-}$ mice, the non-inactivating persistent tetrodotoxin-resistant sodium TTXr-Per current is absent, whereas TTXr-Slow is unchanged. TTXs currents are unaffected by the mutation of $Na_v1.9$. Pain hypersensitivity elicited by intraplantar administration of prostaglandin E_2, bradykinin, interleukin-1β, capsaicin, and $P2X_3$ and P2Y receptor agonists, but not NGF, is either reduced or absent in $Na_v1.9^{-/-}$ mice, whereas basal thermal and mechanical pain sensitivity is unchanged. Thermal, but not mechanical, hypersensitivity produced by peripheral inflammation (intraplanatar complete Freund's adjuvant) is substantially diminished in the null allele mutant mice, whereas hypersensitivity in two neuropathic pain models is unchanged in the $Na_v1.9^{-/-}$ mice. $Na_v1.9$ is, we conclude, an effector of the hypersensitivity produced by multiple inflammatory mediators on nociceptor peripheral terminals and therefore plays a key role in mediating peripheral sensitization.

Key words: **sensory neuron; peripheral sensitization; inflammation; nociceptor; pain; sodium channel**

Introduction

The peripheral terminals of nociceptors are the interface between noxious stimuli and the nervous system. Conversion of the stimuli into ion fluxes across nociceptor membranes is mediated by high-threshold thermal, mechanical, and chemical protein transducers (Julius and Basbaum, 2001). Transduction represents the first step in the generation of nociceptive pain, a sensation that warns of the presence of potentially tissue-damaging stimuli. The second step is initiation of action potentials in the nociceptors by voltage-gated sodium channels (VGSCs). Among the 10 VGSC isoforms, two, $Na_v1.8$ (Akopian et al., 1996; Sangameswaran et al., 1997) and $Na_v1.9$ (Dib-Hajj et al., 1998; Tate et al., 1998), are expressed almost exclusively by small nociceptor primary afferent neurons (Amaya et al., 2000) and are tetrodotoxin insensitive. $Na_v1.8$ generates a tetrodotoxin-resistant (TTXr) current with a threshold of activation of -40 mV that inactivates slowly (TTXr-Slow) (Akopian et al., 1996; Sangameswaran et al., 1997; Renganathan et al., 2002b; John et al., 2004), whereas $Na_v1.9$ is presumed from recordings in $Na_v1.8$ null mice, to produce a persistent current with a more hyperpolarized voltage dependence and ultraslow recovery from inactivation (TTXr-Per) (Cummins et al., 1999; Baker et al., 2003).

In the presence of inflammation, the threshold to elicit pain falls and the response to noxious stimuli is exaggerated. The conversion of high-threshold nociceptive pain to a low-threshold inflammatory pain is contributed to by peripheral sensitization of the nociceptor peripheral terminal (Levine and Reichling, 1999). Inflammatory mediators, including amines, prostanoids, kinins, purines, protons, and nerve growth factor (NGF), sensitize the nociceptor peripheral terminal by producing a reduction in the threshold of transducer channels (McCleskey and Gold, 1999; Julius and Basbaum, 2001), an increase in terminal membrane excitability (Amir et al., 2006), and insertion of receptors into the terminal membrane (Zhang et al., 2005). The high threshold of the transducers are reduced by posttranslational processing in response to activation by the inflammatory mediators of multiple intracellular signaling pathways [PKA, PKC, extracellular signal-regulated kinase, phosphatidylinositol 3 (PI3) kinase, and phospholipase C] (Cesare et al., 1999; Aley et al., 2001; Bautista et al., 2006).

Prostaglandin E_2 (PGE_2), 5-HT, and bradykinin (BK) increase the $Na_v1.8$-like TTXr current in DRG neurons, with an increase in amplitude and rates of activation and inactivation (Gold et al., 1996, 1998, 2002; England et al., 2001). Inflammatory pain sensitivity is, moreover, delayed in $Na_v1.8$ null mutant mice (Akopian et al., 1999). PGE_2 also increases TTXr-Per in DRG neurons,

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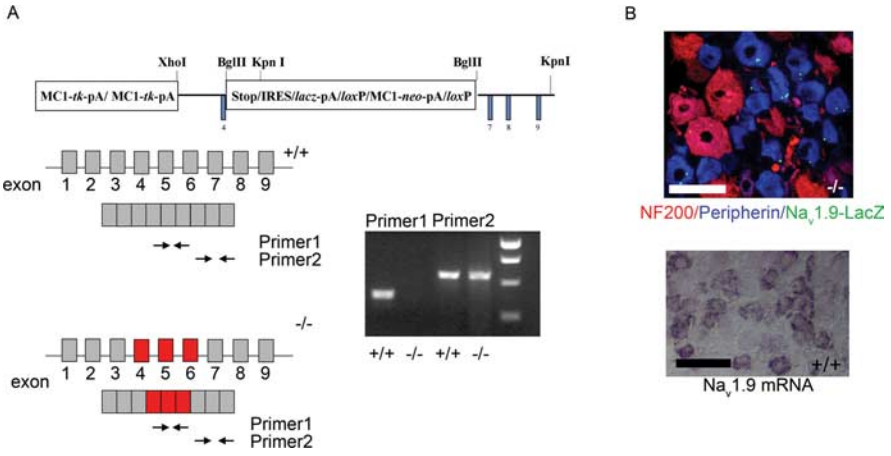

Figure 1. ***A***, Targeting construct for producing $Na_v1.9^{-/-}$ mice by deleting exons 4–6 of *SCN11a*. The region of $Na_v1.9^{-/-}$ mRNA encoded by exon 5 is absent in the knock-out mice (primer1). ***B***, Top, Triple-labeled immunohistochemistry for lacZ (green), NF200 (red), and peripherin (blue) in a DRG section from a $Na_v1.9^{-/-}$ mouse showing lacZ expression in small neurons. Scale bar, 50 μm. Bottom, *In situ* hybridization for $Na_v1.9$mRNA in WT mice showing expression similar to lacZ in −/− mice. Scale bar, 100 μm.

with steady-state activation shifted in the hyperpolarizing direction but no detectable change in channel activation or inactivation kinetics (Rush and Waxman, 2004). TTXr-Per is also increased by PKC activators (Baker, 2005). In an $Na_v1.9$ knock-out (KO) with deletion of exons 2–4, there is loss of TTXr-Per, a reduction in inflammatory pain behavior (intraplantar carrageenan), and the response to peripheral PGE_2 administration, but no disruption in basal or neuropathic pain (Priest et al., 2005). $Na_v1.9$ may, like $Na_v1.8$, play a pivotal role, therefore, in integrating the response of nociceptors to inflammatory mediators. We demonstrate here that $Na_v1.9$ is a major effector of the peripheral pain-producing actions of multiple inflammatory mediators that act on G-protein-coupled receptors (GPCRs), TRP, and ligand-gated ion channels.

Materials and Methods

The SCN11A gene was isolated from a 129/Sv mouse genomic library and a "knock-out vector" generated containing an internal ribosome entry site (IRES)–lacZ reporter followed by a loxP-flanked neomycin resistance cassette to produce a targeting construct (Fig. 1*A*). Homologous recombination of the construct DNA in embryonic stem cells resulted in a disrupted SCN11A gene, and mice carrying the mutation of the $Na_v1.9$ gene were generated according to standard protocols. The neomycin resistance cassette was not deleted. Germ-line chimeras were crossed with C57BL/6J females to generate heterozygotes and intercrossed, giving rise to overtly healthy and fertile mutant offspring in the expected Mendelian ratio. Successful targeting and transmission was confirmed by Southern blot analysis. An additional six backcrosses onto the C57BL/6J strain were performed before producing homozygote KO mice.

Behavior. An automated behavioral analysis system (LABORAS) was used to measure the frequency and duration of locomotor activity, immobility, climbing, grooming, eating, and drinking behavior. Groups of eight male and eight female wild-type (WT) and homozygous $Na_v1.9^{-/-}$ mice (9–12 weeks old) were housed in individual LABORAS cages with access to food and water *ad libitum* for a 24 h observation period.

For sensory testing, mice (between 8 and 15 weeks) were habituated and tests were performed blind to genotype. Plantar paw punctuate mechanical threshold and pinprick sensitivity were determined with von Frey hairs and a safety pin, heat sensitivity by withdrawal latency on a hotplate (50, 52, and 55°C), and reaction to cold on a cold plate and by acetone evaporation (Decosterd and Woolf, 2000). Intraplantar injections were made of BK (10 μl, 300 ng), capsaicin (10 μl, 2.5 μg), $\alpha\beta$-methyleneadenosine 5′-triphosphate ($\alpha\beta$-met-ATP) (10 μl 20 nmol), UTP (10 μl, 100 nmol), prostaglandin E_2 (PGE_2) (10 μl, 100 ng), interleukin-1β (IL-1β) (10 μl, 1 pg), and NGF (2.5S, 10 μl, 50 ng), and total licking time was recorded. Inflammation was produced by intraplantar injection of complete Freund's adjuvant (CFA) (20 μl; Sigma, St. Louis, MO), and paw edema was assessed by Evans blue extravasation (2%, 500 μl/mouse, i.p.) (Coderre et al., 1989). A spared nerve injury neuropathic pain model (Decosterd and Woolf, 2000) was used with section of the common peroneal and tibial nerves, leaving the sural nerve intact.

Histochemistry. Mice were perfused with 4% paraformaldehyde, the tissue was cryoprotected in 20% sucrose, and DRGs (10 μm) were sectioned. Sections were blocked with Image-iT Fx signal enhancer (Invitrogen, Carlsbad, CA) and incubated with rabbit anti-$Na_v1.7$ (1:1000; Alomone Labs, Jerusalem, Israel), anti- $Na_v1.8$ (1:400), rabbit anti-peripherin (1:200; Chemicon, Temecula, CA), mouse anti-neurofilament 200 kDa isoform (NF200) (1:400; Sigma), and goat anti-β-galactosidase (1:300; Biogenesis, Poole, UK) in 0.1 M PBS, 0.025% Tween 20 and 0.5% BSA at 4°C for 2–3 d and were then incubated with rhodamine-conjugated anti-rabbit anti-mouse or anti-goat (1:100; Chemicon) antibodies. Double labeling was performed with rabbit anti-$Na_v1.9$ (1:400) and either goat anti-transient receptor potential vanilloid receptor 1 (TRPV1) (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) or guinea pig anti-$P2X_3$ (1:1000; Chemicon). Double labeling for $Na_v1.9$ immunohistochemistry and fluorescent *in situ* hybridization for the B_2 bradykinin receptor was performed using a DIG-labeled RNA probe specific for B_2 mRNA. The specificity of the $Na_v1.8$ and $Na_v1.9$ antibodies have been established previously (Amaya et al., 2000).

Neuronal culture. DRGs were placed into HBSS (Invitrogen) and digested with 5 mg/ml collagenase 1, mg/ml Dispase II (Roche, Indianapolis, IN), and 0.25% trypsin (Invitrogen) and triturated, the suspension was centrifuged through 10% BSA (Sigma), and the pellet was resuspended in Neurobasal (Invitrogen) containing B27 supplement (Invitrogen), penicillin and streptomycin (Sigma), 10 μM AraC, and 100 ng/ml 2.5S NGF (Promega, Madison, WI).

Electrophysiology. Whole-cell patch-clamp recordings were made using an Axopatch 200A amplifier and pClamp 8 (Molecular Devices, Palo Alto, CA). Small neurons from freshly (<48 h) dissociated DRG cultures (9–12 weeks old) were recorded at room temperature. Average cell size for WT was 24.6 ± 0.4 μm (n = 91) and for $Na_v1.9^{-/-}$ was 27.5 ± 0.9 μm (n = 24). Fire-polished patch pipettes were made from 1.5 mm borosilicate glass (World Precision Instruments, Sarasota, FL) using a Sutter Instruments (Novato, CA) P-97 puller with 2–3 MΩ resistances. Signals were digitized at 10 kHz and filtered at 5 kHz. Capacity currents were cancelled, and series resistance was compensated by the "prediction" method (~80%) and by the 10-μs-lag "feedback" method (70–80%). Linear leakage currents were digitally subtracted on-line with P/4 routines. Currents were recorded within 30 min of establishment of the whole-cell configuration to minimize time-dependent rundown. TTX at 300 nM was added to the bath solution to block TTX-sensitive (TTXs) currents. Bath solution contained the following (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.1 $CdCl_2$, 10 HEPES, 5 glucose, and 10 tetraethylammonium (TEA)-Cl, pH 7.4 with NaOH (312 mOsm). Internal solution contained the following (in mM): 130 CsCl, 10 NaCl, 2 TEA-Cl, 10 EGTA, 0.5 CaCl2, 1 $MgCl_2$, 5 MgATP, 0.5 NaGTP, 10 HEPES, and 2 glucose, pH 7.3 with CsOH (310 mOsm). After establishing whole-cell configuration, neurons were held at −90 mV to remove resting inactivation. A series of depolarizing voltage commands from −100 to 60 mV in steps of 10 mV for 120 ms with 1 s interval were used. To determine the voltage dependence of channel activation, the sodium conductance (G_{Na}) was calculated. The normalized peak current for each voltage step was divided by the driving force ($V_m - V_{rev}$) and plotted against V_m. The voltage dependence of steady-state inactivation was measured by applying a double-pulse protocol consisting of one constant test pulse to 0 mV

for 30 ms after 500 ms prepulses of amplitudes of −120 to 10 mV in steps of 10 mV. Normalized peak Na^+ current during the test pulse was plotted against the prepulse voltage. The curves were fitted to the Boltzmann equation ($y = A2 + (A1 - A2)/(1 + \exp((x - x@y50/dx)$, with $x@Y50$ representing the potential at which half of the channels are activated or inactivated. Peak TTXs current density was measured by subtraction of TTXr current from total sodium current divided by cell capacitance. Peak TTXr-Slow current density in wild type was measured from cells without TTXr-Per currents.

Taqman reverse transcription-PCR. Animal procedures were performed in accordance with the United Kingdom Home Office regulations as outlined in the Animals (Scientific Procedures) Act of 1986. L3, L4, and L5 DRGs were removed, snap frozen in liquid nitrogen, and stored at −80°C. Frozen DRGs were homogenized using a Mixer Mill MM 300 (Qiagen, Hilden, Germany), and total RNA was extracted using the RNeasy mini kit (Qiagen). A DNase step was included to eliminate DNA contamination in the RNA samples. The extracted RNA was quantified using the Agilent Technologies (Palo Alto, CA) Eukaryotic Total RNA assay and Nanodrop ND1000 spectrophotometer (LabTech, Andover, MA). First-strand cDNA was synthesized from 200 ng of total RNA from each sample. Reverse transcription (RT) was performed using Omniscript RT kit (Qiagen) in triplicate. An additional reaction in which the Omniscript reverse transcriptase enzyme was omitted and the value obtained for this sample was subtracted from the triplicate experimental values to correct for DNA contamination. The resulting cDNA products from the reverse transcription were divided into 20 aliquots using a Hydra 96 robot (Robbins Scientific, Sunnyvale, CA) for parallel Taqman PCR reactions using different primer and probe sets. Taqman RT-PCR was performed using an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA) on the first-strand cDNA. The PCR reaction was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. A standard curve relating threshold cycle to template copy number was deduced using known dilutions of mouse genomic DNA (Clontech, Cambridge, UK). Specific PCR primers for genes/regions of interest were used (for sequence details, see supplemental Table 3, available at www.jneurosci.org as supplemental material).

Microarrays. Lumbar DRGs were dissected and rapidly frozen [$n = 3$ animals per sample, 3 samples per tissue type (WT and −/−)]. The tissue samples were homogenized, and total RNA was obtained (TRIzol reagent; Invitrogen). Biotinylated cRNA was produced, hybridized to the Mouse Genome 430 2.0 array using standard methods, and array data were processed by GCOS 1.4 (Affymetrix, Santa Clara, CA). For sodium channels, probe set data are shown only if a present call occurred in at least one array and average probe signal intensity was >500 in at least one tissue type. If two or more qualifying probe sets were present per transcript on the array, the one with the highest average signal intensity across both comparisons was used. To be considered differentially regulated, a probe set had to be defined present, expression of >500, fold difference >1.5, and p value <0.05.

Statistical analysis was performed using one-way ANOVA, followed by Dunnett's or Student's t test, and data are represented as mean ± SEM. Analysis of the Taqman PCR data used ANOVA on log-transformed data.

Table 1. Microarray and QRT-PCR analysis of DRGs

	Arrays			RT-PCR		
	KO/WT			KO/WT		
Transcript	Region	Fold	p value	Region	Fold	p value
$Na_v1.1$	3′	1.1	0.5902			
$Na_v1.2$		ND				
$Na_v1.3$		ND		3′	0.9	0.7389
$Na_v1.4$		ND				
$Na_v1.5$		ND		3′	1.1	0.2221
$Na_v1.6$	3′	1.0	0.4971			
$Na_v1.7$	3′	1.2	0.2932	3′	1.3	0.0225*
$Na_v1.8$	3′	1.2	0.0820	3′	1.1	0.2909
				5′	1.3	0.0597
$Na_v1.9$				KO	0.0	2.2E-06*
	3′	1.0	0.5102	3′	1.1	0.6683
NaG	3′	1.0	0.8580			
$\beta1$	3′	0.8	0.0314*	3′	0.7	0.0068*
$\beta2$	3′	1.3	0.2326	3′	1.3	0.2576
$\beta3$	3′	1.1	0.3429	5′	1.3	0.1678
$\beta4$	3′	0.9	0.0467*	3′	0.6	0.0346*

ND, Not detected with confidence by the array (see Materials and Methods). *$p < 0.05$.

Results

Production of $Na_v1.9^{-/-}$ mice

To investigate the functional role of the $Na_v1.9$ α subunit, an $Na_v1.9$ null allele mutant ($Na_v1.9^{-/-}$) was produced using a "knock-out vector" that disrupted the SCN11A gene with deletion of most of exon 4 and all of exons 5 and 6 (Fig. 1*A*). The $Na_v1.9^{-/-}$ animals have no overt phenotype and appear indistinguishable from WT littermates. Locomotor activity, climbing, grooming, eating, and drinking behavior were measured, and differences between the $Na_v1.9^{-/-}$ and WT animals were not considered significant (data not shown). The mutation of SCN11A does not result in a loss of DRG neurons, as indicated by presence of the nuclear lacZ reporter in adult DRG neurons in the $Na_v1.9^{-/-}$ mice with a frequency similar to that of $Na_v1.9$ mRNA in WT mice (Fig. 1*B*). More than 80% of the Lac-Z positive neurons colocalized with peripherin (a C-fiber marker) and ~20% with NF200, a maker for neurons with myelinated axons (Fig. 1*B*), a similar proportion we find for $Na_v1.9$ mRNA in WT mice. The relative proportion of peripherin- and NF200-labeled neurons in the $Na_v1.9^{-/-}$ mice was not different from that in WT mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Ion channel expression

Quantitative RT (QRT)-PCR analysis revealed that the levels in the DRG of the 5′ and 3′ regions of $Na_v1.9$ mRNA were not different from WT but that exons 4–6 were absent in $Na_v1.9^{-/-}$ mice. $Na_v1.3$, $Na_v1.5$, $Na_v1.7$, and $Na_v1.8$ mRNA levels were not altered in the $Na_v1.9^{-/-}$ DRG relative to WT, as detected by both QRT-PCR and microarrays (Table 1). The relative numbers of $Na_v1.7$- and $Na_v1.8$-immunostained DRG neuronal profiles were also not altered (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). A significant decrease in mRNA for $\beta1$ and $\beta4$ sodium channel subunits was detected in DRGs from $Na_v1.9^{-/-}$ mice, with no change in $\beta2$ and $\beta3$ (Table 1). Of 39,000 probe sets assayed by the microarrays, 111 had differential expression in the DRG between the WT and $Na_v1.9^{-/-}$, with 73 genes higher $Na_v1.9^{-/-}$ than WT and 38 lower (supplemental Table 2, available at www.jneurosci.org as supplemental material).

Basal pain behavior

Basal mechanical and thermal pain behavior was unaffected in the $Na_v1.9^{-/-}$ mice. Mechanical threshold (von Frey), paw-withdrawal latency to 50, 52, and 55°C hotplate simulation, and the number of flinches produced on exposure to a cold plate (0°C) for 5 min were identical in WT, $Na_v1.9^{-/+}$, and $Na_v1.9^{-/-}$ mice (Fig. 2).

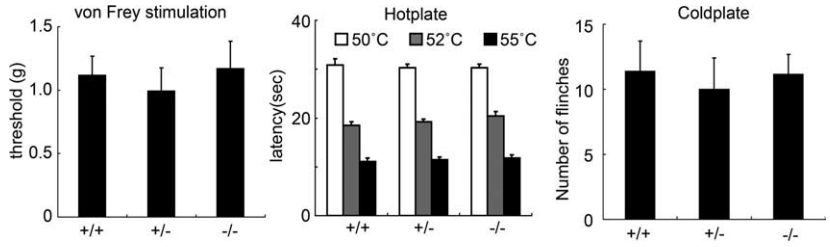

Figure 2. Basal behavioral responses. Responsiveness against mechanical (von Frey threshold), heat (hotplate latency time at 50, 52, and 55°C) and cold (cold plate latency time at 0 °C) stimulations are identical in WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ naive mice ($n = 15$).

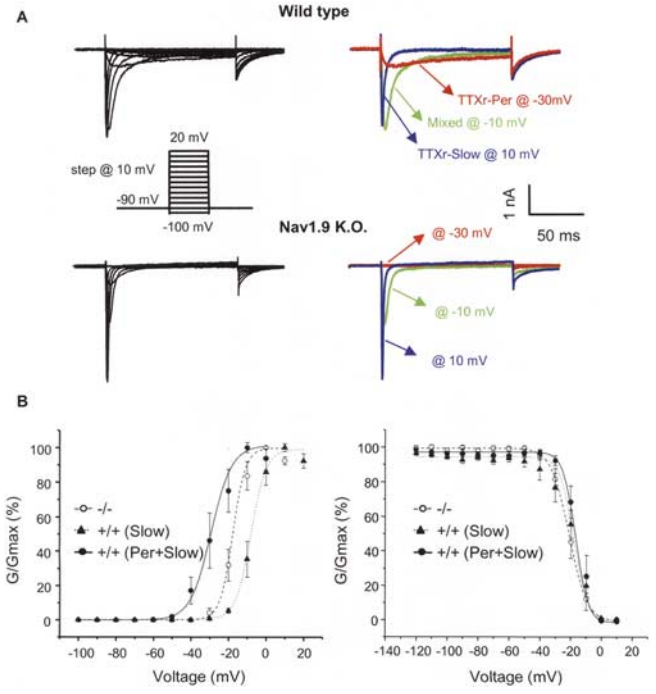

Figure 3. Sodium currents in DRG neurons. ***A***, In WT mice, 45% of small (<25 μm) DRG neurons have a TTXr current that can be separated into TTXr-Per and TTXr-Slow components by voltage steps from −90 mV holding potential to −30 mV and from −90 to 10 mV, respectively. In the *Nav1.9*$^{-/-}$ mice, no small DRG neurons have a TTXr-Per current. ***B***, The voltage-dependent activation in WT mice of TTXr in those small DRG neurons with both TTXr-Slow and TTXr-Per is more hyperpolarized than those cells with just TTXr-Slow. In KO mice, the activation curve reflects a composite of TTXr in small cells that have lost TTXr-Per and those with only TTXr-Slow. Voltage dependence of inactivation of TTXr is similar in WT mice and *Nav1.9*$^{-/-}$ mice.

Sodium currents

Fluoride-based internal solutions are used to stabilize recordings of TTXr-Per (Cummins et al., 1999). However, because fluoride causes a hyperpolarization shift in its voltage dependence (Coste et al., 2004), we preferred to use a chloride-based internal solution and a holding potential of −90 mV to remove resting inactivation. The activation threshold for $Na_v1.9$ (approximately −60 mV) is more negative than that of $Na_v1.8$ (~30 mV) (Renganathan et al., 2002a; Coste et al., 2004), providing a window for observing $Na_v1.9$ currents in WT DRG neurons. Na^+ currents were recorded from 60 WT DRG neurons in the presence of 300 nM TTX using a series of depolarizing voltage commands from −100 to 60 mV for 120 ms. Two distinct subpopulations were identified among the small neurons recorded. One group ($n = 27$, 45%) exhibited both a TTXr-Per current between −60 and −30 mV that lasted the entire duration of the 120 ms voltage step and a TTXr-Slow current at more depolarizing voltage steps, whereas the other group of neurons ($n = 33$) exhibited only TTXr-Slow currents (Fig. 3*A*). In $Na_v1.9^{-/-}$ DRG neurons, we observed only slow TTXr currents in all of the small DRG neurons recorded ($n = 17$) (Fig. 3*A*). We conclude that TTXr-Per currents are mediated by $Na_v1.9$ and that the mice have a null mutation.

To study whether there are compensatory changes in sodium channel function in DRG neurons in the $Na_v1.9^{-/-}$ mice, we subtracted TTXr currents (300 nM TTX) from total sodium currents to obtain TTXs currents. Peak current density of TTXs sodium currents in WT ($n = 12$) did not differ from those in $Na_v1.9^{-/-}$ DRG neurons ($n = 8$, $p = 0.7$), and the TTXr-Slow current density in WT DRGs ($n = 10$) was comparable with that found in $Na_v1.9^{-/-}$ neurons ($n = 10$). There is, therefore, no detectable compensatory change in sodium currents in the DRG of these $Na_v1.9^{-/-}$ mice, in keeping with the unchanged channel mRNA levels. We conclude that the alterations in $\beta 1$ and $\beta 4$ in the knock-out mice are likely not functionally relevant.

Voltage-dependent activation and steady-state inactivation of sodium channels contributes to membrane excitability. We therefore studied the activation and inactivation properties of the TTXr current in both WT and $Na_v1.9^{-/-}$ small DRG neurons. A series of depolarizing voltage commands from −100 to 60 mV were applied to activate sodium currents. Normalized peak conductance at each voltage step was plotted against voltage and fitted with the Boltzmann equation. We separately plotted the two subpopulations of small WT DRG neurons. The group with both TTXr-Per and TTXr-Slow current showed a significant ($p < 0.05$) hyperpolarization shift ($V_{1/2}$ of −29.2 ± 2.7 mV) relative to that in the WT subpopulation with only TTXr-Slow currents ($V_{1/2}$ of −8.1 ± 1.5 mV). Mean activation in $Na_v1.9^{-/-}$ small DRG neurons had an intermediate value ($V_{1/2}$ of −17.7 ± 1.3 mV). This population is a composite of those neurons that do not normally express $Na_v1.9$ and those which lack $Na_v1.9$ as a result of the knock-out (Fig. 3*B*). To study steady-state inactivation, a series of prepulses from −120 to 10 mV was followed by a testing pulse of 0 mV. The normalized peak current was plotted against the prepulse voltage and fitted with the Boltzmann equation. The voltage-dependent inactivation curves are comparable in the two WT and the KO neuronal populations ($p > 0.05$), with $V_{1/2}$ of −17 ± 1 mV for WT DRGs with both TTXr-Per and TTXr-Slow ($n = 11$), $V_{1/2}$ of −18.2 ± 1.2 mV for WT DRGs with only TTXr-slow currents ($n = 15$), and $V_{1/2}$ of −21 ± 1.8 mV for $Na_v1.9^{-/-}$ DRGs ($n = 10$) (Fig. 3*B*).

Pain hypersensitivity

Double staining in DRG neurons for $Na_v1.9$ immunohistochemically and for B_2 bradykinin receptor mRNA by *in situ* hybridization or the TRPV1 or purinergic $P2X_3$ receptor by immunohistochemistry revealed a high degree of colocalization in all cases (Fig. 4) (supplemental Table 1, available at www.jneurosci.org as supplemental material). We then tested whether the channel has a role in the generation of peripheral pain hypersensitivity by examining the effect of the null mutation of the channel on the direct and delayed pain responses to intraplantar injections of the inflammatory mediators bradykinin, PGE_2, IL-1β, and NGF, the chemical irritant capsaicin, and $P2X_3$ and P2Y receptor agonists.

Intraplantar bradykinin (300 ng) induced in WT mice an immediate paw licking/flinching response that was significantly

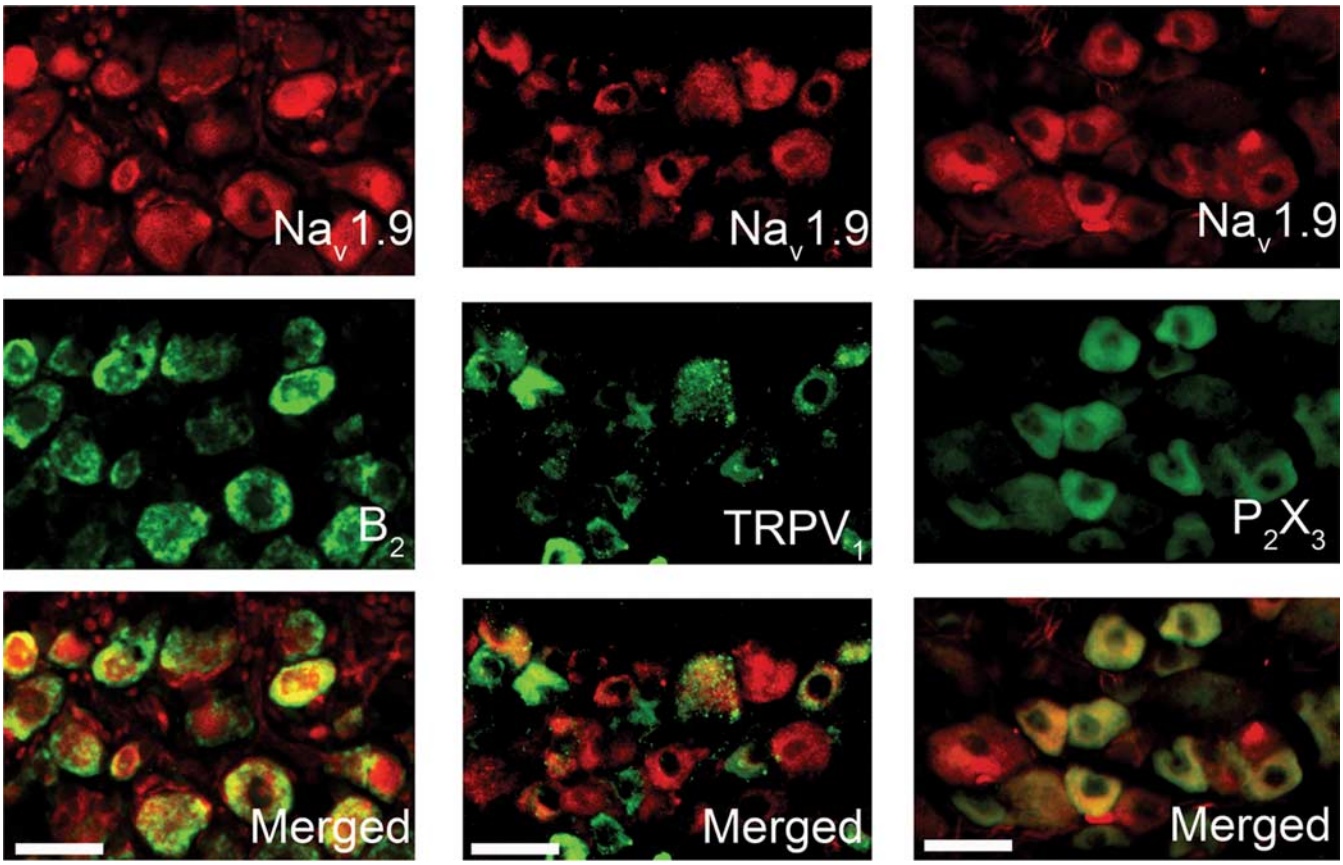

Figure 4. Detection of $Na_v1.9$ expression in the DRG neurons. DRG neurons double labeled for $Na_v1.9$ (red) and B_2 bradykinin, TRPV1 capsaicin, or $P2X_3$ ATP receptors (green). $Na_v1.9$ colocalized well with any of these receptors. Scale bars, 50 μm.

briefer in $Na_v1.9^{-/-}$ mice (Fig. 5*A*). Two hours after the bradykinin injection, WT mice had a reduction in the mechanical threshold for eliciting hindpaw withdrawal that was significantly less in $Na_v1.9^{-/-}$ mice (Fig. 5*B*). Similarly, the reaction time on exposure to a 50°C hotplate decreased significantly after the bradykinin injection (2 h) in WT, but not in $Na_v1.9^{-/-}$, mice (Fig. 5*C*). WT mice exhibited a licking/flinching reaction immediately after intradermal injection of capsaicin (2.5 μg), a TRPV1 agonist, that, like bradykinin, was significantly briefer in $Na_v1.9^{-/-}$ than WT mice (Fig. 5*D*). The capsaicin treatment produced at 25 min a mechanical hypersensitivity of the hindpaw in the WT mice that was significantly less in $Na_v1.9^{-/-}$ mice (Fig. 5*E*). Both the selective P2X agonist $\alpha\beta$-met-ATP (Fig. 6*A*) and the P2Y agonist UTP (Fig. 6*B*) produced on intraplantar injection a rapid but relatively transient thermal hypersensitivity in WT mice that was reduced in $Na_v1.9^{-/-}$ mice. We conclude that multiple inflammatory mediators and nociceptor receptor ligands acting on TRP channels, GPCRs, and ligand-gated ion channels produce on peripheral activation of nociceptors pain-like behavior and pain hypersensitivity that involves or requires $Na_v1.9$.

PGE_2 injection into the hindpaw produced a transient local mechanical allodynia that, like the other inflammatory mediators, was attenuated in the $Na_v1.9^{-/-}$ mice relative to WT (Fig. 7*A*). The thermal hyperalgesia induced in WT mice by peripheral PGE_2 was absent in the $Na_v1.9^{-/-}$ mice (Fig. 7*B*). We also injected PGE_2 into the subarachnoid space of the spinal canal to induce central sensitization (Harvey et al., 2004) (Fig. 7*C*,*D*). Spinal PGE_2 (100 ng) induced an identical increase in thermal and mechanical sensitivity in $Na_v1.9^{-/-}$, $Na_v1.9^{+/-}$, and WT mice (Fig. 7*C*,*D*). We conclude that only the peripheral pain-producing actions of PGE_2 require $Na_v1.9$.

The inflammatory cytokine IL-1β (1 pg) administered into the hindpaw induced mechanical allodynia (Fig. 8*A*) and thermal hyperalgesia (Fig. 8*B*) that was significantly less in $Na_v1.9^{-/-}$ than WT mice, but injection of NGF (50 ng), which is an inflammatory pain modulator (Lewin and Mendell, 1992; Woolf et al., 1994), into the hindpaw produced identical mechanical (Fig. 8*C*) and thermal (Fig. 8*D*) hypersensitivity in WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ mice. Most but not all proinflammatory agents require $Na_v1.9^{-/-}$ to produce peripheral pain hypersensitivity.

Inflammation

Intraplantar injection of CFA in WT mice induced a localized inflammation of the hindpaw, the extent of which, measured by Evans blue extravasation, was similar in WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ mice. The peripheral inflammation produced in WT and $Na_v1.9^{+/-}$ mice a significant reduction in the response latency to a hotplate at 50°C for 1–7 d (Fig. 9*A*). This inflammatory thermal hyperalgesia was absent, however, in the $Na_v1.9^{-/-}$ mice (Fig. 9*A*). Three days after CFA injection, the latency of response to hotplate stimulation at 50, 52, and 55°C was significantly longer in the $Na_v1.9^{-/-}$ mice than in WT mice (Fig. 9*B*). In contrast, WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ mice all developed similar degrees of mechanical hypersensitivity after intraplantar CFA administration (Fig. 10). Relative numbers of $Na_v1.9$-positive profiles in WT L5 DRGs increased from 30% in naive to 50% after the CFA-induced inflammation ($n = 5$; $p < 0.05$) (Fig. 9*C*).

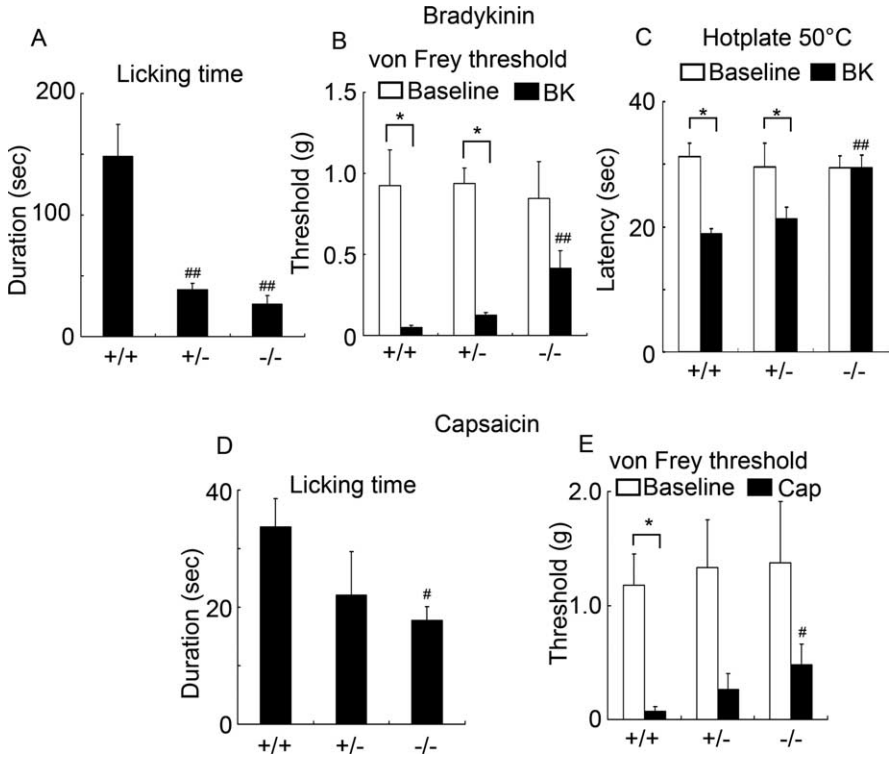

Figure 5. Peripheral pain hypersensitivity in $Na_v1.9$ mice. ***A–C***, In WT mice, intraplantar bradykinin (BK) injection produced immediate licking and flinching (***A***) and a later decrease in mechanical threshold (***B***) and hotplate response latency (***C***). In $Na_v1.9^{-/-}$ mice, BK elicited a reduced licking time and no mechanical or thermal hypersensitivity. ***D***, ***E***, Intraplantar capsaicin produced in WT mice immediate licking (***D***) and delayed reduction in mechanical threshold (***E***). $Na_v1.9^{-/-}$ mice had reduced licking time and a higher mechanical threshold after injection than WT. $^*p < 0.05$, $^{**}p < 0.01$ vs preinjection baseline. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus WT. $n = 7$. Cap, Capsaicin.

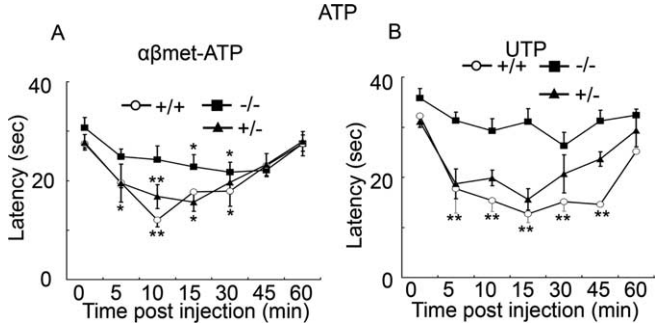

Figure 6. Pain hypersensitivity after purinergic receptor agonist injection. The P2X receptor agonist $\alpha\beta$-met-ATP and the P2Y receptor agonist UTP both produced transient thermal hyperalgesia in WT but not $Na_v1.9^{-/-}$ mice. $^*p < 0.05$, $^{**}p < 0.01$ versus preinjection baseline. $n = 7$.

Nerve injury

The decrease in mechanical threshold, the increase in pinprick response, and the cold allodynia (acetone) that occurs in a mouse spared nerve injury model (Bourquin et al. 2006) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) and in a partial sciatic nerve injury (Seltzer) model (data not shown) were identical in WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ mice.

Discussion

Disruption of exons 4–6 of the SCN11A gene results in a truncated transcript and complete absence of TTXr-Per in DRG neurons. All small (~25 μm) diameter WT DRG neurons exhibit a TTXr-Slow current, and half have a TTXr-Per current as well. We never find any neuron in WT DRG cultures with TTXr-Per but no TTXr-Slow, consistent with our finding in rats that $Na_v1.9$ always colocalizes with $Na_v1.8$ in DRG neurons but that $Na_v1.8$ is, in some rat DRG neurons, expressed without $Na_v1.9$ (Amaya et al., 2000). The activation threshold of TTXr-Per was approximately −60 mV when recording with a chloride-based internal solution, which is relatively depolarized compared with fluoride-based solutions (Cummins et al., 1999). Fluoride, which inhibits phosphatases, produces a hyperpolarizing shift in TTXr-Per in myenteric sensory neurons (Rugiero et al., 2003). The presence in small DRG neurons of TTXr-Per caused a hyperpolarizing shift of activation of the total TTXr current but a similar inactivation. These results support a role for TTXr-Per in increasing membrane excitability and acting as a possible booster of subthreshold electrogenesis. We held the neurons at −90 mV to remove the resting inactivation of $Na_v1.9$ and thereby maximally recruit the channels for analysis. This does not mean, however, that $Na_v1.9$ is inactivated at resting membrane potentials. The half-maximal steady-state inactivation for $Na_v1.9$ is approximately −45 mV (Cummins et al., 1999), and, at −60 or −70 mV, a reasonable fraction of $Na_v1.9$ will still be available. A large number of channels with a low open probability attributable to ultraslow inactivation at the resting state will produce a small but consistent current, shifting the resting potential to a more depolarizing range and in this way will enhance the excitability of the cell (Cummins et al., 1999). QRT-PCR, microarray, and immunohistochemical studies failed to detect any compensatory alterations in other VGSCs in the $Na_v1.9$ null mutant mice, and, although $\beta1$ and $\beta4$ accessory subunits decreased, these alterations did not affect the current density of TTXs and TTXr. Multiple small differences in many genes between WT littermates and the mutant mice detected by array analysis presumably reflect residual background differences, although the animals had been backcrossed for more than five generations. Although the neo cassette was not deleted, we found no indication in the microarray analysis of any change in expression of genes contiguous to SCN11A.

Involvement of $Na_v1.9$ in peripheral nociceptor sensitization

Sensitizing agents act on nociceptor peripheral terminals via specific receptors expressed by the sensory neurons that are coupled with second-messenger systems to produce the changes that underlie peripheral sensitization. $Na_v1.9$ appears from our data to be downstream of several quite distinct signal transduction pathways and to act as a point of convergence of multiple individual inflammatory mediators. Bradykinin evokes pain sensitivity in naive animals via the B_2 bradykinin G-protein-coupled receptor (Dray and Perkins, 1993), which is coupled both to PKC-ε (Cesare and McNaughton, 1996) and TRP ankyrin repeat 1 (Bandell et al., 2004). UTP activates the P2Y purinergic GPCR, which is also coupled to PKC-ε. PKC-ε induces sensitization of nociceptors via changes in TRPV1 (Cesare et al., 1999) and contributes to

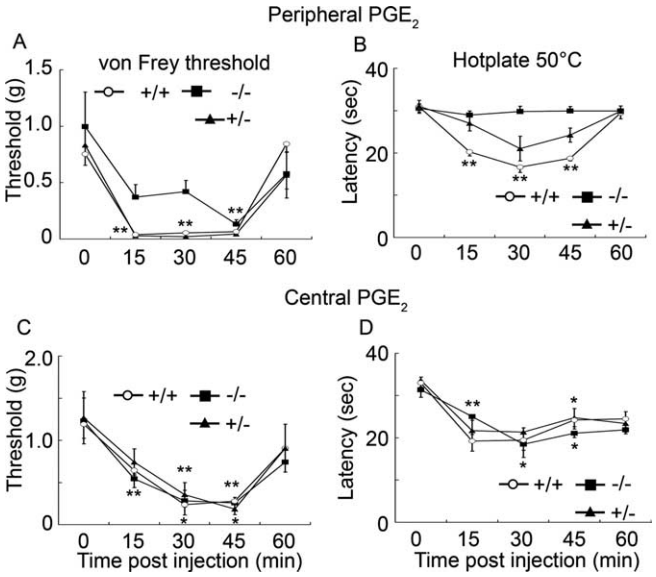

Figure 7. Pain hypersensitivity in response to PGE_2. ***A***, ***B***, Intraplantar PGE_2 induced transient mechanical (***A***) and thermal (***B***) pain hypersensitivity in WT but not in $Na_v1.9^{-/-}$ mice. ***C***, ***D***, Intrathecal PGE_2 produced identical mechanical (***C***) and thermal (***D***) hyperalgesia in WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ mice. $*p < 0.05$, $**p < 0.01$ versus preinjection baseline. $n = 7$.

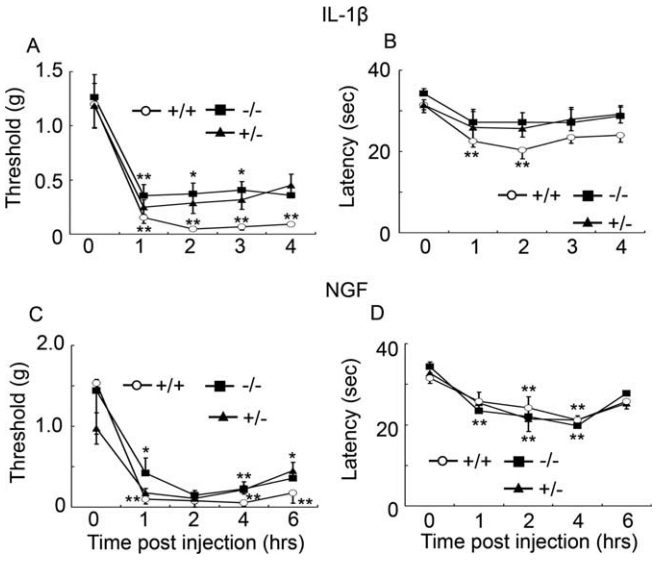

Figure 8. Pain hypersensitivity in response to IL-1β and NGF. ***A***, ***B***, Intraplantar IL-1β induced mechanical (***A***) and thermal (***B***) hyperalgesia in WT mice that was significantly reduced in $-/-$ mice. ***C***, ***D***, Intraplantar NGF induced similar mechanical (***C***) and thermal (***D***) pain hypersensitivity in WT and $Na_v1.9^{-/-}$ mice. $*p < 0.05$, $**p < 0.01$ versus preinjection baseline. $n = 7$.

inflammatory hyperalgesia (Hucho et al., 2005). PKC activators increase TTXr-Per current in rat and mouse DRG neurons (Baker, 2005) so that its sensitizing actions are likely to be attributable to changes in TRP and voltage-gated sodium channels.

PGE_2 has a major role in the development of inflammatory pain and is produced by inflamed tissue as a result of induction of cyclooxygenase-2. There are four PGE_2 G-protein-coupled receptor subtypes expressed by DRG neurons (EP1, EP3, and EP4). EP4 is coupled to G_s, some splice variants of EP3 to G_s and some to G_i, whereas EP1 is coupled to G_q/G_{11}(Narumiya et al., 1999). PGE_2, BK, and 5-HT all increase slow inactivating TTXr currents in DRG neurons with an increase in amplitude and rates of activation and inactivation (England et al., 1996; Gold et al., 1998). PKA potentiates $Na_v1.8$ and depresses $Na_v1.7$ currents, and PKC produces shifts in the steady-state activation of both channels in a depolarizing direction (Saab et al., 2003; Vijayaragavan et al., 2004). PGE_2 increases TTXr-Per twofold in DRG neurons in a GPCR-mediated manner, with steady-state activation shifted 6–8 mV in the hyperpolarizing direction, and availability of the channels increased by 12 mV but with no detectable change in channel activation or inactivation kinetics (Rush and Waxman, 2004). This suggests that both PKC and PKA act on $Na_v1.9$, as they do on $Na_v1.8$. The diminished pain sensitivity we find after PGE_2 treatment in $Na_v1.9$ null mice is similar to that in another $Na_v1.9$ knock-out mouse, with deletion of exons 2–4 (Priest et al., 2005). Because intrathecal application of PGE_2 in the $Na_v1.9^{-/-}$ mice produced similar pain hypersensitivity to that in wild-type mice, we conclude that the sodium channel has a minimal role in the spinal effects of PGE_2, which appear to act mainly via the α3 subunit of glycine receptors (Harvey et al., 2004).

Capsaicin and $\alpha\beta$-met-ATP generate acute pain hypersensitivity through TRPV1 (Caterina et al., 1999) and $P2X_3$ (Burnstock and Wood, 1996) ion channel/receptors, respectively. TRPV1 and $P2X_3$ both increase intracellular calcium concentrations, which activates multiple calcium-sensitive kinases that can phosphorylate multiple ion channels and receptors. Although the expression of $Na_v1.9$ and other receptors differs between mice and rats, $Na_v1.9$ was highly coexpressed with TRPV1 and $P2X_3$ in the mouse. The diminished behavioral response to capsaicin and $\alpha\beta$-met-ATP in the $Na_v1.9$ null mouse indicates that the sodium channel may be downstream of calcium- as well as PKC- and PKA-dependent nociceptor sensitization.

IL-1β sensitizes primary sensory neurons, potentially by facilitating PGE_2 and NGF synthesis (Safieh-Garabedian et al., 1995), in a manner we now show is $Na_v1.9$ dependent. NGF has multiple actions on nociceptors via its TrkA receptor, including an action on TRPV1 receptors via (Chuang et al., 2001) or independent of (Zhang et al., 2005) PI3 kinase as well as slower onset changes that increase the levels of VGSCs or TRPV1 (Ji et al., 2002). We find no reduction in the acute hyperalgesia produced by NGF in $Na_v1.9$ knock-out mice, indicating that its actions in generating peripheral sensitization do not appear in mice to require $Na_v1.9$.

The $Na_v1.9^{-/-}$ mice have markedly diminished thermal hyperalgesia for the week after peripheral inflammation induced by intraplantar CFA. Priest et al. (2005) also find a reduction in inflammatory (24 h after intraplantar carrageenan) thermal pain behavior in their $Na_v1.9$ KO. This suggests that sensitization to heat after inflammation involves mediators that act through $Na_v1.9$, such as BK, PGE_2, or ATP, and that mechanical sensitization either relies on mediators that do not alter this channel (such as NGF) or is more a manifestation of central than peripheral sensitization.

We find that inflammation increases the number of $Na_v1.9$-positive neurons in the DRG, consistent with our previous observation of an induction of $Na_v1.9$ mRNA (Tate et al., 1998). Because neurons with both TTXr-Per and TTXr-Slow are likely to be more excitable than those with only TTXr-Slow, an increased number of $Na_v1.9$-positive cells during inflammation might increase the numbers of neurons activated by peripheral stimuli. Deletion of $Na_v1.8$ results in a delay in the development of inflammatory hyperalgesia (Akopian et al., 1999) but no difference in the magnitude of the hypersensitivity, although compensatory changes in $Na_v1.7$ in the $Na_v1.8^{-/-}$ mice have to be considered. Nociceptor-specific deletion of $Na_v1.7$ induces insensitivity against noxious mechanical stimuli in naive mice and decreased

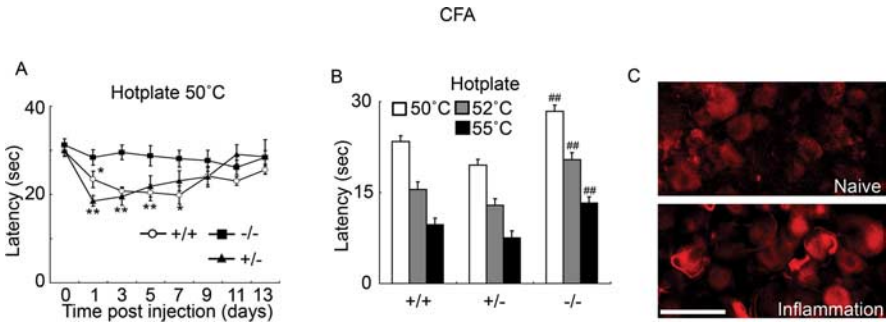

Figure 9. Peripheral inflammation. ***A***, In $Na_v1.9^{-/-}$ mice, inflammatory heat pain hypersensitivity was diminished. CFA injection produced a significant reduction in hotplate latency for 7 d in WT and $Na_v1.9^{+/-}$ mice. However, thermal hyperalgesia was essentially absent in the $Na_v1.9^{+/-}$ mice, with the response latency at 50, 52, and 55°C significantly longer in $Na_v1.9^{-/-}$ mice. $^*p < 0.05$, $^{**}p < 0.01$ versus preinjection baseline. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus WT. $n = 8$. ***C***, The relative numbers of $Na_v1.9$-positive neuron profiles increased in the DRG after inflammation. Scale bar, 100 μm.

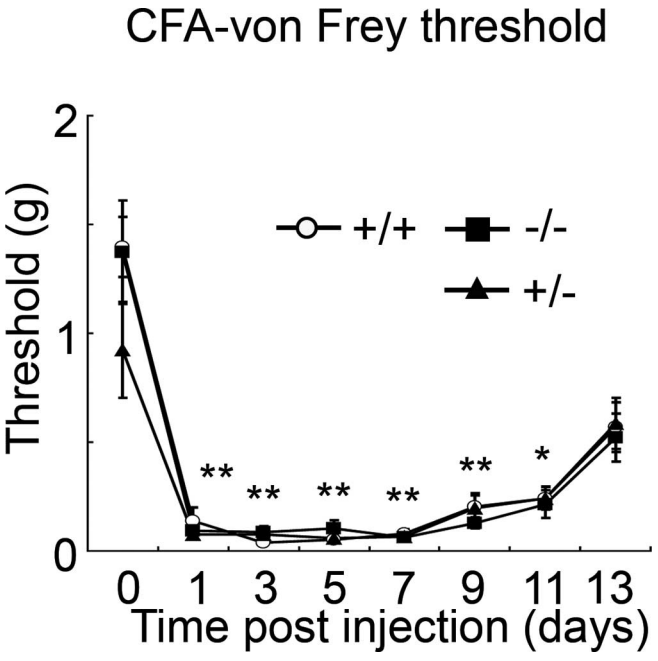

Figure 10. Mechanical sensitivity after peripheral inflammation. Mechanical threshold against von Frey stimulation reduced after peripheral inflammation similarly in WT, $Na_v1.9^{+/-}$, and $Na_v1.9^{-/-}$ mice. $^*p < 0.05$, $^{**}p < 0.01$ versus preinjection baseline. $n = 8$.

inflammatory hyperalgesia (Nassar et al., 2004). $Na_v1.7$ knock-out mice show no hyperalgesia in response to intraplantar NGF, indicating that $Na_v1.7$ is downstream of different inflammatory mediators than $Na_v1.9$.

Our results, like those of Priest et al. (2005), demonstrate that Nav1.9 does not contribute to neuropathic pain hypersensitivity. $Na_v1.9$ expression is downregulated in injured neurons (Tate et al., 1998; Dib-Hajj et al., 1999) without a significant change in neighboring uninjured neurons (Decosterd et al., 2002). A $Na_v1.8$ and $Na_v1.7$ double knock-out mouse continues to show substantial hyperalgesia after nerve injury (Nassar et al., 2005). The data from $Na_v1.8$ and $Na_v1.9$ knock-outs imply that TTXr VGSCs may not contribute to the development of neuropathic pain, although a $Na_v1.8$ knockdown produced by antisense oligodeoxynucleotide administration does reduce behavioral hyperalgesia after nerve injury (Lai et al., 2002).

We conclude that the $Na_v1.9$ VGSC α subunit carries TTXr-Per in small DRG neurons and that it is a downstream effector of the increased pain sensitivity produced by diverse inflammatory mediators on nociceptor peripheral terminals. How the multiple different signal pathways converge on the channel, and the specific changes they produce that alter its properties, need now to be explored. Our data indicate, however, that sodium channel blockers that act on $Na_v1.9$ may prove useful for the treatment of inflammatory pain.

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