**Review**

# Probing ON and OFF Retinal Pathways in Glaucoma Using Electroretinography

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Glaucoma is a progressive neurodegenerative disease involving damage and eventually death of retinal ganglion cells (RGCs) that comprise the optic nerve. This review summarizes current understanding of specific RGC type vulnerability in glaucoma and how electroretinography (ERG) may provide an objective measure of these functional perturbations. There is building evidence to suggest that ON RGCs, which respond to light increments, may be more resilient to elevated intraocular pressure and glaucoma, whereas OFF RGCs, which respond to light decrements, may be more susceptible. ERG experiments in nonhuman primates and mice have also shown that the ON- and OFF-pathways can be separated using a variety of techniques such as pattern ERG and the photopic negative response. Another ERG paradigm of interest to separate the ON and OFF responses is a flicker stimulus at varying temporal frequencies. Response to lower temporal frequencies is associated with the ON-pathway, and ERG response to higher frequencies is associated with the OFF-pathway. In mice, experimental glaucoma models have shown greater decreases in ERG response at higher frequencies, suggesting that the OFF-pathway is more susceptible. We also summarize current clinical ERG protocols used for glaucoma and discuss innovations for developing new types of stimuli that can further separate the ON- and OFF-pathways. Applying these novel paradigms that distinguish ON- and OFF-pathways may ultimately improve glaucoma diagnostics and monitoring of glaucoma progression.

**Translational Relevance:** Based on our current understanding of specific RGC type vulnerability in glaucoma, we explore how ERG may provide an objective measure of ON- versus OFF-pathway functional perturbations.

## Introduction

Glaucoma is a complex set of neurodegenerative diseases that involves damage and subsequent cell death of the retinal ganglion cells (RGCs) that compose the optic nerve. Despite glaucoma being the leading cause of irreversible blindness worldwide with an estimated prevalence of 3.54% for the worldwide population between 40 and 80 years of age, there remain gaps in our understanding of its pathogenesis.[1,2] Glaucoma had been traditionally associated with age-related stressors and elevated intraocular pressure (IOP), with the latter being the only treatable risk factor.[3] Consequently, the majority of research in experimental animal-based models such as nonhuman primates, felines, and rodents has focused on the effects of elevated IOP on RGC health.

Before the development of RGC type-specific labeling techniques based on genetic cell targeting, studies in monkey and feline models suggested that RGCs with the largest somas and axons were most susceptible to injury.[4,5] More recently, improved techniques to identify specific cell types have permitted the discovery of resilient and susceptible RGC types.[6,7] Two main functional groups of RGCs include the ON RGCs that depolarize in response to light and the OFF RGCs that hyperpolarize with light stimulus. These ganglion cells are the final output neuron reflecting the visual signals that segregate into parallel ON- and OFF-pathways. Thus, further elucidating the difference between these RGC types and ON- versus OFF-pathways could provide a novel way to diagnose glaucoma in patients.

There remains a gap, however, between laboratory discoveries and clinical applications. Although

it is important to decipher the pathogenic mechanisms underlying glaucoma, we ultimately want to leverage this understanding to allow for earlier and more accurate diagnosis of glaucoma and for improved progression detection. After all, epidemiologic studies performed in the United States, Australia, and Barbados have all consistently shown that approximately 50% of the people with glaucoma are unaware of their condition,[8–11] suggesting that patients tend to show up late under care because their visual system is able to compensate for RGC loss. In fact, current visual field testing is limited in its ability to detect these subtle functional changes; previous studies estimated between 25% and 50% of RGCs are lost before statistical abnormalities appear in automated visual field testing.[12,13] Much work has been done to develop psychophysical stimuli that have an improved ability to detect early glaucoma and visual field progression,[14,15] but current tests of visual function lag behind structural measurements.[16] Electroretinography (ERG) can provide sensitive and objective information, but it requires identifying a differential functional change in populations of RGCs or in their upstream circuitry. As a result, this review addresses the gap between laboratory research on RGC susceptibility with current clinical paradigms for ERG assessment of glaucoma, highlighting areas of clinical translation using ERG paradigms that distinguish between ON- and OFF-pathways that could lead to improvements in diagnosis and progression detection.

## Specific RGC Types Are More Susceptible to Injury From Elevated IOP

The initial studies addressing RGC susceptibility to IOP elevation damage relied on morphologic differences such as soma size. Early studies with experimental nonhuman primate models (*Macaca fascicularis* and *Macaca mulatta*) have demonstrated that the parasol cells of the magnocellular pathway are more susceptible to cell death compared with the smaller midget cells of the parvocellular pathway.[4,17] Furthermore, in human psychophysical studies, glaucoma subjects were inferred to have selective loss of RGCs in the magnocellular pathway.[18,19] Additional experiments quantifying RGC type vulnerability supported that large RGCs and large axon fibers were more vulnerable compared with other RGCs in an experimental laser-induced *M fascicularis* glaucoma model.[20,21] A follow-up study using human

pathologic specimens with moderate and severe glaucoma similarly found larger optic nerve fibers were more vulnerable.[22]

However, there was not a consensus within the field regarding larger cell susceptibility. Morgan et al.[23,24] did not find that the parasol cells were specifically targeted by elevated IOP, and instead, parasol and midget cells were equally affected in an experimental *M fascicularis* model. The authors concluded that there was an overall loss in cell size during the initial phases of glaucoma, which could suggest that there is a generalized morphologic change affecting a multitude of RGC types in glaucoma, rather than specific RGC type susceptibility.[23,24] Additionally, in human retinas, parasol cells were shown to have similar decreases in arborization as midget cells in the setting of glaucoma,[25] although the authors could not conclude if parasol cells experienced earlier changes than midget cells. Li et al.[26] found in Sprague-Dawley rats that melanopsin-expressing RGCs, which have large somas and intrinsically respond to light, were not susceptible to elevated IOP, even though there was a significant loss of superior colliculus-projecting RGCs. Although, in an experimental cat model of chronic glaucoma larger alpha RGCs were more susceptible than smaller beta RGCs,[5,27] the opposite was found after axotomy or optic nerve transection.[28,29] These findings demonstrate that specific RGC vulnerability may be due to factors beyond just cell size and that these susceptible cells may first undergo morphologic changes.

The development of more specific immunohistochemistry protocols, molecular markers, and functional assessments have resulted in a deeper understanding of RGC type-specific vulnerability.[30] For instance, patch clamp recordings in mice were used to further divide alpha RGCs, which are rich in neurofilament, into three main types: $\alpha$ON-sustained ($\alpha$ON-S), $\alpha$OFF-sustained ($\alpha$OFF-S), and $\alpha$OFF-transient ($\alpha$OFF-T). These names are defined based on their preferential tonic versus phasic response to the onset or offset of a light step stimulus. The dendrites of $\alpha$ON-S, $\alpha$OFF-T, and $\alpha$OFF-S RGCs stratify into distinct sublaminae in the inner plexiform layer, at 30%, 50%, and 70% inner plexiform layer depth from the ganglion cell layer, respectively.[30,31]

When probing how different RGC types respond to elevated IOP, the literature has shown that $\alpha$OFF-T RGCs are a vulnerable cell type with increased rates of cell death and decreased dendritic field area and complexity.[32–37] The receptive field area was also shown to be decreased,[32,38] and the excitatory glutamatergic postsynaptic density was diminished both with transient ocular hypertension as early as 7 days after
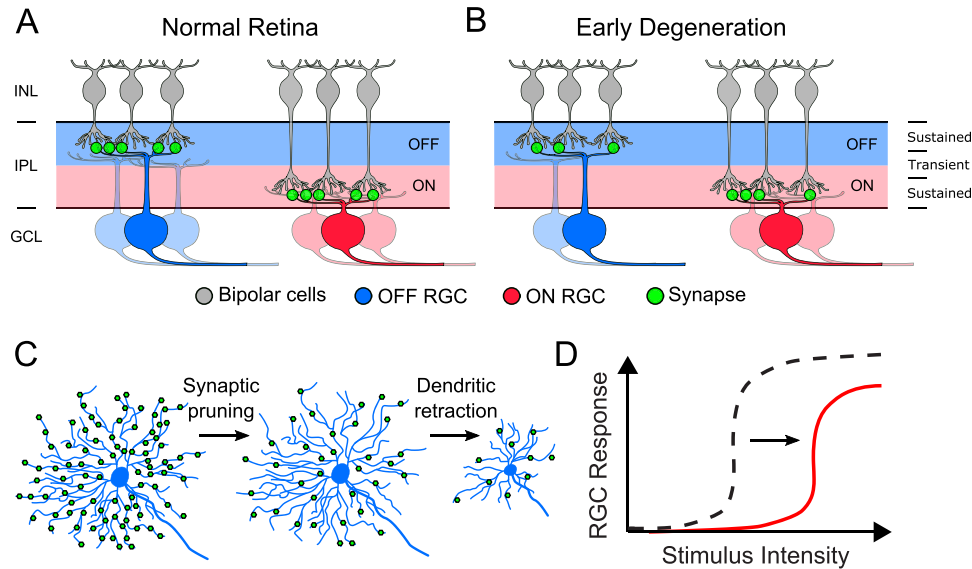
**Figure 1.** Model of early degeneration of RGCs in glaucoma. (**A**) Normal retina with RGC somas in the ganglion cell layer (GCL) and bipolar cell somas in the inner nuclear layer (INL). The RGCs and bipolar cells form synapses in the inner plexiform layer (IPL), where the OFF RGCs stratify closer to the INL and the ON RGCs stratify near the GCL. The IPL can be further divided into zones where sustained RGCs stratify their dendrites near the top and bottom quartiles and transient RGCs stratify their dendrites near the center of the IPL. (**B**) In early degeneration, OFF RGCs exhibit a decrease in number of synapses, dendritic field area, and dendritic complexity. ON RGCs have been shown to be more resilient. (**C**) With elevated IOP, RGCs first undergo synaptic pruning. Continual damage results in dendritic retraction. (**D**) Following elevated IOP, the light responses of RGCs exhibit a reduction in sensitivity and magnitude.

elevated IOP and with sustained elevated IOP (Fig. 1 and Table).[32,33] Other lines of evidence suggest that not only are $\alpha$OFF-T RGCs vulnerable, but also other RGC types stratifying in the OFF sublamina such as $\alpha$OFF-S and M1 RGCs—which are functionally ON RGCs but their dendrites stratify in the OFF sublamina.[32,34] There was also greater loss of presynaptic ribbon density in the OFF sublamina as well,[32] suggesting that the loss of excitatory synapses may precede actual morphologic changes in the dendritic structure of $\alpha$RGCs. However, even though Berry et al.[39] did not isolate different types of $\alpha$RGCs, this group found the loss of synapses and dendritic shrinkage to occur near simultaneously, illustrating that the exact order of RGC degeneration is still not fully understood.

Although all $\alpha$RGCs experience excitatory synapse reduction and OFF RGCs may be more vulnerable, some RGCs stratifying dendrites in the ON sublamina seem to be more resilient. In fact, $\alpha$ON-S RGCs had no significant decrease in dendritic complexity as well as no change in receptive field size when these alterations were already present in $\alpha$OFF RGCs.[32,33] Tran et al.[40] used genetic data from single cell RNA seq in the setting of optic nerve crush injury to identify which RGCs were most resilient among all RGC types. The authors found that $\alpha$ON-S RGCs

were the most resilient cell type, and $\alpha$OFF-S RGCs were more resilient than $\alpha$OFF-T RGCs.[40] This result is consistent with the findings that sustained cells are more resilient than transient cells, and the differential susceptibility of $\alpha$RGCs in order from most to least vulnerable is $\alpha$OFF-T, $\alpha$OFF-S, and $\alpha$ON-S RGCs. A review from Wang et al.[7] provides a discussion on cell autonomous and non-cell-autonomous mechanisms to explain this difference in susceptibility, the latter including mechanisms related to RGC interactions with other neurons, glia, and vasculature that may contribute to the observed ON RGC resiliency.

The finding of ON resiliency versus OFF susceptibility in experimental glaucoma, however, is not found consistently throughout the literature. Risner et al.[41] found in a mouse microbead occlusion experimental model that $\alpha$ON RGCs were equally affected as $\alpha$OFF RGCs, where both had a decreased dendritic area and dendritic complexity. ON and OFF RGCs were additionally found to have similar decreases in light responses,[42] and this decrease may be mediated by the suppression of AII amacrine cells.[43] Feng et al.[44] lacked a large enough sample size to analyze the OFF RGCs, but still showed decreases in the dendritic area of ON RGCs. Moreover, an experimental model with milder IOP elevation of about 3 mm Hg for

**Table.** Pathologic RGC Alterations in Rodent Models of Experimental Glaucoma

| | Synapse Loss | Dendritic Shrinkage | Light Response Impairment | RNA Expression Changes | Rates of Cell Death | IOP Increase and Duration | Rodent Model* |
|---|---|---|---|---|---|---|---|
| Li et al., 2006[26] | | ✓ | | | ✓ | ~9 mm Hg (~70%); 12 weeks | Laser-induced chronic ocular hypertension (Sprague-Dawley rats) |
| Della Santina et al., 2013[33] | ✓ | ✓ | ✓ | | ✓ | ~10 mm Hg (~100%); 15 or 30 days | Microbead injection |
| Feng et al., 2013[44] | | ✓ | ✓ | | | ~8 mm Hg (~80%) at 16 weeks; ~6 mm Hg (~40%) at 24 weeks | Laser-induced chronic ocular hypertension |
| Berry et al., 2015[39] | ✓ | ✓ | | | | NR | DBA/2J mouse model and ONC injury |
| Chen et al., 2015[42] | | | ✓ | | | ~8 mm Hg (~50%); 15–18 weeks | Laser-induced chronic ocular hypertension and microbead injection |
| El-Danaf et al., 2015[34] | | ✓ | | | | ~8 mm Hg (~70%); 7 days | Microbead injection |
| Pang et al., 2015[43] | | | ✓ | | | NR | Laser-induced chronic ocular hypertension and microbead injection |
| Ou et al., 2016[32] | ✓ | ✓ | ✓ | | ✓ | ~15 mm Hg (~100%) after 24 hours; returned to baseline by day 4 | Laser-induced transient ocular hypertension |
| Puyang et al., 2017[35] | | | ✓ | | | NR | ONC injury |
| Sabharwal et al., 2017[38] | | | ✓ | | | ~7 mm Hg (~60%); 11–14 days | Microbead injection |

**Table.** Continued

| | Synapse Loss | Dendritic Shrinkage | Light Response Impairment | RNA Expression Changes | Rates of Cell Death | IOP Increase and Duration | Rodent Model* |
|---|---|---|---|---|---|---|---|
| Daniel et al., 2018[36] | ✓ | | | | ✓ | NR | ONC injury |
| Risner et al., 2018[41] | | ✓ | ✓ | | | ~4 mm Hg (~30%); 2 or 4 weeks | Microbead injection |
| Christensen et al., 2019[46] | | ✓ | | | ✓ | NR | Glutamate agonist injection |
| Daniel et al., 2019[37] | | | | | ✓ | ~8 mm Hg (~60%) at 30 days postnatal; ~23 mm Hg (~175%) at 90 days postnatal | Early onset glaucoma model |
| Tao et al., 2019[45] | | | ✓ | | | ~3 mm Hg (~30%); 2 weeks | Microbead injection |
| Tran et al., 2019[40] | | ✓ | | ✓ | ✓ | NR | ONC injury |
| Yang et al., 2020[47] | | | | | ✓ | NR | ONC injury |

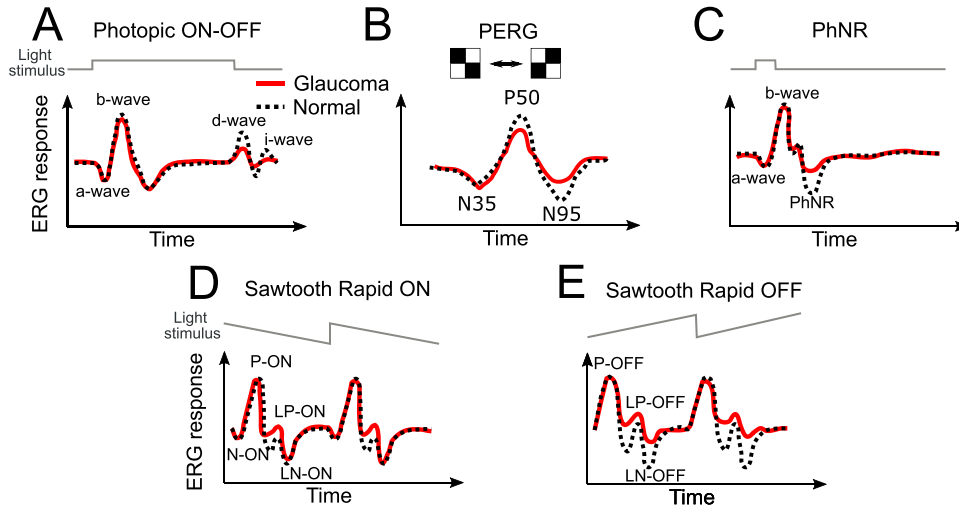*Mouse model unless otherwise indicated. ONC, optic nerve crush; NR, not recorded.

**Figure 2.** Example ERG responses for normal and glaucoma subjects. (**A**) The photopic ON-OFF uses a rod-saturating light background with a long-duration stimulus. In experimental glaucoma models, there is evidence of decreased d- and i-wave amplitudes.[51] (**B**) PERG uses a checkerboard pattern that alternates, and glaucoma subjects have decreased P50 and N95 peaks.[68-71] (**C**) The PhNR also uses a rod-saturating light background. Glaucoma subjects have been shown to have a decreased PhNR amplitude.[70,72-76] (**D**, **E**) The sawtooth stimuli incorporate a slow decrease or increase in light intensity followed by a rapid increase or decrease, respectively. In glaucoma subjects, the sawtooth rapid-on exhibits an elevated late positive-ON (LP-ON) wave, and the sawtooth rapid-off has an elevated late positive-OFF (LP-OFF) and decreased LN-OFF waves.[78]

2 weeks found no change in the ON or OFF RGC receptive field center size.[45] The single cell RNA seq experiments by Tran et al.[40] suggest that there may not be a clear delineation between susceptible versus resilient RGC types based on ON versus OFF status alone. One possible way to reconcile these discrepancies in differential RGC susceptibility may require taking into account that these occur after different types and magnitudes of injury, such as varying degrees and duration of IOP elevation or optic nerve injury, differences in rodent strains, and analysis techniques as highlighted in the Table. For instance, one group used the same mouse line but found differences in RGC type susceptibilities using different experimental models—one model used a glutamate agonist injection and the other used an optic nerve crush model.[46,47] Differences observed across models may reconcile within a larger framework, for example, if there is only a short temporal window to counteract the injuring stimulus before reversible RGC damage triggers degeneration. An additional limitation of examining IOP changes as the main driver of injury is that these experiments only measure snapshots of the IOP. Continuous IOP monitoring may be an area of future study that could be used to better understand changes in experimental glaucoma models and to elucidate the integral role of IOP in RGC type susceptibility.

## Distinguishing the ON Versus OFF Pathway With ERG in Animal Models and Humans

ERG has provided evidence that the ON- and OFF-pathways can be isolated using specific light stimulation paradigms. For instance, the photopic ON-OFF stimulus, which consists of a long-duration stimulus lasting 150 to 200 ms, is now an extended protocol by the International Society for Clinical Electrophysiology and Vision.[48] The long duration photopic ON–OFF stimulus produces at light onset an a-wave followed by a b-wave (Fig. 2A). The b-wave generated in ERG is driven mainly by the ON-pathway, although the OFF bipolar and horizontal cells may affect the shape and amplitude.[48,49] At light offset, there are two additional positive waveforms called the d- and i-waves that are associated with the OFF-pathway response.[49,50] Interestingly, in a laser-induced experimental model of glaucoma in *M fascicularis*, researchers found that, when using a long flash stimulus, the experimental glaucoma eyes had decreased a-wave, d-wave, and i-wave amplitudes,[51] illustrating how the OFF-pathway may be more vulnerable as both the d- and i-wave amplitudes were diminished.

The multifocal ERG (mfERG) is an ERG modality that can assess multiple retinal locations simultaneously, and it may even have the potential to measure local cone ON- and OFF-pathways.[52] The mfERG also generates a negative a-wave and positive b-wave after light onset, and a d-wave after light offset. The authors found that a subject with macular dystrophy consistent with damage to the OFF bipolar cells had a decreased d-wave response.[52] This decrease was particularly noticeable in the central retina. This finding was in contrast with a subject lacking an ON bipolar response, who had a missing b-wave while the a-wave and d-wave were enhanced.[52] A review by Chan et al.[53] describes several studies that also discuss the origin of the mfERG waveforms, but additional research with a larger sample size is needed to identify the effectiveness of mfERG at measuring the ON- and OFF-pathway separately.

One testing modality with increasing interest for glaucoma detection is pattern ERG (PERG). Transient PERG uses a temporally modulated checkerboard pattern in order to cancel out the cone photoreceptor and cone bipolar cell responses to produce an RGC-driven signal with a positive peak and negative peak at 50 and 95 ms called the P50 and N95 peaks, respectively (Fig. 2B).[54] In experimental animal models, there is evidence that the N95 peak is driven primarily, but not exclusively, by the OFF-pathway, and the P50 peak reflects the ON-pathway. In a mouse model with intravitreal injections of *cis*-2,3-piperidinedicarboxylic acid (PDA), which blocks OFF bipolar cells and third-order neurons (ON and OFF amacrine and ganglion cells), the N95 homologue, N2 peak, was decreased.[55] In contrast, the addition of 2-amino-4-phosphonobutyric acid (APB) that blocks ON bipolar cells, eliminated the P50 homologue, P1 peak, whereas the N2 peak increased in amplitude.[55] In a *M mulatta* model, the addition of PDA eliminated the N95 peak, whereas the P50 peak significantly increased.[56] Even though PDA does not exclusively block the OFF-pathway, the ON bipolar cells dominate the post-PDA response, demonstrating how the N95 peak has a greater OFF-pathway contribution. However, the addition of APB resulted in both the P50 and N95 amplitudes to decrease by half, suggesting the N95 peak receives contributions from both the ON- and OFF-pathways.[56]

The photopic negative response (PhNR) is a type of full-field ERG response driven by inner retinal neurons that research teams have examined in experimental glaucoma. PhNR is identified as the slow negative component that follows a b-wave after a photopic flash stimulus (Fig. 2C). In nonhuman primate experimental models, tetrodotoxin eliminated PhNR, suggesting its origin is derived from the inner retina because tetrodotoxin blocks the spiking activity of inner retinal neurons.[57,58] Furthermore, in laser-induced experimental glaucoma with *M mulatta*, the PhNR was greatly reduced.[59] Luo and Frishman[56] also used a long duration stimulus and showed that the addition of PDA eliminated the PhNR after stimulus onset and offset, whereas the addition of APB eliminated only the onset PhNR. This finding demonstrates how a long duration stimulus may also be used to measure the ON- and OFF-pathways, where the onset PhNR and offset PhNR correspond with the ON- and OFF-pathways, respectively.

There is also growing evidence that flicker stimuli can be employed to separate the ON- and OFF-pathways. In *M mulatta*, Kondo and Sieving[60] used sinusoidal, square wave, and brief pulse flicker stimuli to measure the ERG response to different stimulation frequencies, generating frequency response curves. They modulated the light stimulus frequency from 4 to 64 Hz. They also intravitreally injected APB, followed by PDA. In control eyes before injection, they found that the ERG fundamental frequency response curve for the sine- and square-wave stimuli had a bimodal shape with a local minimum around 10 Hz and two maxima at around 4 and 48 Hz (Fig. 3A). When APB was introduced, the ERG response between 6 and 32 Hz was larger than the control, suggesting that the ON-pathway is responsible for the depression of the ERG response in that range. After the injection of both APB and PDA, the ERG response was depressed across all temporal frequencies except around 10 Hz. However, the addition of either APB or PDA minimally affected the brief pulse flicker response amplitude, suggesting that the flicker stimulus elicits ON and OFF events simultaneously unlike the sine or square wave stimuli.[60]

The same group followed up on this experiment by measuring human ERG responses with a sinusoidal stimulus also ranging from 4 to 64 Hz, and they found similar results (Fig. 3B).[61] As in nonhuman primates, the human ERG response followed a pattern with a local minimum at 12 Hz, believed to occur when the phase of the ON- and OFF-pathways cancel out, leaving only the photoreceptor response.[62] The authors also measured the response in patients with complete-type congenital stationary night blindness (CSNB1) with mutations in the *NYX* gene, which is found on chromosome X and encodes for the nyctalopin protein. Patients with this mutation lack ON-pathway function. Similar to the addition of APB in the primates, CSNB1–*NYX* patients had an increased ERG response at lower frequencies and lost the minimum inflection point near 12 Hz, whereas at 32 Hz and higher frequencies the ERG response was largely similar to controls.[61]
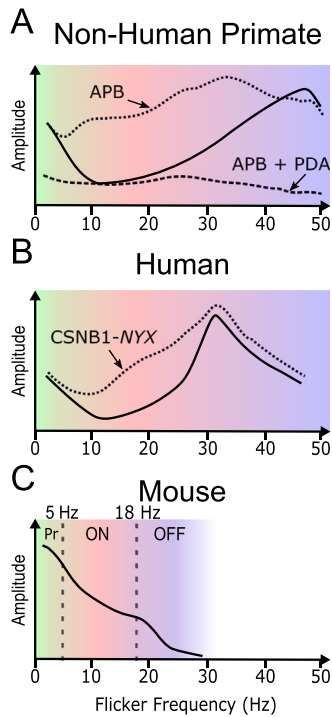
## A Non-Human Primate



## B Human



## C Mouse



**Figure 3.** ERG response amplitude as a function of frequency of the flickering light stimulus. Experiments in nonhuman primates and humans have shown a different ERG response curve that is bimodal. (**A**) Nonhuman primates have a minimum near 10 Hz and a maximum near 48 Hz. The effect of intravitreal injection of APB and APB+PDA are also shown.[60] (**B**) Humans have a minimum near 12 Hz and a maximum near 32 Hz. CSNB1-*NYX* subjects have an elevated ERG amplitude from 8 to 32 Hz.[61] The specific boundaries of the rod photoreceptors, ON-, and OFF-pathways have not been formally identified, but the literature suggests that for both, the OFF-pathway begins to dominate the ERG response near 30 Hz. (**C**) For mice, the ERG response has been more clearly described. The ERG response from 5 Hz and below is correlated mainly with the rod and cone photoreceptors (*green*), between 5-15 Hz the response is driven mainly by the ON-pathway (*red*), and from 18 Hz and above the response is driven by the OFF-pathway (blue). The mouse retina no longer responds to frequencies greater than 30 Hz.[65] Pr, photoreceptors.

Mouse studies also provide evidence that the ON- versus OFF-pathways could be preferentially stimulated using different flicker frequencies at mesopic intensities in full-field ERG recordings. Tanimoto et al.[63] used CNGA3^{−/−}, Rho^{−/−}, and mGluR6^{−/−} mice, which are transgenic mice without functional cones, rods, and ON-bipolar cells, respectively. By stimulating these mice with a wide range of flicker stimulus frequencies, the authors divided the responses into three different regions. From 0.5 to 5.0 Hz, the rod and cone photoreceptors' responses drive the ERG response, whereas above this range the photoreceptor response contribution becomes negligible compared with the bipolar cell response. From 5 to 15 Hz, the response from the ON-pathway is dominant, and

from 18 Hz and above, the OFF-pathway is primarily responsible for the ERG response.[63] Although these temporal frequency ranges may not directly overlap with the human response ranges, it is consistent that the ON-pathway predominates at lower frequencies and the OFF-pathway at higher frequencies across species (Fig. 3C).

In a follow-up study by the same group, three additional experimental mouse models were tested at the same three temporal frequency ranges previously identified that correlate with the different pathways.[64] The mouse models included a *Nyx*[nob] mouse that has deficits in the ON-pathway and is the same genetic mutation identified in the CSNB1 patients from the Khan et al. study.[61] In addition, the authors used an oxygen-induced retinopathy mouse, which has deficits in both ON- and OFF-bipolar cell activity and a *Rs1* knockout mouse that models juvenile retinoschisis and also has deficits in both ON- and OFF-pathways. The *Nyx*[nob] mouse had an attenuated response in the rod and ON-pathway range, but its response to temporal frequencies of greater than 15 Hz were similar to control mice. Both the oxygen-induced retinopathy and *Rs1* knockout mice, however, had attenuated responses across all ranges.[64] These results further suggest that in experimental mouse models, we may be able to use flicker stimuli at varying temporal frequencies to measure changes to the ON- and OFF-pathways.

Additional ERG studies have also shown evidence of differences between the ON- and OFF-pathways. Although Harazny et al.[65] were not focused on distinguishing between ON- versus OFF-pathways in a longitudinal experiment with the DBA/2J mouse, this group showed that, at 2 to 3 months of age, these mice had a decrease in the ERG response to flicker stimuli. Interestingly, these mice had a diminished response before an increase in IOP, which was found to be elevated at about 6 months of age. Axonal death did not present until about 10 months in age. In this study, the mouse retinal response to flicker stimuli that was modulated between 12 and 30 Hz was decreased, and the authors noted that there was an even greater decrease in the amplitude at the higher frequencies.[65] This finding suggests that the OFF-pathway may be more affected in experimental glaucoma and motivates the desire to translate flicker stimuli to measure human responses to detect glaucoma.

## Clinical Use of ERG in Glaucoma

Currently, there has been an increasing emphasis on using ERG to measure retinal cell health and to objectively assess visual function. There is also a growing

role for using ERG in glaucoma assessment, and for a more in-depth discussion of the state of clinical uses of ERGs in glaucoma, we recommend a recent review by Wilsey and Fortune.[54] However, there are still a variety of barriers, such as the length of examination and the lack of equipment, that prevent ERG from being implemented across clinical practices.[14] Here, we briefly describe the evaluation of ON- and OFF-pathways in glaucoma patients as an area of potential research to refine ERG testing that may complement standard automated perimetry.

PERG, which uses spatiotemporal modulation of the light stimulus to isolate RGC responses, has been shown to detect glaucoma 4 years before actual visual changes occurred,[66] and it may be a good predictor of RNFL thinning in glaucoma suspects.[67] In human studies, the N95 peak also demonstrated a greater reduction in human subjects using transient PERG compared with the P50 peak with a diminished N95/P50 ratio.[68,69] Although experimental nonhuman primate glaucoma models suggested that the ON- and OFF-pathways both contribute to the P50 and N95 peaks, elimination of the OFF-bipolar response with the addition of PDA completely eliminated the N95 peak, whereas the P50 peak increased, suggesting that the N95 waveform has a greater OFF-pathway contribution.[56] This decrease in the N95/P50 ratio, therefore, lends further supports that in human glaucoma, there may be more prominent OFF-pathway vulnerability, and PERG may be one paradigm to assess the change in humans. Furthermore, recent work demonstrated that preperimetric and perimetric glaucoma subjects had a decrease in the N95 peak amplitude, whereas only perimetric glaucoma subjects had a decrease in the P50 peak amplitude.[70,71] This finding further supports early OFF-pathway vulnerability in glaucoma.

There is also growing evidence that PhNR may be used to detect early changes in preperimetric glaucoma.[70,72–74] In several clinical trials, there was also a significant decrement of the PhNR amplitude in glaucomatous eyes, while a- and b-wave amplitudes were unchanged.[75,76] Horn et al.[76] also used a long-duration full-field photopic ERG on human subjects and found that PhNR after both the onset and offset of the light stimulus were decreased in glaucoma eyes. Moreover, their results exhibited a greater change in the offset PhNR than the onset PhNR. Thus, this study showed that both the ON- and OFF-pathways may be implicated in human glaucoma, with greater OFF-pathway vulnerability.

A clinical study involving a sawtooth flicker stimulus also demonstrated OFF-pathway susceptibility in glaucoma patients. The sawtooth stimulus was modulated in two ways: one form was rapid-on as the

light gradually decreased in intensity before an abrupt increase in light intensity (Fig. 2D). The other version was rapid-off, where the light gradually increased in intensity before an abrupt decrease in light intensity (Fig. 2E). These protocols were shown to correlate with the ON- and OFF-pathways, respectively.[77] After the abrupt change in light intensity, the rapid-on stimulus generates a waveform with an initial negative peak followed by a large positive waveform. The rapid-off stimulus generates only an initial positive response. After the positive waveform from both stimuli, there are the late positive and late negative (LN) peaks. These authors suspected that the late positive waveform is likely an i-wave homologue, and the LN waveform is likely a PhNR homologue. The late positive and LN peaks can further be described as ON or OFF to define whether it came from the rapid-on or rapid-off stimulus. The researchers found greater changes to the rapid-off waveform versus the rapid-on waveform for perimetric subjects compared with controls, specifically the LN peak was significantly reduced with the rapid-off stimulus.[78] Gowrisankaran et al.[79] similarly showed that subjects with optic atrophy had a decreased LN response with the rapid-off stimulus, but not with the rapid-on stimulus. As a result, these studies suggest that the OFF-pathway is indeed more susceptible, although there may be changes to the ON-pathway in human glaucoma as well. These studies also demonstrate the usefulness of the sawtooth flicker stimulus as a method to elicit differences in the ON- and OFF- pathways, which could be leveraged for early glaucoma detection. Future studies could incorporate the rapid-on and rapid-off sawtooth stimuli as a way to test for early glaucoma. For instance, Norcia et al. used steady-state visual evoked potentials and found that a decrement sawtooth stimulus was better at distinguishing visual responses in glaucoma subjects compared with an increment sawtooth stimulus (Norcia AM et al. IOVS 2019; 60:ARVO E-Abstract 2488). Integrating a sawtooth stimulus in the diagnostic pipeline could thereby provide a better paradigm to isolate the ON- and OFF-pathways to perhaps identify early changes in glaucoma.

Flicker stimuli are another ERG paradigm of growing interest to separate the ON and OFF responses. Modulating the flicker stimulus frequency has been done for subjects with CSNB1 and XLRS,[61,80] but there is still limited research with human glaucoma. Nevertheless, the results from experimental animal models have suggested that higher stimulus frequencies are more associated with the OFF-pathway, which may provide a novel way to detect glaucoma. In a recent study using a sinusoidal stimulus ranging from 0.3 to 50.0 Hz with a handheld ERG device, we found

that for stimulus frequencies between 15 and 50 Hz, glaucoma eyes had significantly reduced ERG amplitudes compared with control eyes (Kong A et al. IOVS 2020; 61:ARVO E-Abstract 4043). Although a large frequency range was shown to be decreased in glaucoma subjects, this study showed that the higher temporal frequencies were predominantly affected, which is consistent with greater OFF-pathway vulnerability. Thus, further developing a modality to more specifically isolate the ON- and OFF-pathways, such as varying the temporal frequency of a flicker stimulus, could be used to better characterize pathway vulnerability in human glaucoma.

## Future Research

Because there is growing evidence that OFF RGCs are more susceptible in experimental mouse models, future experiments should continue to explore the mechanisms underlying type-specific RGC susceptibility, identifying both cell autonomous and non-cell-autonomous mechanisms that may help to explain disease pathogenesis in humans. Although the initiating event in glaucomatous axonopathy is generally IOP-induced strain on the optic nerve head, we hypothesize that RGC type-specific susceptibility may be a function of several different factors such as RGC activity level, direct sensitivity to pressure changes from transient receptor potential channels, and interactions with other cell types and structures, including neurons, glia, and vasculature. Furthermore, one outstanding question is whether the vulnerable RGC types found in mouse have direct counterparts in human, which has fewer RGC types.[81] Consequently, large-scale surveys of specific RGC type vulnerability versus resiliency in human glaucoma are still needed.

The use of ERG has shown promising results as a measure of visual function in glaucoma, and additional studies with clinical ERG can be used to focus on these ON- and OFF-pathway differences. Further longitudinal studies using paradigms such as PERG, PhNR, long-duration photopic ON–OFF stimulus, and sawtooth stimulus could be used to follow the progression of glaucoma and to determine the relationship of ON- and OFF-pathway changes in humans. One limitation of the findings in the Horn et al.[76] and Pangeni et al.[78] studies was that they did not find significant differences between control subjects and either ocular hypertensive subjects or preperimetric subjects, respectively. Although these studies included small sample sizes, the sensitivity for early glaucomatous changes may be improved with the use of ERG paradigms that include a flicker stimulus such as sine or square wave stimuli to measure the fundamental harmonic response. The ability for mfERG to isolate specific parts of the retina is an additional area of interest. For patients with only a hemifield defect, mfERG may contribute interesting data on how retinal pathways may be perturbed within an individual subject. Importantly, by employing ERG to quantify changes to the ON- and OFF-pathways, these studies may be able to identify a more objective measure of visual function in glaucoma.

## Conclusion

The research based on rodent experimental glaucoma models has shown that OFF RGCs may be more susceptible to injury. Indeed, these RGCs showed decreases in dendritic area, dendritic complexity, and density of excitatory synapses. Although there are still conflicting reports on whether RGC susceptibility is driven by ON versus OFF identity, glaucomatous experimental mouse models also exhibited a decrease in ERG response to a flicker stimulus, in particular at a higher frequency, which is more associated with the OFF-pathway in both rodents and humans. There has also been clinical evidence to support the notion that the OFF-pathway may be more affected in human glaucoma. The ERG is increasingly seen as a possible tool to improve glaucoma diagnostics. Currently, there are promising modalities such as PERG and PhNR that may detect early glaucomatous changes, and these studies do lend support that the OFF-pathway is more vulnerable in humans. Moving forward, developing a more specific measure of ON- and OFF-pathway function, such as with a flicker frequency stimulus or using a sawtooth stimulus, may ultimately provide an objective test to measure the OFF-pathway and to allow for earlier diagnosis of glaucoma even before any visual field changes occur.

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