

Depolarizing Na_V and Hyperpolarizing K_V Channels Are Co-Trafficked in Sensory Neurons

Grant P. Higerd-Rusli,^{1,2,3,4} Matthew Alsaloum,^{1,2,3,4} Sidharth Tyagi,^{1,2,3,4} Nivedita Sarveswaran,^{2,3,4}
 Mark Estacion,^{2,3,4} Elizabeth J. Akin,^{2,3,4} Fadia B. Dib-Hajj,^{2,3,4} Shujun Liu,^{2,3,4} Daniel Sosniak,^{2,3,4}
 Peng Zhao,^{2,3,4} Sulayman D. Dib-Hajj,^{2,3,4} and Stephen G. Waxman^{2,3,4}

¹MD/PhD Program, ²Center for Neuroscience and Regeneration Research and, ³Department of Neurology, Yale University School of Medicine, New Haven, Connecticut 06510, and ⁴Rehabilitation Research Center, Veterans Affairs Connecticut Healthcare System, West Haven, Connecticut 06516

Neuronal excitability relies on coordinated action of functionally distinct channels. Voltage-gated sodium (Na_V) and potassium (K_V) channels have distinct but complementary roles in firing action potentials: Na_V channels provide depolarizing current while K_V channels provide hyperpolarizing current. Mutations and dysfunction of multiple Na_V and K_V channels underlie disorders of excitability, including pain and epilepsy. Modulating ion channel trafficking may offer a potential therapeutic strategy for these diseases. A fundamental question, however, is whether these channels with distinct functional roles are transported independently or packaged together in the same vesicles in sensory axons. We have used Optical Pulse-Chase Axonal Long-distance imaging to investigate trafficking of Na_V and K_V channels and other axonal proteins from distinct functional classes in live rodent sensory neurons (from male and female rats). We show that, similar to Na_V1.7 channels, Na_V1.8 and K_V7.2 channels are transported in Rab6a-positive vesicles, and that each of the Na_V channel isoforms expressed in healthy, mature sensory neurons (Na_V1.6, Na_V1.7, Na_V1.8, and Na_V1.9) is cotransported in the same vesicles. Further, we show that multiple axonal membrane proteins with different physiological functions (Na_V1.7, K_V7.2, and TNFR1) are cotransported in the same vesicles. However, vesicular packaging of axonal membrane proteins is not indiscriminate, since another axonal membrane protein (NCX2) is transported in separate vesicles. These results shed new light on the development and organization of sensory neuron membranes, revealing complex sorting of axonal proteins with diverse physiological functions into specific transport vesicles.

Key words: axonal trafficking; ion channels; live microscopy; sensory neurons

Significance Statement

Normal neuronal excitability is dependent on precise regulation of membrane proteins, including Na_V and K_V channels, and imbalance in the level of these channels at the plasma membrane could lead to excitability disorders. Ion channel trafficking could potentially be targeted therapeutically, which would require better understanding of the mechanisms underlying trafficking of functionally diverse channels. Optical Pulse-chase Axonal Long-distance imaging in live neurons permitted examination of the specificity of ion channel trafficking, revealing co-packaging of axonal proteins with opposing physiological functions into the same transport vesicles. This suggests that additional trafficking mechanisms are necessary to regulate levels of surface channels, and reveals an important consideration for therapeutic strategies that target ion channel trafficking for the treatment of excitability disorders.

Received Jan. 9, 2022; revised May 4, 2022; accepted May 5, 2022.

Author contributions: G.P.H.-R., E.J.A., S.D.D.-H., and S.G.W. designed research; G.P.H.-R., M.A., S.T., N.S., and M.E. performed research; G.P.H.-R., M.A., S.T., N.S., and M.E. analyzed data; G.P.H.-R. wrote the first draft of the paper; G.P.H.-R., S.T., S.D.D.-H., and S.G.W. edited the paper; G.P.H.-R., S.D.D.-H., and S.G.W. wrote the paper; E.J.A., F.B.D.-H., S.L., D.S., and P.Z. contributed unpublished reagents/analytic tools.

This work was supported by Merit Review Award B9253-C and BX004899 from the U.S. Department of Veterans Affairs Rehabilitation Research and Development Service and Biomedical Laboratory Research and Development Service to S.G.W. and S.D.D.-H., respectively. The Center for Neuroscience & Regeneration Research is a Collaboration of the Paralyzed Veterans of America with Yale University. G.P.H.-R. was supported by National Institute of Neurological Disorders and Stroke 1F31NS122417-01. G.P.H.-R., M.A., and S.T. were supported by National Institutes of Health/National Institute of General Medical Sciences Medical

Scientist Training Program T32GM007205. M.A. was supported by Yale Stem Cell Center Lo Graduate Fellowship for Excellence in Stem Cell Research. E.J.A. was supported by Paralyzed Veterans of America Research Foundation Grant 3176. We thank Shawn Ferguson, David Zenisek, Emile Boulpaep, Craig Crews, Angeliki Louvi, Fred Gorelick, and Reiko Fitzsimonds for helpful discussions; and Luke Lavis and Jonathan Grimm for the JaneliaFluors and helpful discussions.

The authors declare no competing financial interests.

Correspondence should be addressed to Sulayman D. Dib-Hajj at sulayman.dib-hajj@yale.edu or Stephen G. Waxman at stephen.waxman@yale.edu.

<https://doi.org/10.1523/JNEUROSCI.0058-22.2022>

Copyright © 2022 the authors

Introduction

Neuronal function relies on the coordinated action of different membrane proteins at the cell surface. For example, voltage-gated sodium (Na_V) channels and voltage-gated potassium (K_V) channels play complementary roles in action potential generation and propagation in axons: Na_V channels underlie the depolarizing phase, while K_V channels mediate the repolarization phase (Hodgkin and Huxley, 1952). Electrogenesis in neurons is finely tuned by the expression of multiple Na_V and K_V channels (Waxman and Zamponi, 2014). $\text{Na}_V1.7$ – 1.9 are the primary Na_V isoforms expressed in unmyelinated sensory neurons and contribute to action potential electrogenesis in these cells (Akopian et al., 1996; Black et al., 1996; Dib-Hajj et al., 1998; Cummins et al., 1999; Renganathan et al., 2001; Blair and Bean, 2002; Nassar et al., 2004; Alexandrou et al., 2016; Grubinska et al., 2019). $\text{Na}_V1.6$ is also expressed in sensory neurons but plays a larger role in myelinated neurons (Burgess et al., 1995; Caldwell et al., 2000; Black et al., 2002). Multiple K_V isoforms, including $\text{K}_V7.2$, are expressed in sensory neurons (Tsantoulas and McMahon, 2014).

Mutations in Na_V and K_V channels cause many channelopathies, including syndromes characterized by extreme pain and insensitivity or resilience to pain (Busserolles et al., 2016; Bennett et al., 2019; Dib-Hajj and Waxman, 2019; Mis et al., 2019; Yuan et al., 2021), epilepsy (Kullmann, 2010), movement disorders (Mantegazza et al., 2021), and cardiac arrhythmias (Amin et al., 2010). The transcription and translation of Na_V and K_V channels are dysregulated oppositely in pain states. For example, $\text{Na}_V1.7$ and $\text{Na}_V1.8$ expression is upregulated in sensory neurons in models of inflammation and chemotherapy-induced neuropathy (Black et al., 2004; Gould et al., 2004; Strickland et al., 2008; Liang et al., 2013; Li et al., 2018), whereas $\text{K}_V7.2$ is downregulated in models of nerve injury and inflammation (Linley et al., 2008; Rose et al., 2011).

Because action potential generation in sensory neurons occurs near axonal ends, the intracellular trafficking of these proteins to distal axons is critical to neuronal signaling. Recently, we showed that axonal trafficking of $\text{Na}_V1.7$ is increased in models of inflammation and chemotherapy-induced peripheral neuropathy (Akin et al., 2019, 2021). While the expression and trafficking of channels are important in determining axonal function, it is not known whether axonal trafficking is regulated in a channel-specific manner.

Based on the genetic and functional validation of peripherally expressed Na_V s and, to a lesser extent K_V s, in the pathogenesis of pain, multiple efforts to develop drugs which specifically modulate conductance of channels inserted in the cell membrane are underway (Abd-Elseyed et al., 2019; Alsaloum et al., 2020). However, an alternative strategy to target channel function would be to alter channel trafficking to and from the membrane. The viability of this strategy would depend on the identification of trafficking mechanisms which mediate the transport of specific channels which could be targeted therapeutically with minimal side effects.

Whether different Na_V channels are trafficked together or with other axonal proteins is a fundamental question that has not been adequately answered to date. Some components of micro-domains of myelinated neurons are trafficked independently to axons (Bekku and Salzer, 2020), and different K_V isoforms are sorted specifically in dendrites (Jensen et al., 2014). However, whether Na_V and K_V channels, as well as other axonal proteins with diverse physiological functions in unmyelinated sensory neurons are

independently trafficked in specific vesicles or packaged together in the same vesicles is not known.

Building on our recent advances in high-resolution imaging of Na_V trafficking and surface distribution (Akin et al., 2019, 2021), we engineered multiple tagged, functional, full-length human Na_V and K_V channels, which enabled simultaneous investigation of multiple channel isoforms trafficking in live sensory axons in real time. These studies revealed co-packaging of ion channels with opposing physiological functions into the same transport vesicles, highlighting a limitation for targeting ion channel axonal trafficking as a novel therapeutic strategy for the treatment of excitability disorders.

Materials and Methods

DNA constructs

The plasmid 2A-GFP-h $\text{Na}_V1.7$ was previously described (Yang et al., 2016). Briefly, human $\text{Na}_V1.7$ plasmid was rendered TTX-resistant (TTX-R) by substituting amino acid (a.a.) Tyr362 with serine (Y362S) using QuikChange Lightning site-directed mutagenesis (Agilent Technologies). Subsequently, a modified plasmid (GFP-2A-h $\text{Na}_V1.7R$) was made by fusing EGFP and a StopGo 2A linker in-frame with the N-terminus of the channel.

The codon-optimized Halo- $\text{Na}_V1.7$ construct followed the protocol for the construction of the non-codon-optimized Halo- $\text{Na}_V1.7$ (Akin et al., 2019). The final construct topology is in order from the N-terminus: 1–30 a.a. β_4 signal peptide, $3\times$ myc tag (EQKLISEEDL), Halo-tag enzyme (297 a.a.) (Promega), $3\times$ HA tag (YPYDVPDYA), 21 a.a. transmembrane segment (β_4 163–183), 7 a.a. linker (SGLRSAT), h $\text{Na}_V1.7$ -R. SNAP- $\text{Na}_V1.7$ was derived from the non-codon-optimized Halo- $\text{Na}_V1.7$ construct (Akin et al., 2019) by replacing the Halo-tag with the 182 a.a. SNAP_f (New England BioLabs) using megamutagenesis.

The codon-optimized human $\text{Na}_V1.8$ construct (pcDNA5-SCN10A) was purchased from Genionics, and previously reported (Faber et al., 2012). Codon-optimized Halo- $\text{Na}_V1.8$ was created in a protocol identical to that used to create the Halo- $\text{Na}_V1.7$. Halo- $\text{Na}_V1.8$ -dABM was created by deleting the Ankyrin 9 a.a. binding motif [VPIAEGESD → 997...1005] using QuikChange Lightning site-directed mutagenesis.

The codon-optimized mouse mycHaloHA-TM-m $\text{Na}_V1.6R$ construct was custom-synthesized by GeneWiz and cloned into pcDNA5, and the m $\text{Na}_V1.6R$ construct was made by deleting the mycHaloHA-TM sequences using megamutagenesis.

The plasmid that encodes human $\text{Na}_V1.9$ was previously described (Huang et al., 2014). The Halo-tag enzyme (297 a.a.) and a 7 a.a. linker (SGLRSAT) were inserted at the N-terminus of the channel using QuikChange Lightning site-directed mutagenesis.

EGFP-Rab3A (plasmid #49542) and EGFP-Rab6a (#49469) were obtained from Addgene as a gift from M. Scidmore (Rzomp et al., 2003).

$\text{K}_V7.2$ -Halo was created by inserting 297 a.a. Halo-tag at pos 118 (between a.a. 117 and 118) flanked by a 3 a.a. spacer (GlyAlaGly). The construct was custom synthesized by GenScript.

TNFR1-tEOS (plasmid #98273) was obtained from Addgene as a gift from Mike Heilemann (Heidbreder et al., 2012). TNFR1-SNAP was derived by replacing tEOS with 182 a.a. SNAP (NEB) by megamutagenesis.

NCX2-mCherry: pNCX2 (plasmid #67148) was obtained from Addgene as a gift from Michela Ottolia and Kenneth Philipson (Li et al., 1994). The NCX2 coding sequence was inserted into pEGFP plasmid by mega-mutagenesis, and subsequently the EGFP was replaced with mCherry.

NPY-td-Orange2 (plasmid #83497) was obtained from Addgene as a gift from S. Barg (Gandasi et al., 2015).

Primary DRG neuron culture and transfection

Animal studies followed a protocol approved by the Veterans Administration Connecticut Healthcare System Institutional Animal Care and Use Committee.

Rat DRG neurons. DRG neurons were isolated from 2- to 4-d-old Sprague Dawley rat pups as described previously (Dib-Hajj et al., 2009). Neonatal rats were selected randomly and thus included males and females. Briefly, dissected DRGs were first incubated at 37°C for 20 min in complete saline solution (CSS) [137 mM NaCl, 5.3 mM KCl, 1 mM MgCl₂, 25 mM sorbitol, 3 mM CaCl₂, and 10 mM HEPES (pH 7.2), adjusted with NaOH], supplemented with 0.6 mM EDTA and collagenase A (1.5 mg/ml; Roche). Rat DRGs were then incubated for 20 min at 37°C in CSS containing collagenase D (1.5 mg/ml; Roche), 0.6 mM EDTA, and papain (30 U/ml; Worthington Biochemical). DRGs were centrifuged and triturated in 1 ml of DRG culture medium DMEM/F12 (1:1) with penicillin (100 U/ml), streptomycin (0.1 mg/ml; Invitrogen), 2 mM L-glutamine, and 10% FBS (Hyclone) containing BSA (1.5 mg/ml; low endotoxin, Sigma-Aldrich) and trypsin inhibitor (1.5 mg/ml; Sigma-Aldrich). The cell suspension was filtered through a 70 μ m nylon mesh cell strainer (Becton Dickinson) to remove debris, and the mesh was then washed once with 1 ml of DRG culture medium.

Transfection of DRG neurons was performed as previously described (Dib-Hajj et al., 2009; Akin et al., 2019). Briefly, DRG neurons were pelleted (200 $\times$ g, 3 min) and gently resuspended with 20 μ l of Nucleofector solution; then, the cell suspension was mixed with DNA (for specific amounts of DNA, see Extended Data Table 1-1). Neurons were transfected using Nucleofector IIS Electroporator (Lonza) using protocol SCN-BNP 6 and Amaxa SCN Nucleofector reagents (VSP1-1003, Lonza). After electroporation, 100 μ l of calcium-free DMEM (37°C) was added, and cells were incubated at 37°C for 5 min in a 95% air/5% CO₂ (v/v) incubator to allow neurons to recover. The cell mixture was then diluted with DRG medium containing BSA (1.5 mg/ml) and trypsin inhibitor (1.5 mg/ml). Rat DRGs were carefully seeded onto 35 mm glass-bottom dishes commercially coated with poly-D-lysine (MatTek) and additionally coated with laminin (10 mg/ml for 2 h at 37°C), or onto the somatic chamber of microfluidic chambers (MFCs). DRGs cultured on coverslips were incubated at 37°C for 45 min to allow DRG neurons to attach. Then, DRG medium was added to each well to a final volume of 1.5 ml (in 35 mm glass-bottom dishes), or $\sim$ 350 μ l (in somatic chamber of MFCs) individually. DRG neurons were maintained at 37°C in a 95% air/5% CO₂ (v/v) incubator before use.

MFCs

As described previously (Akin et al., 2019), MFCs (DOC450, two-chamber 450 μ m groove, Xona Microfluidics) were bound to glass-bottom dishes according to the manufacturer's instructions. Briefly, MFCs were soaked in ethanol for 1 min, then air-dried before being placed on 50 mm glass-bottom dishes (P50G-1.55-30-F, MatTek) that were coated with poly-L-lysine (0.5 mg/ml) overnight at 37°C. The glass surface was washed twice with sterile double-distilled water and then air-dried in a sterile hood. The dishes were then coated with laminin (10 mg/ml) for at least 2 h at 37°C, excess laminin was aspirated, and the dishes were air-dried under the hood before MFCs were adhered. Transfected DRG neuron suspension was applied in the soma chamber containing DRG medium with growth factors (50 ng/mg) [NGF and GDNF from PeproTech], and 2 $\times$ growth factors (100 ng/mg) were added to the axonal chamber. Medium was changed to serum-free medium in both chambers after 24 h (Neurobasal medium supplemented with 2% B27, 1% penicillin/streptomycin, same 1:2 ratio of NGF, GDNF in soma and axonal chambers), and 1 μ M uridine/5-fluoro-2-deoxyuridine was added to inhibit the growth of fibroblasts and glia.

Manual patch-clamp recordings

Macroscopic currents were recorded in voltage-clamp mode using an EPC-10 amplifier and the PatchMaster program (HEKA Elektronik) at room temperature. Patch pipettes were pulled from borosilicate glass (1.65/1.1, outside diameter/inside diameter; World Precision Instruments) using a Sutter Instruments P-97 puller and had a resistance of 0.8–1.8 M Ω .

Sodium currents in transfected HEK293 cells and DRG neurons under 25 μ m in diameter were recorded in the whole-cell configuration. Cells with a leak current >200 pA or 10% of their peak current were excluded. Series resistance prediction and compensation (65%–90%) were applied to reduce the voltage errors. Cells were excluded if their

voltage errors were >3 and 5 mV for HEK293 cells and DRG neurons, respectively. The recorded currents were digitized at a rate of 50 kHz after passing through a low-pass Bessel filter setting of 10 kHz. After achieving whole-cell configuration, a 5 min equilibration period was allowed before initiating the recordings.

Peak inward currents were transformed to conductance using the equation $G = \frac{I}{(V_m - E_{Na})}$. The conductance at each voltage was normalized to the maximum conductance and fit with the following Boltzmann equation to derive the activation curve:

$$G = G_{min} + \left(\frac{G_{max} - G_{min}}{1 + e^{\frac{V_{1/2} - V_m}{k}}} \right).$$

Peak current density was calculated as the peak current during the activation pulse normalized to the cellular capacitance.

Peak inward currents from steady-state fast inactivation protocols were also fit with a Boltzmann equation.

Na_v1.7. Human embryonic kidney 293 (HEK293) cells were cotransfected with 0.5 μ g of 2A-GFP-hNa_v1.7, SNAP-hNa_v1.7, or codon-optimized Halo-Na_v1.7 using a LipoJet transfection kit (SignaGen Laboratories). The SNAP-tagged and Halo-tagged Na_v1.7 channels were also cotransfected with 0.5 μ g GFP to identify transfected cells. Cells were plated on coverslips coated with poly-D-lysine/laminin and maintained in DMEM/F12 (1:1) with penicillin (100 U/ml), streptomycin (0.1 mg/ml; Invitrogen), and 10% FBS (Hyclone) at 37°C in a 95% air/5% CO₂ (v/v) incubator for 2 d after transfection before patch-clamp recordings were conducted.

When recording Na_v1.7 currents in HEK293 cells, the extracellular solution contained the following (in mM): 140 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES. TTX was added to the bath at a concentration of 1 μ M, which is sufficient to block endogenous TTX-S currents. Patch microelectrodes were filled with intracellular solution containing the following (in mM): 140 CsF, 10 NaCl, 1.1 EGTA, and 10 HEPES. Both solutions were titrated to a pH of 7.3 and brought to final osmolarity (320 mOsm for extracellular solution and 310 mOsm for intracellular solution) using dextrose.

To measure Na_v1.7 channel activation, HEK293 cells were pulsed to a range of potentials between -80 mV and 40 mV, in 5 mV increments, from a holding potential of -120 mV. Steady-state fast inactivation of Na_v1.7 constructs was assessed in HEK293 cells with a series of 500 ms prepulses (-140 to 0 mV in 10 mV increments) followed by a 20 ms step depolarization to -10 mV to activate the remaining noninactivated channels.

Na_v1.8. DRGs from adult (4- to 8-week-old) Na_v1.8-KO mice were harvested and dissociated as described previously (Dib-Hajj et al., 2009) with minor differences. DRGs were dissociated with a 20 min incubation in CSS containing 0.5 U/ml Liberase TM (Sigma) and 0.6 mM EDTA, followed by a 15 min incubation in CSS containing 0.5 U/ml Liberase TL (Sigma), 0.6 mM EDTA, and 30 U/ml papain (Worthington Biochemical). DRGs were then centrifuged in 0.5 ml of DRG media containing 1.5 mg/ml BSA (low endotoxin) and 1.5 mg/ml trypsin inhibitor (Sigma). After trituration, neurons were transfected with 2.0 μ g of either Halo-tagged human Na_v1.8 or WT human Na_v1.8 and 0.2 μ g GFP using a Nucleofector IIS (Lonza) and Amaxa Basic Neuron SCN Nucleofector Kit (VSP1-1003). Cells were plated on coverslips coated with poly-D-lysine/laminin and maintained in DRG media at 37°C in a 95% air/ 5% CO₂ (v/v) incubator for 2 d after transfection before patch-clamp recordings were conducted. The extracellular recording solution contained the following (in mM): 140 NaCl, 20 -TEA-Cl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 0.1 CdCl₂, 10 HEPES, and 0.001 TTX.

To measure Na_v1.8 channel activation, transfected Na_v1.8-KO DRG neurons were pulsed to a range of potentials between -70 mV and 40 mV from a holding potential of -100 mV. We excluded from analysis cells in which a residual Na_v1.9 current was detected under these recording conditions. To assess steady-state fast inactivation of Na_v1.8 constructs, DRG neurons were prepulsed (-120 to 20 mV in 5 mV increments) for 500 ms to induce fast inactivation, followed by a 40 ms depolarization step to 0 mV to activate the remaining non-inactivated channels.

Na_v1.9. Characterization of Halo-Na_v1.9 was performed in superior cervical ganglion (SCG) neurons from 1- to 5-d-old rats using electroporation to introduce the expression plasmids as previously described (Dib-Hajj et al., 2009). SCG neurons do not express any TTX-resistant sodium channels, and the external and internal solutions were designed to isolate the heterologously expressed Na_v1.9 current. The Na_v1.9 constructs were mixed with mCherry in pEGFP Δ GFPN1 to identify cells likely to be expressing Na_v1.9 by identifying red fluorescent neurons. Cells were plated on coverslips coated with poly-D-lysine/laminin and maintained in DRG media at 37°C in a 95% air/5% CO₂ (v/v) incubator for 2 d after transfection before patch-clamp recordings were conducted.

Electrodes used for the recordings had resistance of <2 MΩ when filled with the internal solution, which consisted of the following (in mM): 135 CsF, 5 CsCl, 10 HEPES, 5 EGTA, and 3 ATP Na-salt (pH 7.3 with CsOH, adjusted to 314 mOsm with dextrose). The external recording solution contained the following (in mM): 140 NaCl, 3 KCl, 10 HEPES-NaOH; 1 MgCl₂, 1 CaCl₂, 20 TEA-Cl, 0.1 CdCl₂, and 1 μM TTX; pH 7.3, adjusted to 320 mOsm with dextrose.

The stimulation pulse protocol started at a holding potential of −100 mV and then stimulated with 100 ms depolarizing pulses starting at −100 mV and increased with an increment of 5 mV ending at 40 mV. Each sweep of the protocol was applied at 5 s intervals and P/6 leak subtraction was used.

K_v7.2. Characterization of K_v7.2-Halo was performed in HEK293 cells transfected using a LipoJet transfection kit (SignaGen Laboratories). After being cultured in 35 mm Petri dishes until 70%–80% confluence, 1.5 μg of K_v7.2-Halo or WT-K_v7.2 DNA was cotransfected with 0.5 μg GFP to identify transfected cells. One day after transfection, the cells were dissociated with TrypLE reagent (Invitrogen) and then plated on coverslips coated with poly-D-lysine/laminin and maintained in DMEM/F12 (1:1) with penicillin (100 U/ml), streptomycin (0.1 mg/ml; Invitrogen), and 10% FBS (Hyclone) at 37°C in a 95% air/5% CO₂ (v/v) incubator. Patch-clamp recordings were performed 2 d after transfection.

Electrodes used for the recordings had resistance of <2 MΩ when filled with the internal solution, which consisted of the following (in mM): 126 K-Gluconate, 4 KCl, 10 HEPES, 0.3 EGTA, 10 phosphocreatine disodium salt, 4 ATP Mg-salt, and 0.3 GTP Na-salt (pH 7.3 with KOH, adjusted to 320 mOsm with dextrose). The external recording solution contained the following (in mM): 140 NaCl, 3 KCl, 10 HEPES-NaOH, 2 MgCl₂, 2 CaCl₂, 15 dextrose, pH 7.3, ~320 mOsm.

The stimulation pulse protocol started at a holding potential of −100 mV and then stimulated with 500 ms depolarizing pulses starting at −80 mV and increased with an increment of 10 mV ending at 80 mV, followed by a hyperpolarizing pulse to −120 mV to record “tail current” as the opened channels close for 50 ms before returning to the interpulse holding potential. Each sweep of the protocol was applied at 5 s intervals and P/4 leak subtraction was used.

Automated high-throughput patch-clamp recordings

Na_v1.6. The gating properties of the Halo-Na_v1.6 construct was evaluated by automated voltage clamp of transiently transfected HEK cells. Halo-Na_v1.6 was introduced into HEK cells using lipid-based transfection kit (Lipojet, SignaGen Laboratories) and following the recommended protocol. The cells were harvested into a cell suspension on the second day after transfection for recordings by automated electrophysiology (Qube 384, Sophion Biosciences). A detailed description of the capabilities and the setting up of modules to perform voltage-step protocols for the Qube instrument has previously been published.

The solutions used when performing experiments with the Qube are as recommended by Sophion (Qian et al., 2020). The extracellular solution contained the following (in mM): 145 NaCl, 4 KCl, 2 CaCl₂ · (2H₂O), 1 MgCl₂ · (6H₂O), 10 HEPES, titrated to pH 7.4 with NaOH, and osmolality was adjusted to 305 mOsm with glucose. The intracellular solution contained the following (in mM): 140 CsF, 1 EGTA, 10 HEPES, 10 NaCl, titrated to pH 7.3 with CsOH, and osmolality was adjusted to 310 mOsm with glucose.

The voltage-clamp pulse protocols were implemented on the Qube instrument to replicate the ones used for manual patch clamp. The series resistance compensation level was set to 90%. Similar to manual patch-

clamp experiments, protocols were applied in a specific order to prevent time-dependent bias, as follows: the voltage dependence of activation protocol started from a holding potential of −120 mV, pulsed to less negative potentials for 100 ms, then returned to the original holding potential. Step depolarizations ranged from −80 to 40 mV, were applied in 5 mV increments, and looped at 5 s intervals. Voltage dependence of fast inactivation was measured by holding the cell at −120 mV, after which conditioning pulses of 500 ms were applied from −120 to 20 mV in 10 mV increments. Following the conditioning pulse, a test pulse to 0 mV was applied for 50 ms to determine the fraction of sodium channels still available for opening. The potential is then returned to the holding potential of −120 mV until the next loop occurring at 5 s intervals.

A cell was deemed positive for Na_v current expression if the peak inward current during the activation IV protocol exceeds −300 pA. Additional inclusion criteria include a reversal potential between 20 and 80 mV of the activation IV curve and a Boltzmann slope factor >4 mV. For activation and fast inactivation, the peak inward currents measured at each stimulus potential were normalized to capacitance and averaged to create the IV curves. Each individual cell's IV curve was fitted to the BoltzIV function to obtain the parameters V-half of activation, activation slope, G_{max}, and reversal potential. The activation IV curves were transformed into GV curves and then normalized.

Imaging system

As described previously (Akin et al., 2019), images were acquired using an Andor Dragonfly spinning disk confocal platform together with a Nikon Eclipse Ti Fluorescence microscope. Images were taken using an Andor iXon 888 electron multiplying charge-coupled device camera through a Plan Apo λ 60× (numerical aperture 1.4 oil objective). The light source is an Andor Integrated Laser Engine containing 150 mW 488 nm, 150 mW 561 nm, and 140 mW 637 nm solid state lasers. Emission filters include 525/50, 600/50, and 700/75 nm. The Nikon perfect focus system was used to maintain focus during time-lapse experiments.

Optical Pulse-chase Axonal Long-distance (OPAL) imaging

All Halo-tag Ligand and SNAP-tag Ligand conjugated JaneliaFluor labels (Grimm et al., 2015; Jonker et al., 2020) were generous gifts of L. D. Lavis and J. B. Grimm (Janelia Research Campus).

All live-cell experiments were performed at 37°C using a stage incubator (Tokai Hit). During labeling and imaging, neurons were kept in DRG neuronal imaging saline (NIS) containing 136 mM NaCl, 3 mM KCl, 1 mM MgSO₄, 2.5 mM CaCl₂, 0.15 mM NaH₂PO₄, 0.1 mM ascorbic acid, 20 mM HEPES, and 8 mM dextrose (pH 7.35) with NaOH (adjusted to 320 mOsm/L).

The OPAL imaging protocol was previously described (Akin et al., 2019). Briefly, DRG neurons transfected with Halo- and/or SNAP-tagged constructs were cultured in MFCs for 5–7 d. For experiments involving measurement of vesicle velocity and behavior, cell permeable JF646-Halo-tag Ligand (100 nM) was added to the soma chamber for 15 min. Time-lapse movies of distal axon ends were acquired between 45 and 55 min after the start of labeling. For co-trafficking experiments, cell-permeable JF646-Halo-tag Ligand (100 nM) and/or JF549-cpSNAP-tag Ligand (100 nM) were added to the somatic chamber for 30 min, and then removed by washing the chamber 3× with NIS. Time-lapse movies were acquired in the distal axon chamber for up to 1 h after labeling. For co-trafficking experiments involving fluorescent protein-tagged proteins together with Halo- or SNAP-Tagged proteins, fluorescent proteins in the FOV were photobleached using either the 488 nm laser (for GFP) or 561 nm laser (for red proteins) before movie acquisition.

Labeling channels at the cell surface

DRG neurons were transfected with Halo- or SNAP-tag channel constructs and cultured in glass-bottom dishes, coated with poly-L-lysine and laminin, as above, for 5 d. The cells were then exposed to the relevant cell-impermeable Halo-tag or SNAP-tag Ligand (100 nM in NIS, JF635i-Halo-tag Ligand for 15 min or JF549i-SNAP-tag Ligand for 30 min). Excess label was then washed away with NIS, and the cells were

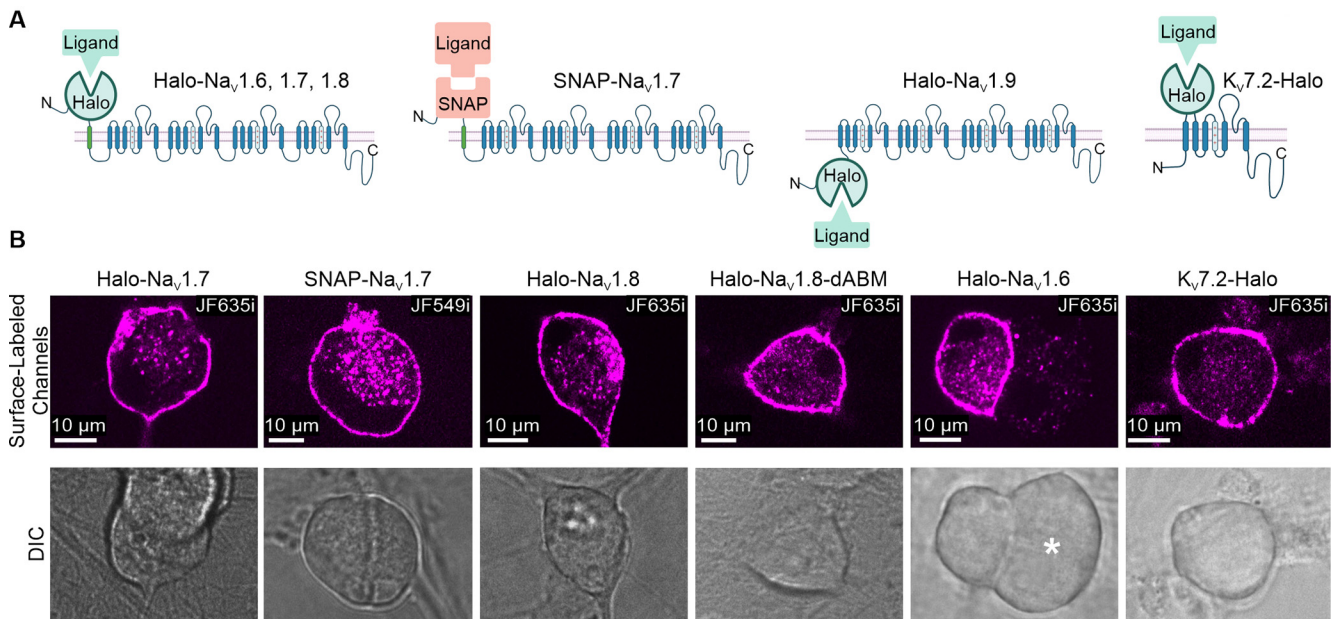


Figure 1. Tagged Na_V and K_V channels are trafficked to the cell surface. **A**, Schematics represent Halo- and SNAP-tagged Na_V and K_V channels and fluorescently conjugated ligands. **B**, DRG neurons were transfected with tagged channel constructs and cultured for 5 d. The cells were then exposed to cell-impermeable Halo-tag or SNAP-tag Ligands (JF635i-Halo-tag Ligand or JF549i-SNAP-tag Ligand), labeling only channels at the cell surface. The cells were then fixed and imaged. Top panels, Confocal slices through somas with surface labeling. Intracellular signal is partly because of channel endocytosis before fixation and some autofluorescence (Akin et al., 2019). Bottom panels, DIC images demonstrating DRG morphology. *Untransfected cell without fluorescent signal, demonstrating specific labeling.

fixed with 4% PFA for 15 min before confocal fluorescence imaging with the imaging system described above.

Kymograph generation and trafficking analysis

Kymographs were generated using KymographClear (Mangeol et al., 2016). Movies acquired using the methods above were opened in ImageJ, and the KymographClear toolset was used to create kymographs of selected axons. Specifically, axons were traced manually using a segmented line, and KymographClear extracts the signal under that line and converts it into a two-dimensional image with distance on the x axis and time on the y axis. Only axons that were separate from other axons were analyzed.

For analysis of vesicle velocity, flux, and pausing behavior, kymographs were generated for both the distal end of the axon (the most distal 15 μm) and a more proximal “mid-axon” portion (the 15 μm segment of axon between 115 and 100 μm from the distal tip). The resulting kymographs were analyzed using the automated kymograph analysis software KymoButler (Jakobs et al., 2019), which uses a machine learning algorithm to trace vesicle tracks. The velocity was calculated as the average over the duration of the track, including pauses and stops. Flux was determined by counting the number of vesicles which crossed the mid-line of the kymograph in either the anterograde or retrograde direction. Pauses were defined as any instance in which a track had zero displacement between two consecutive frames. If a given vesicle paused more than once, the pause durations were averaged to calculate the average pause duration for that vesicle.

For co-trafficking experiments, axons were selected for analysis if there was at least one moving vesicle of each construct visible in the axon, indicating that the neuron had been transfected with both constructs. Kymographs of these axons were created, and vesicles were categorized as either single positive for either construct or double positive.

Image and statistical analysis

Images were processed using either ImageJ or Imaris. Venn diagrams were generated using the Venn Diagram Plotter developed by the Pacific Northwest National Laboratory (<https://omics.pnl.gov/software/>). The elliptical Euler diagram was generated using eulerAPE (Micallef and Rodgers, 2014). Statistical analysis was performed using GraphPad Prism. For *post hoc* pairwise χ^2 tests, a Bonferroni correction, dividing

the α level by the number of comparisons made, was applied to the significance level to compensate for multiple comparisons ($p = 0.05/\text{number of comparisons}$) (Macdonald and Gardner, 2000). Schematics were created with www.BioRender.com.

Results

Self-labeling enzymatic tags enable observation of trafficking of multiple ion channels

Building on a strategy we used previously to tag $\text{Na}_V1.7$ (Akin et al., 2019), we engineered 25 transmembrane $\text{Na}_V1.6$ and $\text{Na}_V1.8$ channels with an N-terminal Halo-tag, an enzymatic tag that reacts with specific synthetic ligands, fused to an extra transmembrane segment to create extracellularly tagged channels (Halo- $\text{Na}_V1.6$ and Halo- $\text{Na}_V1.8$) (Fig. 1A). In addition, we tagged $\text{Na}_V1.9$ by fusing Halo-tag to the intracellular N-terminus (Halo- $\text{Na}_V1.9$) (Fig. 1A).

To enable comparison of $\text{Na}_V1.7$ and other proteins in the same neuron, we created multiple tagged $\text{Na}_V1.7$ constructs. We improved on a previously described Halo- $\text{Na}_V1.7$ construct (Akin et al., 2019) by adding the Halo-tag and extra transmembrane domain to a codon-optimized backbone construct, which encodes the same a.a. sequence but is more efficiently translated in DRG neurons (hereafter referred to as Halo- $\text{Na}_V1.7$). We also replaced the Halo-Tag in the original (non-codon-optimized) Halo- $\text{Na}_V1.7$ construct (Akin et al., 2019) with a SNAP-tag, a different enzymatic tag that functions similarly, but has different cognate ligands, to create SNAP- $\text{Na}_V1.7$ (Fig. 1A). To enable comparison of $\text{Na}_V1.7$ and $\text{K}_V7.2$, we inserted the Halo-tag in the first extracellular loop of $\text{K}_V7.2$ (Fig. 1A), a strategy that has been used previously to tag $\text{K}_V2.1$ (Jensen et al., 2017). Using cell-impermeable Halo-Tag and SNAP-tag ligands (Jonker et al., 2020), we confirmed that the tagged $\text{Na}_V1.6$, $\text{Na}_V1.7$, $\text{Na}_V1.8$, and $\text{K}_V7.2$ constructs are trafficked to the surface of DRG neurons (Fig. 1B). Surface labeling was not possible with Halo- $\text{Na}_V1.9$ because its tag is intracellular.

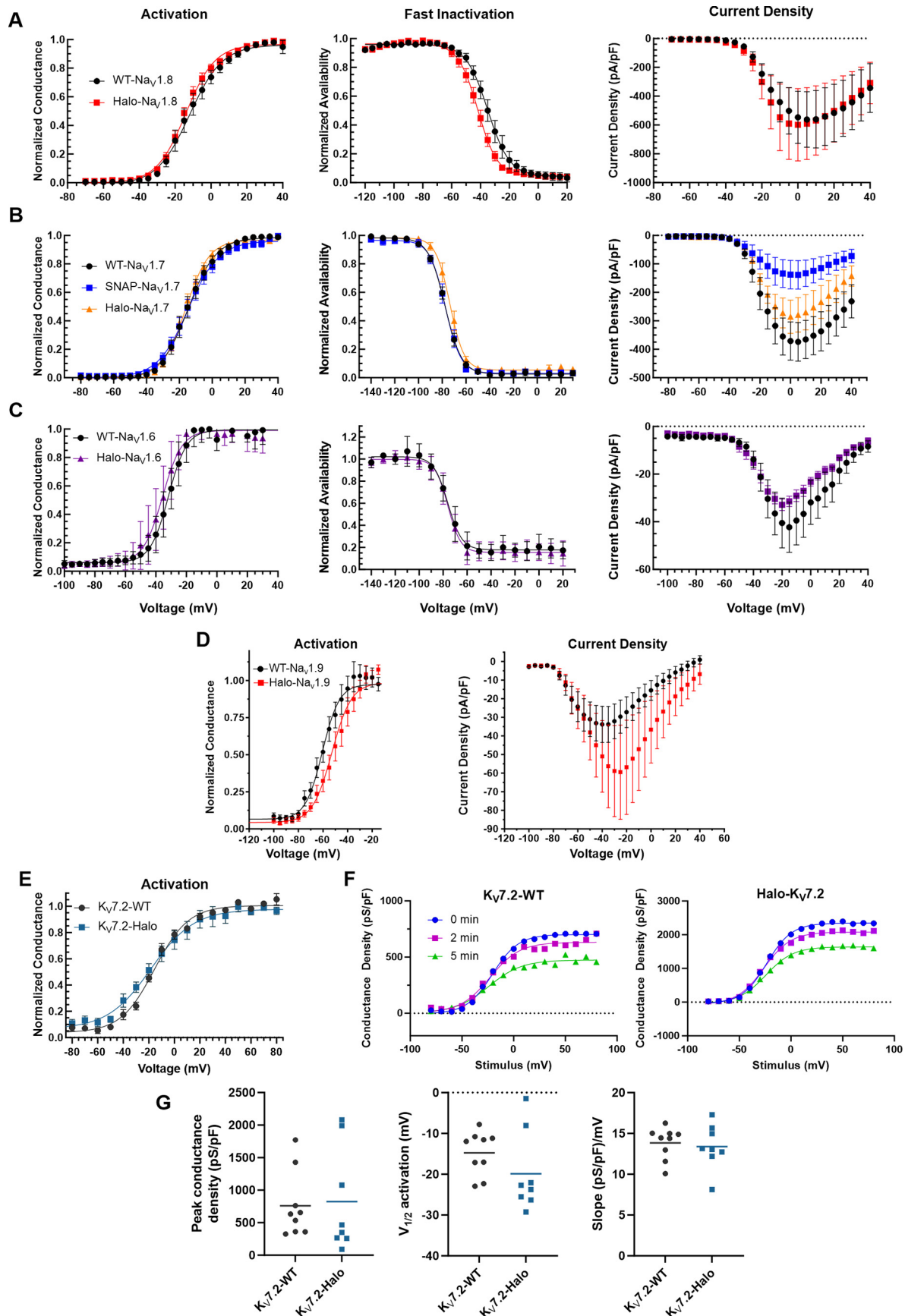


Figure 2. Tagged Na_v and K_v channels retain functional gating properties. **A**, Voltage-clamp recording in DRG neurons from $Na_v1.8$ -KO mice expressing either Halo- $Na_v1.8$ ($n = 7$) or WT- $Na_v1.8$ ($n = 7$) revealed no significant differences between channel activation (left) or current density (right) for the two constructs. Halo- $Na_v1.8$ voltage dependence of fast inactivation was significantly shifted in the hyperpolarizing direction compared with WT- $Na_v1.8$ (middle) ($p = 0.039$). **B**, Voltage-clamp recordings in HEK293 cells expressing WT- $Na_v1.7$ ($n = 7$), SNAP- $Na_v1.7$

Tagged Na_v and K_v constructs retain functional gating properties

To assess whether the Halo-tag altered Na_v1.8 gating properties or the trafficking of the channel to the cell membrane, we transfected either Halo-Na_v1.8 or WT-Na_v1.8 into DRG neurons from Na_v1.8-KO (Na_v1.8-KO) mice and performed whole-cell voltage-clamp recordings (Fig. 2A). The insertion of the HaloTag did not alter the voltage dependence of activation (Fig. 2A, left) of the channel as reflected in similar half-activation voltage ($V_{1/2Act}$) (Halo-Na_v1.8 $V_{1/2Act}$: -13.89 ± 1.75 mV, $n = 7$; WT Na_v1.8 $V_{1/2Act}$: -11.77 ± 2.41 mV, $n = 7$; $p = 0.49$, Student's *t* test). There was a statistically significant hyperpolarizing shift in the half inactivation voltage (Fig. 2A, middle) of Halo-Na_v1.8 relative to WT-Na_v1.8 (Halo-Na_v1.8 $V_{1/2Inact}$: -42.12 ± 1.77 mV, $n = 7$; WT Na_v1.8 $V_{1/2Inact}$: -34.99 ± 2.56 mV, $n = 7$; $p = 0.039$, Student's *t* test). Halo-Na_v1.8 current density was similar to that of WT-Na_v1.8 (Fig. 2A, right) (Halo-Na_v1.8: -604.60 ± 251.89 pA/pF, $n = 7$; WT Na_v1.8: -580.41 ± 196.95 pA/pF, $n = 7$, $p = 0.94$, Student's *t* test).

We evaluated whether the Halo- and SNAPtag channel fusion altered Na_v1.7 biophysical properties and trafficking to the plasma membrane in HEK293 cells (Fig. 2B). The fusion of the Halo- and SNAP-tag to Na_v1.7 did not alter the voltage dependence of activation (Fig. 2B, left) of the tagged channels as reflected in similar $V_{1/2Act}$ (WT-Na_v1.7: -14.20 ± 2.37 mV, $n = 7$; SNAP-Na_v1.7: -14.62 ± 2.33 mV, $n = 4$; $p = 0.99$; Halo-Na_v1.7: -16.00 ± 1.34 mV, $n = 5$; $p = 0.78$; one-way ANOVA with Dunnett's multiple comparisons correction). Similarly, we did not detect a difference in the voltage dependence of fast inactivation (Fig. 2B, middle) between untagged and tagged channels (WT Na_v1.7 $V_{1/2Inact}$: -77.53 ± 1.34 mV, $n = 7$; SNAP-Na_v1.7 $V_{1/2Inact}$: -77.35 ± 2.05 mV, $n = 4$, $p = 0.99$; Halo-Na_v1.7 $V_{1/2Inact}$: -73.50 ± 1.85 mV, $n = 5$, $p = 0.17$). The peak current density (Fig. 2B, right) for WT-Na_v1.7 was -389.20 ± 73.89 pA/

pF ($n = 7$), which was statistically significantly greater than that of SNAP-Na_v1.7 (-140.67 ± 49.59 pA/pF, $n = 4$, $p = 0.0495$), but not statistically different from that of Halo-Na_v1.7 (-285.57 ± 58.50 pA/pF, $n = 5$, $p = 0.46$; one-way ANOVA with Dunnett's correction).

The gating properties of the Halo-Na_v1.6 construct were evaluated by automated voltage-clamp of transiently transfected HEK293 cells (Fig. 2C). The automated pulse protocols were configured to match as closely as possible those used in the manual patch-clamp experiments. There were no significant differences in the voltage dependence of activation for WT-Na_v1.6 ($V_{1/2Act} = -35.5 \pm 2.0$ mV, $n = 10$) compared with Halo-Na_v1.6 ($V_{1/2Act} = -31.6 \pm 1.5$ mV, $n = 8$, $p = 0.16$, Student's *t* test). There were also no significant differences in the voltage dependence of fast inactivation for WT-Na_v1.6 ($V_{1/2Inact} = -76.7 \pm 1.4$ mV, $n = 10$) compared with Halo-Na_v1.6 ($V_{1/2Inact} = -75.8 \pm 2.2$ mV, $n = 8$, $p = 0.7$, Student's *t* test). Although there was a trend toward higher current density (Fig. 2C, right) for WT-Na_v1.6 relative to Halo-Na_v1.6, the differences in current density did not reach significance (42.9 ± 10.6 pA/pF for WT, $n = 8$ vs 34.8 ± 2.4 pA/pF for Halo, $n = 10$, $p = 0.42$, Student's *t* test). The data indicate that the insertion of the Halo-tag into the Na_v1.6 channel results in functional channels in the plasma membrane and the biophysical properties of the Halo-Na_v1.6 channels are similar to WT-Na_v1.6 channels.

The gating properties of the Halo-Na_v1.9 construct were evaluated by voltage-clamp of transiently transfected rat SCG neurons (Fig. 2D). SCG neurons do not express any of the TTX-resistant sodium channels (Rush et al., 2006); thus, the endogenous sodium currents are fully blocked by TTX. Halo-Na_v1.9 was introduced into SCG neurons using electroporation as previously described (Dib-Hajj et al., 2009). Using external and internal solutions designed to isolate TTX-resistant Na currents, the currents of heterologously expressed Halo-Na_v1.9 or Na_v1.9-WT channels were recorded in voltage-clamp mode. The slow activation and inactivation kinetics observed are consistent with previously described Na_v1.9 currents (Cummins et al., 1999; Dib-Hajj et al., 1999). The peak inward currents measured at each stimulus potential were normalized to capacitance and averaged to create the IV curves (Fig. 2D, right). Each individual cell's IV curve was fit with the BoltzIV function to obtain the reversal potential and G_{max} and then transformed into a GV curve and normalized (Fig. 2D, left). Each GV curve was then fitted to the Boltzmann function to obtain the parameters $V_{1/2}$ of activation and activation slope. Although there was a trend toward higher current density (Fig. 2D, right) for Halo-Na_v1.9 relative to WT-Na_v1.9, the difference did not reach significance (2.8 ± 0.8 pA/pF for WT, $n = 9$ vs 7.8 ± 5.4 pA/pF for Halo, $n = 11$, $p = 0.4$, Student's *t* test). Additionally, there were no significant differences between the Halo-Na_v1.9 to Na_v1.9-WT for $V_{1/2}$ activation voltage or activation slope. The data indicate that the insertion of the Halo-tag into the Na_v1.9 channel results in functional channels in the plasma membrane and the biophysical properties of the Halo-Na_v1.9 channels are similar to Na_v1.9-WT channels.

The gating properties of the K_v7.2-Halo construct were evaluated by voltage clamp of transiently transfected HEK cells. Native M-current is composed of heteromeric mixtures of K_v7.2 and K_v7.3 α subunits. However, to unequivocally assign the observed current to the K_v7.2-Halo construct, we only transfected with this construct and any observed current should arise from K_v7.2-Halo homomeric channels. The expression of K_v7.2-WT or K_v7.2-Halo in separate transfections elicits slowly activating outward currents in response to

←

($n = 4$), or Halo-Na_v1.7 ($n = 5$) were analyzed to evaluate effect of tags on channel function. There were no significant differences in the voltage dependence of channel activation (left) or inactivation (middle) between either SNAP-Na_v1.7 or Halo-Na_v1.7 and WT-Na_v1.7. HEK293 cells expressing SNAP-Na_v1.7 displayed a significantly reduced peak current density (right), relative to WT-Na_v1.7. This is likely because the WT and Halo-Na_v1.7 channels have the codon-optimized sequence, which produces more protein than the nonoptimized SNAP-Na_v1.7 channel. However, there was no statistically significant difference in peak current density between WT-Na_v1.7 and Halo-Na_v1.7. **C**, Voltage-clamp recordings in HEK293 cells transiently transfected with mouse Na_v1.6 (either WT ($n = 10$) or Halo- ($n = 8$)) were analyzed to evaluate effect of the Halo-tag on channel function. There were no significant differences in the voltage dependence of channel activation (left) or inactivation (middle) between WT-Na_v1.6 and Halo-Na_v1.6. Although there was a trend toward higher current density (right) for WT-Na_v1.6 relative to Halo-Na_v1.6, the difference did not reach statistical significance ($p = 0.42$). **D**, Voltage-clamp recordings in SCG neurons expressing either Halo-Na_v1.9 ($n = 11$) or WT-Na_v1.9 ($n = 9$). There was no significant difference in channel activation between Halo-Na_v1.9 and WT-Na_v1.9 (left). Although there was a trend toward higher current density (right) for Halo-Na_v1.9 relative to WT-Na_v1.9, the difference did not reach significance ($p = 0.4$). **E**, The gating properties of the K_v7.2-Halo construct were evaluated by voltage clamp of transiently transfected HEK293 cells. The peak inward tail currents of K_v7.2-WT ($n = 9$) and K_v7.2-Halo ($n = 10$) were measured and transformed into conductance as a function of the preceding stimulus voltage and plotted, resulting in G-V curves, which were fitted to a Boltzmann function. There was no significant difference in the voltage dependence of channel activation between K_v7.2-WT and K_v7.2-Halo. **F**, The activation pulse protocol was repeated at various times after initiating whole-cell configuration, and the resulting G-V curves are shown in different colors as indicated. **G**, The values of the Boltzmann fit parameters (including the maximum conductance, half-activation voltage, and slope) for individual cells in each group are plotted. None of the parameters comparing K_v7.2-WT to K_v7.2-Halo was significantly different. Error bars indicate SEM.

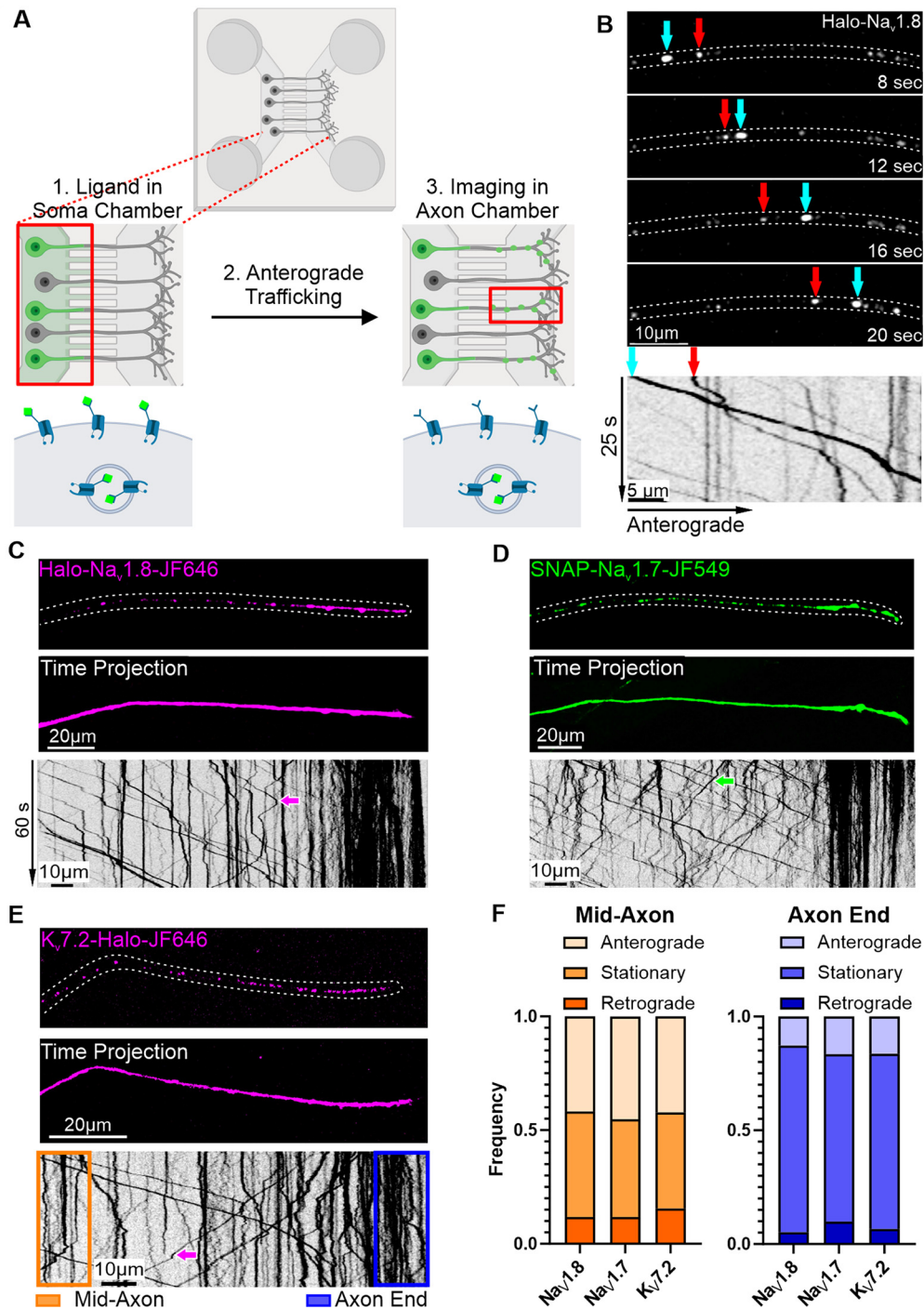


Figure 3. Vesicles containing Na_v or K_v channels are trafficked to distal axons with similar behavior. **A**, Schematic represents the OPAL technique used to visualize tagged channel trafficking. DRG neurons expressing Halo- or SNAP-tagged channels were cultured in MFCs for 5–7 d. Cell-permeable Halo- or SNAP-tag ligand conjugated fluorophore was added to the soma chamber, and axons in the distal chamber were imaged using spinning-disk confocal microscopy to capture anterogradely moving channels. **B**, Anterograde trafficking of Halo- $\text{Na}_v1.8$. Shown are multiple images from a single-color time-lapse movie of an axon (outlined with the dotted lines) in the axonal chamber after the labeling protocol described in **A**. Two Halo- $\text{Na}_v1.8$ -positive vesicles marked by colored arrows can be seen in each frame of the movie, moving anterogradely over time. Bottom, Kymograph generated from a movie of the above axon, plotting the distance along the axon on the x axis and elapsed time on the y axis. In this way, vesicles moving along the axon in the anterograde direction are seen as lines moving from the top left to bottom right, whereas stationary vesicles appear as vertical lines. **C–E**, Top panels, Still images of distal axons expressing Halo- $\text{Na}_v1.8$ (**C**), SNAP- $\text{Na}_v1.7$ (**D**), or $\text{K}_v7.2$ -Halo (**E**), labeled with Halo-tag or SNAP-tag ligands and imaged by OPAL showing discrete labeled puncta. Middle panels, Maximum intensity projections over time (60 s) of the axons above showing the outline and terminal end of the axons. Bottom, Kymographs from the above axons, showing anterograde motion of labeled channels, accumulation of channel at the distal end, and occasional reversal of channel direction (arrows). **F**, Average velocity (including pauses) of vesicles carrying each channel was measured in two regions of axons: the distal end and a more proximal section of mid-axon (boxed in blue and orange, respectively, in **E**). Vesicle motion was categorized as anterograde ($>0.5 \mu\text{m/s}$), stationary (-0.5 to $0.5 \mu\text{m/s}$), or retrograde ($<-0.5 \mu\text{m/s}$). The majority of vesicles ($\sim 80\%$) in axon ends are stopped, while more vesicles in the mid-axon have anterograde velocity. Few vesicles have retrograde velocity denoted by negative velocities. The behavior of vesicles carrying the different ion channels was similar (for detailed velocity analysis, see Fig. 4).

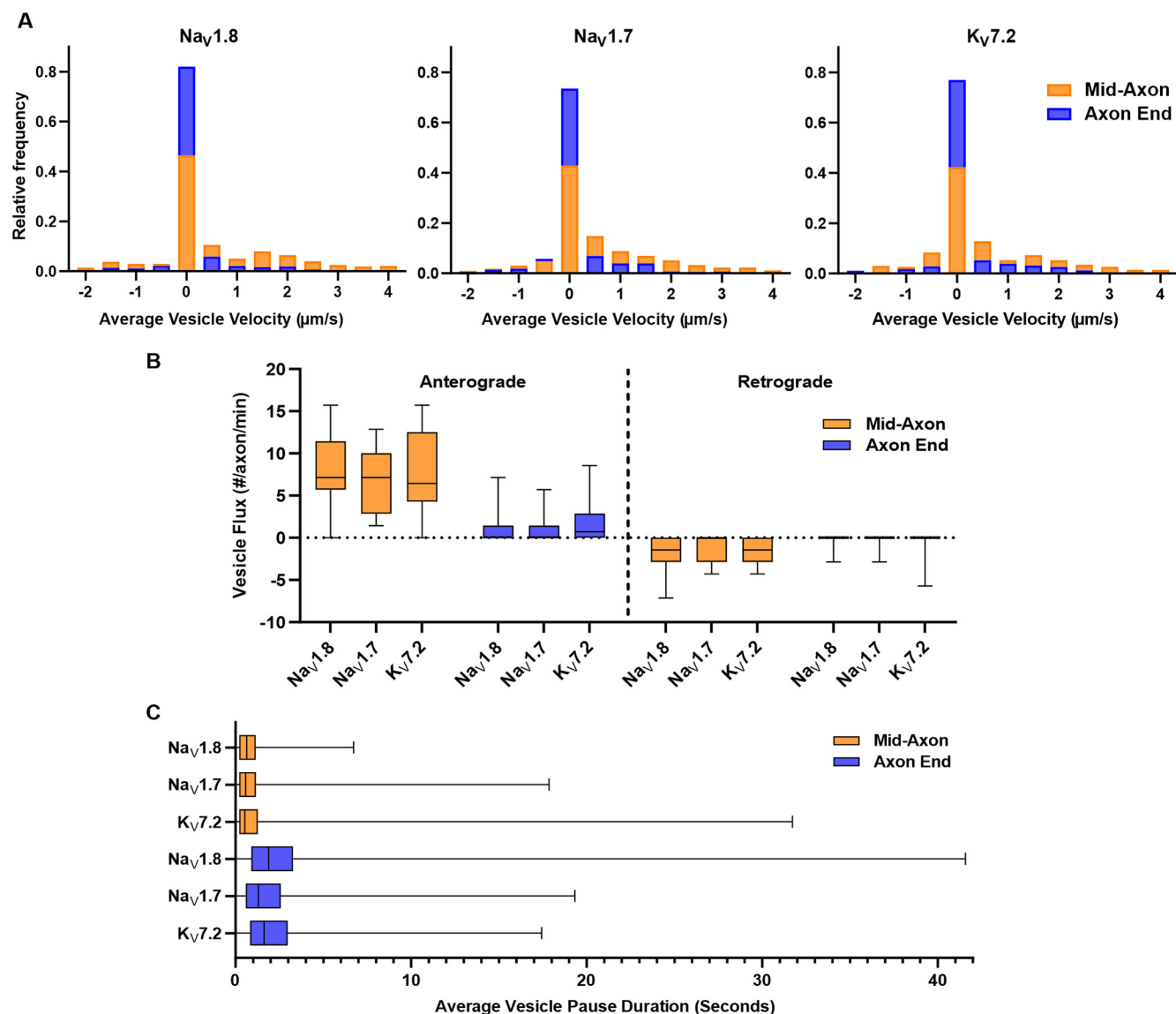


Figure 4. Vesicles containing Na_V or K_V channels are trafficked to distal axons with similar velocity, flux, and pausing behavior. Additional analysis of experiments shown in Figure 3F. **A**, Average velocity (including pauses) of vesicles carrying each channel was measured in two regions of axons: the distal end and a more proximal section of mid-axon. Shown are superimposed histograms of vesicle velocities in axon ends and mid-axons. The velocity distributions of axon ends compared with mid-axons were significantly different for each ion channel ($p < 0.0001$, Kolmogorov–Smirnov test). The velocity distributions of the different ion channels were similar ($p = 0.98$ and 0.53 for axon ends and mid-axons, respectively, Kruskal–Wallis test, $N = 3$ cultures, 24 axons, and 378–490 vesicles for each ion channel). **B**, The number of vesicles passing through a given axon segment (flux) in the anterograde direction was greater in the mid-axon than the axon end (Na_V1.8: $p < 0.0001$, Na_V1.7: $p < 0.0001$, K_V7.2: $p = 0.0002$). Flux in the retrograde direction trended toward being slightly greater in the mid-axon than axon end (Na_V1.8: $p = 0.12$, Na_V1.7: $p = 0.65$, K_V7.2: $p = 0.002$), although retrograde flux was less than anterograde flux for both mid-axon and axon end. Vesicles carrying the different ion channels had similar flux (Ant. Mid-Axon: $p = 0.94$, Ant. Axon End: $p = 0.31$, Ret. Mid-Axon: $p = 0.42$, Ret. Axon End: $p = 0.92$). **C**, The average duration of vesicle pauses was longer within the axon end compared with pauses in the mid-axon ($p < 0.0001$ for each ion channel). Vesicles carrying the different ion channels paused for similar durations in the mid-axon. There was a small but significant difference in pause durations between channels in the Axon End (Mid-Axon $p = 0.95$, Axon End $p = 0.002$). Plots show 25th, 50th, and 75th percentile, max, and min. $N = 3$ cultures, 24 axons, and 378–490 vesicles for each ion channel. Distributions of vesicles carrying a given ion channel were compared across axonal compartments using the Kolmogorov–Smirnov test, and distributions of vesicles carrying each of the three ion channels were compared within an axonal compartment using the Kruskal–Wallis test.

depolarizing voltage pulses. The conductance–voltage (G–V) curve is determined by measuring the peak “tail current” during the -120 mV pulse, which is proportional to the conductance achieved at the end of the preceding stimulation pulse. There was no significant difference in the voltage dependence of channel activation between K_V7.2-WT and K_V7.2-Halo (Student’s t test, Fig. 2E). To determine whether the gating properties vary with time after initiating whole-cell configuration, the activation stimulation protocol was repeated at various times, and the resulting G–V curves were fitted to the Boltzmann function and

are plotted as shown (Fig. 2F). The parameters for the Boltzmann functions fitted to the G–V curves are plotted (Fig. 2G). After initiation of whole-cell configuration, there is a time-dependent decrease in current density which was seen for both channels. There were no significant differences between K_V7.2-Halo and K_V7.2-WT in any of the fit parameters (Student’s t test). The data indicate that the insertion of the Halo-tag into the K_V7.2 channel results in functional channels in the plasma membrane and the biophysical properties of the K_V7.2-Halo current are similar to K_V7.2-WT current.

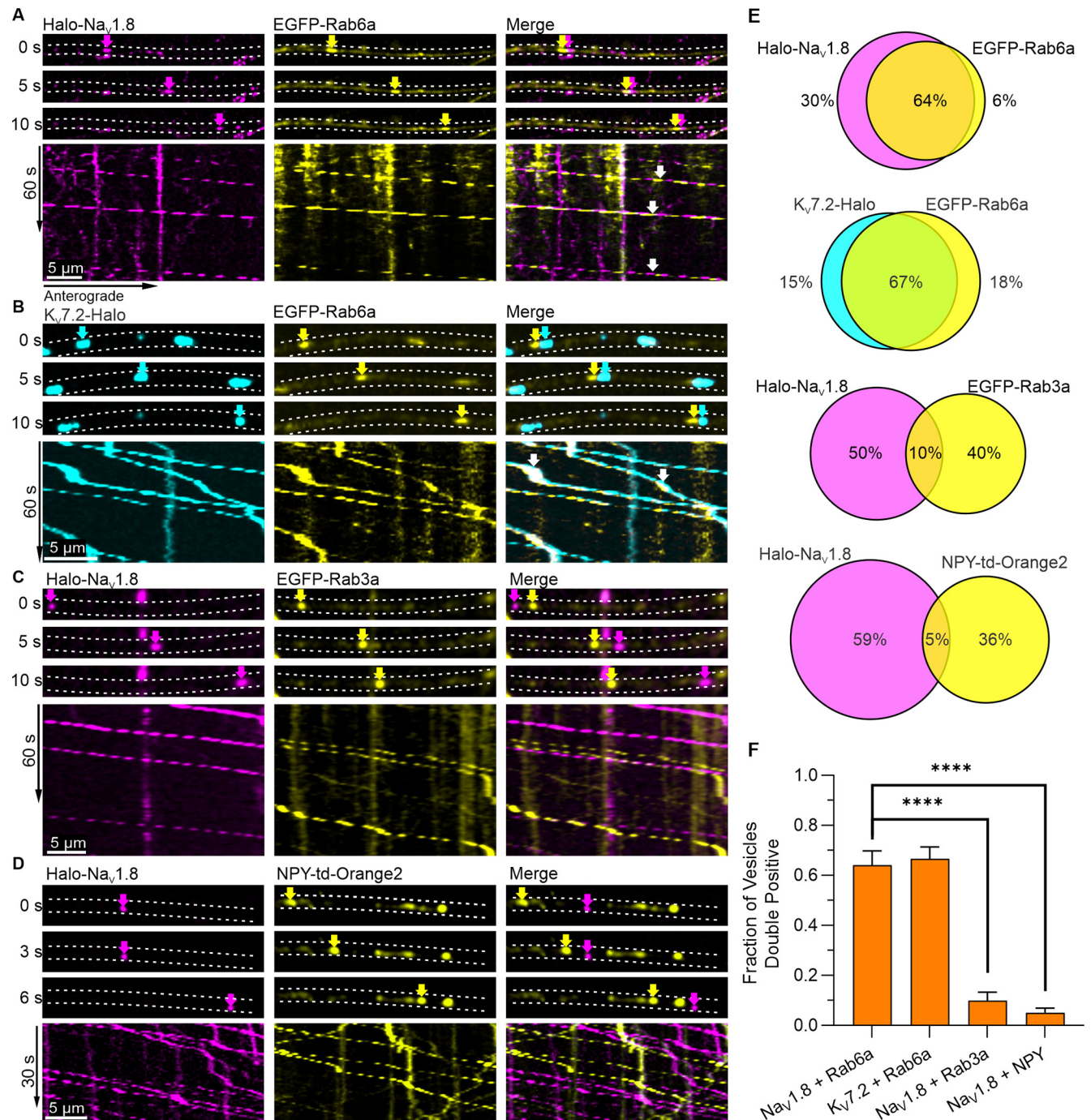


Figure 5. Na_v1.8 and K_v7.2 are trafficked anterogradely in Rab6a-positive vesicles. DRG neurons were transfected with tagged constructs and cultured in MFCs. Two-color, time-lapse OPAL imaging was performed using JF646-Halo-tag Ligand. Only axons expressing both transfected proteins were analyzed. **A–D**, Top panels, Selected frames from movies of outlined axons. Arrows point to vesicles as they move along the axon over time. Bottom panels, Two-color kymographs generated from the axons above. **A**, Halo-Na_v1.8 (pseudocolored magenta) and EGFP-Rab6a (pseudocolored yellow) colocalize in time and space. Right, Merged images represent close colocalization of Halo-Na_v1.8 and EGFP-Rab6a signals. The two-color kymograph represents three anterograde vesicles with overlapping signals for both proteins (white arrows) (see also Movie 1). **B**, K_v7.2-Halo and EGFP-Rab6a colocalize in time and space, moving and stopping together (white arrows). **C**, **D**, Halo-Na_v1.8 is trafficked independently of EGFP-Rab3a and NPY-td-Orange2. Right, Merged images and kymographs represent independent motion of Halo-Na_v1.8 (magenta) and EGFP-Rab3a (yellow) (**C**) or NPY-td-Orange2 (yellow) signals (**D**). **E**, **F**, Kymographs were analyzed via visual inspection, and vesicles were classified as either double-positive or single-positive for one of the tagged proteins: 64% of observed vesicles were Halo-Na_v1.8 and EGFP-Rab6a co-positive (159 of 248 vesicles, 19 axons); 67% of observed vesicles were K_v7.2-Halo and EGFP-Rab6a co-positive (236 of 354 vesicles, 25 axons); 10% and 5% of vesicles were Halo-Na_v1.8 and EGFP-Rab3a co-positive (39 of 394 vesicles, 21 axons) or Halo-Na_v1.8 and NPY-td-Orange2 co-positive (646 of 680 vesicles, 21 axons), respectively. **F**, The proportion of vesicles carrying Na_v1.8 and Rab6a is greater than the proportion carrying Na_v1.8 and Rab3a or Na_v1.8 and NPY (χ^2 , $p < 0.0001$). Error bars indicate 95% CIs calculated by the Wilson–Brown method. All experiments were performed with three independent cultures.

Vesicles containing Na_v or K_v channels are trafficked to distal axons with similar velocity and movement behavior

In a recent study (Akin et al., 2019), we developed the method of OPAL imaging, which allowed the study of channel trafficking in

real time with unprecedented resolution. This technique utilizes the MFC system to isolate tagged Na_v-expressing DRG somata from their distal axons. Importantly, the vast majority of the DRG neurons obtained by this protocol are unmyelinated C

fibers as determined by their expression of the neurofilament peripherin (Akin et al., 2019). After addition of brightly fluorescent, cell-permeable JaneliaFluor tag ligands (Grimm et al., 2015) to the soma chamber, channels are labeled, and those that are trafficked to the distal axon chamber are visualized with very low background fluorescence (Fig. 3A). This substantially improves the signal-to-noise ratio, allowing the detection of dim particles carrying few labeled channels. Using this technique, we have previously demonstrated that Halo-Nav1.7 is transported to distal axons in vesicles that traffic along microtubules (Akin et al., 2019).

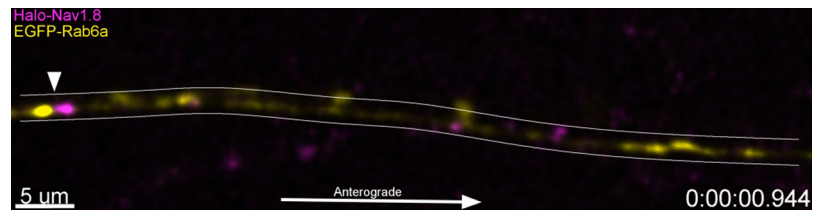
In order to test whether Nav1.8 are trafficked in a similar manner to Nav1.7, we conducted OPAL imaging using Halo-Nav1.8-transfected DRG neurons. Minutes after labeling in the soma chamber, tagged Halo-Nav1.8 channels were observed proceeding along axons in the distal axonal chamber. Figure 3B shows examples of two such vesicles, their positions over time denoted by the cyan and red arrows. The movement of vesicles along axons is shown as kymographs, which display distance along the axon on the *x* axis and elapsed time along the *y* axis. Moving vesicles appear as slanted lines, while stationary vesicles appear as vertical lines (Fig. 3B).

Channels proceeded in the anterograde direction, with periods of forward motion interrupted by pausing until they reached the distal end, where they accumulated at high concentrations (Fig. 3C). Kymograph analysis shows most vesicles moving in the anterograde direction until they reach the end, where they remain in this region over the time observed. However, some of the channels reversed direction and moved retrogradely, a pattern that increases with time after labeling. This behavior is similar to what we observed previously with Halo-Nav1.7 (Akin et al., 2019), as well as the newly characterized SNAP-Nav1.7 (Fig. 3D) and Kv7.2-Halo (Fig. 3E).

We compared the behavior of vesicles containing various ion channels both at the distal end of the axon and in a segment of the mid-axon, 100 μ m away from the distal end. We observed that many vesicles in the mid-axon had anterograde velocity, while most of the vesicles in the distal end had stopped and few vesicles in either region moved retrogradely (Figs. 3F, 4A). The number of vesicles moving through a segment of axon (flux) in the anterograde direction was greater in the mid-axon than the axon end. Flux in the retrograde direction was also greater in the mid-axon than axon end, but retrograde flux was less than anterograde flux in both the mid-axon and axon end (Fig. 4B). Finally, analysis of vesicle pausing behavior revealed that vesicles were stationary for longer periods of time in the axon end than in the mid-axon (Fig. 4C). Importantly, the velocity, flux, and pausing behavior of vesicles carrying the different tagged channels were similar to each other. Together, these results show that vesicles containing Nav1.7, Nav1.8, or Kv7.2 similarly travel to distal axons over long distances, pause in axon ends, and occasionally reverse direction.

Nav1.8 and Kv7.2 are trafficked anterogradely in Rab6a-positive vesicles

After observing that Nav1.7, Nav1.8, and Kv7.2 are trafficked over long distances to distal axons in a similar manner, we investigated whether they could be transported with the same protein partners. In our previous screen of Rab proteins, small cytoplasmic guanosine triphosphatases (GTPases) involved in vesicular



Movie 1. Related to Figure 5. Nav1.8 is co-trafficked anterogradely in Rab6a-positive vesicles. DRG neurons were transfected with Halo-Nav1.8 and EGFP-Rab6a and cultured in MFCs. Two-color, time-lapse OPAL imaging was performed using cell-permeable Halo-tag ligand-JF646. White arrowheads indicate Halo-Nav1.8 and EGFP-Rab6a double-positive vesicles as they move along the axon outlined in white. The $\sim 1 \mu$ m separation of the EGFP and Halo signals is because of the continued motion of the vesicle during the time between acquisition of images in separate channels. Importantly, the EGFP signal is always “behind” the Halo signal relative to the direction of motion because the EGFP (488 nm) channel is acquired first; 66% of observed vesicles are double-positive. [View online]

trafficking and markers of different vesicle populations (Hutagalung and Novick, 2011), Halo-Nav1.7 was shown to preferentially co-traffic with Rab6a, compared with 7 other Rabs expressed in DRG neurons (Akin et al., 2019). Here, we used OPAL imaging to visualize anterogradely trafficking Halo-Nav1.8 or Kv7.2-Halo containing vesicles in DRG neurons cotransfected with EGFP-Rab constructs. Halo-tagged channels were labeled using the far-red JF646-Halo-tag Ligand and simultaneously imaged with the EGFP-Rab by rapid laser and color filter switching; only axons expressing both labeled proteins were analyzed for the co-trafficking experiments. Dual-color time-lapse movies show vesicles containing both Halo-Nav1.8 and EGFP-Rab6a overlaying each other and traveling with the same trajectory (Fig. 5A; Movie 1). The $\sim 1 \mu$ m discrepancy between the Halo and Rab fluorescent signals visible in Figure 5A (Merge) is explained by continued motion of the vesicle during the 333 ms delay between acquisition of the green signal (488 nm excitation) and the far-red signal (637 nm excitation). Importantly, the green image (pseudocolored yellow) was acquired before the far-red image (pseudocolored magenta), which is consistent with the observation that the green signal is slightly behind the far-red signal relative to the direction of motion. Because these vesicles are exceedingly dim, true simultaneous two-color imaging has thus far been difficult because of loss of signal through light-splitting dichroic filters. However, the close colocalization and identical trajectory of the signals are strong evidence that the two proteins are trafficked together.

The majority of vesicles observed were positive for both Halo-Nav1.8 and EGFP-Rab6a, with 64% of vesicles being doubly positive (Fig. 5A,E,F). In a similar experiment, we observed a high level of co-trafficking of Kv7.2-Halo and EGFP-Rab6a (67% double positive, Fig. 5B,E,F). In contrast, Halo-Nav1.8 vesicles rarely contained EGFP-Rab3a, with only 10% of vesicles being doubly positive (Fig. 5C,E,F), which is similar to what was observed with Nav1.7 (Akin et al., 2019).

Since Rab6a has been associated with vesicles containing neuropeptides, such as neuropeptide Y (NPY) that are destined for the axon terminals (Gumy et al., 2017), we investigated whether Halo-Nav1.8 is co-trafficked with NPY. We repeated the co-trafficking experiment as above, cotransfecting rat DRGs with Halo-Nav1.8 and NPY-td-Orange2 (Gandasi et al., 2015), and culturing them in MFCs for 5–7 d before performing OPAL imaging. NPY-td-Orange2 clearly labels vesicles that are trafficked along the axon in both directions (Fig. 5D). Since primarily anterogradely moving Halo-Nav1.8 vesicles are visible in our assay, only anterogradely moving NPY vesicles were scored. Almost all (95%) of anterogradely moving Halo-Nav1.8 and NPY-td-Orange2 vesicles are trafficked independently (Fig. 5E,F).

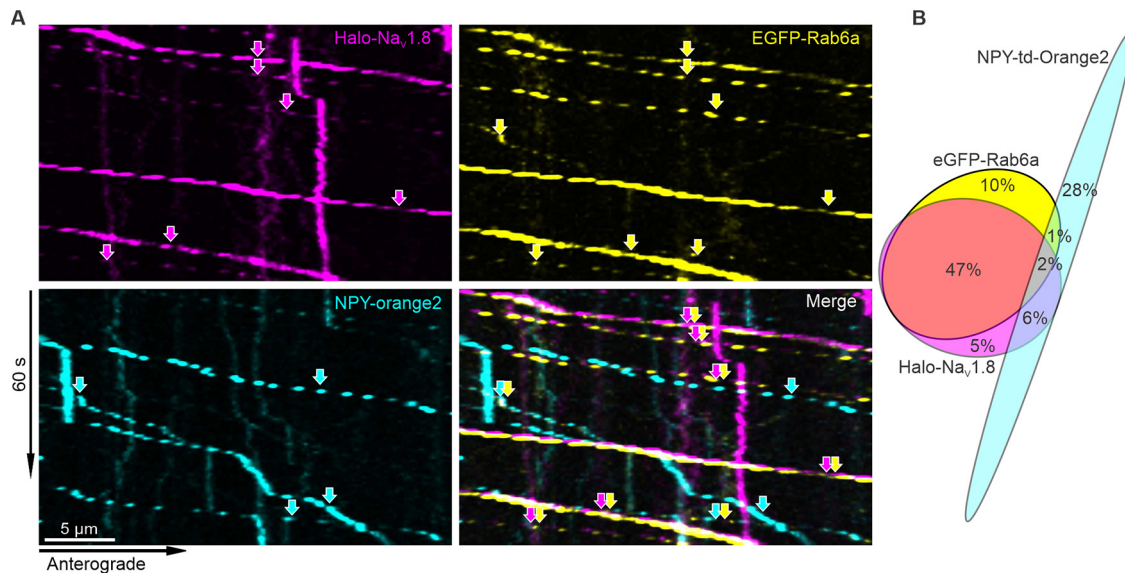


Figure 6. Na_v1.8 is trafficked with Rab6a and independently of NPY. DRG neurons were transfected with Halo-Na_v1.8, EGFP-Rab6a, and NPY-td-Orange2 and cultured in MFCs before three-color OPAL imaging was performed. Axons expressing all three proteins were analyzed. **A**, A three-color kymograph shows multiple vesicles containing pseudocolored Halo-Na_v1.8 (magenta), EGFP-Rab6a (yellow), and NPY-td-Orange2 (cyan). **B**, An area-proportional Venn diagram demonstrates that the majority of vesicles containing Halo-Na_v1.8 or Rab6a contain both (49% of total), while few vesicles that contain Halo-Na_v1.8 or EGFP-Rab6a contain NPY-td-Orange2 (9% of total). A total of 500 vesicles were observed in 17 axons from 3 cultures.

To compare the Na_v1.8, Rab6a, and NPY-containing vesicle populations directly, we repeated the experiment while transfecting neurons with Halo-Na_v1.8, EGFP-Rab6a, and NPY-td-Orange2. In agreement with the previous experiments, Na_v1.8 and Rab6a were mostly transported together (49% of total vesicles), while few vesicles that contained Na_v1.8 or Rab6a contained NPY (9% of total vesicles) (Fig. 6A,B). These results suggest that Na_v- and Rab6a-positive vesicles are not canonical neuropeptide-containing vesicles. This experiment also functions as a negative control, confirming that overexpression of proteins alone does not cause co-trafficking in the same vesicles.

Together, these results show that both Na_v channels and K_v channels are sorted into Rab6a-positive vesicles before transport to distal axons. Further, this vesicular sorting is selective, since Na_v channels are excluded from Rab3a- and NPY-carrying vesicles.

Na_v α and β subunits are trafficked to distal axons in the same vesicles, independent of the ankyrin binding motif

Our data showing that both Halo-Na_v1.7 (Akin et al., 2019) and Halo-Na_v1.8 (Fig. 5) are trafficked with Rab6a suggest the possibility that multiple Na_v isoforms might be trafficked together in the same vesicles. We tested this directly by making use of multiple compatible tagging strategies in dual-color OPAL imaging.

First, we performed a control experiment in neurons transfected with SNAP-Na_v1.7 and Halo-Na_v1.7, which are identical proteins apart from their tags. We reasoned that this would allow us to measure the highest expected rate of colocalization for two tagged proteins in this assay. Halo-Na_v1.7 was labeled using the far-red JF646-Halo-tag Ligand, while SNAP-Na_v1.7 was labeled using the red JF549-cpSNAP-tag Ligand. Only axons expressing both channels were analyzed. The majority of observed vesicles were positive for the two Na_v1.7 constructs, with 68% being doubly positive (Fig. 7A). That the rate of co-trafficking of these two nearly identical proteins is not complete is likely because both the Halo-tag and SNAP-tag proteins do not react with their cognate ligands with complete efficiency (estimates of Halo-tag and SNAP-tag labeling efficiency range from 80% to 33% and 65% to 16%, respectively) (Latty et al., 2015; Virant et al., 2018; Lepore et al., 2019).

Further, we confirmed that the Halo-tag and SNAP-tag ligands bind selectively by showing that they do not label DRG neurons transfected with GFP only (Fig. 8A) and that they are not trafficked anterogradely in the absence of their receptors (Fig. 8B,C).

Next, we cotransfected rat DRG neurons with both SNAP-Na_v1.7 and Halo-Na_v1.8 and cultured them in MFCs before performing dual-color OPAL imaging. Indeed, dual-color time-lapse imaging revealed Halo-Na_v1.8 channels and SNAP-Na_v1.7 traveling along the axon together with identical trajectories (Fig. 7B; Movie 2). The majority of observed vesicles are positive for both SNAP-Na_v1.7 and Halo-Na_v1.8, with 62% of vesicles being doubly positive (Fig. 7B).

A recent study showed that NF186 and NrCAM (components of nodes of Ranvier, which are structures defined in part by the cytoskeletal scaffold protein Ankyrin G), are cotransported along myelinated axons, and that this common vesicular sorting is driven by an Ankyrin binding motif (ABM) present in these proteins (FIGQY) (Bekku and Salzer, 2020). A different ABM ((V/A)P(I/L)AXXE(S/D)D) (Lemaitre et al., 2003) is present in Na_v channels (even in those that are not commonly found at nodes of Ranvier) (Gasser et al., 2012), and Na_v1.8 was suggested to bind constitutively to Ankyrin G (Monterisino et al., 2014). Thus, we tested whether this motif is necessary for the co-trafficking of Na_v1.7 and Na_v1.8 by deleting it from Na_v1.8 (Halo-Na_v1.8-dABM). In axons expressing both SNAP-Na_v1.7 and Halo-Na_v1.8-dABM, we observed that 66% of vesicles were double-positive (Fig. 7C). This suggests that the co-sorting of these proteins occurs independently of binding to Ankyrin.

We next investigated the trafficking of Na_v1.7 and the other Na_v channels expressed in mature sensory neurons, Na_v1.9 and Na_v1.6. We saw that 61% of observed vesicles were positive for both SNAP-Na_v1.7 and Halo-Na_v1.9 (Fig. 7D). Similarly, we found that 65% of observed vesicles were positive for both SNAP-Na_v1.7 and Halo-Na_v1.6 (Fig. 7E).

Na_v channels are composed of a pore-forming α subunit (Na_v1.1–1.9) and associated β subunits (Na_vβ 1–4), which modulate various aspects of Na_v function and neuronal excitability (O'Malley and Isom, 2015; Alsouloum et al., 2019, 2021). Thus, we

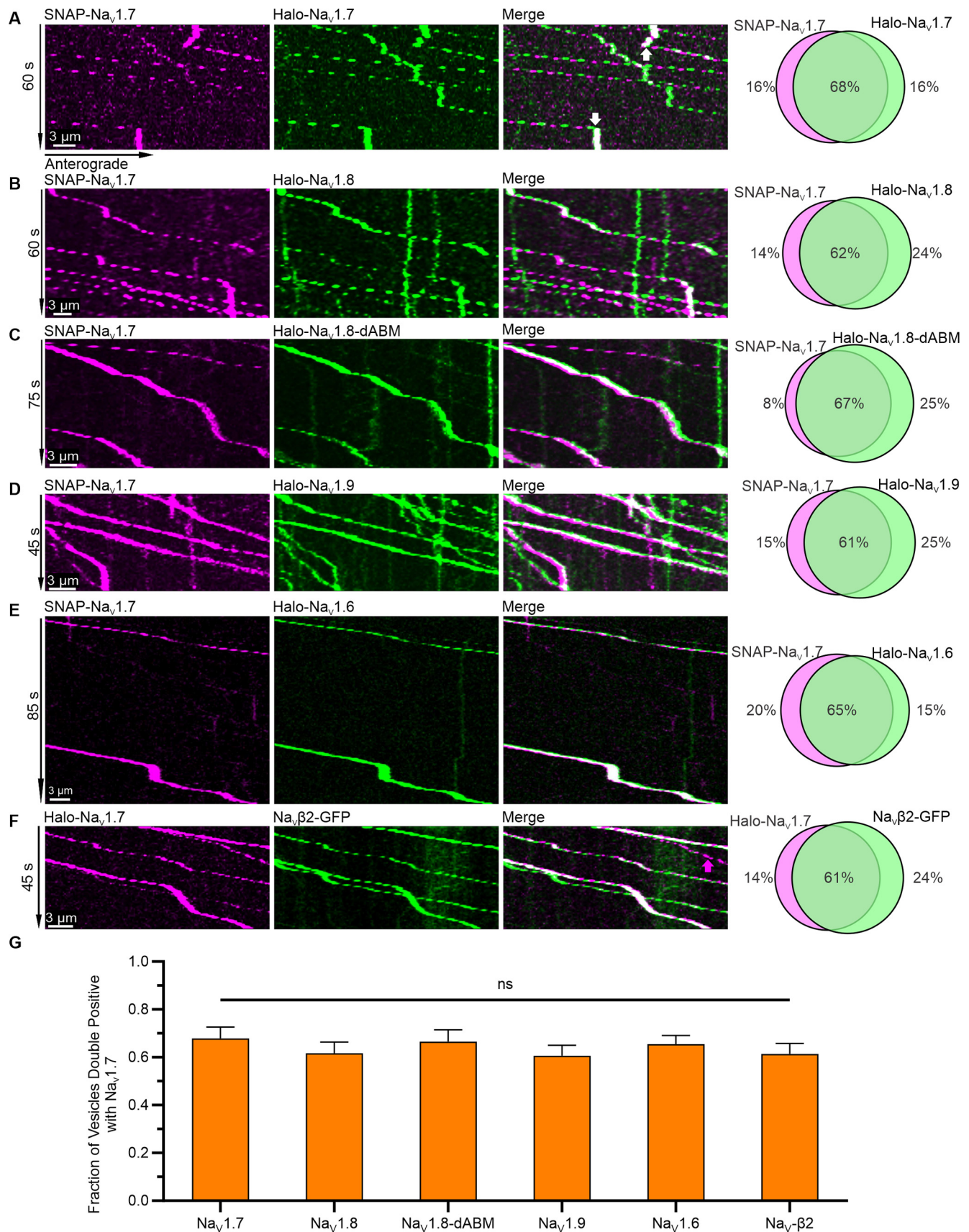


Figure 7. $\text{Na}_V1.7$ is trafficked to distal axons in the same vesicles as other Na_V isoforms, independent of the ABM. DRG neurons were transfected with $\text{Na}_V1.7$ and other constructs and cultured in MFCs. Two-color time-lapse OPAL imaging was performed. Only axons expressing both constructs were analyzed. From left to right, each panel shows a two-color kymograph with colors separated and then merged, and a Venn diagram illustrating the proportion of vesicles classified as positive for either construct or both. **A**, To measure the highest expected level of co-trafficking for this assay, the experiment was performed using Halo- $\text{Na}_V1.7$ and SNAP- $\text{Na}_V1.7$, which are identical proteins apart from their tags. SNAP- $\text{Na}_V1.7$ and Halo- $\text{Na}_V1.7$ traveled together in 68% of observed

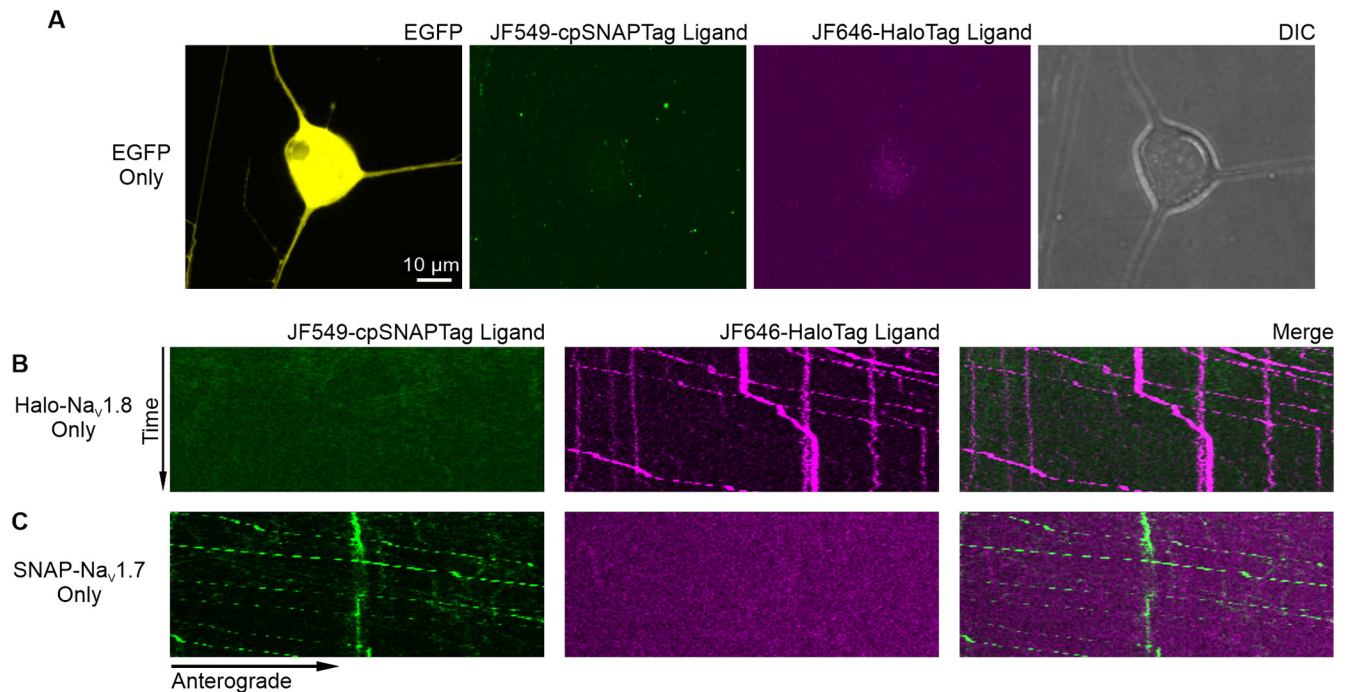


Figure 8. Fluorescent Halo-tag and SNAP-tag Ligands lack nonspecific binding, are spectrally separated, and are not trafficked anterogradely in the absence of their receptors. **A**, DRG neurons were transfected with EGFP only and cultured for 3 d. The cells were exposed to JF646-Halo-tag Ligand and JF549-cpSNAP-tag Ligand, washed, and then imaged. Shown is a confocal z stack of a DRG neuron in different spectral channels: 488 nm channel showing EGFP fluorescence (pseudocolored yellow), 561 nm channel showing lack of JF549 fluorescence (pseudocolored green), 637 nm channel showing lack of JF646 fluorescence (pseudocolored magenta), and DIC image showing normal DRG morphology. DRG neurons were transfected with Halo- $\text{Na}_V1.8$ only (**B**) or SNAP- $\text{Na}_V1.7$ only (**C**) and cultured in MFCs for 5 d before OPAL imaging. The soma chamber was exposed to both JF549-cpSNAP-tag Ligand and JF646-Halo-tag Ligand, washed, and then imaged. **B**, A kymograph of an axon transfected with Halo- $\text{Na}_V1.8$ only shows that the SNAP-tag ligand is not trafficked anterogradely in the absence of SNAP-tag, and the JF646 signal is absent in the 561 nm channel (pseudocolored green). **C**, A kymograph of an axon transfected with SNAP- $\text{Na}_V1.7$ only shows that the Halo-tag Ligand is not trafficked anterogradely in the absence of Halo-tag, and the JF549 signal is absent in the 637 nm channel (pseudocolored magenta).

tested whether Halo- $\text{Na}_V1.7$ and $\text{Na}_V\beta 2$ -GFP are trafficked together. We saw that 61% of vesicles were positive for both proteins (Fig. 7F). This result is perhaps unsurprising, given the known covalent binding of these proteins.

We statistically compared the cotransport of $\text{Na}_V1.7$ and other Na_V isoforms versus cotransport of Halo- $\text{Na}_V1.7$ and SNAP- $\text{Na}_V1.7$ (Fig. 7A) and show that all of the tested proteins were detected in a similar fraction of axonal vesicles (Fig. 7G). These data show that Na_V channels are not sorted into isoform-specific vesicles for long-distance axonal trafficking.

Na_V channels are trafficked to distal axons together with a specific group of neuronal membrane proteins from different classes

Next, we investigated whether $\text{Na}_V1.7$ channels are cotransported in axons with members of different classes of membrane

proteins with opposite or unrelated functions. To compare trafficking of ion channels with opposite but complementary function, we investigated sorting of $\text{Na}_V1.7$ and $\text{K}_V7.2$. In axons expressing both SNAP- $\text{Na}_V1.7$ and $\text{K}_V7.2$ -Halo, 66% of observed vesicles contained both proteins (Fig. 9A). Next, we tested two axonal membrane proteins that are expressed in DRG neurons but are not voltage-gated ion channels or subunits: Tumor Necrosis factor Receptor 1 (TNFR1) (Wheeler et al., 2014) and Sodium Calcium Exchanger 2 (NCX2) (Persson et al., 2010). Halo- $\text{Na}_V1.7$ and TNFR1-SNAP traveled together in 63% of observed vesicles (Fig. 9B). In contrast, Halo- $\text{Na}_V1.7$ and NCX2-mCherry traveled together in only 4% of observed vesicles (Fig. 9C). We then compared the cotransport of $\text{Na}_V1.7$ and other proteins versus cotransport of Halo- $\text{Na}_V1.7$ and SNAP- $\text{Na}_V1.7$ (the same control data as in Fig. 7A,G) and show that $\text{K}_V7.2$ and TNFR1 are co-trafficked with $\text{Na}_V1.7$ at similar rates, while NCX2 is trafficked independently (Fig. 9D).

Together, these results show that multiple ion channels and other axonal proteins can be trafficked together in the same vesicles, but that vesicular sorting is not indiscriminate, with several proteins (Rab3, NPY, and NCX2) being packaged into separate vesicles from Na_V channels. Importantly, ion channels are not segregated into distinct vesicle populations based on their physiological functions, but instead trafficked together (Fig. 10).

Discussion

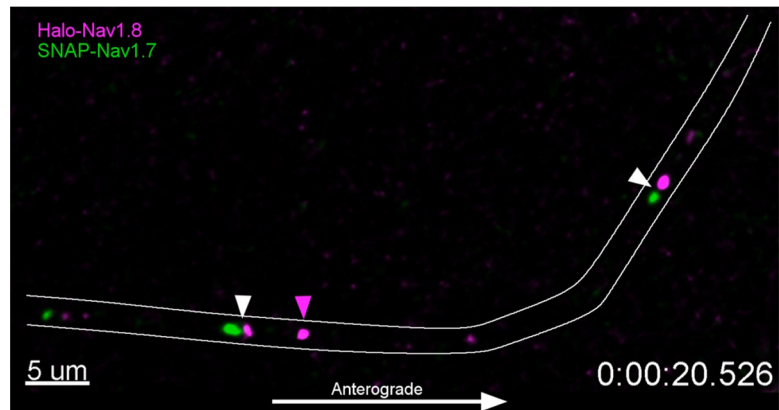
The principal question addressed by this study is whether axonal proteins with diverse functions are sorted into different vesicles or transported together within the same vesicles. There is

vesicles (339 vesicles in 21 axons from 3 cultures). White arrows indicate points where vesicles stop, and the two signals colocalize perfectly. Having identical trajectories through time and space strongly suggests that both proteins are in the same vesicle. **B**, SNAP- $\text{Na}_V1.7$ and Halo- $\text{Na}_V1.8$ traveled together in 62% of observed vesicles (402 vesicles in 22 axons from 3 cultures) (see also Movie 2). **C**, SNAP- $\text{Na}_V1.7$ and Halo- $\text{Na}_V1.8$ -DABM traveled together in 66% of observed vesicles (326 vesicles in 21 axons from 3 cultures). **D**, SNAP- $\text{Na}_V1.7$ and Halo- $\text{Na}_V1.9$ traveled together in 61% of observed vesicles (460 vesicles in 21 axons from 3 cultures). **E**, SNAP- $\text{Na}_V1.7$ and Halo- $\text{Na}_V1.6$ traveled together in 65% of observed vesicles (625 vesicles in 21 axons from 3 cultures). **F**, Halo- $\text{Na}_V1.7$ and $\text{Na}_V\beta 2$ -GFP traveled together in 61% of observed vesicles (456 vesicles in 21 axons from 3 cultures). Magenta arrow indicates a vesicle positive for Halo- $\text{Na}_V1.7$ only. **G**, Proportions of vesicles carrying both channels were compared by an omnibus χ^2 test ($p = 0.15$). Error bars indicate 95% CIs calculated by the Wilson–Brown method.

evidence for both co-trafficking (Maas et al., 2012) and independent trafficking of neuronal proteins (Jensen et al., 2014; Bekku and Salzer, 2020). In this study, we saw that multiple types of proteins, including ion channels with diverse physiological functions, are sorted into common vesicles (Fig. 9). First, we show that all of the major Na_V channels in sensory neurons are transported in the same vesicles, indicating that Na_V channel trafficking to distal axons is not isoform-dependent. This lack of specificity of channel transport extends to a related class of voltage-gated ion channel, $\text{K}_V7.2$, and to an unrelated membrane protein, TNFR1. However, it appears that there is a degree of specificity in vesicular sorting, since Na_V channels are sorted into vesicles distinct from those carrying Rab3, NPY, or the membrane protein NCX2. Thus, while multiple proteins with diverse physiological functions are trafficked within sensory axons in the same vesicles, this process is not indiscriminate and a degree of selectivity in sorting these axonal proteins exists.

Our current limited understanding of Na_V channel trafficking stems from difficulty visualizing channels in live neurons, especially at a distance from the soma. Studying tagged Na_V channels was historically difficult because previous tagging strategies disrupted the channel's physiological tuning and low levels of expression led to dim fluorescent signals. Recently, however, we succeeded in tagging full-length human Na_V channels without impairing their function, and developed novel imaging strategies which have enabled high-resolution imaging of full-length $\text{Na}_V1.7$ trafficking and surface distribution in live DRG axons in real time (Akin et al., 2019, 2021). Building on these advances, the present study represents the first investigation of the trafficking of multiple Na_V channel isoforms together with other axonal proteins in live neurons. We show that $\text{Na}_V1.8$ and $\text{K}_V7.2$ are trafficked in Rab6a-positive vesicles, but not Rab3a-positive vesicles, as we previously showed for $\text{Na}_V1.7$ (Akin et al., 2019). Further, we demonstrate that $\text{Na}_V1.8$ and Rab6a containing vesicles generally do not contain NPY in DRG axons. We also show that $\text{Na}_V1.6$, $\text{Na}_V1.7$, $\text{Na}_V1.8$, and $\text{Na}_V1.9$ (the primary Na_V channel isoforms expressed in unmyelinated DRG neurons, which constitute the vast majority of the neurons in our culture system) (Akin et al., 2019) are transported in the same vesicles, indicating that Na_V channel delivery to distal axons is not isoform-dependent, and that this sorting is not dependent on the conserved Na_V Ankyrin binding motif. We also show that both Na_V and K_V channels can be transported in the same axonal vesicles. Finally, we demonstrate that an integral membrane protein from a distinct functional class (TNFR1) is transported to axons in the same vesicles as $\text{Na}_V1.7$, whereas another membrane protein, NCX2, is transported independently. These data demonstrate the power of the OPAL method to investigate cell biological questions that were previously intractable.

While one might have expected proteins that contribute to the depolarizing upstroke (Na_V channels) and repolarizing downstroke (K_V channels) of the action potential to be transported separately from each other so that a neuron could modulate its excitability by adjusting the trafficking of either set of



Movie 2. Related to Figure 7. $\text{Na}_V1.7$ and $\text{Na}_V1.8$ are co-trafficked to distal axons in the same vesicles. DRG neurons were transfected with both SNAP- $\text{Na}_V1.7$ and Halo- $\text{Na}_V1.8$ and cultured in MFCs. Two-color time-lapse OPAL imaging was performed using cell-permeable Halo-tag ligand-JF646 (magenta) and SNAP-tag ligand-JF549 (green). White arrowheads indicate Halo- $\text{Na}_V1.8$ and SNAP- $\text{Na}_V1.7$ double-positive vesicles as they move anterogradely along the axon outlined in white. Magenta and green arrowheads indicate less frequently observed vesicles that are single-positive for Halo- $\text{Na}_V1.8$ or SNAP- $\text{Na}_V1.7$, respectively. As described previously, the $\sim 1 \mu\text{m}$ separation of the SNAP and Halo signals is because of the continued motion of the vesicle during the time between acquisition of images in separate channels. When the vesicle is moving, the SNAP signal is always “behind” the Halo signal because the SNAP (561 nm) channel is acquired first. When the vesicle stops, however, the two signals overlap completely; 61% of observed vesicles are double-positive. [View online]

proteins independently, the co-trafficking of Na_V and K_V channels seen here shows that this is not the case. Despite the co-trafficking of these different classes of voltage-gated ion channels, the neuron may regulate the relative amounts of these proteins at their sites of action by multiple other mechanisms, including transcription, translation, vesicular loading, membrane insertion, and endocytosis to balance the relative abundance of these proteins to maintain homeostasis of excitability.

A recent study has reported that some components of myelinated neurons are trafficked independently of each other, while others are trafficked together in an Ankyrin-dependent manner (Bekku and Salzer, 2020). Importantly, the authors of that study examined protein components of nodes of Ranvier, which depend on Ankyrin binding to localize to their site of function at nodes in myelinated neurons. In contrast, our study focused on proteins which are not always associated with Ankyrin, and our experiments were conducted in unmyelinated neurons where Ankyrin is not thought to play as important of a role in membrane organization. Thus, while Ankyrin binding may play an important role in vesicular sorting of proteins which depend on it for proper localization at specific neuronal compartments (the axon initial segment and nodes of Ranvier) in cells which use it for membrane organization, such as myelinated axons, it is less important for sorting in cells that do not rely on this mechanism. It is possible that the conserved ABM found in $\text{Na}_V1.7$ and $\text{Na}_V1.8$ may be important for their trafficking and localization at some nodes of Ranvier (Henry et al., 2005; Black et al., 2012) in myelinated neurons, whereas their transport in unmyelinated fibers and soma surface is independent of the ABM, similar to the role of the ABM in promoting delivery of $\text{Na}_V1.6$ to the axon initial segment but not the soma (Akin et al., 2015).

To our knowledge, the present studies are the only observations of the trafficking of multiple full-length Na_V channels in living cells to date. The paucity of data in this area is because of the inherent challenges of visualizing the dim fluorescent signals of low-abundance Na_V channels. However, there are several limitations of these experiments. First, it is possible that overexpressed

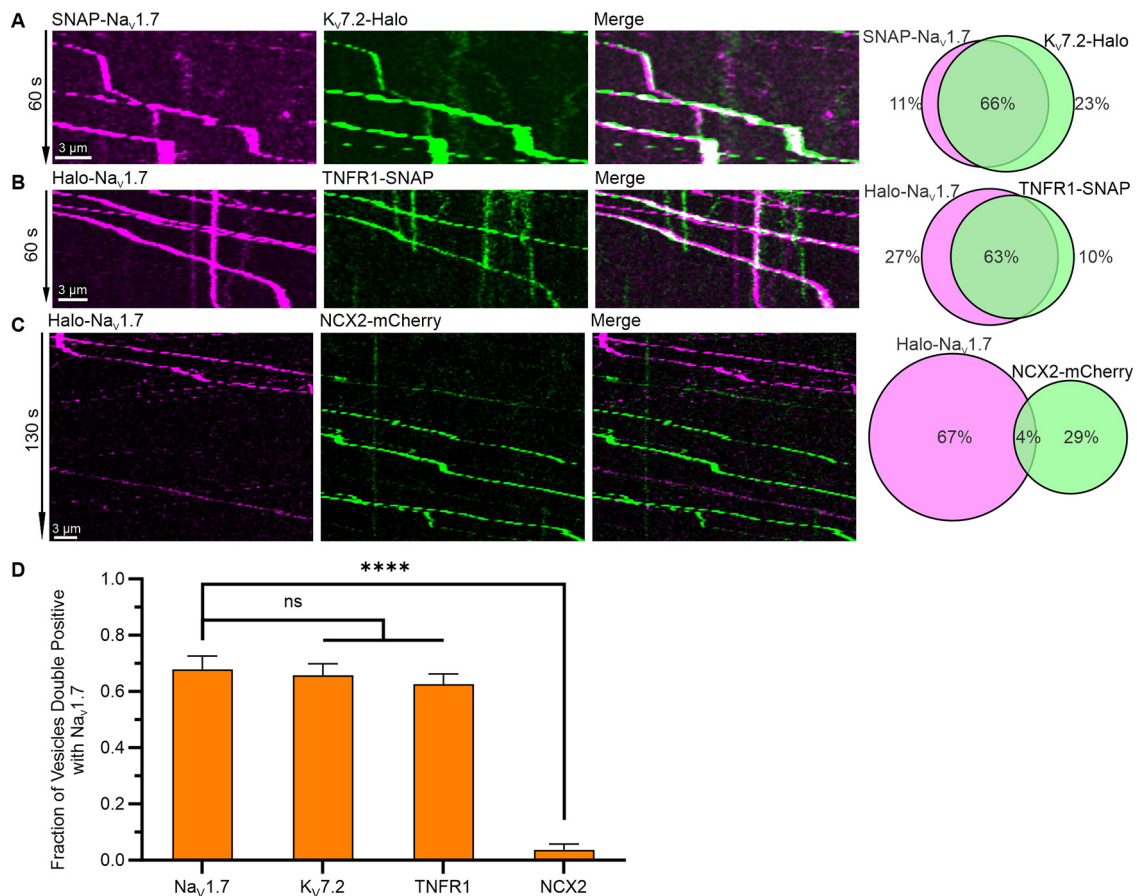


Figure 9. Nav_v1.7 is trafficked to distal axons in the same vesicles as Kv7.2 and TNFR1, but separately from NCX2. Experiments similar to those shown in Figure 7 were performed. **A**, SNAP-Nav_v1.7 and Kv7.2-Halo traveled together in 66% of observed vesicles (329 of 500 vesicles in 18 axons from 3 cultures). **B**, Halo-Nav_v1.7 and TNFR1-SNAP traveled together in 63% of observed vesicles (423 of 675 vesicles in 19 axons from 3 cultures). **C**, Halo-Nav_v1.7 and NCX2-mCherry traveled together in 4% of observed vesicles (17 of 469 vesicles in 16 axons from 3 cultures). **D**, Proportions of vesicles carrying both channels were compared by an omnibus χ^2 test ($p < 0.0001$), followed by pairwise χ^2 comparisons against the Nav_v1.7 versus Nav_v1.7 control (the same control data as that shown in Fig. 7G) with Bonferroni correction. ns, $p > 0.05$. **** $p < 0.0001$. Error bars indicate 95% CIs calculated by the Wilson–Brown method.

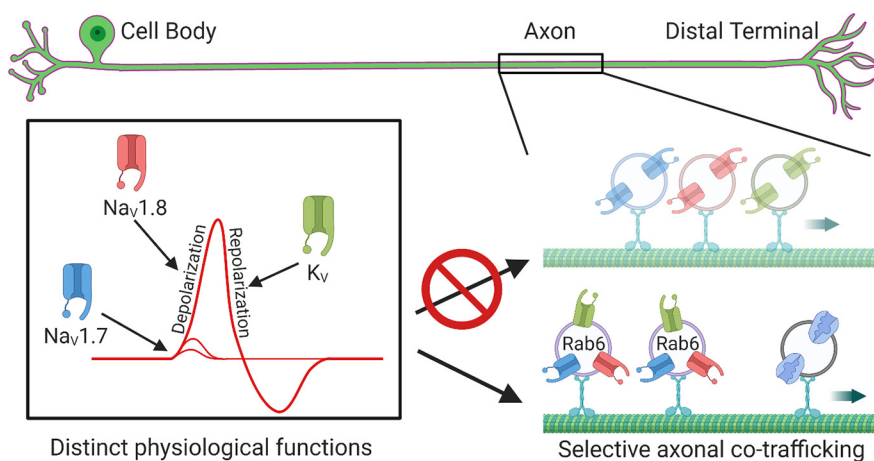


Figure 10. Depolarizing Nav_s and hyperpolarizing K_s are trafficked to distal axons together in the same vesicles. Multiple Nav_v and Kv channels with distinct physiological function are transported together in the same Rab6-positive vesicles. However, vesicular sorting of ion channels is also specific, since several proteins (Rab3, NPY, and NCX2) are packaged into distinct vesicle populations.

tagged proteins could be loaded into vesicles indiscriminately. However, our observations that Nav_v1.7 and Nav_v1.8 traffic independently of Rab3 and NPY, and that Nav_v1.7 moves independently of NCX2 (Akin et al., 2019; present study) demonstrate that overexpression itself does not cause proteins to be

nonspecifically packaged in the same axonal vesicles. Another limitation is that our reductionist model system is comprised of neurons growing *in vitro* without support cells, target cells, or extracellular matrix that may provide physiologically important cues (Zweifel et al., 2005). Nevertheless, this allows for strict experimental control and has the advantage that the observed patterns can be understood as neuron-intrinsic, requiring no extracellular influence. Further, the processes of anterograde vesicular sorting observed here are likely independent of extracellular cues by virtue of their intracellular compartmentalization.

The burden of pain is significant, and current pain treatments are often ineffective and addictive (Benyamin et al., 2008; Stahl et al., 2009). Alternatives are urgently needed. Of the Nav_v isoforms expressed in sensory neurons, Nav_v1.7, Nav_v1.8, and Nav_v1.9 are of particular interest because of their preferential expression in peripheral neurons and genetic validation in human pain syndromes (Bennett et al., 2019; Dib-Hajj and Waxman, 2019). Also of special interest is Kv7.2,

which acts to dampen DRG excitability and is associated with resilience to pain (King et al., 2014; Tsantoulas and McMahon, 2014; Mis et al., 2019). These factors have spurred efforts toward development of specific blockers of $\text{Na}_V1.7$ and $\text{Na}_V1.8$ (Alsalamoun et al., 2020) and, to a lesser extent, K_V7 activators for treatment of pain (Abd-Elseyed et al., 2019). However, ongoing efforts to develop modulators of Na_V and K_V conductance at the cell membrane have not yet resulted in new therapies. An alternative strategy for control of ion channel function is to alter the number of channels at the cell surface by modulating their trafficking to and from the cell membrane. Achieving this goal without causing side effects would require identifying and modulating mechanisms that mediate the trafficking of channels of interest but not other proteins. Our data demonstrate that multiple Na_V channels and K_V channels undergo long-distance transport together in the same vesicles. Thus, interfering with the trafficking of these shared vesicles would be expected to cause undesired side effects and would not be a promising therapeutic strategy. However, it is possible that other aspects of channel trafficking (e.g., exocytosis, endocytosis, and recycling) might be targeted more specifically. The mechanisms mediating these processes for Na_V channels are poorly understood and require further study.

Together, our results provide new insights into the development of excitable membranes, revealing complex sorting of axonal membrane proteins with diverse physiological functions into specific transport vesicles. Our recently developed OPAL imaging in live neurons has permitted the examination of specificity of ion channel trafficking, revealing co-packaging of axonal proteins with opposing physiological functions into the same transport vesicles. This suggests that additional mechanisms are necessary to regulate levels of surface channels and other membrane proteins. Our results thus reveal an important consideration for therapeutic strategies that target ion channel trafficking for the treatment of excitability disorders.

References

- Abd-Elseyed A, Jackson M, Gu SL, Fiala K, Gu J (2019) Neuropathic pain and K_V7 voltage-gated potassium channels: the potential role of K_V7 activators in the treatment of neuropathic pain. *Mol Pain* 15:1744806919864256.
- Akin EJ, Solé L, Dib-Hajj SD, Waxman SG, Tamkun MM (2015) Preferential targeting of $\text{Nav}1.6$ voltage-gated Na^+ channels to the axon initial segment during development. *PLoS One* 10:e0124397.
- Akin EJ, Higerd GP, Mis MA, Tanaka BS, Adi T, Liu S, Dib-Hajj FB, Waxman SG, Dib-Hajj SD (2019) Building sensory axons: delivery and distribution of $\text{Nav}1.7$ channels and effects of inflammatory mediators. *Sci Adv* 5:eaax4755.
- Akin EJ, Alsalamoun M, Higerd GP, Liu S, Zhao P, Dib-Hajj FB, Waxman SG, Dib-Hajj SD (2021) Paclitaxel increases axonal localization and vesicular trafficking of $\text{Nav}1.7$. *Brain* 144:1727–1737.
- Akopian AN, Sivilotti L, Wood JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* 379:257–262.
- Alexandrou AJ, et al. (2016) Subtype-selective small molecule inhibitors reveal a fundamental role for $\text{Nav}1.7$ in nociceptor electrogenesis, axonal conduction and presynaptic release. *PLoS One* 11:e0152405.
- Alsalamoun M, Estacion M, Almomani R, Gerrits MM, Bönhof GJ, Ziegler D, Malik R, Ferdousi M, Lauria G, Merkies IS, Faber CG, Dib-Hajj S, Waxman SG, PROPANE Study Group (2019) A gain-of-function sodium channel $\beta 2$ -subunit mutation in painful diabetic neuropathy. *Mol Pain* 15:1744806919849802.
- Alsalamoun M, Higerd GP, Effraim PR, Waxman SG (2020) Status of peripheral sodium channel blockers for non-addictive pain treatment. *Nat Rev Neurol* 16:689–705.
- Alsalamoun M, Labau JL, Sosniak D, Zhao P, Almomani R, Gerrits M, Hoeijmakers JG, Lauria G, Faber CG, Waxman SG, Dib-Hajj S (2021) A novel gain-of-function sodium channel $\beta 2$ subunit mutation in idiopathic small fiber neuropathy. *J Neurophysiol* 126:827–839.
- Amin AS, Asghari-Roodsari A, Tan HL (2010) Cardiac sodium channelopathies. *Pflugers Arch* 460:223–237.
- Bekku Y, Salzer JL (2020) Independent anterograde transport and retrograde cotransport of domain components of myelinated axons. *J Cell Biol* 219:e201906071.
- Bennett DL, Clark AJ, Huang J, Waxman SG, Dib-Hajj SD (2019) The role of voltage-gated sodium channels in pain signaling. *Physiol Rev* 99:1079–1151.
- Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R (2008) Opioid complications and side effects. *Pain Physician* 11:S105–S120.
- Black JA, Dib-Hajj S, McNabola K, Jeste S, Rizzo M, Kocsis J, Waxman S (1996) Spinal sensory neurons express multiple sodium channel α -subunit mRNAs. *Mol Brain Res* 43:117–131.
- Black JA, Renganathan M, Waxman SG (2002) Sodium channel $\text{Nav}1.6$ is expressed along nonmyelinated axons and it contributes to conduction. *Mol Brain Res* 105:19–28.
- Black JA, Liu S, Tanaka M, Cummins TR, Waxman SG (2004) Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. *Pain* 108:237–247.
- Black JA, Frézel N, Dib-Hajj SD, Waxman SG (2012) Expression of $\text{Nav}1.7$ in DRG neurons extends from peripheral terminals in the skin to central preterminal branches and terminals in the dorsal horn. *Mol Pain* 8:82.
- Blair NT, Bean BP (2002) Roles of tetrodotoxin (TTX)-sensitive Na^+ current, TTX-resistant Na^+ current, and Ca^{2+} current in the action potentials of nociceptive sensory neurons. *J Neurosci* 22:10277–10290.
- Burgess DL, Kohrman DC, Galt J, Plummer NW, Jones JM, Spear B, Meisler MH (1995) Mutation of a new sodium channel gene, *Scn8a*, in the mouse mutant ‘motor endplate disease.’ *Nat Genet* 10:461–465.
- Busserolles J, Tsantoulas C, Eschalier A, López García JA (2016) Potassium channels in neuropathic pain. *Pain* 157:S7–S14.
- Caldwell JH, Schaller KL, Lasher RS, Peles E, Levinson SR (2000) Sodium channel $\text{Nav}1.6$ is localized at nodes of Ranvier, dendrites, and synapses. *Proc Natl Acad Sci USA* 97:5616–5620.
- Cummins TR, Dib-Hajj SD, Black JA, Akopian AN, Wood JN, Waxman SG (1999) A novel persistent tetrodotoxin-resistant sodium current in SNS-null and wild-type small primary sensory neurons. *J Neurosci* 19:RC43.
- Dib-Hajj SD, Waxman SG (2019) Sodium channels in human pain disorders: genetics and pharmacogenomics. *Annu Rev Neurosci* 42:87–106.
- Dib-Hajj SD, Tyrrell L, Black JA, Waxman SG (1998) Na_V , a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. *Proc Natl Acad Sci USA* 95:8963–8968.
- Dib-Hajj SD, Tyrrell L, Cummins TR, Black JA, Wood PM, Waxman SG (1999) Two tetrodotoxin-resistant sodium channels in human dorsal root ganglion neurons. *FEBS Lett* 462:117–120.
- Dib-Hajj SD, Choi JS, Macala LJ, Tyrrell L, Black JA, Cummins TR, Waxman SG (2009) Transfection of rat or mouse neurons by biolistics or electroporation. *Nat Protoc* 4:1118–1126.
- Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, Persson AK, Hoeijmakers JG, Gerrits MM, Pierrro T, Lombardi R, Kapetis D, Dib-Hajj SD, Waxman SG (2012) Gain-of-function $\text{Nav}1.8$ mutations in painful neuropathy. *Proc Natl Acad Sci USA* 109:19444–19449.
- Gandasi NR, Vestö K, Helou M, Yin P, Saras J, Barg S (2015) Survey of red fluorescence proteins as markers for secretory granule exocytosis. *PLoS One* 10:e0127801.
- Gasser A, Ho TS, Cheng X, Chang KJ, Waxman SG, Rasband MN, Dib-Hajj SD (2012) An AnkyrinG-binding motif is necessary and sufficient for targeting $\text{Nav}1.6$ sodium channels to axon initial segments and nodes of Ranvier. *J Neurosci* 32:7232–7243.
- Gould HJ, England JD, Soignier RD, Nolan P, Minor LD, Liu ZP, Levinson SR, Paul D (2004) Ibuprofen blocks changes in $\text{Nav}1.7$ and $\text{Nav}1.8$ sodium channels associated with complete Freund’s adjuvant-induced inflammation in rat. *J Pain* 5:270–280.
- Grimm JB, English BP, Chen J, Slaughter JP, Zhang Z, Revyakin A, Patel R, Macklin JJ, Normanno D, Singer RH, Lionnet T, Lavis LD (2015) A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nat Methods* 12:244–250.
- Grubinska B, Chen L, Alsalamoun M, Rampal N, Matson DJ, Yang C, Taborn K, Zhang M, Youngblood B, Liu D, Galbreath E, Allred S, Lephed M,

- Ferrando R, Kornecook TJ, Lehto SG, Waxman SG, Moyer BD, Dib-Hajj S, Gingras J (2019) Rat Nav1.7 loss-of-function genetic model: deficient nociceptive and neuropathic pain behavior with retained olfactory function and intra-epidermal nerve fibers. *Mol Pain* 15:1744806919881846.
- Gumy LF, Katrukha EA, Grigoriev I, Jaarsma D, Kapitein LC, Akhmanova A, Hoogenraad CC (2017) MAP2 defines a pre-axonal filtering zone to regulate KIF1- versus KIF5-dependent cargo transport in sensory neurons. *Neuron* 94:347–362.e347.
- Heidbreder M, Zander C, Malkusch S, Wiedera D, Kaltschmidt B, Kaltschmidt C, Nair D, Choquet D, Sibarita JB, Heilemann M (2012) TNF- α influences the lateral dynamics of TNF receptor I in living cells. *Biochim Biophys Acta* 1823:1984–1989.
- Henry MA, Sorensen HJ, Johnson LR, Levinson SR (2005) Localization of the Nav1.8 sodium channel isoform at nodes of Ranvier in normal human radicular tooth pulp. *Neurosci Lett* 380:32–36.
- Hodgkin AL, Huxley AF (1952) Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J Physiol* 116:449–472.
- Huang J, Han C, Estacion M, Vasylyev D, Hoeijmakers JG, Gerrits MM, Tyrrell L, Lauria G, Faber CG, Dib-Hajj SD, Merkies IS, Waxman SG, PROPANE Study Group (2014) Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy. *Brain* 137:1627–1642.
- Hutagalung AH, Novick PJ (2011) Role of Rab GTPases in membrane traffic and cell physiology. *Physiol Rev* 91:119–149.
- Jakobs MA, Dimitracopoulos A, Franze K (2019) KymoButler, a deep learning software for automated kymograph analysis. *eLife* 8:e42288.
- Jensen CS, Watanabe S, Rasmussen HB, Schmitt N, Olesen SP, Frost NA, Blanpied TA, Misonou H (2014) Specific sorting and post-Golgi trafficking of dendritic potassium channels in living neurons. *J Biol Chem* 289:10566–10581.
- Jensen CS, Watanabe S, Stas JL, Klaphaak J, Yamane A, Schmitt N, Olesen SP, Trimmer JS, Rasmussen HB, Misonou H (2017) Trafficking of Kv2.1 channels to the axon initial segment by a novel nonconventional secretory pathway. *J Neurosci* 37:11523–11536.
- Jonker CT, Deo C, Zager PJ, Tkachuk AN, Weinstein AM, Rodriguez-Boulton E, Lavis LD, Schreiner R (2020) Accurate measurement of fast endocytic recycling kinetics in real time. *J Cell Sci* 133:jcs21225.
- King CH, Lancaster E, Salomon D, Peles E, Scherer SS (2014) Kv7.2 regulates the function of peripheral sensory neurons. *J Comp Neurol* 522:3262–3280.
- Kullmann DM (2010) Neurological channelopathies. *Annu Rev Neurosci* 33:151–172.
- Latty SL, Felce JH, Weimann L, Lee SF, Davis SJ, Klenerman D (2015) Referenced single-molecule measurements differentiate between GPCR oligomerization states. *Biophys J* 109:1798–1806.
- Lemaitre G, Walker B, Lambert S (2003) Identification of a conserved Ankyrin-binding motif in the family of sodium channel α subunits. *J Biol Chem* 278:27333–27339.
- Lepore A, Taylor H, Landgraf D, Okumus B, Jaramillo-Riveri S, McLaren L, Bakshi S, Paulsson J, Karoui ME (2019) Quantification of very low-abundant proteins in bacteria using the HaloTag and epi-fluorescence microscopy. *Sci Rep* 9:7902.
- Li Y, North RY, Rhines LD, Tatsui CE, Rao G, Edwards DD, Cassidy RM, Harrison DS, Johansson CA, Zhang H, Dougherty PM (2018) DRG voltage-gated sodium channel 1.7 is upregulated in paclitaxel-induced neuropathy in rats and in humans with neuropathic pain. *J Neurosci* 38:1124–1136.
- Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM, Burke EP, Lifton RP, Philipson KD (1994) Cloning of the NCX2 isoform of the plasma membrane Na(+)-Ca²⁺ exchanger. *J Biol Chem* 269:17434–17439.
- Liang L, Fan L, Tao B, Yaster M, Tao YX (2013) Protein kinase B/Akt is required for complete Freund's adjuvant-induced upregulation of Nav1.7 and Nav1.8 in primary sensory neurons. *J Pain* 14:638–647.
- Linley JE, Rose K, Patil M, Robertson B, Akopian AN, Gamper N (2008) Inhibition of M current in sensory neurons by exogenous proteases: a signaling pathway mediating inflammatory nociception. *J Neurosci* 28:11240–11249.
- Maas C, Torres VI, Altmann WD, Leal-Ortiz S, Wagh D, Terry-Lorenzo RT, Fejtova A, Gundelfinger ED, Ziv NE, Garner CC (2012) Formation of Golgi-derived active zone precursor vesicles. *J Neurosci* 32:11095–11108.
- Macdonald PL, Gardner RC (2000) Type I error rate comparisons of post hoc procedures for I j chi-square tables. *Educ Psychol Meas* 60:735–754.
- Mangeol P, Prevo B, Peterman EJ (2016) KymographClear and KymographDirect: two tools for the automated quantitative analysis of molecular and cellular dynamics using kymographs. *Mol Biol Cell* 27:1948–1957.
- Mantegazza M, Cestèle S, Catterall W (2021) Sodium channelopathies of skeletal muscle and brain. *Physiol Rev* 101:1633–1689.
- Micallef L, Rodgers P (2014) eulerAPE: drawing area-proportional 3-Venn diagrams using ellipses. *PLoS One* 9:e101717.
- Mis MA, Yang Y, Tanaka BS, Gomis-Perez C, Liu S, Dib-Hajj F, Adi T, Garcia-Milian R, Schulman BR, Dib-Hajj SD, Waxman SG (2019) Resilience to pain: a peripheral component identified using induced pluripotent stem cells and dynamic clamp. *J Neurosci* 39:382–392.
- Monterisino A, Brachet A, Ferracci G, Fache MP, Angles D'Ortoli S, Liu W, Rueda-Boroni F, Castets F, Dargent B (2014) Tetrodotoxin-resistant voltage-gated sodium channel Nav1.8 constitutively interacts with ankyrin G. *J Neurochem* 131:33–41.
- Nassar MA, Stirling LC, Forlani G, Baker MD, Matthews EA, Dickenson AH, Wood JN (2004) Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Proc Natl Acad Sci USA* 101:12706–12711.
- O'Malley HA, Isom LL (2015) Sodium channel β subunits: emerging targets in channelopathies. *Annu Rev Physiol* 77:481–504.
- Persson AK, Black JA, Gasser A, Cheng X, Fischer TZ, Waxman SG (2010) Sodium-calcium exchanger and multiple sodium channel isoforms in intra-epidermal nerve terminals. *Mol Pain* 6:84.
- Qian B, Park SH, Yu W (2020) Screening assay protocols targeting the Nav1.7 channel using Qube high-throughput automated patch-clamp system. *Curr Protoc Pharmacol* 89:e74.
- Renganathan M, Cummins TR, Waxman SG (2001) Contribution of Na(v)1.8 sodium channels to action potential electrogenesis in DRG neurons. *J Neurophysiol* 86:629–640.
- Rose K, Ooi L, Dalle C, Robertson B, Wood IC, Gamper N (2011) Transcriptional repression of the M channel subunit Kv7.2 in chronic nerve injury. *Pain* 152:742–754.
- Rush AM, Dib-Hajj SD, Liu S, Cummins TR, Black JA, Waxman SG (2006) A single sodium channel mutation produces hyper- or hypoexcitability in different types of neurons. *Proc Natl Acad Sci USA* 103:8245–8250.
- Rzomp KA, Scholtes LD, Briggs BJ, Whittaker GR, Scidmore MA (2003) Rab GTPases are recruited to chlamydial inclusions in both a species-dependent and species-independent manner. *Infect Immun* 71:5855–5870.
- Staahl C, Olesen AE, Andresen T, Arendt-Nielsen L, Drewes AM (2009) Assessing efficacy of non-opioid analgesics in experimental pain models in healthy volunteers: an updated review. *Br J Clin Pharmacol* 68:322–341.
- Strickland IT, Martindale JC, Woodhams PL, Reeve AJ, Chessell IP, McQueen DS (2008) Changes in the expression of Nav1.7, Nav1.8 and Nav1.9 in a distinct population of dorsal root ganglia innervating the rat knee joint in a model of chronic inflammatory joint pain. *Eur J Pain* 12:564–572.
- Tsantoulas C, McMahon SB (2014) Opening paths to novel analgesics: the role of potassium channels in chronic pain. *Trends Neurosci* 37:146–158.
- Virant D, Traenkle B, Maier J, Kaiser PD, Bodenhöfer M, Schmees C, Vojnovic I, Pisak-Lukáts B, Endesfelder U, Rothbauer U (2018) A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. *Nat Commun* 9:930.
- Waxman SG, Zamponi GW (2014) Regulating excitability of peripheral afferents: emerging ion channel targets. *Nat Neurosci* 17:153–163.
- Wheeler AM, Heffner LD, Kim S, Espy MS, Spano JA, Cleland LC, Deppmann DC (2014) TNF- α /TNFR1 signaling is required for the development and function of primary nociceptors. *Neuron* 82:587–602.
- Yang Y, Huang J, Mis MA, Estacion M, Macala L, Shah P, Schulman BR, Horton DB, Dib-Hajj SD, Waxman SG (2016) Nav1.7-A1632G mutation from a family with inherited erythromelalgia: enhanced firing of dorsal root ganglia neurons evoked by thermal stimuli. *J Neurosci* 36:7511–7522.
- Yuan JH, Estacion M, Mis MA, Tanaka BS, Schulman BR, Chen L, Liu S, Dib-Hajj FB, Dib-Hajj SD, Waxman SG (2021) KCNQ variants and pain modulation: a missense variant in Kv7.3 contributes to pain resilience. *Brain Commun* 3:fcab212.
- Zweifel LS, Kuruville R, Ginty DD (2005) Functions and mechanisms of retrograde neurotrophin signalling. *Nat Rev Neurosci* 6:615–625.