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

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Abstract

Gain-of-function missense mutations in sodium channel Nav1.7 have been linked to small fiber neuropathy (SFN), which is characterized by burning pain, dysautonomia and a loss of intra-epidermal nerve fibers (IENF). However, the mechanistic cascades linking Nav1.7 mutations to axonal 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 these cells. We report here that cell bodies and neurites of DRG neurons transfected with G856D display increased levels of [Na⁺], and [Ca²⁺], following stimulation with high [K⁺] compared to WT Nav1.7-expressing neurons. Blockade of reverse mode of the sodium-calcium exchanger (NCX) or of sodium channels attenuates Ca²⁺ transients evoked by high [K⁺] in G856D-expressing DRG cell bodies and neurites. We also show that treatment of WT or G856D-expressing neurites with high [K⁺] or 2-deoxyglucose (2-DG) does not elicit degeneration of these neurites, but that high [K⁺] and 2-DG in combination evokes degeneration of G856D neurites but not WT neurites. Our results also demonstrate that Ca²⁺ or blockade of reverse mode of NCX protects G856-expressing neurites from degeneration when exposed to high [K⁺] and 2-DG. These results point to [Na⁺] overload in DRG neurons expressing mutant G856D Nav1.7 which triggers reverse mode of NCX and contributes to Ca²⁺ toxicity, and suggest subtype-specific blockade of Nav1.7 or inhibition of reverse NCX as strategies that might slow or prevent axon degeneration in SFN.

Key words: axon degeneration; calcium transient; sodium channels; sodium-calcium exchanger
Introduction

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 NCX2 (Persson et al. 2010). Gain-of-function missense mutations in Na, 1.7 channels have been linked to idiopathic small fiber neuropathy (Faber et al. 2012; Han et al. 2012). Small fiber neuropathy (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, intra-epidermal 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⁺ overload and abnormal increases in intra-axonal Ca²⁺ due to reverse Na-Ca exchange in DRG neurons expressing the G856D Nav1.7 mutation, which may trigger downstream degenerative cascades.

The G856D mutation in Nav 1.7 was identified in a multigenerational family with severe pain and dysautonomia due to small fiber neuropathy (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 supra-threshold 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 to other Nav1.7 mutations associated with small fiber neuropathy (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⁺] stimulated Na⁺ load in DRG neurons expressing mutant G856D channels leads to Ca²⁺ overload in cell bodies and neurites. We show that blocking of reverse action of the
sodium-calcium exchanger (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 Nav1.7 channels, triggers neurite degeneration in DRG neurons expressing mutant G856D channels. Our data suggest that Ca\textsuperscript{2+} toxicity induced by hyperactive mutant Nav1.7 channels and reverse sodium-calcium exchange contribute to degeneration of axons in Nav1.7 mutation-associated small fiber neuropathy.
Materials and Methods

DRG culture

Experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals; all animal protocols were approved by the Institutional Animal Care and Use Committee of VA Connecticut Healthcare System, West Haven, Connecticut. Adult Sprague-Dawley rats (4-6 weeks) 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 (CSS), 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 minutes at 37°C in CSS containing Collagenase D (1.5 mg/ml) and Papain (30 U/ml). DRG were centrifuged and re-suspended in DRG media (DMEM/F12 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA) and 10% 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 rcf for 10 minutes to remove non-neuronal 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-GFP or mCherry (channel:GFP/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 minutes 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 was 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 NGF (2.5S, Harlan Bioproducts) and GDNF (Peprotec, Rocky Hill, NJ), was added and cells were maintained at 37°C in a 5% CO₂ incubator. After twenty four hours, DRG media was replaced with Neurobasal A media containing 0.02% B27, 50 ng/ml NGF, 1 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 50mM KCl (Sigma) and/or 25mM 2-deoxyglucose (2-DG, Sigma) for 4 days. To assess the role of reverse Na⁺-Ca²⁺ exchange and calcium overload, we examined the protective effects of inhibition of reverse sodium-calcium exchange 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 wild type (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 (Alobuia 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 Nav 1.7 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**
[Na⁺]ᵢ levels were measured using the intracellular sodium indicator CoroNa Green, which increases in fluorescence emission intensity upon binding Na⁺. 18 DIV cultured DRG neurons expressing either WT or mutant Nav1.7 channels were loaded with 10 μM CoroNa Green for 40 minutes in standard bath solution (SBS) containing (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 channels. 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 20x objective (Super Fluor; Nikon).

After recording the baseline [Na⁺]ᵢ, 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 one minute, control SBS perfusion was started for 2 minutes to establish a baseline. The neurons were first depolarized by exposure to 25 mM KCl solution for one minute, which was followed by three minutes of SBS. After three minutes of SBS, neurons were progressively depolarized by exposure with 50 mM, 100 mM, and 150 mM KCl, separated by three minute 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 ROI. After background correction the change in fluorescence intensity was calculated for each time frame. To facilitate comparison between regions of interest 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 delta F/F. Control experiments revealed slow loss of fluorescence over time that could be fit with a combination of linear and exponential decay segments. The baseline period of each ROI was extended using these functions and used as the denominator to calculate the delta F/F data shown.

**Ca\(^{2+}\) imaging**

\([\text{Ca}^{2+}]\) levels were assayed by using the ratiometric intracellular calcium indicator Fura-2-AM (Invitrogen). DRG neurons cultured for 18 days were loaded with 2 µM Fura-2-AM in standard bath solution (SBS) containing the following (in mM): 140 NaCl, 3 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), and 10 HEPES, pH 7.3 with 0.02% Pluronic (Invitrogen) at room temperature for 50 min. Neuronal cultures were illuminated with 554 nm light to localize the neurons which were expressing mCherry co-transfected with mutant G856D or WT Na\(_v\)1.7 channels. Neuronal cell bodies and neurites identified from the mCherry signal were selected for Ca\(^{2+}\) imaging. Neurons were illuminated every 2 s with alternatively 340 and 380 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 20x objective (Super Fluor; Nikon).

**Stimulation protocol for Ca\(^{2+}\) imaging**

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

**Ca\(^{2+}\) 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 µm long segment of the binary mask overlaying the neurites was defined as the ROI. \(F_{340}\) and \(F_{380}\) mean pixel intensities were measured for every
2s frame. After background correction, the ratio of $F_{340}/F_{380}$ 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\(^+\)]\(_i\)

We have shown earlier that the G856D mutation in sodium channel Nav1.7, identified in a family with small fiber neuropathy (SFN), enhances activation, slows deactivation, and increases ramp and persistent currents in this channel, an ensemble of gain-of-function changes that contribute 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\(^+\)]\(_i\) levels in DRG neurons expressing these channels, which in turn trigger detrimental downstream effects, including altered [Ca\(^{2+}\)]\(_i\) dynamics and axonal degeneration.

In order 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\(^+\). Our results demonstrated that compared to WT Nav1.7-expressing neurons G856D-expressing neurons exhibited higher [K\(^+\)]-stimulated transient levels of cytosolic Na\(^+\) (Fig. 1). After recording the baseline [Na\(^+\)]\(_i\), membrane depolarization was induced by perfusion with gradients of high [K\(^+\)] concentration solution (25mm to 150 mM KCl) to measure [Na\(^+\)]\(_i\) transients in depolarized neurons. In G856D-expressing DRG neuronal cell bodies, peak [Na\(^+\)]\(_i\), (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\(^{2+}\) in neuronal cell bodies

An increase in intracellular neuronal Na\(^+\) levels can elicit reverse operation of the sodium-calcium exchanger (NCX) (Annunziato et al. 2004), which may lead to neuronal [Ca\(^{2+}\)]\(_i\) overload. Because we observed a higher Na\(^+\) load in G856D-expressing compared to WT Nav1.7-expressing neurons, we posited that there would be higher intracellular levels of Ca\(^{2+}\) in these neurons compared to neurons expressing WT Nav1.7 channels. To test this hypothesis, we assessed [Ca\(^{2+}\)]\(_i\) levels in neuronal cell bodies and neurite processes by measuring the ratio of Fura-2 emission intensities evoked with 340 (F340) and 380 (F380) nm excitations. Baseline
levels of \([Ca^{2+}]_i\) in WT and G856D expressing neuronal cell bodies were similar (Fig. 2A). After recording the base-line \([Ca^{2+}]_i\), membrane depolarization was induced by perfusion with a high \([K^+]\) concentration solution (50 mM KCl) to measure \([Ca^{2+}]_i\) transients in activated neurons. In G856D-expressing DRG neuronal cell bodies, the peak in R340/380 was significantly higher than that of WT Nav1.7-expressing DRG neuron cell bodies (Fig. 2A). WT Nav1.7-expressing DRG neurons exhibited a R Peak value of 0.88±.11 while, for the G856D-expressing neurons, the R Peak value was 1.81 ± 0.08 (p<0.05; Fig. 2A). Similar to \([Na^+]_i\) transients, the AUC from t=10 s to t= 240 s for the \([Ca^{2+}]_i\) transient was significantly greater in G856D-expressing than in WT Nav1.7-expressing neurons (410±40 AUC vs. 304±21 AUC, respectively; 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 R340/380 that was significantly higher than that of WT Nav1.7-expressing neurites (0.41 ± 0.06 vs 0.22±0.02, respectively; 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, respectively; p<0.05; Fig. 2D).

Reverse function of NCX contributes to \([Na^+]_i\) levels in G856D Nav1.7-expressing neurons

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 to WT Nav1.7-expressing neurons, we hypothesized that these neurons 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 to vehicle-treated parallel cultures (Fig. 3).
Reverse function of NCX contributes to \([Ca^{+2}]\) overload in G856D-expressing neurons

We found that both neuronal cell bodies and neurites of G856D-expressing neurons exhibited higher \([Ca^{+2}]\) transients in response to high \([K^+]\) perfusate (cf Fig. 2) To determine whether NCX contributes to the observed \([Ca^{+2}]\) overload in G856D-expressing DRG neurons, G856D-expressing DRG neurons 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 \([Ca^{2+}]\), compared to 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 KB-R7943-treated group the R peak value was significantly lower at 0.83±0.08 (p < 0.01, Fig. 4A). The AUC of the \([Ca^{2+}]\) transient was significantly smaller in G856D-expressing neurons treated with KB-R7943 than in untreated neurons (303±48 AUC vs 422±34 AUC; p < 0.01; Fig. 4B).

Neurites of KB-R7943-treated G856D-expressing DRG neurons had lower basal and high \([K^+]\)-stimulated transient levels of \([Ca^{2+}]\), than untreated G856D-expressing neurons. Quantitative analysis revealed that basal levels of \([Ca^{+2}]\), were significantly lower in KB-R7943-treated neurites of G856D-expressing neurons compared to untreated G856D neurons (0.10±0.01 vs 0.17±0.02, respectively; p < 0.01; Fig. 4C). After stimulation with high \([K^+]\), KB-R7943-treated G856D-expressing neurites exhibited a R Peak value of 0.28±0.03, whereas in the untreated group the R Peak value was significantly higher at 0.41±0.04 (p < 0.01, Fig. 4C). The AUC of the \([Ca^{2+}]\) transient in neurites was also significantly smaller in G856D-expressing neurons treated with KB-R7943 than in untreated neurons (136±38 AUC vs 224±36, respectively; p < 0.01; Fig. 4D).

Sodium channels contribute to calcium overload in G856D-expressing neurons

We observed that DRG neurons expressing mutant G856D channels exhibited significantly higher stimulated intracellular \([Ca^{2+}]\) levels than WT Nav1.7-expressing neurons. Previous studies have shown that sodium channel activity can contribute to calcium overload and axonal dysfunction under conditions of energetic stress induced by anoxia (Stys et al. 1992) and mitochondrial complex 1 inhibition by rotenone (Persson et al. 2013a). Our results suggest that enhanced levels of \([Ca^{2+}]\), in G856D-expressing neuronal cell bodies and neurites compared to WT Nav1.7 neurons is a consequence of the increased excitability of these G856D channels. To
examine directly whether the activity in sodium channels contributes to higher \([\text{Ca}^{2+}]\) levels in G856D-expressing neurons, we treated G856D-expressing neurons with the sodium channel blocker, tetrodotoxin (TTX), at a concentration of 0.3 \(\mu\text{M}\) (a dose known to block TTX-sensitive sodium currents including Nav1.7; Catterall et al. 2005). High [K\(^+\)]-stimulated peak transient levels of \([\text{Ca}^{2+}]\) were substantially lower in TTX-treated neurons than in untreated G856D neurons (Fig. 5A). Quantitative analysis revealed that after stimulation, G856D-expressing neurons exhibited \(R\) Peak value 1.91±0.13, whereas in the TTX-treated 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 to untreated neurons (247±38 AUC vs 444±59 AUC, respectively; \(p<0.01\); Fig. 5B).

Neurites of TTX-treated G856D-expressing DRG neurons displayed a slightly lower basal \([\text{Ca}^{2+}]\) level compared to untreated neurites, consistent with a basal level of G856D channel activity, and also a reduced high [K\(^+\)]-stimulated transient \([\text{Ca}^{2+}]\) (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 to untreated neurites (117±17 AUC vs 224±34 AUC, respectively; \(p<0.01\); Fig. 5D).

Blockade of Nav1.7-WT channels with TTX is expected to have a smaller effect compared to blockade of G856D mutant channels. Indeed, the reduction of the peak calcium transient in response to high [K\(^+\)]-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\(^+\)] (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 to G856D-expressing neurites. The reduction of the peak calcium transient in response to high [K\(^+\)]-stimulation was small or subtle with a peak ratio of 1.4 ± 0.1 for untreated
WT-expressing neurites which was reduced to $1.2 \pm 0.1$ 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 small fiber neuropathy, 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 small fiber neuropathy (Hoeijmakers et al. 2012b), increased $[\text{Na}^+]_i$, and triggers NCX-mediated $[\text{Ca}^{2+}]_i$ 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 these cells. We did not observe significant neurite degeneration in WT and G856D-expressing neurons for up to 18 DIV (Fig. 7A,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. 7A,B and Fig. 8). To determine whether depolarization would induce greater neurite degeneration in G856D-expressing neurons compared to 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. 7C,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-deoxyglucose (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, respectively Fig. 7E,F and Fig. 8).
As noted above, we observed that neurites of G856D-expressing neurons exhibited higher basal levels of $[\text{Ca}^{2+}]_i$ and higher levels of $[\text{Ca}^{2+}]_i$ upon depolarization. Axonal $\text{Ca}^{2+}$ is known to activate calpains that drive cytoskeletal degradation and compromise mitochondria function. Since neurons in which mitochondrial function is compromised can survive on glycolysis (Balog 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 Na\textsubscript{v}1.7-expressing neurons by exposure to 50 mM KCl and 25 mM 2-DG. Compared to WT Na\textsubscript{v}1.7-expressing neurons, inhibition of glycolysis in depolarizing conditions for 4 days caused a significantly increased level of neurite degeneration in G856D-expressing neurons (Fig. 7 H 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 Na\textsubscript{v}1.7 expressing neurons resulted in degeneration of only 13 ±2 % neurites ($p < 0.01$, Fig. 7G,H and Fig. 8).

**Reverse Na-Ca exchange-mediated calcium overload contributes to neurite degeneration in G856D-expressing neurons.**

Since increased Na$^+$ influx can trigger reverse operation of NCX causing $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ from the medium and treatment with 100 μm EGTA, a calcium chelator, and 0 $\text{Ca}^{2+}$ 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 versus 30±4 % fragmented neurites in G856D-expressing neurons without EGTA treatment, Fig. 9 F,G,I). Because we observed that $\text{Ca}^{2+}$ 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 Na-Ca exchange, 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 versus 30±4 % fragmented neurites in G856D-expressing neurons...
not treated with KB-R7943; Fig. 9 F,H,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,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 small fiber neuropathy (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 those produced by wild-type 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, D623N, that enhanced persistent current supports a sustained Na⁺ influx in DRG neurons.

The Nav1.7 channel is co-expressed with the NCX2 sodium-calcium exchanger 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 Ca²⁺ 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, 2011; 6.8 mV, G856D, Hoeijmakers et al. 2012b), which would be expected to bias NCX away from forward (Ca²⁺ extruding) and toward reverse (Ca²⁺ 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.
These earlier studies did not, however, assess intra-cellular levels of Na\(^+\) or Ca\(^{2+}\), 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 versus 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\(^{2+}\)]\(_i\) levels 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 NMDA-induced [Ca\(^{2+}\)] fluxes and 2,4 DNP-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\(^+\)]\(_i\) in G856D- or WT-expressing neurites. At present, there is a paucity of reports that have assayed [Na\(^+\)]\(_i\) 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 (SBFI), in lizard peripheral myelinated axons, which have inner axonal diameters of 14 mm (nearly 30-fold larger than the axons studied here), to assess spatiotemporal changes in $[Na^+]_i$. SBFI has also been introduced into rat pyramidal neurons via the internal patch-pipette solution, in order to measure $Na^+$ 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 $[Na^+]_i$ that paralleled increased $[Ca^{2+}]_i$ following ischemia. The inability to detect $[Na^+]_i$ transients that paralleled increased $[Ca^{2+}]_i$ in our cultured DRG neurites compared to the optic nerve axons may result from the three-fold smaller diameter of the DRG neurites that we studied (~0.5 $\mu$m) compared to the optic nerve axons (>1.5 $\mu$m) assessed by Nikolaeva et al. (2005) producing a lower signal: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 versus G856D-expressing cells, suggesting that it is likely that G856D-expressing neurites exhibit elevated levels of $[Na^+]_i$ compared to 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. Rather, metabolic stress with 2-DG, combined with depolarization, triggered neurite degeneration in G856D-expressing DRG neurons. This finding has a theoretical basis in the requirement for energetic stores to maintain membrane potential and transmembrane ionic gradients in excitable cells such as neurons (Ames et al. 1992; Hertz and Schousboe 1975; Sokoloff 1999). 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 mtDNA damage accumulates in distal mitochondria of long axons, especially in patients with HIV-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 -25mV) 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 intra-epidermal 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 small fiber neuropathy 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 Na\(^+\)-Ca\(^{2+}\) exchange and Ca\(^{2+}\) toxicity as a contributor to axonal injury in small fiber neuropathy associated with Nav1.7 mutations. Our results suggest that subtype-specific block of Nav1.7 channels, and inhibition of reverse Na\(^+\)-Ca\(^{2+}\) exchange, merit exploration as potential strategies that might slow or halt axonal degeneration in small fiber neuropathy.

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References


Figure Legends

**Figure 1** Mutant Nav1.7 G856D-expressing DRG neurons exhibit higher stimulated levels of [Na$^+$]$_i$. DRG neurons expressing hNav1.7-WT or –G856D mutant channels were identified by positive fluorescence for mCherry, the co-transfection marker, loaded with CoroNa Green sodium-sensitive dye and imaged as described in Methods. The fluorescent signals from the soma were transformed to delta F/F and averaged together to give the traces shown. Neurons expressing hNav1.7-WT (gray trace) and hNav1.7-G856D mutant (black trace) channels both showed progressively increasing levels of intracellular sodium with increasing pulsed concentrations of extracellular potassium (indicated by gray to black boxes) to induce depolarizations. Each stimulated response was significantly higher than its prestimulation baseline for WT and G856D channels. Quantitative comparison of the two groups by ANOVA indicates that the G856D responses are significantly higher than the WT responses ($p<0.001$).

**Figure 2** Mutant Nav1.7 G856D-expressing DRG neuronal cell bodies and neurites exhibit higher stimulated levels of [Ca$^{2+}$]$_i$. A. Mutant G856D Nav1.7-expressing neuronal cell bodies exhibit significantly greater rises in [Ca$^{2+}$]$_i$ levels after 50 mM [K$^+$]-induced membrane depolarization compared with WT Nav1.7-expressing neurons. Traces represent means of n = 30 WT and n = 30 mutant Nav1.7-expressing neurons. B. Quantification of [Ca$^{2+}$]$_i$ after depolarization demonstrates that the AUC for mutant Nav1.7 expressing neuronal cell bodies is significantly higher than that WT Nav1.7-expressing neurons (*$p<0.001$). C. Mutant G856D Nav1.7-expressing neurites exhibit higher basal [Ca$^{2+}$]$_i$ levels and significantly greater peaks in [Ca$^{2+}$]$_i$ after 50 mM [K]-induced membrane depolarization compared with WT Nav1.7-expressing neurites. Traces represent means of n = 30 WT and n = 30 mutant G856D Nav1.7-expressing neurites. D. Quantification of [Ca$^{2+}$]$_i$ after depolarization demonstrates that the AUC for mutant Nav1.7-expressing neurites is significantly higher than that WT Nav1.7 expressing neurons (*$p<0.001$).

**Figure 3** Inhibition of reverse sodium-calcium exchange increases stimulated levels of [Na$^+$]$_i$ in G856D Nav1.7-expressing neurons. Neurons expressing hNav1.7-G856D mutant channels were identified by positive mCherry fluorescence, and were loaded with CoroNa

25
sodium-sensitive dye and imaged as described in Methods. The fluorescence signals from the soma were transformed to delta F/F and averaged together to give the traces shown. Neurons were pretreated for 24-hr with either vehicle (0.1% DMSO gray trace) or 0.5 μM KB-R7943 (black trace). Both showed progressively larger elevations of intracellular sodium with increasing elevations of extracellular potassium (indicated by gray to black boxes) to induce RMP depolarizations. Each stimulated response was significantly higher than its prestimulation baseline for either treatment. Comparison of the two groups by ANOVA shows that the KB-R7943 responses are significantly higher than the vehicle control responses (p<0.001).

**Figure 4** Inhibition of reverse sodium-calcium exchange attenuates stimulated levels of [Ca\(^{2+}\)]\(_i\) in neuronal somata and neurites of G856D mutant Nav1.7- expressing neurons. Inhibition of reverse function of NCX triggers a significant decrease in high [K\(^+\)]-stimulated transient [Ca\(^{2+}\)]\(_i\) levels in both the cell bodies (A.) and neurites (C.) of mutant G856D Nav1.7-expressing neurons. Inhibition of reverse function of NCX effects a significant decrease in basal [Ca\(^{2+}\)]\(_i\) levels in the neurites of mutant G856D Nav1.7-expressing neurons (C.). Traces represent means of n = 30 G856D neurons without KB-R7943 and n = 20 G856D with KB-R7943. Quantification of [Na\(^+\)]\(_i\) after depolarization demonstrates that the AUC for cell bodies (B.) and neurites (D.) of KB-R7943-treated neurons is significantly lower than that of vehicle treated mutant Nav1.7 expressing-neurons (*p<0.001).

**Figure 5** Sodium channels contribute to increased [Ca\(^{2+}\)]\(_i\) in G856D Nav1.7-expressing neurons. TTX treatment triggers a significant decrease in transient [Ca\(^{2+}\)]\(_i\) levels after high [K\(^+\)]-induced membrane depolarization in both cell bodies (A.) and neurites (C.) of mutant G856D Nav1.7-expressing neurons. Traces represent means of n = 30 G856D neurons without TTX and n = 18 G856D neurons with TTX. Quantification of [Ca\(^{2+}\)]\(_i\) after depolarization demonstrates that the AUCs for cell bodies (B.) and neurites (D.) of TTX-treated neurons are significantly lower than that of vehicle treated mutant Nav1.7-expressing neurons (*p<0.001).

**Figure 6** Sodium channels contribute to increased [Ca\(^{2+}\)]\(_i\) in wild-type Nav1.7-expressing cell bodies but not neurites. TTX treatment triggers a significant decrease in transient [Ca\(^{2+}\)]\(_i\) levels after high [K\(^+\)]-induced membrane depolarization in cell bodies (A.) but not neurites (C.)
of wild-type Nav1.7-expressing neurons. Traces represent means of \( n = 16 \) WT neurons without TTX and \( n = 18 \) WT neurons with TTX. Quantification of \([\mathrm{Ca}^{2+}]_i\), after depolarization demonstrates that the AUCs for cell bodies (B.) but not neurites (D.) of TTX-treated neurons are significantly lower than that of vehicle treated mutant Nav1.7-expressing neurons (*p<0.05).

**Figure 7** Inhibition of glycolysis in Nav1.7 mutant G856D-expressing DRG neurons maintained in a depolarizing condition induces neurite degeneration. After 18 DIV culture neurites of both WT (A.) and mutant G856D (B.) Nav1.7 expressing-neurons exhibited very limited neurite degeneration. At 18 DIV, neurons were depolarized with 50 mM KCl and maintained for additional 4 days in the presence of 50 mM KCl. This treatment did not increase neurite degeneration in neurons expressing either WT Nav1.7 (C.) or mutant G856D Nav1.7 channels (D.). Inhibition of glycolysis in 18 DIV neurons with 2-deoxyglucose (2-DG) for 4 days also failed to increase degeneration in neurons expressing either WT Nav1.7 (E.) or mutant G856D Nav1.7 (F.) channels. Inhibition of glycolysis for 4 days in the presence of 50 mM KCl did not evoke neurite degeneration in WT Nav1.7 expressing-neurons (G.). In contrast, inhibition of glycolysis for 4 days in the presence of 50 mM KCl evoked significant neurite fragmentation and/or blebbing in Nav1.7 G856D expressing neurons (H.).

**Figure 8** Metabolic stress in a depolarizing condition causes significant neurite degeneration in Nav1.7 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 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 co-expressed GFP (\( n \geq 4 \) wells per condition from triplicate experiments). Quantitative analysis demonstrated a significant difference in neurite degeneration in WT Nav1.7 versus G856D Nav1.7- expressing neurons after 18 days in culture. Treatment with 50 mM KCl or inhibition of glycolysis by 2-deoxyglucose (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 to WT Nav1.7 channels (*P<0.05).

Figure 9  Reverse sodium-calcium exchange (NCX)-mediated rise in calcium plays a critical role in neurite degeneration in Nav1.7 G856D expressing DRG neurons. Neurites of both WT Nav1.7 (A.) and mutant G856D Nav1.7 (B.) expressing neurons exhibit limited neurite degeneration at 18 DIV. Inhibition of glycolysis for 4 days in the presence of 50mM KCl induces substantial neurite degeneration in mutant G856D Nav1.7 expressing neurons (F.). Omission of Ca^{2+} from the medium and treatment with EGTA, or treatment with KB-R7943, attenuates neurite degeneration in metabolically stressed and depolarized Nav1.7 G856D-expressing DRG neurons. Representative images are shown of DRG neurons treated with 2-DG and 50 mM KCl for 4 days with omission of C^{2+} from the medium and treatment with 100 μM EGTA (G.) or treatment with 0.5 μM KB-R7943 (H). WT Nav1.7 neurites exhibit limited neurite degeneration following treatment with 50 mM KCl and 2-DG (C.), which persists when treated with EGTA (D.) or 2-DG (E.). Quantitative analysis demonstrates that omission of Ca^{2+} and calcium chelation by EGTA, and inhibition of NCX activity, both attenuate neurite degeneration in G856D-expressing DRG neurons. n≥6 wells per condition from triplicate experiments, (*significantly different from WT (p<0.001); # significantly different from 2-DG and KCl treated neurons, p<0.001).
Cell body $[\text{Ca}^{2+}]$

(A) Graph showing $R_{340/380}$ over time with high [K$^+$], SBS, G856D, and TTX.

(B) Bar graph showing AUC with G856D and G856D + TTX.

Neurites $[\text{Ca}^{2+}]$

(C) Graph showing $R_{340/380}$ over time with high [K$^+$], SBS, G856D, and TTX.

(D) Bar graph showing AUC with G856D and G856D + TTX.
Cell body $[\text{Ca}^{2+}]$

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