Axogenic mechanism enhances retinal ganglion cell excitability during early progression in glaucoma

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Diseases of the brain involve early axon dysfunction that often precedes outright degeneration. Pruning of dendrites and their synapses represents a potential driver of axonopathy by reducing activity. Optic nerve degeneration in glaucoma, the world’s leading cause of irreversible blindness, involves early stress to retinal ganglion cell (RGC) axons from sensitivity to intraocular pressure (IOP). This sensitivity also influences survival of RGC dendrites and excitatory synapses in the retina. Here we tested in individual RGCs identified by type the relationship between dendritic organization and axon signaling to light following modest, short-term elevations in pressure. We found dendritic pruning occurred early, by 2 wk of elevation, and independent of whether the RGC responded to light onset (ON cells) or offset (OFF cells). Pruning was similarly independent of ON and OFF in the DBA/2J mouse, a chronic glaucoma model. Paradoxically, all RGCs, even those with significant pruning, demonstrated a transient increase in axon firing in response to the preferred light stimulus that occurred on a backdrop of generally enhanced excitability. The increased response was not through conventional presynaptic signaling, but rather depended on voltage-sensitive sodium channels that increased transiently in the axon. Pruning, axon dysfunction, and deficits in visual acuity did not progress between 2 and 4 wk of elevation. These results suggest neurodegeneration in glaucoma involves an early axogenic response that counters IOP-related stress to excitatory dendritic architecture to slow progression and maintain signaling to the brain. Thus, short-term exposure to elevated IOP may precondition the neural system to further insult.

Significance

Identifying new therapies for neurodegenerative disease requires understanding how neurons respond to stress and whether this response includes adaptation to slow progression. Because neurodegeneration affects both axons and dendrites, with their synaptic contacts, adaptation could involve both compartments. We investigated this question in experimental glaucoma, the world’s leading cause of irreversible vision loss. Glaucoma attacks retinal ganglion cell neurons and their axons, which comprise the optic nerve. We found that elevations in ocular pressure, a prominent risk factor for glaucoma, caused a paradoxical increase in ganglion cell excitability, including response to light, even in cells with substantial dendritic pruning. This adaptation arose from voltage-dependent mechanisms in the axon and may help maintain signaling to the brain to preserve vision.

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transient phase, conventional AMPA receptor-mediated glutamatergic signaling remained constant despite dendritic pruning, possibly through increased presynaptic active zones. However, isolating AMPA receptor signaling eradicated the enhanced light response, which depended on activation of voltage-sensitive sodium channels (NaV). Localization of the NaV1.6 subunit, which supports higher rates of action potential generation (14, 15), increased during the transient phase in the unmyelinated axon segment, including within varicosities that increased in density, size, and proximity to the RGC body with elevated IOP. Antagonism of NaV1.6 suppressed enhancement. Dendritic pruning, axon dysfunction, and deficits in visual acuity did not progress further between 2 and 4 wk of elevation following transient enhancement. Thus, early progression in glaucoma involves a compensatory or preconditioning mechanism that includes an axodendritic response to counter IOP-dependent challenges to excitatory dendritic architecture and help RGCs maintain signaling to the brain.

Results
Dendritic Pruning in Glaucoma Affects both ON and OFF RGC Types.

We elevated IOP in mice using microbead occlusion of anterior fluid (16–18). An injection raised IOP compared with control for either 2 wk (32%, \( p < 0.001 \)) or 4 wk (26%, \( p < 0.001 \)). We identified first αON-sustained (αON-S) RGCs (Fig. 1A). These cells have large cell bodies (area ≥ 180 \( \mu \text{m}^2 \)), a signature sustained response to light, strong expression of staining for nonphosphorylated neurofilament H protein (SMI32), and narrow dendritic ramification in the ON sublamina relative to bands of choline acetyltransferase (ChAT; Fig. S1 A and B) (7, 9, 10, 13, 19). Compared with control, the dendritic arbors of αON-S RGCs appeared to diminish in size and complexity by 2 wk of elevated IOP (Fig. 1B). When quantified using Sholl analysis, even this brief elevation in IOP significantly reduced dendritic complexity (Fig. 1C). This involved decreased mean dendritic field area, which diminished by 35% on average, and total dendrite length, which diminished by 23%, compared with control (\( P ≤ 0.05 \); Fig. 1D). Pruning did not progress between 2 and 4 wk of elevated IOP, with indications of modest recovery in both field size and dendrite length (Fig. 1D).

Next, we identified a type of RGC with dual dendritic arbors in the ON and OFF sublaminas (Fig. 2 A and B). This RGC is distinguished by lack of staining for SMI32 (11), a characteristic transient response to both onset and offset of light, and narrow dendritic stratification near bands of ChAT (Fig. S1 C and D). Based on morphology and light response, these likely correspond to the direction-selective RGC type identified in other studies (12, 20). The reconstructed dendritic arbors of ON-OFF RGCs appeared to diminish in size by 2 wk of elevation, but only in the ON sublamina (Fig. 2B). Sholl analysis of complexity for ON dendrites demonstrated significant pruning within a 120- to 230- \( \mu \text{m} \) radius from the soma, which, like αON-S RGCs, did not progress between 2 and 4 wk of elevated IOP (Fig. 2C). Pruning at 2 wk corresponded to a decrease in both mean dendritic field area (59%) and total dendrite length (32%) compared with control RGCs of the same type (Fig. 1D; \( P = 0.02 \)). Neither measure progressed between 2 to 4 wk; both ON arbor size and dendrite length at 4 wk did not differ from control RGCs (\( P ≥ 0.07 \)). In contrast to ON dendrites, elevated pressure had little influence on complexity of the dendritic arbor in the OFF sublamina (Fig. 2E), without a significant decline in dendritic field area or total length (Fig. 2F).

The results for ON-OFF RGCs suggest that ON arbors rather than OFF are more susceptible to IOP early in progression. To test this, we identified two types of OFF αRGC (Fig. S2), again based on dendritic stratification, response to light offset, large cell bodies (area ≥ 180 \( \mu \text{m}^2 \)), and expression of nonphosphorylated neurofilament (7, 10). Unlike their ON counterparts, αOFF-S RGC cells showed very little change in dendritic complexity with elevated IOP (Fig. S3). However, the dendritic arbors of αOFF-transient (αOFF-T) RGCs demonstrated dramatically reduced size and complexity following 2 wk of elevated IOP (Fig. S4). We also found similar pruning for ON, OFF, and ON-OFF RGCs in the DBA/2J mouse (Figs. S5 and S6), which develops a glaucoma-like phenotype over a period of months as they age (21–23).

Paradoxical Transient Increase in Excitability Accompanies Dendritic Pruning.

Next, we measured how IOP influences the response to the preferred light stimulus for RGCs characterized morphologically. The αON-S RGCs we examined (Fig. 1) responded to light with a sustained train of action potentials for the duration of the stimulus (Fig. 3A), following the pattern of inward current (Fig. S1A). Two weeks of elevated IOP appeared to increase depolarization of the resting membrane potential as well as firing rate to the preferred stimulus (Fig. 3A, Center). The average light response measured demonstrated a sharp, transient peak followed by the sustained component (Fig. 3B), again following the profile of the inward current (Fig. S1). Two weeks of elevated IOP increased the pattern of light-induced excitability, despite significant pruning of the dendritic arbor in the ON sublamina (Fig. 1). Accordingly, compared with control cells, the mean response rate over the duration of the light stimulus increased significantly after 2 wk of elevated pressure (30.2 ± 1.9 vs. 20.6 ± 1.5 spikes/s; \( P < 0.001 \); Fig. 3C, Left). Indeed, the median firing
diminish ($P = 0.07$), resting membrane potential returned to control cell levels ($P = 0.12; -55.1 \pm 1.8$ mV).

The ON-OFF RGCs described in Fig. 2 demonstrated a transient burst of action potentials both at the onset and offset of the light stimulus (Fig. 3D), each corresponding to transient inward current as shown (Fig. S1C). Two weeks of elevated IOP increased the response to light onset in both magnitude and duration (Fig. 3E). Like αON-S RGCs, this increase occurred despite significant pruning of the dendritic arbor in the ON sublamina (Fig. 2). Accordingly, compared with control cells, the mean response rate over the duration of the light stimulus nearly doubled after 2 wk of elevated pressure ($4.6 \pm 0.6$ vs. $2.6 \pm 0.4$ spikes/s; $P < 0.001$; Fig. 3F). Like αON-S RGCs, this increase was transient, because for 4-wk ON-OFF RGCs the mean firing rate to light onset decreased by $38\%$ compared with control cells of the same type ($1.6 \pm 0.4$ spikes/s; $P = 0.001$). This corresponded to a significant decrease in the integrated ON response for 4-wk cells ($P = 0.03$), whereas 2-wk cells demonstrated a modest increase (Fig. 3G). The OFF response to light was unchanged for both 2 and 4 wk of elevated IOP, as were the dendrites in the OFF sublamina (Fig. 2).

Like their ON counterparts, αOFF-S RGCs respond to their preferred light stimulus with a sustained volley of action potentials (Fig. 4A), but in this case to the offset of light. This too follows the pattern of inward current (Fig. S2). However, unlike αON-S RGCs, αOFF-S RGCs did not demonstrate appreciable dendritic pruning with elevated IOP (Fig. S3). Even so, 2 wk of elevated IOP enhanced the average αOFF-S RGC response to its preferred stimulus (Fig. 4B), as it did for αON-S RGCs. The mean firing rate at light offset nearly doubled compared with control cells ($13.0 \pm 0.9$ vs. $7.3 \pm 0.6$ spikes/s, $P < 0.001$; Fig. 4C). Like the other cell types we examined, the increased average response was transient, because 4 wk of elevated IOP nearly halved the mean firing rate compared with control ($P < 0.001$; Fig. 4C). The integrated response to light tended to reflect these changes (Fig. 4C, Center). Like their ON counterparts, αOFF-S RGCs also demonstrated a more depolarized resting membrane potential compared with control cells: $-52.1 \pm 1.3$ vs. $-57.5 \pm 1.0$ mV, $P = 0.005$; Fig. 4C, Right). Similarly, αOFF-T RGCs also respond to light offset, but with a transient burst of action potentials that quickly dissipates (Fig. 4D). Interestingly, although 2 wk of elevated IOP did dramatically reduce their dendritic arbors (Fig. S4), the average response of αOFF-T RGCs to their preferred stimulus did not diminish (Fig. 4E and F). However, by 4 wk, the mean firing rate decreased by over half compared with control ($P = 0.03$; Fig. 4F). Accordingly, the mean integrated response during light offset also diminished for 4-wk cells ($P = 0.04$). Although the peak firing rate at 2 wk increased modestly compared with control cells ($20.6 \pm 2.5$ vs. $13.5 \pm 2.8$ spikes/s, respectively; $P = 0.07$), the response to light offset was significantly faster (Fig. 4E, Inset). Two weeks of elevation reduced time to the first action potential by $36\%$ compared with control: $94.8 \pm 4.8$ vs. $148.3 \pm 13.1$ ms, respectively ($P = 0.03$; Fig. 4F). This change too was transient, returning to control by 4 wk ($P = 0.20$).

Thus, no RGC type we examined demonstrated a reduced response to its preferred light stimulus after 2 wk of elevated IOP, even for those cells with dendritic pruning within that time (αON-S, ON-OFF, and αOFF-T). Rather, for each type, elevated IOP led to transient enhancement of excitability by multiple measures that varied in significance between RGC types. To determine more accurately how enhancement generalizes across types and IOP exposures, we normalized each physiological parameter for each RGC type by the mean value for the corresponding control cell and pooled the data according to IOP. When normalized in this way, RGCs exposed to 2 wk of elevated IOP demonstrated significantly increased mean (+60\%, $P = 0.005$) and peak (+39\%, $P = 0.007$) firing rates and integrated response (+71\%, $P = 0.005$) to the preferred light stimulus.

Fig. 2. ON sublamina dendrites for ON-OFF RGCs are targeted early. (A) ON-OFF RGCs following intracellular filling (AL555) from CTRL, 2-wk, and 4-wk retinas. (B) Reconstructions indicate shrinkage in the ON sublamina with elevated IOP. (Scale bars: A and B, 40 μm.) (C) Sholl analysis averaged from CTRL ($n = 24$), 2 wk ($n = 15$), and 4 wk ($n = 12$) RGCs show complexity reduced at 2 wk within a radius of 120–230 μm from the soma compared with CTRL (⁎$P < 0.03$, two-way ANOVA). These reductions remained significant after 4 wk for about the same radius (150, 210–230, and 230 μm; †$P < 0.04$, two-way ANOVA). (D) Reduced complexity corresponded to smaller ON dendritic fields with decreased total dendrite length for 2-wk cells (⁎, area: $P = 0.03$, t-test; length: $P = 0.02$, t-test). (E) Sholl analysis of OFF dendritic arbors shows only minor changes for both 2- and 4-wk ON-OFF RGCs that were not significant at any distance from the soma ($P = 0.23$, two-way ANOVA); neither field area nor total length declined significantly (†, area: $P = 0.25$, t-test; length: $P = 0.48$, t-test). Data: mean ± SEM.

rate for 2-wk cells increased from 12.5 to 28.3 spikes/s ($P < 0.001$), and the integrated response defined by the area under the curve for firing rate during the light stimulus also tended to increase (Fig. 3C, Center). This increase accompanied a significant increase in depolarization of the resting membrane potential from −58.4 ± 1.1 mV for control to −52.9 ± 1.7 mV for 2-wk cells ($P = 0.007$; Fig. 3C, Right). This increase in excitability for αON-S RGCs was transient, because for 4-wk cells the response profile diminished (Fig. 3B) and the mean firing rate decreased by 70\% (6.2 spikes/s) compared with control cells ($P < 0.001$; Fig. 3C). Although the integrated response to light also tended to
compared with control (Fig. 4 G–I). As expected, each of these measures diminishing significantly compared with control cells by 4 wk of elevated IOP ($P \leq 0.02$). Elevated IOP also led to a transient increase in depolarization of resting membrane potential compared with control ($+6\%$, $P < 0.001$; Fig. 4J), as well as a quicker response to the preferred stimulus through reduced latency ($−24\%$, $P = 0.006$; Fig. 4F). Finally, we noted additional noise in resting membrane potential that accompanied enhanced excitation. For example, $\alpha$ON-S RGCs had a $40\%$ greater coefficient of variance at rest compared with control cells following 2 wk of elevated pressure ($P = 0.03$). This effect can be gleaned from our recordings (Fig. 3A). This increase in membrane noise also generalized to all RGCs when normalized by each type’s control value ($P = 0.013$; Fig. 4L). Resting potential, response latency, and coefficient of variance all returned to control levels by 4 wk of elevated IOP.

**Enhanced Light Response Depends on Voltage-Dependent Activity.**

Our physiological recordings indicate that both ON and OFF RGC types demonstrate transient enhancement in excitability after 2 wk of elevated IOP. This was so even for the three types with early and significant dendritic pruning (Figs. 1 and 2 and Fig. S4). Importantly, enhancement includes a transient increase in response to the preferred light stimulus, which reverses to below control levels by 4 wk (Fig. 4 G–I). Next, we sought to understand possible mechanisms underlying this response.

Light-induced excitation of RGCs arises from glutamate released from bipolar cell axon terminals that binds ionotropic receptors localized to postsynaptic dendrites (24). Upon binding glutamate, excitation through AMPA-sensitive glutamate receptors enables activation of voltage-dependent channels that contribute to propagation of depolarization in the RGC (25, 26).
We isolated pharmacologically the influence of excitatory AMPA receptor signaling in RGCs by silencing other glutamatergic receptors and voltage-gated sodium channels under voltage clamp near the equilibrium potential of chloride to reduce inhibitory currents (27) (Fig. S7). Under these conditions, all four types of RGC both (i) failed to demonstrate an enhanced response to the preferred stimulus and (ii) did not demonstrate a reduction in response compared with control cells after 2 wk of elevated IOP. For ON-OFF and αOFF-T RGCs, the AMPA-mediated light response did diminish by 4 wk (Fig. S7 A–D). Finally, the response of αON-S and ON-OFF RGCs to AMPA delivered directly to the cell by puff pipette, which reflects the activity of postsynaptic AMPA receptors (26), decreased progressively between 2 and 4 wk of IOP elevation (Fig. S7E). Thus, AMPA receptor-mediated signaling does not underlie the increased light response, because isolating AMPA currents eradicated the increase for each RGC type’s preferred stimulus, and delivery of AMPA directly to αON-S and ON-OFF RGCs did, in fact, diminish progressively between
2 and 4 wk of elevated IOP, indicating that dendritic degradation does eventually produce a diminished postsynaptic response.

We asked next what could explain the persistence of the AMPA receptor-mediated contribution to the light response after 2 wk despite dendritic pruning in αON-S, ON-OFF, and αOFF-T RGCs. We found that localization of PSD95, which stabilizes the glutamatergic postsynaptic density on RGC dendrites (24, 28), diminished between 2 and 4 wk of IOP elevation (Fig. S8 A and B). A presynaptic ribbon marks each glutamatergic active zone in the bipolar cell axon terminal, which can be visualized through immunolabeling for the synaptic ribbon protein RIBEYE (29). Surprisingly, we found that 2 wk of elevated pressure increased RIBEYE significantly (Fig. S8 C and D). We also found that GABA/glycine-mediated inhibitory currents do not differ between control and 2-wk RGCs (Fig. S8 G and H).

Our pharmacological experiments indicate that quenching voltage-dependent channels with TTX prevented the enhanced RGC response at 2 wk (Figs. S7 and S8). To explore possible mechanisms of this effect, we examined changes in localization of the voltage-dependent sodium channel NaV1.6, which activates at relatively negative membrane potentials and contributes to action potential initiation and to repetitive firing (15). In mouse retina, NaV1.6 localization within RGCs increased with 2 wk of elevated IOP (Fig. 5 A and D). Expression seemed to be restricted to somatic and axonal compartments, including within the varicosities most proximal to the soma (Fig. 5E). Perhaps most interesting, the axonal varicosities themselves changed. Two weeks of elevated ocular pressure caused the varicosities along the proximal axon segment to translocate closer to the RGC body, increase in density by 41%, and expand in size (Fig. 5F). Finally, we tested how NaV1.6 influences RGC physiology with elevated IOP by measuring the response to light before and after application of the NaV1.6-specific antagonist, α-BTX (30). An independent sample of αON-S (Fig. 5G, Left) and αOFF (Fig. 5G, Right) RGCs demonstrated the expected enhanced response after 2 wk of IOP elevation. For CTRL RGCs in each group, both 300 and 500 nM α-BTX were equally efficacious in suppressing the preferred light response (P = 0.99; Fig. 5G). However, for 2-wk RGCs, 300 nM was significantly less effective in suppressing the light current (Fig. 5G). The difference in light response between 2-wk and control αON-S RGCs—the enhanced light response—was reduced by 75% by 500 nM α-BTX and by 86% after light offset for αOFF RGCs (Fig. 5G, Inset). When normalized with respect to the degree of suppression for control RGCs and pooled, 300 nM α-BTX left a 30% residual preferred light response for 2-wk RGCs (P < 0.001), whereas 500 nM was not significantly different from control (Fig. 5H).

Enhanced Light Response May Help Slow Axonopathy. A significant element of RGC axon injury in glaucoma is progressive loss of axonal antegrade transport between the retina and superior colliculus (SC), the primary RGC target in rodents (23). Because the transient enhancement of RGC excitation occurs by 2 wk of elevated IOP, we asked if this phenomenon correlates with an axonal outcome at the same time. By 2 wk of elevated IOP, the level of intact transport in the SC degraded by 40% compared with control eyes (P = 0.018; Fig. 6 A and B). Although degradation by 4 wk of elevation was also significant compared with control (P = 0.008), deficits in transport did not progress between 2 and 4 wk (P = 0.24). For comparison, we also measured the progression of deficits in spatial frequency sensitivity (acuity) by comparing optokinetic tracking (OKT) between control and microbead eyes. Deficits in acuity with elevated IOP occurred very early, with a 7% decline by 3 d post microbead injection that was significant compared with control (Fig. 6C). Elevated IOP caused a 27% decrease over 10 d that was interrupted at 2 wk by a significant 25% rebound in acuity that was significant compared with performance at 10 d (P = 0.02). Although improvement at 2 wk was transient, following this point acuity did not significantly degrade further compared with 10 d (P ≥ 0.22).

Discussion

Glucomatous neurodegeneration involves both an early distal component, affecting RGC axon function in the optic projection (23), and a proximal component involving loss of RGC dendrites and their synapses (31–33). Our fundamental finding is that modestly elevated IOP—the only treatable risk factor in glaucoma—induces a paradoxical increase in RGC light response, even in RGC types with demonstrable dendritic pruning. This enhancement, which is transient, is part of a more general increase in excitability displayed by each of the four types of RGC we identified morphologically and by their characteristic response to a preferred light stimulus (Figs. 1 and 2 and Figs. S1–S4). Dendritic pruning did not depend on response polarity, either to light onset or offset, with both ON and OFF dendrites showing susceptibility depending on RGC type. Enhancement is not through conventional AMPA-sensitive glutamatergic signaling or changes in inhibitory currents, but rather depends on voltage-sensitive sodium channels with the NaV1.6 subunit contributing substantially to the increased light response. This subunit localized mostly in axons, especially within varicosities that increased in size, density, and proximity to the RGC body with elevated pressure. We propose that this axogenic mechanism in the retina boosts RGC excitation even as stress due to IOP challenges conventional glutamatergic signaling.

Ganglion Cell Pruning Is Independent of Response Polarity. An important question in neurodegenerative disease is whether and how certain types of neuron demonstrate increased susceptibility to disease-relevant stressors. Our experiments combining morphological and physiological measurements from single RGCs show 2 wk of moderate (+26%) elevations in IOP induce early pruning of RGC dendritic arbors. This is consistent with previous studies describing dendritic pruning following brief elevations in IOP for some RGC types (6, 7). A key identifier for the 30 or so types is response polarity and dendritic stratification in the corresponding ON or OFF sublamina of the inner plexiform layer (8, 9). Recent evidence suggests that OFF RGCs, in particular αOFF-T RGCs, may be more susceptible to IOP-related stress (5–7). Although accelerated degeneration could be supported through known homotypic coupling between RGCs of this type (34–36), IOP elevation in these studies was far more acute, reaching peaks of 70–80% above control (5–7).

With moderate elevations more typical of human glaucoma (16), we found that pruning was independent of response polarity. Pruning for αON-S RGCs after 2 wk was similar to that of ON-OFF RGCs in the ON sublamina (Figs. 1 and 2). This type of ON-OFF RGC likely corresponds to a directional selective cell identified in previous studies (12, 20). For this RGC, 2 wk of elevation did not influence dendrites in the OFF sublamina, which showed insignificant signs of pruning after even 4 wk (Fig. 2). This result differs from that of El-Danaf and Huberman (6), who found that for anterior motion-selective ON-OFF RGCs, the ON tree expanded slightly, whereas the OFF tree demonstrated modest pruning after 7 d. This difference again could be due to the
short-term, acute nature of the IOP elevation (peaking at +70%) which may have induced rapid adaption, or to the fact that our sample did not distinguish between directions of motion selectivity. Although the dendrites of αOFF-S RGCs did not change even after 4 wk of elevated IOP (Fig. S3), αOFF-T RGCs demonstrated reduced arbor size and total dendrite length after only 2 wk (Fig. S4). This is similar to the findings of Della Santina et al. (5), but contrary to those of Ou et al. (7), who described comparable pruning for αOFF-S and αOFF-T RGCs. Dendritic pruning also did not depend on response polarity for RGCs identified morphologically in retinas of the DBA/2J mouse model of hereditary glaucoma (Figs. S5 and S6). This model presents significant loss of RGCs and their axons with age-related increases in IOP (21–23). We found that pruning was

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significant at 10 mo of age and similar in magnitude among ON, OFF, and ON-OFF dendritic arbors, although we did not discriminate specific types. This result is consistent with an earlier comparison between RGCs in the DBA/2J retina (31).

An Axogenic Mechanism to Boost Excitation. We found that even with early dendritic pruning at 2 wk, no RGC type had a diminished response. Rather, each demonstrated an increased response to the preferred light (ON or OFF). For ON-OFF RGCs, the enhanced response was restricted to light onset (Fig. 3), just as dendritic pruning was restricted to the ON sublamina (Fig. 2). When normalized to their respective control group, each of the four RGC types examined had increased mean and peak firing rates and a faster response to the preferred light stimulus at 2 wk (Fig. 4). For αOFF-S RGCs the dendritic arbors remained intact (Fig. S1), although the light response did increase (Fig. 4). Interestingly, Ou et al. (7) also found an increased response, but with dendritic pruning. The response increase we report was transient for all RGCs, occurring by 2 wk of elevated IOP but decreasing to below control RGC levels by 4 wk (Fig. 4). This eventual decline is consistent with studies that followed the response for longer periods of IOP elevation (37, 38). Finally, the change in light response we document here took place on a backdrop of transiently enhanced excitability that included increased depolarization of the resting membrane potential with increased variability in membrane noise (Fig. 4).

Light-induced excitation of RGCs arises via glutamate from bipolar cell presynaptic ribbons that bind ionotropic glutamate receptors stabilized by PSD95 in RGC dendrites (24, 28). Bound glutamate depolarizes RGCs primarily through AMPA-sensitive receptors (Fig. S7A), thereby activating voltage-gated channels that propagate excitation (25, 26). The AMPA-driven contribution to the light response did not diminish after 2 wk of elevated IOP (Fig. S7B–D), even with dendritic pruning in ON-OFF, αON-S, and αOFF-T RGCs. Only after 4 wk of elevation did the AMPA-mediated response show signs of decline, especially in ON-OFF and αOFF-T RGCs (Fig. S7D). This is commensurate with the decrease in levels of PSD95 in the inner plexiform layer we saw at 4 wk (Fig. S8A and B). This result does not contradict reports of diminished PSD95 on individual RGC dendritic arbors before demonstrable pruning (5, 7). Our measurements reflect the entire inner plexiform layer with its great variety of glutamate receptor-expressing processes. Indeed for αON-S and ON-OFF RGCs, we found diminished response to AMPA delivered directly by puff pipette (Fig. S7E and F). This measurement, reflecting the activity of postsynaptic receptors (26), decreased progressively between 2 and 4 wk of IOP elevation as did PSD95, even though dendritic pruning did not significantly change (Figs. 1 and 2).

Interestingly, 2 wk of IOP elevation increased immunolabeling for the presynaptic ribbon protein RIBEYE throughout the inner plexiform layer (Fig. S8C and D). This effect too was transient, declining to control levels by 4 wk. The transient increase in RIBEYE at 2 wk contrasts with reduced numbers of synaptic ribbons found using a different marker (7). Colocalization with PSD95 did not change with elevated IOP but remained comparable with control levels (Fig. S8E and F). Because PSD95 did not change at 2 wk, this may indicate multiple presynaptic ribbons for a given postsynaptic active zone. Increased RIBEYE in the inner retina may reflect a mechanism to stabilize the amplitude of AMPA receptor-mediated responses through presynaptic glutamatergic machinery in bipolar cell axons. Even as response amplitude persists due to presynaptic changes, ON and OFF RGC receptive fields should decrease in size as dendritic field area diminishes, effectively reducing the RGC sampling aperture in space. Recordings of the RGC spatial response to light in glaucoma models have confirmed this prediction (38). Perhaps more importantly, we found that isolating AMPA-mediated currents in RGCs while quenching voltage-gated sodium channels eradicated the transient increase in light response for all RGCs tested (Fig. S7E–D). This result, coupled with diminished response to puffed AMPA (Fig. S7E and F), demonstrates that conventional glutamatergic signaling between bipolar cells and RGCs does not underlie the increased light response. Similarly, we found that changes in inhibitory chloride currents also cannot explain the increased light response (Fig. S8G and H), although the modestly diminished magnitude at 2 wk is consistent with earlier changes in GABAergic signaling observed in other glaucoma models (39, 40).
To explore further the voltage dependence of the increased light response, we examined the localization of NaV1.6, a voltage-gated sodium channel involved in action potential initiation and propagation (15). In the retina, NaV1.6 localizes predominantly in RGCs and their axons (41–44), a finding consistent with its bio-
physical function and one we now corroborate because localization in the inner plexiform layer was undetectable (Fig. 5 B and C). At 2 wk of IOP elevation, NaV1.6 increased in RGC axons, especially within varicosities that increased in size, density, and proximity to the RGC body (Fig. 5 D–F). This change coincided with the enhanced light response (Figs. 3 and 4). By 4 wk of elevation, NaV1.6 declined throughout the RGC to below control levels (Fig. 5E), again following precisely the transient pattern of the increased light response.

The NaV1.6 subunit produces a large persistent excitatory current that sustains generation of higher firing rates compared with other subunits (14). We propose that increased NaV1.6 in the RGC axon supports the enhanced light response at 2 wk of IOP elevation by bolstering excitation originating from remaining glutamatergic synapse in the dendritic arm. Even the small (−5%) shift in RGC resting membrane potential that we observed (Figs. 3C and 4J) is sufficient to move axonal NaV1.6 from an inactivated to activated state (15). Furthermore, phosphorylated p38 mitogen-activated protein kinase, which we have shown increases in RGCs in glaucoma, directly activates NaV1.6 (45). Thus, increasing NaV1.6 in RGC axons should facilitate activation and increase firing, which could also explain the shorter response latency and increased membrane noise we observed at 2 wk (Fig. 4 K and L).

Activation of NaV channels close to cell bodies requires more depolarization (15). Because axonal varicosities are rich in mitochondria (46), increased size may hold more mitochondria, which would help support additional NaV1.6 activity. Finally, our physiological recordings using the NaV1.6-selective antagonist αTTX demonstrates a critical role for this subunit in driving action potential generation in response to the prefered light stimulus. For control RGCs its presence nearly eradicated the excitatory light response (Fig. 5G). For 2-wk ON- and OFF-S/T RGCs, although αTTX significantly suppressed the enhanced light response, even a 40% higher concentration left some residual response (Fig. 5H). This is consistent with the increased levels of NaV1.6 observed in this IOP elevation (Fig. 5D and E). Even so, we cannot exclude the possibility of additional voltage-dependent mechanisms contributing to enhancement, including the NaV1.2 subunit also expressed by RGCs (47).

Increased firing rate can improve correlated signaling between RGCs to help encode information embedded in a light stimulus (48, 49). We propose that RGCs that fail to do so may be more susceptible to early axonal degeneration, which is characteristic of glaucoma (23). Consistent with this idea, RGCs in mice lacking the transient receptor potential vanilloid-1 channel (TRPV1−/−) not only have a higher threshold for excitation, but also undergo accelerated axon degeneration with elevated IOP (17, 18). Although transient increases in axonal NaV1.6 may promote RGC signaling, long-term changes could be deleterious for axons already challenged by glaucomatous stress. Where NaV1.6 colocalizes with the Na+/Ca2+ exchanger in injured axons, its large persistent Na+ current could reverse the exchanger, increasing intra-axonal Ca2+ to protease-
activating levels (14, 50, 51). Increased persistent current through NaV1.6 channels contributes to neuronal hyperexcitability in epi-leptogenesis (30), which may be similar to the enhanced excitability we observed here (Fig. 4 G–L). That both the light response and Nav1.6 localization in axons diminished below control levels by 4 wk (Figs. 4 and 5E) suggests a delicate balance between the benefits of axogenic compensation and increased excitation and accelerated cytoskeletal degradation.

Although our results demonstrate that RGCs respond to IOP-related stress with a voltage-dependent mechanism to boost ex-
citation, we cannot ascertain yet whether this mechanism is protective or ultimately deleterious to survival. Because the effect is transient, it is possible short-term elevations in IOP provide a preconditioning stimulus to slow progression. In the retina, although RGC dendritic arbors shrank by 33–59% at 2 wk of elevation for αON-S, ON-OFF, and αOFF-T RGCs, pruning did not progress between 2 and 4 wk (Figs. 1 and 2 and Fig. S4). Similar persistence has occurred even with sharper elevations in IOP (7). Similarly, in the distal optic projection, we found that deficits in axonal anterograde transport to the SC did not progress between 2 and 4 wk of elevation (Fig. 6B). Finally, our measurements of spatial acuity using OKT demonstrate a dra-
matic and steady decline up to 2 wk of elevated IOP, at which point performance significantly improves and then stabilizes (Fig. 6C). Thus, the enhanced excitation in individual RGCs in response to disease-relevant elevations in IOP seems to translate to a broader influence on the optic projection as a whole.

Methods

Animals. The Vanderbilt University Medical Center Institutional Animal Use and Care Committee approved all experimental procedures. Young adult (2 mo) C57 mice (C57BL/6, male; Charles River Laboratory) were maintained in a 12-h light/dark cycle with standard rodent chow and water available ad libitum. We used unilateral microbead occlusion to elevate IOP in C57 mice, with the fellow eye receiving an equal volume saline injection as internal control (16–18). We measured IOP biweekly using rebound tonometry (Tono-Pen XL; Medtronic Solan), as described (16–18) (Fig. S9). For physio-
logical recordings and intracellular filling, retinae were free of long-wavelength light (630 nm, 800 μW/cm²; Ushio FND/FG). A subset of animals was bilaterally injected intravitreally with 1 μl of 0.5 mg chole-
sterol toxin subunit B (CTB) conjugated to Alexa Fluor 488 (Invitrogen). Intact transport within serial coronal superior colliculus sections was quantified (18, 23). For tissue required for immunolabeling of vertical sections and analysis of anterograde transport, mice were perfused transcardially with 4% paraformaldehyde.

Ex Vivo Retinal Preparation. Retinas were placed in the dark into a physio-
logical chamber mounted on an upright microscope, maintained at 30 °C (TC-344B; Warner Instruments), and perfused (2 ml/min) with oxygenated bicarbonate buffered Ames’ medium supplemented with 20 mM glucose (pH 7.4, Osm 290). For intracellular filling and patch-clamp recordings, we used fire-polished borosilicate glass pipettes containing (in mM) 125 K-gluconate, 10 KCl, 10 Hepes, 4 Mg-ATP, 1 Na-GTP, and 0.1 ALEXA 555 (Invitro-
gen) or 1 Lucifer Yellow (pH 7.35, Osm 285). Access resistance was ≤30 MΩ. Whole-cell RGC current and voltage signals were amplified (Multiclamp 700B; Molecular Devices) and digitized at a sampling rate of 10 kHz (Digidata 1550A; Molecular Devices). We measured resting membrane potential within 1 min after whole-cell conformation and calculated the coefficient of variation during a 5-s interval in current-clamp mode. Light responses were obtained using full-field light flashes of 365 nm (300 μW/cm²), 3-s duration; Roithner Laser-
technik) generated by a light-emitting diode delivered through a shutter in the microscope condenser. The mouse retina produces a robust response to this wavelength based on the electroretinogram (S2).

Following physiology, retinas were fixed overnight in 4% paraformalde-
hyde and immunolabeled for nonphosphorylated neurofilament (SMI-32; 1:1,000; BioLegend) and ChAT (1:100; Millipore). All RGCs were from mid-
peripheral retina, 1,226 ± 35 μm from the optic nerve head, and equally distributed between superior vs. inferior and nasal vs. temporal quadrants without bias for the four types identified (P ≥ 0.10). Confocal micrographs of all RGC dendritic arbors were obtained en montage using an Olympus FV-
1000 inverted microscope and Sholl analysis of skeletonized arbors analyzed using ImageJ (Version 1.51i).

Optokinetic Tracking. Mice were placed on an elevated platform surrounded by four adjoining LCD monitors as described (53) (OptoMotry; CerebralMechanics Inc.). Visual response to drifting sine-wave gratings was measured through OKT reflex to the drifting sine-wave gratings of 100% contrast. Spatial frequency of the drifting grating was systematically adjusted to threshold depending on the animal’s OKT response. Each grating was presented until a response (tracking) or no response (no tracking) was noted by the naïve experimenters.

Statistics. We performed one- or two-way ANOVAs (SigmaPlot 12.5; Systat). ANOVAs showing significance (P < 0.05) were followed by Tukey’s (parametric)
or Dunn’s (nonparametric) post hoc comparisons using independent samples (t tests). In some instances, when data distributions violated two or more of the assumptions of ANOVA (normality of residuals, equal variances, and independent sampling), we performed t tests or ANOVAs on ranked data. Before statistical analysis of dependent variables, we assessed the data for outliers, using Grubbs’ test (GraphPad).

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