Elevated intraocular pressure decreases response sensitivity of inner retinal neurons in experimental glaucoma mice

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Glaucoma is the second leading cause of blindness in the United States and the world, characterized by progressive degeneration of the optic nerve and retinal ganglion cells (RGCs). Glaucoma patients exhibit an early diffuse loss of retinal sensitivity followed by focal loss of RGCs in sectorized patterns. Recent evidence has suggested that this early sensitivity loss may be associated with dysfunctions in the inner retina, but detailed cellular and synaptic mechanisms underlying such sensitivity changes are largely unknown. In this study, we use whole-cell voltage-clamp techniques to analyze light responses of individual bipolar cells (BCs). All amacrine cells (AIIACs), and ON and sustained OFF alpha-ganglion cells (ONxGCs and sOFFxGCs) in dark-adapted mouse retinas with elevated intraocular pressure (IOP). We present evidence showing that elevated IOP suppresses the rod ON BC inputs to AIIACs, resulting in less sensitive AIIACs, which alter AIIAC inputs to ONxGCs via the AIIAC→cone ON BC→ONxGC pathway, resulting in lower ONxGC sensitivity. The altered AIIAC response also reduces sOFFxGC sensitivity via the AIIAC→sOFFxGC chemical synapses.

This sensitivity decreases in outer retinal neurons were found in mice with elevated IOP for 3–7 wk, a stage when little RGC or optic nerve degeneration was observed. Our finding that elevated IOP alters neuronal function in the inner retina before irreversible structural damage occurs provides useful information for developing new diagnostic tools and treatments for glaucoma in human patients.

Intraocular pressure | glaucoma | ganglion cells | bipolar cells | All amacrine cells

Glaucoma is a leading cause of irreversible blindness in the United States and the world (1, 2), and is characterized by optic nerve cupping (thinning of the neuroretinal rim at the optic nerve head) and progressive optic nerve and retinal ganglion cell (RGC) degeneration as well as functional deficit revealed by psychophysical tests (3, 4). Although factors causing the eventual RGC death and blindness remain controversial (1, 5–8), increasing evidence from human patients and animal models has shown that the disease is associated with an early mild diffuse loss of retinal sensitivity or inner retinal response decrease (9–14). Although it is unclear whether these functional changes are a prelude or even causal to RGC death and blindness, elucidating the underlying synaptic and cellular mechanisms for such sensitivity/response decline will nevertheless provide novel insights pertaining to early detection and treatment of human glaucoma.

Multiple risk factors are associated with glaucomatous diseases, among which elevated intraocular pressure (IOP) is widely accepted as the most significant for both disease onset and progression (2, 15). Because high IOP (H-IOP) is an important risk factor, many experimental animal models of elevated IOP have been developed in multiple species including monkeys, rats, and mice (16–22). Most experiments performed in animal models have focused on anatomical and histopathological analyses of RGC death, axon loss, and changes to axonal projections to higher visual centers in the brain (23–25). Only a few studies have attempted to address whether function and light sensitivity of retinal neurons are affected. Some reports have suggested a possible but inconclusive involvement of amacrine cells (26, 27). A recent study examining the scotopic threshold responses (STRs) in an elevated IOP mouse model generated by the microbead occlusion method (28) has suggested that the voltage gains (ratio of post/presynaptic signals) of the negative STR [possibly representing AII amacrine cell (AIIAC) responses (29)] and positive STR [possibly representing ON GC responses (30)] are both reduced at stages before morphological changes or RGC death occurs (12). However, no changes in single RGC or their presynaptic bipolar cell (BC)/AIIAC responses have been reported in experimental glaucoma models. Studies using electroretinogram, STR, and optic nerve recording techniques lack the power to identify or establish cellular and synaptic sites of retinal dysfunctions (27, 31, 32), leaving a disabling gap preventing us from knowing how elevated IOP affects light responses of individual retinal neurons. In this study, we fill this gap by using whole-cell voltage-clamp techniques to study light responses of individual alpha-RGCs (αGCs) and AIIACs, as well as their presynaptic BCs, in two experimental glaucoma mouse models.

It has been shown that light responses of mammalian AIIACs are mediated by rod bipolar cell (DBC_R) inputs via a 6,7-dinitroquinoxaline-2,3-dione (DNOX)–sensitive glutamatergic synapse and certain types of cone depolarizing bipolar cell [DBC_R/MC; ON bipolar cells with mixed rod and M-cone inputs (33), or B6-7] input via a connexin36 (Cx36)-dependent electrical synapse (34–36). AIIACs are perhaps the most sensitive (with the lowest response threshold) neurons in the mouse retina (37, 38), and thus they send highly sensitive output signals to postsynaptic neurons such as certain types of cone hyperpolarizing bipolar cells [HBC_R/MC; OFF bipolar cells with mixed rod and M-cone inputs (39), or B1-2] and OFF GCs (37, 40). ON and sustained positive STR [possibly representing AII amacrine cell (AIIAC) responses (29)] and positive STR [possibly representing ON GC responses (30)] are both reduced at stages before morphological changes or RGC death occurs (12). However, no changes in single RGC or their presynaptic bipolar cell (BC)/AIIAC responses have been reported in experimental glaucoma models. Studies using electroretinogram, STR, and optic nerve recording techniques lack the power to identify or establish cellular and synaptic sites of retinal dysfunctions (27, 31, 32), leaving a disabling gap preventing us from knowing how elevated IOP affects light responses of individual retinal neurons. In this study, we fill this gap by using whole-cell voltage-clamp techniques to study light responses of individual alpha-RGCs (αGCs) and AIIACs, as well as their presynaptic BCs, in two experimental glaucoma mouse models.

Significance

Glaucoma is a leading cause of blindness, associated with elevated intraocular pressure (IOP) and progressive loss of the optic nerve and retinal ganglion cells (RGCs). Glaucoma patients exhibit diffuse loss of visual sensitivity, but the cellular origins of such sensitivity loss is unknown. In this study, we present evidence showing that elevated IOP decreases the efficacy of the rod bipolar cell to the AII amacrine cell synapse, resulting in reduction of RGC sensitivity. These findings, for the first time to our knowledge, identify the synaptic loci mediating visual sensitivity loss in early glaucoma, and can be used to develop new diagnostic tools and treatments for this blinding disease.


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OFF alpha-GCs (ONα-GCs and sOFFα-GCs) are two primary types of GCs in the mouse retina, and their synaptic circuitries include many major types of mammalian BCs and ACs (40–42). By studying the effects of elevated IOP on these GCs and their presynaptic neurons, we will be able to gain insights into general mechanisms underlying inner retina dysfunction in glaucoma. ONα-GCs and sOFFα-GCs exhibit characteristic morphology (large somas and dendritic trees) and light response signatures (40). ONα-GCs have no or very little spike activity in darkness, increased spikes in light, inward light-evoked cation current (ΔIC; mediated by DBCs inputs), and outward light-evoked chloride current (ΔICl; mediated by AC inputs) (40, 43). sOFFα-GCs exhibit maintained spike activity in darkness, sustained decrease of spikes in light, outward ΔICl (mediated by AIIAC/AC inputs) (40, 44). Fig. 1 is a schematic diagram of synaptic connections between ONα-GCs/sOFFα-GCs and their primary pre-synaptic neurons: DBCs, DBC\textsubscript{RMCs}, HBC\textsubscript{RMCs}, and AIIACs (key synapses are labeled 1–5 in the figure; see below). In this report, we analyzed light responses of these retinal neurons in treated mice (in which H-IOP was induced; Materials and Methods) and compared them with the corresponding responses measured in nontreated mice with normal IOP (n-IOP).

Results

Effects of Elevated IOP on Light-Evoked Spike Responses and Cation and Chloride Currents in ON and OFF Ganglion Cells. We first studied how elevated IOP affects light-evoked spike responses and the BC and AC inputs (represented by ΔISC and ΔICl, respectively) to ON and OFF GCs. Fig. 2 shows the light-evoked spike activities (Fig. 2B) and ΔIC and ΔICl (Fig. 2C and D, respectively) of an ONα-GC to light steps of various intensities in a dark-adapted flat-mounted retina of a mouse with 5-wk elevated IOP (15–24 Hg) (12, 16). The average response–intensity (R-Log I) relations of six ONα-GCs in H-IOP mouse retinas (with 3- to 7-wk elevated IOP, four with the laser method and two with the microbead method; Materials and Methods) are shown as dotted curves in Fig. 2E, whereas the corresponding R-Log I relations obtained from ONα-GCs in n-IOP (10–15 Hg) mice (n = 18) are shown as solid curves for comparison. ONα-GCs were initially identified by their characteristic large soma size in flat-mounted retinas, their characteristic spike, ΔIC, and ΔICl response waveforms (40), and subsequently confirmed by their characteristic morphology [including soma size, dendritic pattern in the flat mount, and levels of stratification by z-axis rotation (40, 45, 46)] revealed by neurobiotin (NB) or Lucifer yellow (LY) fluorescence after the experiment (Fig. 2A, B). It is evident from Fig. 2E that light-evoked spike responses and ΔIC of ONα-GCs in the H-IOP retina (black and red dotted curves) are about 2 log units less sensitive (right shifts of the R-Log I curves; thin black arrow and thick red/yellow arrow) than the corresponding responses of the ONα-GCs in n-IOP mice (black and red solid curves). The average light response thresholds, defined as the light intensity eliciting 5% of the maximum response of spike responses, ΔIC, and ΔICl in control and H-IOP mouse retinas; the significance levels of the threshold differences are given by the P values of the t test.

Fig. 1. Schematic diagram of major synaptic connections in the ON and OFF ganglion pathways in the mouse retina. Green, rods and rod BCs; blue, M cones and mixed rod/M-cone BCs; orange, AIIACs; gray, nGCs; arrows, chemical synapses (red, glutamatergic; blue, glycinergic; +, sign-preservation; −, sign-inverting); zigzag (red), electrical synapses. a, sublamina a; b, sublamina b; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PRL, photoreceptor layer. Synapses directly relevant to this study are marked with numbers in circles: 1: DBCs→AIIAC glutamatergic; 2: DBCs→AIIAC electrical; 3: DBCs\textsubscript{RMC}→HBC\textsubscript{RMC}→ONα-GCs/sOFFα-GCs glutamatergic; 4: AIIAC→HBC\textsubscript{MCs} glycinergic; and 5: AIIAC→sOFFα-GCs glycinergic.

Fig. 2. Light responses of ONα-GCs in high-IOP and normal-IOP mice. (A) Confocal image of an LV-filled ONα-GC in an H-IOP mouse (white arrow, axon). (Scale bar, 20 μm.) (B–D) The cell’s light-evoked spike responses, cation current, and chloride current to 500-nm light steps of various intensities are shown in B–D, respectively. (E) The normalized, average response–intensity relations of six ONα-GCs in H-IOP mice (population R\textsubscript{max} (mean ± SE) for spike, ΔIC, and ΔICl responses: 88 ± 37 per s, 203 ± 16 pA, and 194 ± 18 pA) are shown as dotted curves, whereas the corresponding R-Log I relations obtained from ONα-GCs in normal mice (n = 18, population R\textsubscript{max} (mean ± SE) for spike, ΔIC, and ΔICl responses: 97 ± 42 per s, 218 ± 21 pA, and 183 ± 13 pA) are shown as solid curves for comparison. Black, spike responses; red, ΔIC green, ΔICl. Arrows indicate H-IOP–induced shifts of the R-Log I relations. (F) Bar graphs of the average light response thresholds (mean ± SE), defined as the light intensity eliciting 5% of the maximum response of spike responses, ΔIC, and ΔICl in control and H-IOP mouse retinas; the significance of the threshold differences are given by the P values of the t test.
that the average spike R-Log I curve (black solid curve) lies between the average ΔC₁ R-Log I (solid green) and the ΔC₂ R-Log I (solid red), with the low-intensity ends very close to the ΔC₂ R-Log I. The average light response thresholds of spike responses, ΔC₁ and ΔC₂ in n-IOP and H-IOP mouse retinas are shown as bar graphs in Fig. 3F. The differences in spike and ΔC₂ thresholds between the H-IOP and n-IOP mice are highly significant (P < 0.001, t test), and the difference in ΔC₁ thresholds between the two groups is not (P = 0.122). These results suggest that the spike responses of mouse sOFFGCs at low light intensities are largely mediated by an AC input of high sensitivity. The most likely ACs with such high sensitivity are the AIACs (37, 38). The reason why both the ΔC₁ and ΔC₂ contribute to the sOFFGC spike activity is that the dark resting potential of the mouse sOFFGCs is about 10 mV positive to E_C (in contrast to the near E_C dark membrane potential of the ONGCs described in Fig. 2) (40), and thus both ΔC₁ and ΔC₂ have enough driving force to contribute to the spike generator potentials in sOFFGCs. Because AIACs make chemical synapses on sOFFGCs (synapse 5 in Fig. 1) (40, 44), it is possible that the H-IOP-induced sOFFGC ΔC₂ and spike response sensitivity decreases are mediated by AIACs.

Elevated IOP Does Not Significantly Alter the ON and OFF Bipolar Cell Light Sensitivities. We next examined the effects of elevated IOP on the three types of BCs pre-synaptic to ONGCs and sOFFGCs: the HBCR₃MC, DBCRₛ, and DBCR (synapses 3 and 1 in Fig. 1). Fig. 4A shows the morphology and ΔC₃ of an HBCR₃MC, DBCR₃MC, and DBCR to light steps of various intensities in dark-adapted living retinal slices of mice with 5-, 3-, and 7-wk elevated IOP (17–24 Hg), respectively. Retinal slices such as that shown in the left panel (DBC₃R) were counterstained with the anti-PKD₃ antibody [red; labels all DBC₃ (33)] to demonstrate that the recorded cells were DBC₃. The HBCR₃MC and DBCR₃MC were identified by their characteristic morphology (including soma size/shape and patterns/levels of axon terminal stratification), response waveforms, thresholds, and dynamic ranges (33, 39). The average R-Log I relations of 4 HBCR₃MC₁, 3 DBCR₃MC₁, and 5 DBC₃ in H-IOP mouse retinas (with 3- to 7-wk elevated IOP, 16–24 Hg, 2 HBCR₃MC₁, 1 DBCR₃MC, and 2 DBC₃ with the laser method and 2, 2, and 3 with the microbead method; Materials and Methods) are shown as dotted curves in Fig. 4C, whereas the corresponding R-Log I relations obtained from 7 HBCR₃MCₛ, 6 DBCR₃MCₛ, and 11 DBCₛ in n-IOP mice are shown as solid curves for comparison. The average light response thresholds of ΔC₃ of the three types of BCs in n-IOP and H-IOP mouse retinas are shown as bar graphs in Fig. 4D. The differences in HBCR₃MC, DBCR₃MC, and DBC₃ ΔC₃ thresholds between the H-IOP and n-IOP mice are not significant (P = 0.667, 0.422, and 0.180, respectively, t test). Because dark resting potentials of the mouse BCs are very close to E_C (37), the light-evoked voltage responses of the three types of BCs are mainly derived from the ΔC₃ contribution. Therefore, the BC responses pre-synaptic to ONGC and sOFFGC ΔC₃ are not significantly altered by elevated IOP.

Elevated IOP Suppresses Rod Bipolar Cell Synaptic Inputs to AIACs. Because results in Figs. 2 and 3 suggest that the H-IOP-induced sensitivity loss in ON and OFF GCs is likely to be mediated by AIACs, we examined light-evoked rod and cone DBC (DBCₛ and DBCₛ) inputs to AIACs (synapses 1 and 2 in Fig. 1) in mice with elevated IOP. Fig. 5 shows the confocal image and light responses of an AIAC in an H-IOP mouse with 3 wk of elevated IOP. Similar to the AIACs recorded in retinal slices of the n-IOP mice (34, 37), AIACs in retinal slices of H-IOP mice are reasonably well clamped. The current–voltage responses and cation current responses (ΔCₐ) to 24 Hg inputs to AIACs (34), and in the presence of 100 μM DNOX [an AMPA/kainate receptor blocker that suppresses DBCₛ inputs to AIACs (34)], and in the presence of 100 μM DNOX + 100 μM MFA [methylene fluoride; a gap-junction blocker that suppresses DBCₛ inputs to AIACs (48–50)] are shown in Fig. 5B–E, respectively. The average R-Log I relations measured under various conditions in H-IOP mice are plotted as dashed curves in Fig. 5F: numbers of AIACs under each condition in H-IOP mice (3–7 wk of elevated IOP, 15–22 Hg, four with the laser method and two with the microbead method; Materials and Methods) are given in the Fig. 5 legend, and all AIACs were identified by their characteristic morphology and light responses (34, 37). By comparing these R-Log I relations with the corresponding results from AIACs in n-IOP mice (34), it is evident that the AIACs in the H-IOP mice exhibit lower light sensitivity than the AIACs in the n-IOP mice. We also applied 100 μM MFA [methylene fluoride; a gap-junction blocker that suppresses DBCₛ inputs to AIACs (48–50)] to these photoreceptors to show that light responses (ΔCₐ) to 24 Hg inputs to AIACs (34), and in the presence of 100 μM DNOX + 100 μM MFA + 100 μM MFA are not significantly altered by elevated IOP.

Fig. 3. Light responses of sOFFGCs in H-IOP and n-IOP mice. (A) Confocal image of an LY-filled (yellow) HBCR₃MC in an H-IOP mouse (white arrow, axon). (Scale bar, 20 μm.) (B–D) The cell’s light-evoked spike responses, ΔC₃, and ΔC₄ to 500-nm light steps of various intensities are shown in B–D, respectively. (E) The normalized, average R-Log I relations of seven sOFFGCs in H-IOP mice [population R₃MC (mean ± SE) for spike, ΔC₃, and ΔC₄ responses: 12 ± 8 pA, 181 ± 15 μA, and 250 ± 25 μA] are shown as dotted curves, whereas the corresponding R-Log I relations obtained from sOFFGCs in n-IOP mice [n = 15, population R₃MC (mean ± SE) for spike, ΔC₃, and ΔC₄ responses: 14 ± 8 pA, 162 ± 16 μA, and 241 ± 33 μA] are shown as solid curves for comparison. Black, spike responses; red, ΔC₃ green, ΔC₄. Colored arrows indicate H-IOP-induced shifts of the R-Log I relations. (F) Bar graphs of the average light response thresholds (mean ± SE) of spike responses, ΔC₃, and ΔC₄ in n-IOP and H-IOP mouse retinas; the significance levels of the threshold differences are given by the P values of the t test.
DNQX to block the DBC$_R$→AIIAC glutamatergic synaptic inputs (34, 35, 51, 52) (Fig. 5D). DNQX substantially reduced the response amplitude and sensitivity of the AIIACs in the n-IOP mice (red solid R-Log I curve in Fig. 5F) (34) but does not significantly alter the AIIAC response amplitude and sensitivity of the AIIAC in the H-IOP mice (Fig. 5C and D and the dashed red curve in Fig. 5F), suggesting that AIIACs in the H-IOP retinas have very little DBC$_R$ input. Application of 100 µM MFA almost completely abolished the AIIAC response in the H-IOP retinas (Fig. 5E and green dashed curve in Fig. 5F), indicating that the responses in H-IOP mice are mediated by DBC$_C$ inputs, because it has been shown that the Cx36/DBC$_C$-mediated responses in AIIACs are very similar to the DNQX-resistant responses (34). Our results suggest that elevated IOP suppresses AIIAC response sensitivity by mainly affecting the DBC$_R$→AIIAC synapses. The H-IOP→induced sensitivity decrease of DBC$_R$→AIIAC signals is likely to be mediated by suppression of synaptic efficacy, rather than by changes in the DBC$_R$ output synapses to AIIACs.

**Discussion**

**Suppression of the Rod BC Inputs to AIIACs Is a Primary Cause of Light Response Sensitivity Decrease of Retinal Ganglion Cells.** In this study, we provide evidence demonstrating that elevated IOP in experimental glaucoma mouse models significantly decreases light-evoked spike response sensitivity of ONaGCs and OFFr GCs (i.e., raises thresholds by 1.5–2.5 log units), and that the decrease is primarily caused by a sensitivity reduction of ON cone bipolar cell (DBC$_{R/MC}$) signals to the ONaGCs ($ΔI_{s}$) and a sensitivity reduction of amacrine cell signals to the OFFr GCs ($ΔI_{s}$). We also show that the soma responses of the ON and OFF bipolar cells presynaptic to ONa GCs and OFFr GCs (DBC$_{R/MC}$ and HBC$_{R/MC}$, respectively) (41, 53) are not significantly altered by elevated IOP but that the sensitivity of the output signals of the DBC$_{R/MC}$ axon terminals to ONaGCs (Δ$I_{s}$: synapse 3 in Fig. 1, Right) is significantly reduced. This may suggest that somas and axon terminals of DBC$_{R/MC}$s are not isopotential and that the high DBC$_{R/MC}$ soma sensitivity may reflect rod inputs to the dendrites of these cells (33). Anatomical and physiological evidence has suggested that AIIACs make electrical synaptic contacts with DBC$_{R/MC}$ synaptic terminals (synapse 2 in Fig. 1) (47, 54), and that the AIIAC inputs to DBC$_{R/MC}$ synaptic terminals contribute to the DBC$_{R/MC}$ outputs to ONaGCs (Δ$I_{s}$: via synapses 2 and 3 in Fig. 1) (41). Therefore, it is possible that the sensitivity reduction of DBC$_{R/MC}$ output signals to ONaGCs in H-IOP mice is mediated by the AIIACs, which send light responses of lower sensitivity to DBC$_{R/MC}$ axon terminals, resulting in an output signal ($ΔI_{s}$) of reduced sensitivity. Our observation that $ΔI_{s}$ in ONaGCs was not significantly reduced by H-IOP suggests that AIIAC inputs to other ACs that make inhibitory synapses on ONaGCs are relatively minor. It has also been suggested that AIIACs make inhibitory chemical synapses onto OFFr GC dendrites (synapse 5 in Fig. 1) (44, 45, 55), and thus the H-IOP→induced sensitivity reduction of DBC$_{R}$ in OFFr GCs may also be mediated by AIIACs. Our result in Fig. 5 shows that elevated IOP indeed reduces AIIAC response sensitivity by about 2 log units, supporting the assertion that the H-IOP→induced sensitivity loss in ON and OFF r GCs is mediated by AIIACs. Although anatomical studies have also indicated that AIIACs make chemical synapses on HBC axon terminals (47), our results that $ΔI_{s}$ in OFFr GCs is less affected than $ΔI_{s}$ by H-IOP are consistent with the notion that AIIAC feedback synapses on HBCs are weaker than the feedforward synapses on OFFr GCs (40, 44). The single-cell AIIAC, ON, and OFFr GC results in this report are also consistent with the observation that elevated IOP reduces the voltage gains (ratio of post/presynaptic signals) of the positive and negative scotopic threshold responses (representing the GC and AIIAC responses, respectively) in living mice (12).

Experiments in Fig. 5 indicate that the reduction of AIIAC response sensitivity is primarily mediated by suppression of the rod BC (DBC$_R$) inputs to AIIACs (synapse 1 in Fig. 1), because the average AIIAC light responses in H-IOP mice were shifted to the same level as the average AIIAC responses of the n-IOP mice when DBC$_R$ inputs were blocked by DNQX (black dashed curve and solid red curve in Fig. 5F). The average light-evoked current responses of the DBC$_R$s in H-IOP mice do not significantly differ from DBC$_R$ responses in n-IOP mice (solid and dashed red curves in Fig. 4C), suggesting that the reduction of AIIAC response sensitivity is not mediated by decrease of DBC$_R$s’ soma responses but by the efficacy of the DBC$_R$ output synapses to AIIACs. Our observation that no significant morphological changes occur in H-IOP mice within the period of our study suggests that the changes in synaptic efficacy are mediated by physiological factors, rather than the reduction of numbers of DBC$_R$ axon terminals or synaptic contacts. One possible element mediating such physiological changes is the BK channel in A17 ACs, which are the cell dendrites that mediate inputs from DBR$_{R/MC}$s at the axon terminal dyads (56). BK channels are known to be mechanosensitive (57), and thus chronic elevation of IOP may affect these channels and impede the efficacy of the DBC$_R$→AIIAC synapses. It is also interesting to note that the DBC$_R$→AIIAC and A17AC→DBC$_R$ synapses are among the most proximally located chemical synapses in the mammalian retina (47, 58), and thus they may be most susceptible to chronic high IOP.

**Sensitivity Loss in Inner Retinal Neurons Occurs Before Observable Structural Damage, and Thus It Is a Useful Early Diagnostic Tool for Glaucoma at Its Reversible Stages.** Our results have shown that functional changes of the DBC$_R$→AIIAC synapses are likely to be a primary source of RGC sensitivity loss in mice with elevated IOP. We found such sensitivity reduction in ON and OFF r GCs as well as in AIIACs in mice with elevated IOP at stages before significant RGC or optic nerve degeneration is observed (12, 16). This suggests that physiological response sensitivity changes may occur before structural damage in early stages of glaucoma, and thus measuring RGC and AIIAC response sensitivity changes may be used as a diagnostic tool for glaucoma at its early stages before any irreversible structural damage occurs. It is possible, for example, to develop new human scotopic optokinetic response apparatuses to screen patients with early signs of glaucoma and to determine whether therapeutic treatments are needed before.
RGC/optic nerve damage and/or visual field defects are detected. From our finding that defective DBC \( \rightarrow \) AIIAC signal transmission is a primary source of sensitivity loss in inner retinal neurons, functionally repairing the DBC \( \rightarrow \) AIIAC synapses, such as targeted expression of specific synaptic proteins, ion channels [e.g., BK channels in A17 ACs (56)], and neurotrophins [e.g., BDNF (59, 60)] capable of restoring the efficacy of the DBC \( \rightarrow \) AIIAC synapses, may be a useful preventive strategy against glaucomatous degeneration. Although a large amount of work is needed to identify these synaptic molecular targets, by determining synaptic sites most vulnerable to functional changes our study provides useful information in narrowing down the scope of such a research endeavor.

Materials and Methods

Preparations. The wild-type mouse used in this project was the C57BL6J from the Jackson Laboratory. All animals were handled in accordance with Baylor College of Medicine’s policies on the treatment of laboratory animals and conform to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and the Guide for the Care and Use of Laboratory Animals (61). Mice were dark-adapted for 1–2 h before the experiment. To maintain the retina in the fully dark-adapted state, all further procedures were performed under infrared illumination with dual-unity NiteWare (B.E. Meyers) infrared scopes. Animals were killed by a lethal injection of ketamine (80 mg/kg), xylazine (16 mg/kg), and acepromazine (1.2 mg/kg). Detailed procedures of this method are described in our previous publications (33, 40, 62, 63). All pharmacological agents (purchased from Sigma) were dissolved in Ames medium with a superfusion time of 45–80 s, and the superfusion and puff drug application procedures were described in previous publications (64–66). We found that a high dose has devastating side effects on the retina, and thus carefully calibrated the dose and found that 100 \( \mu \)M suppresses the gap junction and enables partial recovery after washing in retinal slices. This dose was used in tiger salamander retinal slices (67) and in mouse retinal slices in this study.

Ocular hypertension was induced on the right eye of the C57BL6J mouse line with one of two methods, as follows. (i) Microbead occlusion method: Six-week-old C57BL6J mice were anesthetized with weight-based i.p. injection of ketamine (80 mg/kg), xylazine (16 mg/kg), and acepromazine (1.2 mg/kg). Detailed procedures of this method are described in our previous publication (12). (ii) Laser/cautery coagulation method: Six-week-old C57BL6J mice were anesthetized the same way as described in the first method. One eye per animal underwent argon laser coagulation of the episceral and limbal areas. Detailed procedures of this method are described in our previous publications (16, 68). Both methods induced sustained high IOP for over 2 mo, although RGC degeneration in eyes treated with the first method appeared at an earlier time (5–6 wk after treatment) than in eyes treated with the second method (8–12 wk after treatment) (12, 16). To assess GC, AIIAC, and BC sensitivity loss before substantial GC degeneration, all cells in this study were recorded from eyes with elevated IOP for 3–4 wk treated with the laser method or from eyes with elevated IOP for 6–7 wk treated with the microbead method. IOP was measured with a TonoLab rodent tonometer (TioLat) when the mice were anesthetized (one drop of topical proparacaine 1% solution was applied to each eye and IOP was measured 10 times). IOP was again measured at week 1 after treatment and again weekly through week 8 using the same technique. IOP measurements were averaged at each time point to establish a curve of IOP change compared with untreated fellow eyes.

Recordings, Cell Morphology, and Immunocytochemistry. Whole-cell voltage-clamp and loose-patch experiments were carried out using the Axon MultiClamp 700A amplifier connected to a Digidata 1200 interface and pClamp 10 software (Axon Instruments); the procedures for making whole-cell patch, loose-patch, and granamicidin-filled perforated patch pipettes, as well as verification of the liquid junction potentials, have been described in previous papers (40, 64, 65). The internal solution (with 0.8 mM Lucifer yellow and/or 0.8 mM neurobiotin) for standard whole-cell patch-clamp pipettes and the external Ames medium yielded an \( E_C \) of about –60 mV. Three-dimensional cell morphology in retinal slices and flat-mounted retinas was visualized through the use of LY fluorescence or NB-conjugated fluorescence with a confocal microscope (Zeiss; S10). The procedures of 2-axis rotation and preparation for viewing have been described in our previous publications (33, 40, 69, 70).

Light Stimulation. The retinal slices and flat-mounted retinas were stimulated with a photostimulator that delivers light spots of various wavelengths and intensities to the retina via the epilluminator of the microscope. Because we delivered an uncollimated stimulus light beam through an objective lens with large numerical aperture (Zeiss; 40×/0.75 water), the incident light entered the retinal slice from many directions, and thus the effect of photoreceptor self-screening was minor (71). The intensity of unattenuated 500-nm light (log 1 0) is 1.4 \( \times 10^7 \) photons-\( \mu \)m\(^2\) s\(^{-1}\). The number of photoisomerizations per rod per second was calculated by using a rod cross-section of 0.45 \( \mu \)m\(^2\) (72, 73). The light-evoked responses were plotted against light stimulus intensity, and data points were fitted by the Hill equation, \( R = R_{max} (1 + tanh (k \cdot log(I_0) - log(N))) \); where \( R \) is the current response amplitude, \( R_{max} \) is the maximum response amplitude, \( k \) is the light intensity that elicits a half-maximal response, and \( N \) is the Hill coefficient (74, 75). Normalization was done cell by cell: \( R_{max} \) of each cell was set to unity, and the \% responses to a given light intensity of a given group of cells were averaged and fitted by the Hill equation. Response thresholds are defined as the light intensity that generates 5\% of the maximum responses. For current responses of OFF/ON GCs, AIIACs, and BCs, the peak sustained outward/inward currents (in light) compared with the baseline current (before light) were measured. For the spike response of OFF/ON GCs, the spike decrement/increment during light (number of spikes per second in light — number of spikes per second before light) was measured. Significance in threshold differences between various groups of responses (P values) was computed by the Student’s t test. The intensity of light beams was calibrated with a radiometric detector (United Detector Technology).

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