



Elevated ocular pressure reduces voltage-gated sodium channel NaV1.2 protein expression in retinal ganglion cell axons

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ABSTRACT

Glaucoma is an age-related neurodegenerative disease that is commonly associated with sensitivity to intraocular pressure. The disease selectively targets retinal ganglion cells (RGCs) and constituent axons. RGC axons are rich in voltage-gated sodium channels, which are essential for action potential initiation and regeneration. Here, we identified voltage-dependent sodium channel, NaV1.2, in the retina, examined how this channel contributes to RGC light responses, and monitored NaV1.2 mRNA and protein expression in the retina during progression of modeled glaucoma. We found NaV1.2 is predominately localized in ganglion cell intraretinal axons with dispersed expression in the outer and inner plexiform layers. We showed Phrixotoxin-3, a potent NaV1.2 channel blocker, significantly decreased RGC electrical activity in a dose-dependent manner with an I_{C50} of 40 nM. Finally, we found four weeks of raised intraocular pressure (30% above baseline) significantly increased NaV1.2 mRNA expression but reduced NaV1.2 protein level in the retina up to 57% ($p < 0.001$). Following prolonged intraocular pressure elevation, NaV1.2 protein expression particularly diminished at distal sections of ganglion cell intraretinal axons ($p \leq 0.01$). Our results suggest NaV1.2 might be a therapeutic target during disease progression to maintain RGC excitability, preserving presynaptic connections through action potential backpropagation.

Glaucoma (glaucomatous optic neuropathy) is an age-related neurodegenerative disease worsened by sensitivity to intraocular pressure, which is the symptom typically treated (Heijl et al., 2002; Calkins, 2012). The defining feature of glaucoma is selective degeneration of retinal ganglion cells (RGCs) and their axons. Prior to outright RGC death, axons show degenerative signatures, including anterograde transport deficits, neurofilament loss, reduced optic nerve compound action potentials, and axon dropout (Buckingham et al., 2008; Baltan et al., 2010; Crish et al., 2010). Ganglion cell intraretinal axons are particularly sensitive to pressure due to their tortuous path. Intraretinal axons collect into axon bundles and exit the optic disk at right angles, forming the optic nerve head (Soto et al., 2008). RGC intraretinal axons are also vulnerable to stress due to high energy demands of voltage-gated sodium channels within unmyelinated axon sections (Calkins, 2012).

Of the 10 identified voltage-sensitive sodium (NaV1.1–NaVx) channel isoforms, NaV1.1, NaV1.2, NaV1.3, and NaV1.6 have been found in various tissues of the central nervous system, including the retina (Fjell et al., 1997; Caldwell et al., 2000; Boiko et al., 2003; Van

Wart et al., 2005, 2007; Mojumder et al., 2007; Catterall, 2012). Previously, we reported expression of NaV1.6, which is essential for action potential initiation and propagation, transiently increased following modest short-term intraocular pressure elevation (Rush et al., 2005; Hu et al., 2009; Risner et al., 2018). Prolonged (four weeks) ocular hypertension led to reduced NaV1.6 expression (Risner et al., 2018). Here, we examined how modeled glaucoma affects NaV1.2, which has been shown to colocalize with NaV1.6 within ganglion cell intraretinal axons and mediate action potential backpropagation (Boiko et al., 2003; Hu et al., 2009). We found NaV1.2 is expressed predominately within intraretinal ganglion cell axons. The NaV1.2 blocker, Phrixotoxin-3, significantly suppressed light responses of physiologically-identified α ON-sustained RGCs in a dose-dependent manner. Raising intraocular pressure for four weeks reduced NaV1.2 protein but increased mRNA expression in the retina. Our results, taken together, confirm the architectural expression of NaV1.2 in the retina, show NaV1.2 significantly contributes to RGC electrical activity, and implicates NaV1.2 as a potential therapeutic target in glaucoma.

All methods employed in this study were approved by the

Abbreviations: RGC, Retinal ganglion cell; NaV, Voltage-gated sodium; Phx3, Phrixotoxin-3; mRNA, messenger ribonucleic acid

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Vanderbilt University Medical Center Institutional Animal Care and Use Committee. We modeled glaucoma in adult (2 months old) C57Bl/6 male mice (C57, Charles River Laboratory) by raising intraocular pressure (IOP) using the microbead occlusion model (Sappington et al., 2010; Calkins et al., 2018). Polystyrene microbeads (1.5 μ L volume, 15 μ m bead size, Invitrogen, Carlsbad CA) were injected into the anterior chamber of one eye and an equal volume of saline into the fellow eye while animals were anesthetized (2.5% isoflurane). Intraocular pressure was measured in anesthetized animals 2–3 times per week for up to 4 weeks by rebound tonometry (Tonopen XL, Medtronic Solan, Waltham MA). Microbead injection significantly raised eye pressure up to 30% in the cohort of animals used for immunohistochemical analysis of protein level (two week cohort (mean $\pm$ sem): 19.1 ± 2 vs 14.6 ± 1.7 mmHg; four week cohort: 19 ± 1.3 vs 14.9 ± 1.3 mmHg; see Supplementary Results in (Risner et al., 2018)). Microbead injection also significantly raised intraocular pressure up to 30% in animals used for mRNA expression quantification (two-week cohort (mean $\pm$ sem): 21.5 ± 1.3 vs 14.9 ± 1.1 mmHg, three-week cohort: 22.75 ± 1.25 vs 16.6 ± 1 mmHg, four-week cohort: 22.3 ± 1.9 vs 16.7 ± 1.6 mmHg).

For immunohistochemical experiments, animals were transcardially perfused with 4% paraformaldehyde in PBS and retinas were dissected out of the eyes. Retinas from each cohort (two and four week) and condition (microbead and saline) were vertically cryosectioned (10 μ m thick) or kept whole. Retinal sections were labeled with anti-NaV1.2 (1:200, Alomone Labs, Jerusalem Israel), anti-choline acetyltransferase (ChAT, 1:100, Millipore, Burlington MA), and mounted using Fluoromount medium with DAPI (SouthernBiotech, Birmingham AL). Whole retinas were labeled with anti-NaV1.2, anti-Pou domain transcription factor (Brn3a, 1:200 Santa Cruz Biotechnology, Dallas TX), and anti-neurofilament H (SMI32, 1:1000, BioLegend, San Diego CA). Retinal sections and whole mounts were imaged (0.72 μ m z slices) using an Olympus FV-1000 confocal microscope with a 60X objective (Vanderbilt Cell Imaging Shared Resource). Confocal laser transmissivity and exposure settings (4 μ s/pixel) were the same for all imaging sessions: SMI32 = 488 nm 5% transmissivity, NaV1.2 = 543 nm 25% transmissivity, Brn3a = 633 nm 25% transmissivity. Individual RGCs, marked by SMI32 labeling, were imaged using a 60X objective with 3X magnification. Images were analyzed using ImageJ (Fuji, Version 1.51i). Colocalization analysis was performed using the Coloc2 plugin within ImageJ. We measured the proportion of colocalization between SMI32 and NaV1.2 (tM1) and NaV1.2 and SMI32 (tM2 (Manders et al., 1993)). We also assessed NaV1.2 intensity distributions along SMI32-labeled axon proximal segments. NaV1.2 intensity profiles along each separate axon segment were normalized relative to the total length of that segment. Normalized segments were binned into 0.02 sections. NaV1.2 intensities were then averaged within each bin (Duflocq et al., 2011).

We also assessed the influence of elevated intraocular pressure on NaV1.2 mRNA expression. Total RNA was extracted from each retina according to the SV Total RNA Isolation System kit protocol (Promega, Madison, WI). RNA concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm using a NanoDrop 8000 (Thermo Scientific, Wilmington, DE). mRNA was then reverse-transcribed into cDNA using the 1st Strand cDNA Synthesis System for quantitative RT-PCR (Origene, Rockville, MD) following the manufacturer's instructions. Reaction mixtures were diluted 2-fold and real-time PCR was performed using QuantStudio 3 Real Time PCR System (Applied Biosystem, Waltham, MA) using PowerUp SYBR Green master mix (Applied Biosystem) and the following primers: NaV1.2 (Fw) 5'-GGGAACGCCCATCAAAGAAG-3' (Rev) 5'-ACGCTATCGTAGGAAGTGG-3'; GAPDH (Fw) 5'-TCCATGACAACCTTGGCATTG-3' (Rev) 5'-CAGTCTTCTGGGTGGCAGTGA-3' (Laedermann et al., 2014). Cycling conditions were: 95 $^{\circ}$ C for 2 min and 40 cycles of 95 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s. Samples were run in triplicate and analysis was performed using the comparative CT method, using GAPDH mRNA

as endogenous controls.

In a subset of experiments, we examined NaV1.2 channel contribution to the light-evoked spike rate of RGCs from naïve C57 mice (1.5–2 months old). Retinas were dissected out of the eye and placed in a physiological chamber continually perfused (2 mL/min) with oxygenated Ames' medium (pH = 7.4, Osmolarity = 290, U.S. Biologicals, Salem MA), supplemented with 20 mM glucose and heated to 35 $^{\circ}$ C (TC-344C, Warner Instruments). RGCs with large somas (> 18 μ m in diameter) were targeted for recording with pipettes filled with (in mM) 125 K-Gluconate, 10 KCl, 10 HEPES, 10 EGTA, 4 Mg-ATP, and 1 Na-GTP (pH = 7.35, Osmolarity = 285). Pipette resistance ranged from 4 to 8 M Ω . Electrical signals were amplified (MultiClamp 700B, Molecular Devices, Sunnyvale CA), digitized (DigiData 1550A, Molecular Devices), and sampled at 50 kHz. Light responses were evoked using a full-field flash produced by a 365 nm 300 μ W/cm² light-emitting diode (Roithner Lasertechnik, Vienna Austria). NaV1.2 channels were modulated by bath application of 25–400 nM Phrixotoxin-3 (Alomone labs (Bosmans et al., 2006)). Physiological data were analyzed using Clampfit 10.6 (Molecular Devices). Statistical analysis was performed with SigmaPlot Version 14.0 (Systat Software Inc., San Jose CA).

NaV1.2 is localized within RGC axon initial segments of adult mice (Boiko et al., 2003). To confirm this finding, we assessed NaV1.2 expression within individual intraretinal ganglion cell axons. RGCs and their axons were identified by NaV1.2 and SMI32 immunolabeling, and axons were distinguished from dendrites by the axon hillock and z-axis depth. RGC proximal axon segments were clearly labeled by anti-NaV1.2 (Fig. 1A dashed line). We examined the expression pattern of NaV1.2 within RGC axon proximal segments by manually tracing the axon, using the segmented line tool in ImageJ. The line width was 0.7 μ m. The average axon proximal segment length was 35 ± 1.7 μ m, which is similar to axon initial segment lengths previously reported (up to 30 μ m (Boiko et al., 2003)). The intensity profile of NaV1.2 labeling appeared to form a normal distribution (Fig. 1B). We also found NaV1.2 expression within distal RGC axons (Fig. 1A arrowheads). NaV1.2 is particularly localized within axon varicosities of distal RGC intraretinal axons, as evidenced by the spatial pattern of NaV1.2 immunolabeling and the overlap of NaV1.2 and SMI32 signals (Fig. 1C–D). In addition, we found NaV1.2 labeling defined the perimeter of many RGC somas (Fig. 1E). NaV1.2 intensity profiles show NaV1.2 staining outlined the cell body, reduced labeling in surrounding vicinities, and sparse NaV1.2 localization within Brn3a-positive areas (Fig. 1F).

NaV1.2 has previously been shown to distinctively promote action potential backpropagation in pyramidal neurons of the rodent prefrontal cortex (Hu et al., 2009). Action potential backpropagation significantly regulates dendritic synaptic strength (Eilers and Konnerth, 1997). RGC dendritic remodeling and pruning is observed in several models of glaucoma (Della Santina et al., 2013; El-Danaf and Huberman, 2015; Ou et al., 2016; Risner et al., 2018). Thus, NaV1.2 channels might mediate RGC dendritic organization via action potential backpropagation during disease progression. However, it is unknown if NaV1.2 channels significantly modulate RGC electrical activity.

To address the functional contribution of NaV1.2 channels on RGC light responses, we measured the influence of the NaV1.2 channel blocker, Phrixotoxin-3 (Phx3). We targeted RGCs with large cell bodies (20 ± 0.3 μ m diameter) that produced a sustained spike train during light presentation (Fig. 1G, cell n = 10, animal n = 3). These cells have been categorized as α ON-sustained (α ON-S) RGCs (Pang et al., 2003; Risner et al., 2018). We assessed each cell's light response by counting the number of spikes during the 3 s of light onset and dividing by the number of trials (≥ 6 trials) and time (i.e., referred to as spike rate or spikes/s). α ON-S RGCs produced 51 ± 9.5 spikes/s during light onset. To determine the potency of Phx3 on blocking spike activity, we bath-applied the drug from 25 to 400 nM for 2 min and measured the effects of the agent on the light response for each concentration tested (Fig. 1H). Application of just 25 nM Phx3 significantly decreased light-

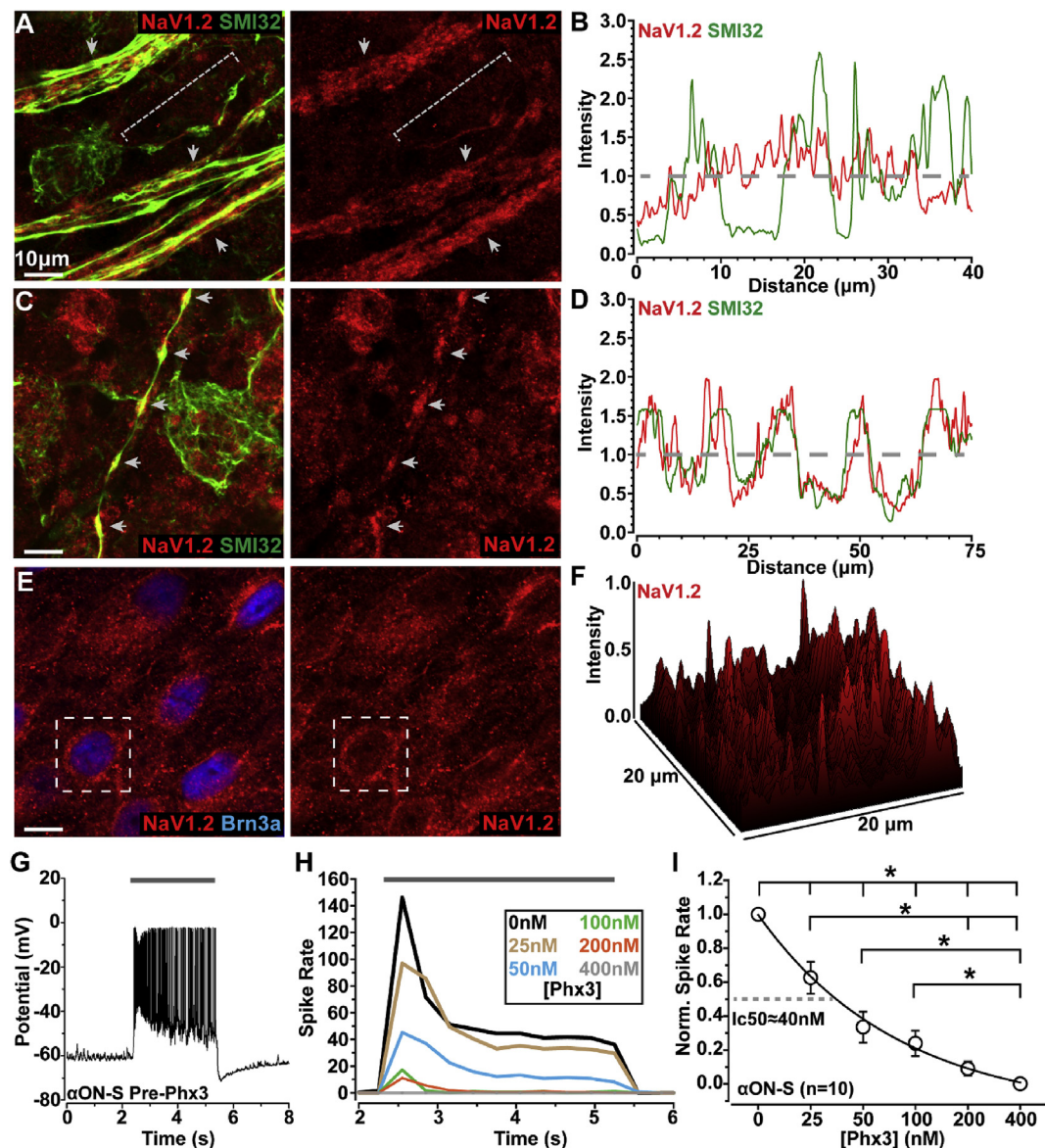


Fig. 1. Assessment of NaV1.2 channel protein level and physiology in retinal ganglion cells. (A) NaV1.2 (red) is expressed along retinal ganglion cell (RGC) axon proximal sections (dashed line segment) as identified by SMI32 (green) and z-axis depth within whole mount retinas. NaV1.2 is also localized within distal axons (arrowheads) marked by SMI32. (B) Intensity profiles of NaV1.2 and SMI32 along the axon proximal segment indicated in A. The heavy dashed horizontal line indicates mean intensity for each signal. (C) NaV1.2 is localized within varicosities of distal axons (arrowheads). (D) Overlap of NaV1.2 and SMI32 signals within distal axon example shown in C. The heavy dashed horizontal line marks the mean intensity for each signal. (E) NaV1.2 outlines Brn3a-positive (blue) somas. (F) NaV1.2 intensity profile of example RGC soma (dashed square) shown in E. Scale bar = 10 μm. (G) Example light-evoked trace from a typical αON-sustained RGC prior to blockade of NaV1.2. (H) The NaV1.2-specific blocker, Phrixotoxin-3 (Phx3), reduces light-evoked spiking in a dose-dependent manner in an example αON-sustained RGC (histogram bin width = 300 ms). (I) Light-evoked spike rate was normalized to pre-drug light response for each cell (n = 10). Spike rate was significantly (*, p ≤ 0.04) reduced by application of 25 nM Phx3, and spiking was completely abolished by 400 nM for all cells analyzed. Solid line is the best-fit exponential decay (R² = 0.88). Based on the exponential decay function 40 nM Phx3 would reduce the light-evoked spike rate by 50% (I_{c50}, dashed gray line). The heavy solid gray horizontal lines in G and H indicates light duration (3 s). Data represented as mean ± sem. Statistics: One-Way ANOVA, Tukey Post-hoc comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

evoked activity by 38% (p = 0.001). Raising the concentration of Phx3 to 50 nM suppressed light-induced firing by 67% (p < 0.001). Light-evoked spiking was completely abolished by 400 nM Phx3 (Fig. 1I). We determined the I_{c50} of Phx3 on the light-evoked spike rate using interpolated values from a best-fit exponential decay function. This analysis revealed 40 nM of Phx3 reduced the light-evoked spike rate by half (Fig. 1I), interestingly this is the concentration used to probe NaV1.2 channels in pyramidal neurons of the prefrontal cortex (Yin et al., 2017). Following Phx3 washout (> 20 min), light responses returned 72 ± 7% of pre-drug firing rates for all but one cell, which failed to recover from the highest dose of Phx3. This reduction in light-evoked

activity following drug washout may be due to cell run-down as experiments typically lasted about 1.5 h, including all washout cycles.

In a previous report, we found two weeks of modest intraocular pressure elevation (30% above baseline) led to a transient increase in NaV1.6 expression within RGC intraretinal axons. After four weeks of ocular hypertension, NaV1.6 abundance decreased (Risner et al., 2018). Here, we explore how modeled glaucoma influences NaV1.2 mRNA and protein expression within the retina.

We first examined the potential influence of raising eye pressure on NaV1.2 mRNA expression in the retina. We found NaV1.2 expression is dependent on the duration of raised intraocular pressure. Elevating

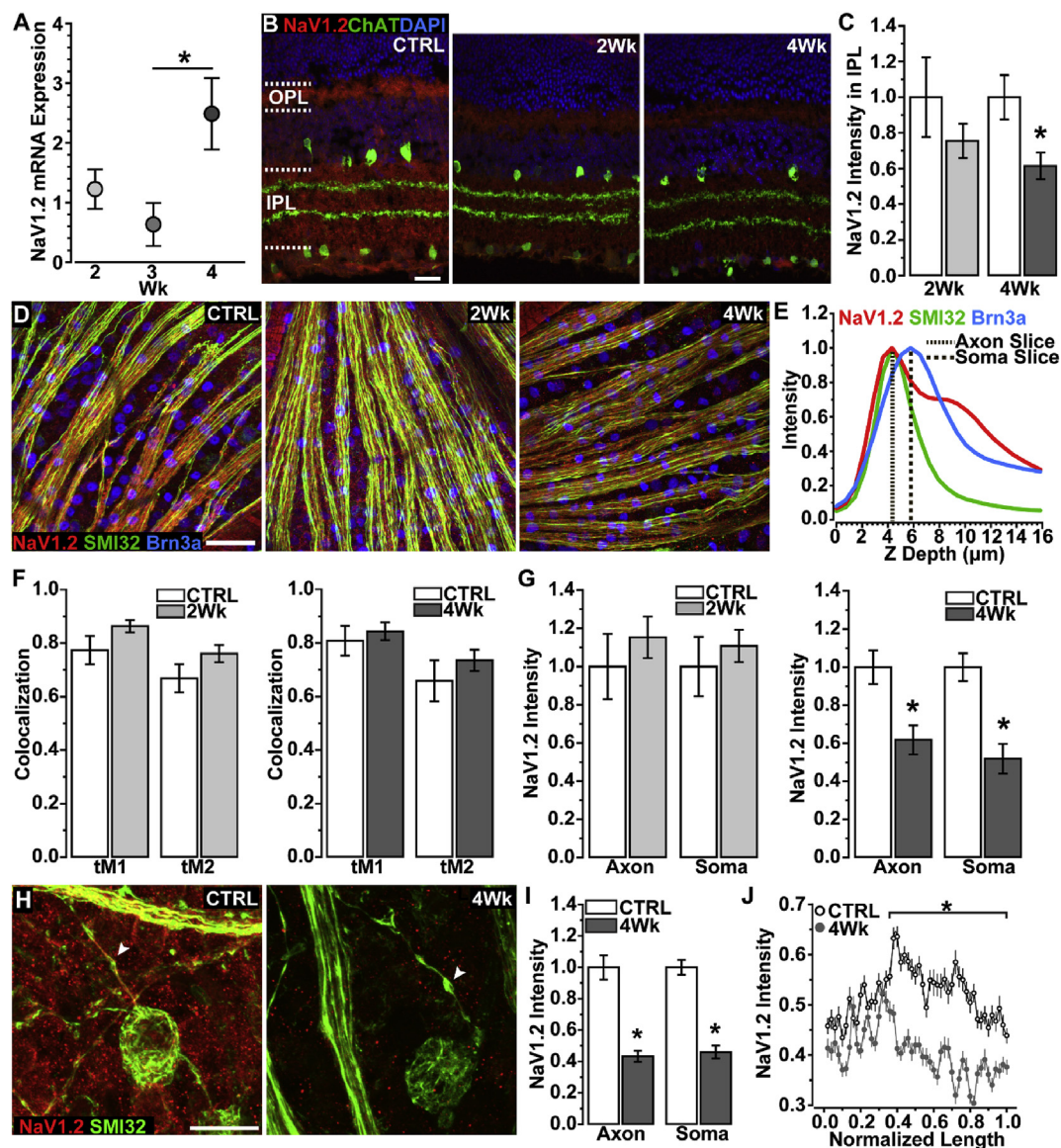


Fig. 2. Retinal NaV1.2 channel protein expression decreases following increased IOP. (A) Relative NaV1.2 mRNA expression (microbead:saline) following two ($n = 4$), three ($n = 4$), and four ($n = 4$) weeks of raised intraocular pressure (*, $p = 0.039$). (B) Retinal vertical sections labeled for NaV1.2 (red), ChAT (green), and DAPI (blue) in saline-injected control, 2-week microbead, and 4-week microbead retinas. OPL, outer plexiform layer, IPL, inner plexiform layer. Scale bar = 20 μ m. (C) Quantification of NaV1.2 intensity within the IPL relative to control retinas revealed no significant difference in protein level after 2 weeks ($p = 0.31$) but reduced expression after 4 weeks (*, $p = 0.02$) of raised IOP (30% above baseline). (D) Maximum projection micrographs of whole-mounted retinas show 4 weeks of elevated IOP decreases NaV1.2 (red) staining within RGCs co-labeled by SMI32 (cell bodies and axon bundles, green) and the transcription factor Brn3a (cell bodies, blue). Scale bar = 50 μ m. (E) NaV1.2 expression was analyzed in RGC axons within the plane of most intense SMI32 labelling and somas within the plane of densest Brn3a staining. (F) Colocalization of SMI32 to NaV1.2 (tM1) and NaV1.2 to SMI32 (tM2) is not influenced by increased IOP ($p \geq 0.11$). (G) Quantification of NaV1.2 within SMI32-positive axon layer and Brn3a-positive soma layer show expression significantly diminishes after 4 weeks of elevated IOP (*, $p \leq 0.003$). (H) NaV1.2 (red) localizes in SMI32-labeled (green) RGC somas and axons (arrowheads). Scale bar = 20 μ m. (I) Following 4 weeks of raised IOP, NaV1.2 intensity significantly decreases within RGC axon varicosities and somas (*, $p < 0.001$). (J) NaV1.2 intensity distribution normalized by length (bin width = 0.02 μ m). NaV1.2 significantly decreases within distal axon sections (*, $p \leq 0.01$). Data represented as mean $\pm$ sem. Statistics: A: One-way ANOVA, Tukey Post hoc; C–J: Independent samples t-tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pressure for two weeks did not alter NaV1.2 mRNA levels ($p = 0.81$). Three weeks of intraocular pressure elevation decreased NaV1.2 mRNA abundance by 37% ($p = 0.33$). Raising pressure for four weeks, however, increased NaV1.2 mRNA expression by 93% compared to retinas of saline-injected eyes ($p = 0.06$). Given that mRNA expression appears to be altered by the duration of raised pressure, we compared NaV1.2 levels between each time point. We observed an approximate 3-fold increase in NaV1.2 mRNA expression between three and four weeks of raised eye pressure (*, $p = 0.039$ Fig. 2A).

Next, we assessed the impact of intraocular pressure elevation on

NaV1.2 protein expression within the outer and inner plexiform layers of the retina (OPL, IPL). Following IOP elevation, retinal cryosections were immunolabeled for NaV1.2, ChAT, and DAPI. The outer plexiform layer was identified based on the lack of DAPI staining between the outer and inner nuclear layers. Similar to Mojumder et al. (2007), we find dispersed NaV1.2 labeling within the OPL (Fig. 2B). We quantified NaV1.2 expression by manually outlining the OPL within each retinal section ($n = 4$ sections/retina, animal $n = 6$) and computing the average channel intensity for the region of interest using ImageJ. Raising eye pressure modestly decreased NaV1.2 expression within the

OPL at both two (25%, $p = 0.12$) and four weeks (35%, $p = 0.18$). Next, we assessed NaV1.2 expression within the IPL, which was identified by ChAT labeling and lack of DAPI staining. Comparable to the OPL, the IPL exhibited dispersed NaV1.2 labeling. NaV1.2 expression within the IPL was unaffected by two weeks of elevated pressure ($p = 0.33$). However, following four weeks of elevated pressure, NaV1.2 staining within the IPL was significantly reduced by 39% ($p = 0.02$, Fig. 2C).

We then broadly evaluated the influence of intraocular pressure on NaV1.2 channel protein localization within RGC axon and soma sub-layers from large patches (212 μm^2) of whole-mounted retinas from all quadrants ($n = 4$ images/retina, animal $n = 6$). The axon layer, marked by predominate SMI32 staining of axon bundles, possesses intense NaV1.2 expression, colocalizing with SMI32 by $72 \pm 3\%$ (Fig. 2D, F). The RGC soma layer appears weak compared to the axon layer but more punctate relative to the plexiform layers. Following four weeks of intraocular pressure elevation, NaV1.2 appears to be reduced (Fig. 2D, right). We separately measured NaV1.2 expression within RGC soma and axon layers by identifying the plane with the densest SMI32 (axon layer) and Brn3a (soma layer) labeling and computing the average intensity of the NaV1.2 channel (Fig. 2E). Following two weeks of raised pressure, we did not detect a change in NaV1.2 protein expression within RGC axon or soma layers (axon layer $p = 0.37$, soma layer $p = 0.16$, Fig. 2G, left). However, after four weeks of elevated pressure, NaV1.2 expression significantly decreased in both RGC axon (38%, $p = 0.003$) and soma layers (48%, $p = 0.002$, Fig. 2G, right).

To confirm our analysis of the impact of ocular hypertension on NaV1.2 expression within RGC somas and axons from large retinal images, we examined NaV1.2 localization in individual SMI32-positive RGCs from the same images ($n \geq 19$ cells for each condition). Visually, NaV1.2 intensity was drastically reduced after four weeks of elevated IOP (Fig. 2H). NaV1.2 channel expression was analyzed in ImageJ using the freehand draw function to outline somas and the line segment tool (0.7 μm line width) to mark axons. NaV1.2 channel mean intensity was computed for each region of interest. There was no significant difference in proximal axon segment lengths between microbead- and saline-injected eyes (saline = $35 \pm 1.7 \mu\text{m}$, microbead = $39 \pm 1.8 \mu\text{m}$, $p = 0.09$). Like the results from our analysis of large retinal patches (Fig. 2D, G), NaV1.2 expression is unaltered after two weeks of elevated pressure in either RGC somas ($p = 0.29$) or axons ($p = 0.84$). Four weeks of raised intraocular pressure, however, significantly reduced NaV1.2 intensity within RGC somas (54%, $p < 0.001$) and their axons (57%, $p < 0.001$, Fig. 2I). To further analyze where the NaV1.2 dropout occurs within axon proximal segments, we plotted the distribution of NaV1.2 intensities normalized by length. NaV1.2 expression significantly decreased within distal sections of the axon following four weeks of elevated pressure ($p \leq 0.01$, Fig. 2J).

Our results confirm NaV1.2 expression within the retina, and we identify dense NaV1.2 localization within ganglion cell intraretinal axons (Boiko et al., 2003). Furthermore, for the first time, we show NaV1.2 channels significantly contribute to RGC light-evoked spiking. Finally, we find retinal NaV1.2 mRNA expression is increased but protein is reduced after prolonged intraocular pressure elevation.

At steady-state, mRNA concentration is a weak predictor of protein abundance (Gygi et al., 1999; Vogel and Marcotte, 2012). Protein availability is determined by dynamically linked processes controlling production and degradation of mRNA and protein, including translation rates, modification of protein turnover, protein synthesis time, protein localization, and cellular state. When the cellular environment is perturbed, the relationship between mRNA levels and protein abundance is often temporally discordant (Vogel et al., 2011; Liu et al., 2016; Martin-Perez and Villén, 2017).

Here, we found elevating pressure for two weeks does not change NaV1.2 mRNA relative expression and protein in the retina (Fig. 2A, C, G). After three weeks of pressure elevation, we found a modest decrease in mRNA expression. Following four weeks of increased intraocular

pressure, NaV1.2 mRNA increases while local protein expression decreases (Fig. 2A, C, G, I). Based on our current results, we suggest the modest decrease in NaV1.2 mRNA expression noted after three weeks of raised pressure contribute to the reduction of protein observed following four weeks of ocular hypertension, and the significant increase in NaV1.2 mRNA at four weeks post microbead injection might reveal an on-going compensatory reaction to IOP-related reduction in NaV1.2 protein. A lengthy delay between changes in transcription and protein levels is possible given the reported half-life of NaV channels is approximately 30 h (Maltsev et al., 2008).

Previously, we found four to five weeks of elevated IOP decreased RGC spike activity and decreased RGC dendritic complexity, suggesting a positive correlation between spike rate and dendritic arbor maintenance (Weitlauf et al., 2014; Risner et al., 2018). In our previous studies, intracellular recordings of action potentials were performed from RGC somas, which is a measure of spike backpropagation. Recent evidence suggests a direct relationship between NaV1.2-mediated backpropagation and synaptic strength (Shin et al., 2019; Spratt et al., 2019).

Heterozygous loss of *Scn2a* (+/-), the gene that encodes the α subunit of NaV1.2 channels, significantly decreases excitability of hippocampal CA1 and prefrontal cortical (PFC) pyramidal neurons (Shin et al., 2019; Spratt et al., 2019). In PFC pyramidal neurons of *Scn2a*^{+/-} mice, somatic current injections fail to depolarize dendrites and these dendrites have less Ca^{2+} influx, suggesting a reduction in synaptic strength considering intracellular Ca^{2+} levels predict long-term potentiation (LTP) (Eilers and Konnerth, 1997; Spratt et al., 2019). In agreement with this notion, *Scn2a*^{+/-} mice show reduced LTP strength and frequency of spontaneous excitatory postsynaptic currents (Shin et al., 2019; Spratt et al., 2019). Finally, *Scn2a*^{+/-} PFC pyramidal neuron dendrites have thinner, immature dendritic spines, indicating weaker synaptic connections with presynaptic neurons (Spratt et al., 2019).

Since NaV1.2 channels support action potential electrogenesis, backpropagation, and consequently, synaptic strength, our current results suggest if RGC electrical activity can be maintained during the early stages of modeled glaucoma, synaptic integrity may be preserved. Previously, we have found four weeks of raised IOP led to significant RGC dendritic pruning, but did not cause outright RGC degeneration (Risner et al., 2018). However, two and four weeks of raised IOP decreases anterograde transport of cholera toxin subunit B to the superior colliculus, indicating progression of axonopathy (Risner et al., 2018). Following eight weeks of microbead-induced elevated pressure, we find significant RGC cell body dropout and axon loss in the optic nerve (Sappington et al., 2010; Chen et al., 2011). These results suggest RGC-bipolar cell synapse loss precedes RGC death and axon dropout. Activating NaV1.2 channels might be one way to maintain the backward-propagating spread of depolarization in order maintain presynaptic connections in addition to pressure-lowering treatment.

Author contributions

M.L.R., N.R.M., S.P., W.S.L., D.J.C. designed research; M.L.R., N.R.M., S.P., W.S.L. performed research; M.L.R., N.R.M., S.P. analyzed data; M.L.R. N.R.M, D.J.C. wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exer.2019.107873>.

References

- Baltan, S., Inman, D.M., Danilov, C.A., Morrison, R.S., Calkins, D.J., Horner, P.J., 2010. Metabolic vulnerability disposes retinal ganglion cell axons to dysfunction in a model of glaucomatous degeneration. *J. Neurosci.* 30, 5644–5652.
- Boiko, T., Van Wart, A., Caldwell, J.H., Levinson, S.R., Trimmer, J.S., Matthews, G., 2003. Functional specialization of the axon initial segment by isoform-specific sodium channel targeting. *J. Neurosci.* 23, 2306–2313.
- Bosmans, F., Rash, L., Zhu, S., Diocot, S., Lazdunski, M., Escoubas, P., Tytgat, J., 2006. Four novel tarantula toxins as selective modulators of voltage-gated sodium channel subtypes. *Mol. Pharmacol.* 69, 419–429.
- Buckingham, B.P., Inman, D.M., Lambert, W., Oglesby, E., Calkins, D.J., Steele, M.R., Vetter, M.L., Marsh-Armstrong, N., Horner, P.J., 2008. Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. *J. Neurosci.* 28, 2735–2744.
- Caldwell, J.H., Schaller, K.L., Lasher, R.S., Peles, E., Levinson, S.R., 2000. Sodium channel Na(v)1.6 is localized at nodes of ranvier, dendrites, and synapses. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5616–5620.
- Calkins, D.J., 2012. Critical pathogenic events underlying progression of neurodegeneration in glaucoma. *Prog. Retin. Eye Res.* 31, 702–719.
- Calkins, D.J., Lambert, W.S., Formichella, C.R., McLaughlin, W.M., Sappington, R.M., 2018. The microbead occlusion model of ocular hypertension in mice. *Methods Mol. Biol.* 1695, 23–39.
- Catterall, W.A., 2012. Voltage-gated sodium channels at 60: structure, function and pathophysiology. *J. Physiol.* 590, 2577–2589.
- Chen, H., Wei, X., Cho, K.S., Chen, G., Sappington, R., Calkins, D.J., Chen, D.F., 2011. Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. *Investig. Ophthalmol. Vis. Sci.* 52, 36–44.
- Crish, S.D., Sappington, R.M., Inman, D.M., Horner, P.J., Calkins, D.J., 2010. Distal axonopathy with structural persistence in glaucomatous neurodegeneration. *Proc. Natl. Acad. Sci. U. S. A.* 107, 5196–5201.
- Della Santina, L., Inman, D.M., Lupien, C.B., Horner, P.J., Wong, R.O., 2013. Differential progression of structural and functional alterations in distinct retinal ganglion cell types in a mouse model of glaucoma. *J. Neurosci.* 33, 17444–17457.
- Duflocq, A., Chareyre, F., Giovannini, M., Couraud, F., Davenne, M., 2011. Characterization of the axon initial segment (AIS) of motor neurons and identification of a para-AIS and a juxtapara-AIS, organized by protein 4.1B. *BMC Biol.* 9, 66–66.
- Eilers, J., Konnerth, A., 1997. Dendritic signal integration. *Curr. Opin. Neurobiol.* 7, 385–390.
- El-Danaf, R.N., Huberman, A.D., 2015. Characteristic patterns of dendritic remodeling in early-stage glaucoma: evidence from genetically identified retinal ganglion cell types. *J. Neurosci.* 35, 2329–2343.
- Fjell, J., Dib-Hajj, S., Fried, K., Black, J.A., Waxman, S.G., 1997. Differential expression of sodium channel genes in retinal ganglion cells. *Brain Res. Mol. Brain Res.* 50, 197–204.
- Gygi, S.P., Rochon, Y., Franza, B.R., Aebersold, R., 1999. Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* 19, 1720–1730.
- Heijl, A., Leske, M.C., Bengtsson, B., Hyman, L., Hussein, M., 2002. Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial. *Arch. Ophthalmol.* 120, 1268–1279.
- Hu, W., Tian, C., Li, T., Yang, M., Hou, H., Shu, Y., 2009. Distinct contributions of Na(v)1.6 and Na(v)1.2 in action potential initiation and backpropagation. *Nat. Neurosci.* 12, 996–1002.
- Laedermann, C.J., Pertin, M., Suter, M.R., Decosterd, I., 2014. Voltage-gated sodium channel expression in mouse DRG after SNI leads to re-evaluation of projections of injured fibers. *Mol. Pain* 10, 19–19.
- Liu, Y., Beyer, A., Aebersold, R., 2016. On the dependency of cellular protein levels on mRNA abundance. *Cell* 165, 535–550.
- Maltsev, V.A., Kyle, J.W., Mishra, S., Undrovinas, A., 2008. Molecular identity of the late sodium current in adult dog cardiomyocytes identified by Nav1.5 antisense inhibition. *Am. J. Physiol. Heart Circ. Physiol.* 295, H667–H676.
- Manders, E.M.M., Verbeek, F.J., Aten, J.A., 1993. Measurement of co-localization of objects in dual-colour confocal images. *J. Microsc.* 169, 375–382.
- Martin-Perez, M., Villén, J., 2017. Determinants and regulation of protein turnover in yeast. *Cell Syst.* 5, 283–294 e285.
- Mojumder, D.K., Frishman, L.J., Otteson, D.C., Sherry, D.M., 2007. Voltage-gated sodium channel alpha-subunits Na(v)1.1, Na(v)1.2, and Na(v)1.6 in the distal mammalian retina. *Molecular Vision* 13, 2163–2182.
- Ou, Y., Jo, R.E., Ullian, E.M., Wong, R.O., Della Santina, L., 2016. Selective vulnerability of specific retinal ganglion cell types and synapses after transient ocular hypertension. *J. Neurosci.* 36, 9240–9252.
- Pang, J.J., Gao, F., Wu, S.M., 2003. Light-evoked excitatory and inhibitory synaptic inputs to ON and OFF alpha ganglion cells in the mouse retina. *J. Neurosci.* 23, 6063–6073.
- Risner, M.L., Pasini, S., Cooper, M.L., Lambert, W.S., Calkins, D.J., 2018. Axogenic mechanism enhances retinal ganglion cell excitability during early progression in glaucoma. *Proc. Natl. Acad. Sci. U. S. A.* 115, E2393–e2402.
- Rush, A.M., Dib-Hajj, S.D., Waxman, S.G., 2005. Electrophysiological properties of two axonal sodium channels, Nav1.2 and Nav1.6, expressed in mouse spinal sensory neurones. *J. Physiol.* 564, 803–815.
- Sappington, R.M., Carlson, B.J., Crish, S.D., Calkins, D.J., 2010. The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Investig. Ophthalmol. Vis. Sci.* 51, 207–216.
- Shin, W., Kweon, H., Kang, R., Kim, D., Kim, K., Kang, M., Kim, S.Y., Hwang, S.N., Kim, J.Y., Yang, E., Kim, H., Kim, E., 2019. Scn2a haploinsufficiency in mice suppresses hippocampal neuronal excitability, excitatory synaptic drive, and long-term potentiation, and spatial learning and memory. *Front. Mol. Neurosci.* 12, 145.
- Soto, I., Oglesby, E., Buckingham, B.P., Son, J.L., Roberson, E.D., Steele, M.R., Inman, D.M., Vetter, M.L., Horner, P.J., Marsh-Armstrong, N., 2008. Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model. *J. Neurosci.* 28, 548–561.
- Spratt, P.W.E., Ben-Shalom, R., Keshen, C.M., Burke Jr., K.J., Clarkson, R.L., Sanders, S.J., Bender, K.J., 2019. The autism-associated gene Scn2a contributes to dendritic excitability and synaptic function in the prefrontal cortex. *Neuron* 103, 673–685 e675.
- Van Wart, A., Boiko, T., Trimmer, J.S., Matthews, G., 2005. Novel clustering of sodium channel Na(v)1.1 with ankyrin-G and neurofascin at discrete sites in the inner plexiform layer of the retina. *Mol. Cell. Neurosci.* 28, 661–673.
- Van Wart, A., Trimmer, J.S., Matthews, G., 2007. Polarized distribution of ion channels within microdomains of the axon initial segment. *J. Comp. Neurol.* 500, 339–352.
- Vogel, C., Marcotte, E.M., 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* 13, 227–232.
- Vogel, C., Silva, G.M., Marcotte, E.M., 2011. Protein expression regulation under oxidative stress. *Mol. Cell. Proteom.* 10, M111.009217.
- Weitlauf, C., Ward, N.J., Lambert, W.S., Sidorova, T.N., Ho, K.W., Sappington, R.M., Calkins, D.J., 2014. Short-term increases in transient receptor potential vanilloid-1 mediate stress-induced enhancement of neuronal excitation. *J. Neurosci.* 34, 15369–15381.
- Yin, L., Rasch, M.J., He, Q., Wu, S., Dou, F., Shu, Y., 2017. Selective modulation of axonal sodium channel subtypes by 5-HT1A receptor in cortical pyramidal neuron. *Cerebr. Cortex* 27, 509–521 New York, NY : 1991.