# Mechanism of sodium channel Na<sub>V</sub>1.9 potentiation by G-protein signaling

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Tetrodotoxin (TTX)-resistant voltage-gated Na (Na<sub>V</sub>) channels have been implicated in nociception. In particular, Na<sub>V</sub>1.9 contributes to expression of persistent Na current in small diameter, nociceptive sensory neurons in dorsal root ganglia and is required for inflammatory pain sensation. Using ND7/23 cells stably expressing human Nay1.9, we elucidated the biophysical mechanisms responsible for potentiation of channel activity by G-protein signaling to better understand the response to inflammatory mediators. Heterologous Na<sub>V</sub>1.9 expression evoked TTX-resistant Na current with peak activation at -40 mV with extensive overlap in voltage dependence of activation and inactivation. Inactivation kinetics were slow and incomplete, giving rise to large persistent Na currents. Single-channel recording demonstrated long openings and correspondingly high open probability  $(P_0)$  accounting for the large persistent current amplitude. Channels exposed to intracellular GTPyS, a proxy for G-protein signaling, exhibited twofold greater current density, slowing of inactivation, and a depolarizing shift in voltage dependence of inactivation but no change in activation voltage dependence. At the single-channel level, intracellular GTP $\gamma$ S had no effect on single-channel amplitude but caused an increased mean open time and greater  $P_0$ compared with recordings made in the absence of GTP<sub>γ</sub>S. We conclude that G-protein activation potentiates human Na<sub>V</sub>1.9 activity by increasing channel open probability and mean open time, causing the larger peak and persistent current, respectively. Our results advance our understanding about the mechanism of Na<sub>V</sub>1.9 potentiation by G-protein signaling during inflammation and provide a cellular platform useful for the discovery of Nav1.9 modulators with potential utility in treating inflammatory pain.

#### INTRODUCTION

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Voltage-gated Na ( $Na_V$ ) channels are critical for the initiation and propagation of action potentials in excitable tissues, including the brain and peripheral nerves. Two specific isoforms,  $Na_V1.8$  and  $Na_V1.9$  (also known as SNS and NaN, respectively), are tetrodotoxin (TTX)-resistant  $Na_V$  channels expressed in the peripheral nervous system (Dib-Hajj et al., 1998; Tate et al., 1998; Akopian et al., 1999; Persson et al., 2010). These channels are also found in the central nervous system (Jeong et al., 2000; Blum et al., 2002; O'Brien et al., 2008). Both  $Na_V1.8$  and  $Na_V1.9$  have been implicated in nociception, including neuronal pain signaling triggered by inflammation (Lai et al., 2004).

 $Na_V 1.8$  channels are expressed in retinal amacrine and ganglion cells (O'Brien et al., 2008), small and medium-sized dorsal root ganglion (DRG) neurons, and their nociceptive afferent fibers (Benn et al., 2001). In nociceptive fibers,  $Na_V 1.8$  channels are responsible for slowly inactivating Na currents that contribute to the depolarizing phase of action potentials in C-type small DRG neurons (Renganathan et al., 2001).  $Na_V 1.9$  channels are found in the hippocampus, cortex (Jeong

et al., 2000; Blum et al., 2002), photoreceptors and Müller glia (O'Brien et al., 2008), small diameter, nociceptive sensory neurons in DRG (Fang et al., 2002), trigeminal ganglia, and in the intrinsic sensory neurons of the gut (Rugiero et al., 2003; Padilla et al., 2007). Compared with Na<sub>v</sub>1.8 and TTX-sensitive neuronal channels, Na<sub>v</sub>1.9 exhibits unique biophysical properties that include a hyperpolarized voltage-dependent activation, activation and inactivation curves that overlap near the resting membrane potential, slow activation and inactivation kinetics, and a very large persistent current (Cummins et al., 1999; Dib-Hajj et al., 2002; Coste et al., 2004). Persistent current generated by Na<sub>V</sub>1.9 has been proposed to set thresholds for excitability of nociceptive sensory neurons by modulating both the resting potential and responses to subthreshold stimuli (Herzog et al., 2001; Priest et al., 2005; Ostman et al., 2008). A link between Na<sub>V</sub>1.9-associated persistent current and pain sensation was demonstrated in neurons from Na<sub>v</sub>1.9 knockout mice that lack persistent Na current and have greatly reduced inflammatory hyperalgesia (Priest et al., 2005; Amaya et al., 2006).

Correspondence to Alfred L. George Jr.: al.george@vanderbilt.edu Abbreviations used in this paper: DRG, dorsal root ganglion; Na<sub>V</sub>, voltagegated Na; RT, room temperature; TTX, tetrodotoxin.

Inflammation caused by tissue damage results in pain, reflecting an increase in excitability of the primary afferent neurons innervating the area. Inflammatory agents such as bradykinin, ATP, histamine, prostaglandin E2, and norepinephrine potentiate Na<sub>v</sub>1.9 current, increasing the excitability of DRG neurons, but these mediators fail to sensitize sensory neurons in Na<sub>V</sub>1.9null mice (Maingret et al., 2008a; Ritter et al., 2009). Studies using prostaglandin E2, protein kinase C, or G proteins also have demonstrated a link between inflammatory pathways and Na<sub>V</sub>1.9-mediated nociceptor excitability (Baker et al., 2003; Baker, 2005). Together, these results indicate that Na<sub>V</sub>1.9 contributes to the hyperexcitability of nociceptors observed during inflammatory pain. Inflammatory mediators that activate G-proteindependent signal transduction increase the Na<sub>V</sub>1.9 Na current (Rush and Waxman, 2004). Moreover, intracellular GTP<sub>γ</sub>S (a hydrolysis-resistant GTP analogue) also potentiates persistent TTX-resistant Na current in wildtype and Na<sub>V</sub>1.8-null mice sensory neurons (Baker et al., 2003) but does not increase persistent TTX-resistant Na current in small sensory neurons from Na<sub>v</sub>1.9 knockout mice (Ostman et al., 2008). Discerning the mechanism for GTPyS potentiation of Na<sub>v</sub>1.9 is important for understanding the encoding of painful sensations mediated by G-protein activation.

Heterologous expression of  $Na_V1.9$  has proven difficult, and this has limited opportunities to elucidate functional properties of the channel and to implement small molecule screening assays to discover  $Na_V1.9$  modulators. In this study, we used a highly efficient transposon system (Kahlig et al., 2010) to achieve stable expression of human  $Na_V1.9$  in heterologous cells. This expression system allowed us to elucidate detailed biophysical properties of the channel, including the single-channel mechanism underlying potentiation by GTP $\gamma$ S. Our work further illustrates a cellular platform useful for the discovery of  $Na_V1.9$  modulators that may have utility in treating chronic and inflammatory pain.

### MATERIALS AND METHODS

# Cell culture

All experiments were conducted using ND7/23 cells (Sigma-Aldrich) grown at 37°C with 5% CO $_2$  in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (ATLANTA Biologicals), 2 mM L-glutamine, and 50 U/ml penicillin–50 µg/ml streptomycin. Unless otherwise stated, all tissue culture media was obtained from Life Technologies.

#### Plasmids and cell transfection

A full-length, human  $Na_V1.9$  (GenBank accession no. NM\_014139) cDNA including a C-terminal triple FLAG epitope (SRDYKDHDGDYKDHDIDYKDDDDKSR) was synthesized by DNA2.0 Inc. without codon optimization and cloned into a low copy number plasmid (pJ251). The FLAG epitope does not

prevent plasma membrane trafficking of other neuronal Na<sub>V</sub> channel proteins expressed heterologously or in vivo (Kearney et al., 2001; Kahlig et al., 2008). Stable expression of Na<sub>V</sub>1.9-3xFLAG in ND7/23 cells was achieved using the *piggyBac* transposon system as previously described (Wilson et al., 2007; Kahlig et al., 2010) using a transposon vector containing a puromycin resistance gene (pB-Na<sub>V</sub>1.9-3xFLAG-PuroR). ND7/23 cells were transfected with pCMV-hyPBase encoding a hyperactive version of the *piggyBac* transposase (Yusa et al., 2011) with pB-Na<sub>V</sub>1.9-3xFLAG-PuroR using FUGENE-6 (Roche). Stable Na<sub>V</sub>1.9-expressing cells were selected with 3 μg/ml puromycin (GIBCO/Invitrogen), and individual cell colonies were isolated using cloning discs and expanded.

#### Na<sub>v</sub>1.9 protein expression

Cells were incubated at 37°C or 28°C with 5% CO<sub>2</sub> for ~24 h before protein isolation. Na<sub>V</sub>1.9 was detected using a monoclonal anti-FLAG antibody (Sigma-Aldrich). Na<sub>V</sub>1.9-expressing cells (60-mm dishes) were washed twice with Dulbecco's PBS supplemented with CaCl<sub>2</sub> and MgCl<sub>2</sub> and then incubated with 1.5 mg/ml membraneimpermeant sulfo-NHS-biotin reagent (Thermo Fisher Scientific) for 1 h on ice. The cells were lysed with 500 µl RIPA buffer (150 mm NaCl, 50 mm Tris-Base, pH 7.5, 1% IGEPAL, 0.5% Na deoxycholate, and 0.1% SDS) supplemented with a Complete mini protease inhibitor tablet (Roche) and centrifuged at 14,000 g for 30 min at 4°C, and the supernatant fraction was collected and retained as the total lysate protein fraction. Protein lysates were quantified using the Bradford reagent (Bio-Rad Laboratories), and equal amounts of proteins were used in the immunoprecipitation experiments. The lysates were incubated with 50 µl ImmunoPure Immobilized Streptavidin beads (Thermo Fisher Scientific) overnight at 4°C. The samples were centrifuged for 10 s at 10,000 g and then washed three times with RIPA buffer at 4°C. The biotinylated proteins were eluted with 57 ul Laemmli sample buffer (Bio-Rad Laboratories) supplemented with 5% 2-mercaptanol (β-ME) for 30 min at room temperature (RT). Western blot analysis was performed by incubation for 1 h at RT with anti-FLAG diluted 1:15,000 for Na<sub>V</sub>1.9 detection or anti-transferrin (GIBCO/Invitrogen) diluted 1:5,000 as a gel loading control. The secondary antibody (goat anti-mouse, same for both primary antibodies, diluted 1:10,000; Santa Cruz Biotechnology, Inc.) was horseradish peroxidase conjugated and incubated at RT for 1 h. Densitometry of protein bands was used to quantify protein levels (ImageJ software; National Institutes of Health). The Na<sub>V</sub>1.9 band was normalized to the transferrin band to normalize for protein loading.

# Electrophysiology

Cells were incubated at  $28^{\circ}$ C with 5% CO<sub>2</sub> for  $\sim$ 24 h before their use in electrophysiology experiments. Cells were dissociated by trituration, resuspended in supplemented DMEM medium (without puromycin), plated on glass coverslips, and allowed to recover for  $\sim$ 2 h before electrophysiological experiments. Cells were first maintained for 1 h at  $37^{\circ}$ C in 5% CO<sub>2</sub> and then at  $28^{\circ}$ C with 5% CO<sub>2</sub> until used in the experiments. For single-channel experiments, the glass coverslips were pretreated with CELL-TAK cell and tissue adhesive (Collaborative Biomedical Products).

Na currents were recorded at RT in the whole cell or excised, outside-out patch configuration of the patch-clamp technique (Hamill et al., 1981) using an Axopatch 200B series amplifier (Molecular Devices). Whole-cell currents were acquired at 20 kHz and filtered at 5 kHz. Pulse generation and data collection were performed with Clampex 9.2 (Molecular Devices). Bath solution contained (in mM) 145 NaCl, 4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 HEPES (*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanosulphonic acid), pH 7.35, 310 mOsm/kg. 150 nM TTX was present in the bath to block endogenous Na currents. The

composition of the pipette solution was (in mM) 10 NaF, 110 CsF, 20 CsCl, 2 EGTA (ethyleneglycol-bis-( $\beta$ -aminoethylether), and 10 HEPES, pH 7.35, 310 mOsm/kg. Osmolarity and pH values were adjusted with sucrose and NaOH, respectively. Whole-cell patch pipettes were pulled from thin-wall borosilicate glass, and single-channel patch pipettes were pulled from standard-wall borosilicate glass (Warner Instruments, LLC) with a multistage P-97 Flaming-Brown micropipette puller (Sutter Instrument) and fire-polished with a Micro Forge MF 830 (Narashige). Pipette resistance was  $\sim$ 2 M $\Omega$  for whole-cell recording and 4–6 M $\Omega$  for outside-out patch recording. Single-channel recording pipettes were coated with Sylgard 184 (Dow Corning). 2% agar bridges containing bath solution served as reference electrodes.

Cells were allowed to equilibrate for 10 min in bath solution before obtaining seals. Peak currents were measured using 50-ms pulses to between -100 and 40 mV every 5 s from a holding potential of -120 mV. The peak current was normalized for cell capacitance and plotted against voltage to generate peak current density-voltage relationships. For recording of Na<sub>V</sub>1.9 currents in low Na<sup>+</sup> bath solution ([Na<sup>+</sup>]<sub>o</sub> = 20 mM), NaCl was replaced with equimolar N-methyl-D-glucamine-chloride (NMDG-Cl). The liquid junction potential generated upon reducing extracellular Na<sup>+</sup> was calculated using the Junction Potential Calculator in Clampex 9.2. The calculated junction potential was 4.5 mV. Whole-cell conductance was calculated from the peak current amplitude using the formula  $G_{Na} = I_{Na}/(V - E_{rev})$ , where  $E_{rev}$  is the estimated Na+ reversal potential, and then normalized to the maximal conductance recorded between -80 and 20 mV. The normalized G-V  $V_{1/2}/k$ , to determine the voltage for half-maximal channel activation  $(V_{1/2})$  and slope factor (k). The voltage dependence of channel availability was assessed after a 300-ms prepulse to various potentials followed by 50-ms pulse to -40 mV, the voltage at which peak Na<sub>v</sub>1.9 currents were measured. The normalized current was plotted against the voltage, and the steady-state channel availability curves were fit with Boltzmann functions (I/Imax = 1/Imax = $(1 + exp[(V - V_{1/2})/k])$  to determine the voltage for half-maximal channel inactivation  $(V_{1/2})$  and slope factor (k). Recovery from fast inactivation was examined as fractional recovery against the recovery period and fitted to a five-parameter double exponential function,  $I/I_{max} = A_f \times [1 - exp(-t/\tau_f)] + A_s \times [1 - exp(-t/\tau_s)] + C$ , where  $\tau_f$  and  $\tau_s$  denote time constants (fast and slow components, respectively) and A<sub>f</sub> and A<sub>s</sub> represent the fast and slow fractional amplitudes. Single-channel behavior was examined during a -40-mV pulse from a holding potential of -120 mV in the presence of 150 nM external TTX. Single-channel currents were filtered at 2 kHz and acquired at 20 kHz. Each test pulse was followed by a 10-s recovery period at -120 mV. Linear leak and capacitive transients were subtracted using the mean of blank traces, and no corrections were made for missed events. The number of channels in each patch was determined as the maximum number of simultaneously open channels observed in all sweeps at a given voltage step. Only membrane patches without channel activity at the holding potential (-120 mV) or outward openings at the test potential (-40 mV) were analyzed, and this excluded contaminating currents from the other possible charge carriers present in the solutions (i.e., Ca<sup>2+</sup>, F<sup>-</sup>, and Cl<sup>-</sup>).

#### Data analysis

Data were analyzed and plotted using a combination of Clampfit 9.2 (Molecular Devices), SigmaPlot 10 (Systat Software, Inc.) and Origin 7.0 (OriginLab). Statistical analyses were performed using SigmaStat 2.03 (Systat Software, Inc.), and statistical significance was determined using unpaired Student's t test. Data are represented as means  $\pm$  standard error of the mean. The number of cells/patches used for each experimental condition is given in the figure legends.

#### RESULTS

# Stable expression of human Na<sub>V</sub>1.9

We generated a stable cell line expressing full-length, FLAG-tagged human  $Na_V1.9$  cDNA ( $Na_V1.9$ -3xFLAG) in ND7/23 cells using a previously described transposon system (Wilson et al., 2007; Kahlig et al., 2010). We selected ND7/23 cells because of their chimeric derivation from rat DRG and mouse neuroblastoma cells and because our attempts to generate stably transfected HEK-293 and Chinese hamster ovary cells were unsatisfactory.

Initial electrophysiological analysis of Na<sub>v</sub>1.9-expresing cells (performed in the presence of 150 nM TTX to block an endogenous TTX-sensitive Na<sub>V</sub> channel) demonstrated small TTX-resistant currents (Fig. 1 A, top). Based on prior reports that low-temperature incubation of heterologous cells increased the functional expression of ion channels (e.g., CFTR and hERG; Denning et al., 1992; Zhou et al., 1999), we tested this strategy with Na<sub>v</sub>1.9. Overnight incubation of Na<sub>v</sub>1.9-ND7/23 cells at 28°C increased the current magnitude considerably (Fig. 1, A and B), whereas incubation of nontransfected ND7/23 cells at 28°C did not generate TTX-resistant currents. The Na<sub>V</sub>1.9-dependent currents were markedly attenuated when the extracellular Na<sup>+</sup> concentration was reduced, consistent with an Na-selective channel (peak current: 145 mM external [Na $^+$ ], 37.4  $\pm$  13.2 pA/pF; 20 mM external [Na<sup>+</sup>],  $6.5 \pm 1.9 \text{ pA/pF}$ ; n = 5). Further, application of 30 µM TTX blocked peak current recorded at -40 mV by a mere  $12.8 \pm 2.9\%$  (n = 4), consistent with a TTX-resistant channel.

Biochemical analysis of total and cell surface proteins isolated from Na<sub>v</sub>1.9 stable cells cultured at 37°C and 28°C showed a prominent immunoreactive band ( $\sim$ 205 kD; Fig. 1 C) approximately the size predicted for native Na<sub>v</sub>1.9. Previous analysis of Na<sub>v</sub>1.9 protein from adult rat DRG neurons indicated that the channel is lightly glycosylated (Tyrrell et al., 2001), and this is consistent with our observation. Densitometry analysis of Na<sub>V</sub>1.9 immunoblots (Fig. 1 C) demonstrated that total and cell surface Na<sub>V</sub>1.9 protein expression were increased approximately twofold after overnight incubation of the cells at 28°C, but the molecular mass did not change (Fig. 1 D). The mean ratio of surface to total protein trended higher for cells incubated at 28°C (37°C =  $0.8 \pm$ 0.3;  $28^{\circ}$ C = 1.1 ± 0.3; n = 3), but the difference was not statistically significant.

# Biophysical properties of human Na<sub>V</sub>1.9

After establishing stable heterologous expression of  $Na_V 1.9$ , we proceeded with determining more detailed functional properties of this unusual Na channel. As illustrated in Fig. 1 A, the kinetics of channel gating, especially inactivation, are markedly slow and have substantial voltage dependence. Furthermore, inactivation

at most test potentials is incomplete, producing large persistent Na currents. Activation of Na<sub>V</sub>1.9 whole-cell current follows an exponential time course that is much slower than other mammalian Na<sub>V</sub> channels (Fig. 2, A and B). For example, the time to peak activation for  $Na_V 1.9$  (9.4 ms at -30 mV; Fig. 2 B) is several fold slower than the TTX-sensitive Na<sub>V</sub>1.1 (1.1 ms at -30 mV; Kahlig et al., 2008). Inactivation of Na<sub>V</sub>1.9 is also slow and highly voltage sensitive (Fig. 2 C). At some test potentials (e.g., -80 mV), there was no measurable current decay, and the time course for the current recorded at this voltage was best fit with a linear equation. The time course of inactivation at other voltages was best fit with a single-exponential function from which we determined time constants. Inactivation is much slower than that reported for TTX-sensitive Na<sub>V</sub> channels. For example, inactivation time constants at -40 mVfor Na<sub>v</sub>1.1 and Na<sub>v</sub>1.2 are in the range of 1–2 ms (Lossin et al., 2002; Misra et al., 2008) as compared with  $\sim$ 70 ms for  $Na_V 1.9$  (Fig. 2 D).

Steady-state inactivation and conductance-voltage (activation) curves have considerable overlap and intersect near -50 mV (Fig. 2 E). The intersection of these relationships at voltages near the resting potential of neurons predict a large window current, and this is consistent with previous explanations for the persistent Na current observed in DRG neurons (Herzog et al., 2001; Priest et al., 2005). The conductance-voltage relationship for Na<sub>V</sub>1.9 (Fig. 2 E) exhibits a peculiar descent at potentials more positive than -30 mV. This phenomenon was previously observed in mouse DRG neurons (Cummins et al., 1999) but not in rat DRG or myenteric neurons (Tyrrell et al., 2001; Coste et al., 2004). We also

measured recovery from inactivation after a 300-ms depolarization pulse to -40 mV. The recovery rate exhibited two exponential components with a predominant fast and a smaller slow component. Quantified biophysical parameters for the voltage dependence of activation, steady-state inactivation, and recovery from fast inactivation are provided in Table 1.

# Na<sub>V</sub>1.9 activation by GTP<sub>γ</sub>S

Inflammatory mediators acting through G-proteincoupled receptors are known to potentiate Na<sub>V</sub>1.9 channel activity and increase TTX-resistant persistent Na current in sensory neurons (Baker et al., 2003; Maingret et al., 2008b; Ostman et al., 2008; Ritter et al., 2009). These effects can be mimicked by intracellular application of GTPyS (a hydrolysis-resistant GTP analogue), and we tested whether this phenomenon could be replicated in Na<sub>v</sub>1.9-expressing ND7/23 cells. Fig. 3 A illustrates the mean whole-cell currents recorded from Na<sub>V</sub>1.9-expressing cells in the absence and presence of 200 μM of intracellular GTPγS. Application of intracellular GTPyS had a concentration-dependent effect on Na<sub>V</sub>1.9 current density (Fig. 3, B and C). Addition of 20 μM GTPγS had no effect, whereas 63 μM increased the current density by 40% and 200 µM approximately doubled Na<sub>v</sub>1.9 current density (Fig. 3, B and C). All subsequent experiments were performed with 200 µM GTP<sub>γ</sub>S. The doubling of Na<sub>v</sub>1.9 current density by GTP<sub>γ</sub>S was evident 2 min after rupture of the membrane patch and intracellular dialysis with the pipette solution (Fig. 3 D). The increase in current density was accompanied by a depolarizing shift in the voltage dependence of inactivation without any effect on the

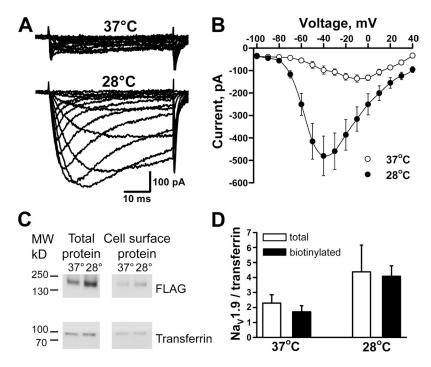


Figure 1. Incubation at  $28^{\circ}\text{C}$  increases  $Na_V1.9$  cell surface expression and current magnitude. (A) Averaged whole-cell currents recorded from cells incubated at  $37^{\circ}\text{C}$  (n=8) or  $28^{\circ}\text{C}$  (n=9). (B) Current-voltage relationships measured from  $Na_V1.9$ -expressing cells at  $37^{\circ}\text{C}$  or  $28^{\circ}\text{C}$ . (C) Total and cell surface  $Na_V1.9$  protein isolated from cells grown at  $37^{\circ}\text{C}$  or  $28^{\circ}\text{C}$ . (D) Densitometric analysis of total and cell surface  $Na_V1.9$  bands obtained from three different protein isolations. Data are represented as means  $\pm$  standard error of the mean.

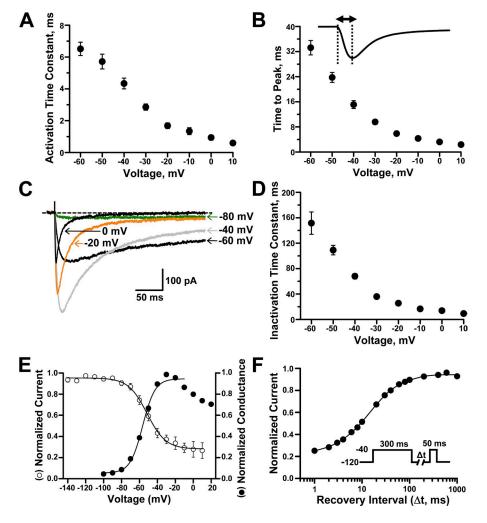


Figure 2. Biophysical properties of heterologously expressed human Na<sub>V</sub>1.9. (A) Voltage dependence of activation time constants (n = 14-17). (B) Voltage dependence of time to peak current (n = 18). Solid line illustrates a representative inward current trace, dashed lines point to the beginning and peak of the current, and the double-headed arrow indicates the time to peak measurement period. (C) Mean whole-cell Na<sub>V</sub>1.9 currents recorded during 300-ms pulses to -80, -60, -40, -20, and 0 mV(n = 12). Dashed line indicates the zero current level. (D) Voltage dependence of inactivation time constants (n = 13-17). (E) Superimposed steadystate channel availability (open circles) and conductance-voltage relationship (closed circles) for  $Na_V 1.9$  currents (n =7 and 16, respectively). Solid lines represent data fitted with Boltzmann functions. (F) Time course of recovery from fast inactivation (holding potential was -120 mV). Line represents data fitted with a two-exponential function (n = 8). Data are represented as means ± standard error of the mean.

voltage dependence of activation or the time course of recovery from inactivation (Fig. 3, E and F; and Table 1). Importantly, application of intracellular GTP $\gamma$ S did not evoke expression of TTX-resistant currents in nontransfected ND7/23 cells.

More detailed analyses revealed that intracellular  $GTP\gamma S$  slightly decreased the slope factor for voltage

dependence of activation and increased the time constant corresponding to the slow component of recovery from fast inactivation (Table 1). Whole-cell activation kinetics were not affected by intracellular GTP $\gamma$ S; both activation time constants (Fig. 4 A) and time to peak (Fig. 4 B) were similar to control values. However, GTP $\gamma$ S did affect inactivation kinetics at potentials

TABLE 1
Biophysical properties of  $Na_V 1.9$  in the absence (control) or presence of intracellular GTPyS

Condition	Peak current density (-40 mV)		Voltage dependence of activation			Steady-state inactivation			Recovery from inactivation		
	pA/pF	$\overline{n}$	$ V_{1/2}$	k	n	$ ule{V_{1/2}}$	k	$\overline{n}$	$\tau_1$	$\tau_2$	$\overline{n}$
			mV			mV			ms	ms	
Control	$-23.2 \pm 3.4$	16	$-56.9 \pm 0.6$	$6.7 \pm 0.3$	16	$-52.1 \pm 2.6$	$9.2 \pm 0.7$	7	$13.6 \pm 2.2$ (52.3 ± 7.3%)	$77.9 \pm 11.5$ (24.4 ± 6.9%)	8
GTPγS	$-38.9 \pm 5.1^{a}$	20	$-54.3 \pm 1.1$	$5.8 \pm 0.3^{\rm b}$	20	$-43.6 \pm 2.0^{\circ}$	$10.4 \pm 0.7$	8	$16.0 \pm 1.0$ $(61.7 \pm 3.9\%)$	$466.9 \pm 151.9^{d}$ (18.0 ± 3.2%)	6

Steady-state inactivation was determined using a 300-ms prepulse to various potentials followed by a 50-ms pulse to -40 mV. Recovery from inactivation was determined after a 300-ms prepulse to -40 mV.

 $<sup>^{</sup>a}P = 0.025$  (GTP $\gamma$ S vs. control using Student's t test).

 $<sup>{}^{\</sup>mathrm{b}}\mathrm{P} = 0.015$  (GTP $\gamma\mathrm{S}$  vs. control using Student's t test).

 $<sup>^{</sup>c}P = 0.021$  (GTP $\gamma$ S vs. control using Student's t test).

 $<sup>^{</sup>d}P = 0.011$  (GTP $\gamma$ S vs. control using Student's t test).

positive to -50 mV. Fig. 4 C shows whole-cell current inactivation of Na<sub>V</sub>1.9 in the presence of intracellular GTP $\gamma$ S. Specifically, the inactivation time course determined between -40 mV and 10 mV exhibited a biexponential decay (Fig. 4 D) with voltage-dependent time constants and fractional amplitudes. At -40 mV, both the slow and fast components were equally proportioned, whereas at more positive potentials the fast component fraction predominated ( $\sim$ 65% at -20 mV and  $\sim$ 80% at 10 mV, Fig. 4 E). These data indicated that GTP $\gamma$ S evoked changes in Na<sub>V</sub>1.9 gating, but these findings from whole-cell recording experiments did not provide a clear explanation for increased current amplitude.

# Single-channel mechanism of Na<sub>V</sub>1.9 potentiation by GTP $\gamma$ S

To further elucidate biophysical mechanisms that explain GTP $\gamma$ S potentiation of Na<sub>V</sub>1.9 current, we performed single-channel analysis on Na<sub>V</sub>1.9-ND7/23 cells in the absence or presence of intracellular GTP $\gamma$ S. These experiments were performed in the presence of 150 nM external TTX to block endogenous Na<sub>V</sub> channels.

Fig. 5 illustrates representative single-channel traces recorded at -40 mV from excised outside-out membrane patches dialyzed with either control intracellular solution or with solution containing 200 µM GTPyS. Singlechannel amplitudes were similar for both conditions (control,  $0.69 \pm 0.05$  pA; +GTP $\gamma$ S,  $0.72 \pm 0.04$  pA; Fig. 5 C). This observation excluded a fundamental change in ion permeation as the cause for increased whole-cell current amplitude evoked by GTPyS. In contrast, exposure of Na<sub>V</sub>1.9 to intracellular GTP<sub>Y</sub>S was associated with an  $\sim$ 2.7-fold larger nP<sub>o</sub> (control, 0.23 ± 0.07, n = 4; +GTP $\gamma$ S,  $0.63 \pm 0.05$ , n = 4; P = 0.004; Fig. 5 D) and a significantly greater mean open time deduced from open time histograms (Fig. 5 E). By dividing the nP<sub>o</sub> value calculated for each outside-out patch by the maximum number of channels observed in the same patch, we calculated an  $\sim$ 3.3-fold larger P<sub>o</sub> for channels in the presence of GTP $\gamma$ S (P<sub>o</sub>: control = 0.06 ± 0.01 [4.0 ± 1.3 channels/ patch]; +GTP $\gamma$ S = 0.19 ± 0.02 [3.2 ± 0.2 channels/ patch]). These findings indicated that changes in gating were primarily responsible for GTPyS potentiation of Na<sub>v</sub>1.9 current.

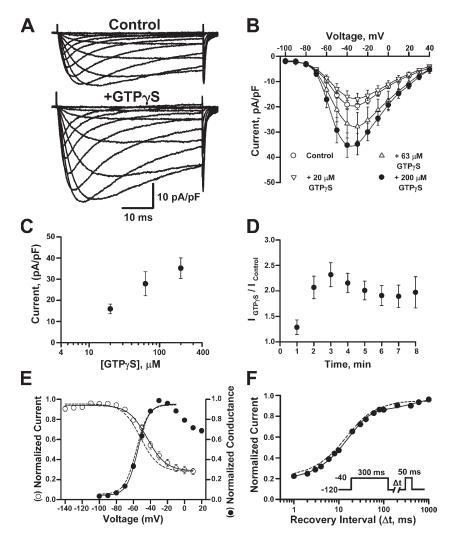


Figure 3. Intracellular GTPγS potentiates Na<sub>V</sub>1.9 activity. (A) Averaged whole-cell currents recorded from Na<sub>V</sub>1.9-expressing cells in the absence (control) or presence (+GTPyS) of 200 µM intracellular GTPyS. (B) Mean current-voltage relationships measured with control intracellular solution (n =33) or with internal GTP $\gamma$ S at 20  $\mu$ M (n = 9), 63  $\mu$ M (n = 9), or 200  $\mu$ M (n = 21). (C) Mean current density measured at -40 mV with control pipette or pipette plus GTPyS at 20 µM (n = 9), 63 µM  $(\hat{n} = 9)$ , or 200 µM (n = 21). (D) Whole-cell currents measured at -40 mV after rupture of membrane patch from cells dialyzed with 200 μM of intracellular GTPγS and normalized to the mean whole-cell current measured in cells dialyzed with control pipette solution (n = 8). (È) Superimposed steady-state channel availability (open circles) and conductance-voltage relationship (closed circles) recorded in the presence of intracellular GTP $\gamma$ S (n = 8 and 20, respectively). Dotted lines illustrate activation and inactivation curves determined in control solution (from Fig. 2 E). (F) Time course of recovery from fast inactivation (n = 8). Solid line represents data fitted with a two-exponential function. Dotted line depicts the recovery from inactivation recorded using control intracellular solution (from Fig. 2 F). Data are represented as means ± standard error of the mean.

#### DISCUSSION

In this study, we sought to determine the functional properties of recombinant human  $Na_v1.9$  and the mechanism by which G-protein signaling increases  $Na_v1.9$  activity. Because this  $Na_v$  channel has been implicated in nociception, our findings have implications for understanding the physiology of pain and could provide the basis for new analgesic strategies.

#### Heterologous functional expression of human Na<sub>V</sub>1.9

Our experiments were enabled by successful and robust stable expression of human  $Na_V1.9$  in heterologous cells. Previously, heterologous expression of  $Na_V1.9$  has been difficult (Dib-Hajj et al., 2002), leading to speculation this is a not a voltage-gated channel, but rather a ligand-gated channel (Blum et al., 2002). However, the absence of TTX-resistant persistent Na current in mouse Scn11A-null neurons and its restoration by transfection of human  $Na_V1.9$  in these cells (Ostman et al., 2008) eliminated doubt regarding the true nature of this protein.

Our success in achieving stable heterologous expression of Na<sub>v</sub>1.9 can be attributed to three factors. First, we engineered Na<sub>v</sub>1.9 stable cells using a highly efficient transposon system capable of integrating transgenes into multiple loci within a host cell genome (Kahlig et al., 2010). This approach may also bias integration events to regions of active chromatin increasing the likelihood of transgene expression (Wilson et al., 2007). Second, use of ND7/23 cells may provide intracellular factors expressed in neurons that are absent in other commonly used heterologous cells (e.g., HEK-293). For example, contactin has been demonstrated to bind to Na<sub>V</sub>1.9 and increase surface expression and current density of Na<sub>V</sub>1.9 channels in DRG neurons (Liu et al., 2001; Rush et al., 2005). Finally, we used low-temperature (28°C) incubation of cells to promote higher levels of functional Na<sub>v</sub>1.9 expression. The efficacy of 28°C incubation to boost channel activity may reflect a certain degree of impaired Na<sub>v</sub>1.9 trafficking in heterologous cells. However, our biochemical data did not demonstrate a clear change in the ratio of cell surface to total cellular protein consistent with

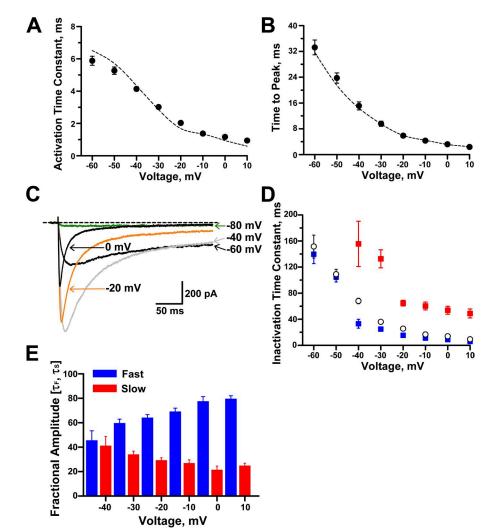


Figure 4. Effects of intracellular GTPyS on Na<sub>V</sub>1.9 kinetics. (A) Time constants for activation in the presence of intracellular GTP $\gamma$ S (n = 13-14). (B) Time to peak current in the presence of intracellular GTP $\gamma$ S (n = 21). Dotted lines illustrate data for control solution (from Fig. 2). (C) Averaged whole-cell Na<sub>v</sub>1.9 currents recorded during 300-ms pulses to -80, -60, -40, -20, and 0 mV. Dotted line indicates the zero current level (n =10). (D) Inactivation time constants (fast, blue; slow, red) recorded in the presence of intracellular GTP $\gamma$ S (n = 8-14). Inactivation time constants for control pipette solution (open circles; n =13–17) are also shown (from Fig. 2 D). (E) Fractional amplitudes for inactivation time constants determined in the presence of intracellular GTPyS for -40 to 10 mV (n = 8-14). Data are represented as means ± standard error of the mean.

redistribution of channels to the plasma membrane. Alternative explanations for the effects of low temperature include more efficient translation of  $Na_V1.9$  or slower protein turnover. These various maneuvers appear to overcome the natural tendency for  $Na_V1.9$  to express small Na currents, an intrinsic property of the channel which may protect cells from intracellular  $Na^+$  overload or excessive membrane depolarization.

# Biophysical properties of human Na<sub>V</sub>1.9

Using stably expressing heterologous cells, we determined the biophysical properties of human  $Na_V1.9$  using both whole-cell and single-channel recording strategies. Among the more characteristic features of  $Na_V1.9$  is its unusually slow gating, especially inactivation. In single-channel recordings, long openings, and tendency for reopenings account for the sluggish nature of inactivation. This characteristic of  $Na_V1.9$  inactivation stems in part from the large overlap between the voltage dependence

of activation and inactivation with an expanded range of voltages in which channels are active but not subject to strong inactivation tendencies ("window" current). Furthermore, the voltage midpoint  $(V_{1/2})$  for activation (-56 mV) is similar to that for inactivation (-52 mV). In contrast, fast-gating Na<sub>V</sub> channels have inactivation V<sub>1/2</sub> values that are typically more negative than activation. The effect of this relationship is illustrated at -80 mV (Fig. 2 C), a voltage at which Na<sub>V</sub>1.9 is activated but exhibits very little inactivation. Our findings are in agreement with previous experiments associating this channel with subthreshold Na current observed in sensory neurons. Some species-related differences in the reported voltage dependence of activation and inactivation for Na<sub>v</sub>1.9 are evident when comparing our data with recordings from rats and mice (Cummins et al., 1999; Coste et al., 2004; Rush and Waxman, 2004), but the general trends are similar. However, the structural basis for the unusual gating behavior of Na<sub>v</sub>1.9 is unclear.

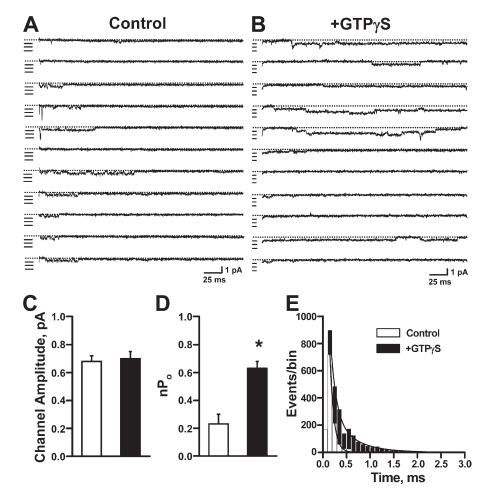


Figure 5. Effect of GTPγS on Na<sub>V</sub>1.9 single-channel activity. Single Na<sub>V</sub>1.9 channel openings were elicited by 300-ms pulses to -40 mV from a holding potential of -120 mV with 150 nM TTX present in the bath solution. (A) Single-channel traces from excised, outside-out patches dialyzed with control intracellular solution. Dotted lines indicate the zero current level, and individual single-channel amplitudes are marked by horizontal lines. The illustrated patch recordings contain at least six channels. (B) Single-channel traces from excised, outside-out patches dialyzed with pipette solution containing 200 μM GTPγS. The illustrated patch recordings contain at least four channels. (C) Single-channel amplitudes measured in the absence (control, n = 4) or presence (+GTP $\gamma$ S, n = 4) of 200  $\mu$ M GTPγS. (D) Values for nPo calculated from single-channel recordings from control and +GTPyS patches. Intracellular GTP $\gamma$ S increased nP<sub>o</sub>  $\sim$ 2.5 (\*, P = 0.004). (E) Open time histograms plotted for single-channel recordings performed in the absence or presence of GTPyS. The mean open times calculated for control solution were  $\tau_1 = 0.09$  $\pm$  0.01 ms (A<sub>1</sub> = 95.7  $\pm$  1.8%) and  $\tau_2$  =  $0.47 \pm 0.07$  ms (A<sub>2</sub> =  $4.3 \pm 1.8\%$ ; n = 4). In the presence of GTP<sub>γ</sub>S, mean open times were  $\tau_1 = 0.24 \pm 0.05$  ms (A<sub>1</sub> = 91.5  $\pm$  2.4%) and  $\tau_2$  = 1.55  $\pm$  0.35 ms (A<sub>2</sub> =  $8.5 \pm 2.4\%$ ; n = 4). The differences in  $\tau_1$  and  $\tau_2$  values between control and +GTP $\gamma$ S were significant, P = 0.026 and P = 0.023, respectively. Data are represented as means ± standard error of the mean.

# Mechanisms of GTPγS potentiation of human Na<sub>V</sub>1.9

Previous studies have demonstrated that G-protein activation increases TTX-resistant, persistent Na current in sensory neurons and that this phenomenon requires Na<sub>v</sub>1.9 (Baker et al., 2003; Rush and Waxman, 2004; Ostman et al., 2008). In this study, we demonstrated that heterologously expressed human Na<sub>V</sub>1.9 can also be potentiated by intracellular GTPyS, a nonselective activator of G-protein signaling. We observed that exposure to GTPyS caused am approximate twofold greater current density accompanied by an ~10-mV depolarizing shift in the voltage dependence of inactivation but without a change in activation voltage dependence. The unchanged voltage dependence of activation in combination with a more positive voltage dependence of inactivation yields a larger window current after GTPyS exposure. Additionally, GTP<sub>\gammaS</sub> slowed inactivation by introducing a slower component readily discernable in a physiological voltage range. In contrast, study of murine Na<sub>v</sub>1.9 demonstrated that Na<sub>v</sub>1.9 potentiation by PGE<sub>2</sub> was accompanied by hyperpolarizing shifts in the voltage dependence of activation and inactivation but no effects on gating kinetics (Rush and Waxman, 2004). The different effects induced by G-protein activation on human or murine Na<sub>v</sub>1.9 biophysical properties may be species or agonist specific. For example, GTPyS is a nonselective activator of G proteins, whereas PGE2 signals through G<sub>i/o</sub> (Rush and Waxman, 2004). Our experiments were performed in the presence of intracellular fluoride, which may activate G proteins after binding to trace aluminum (Matzel et al., 1996). However, this did not prevent GTPyS modulation of Na<sub>v</sub>1.9 channel activity.

To further elucidate the mechanism responsible for effects of intracellular GTP<sub>γ</sub>S on Na<sub>v</sub>1.9, we performed single-channel analysis. Our findings excluded a major change in permeation properties as the source for increased current density evoked by GTPyS. However, intracellular GTPyS did cause a significantly greater mean open time, which could account for the slower time course of inactivation and thus larger persistent current evoked by G-protein activation. We also examined whether channel open probability was affected. We determined that  $nP_0$  was  $\sim 2.7$  times greater in the presence than absence of intracellular GTPyS. Further analysis revealed that a greater P<sub>o</sub> associated with GTPyS exposure closely approximates the fold increase in nP<sub>o</sub> and whole-cell current density. Together, these results indicated that G-protein activation potentiates human Na<sub>v</sub>1.9 by increasing channel open probability and lengthening mean open time, causing the larger peak and persistent current after exposure to inflammatory mediators.

In summary, we have developed a stable cell line expressing human Na<sub>v</sub>1.9 and overcame previous challenges to investigating this channel in heterologous cells.

We analyzed the biophysical properties of human  $Na_V1.9$  and determined the mechanism by which GTP $\gamma$ S potentiates channel activity. Our results demonstrate a cellular platform that will be useful for the discovery of  $Na_V1.9$  blockers or modulators and to understand how inflammatory agents regulate  $Na_V1.9$  current. Furthermore, our findings may contribute to the development of new treatment strategies for chronic and inflammatory pain.

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