Ca\textsuperscript{2+} toxicity due to reverse Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange contributes to degeneration of neurites of DRG neurons induced by a neuropathy-associated Nav1.7 mutation

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Ca\textsuperscript{2+} toxicity due to reverse Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange contributes to degeneration of neurites of DRG neurons induced by a neuropathy-associated Nav1.7 mutation. J Neurophysiol 114: 1554–1564, 2015. First published July 8, 2015; doi:10.1152/jn.00195.2015.—Gain-of-function missense mutations in voltage-gated sodium channel Nav1.7 have been linked to idiopathic small-fiber neuropathy (SFN) (Faber et al. 2012; Han et al. 2012). SFN is characterized clinically by burning pain and autonomic abnormalities, and anatomically by degeneration of unmyelinated and thinly myelinated peripheral nerve fibers, including the loss of distal, intraepidermal nerve fibers (Hoeijmakers et al. 2012a). Although there is substantial interest in axonal degeneration in neuropathies, mechanistic details of the molecular cascade linking Nav1.7 mutations to nerve fiber degeneration are incompletely understood. The G856D mutation in Nav1.7 produces robust changes in channel biophysical properties, including hyperpolarized activation, depolarized inactivation, and enhanced ramp and persistent currents, which contribute to the hyperexcitability exhibited by neurons containing Nav1.8. We report here that cell bodies and neurites of dorsal root ganglion (DRG) neurons transfected with G856D display increased levels of intracellular Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]) and intracellular Ca\textsuperscript{2+} following stimulation with high [K\textsuperscript{+}] compared with wild-type (WT) Nav1.7-expressing neurons. Blockade of reverse mode of the sodium/calcium exchanger (NCX) or of sodium channels attenuates Ca\textsuperscript{2+} transients evoked by high [K\textsuperscript{+}] in G856D-expressing DRG cell bodies and neurites. We also show that treatment of WT or G856D-expressing neurites with high [K\textsuperscript{+}] or 2-deoxyglucose (2-DG) does not elicit degeneration of these neurites, but that high [K\textsuperscript{+}] and 2-DG in combination evokes degeneration of G856D neurites but not WT neurites. Our results also demonstrate that 0 Ca\textsuperscript{2+} or blockade of reverse mode of NCX protects G856D-expressing neurites from degeneration when exposed to high [K\textsuperscript{+}] and 2-DG. These results point to [Na\textsuperscript{+}] overload in DRG neurons expressing mutant G856D Nav1.7, which triggers reverse mode of NCX and contributes to Ca\textsuperscript{2+} toxicity, and suggest subtype-specific blockade of Nav1.7 or inhibition of reverse NCX as strategies that might slow or prevent axon degeneration in small-fiber neuropathy.

VOLTAGE-GATED SODIUM CHANNEL Nav1.7 is abundantly expressed within peripheral dorsal root ganglion (DRG) and sympathetic ganglion neurons (Rush et al. 2006; Toledo-Aral et al. 1997) and their axons, where it is co-expressed with the Na/Ca exchanger (NCX) 2 (Persson et al. 2010). Gain-of-function missense mutations in Nav1.7 channels have been linked to idiopathic small-fiber neuropathy (SFN) (Faber et al. 2012; Han et al. 2012). SFN is characterized clinically by burning pain and autonomic abnormalities, and anatomically by degeneration of unmyelinated and thinly myelinated peripheral nerve fibers, including the loss of distal, intraepidermal nerve fibers (Hoeijmakers et al. 2012a). Although there is substantial interest in axonal degeneration in neuropathies, mechanistic details of the molecular cascade linking Nav1.7 mutations to nerve fiber degeneration are incompletely understood. In this study, we provide evidence demonstrating Na\textsuperscript{+} overload and abnormal increases in intra-axonal Ca\textsuperscript{2+} due to reverse NCX in DRG neurons expressing the G856D Nav1.7 mutation, which may trigger downstream degenerative cascades.

The G856D mutation in Nav1.7 was identified in a multi-generational family with severe pain and dysautonomia due to SFN (Hoeijmakers et al. 2012b). No other cause was identified for the neuropathy, and the mutation segregated with disease phenotype (Hoeijmakers et al. 2012b). The G856D mutation was found to markedly enhance channel activation, shifting its voltage dependence 10–15 mV in a hyperpolarizing direction. The mutation also depolarizes the voltage dependence of inactivation by 6.2 mV, and it slows deactivation and markedly enhances the ramp current and persistent current produced by the channel. DRG neurons expressing the mutant G856D channel were found to have a resting membrane potential that was depolarized and displayed an increased incidence of spontaneous firing, together with decreased current threshold and increased frequency of firing in response to graded suprathreshold stimulation (Hoeijmakers et al. 2012b). We chose to assess the G856D mutation in this study because it induces relatively robust changes in channel function, compared with other Nav1.7 mutations associated with SFN (Faber et al. 2012; Han et al. 2012). In this study, we expressed the G856D mutant in cultured DRG neurons. We provide evidence that high K\textsuperscript{+} concentration ([K\textsuperscript{+}]) stimulated Na\textsuperscript{+} load in DRG neurons expressing mutant G856D channels leads to Ca\textsuperscript{2+} overload in cell bodies and neurites. We show that blocking of reverse action of the NCX2 attenuates Ca\textsuperscript{2+} overload. We further demonstrate that metabolic stress coupled with depolarization, at levels that do not evoke neurite degeneration in DRG neurons expressing wild-type (WT) Nav1.7 channels, triggers axon degeneration; calcium transient; sodium channels; sodium-calcium exchanger.

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neurite degeneration in DRG neurons expressing mutant G856D channels. Our data suggest that Ca2+ toxicity induced by hyperactive mutant Nav1.7 channels and reverse NCX contribute to degeneration of axons in Nav1.7 mutation-associated SNF.

**MATERIALS AND METHODS**

**DRG culture.** Experiments were carried out in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals; all animal protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Connecticut Healthcare System, West Haven, Connecticut. Adult Sprague-Dawley rats (4–6 wk) were deeply anesthetized by CO2 narcosis and decapitated. DRG were isolated and dissociated as described previously (Persson et al. 2013b). In brief, dissected ganglia were placed in ice-cold oxygenated complete saline solution, containing the following (in mM): 137 NaCl, 5.3 KCl, 1 MgCl2, 25 sorbitol, 3 CaCl2, 10 HEPES, pH 7.2. DRG were digested for 20 min at 37°C in complete saline solution containing collagenase D (1.5 mg/ml) and papain (30 U/ml). DRG were centrifuged and resuspended in DRG media [DMEM/F12 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA) and 1% fetal bovine serum (Sigma-Aldrich, St. Louis, MO)]. 1.5 mg/ml bovine serum albumin and 1.5 mg/ml trypsin inhibitor (Sigma)]. DRG were triturated in DRG media and centrifuged. The cell pellet was resuspended in DRG media, placed on a cushion of 15% bovine serum albumin in DRG media and centrifuged at 200 relative centrifugal force for 10 min to remove nonneuronal cells.

**Plasmids.** Human WT Nav1.7 and mutant G856D Nav1.7 plasmids were previously described (Hoeijmakers et al. 2012b). The G856D mutation was introduced into Nav1.7 complementary DNA (Herzog et al. 2003) using QuickChange XL site-directed mutagenesis, as described by Hoeijmakers et al. (2012b).

**Transfection of DRG neurons.** Dissociated DRG neurons were transfected by electroporation with WT Nav1.7 or mutant G856D constructs (AL, adult long splice isoform), along with enhanced green fluorescent protein (GFP) or mCherry (channel: GFP-to-mCherry ratio 10:2) using Nucleofector II (program G-013; Amaxa, Gaithersburg, MD) (Persson et al. 2013b). The transfected neurons were allowed to recover for 5 min at 37°C in 0.5 ml of Ca2+-free DMEM. The cell suspension was diluted with DRG media containing 1.5 mg/ml bovine serum albumin and 1.5 mg/ml trypsin inhibitor, and 95 μl of the cell solution were placed on 12-mm circular poly-D-lysine/laminin-coated coverslips (BD Biosciences, Bedford, MA) and incubated at 37°C in 5% CO2, for 45 min. DRG media, supplemented with 50 ng/ml each of nerve growth factor (NGF) (2.5S, Harlan Bioproducts) and glial cell line-derived neurotrophic factor (Peprotec, Rocky Hill, NJ), was added, and cells were maintained at 37°C in a 5% CO2 incubator. After 24 h, DRG media was replaced with neurobasal A media containing 0.02% B27, 50 ng/ml NGF, 100 μM 5-fluoro-2'-deoxyuridine (Sigma), and 1 μM uridine (Sigma).

**Reagents and treatment.** DRG neurons were cultured for 18 days and then treated with 50 mM KCl (Sigma) and/or 25 mM 2-deoxyglucose (2-DG, Sigma) for 4 days. To assess the role of reverse NCX and calcium overload, we examined the protective effects of inhibition of reverse NCX in 50 mM KCl and 25 mM 2-DG treated neurons with 0.5 μM KB-R7943. We also examined the effect of omitting Ca2+ from the medium, together with calcium chelation with 100 μM EGTA. For these studies, Nav1.7 WT and G856D-expressing DRG neurons were cultured for 18 days. On day 18, 2-DG and KCl in media (DMEM containing B-27 supplement, NGF, glutamine and other components of regular culture media) were added to Nav1.7 WT and G856D-expressing cultures. In a parallel set cultures with Nav1.7 WT and G856D-expressing neurons, on day 18 these groups were cultured in the presence of 2-DG and KCl in calcium-free DMEM containing EGTA or 0.5 μM KB-R7943 for 4 days, with B-27, NGF and other components of regular culture media, to test the role of Ca2+ in neurite degeneration.

**Neurite degeneration.** Adult DRG neurons expressing Nav1.7 WT or mutant G856D channels were cultured for 18 days and then subjected to experimental conditions for 4 days. Fluorescent microscopy was used to monitor neurite degeneration (Aloisua et al. 2013). Neurons were imaged using a ×20 objective lens. NIH ImageJ was utilized to create a grid over each image, and a cell counting plugin was used to score each neurite. Degenerating and healthy axons were counted in at least five fields per image (4 corners and center) for each well from GFP-expressing WT or G856D Nav1.7-expressing neurons (n ≥ 4 wells per condition from triplicate experiments). Neurite segments were considered degenerated if they displayed fragmentation and/or blebbing. Differences between experimental groups were analyzed by Student’s t-test, and P < 0.05 was considered significant.

**Na+ imaging.** Intracellular [Na+]i ([Na+]i) levels were measured using the intracellular sodium indicator CoroNa Green, which increases in fluorescence emission intensity upon binding Na+. Eighteen-day in vitro cultured DRG neurons expressing either WT or mutant Nav1.7 channels were loaded with 10 μM CoroNa Green for 40 min in standard bath solution (SBS) containing the following (in mM): 140 NaCl, 3 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES, pH 7.3, with 0.02% Pluronic (Invitrogen) at room temperature. Neuronal cultures were illuminated with 554-nm light to localize the neurons that were expressing mCherry co-transfected with mutant G856D or WT Nav1.7 neurons. Neuronal cell bodies identified from the mCherry signal were selected for [Na+]i imaging. Neurons were illuminated every 2 s with 492-nm light using a Nikon Ti-E inverted microscope equipped with a fast switching xenon light source (Lambda DG-4; Sutter Instruments). Images were captured using a QuantEM CCD camera (Princeton Instruments) and a UV transmitting ×20 objective (Super Fluor; Nikon).

After the baseline [Na+]i was recorded, membrane depolarization was induced by perfusion with high [K+] solution to measure [Na+]i, transients in activated neurons. To measure Na+ transients in activated neuronal cell bodies, membrane depolarization was induced by perfusion with different gradients of high [K+] solutions (SBS containing 25 mM KCl, 50 mM KCl, 100 mM KCl, and 150 mM KCl), according to the following perfusion protocol. After 1 min, control SBS perfusion was started for 2 min to establish a baseline. The neurons were first depolarized by exposure to 25 mM KCl solution for 1 min, which was followed by 3 min of SBS. After 3 min of SBS, neurons were progressively depolarized by exposure with 50 mM, 100 mM, and 150 mM KCl, separated by 3-min washes in SBS. The specificity of the increased fluorescent intensity of Na+ was verified by exposing neurons to 30-μm veratridine; exposure of veratridine caused a sharp rise in CoroNa Green fluorescent intensity (data not shown).

**Na+ imaging data analysis.** Acquired images were digitized and analyzed with NIS-Elements software (Nikon). Based on mCherry signal, images were thresholded, and a binary mask created over mCherry-positive neuronal cell bodies. Binary mask overlaying the each neuronal cell body was defined as a region of interest (ROI). After background correction, the change in fluorescence intensity was calculated for each time frame. To facilitate comparison between ROIs of DRG neuronal cell bodies, and since CoroNa is not a ratiometric indicator dye, the signal was normalized to its own fluorescence and displayed as ΔF/F. Control experiments revealed slow loss of fluorescent calcium for 4 days. We present a combination of linear and exponential decay segments. The baseline period of each ROI was extended using these functions and used as the denominator of linear and exponential decay segments. The baseline period of each ROI was extended using these functions and used as the denominator of linear and exponential decay segments. The baseline period of each ROI was extended using these functions and used as the denominator of linear and exponential decay segments. The baseline period of each ROI was extended using these functions and used as the denominator of linear and exponential decay segments. The baseline period of each ROI was extended using these functions and used as the denominator of linear and exponential decay segments.
room temperature for 50 min. Neuronal cultures were illuminated with 554-nm light to localize the neurons that were expressing mCherry cotransfected with mutant G856D or WT Nav1.7 channels. Neuronal cell bodies and neurites identified from the mCherry signal were selected for Ca\textsuperscript{2+} imaging. Neurons were illuminated every 2 s with alternatively 340- and 38-nm light using a Nikon Ti-E inverted microscope equipped with a fast switching xenon light source (Lambda DG-4; Sutter Instruments). Images were captured using a QuantEM CCD camera (Princeton Instruments) and a UV transmitting \times 20 objective (Super Fluar; Nikon).

**Stimulation protocol for Ca\textsuperscript{2+} imaging.** Neuronal culture dishes were microperfused at a constant flow rate using a computerized valve system (ValveLink 8.2; AutoMate Scientific). To measure [Ca\textsuperscript{2+}], transients in activated neuronal cell bodies and neurites, membrane depolarization was induced by perfusion with high [K\textsuperscript{+}] solution (SBS containing 50 mM KCl/50 mM NaCl). After measuring the basal level of [Ca\textsuperscript{2+}] in SBS, neurons were exposed to 50 mM KCl, and this time was designated as \( t = 0 \). After 60 s of exposure to high [K\textsuperscript{+}] solution, flow was returned to SBS, and recordings were made until \( t = 240 \) s.

**Ca\textsuperscript{2+} imaging data analysis.** Acquired images were digitized and analyzed with NIS-Elements software (Nikon). Based on mCherry signal, images were thresholded, and a binary mask created over mCherry-positive neuronal cell bodies and neurites. A binary mask overlaying each neuronal cell body was defined as a ROI. For neurites, each 50-\mu m-long segment of the binary mask overlaying the neurites was defined as the ROI. Fluorescence at 340-nm (F\textsubscript{340}) and 380-nm excitation (F\textsubscript{380}) mean pixel intensities were measured for every 2-s frame. After background correction, the ratio of F\textsubscript{340}/F\textsubscript{380} was calculated for each time frame. Mean values from neuronal cell bodies and neurites are depicted in graphs.

The area under the curve (AUC) was calculated from \( t = 10 \) s to \( t = 240 \) s using NIH ImageJ software. Differences between experimental groups were analyzed by Student’s t-test, and \( P < 0.05 \) was considered significant.

**RESULTS**

**DRG neurons expressing human Nav1.7 G856D mutant channels exhibit higher transient levels of [Na\textsuperscript{+}].** Our laboratory has shown earlier that the G856D mutation in sodium channel Nav1.7, identified in a family with SFN, enhances activation, slows deactivation, and increases ramp and persistent currents in this channel, an ensemble of gain-of-function changes that contributes to spontaneous firing and hyperexcitability in DRG neurons (Hoeijmakers et al. 2012b). We hypothesized that the gain-of-function changes in G856D mutant channels might result in higher [Na\textsuperscript{+}], levels in DRG neurons expressing these channels, which in turn trigger detrimental downstream effects, including altered [Ca\textsuperscript{2+}], dynamics and axonal degeneration.

To test this hypothesis, WT Nav1.7 and mutant G856D Nav1.7-expressing DRG neurons were loaded with an intracellular sodium indicator, CoroNa Green, which exhibits an increase in fluorescent emission intensity upon binding Na\textsuperscript{+}. Our results demonstrated that, compared with WT Nav1.7-expressing neurons, G856D-expressing neurons exhibited higher [K\textsuperscript{+}]-stimulated transient levels of cytosolic Na\textsuperscript{+} (Fig. 1). After the baseline [Na\textsuperscript{+}], was recorded, membrane depolarization was induced by perfusion with gradients of high [K\textsuperscript{+}] solution (25 mm to 150 mM KCl) to measure [Na\textsuperscript{+}], transients in depolarized neurons. In G856D-expressing DRG neuronal cell bodies, peak [Na\textsuperscript{+}], (as measured by increased in CoroNa Green fluorescent intensity) was significantly higher than that of WT Nav1.7-expressing DRG neuron cell bodies (Fig. 1).

**Expression of mutant G856D channels induces higher stimulated levels of Ca\textsuperscript{2+} in neuronal cell bodies.** An increase in intracellular neuronal Na\textsuperscript{+} levels can elicit reverse operation of the NCX (Annunziato et al. 2004), which may lead to neuronal [Ca\textsuperscript{2+}] overload. Because we observed a higher Na\textsuperscript{+} load in G856D-expressing compared with WT Nav1.7-expressing neurons, we postulated that there would be higher intracellular levels of Ca\textsuperscript{2+} in these neurons compared with neurons expressing WT Nav1.7 channels. To test this hypothesis, we assessed [Ca\textsuperscript{2+}], levels in neuronal cell bodies and neurite processes by measuring the ratio of fura 2 emission intensities evoked with 340 (F\textsubscript{340}) and 380-nm (F\textsubscript{380}) excitations. Baseline levels of [Ca\textsuperscript{2+}], in WT and G856D-expressing neuronal cell bodies were similar (Fig. 2A). After recording the baseline [Ca\textsuperscript{2+}], membrane depolarization was induced by perfusion with a high [K\textsuperscript{+}] solution (50 mM KCl) to measure [Ca\textsuperscript{2+}], transients in activated neurons.

In G856D-expressing DRG neuronal cell bodies, the peak in the ratio of F\textsubscript{340} to F\textsubscript{380} was significantly higher than that of WT Nav1.7-expressing DRG neuron cell bodies (Fig. 2A). WT Nav1.7-expressing DRG neurons exhibited an R peak value of 0.88 ± 0.11, while, for the G856D-expressing neurons, the R peak value was 1.81 ± 0.08 (\( P < 0.05 \); Fig. 2A). Similar to [Na\textsuperscript{+}], transients, the AUC from \( t = 10 \) s to \( t = 240 \) s for the [Ca\textsuperscript{2+}], transient was significantly greater in G856D-expressing than in WT Nav1.7-expressing neurons (410 ± 40 AUC vs. 304 ± 21 AUC; \( P < 0.05 \); Fig. 2B).
Expression of Nav1.7 G856D channels induces higher basal and stimulated levels of [Ca$^{2+}$]$_i$ in neurites of DRG neurons. Neurites of G856D-expressing DRG neurons displayed basal levels of [Ca$^{2+}$]$_i$ that were significantly higher than in the neurites of WT Nav1.7-expressing DRG neurons (0.1 ± 0.02 vs. 0.20 ± 0.02, $P < 0.01$; Fig. 2C). In response to high [K$^+$]-induced depolarization, G856D-expressing neurites exhibited a peak in the ratio of F$_{340}$ to F$_{380}$ that was significantly higher than that of WT Nav1.7-expressing neurites (0.41 ± 0.06 vs. 0.22 ± 0.02; $P < 0.01$; Fig. 2C). Like [Ca$^{2+}$]$_i$, transients in neuronal cell bodies, the AUC from $t = 10$ s to $t = 240$ s for the [Ca$^{2+}$]$_i$ transient in neurites was significantly greater in G856D-expressing neurons than in WT Nav1.7-expressing neurons (220 ± 28 AUC vs. 107 ± 26 AUC; $P < 0.05$; Fig. 2D).

Reverse function of NCX contributes to [Ca$^{2+}$]$_i$ overload in G856D-expressing neurites. Increased Na$^+$ influxes can trigger reverse operation of NCX that results in higher intracellular [Ca$^{2+}$]$_i$ levels. Based on our observations of significantly higher levels of neuronal [Na$^+$]$_i$ and [Ca$^{2+}$]$_i$ in G856D-expressing neurons compared with WT Nav1.7-expressing neurons, we hypothesized that these neurites exhibit [Ca$^{2+}$]$_i$ overload due to reverse function of NCX. Thus inhibition of reverse NCX would be predicted to increase [Na$^+$]$_i$ and decrease [Ca$^{2+}$]$_i$ in G856D-expressing neurons. To test this hypothesis, G856D-expressing DRG neurons were cultured for 18 days and then treated with 0.5 μM KB-R7943, a concentration that inhibits the reverse function of NCX (Watanabe et al. 2006). Twenty-four hours after treatment with KB-R7943, G856D-expressing DRG neurons exhibited higher [Na$^+$]$_i$ levels in response to high [K$^+$]-stimulation compared with vehicle-treated parallel cultures (Fig. 3).

Reverse function of NCX contributes to [Ca$^{2+}$]$_i$ overload in G856D-expressing neurons. We found that both neuronal cell bodies and neurites of G856D-expressing neurons exhibited higher [Ca$^{2+}$]$_i$ transients in response to high [K$^+$] perfusate (cf., Fig. 2) To determine whether NCX contributes to the observed [Ca$^{2+}$]$_i$ overload in G856D-expressing DRG neurons, G856D-expressing DRG neurons were cultured for 18 days and then treated with 0.5 μM KB-R7943. Twenty-four hours after treatment, cell bodies of G856D-expressing DRG neurons exhibited similar basal levels and decreased high [K$^+$]-stimulated transient levels of [Ca$^{2+}$]$_i$, compared with untreated G856D neurons (Fig. 4A). Quantitative analysis revealed that, after stimulation, G856D-expressing neurons exhibited an R peak value of 1.20 ± 0.15, whereas in the...
pressing neurites exhibited a R peak value of 0.28 whereas in the untreated group the R peak value was significantly lower at 0.54 ± 0.11 (P < 0.01, Fig. 5A). TTX treatment also significantly decreased the AUC exhibited by G856D neurons compared with untreated neurons (247 ± 38 AUC vs. 444 ± 59 AUC; P < 0.01; Fig. 5B).

Neurites of TTX-treated G856D-expressing DRG neurons displayed a slightly lower basal [Ca2+]i level compared with untreated neurites, consistent with a basal level of G856D channel activity, and also a reduced high [K+]i-stimulated transient [Ca2+]i (Fig. 5C). Quantitative analysis revealed that, after stimulation, TTX-treated G856D-expressing neurites exhibited R peak value 0.30 ± 0.02, whereas in the untreated group the R peak value was significantly higher at 0.40 ± 0.04 (P < 0.01, Fig. 5C). Similar to the neuronal cell body, TTX treatment also significantly decreased the AUC exhibited by G856D neurites compared with untreated neurites (117 ± 17 AUC vs. 224 ± 34 AUC; P < 0.01; Fig. 5D).

Blockade of Nav1.7-WT channels with TTX is expected to have a smaller effect compared with blockade of G856D mutant channels. Indeed, the reduction of the peak calcium transient in response to high [K+]i stimulation showed an average ratio of 1.7 ± 0.2 for untreated WT-expressing cell bodies, which was reduced to 1.2 ± 0.2 for TTX-treated cell bodies (P < 0.05, Fig. 6A). Analysis using AUC confirmed that TTX significantly reduced the response of DRG neurons expressing Nav1.7-WT channels to high [K+]i (Fig. 6B), but to a smaller degree than we observed for DRG neurons expressing Nav1.7-G856D. This 30% reduction of peak response of Nav1.7-WT-expressing cell bodies is less than the 50% reduction of peak response of Nav1.7-G856D-expressing cell bodies.

The effect of TTX on Nav1.7 WT-expressing neurites is also predicted to have a reduced effect compared with G856D-expressing neurites. The reduction of the peak calcium transient in response to high [K+]i stimulation was small or subtle with a peak ratio of 1.4 ± 0.1 for untreated WT-expressing neurites, which was reduced to 1.2 ± 0.1 for TTX-treated neurites (Fig. 6C). Analysis using AUC did not reveal a significant effect on the overall response of Nav1.7 WT-expressing neurites when treated with TTX (Fig. 6D).

**Combined metabolic stress and depolarization elicit neurite degeneration in G856D-expressing DRG neurons.** Axonal degeneration is a hallmark of SFN, but the mechanisms responsible for this degeneration are not fully understood. We have observed that expression of the mutant G856D Nav1.7 channel, identified in a family with SFN (Hoeijmakers et al. 2012b), increased [Na+]i, and triggers NCX-mediated [Ca2+]i overload in the cell body and neurites of DRG neurons. Since calcium overload is considered to be a key initiator of axonal degeneration, we examined whether expression of mutant G856D channels in DRG neurons would induce neurite degeneration in

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these cells. We did not observe significant neurite degeneration in WT and G856D-expressing neurons for up to 18 days in vitro (Fig. 7, A and B): 8 ± 2% of neurites were fragmented/blebbed in cultures of neurons expressing G856D, while 7 ± 3% of neurites were fragmented/blebbed in cultures of DRG neurons expressing WT Nav1.7 (Fig. 7, A and B, and Fig. 8). To determine whether depolarization would induce greater neurite degeneration in G856D-expressing neurons compared with WT Nav1.7-expressing cells, G856D-expressing and WT Nav1.7-expressing neurons were cultured for 18 days and then exposed to 50 mM KCl for 4 days. We found that under these depolarizing conditions G856D-expressing neurons exhibited 9.8 ± 2% fragmented/blebbed neurites, while neurons expressing WT Nav1.7 exhibited 9.6 ± 2% fragmented/blebbed neurites (Fig. 7, C and D, and Fig. 8).

To explore the possibility that increased sodium influx due to the gain-of-function G856D mutation leads to mitochondrial failure followed by depletion of neuronal ATP, we metabolically stressed WT Nav1.7-expressing and G856D-expressing DRG neurons with 25 mM 2-DG, an inhibitor of glycolysis, for 4 days. Similar to prolonged depolarization, treatment with 2-DG did not induce significant degeneration in WT Nav1.7-expressing or G856D-expressing neurites (11 ± 2% vs. 10 ± 3% fragmented neurites, Fig. 7, E and F, and Fig. 8).

As noted above, we observed that neurites of G856D-expressing neurons exhibited higher basal levels of [Ca^{2+}]_{i} and higher levels of [Ca^{2+}]_{i} upon depolarization. Axonal Ca^{2+} is known to activate calpains that drive cytoskeletal degradation and compromise mitochondrial function. Since neurons in which mitochondrial function is compromised can survive on glycolysis (Baloh et al. 2007; Summers et al. 2014), and because sensory neurons must maintain their resting potential and transmembrane gradients and consume energy in the steady state, we simultaneously depolarized and inhibited glycolysis in G856D-expressing and WT Nav1.7-expressing neurons by exposure to 50 mM KCl and 25 mM 2-DG. Compared with WT Nav1.7-expressing neurons, inhibition of glycolysis in depolarizing conditions for 4 days caused a significantly increased level of neurite degeneration in G856D-expressing neurons (Fig. 7H and Fig. 8). Inhibition of glycolysis, together with exposure to 50 mM KCl for 4 days, resulted in degeneration in 23 ± 3% of the neurites in cultures of G856D-expressing DRG neurons. In contrast, the inhibition of glycolysis and exposure to 50 mM KCl for 4 days in cultures of WT Nav1.7 expressing neurons resulted in degeneration of only 13 ± 2% neurites (P < 0.01, Fig. 7, G and H, and Fig. 8).

Reverse NCX-mediated calcium overload contributes to neurite degeneration in G856D-expressing neurons. Since increased Na^{+} influx can trigger reverse operation of NCX causing Ca^{2+} overload and cellular dysfunction, we tested the involvement of calcium in neurite degeneration in 50 mM KCl and 2-DG-treated cultures of WT and G856D-expressing neurons. We found that omission of Ca^{2+} from the medium and treatment with 100 μM EGTA, a calcium chelator, and 0 Ca^{2+}
in the perfusate prevented neurite degeneration in 50 mM KCl and 2-DG-treated cultures (13 ± 2% fragmented neurites in EGTA-treated G856D-expressing neurons vs. 30 ± 4% fragmented neurites in G856D-expressing neurons without EGTA treatment, Fig. 9, F, G, and I). Because we observed that Ca\(^{2+}\) overload in G856D-expressing neurons is at least in part mediated by reverse function of NCX, we investigated the effect of KB-R7943, at a concentration that blocks reverse function of NCX, on neurite degeneration in 50 mM KCl and 2-DG-treated cultures of G856D-expressing neurons. We found that inhibition of reverse function of NCX prevented neurite degeneration under these culture conditions (18 ± 3% fragmented neurites in KB-R7943-treated G856D-expressing neurons vs. 30 ± 4% fragmented neurites in G856D-expressing neurons not treated with KB-R7943; Fig. 9, F, H, and I). WT Nav1.7 neurons incubated with 50 mM KCl + 2-DG, 50 mM KCl + 2-DG + EGTA, or 50 mM KCl + 2-DG + KB-R7943 exhibited low (<10%) levels of neurite degeneration (Fig. 9, C, D, E, and I).

**DISCUSSION**

The Nav1.7 sodium channel is a major focus of pain research due to its preferential expression in peripheral DRG and sympathetic ganglion neurons (Rush et al. 2006; Toledo-Aral et al. 1997) and their axons (Persson et al. 2010) and its biophysical properties, which poise it to amplify small depolarizations that are subthreshold with respect to the action potential (Cummins et al. 1998; Rush et al. 2007). Gain-of-function mutations of Nav1.7 have recently been identified in patients with painful SFN and have been shown to increase the excitability of DRG neurons (Faber et al. 2012; Han et al. 2012). One of these mutations, G856D, has a particularly strong effect on channel function, hyperpolarizing the voltage dependence of activation by 10–15 mV, depolarizing fast inactivation by 6.2 mV, slowing deactivation and robustly enhancing ramp currents. As expected from the increased overlap (window) of activation and depolarized inactivation (Estacion and Waxman 2013), persistent currents of the G856D mutant channels are increased 10- to 11-fold compared with those produced by WT Nav1.7 channels (Hoeijmakers et al. 2012b). While not linking window current to neuronal injury, Vasylyev et al. (2014), using dynamic clamp, showed for another Nav1.7 mutation, L858H, that enhanced persistent current supports a sustained Na\(^{+}\) influx in DRG neurons.

The Nav1.7 channel is co-expressed with the NCX2 in peripheral nerve axons (Persson et al. 2010). Previous studies have shown that, in the anoxic optic nerve and anoxic peripheral nerve, Na\(^{+}\) enters axons via voltage-gated Na\(^{+}\) channels, and subsequent increases in axoplasmic Na\(^{+}\) are coupled functionally to Ca\(^{2+}\) influx mediated by reverse operation of NCX (Lehning et al. 1996; Stys et al. 1992). We previously showed that the neuropathy-associated Nav1.7 mutation, I228M, impairs the integrity of the axons of DRG neurons (Persson et al. 2013b). Mutations G856D and I228M both depolarize resting potential (4.8 mV, I228M, Estacion et al. 2013) yet are associated with increased axonal injury (Persson et al. 2013b; Hoeijmakers et al. 2012b). The Nav1.7 channel is a major focus of pain research due to its preferential expression in peripheral DRG and sympathetic ganglion neurons (Rush et al. 2006; Toledo-Aral et al. 1997) and their axons (Persson et al. 2010).
2011; 6.8 mV, G856D, Hoeijmakers et al. 2012b), which would be expected to bias NCX away from forward (Ca\(^{2+}\)/H\(^{+}\)) extruding and toward reverse (Ca\(^{2+}\)/H\(^{+}\)) importing operation. We have shown that sodium channels contribute to degeneration of DRG neuron axons induced by rotenone, which induces mitochondrial dysfunction, but not to degeneration of these axons induced by vincristine, which selectively injures microtubules (Persson et al. 2013a). These earlier studies did not, however, assess intracellular levels of Na\(^{+}/H^{+}\) or Ca\(^{2+}/H^{+}\), or the role of sodium channels or NCX in triggering changes in the intracellular levels of these ions, in DRG neurons or axons after expression of neuropathy-associated Nav1.7 mutations. In this study, we assessed DRG neurons expressing WT vs. G856D mutant channels and show that DRG neurons expressing G856D mutant channels express higher depolarization-evoked transient levels of [Na\(^{+}\)], and higher stimulated levels of [Ca\(^{2+}\)]. Using KB-R7943, we present evidence that reverse function of NCX contributes to increased [Ca\(^{2+}\)] in DRG neurons expressing the mutant Nav1.7 channels. We also demonstrate that metabolic stress and depolarization trigger neurite degeneration in DRG neurons that express G856D mutant, but not WT Nav1.7, channels.

The regulation of Ca\(^{2+}\) within DRG neurons is complex and involves multiple mechanisms. Off-target effects of KB-R7943 include inhibition of N-methyl-D-aspartate-induced [Ca\(^{2+}\)] fluxes and 2,4-dinitrophenol-stimulated respiration; however, these occur with higher concentrations (IC\(_{50}\) = 13.4 \(\mu\)M and 11.4 \(\mu\)M, respectively; Brustovetsky et al. 2011) than the 0.5 \(\mu\)M concentration used in our experiments, a level that has been shown to inhibit reverse operation of NCX (Watanabe et al. 2006). A role of NCX in mitochondria cannot be ruled out, since pharmacological modulation could cause changes in calcium flux from this pool of calcium (Svichar et al. 1999). There is evidence that KB-R7943 can enhance Ca-dependent potassium conductance (Rahman et al. 2012), which could contribute to modulation of neurite degeneration, although this occurs with higher concentrations of the drug, e.g., 30 \(\mu\)M (Rahman et al. 2012). While not excluding a contribution via these other routes, our data point to a role of reverse NCX, triggered by activity of sodium channels, as a contributor to calcium overload in G856D-expressing DRG neurons.

Our results demonstrate that G856D-expressing neuronal cell bodies exhibit higher high [K\(^{+}\)]-stimulated intracellular levels of [Na\(^{+}\)] than WT neurons. We were, however, unable to assess [Na\(^{+}\)], in G856D- or WT-expressing neurites. At present, there is a paucity of reports that have assayed [Na\(^{+}\)] in axons. The available Na\(^{+}\)-sensitive dyes have low ion selectivity over K\(^{+}\) (1:2). Due to this poor selectivity, it is difficult to specifically measure Na\(^{+}\) influx with moderately high K\(^{+}\) outside (25 and 50 mM), which also leads to K\(^{+}\) efflux from the cells/neurites, and under such condition the dye intensity gain from Na\(^{+}\) influx is countered by the dye intensity loss from K\(^{+}\) efflux. David et al. (1997) ionophoretically-
injected the Na\(^+\)-sensitive ratiometric dye, sodium-binding benzofuran isophthalate, in lizard peripheral myelinated axons, which have inner axonal diameters of 14 \(\mu\)m (nearly 30-fold larger than the axons studied here), to assess spatiotemporal changes in [Na\(^+\)]. Sodium-binding benzofuran isophthalate has also been introduced into rat pyramidal neurons via the internal patch-pipette solution, to measure Na\(^+\)/H\(^+\) fluxes in cell bodies, axon initial segments, and dendrites following soma current injection (Fleidervish et al. 2010). Similar to the present study, Nikolaeva et al. (2005) loaded adult rat optic nerve axons with CoroNa Green to demonstrate increased [Na\(^+\)] that paralleled increased [Ca\(^{2+}\)] following ischemia. The inability to detect [Na\(^+\)] transients that paralleled increased [Ca\(^{2+}\)] in our cultured DRG neurites compared with the optic nerve axons may result from the three-fold smaller diameter of the DRG neurites that we studied (0.5 \(\mu\)m) compared with the optic nerve axons (1.5 \(\mu\)m) assessed by Nikolaeva et al. (2005), producing a lower signal-to-noise ratio. Ca\(^{2+}\) imaging, which employs more robust indicator dyes than available for Na\(^+\) imaging (Ross et al. 2013), detected similar responses in DRG neuronal cell bodies and neurites for WT vs. G856D-expressing cells, suggesting that it is likely that G856D-expressing neurites exhibit elevated levels of [Na\(^+\)] compared with WT.

In the present study, we show that inhibition of glycolysis or application of high KCl individually did not induce neurite degeneration in G856D-expressing DRG neurons. Adult DRG neurons were transfected with Nav1.7 WT or mutant G856D channels and were cultured for 18 days. Neurons were imaged using \(\times 20\) objective lens. Degenerating and healthy neurites were counted in at least five fields per image (4 corners and center) for each well from WT or G856D Nav1.7-expressing neurons that coexpressed green fluorescent protein (\(n = 4\) wells per condition from triplicate experiments). Quantitative analysis demonstrated a significant difference in neurite degeneration in WT Nav1.7 vs. G856D Nav1.7-expressing neurons after 18 days in culture. Treatment with 50 mM KCl or inhibition of glycolysis by 2-DG for 4 days beginning at 18 DIV did not cause significant neurite degeneration in neurons expressing either WT Nav1.7 or G856D Nav1.7 channels. Inhibition of glycolysis for 4 days after 18 DIV in the presence of 50 mM KCl evoked significant neurite fragmentation and/or blebbing in DRG neurons expressing Nav1.7 G856D channels compared with WT Nav1.7 channels (*\(P < 0.05\)).
There is precedent for a mechanistic link between mitochondrial dysfunction and degeneration of sensory peripheral axons from studies on the effects of the mitochondrial toxin rotenone on the neurites of DRG neurons in vitro (Persson et al. 2013a) and from studies showing that mitochondrial DNA damage accumulates in distal mitochondria of long axons, especially in patients with human immunodeficiency virus-associated sensory neuropathy (Lehmann et al. 2011).

The onset of symptoms of SFN in patients with variant or mutated Nav1.7 channels generally occurs in the third or fourth decade of life, despite the presence of these aberrant channels from birth (Faber et al. 2012). In our assay, in which we followed neurites for weeks, we only observed degeneration of neurites when G856D-expressing neurons were challenged with two harsh stressors: extended depolarization produced by 50 mM KCl (which would be expected to depolarize resting potential to \(-25\) mV) and inhibition of glycolysis. Neurite degeneration was only observed in G856D-expressing, and not WT Nav1.7-expressing, neurons. These observations suggest that the additional influx of Na\(^{+}\) through the G856D channels is sufficient to tip the balance of the neurite’s ability to prevent degenerative cascades from being initiated following challenge. We suggest that, in vivo, the expression of variant/mutated Nav1.7 channels, in combination with additional stressor(s), initiates a cumulative burden that leads to the onset of SFN symptoms and loss of intraepidermal nerve fibers.

Studies carried out to date (Estacion et al. 2011; Faber et al. 2012; Han et al. 2012; Hoeijmakers et al. 2012b) have demonstrated that Nav1.7 mutations associated with SFN produce spontaneous firing and hyperexcitability in DRG neurons and provide a mechanistic basis for the pain that characterizes these neuropathies. However, these earlier studies do not provide a mechanistic explanation for axonal degeneration. The present observations on a neuropathy-associated Nav1.7 mutant channel point to increased intracellular levels of Na\(^{+}\) that trigger reverse NCX and Ca\(^{2+}\) toxicity as a contributor to axonal injury in SFN associated with Nav1.7 mutations. Our results suggest that subtype-specific block of Nav1.7 channels, and inhibition of reverse NCX, merit exploration as potential strategies that might slow or halt axonal degeneration in SFN.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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