The Photopic Negative Response of the Mouse Electroretinogram: Reduction by Acute Elevation of Intraocular Pressure

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PURPOSE. To determine the presence and magnitude of the photopic negative response (PhNR) component of the electroretinogram (ERG) in the mouse eye and to test if it is altered by short-term elevation of intraocular pressure (IOP).

METHODS. Photopic and scotopic ERGs were recorded from 12-month-old C57BL/6J mice and analyzed for photoreceptor responses (a-wave), bipolar cell responses (b-wave), scotopic threshold responses (STRs), and PhNRs. Electroretinogram signals were measured before and after short-term subischemic elevation of IOP (50 mm Hg for 30 minutes) induced by cannulation of the anterior chamber. Retinas were subsequently assessed for signs of retinal stress and cell survival using immunohistochemistry and quantitative PCR.

RESULTS. The corneal negative PhNR of the photopic ERG was elicited in the mouse eye, and its amplitudes correlated with amplitudes of the positive STR (pSTR). Elevation of IOP significantly reduced amplitudes of both the PhNR and pSTR, while scotopic a-waves, scotopic b-waves, and photopic b-waves were unchanged. Pressure elevation was associated with upregulation of glial fibrillary acidic protein and heme oxygenase 1 expression in retinal macroglia in the absence of retinal cell death.

CONCLUSIONS. The PhNR component of the full-field ERG can be recorded in mice and is sensitive to elevation of IOP. Correlation between PhNR and pSTR signals before and after IOP elevation suggests that the PhNR depends on inner retinal integrity and provides a means for evaluating inner retinal function in mouse models.

Keywords: electroretinogram, ganglion cell, intraocular pressure

Sensitive and specific measures of optic nerve function are vital for detecting and monitoring optic neuropathies and other diseases characterized by inner retinal dysfunction. The full-field flash electroretinogram (ERG) is an established noninvasive tool that records summed electrical activities of retinal neurons and can be measured serially in subjects to evaluate longitudinal changes. While ERG responses derived from photoreceptors and bipolar cells are well characterized, the smaller contributions from innermost cells of the retina, namely, retinal ganglion cells (RGCs), are less well defined.

The photopic negative response (PhNR) is a corneal negative potential after the b-wave of the photopic ERG that was first identified in 1999 by Viswanathan et al.1 There is good evidence that the PhNR signal originates directly from the spiking activity of RGCs or through mediation by amacrine cells or glia. Studies in rodents and primates have shown reduced PhNR amplitudes in response to optic nerve transection,2 pharmacological blockade of inner retinal signaling,3,4 and laser-induced ocular hypertension (OHT)5 in correlation with RGC loss.6 There is also evidence that the PhNR can be used to detect inner retinal dysfunction in humans with ocular pathologies of varying etiology. Reduced PhNR amplitudes were seen in patients with open-angle glaucoma,7,8 optic nerve atrophy,8 anterior ischemic optic neuropathy,9 autosomal dominant optic atrophy,9 and diabetic retinopathy.6 The magnitude of PhNR signals in patients with inner retinal pathology significantly correlated with clinical parameters and nerve fiber layer thickness.8,5 Because the PhNR is reduced when visual sensitivity losses are still mild, it holds promise as a tool for early detection of disease.6 Most recently, we showed that IOP lowering improves PhNR amplitudes in a cohort of glaucoma and OHT patients, suggesting that the PhNR can act as a reversible measure of inner retinal function.10

Despite growing interest in the PhNR and its clinical applications, the PhNR signal has not yet been described in the mouse to our knowledge. Due to well-defined genetics, amenability to genetic manipulation, high fecundity, and low housing costs, the mouse is one of the most commonly used and important animal models in disease research. Hence, the aim of the present study was to characterize the PhNR of the mouse eye in order to establish a common measure of inner retinal health in mouse and human studies. To address this aim, we determined the presence of the PhNR in the normal mouse retina and tested its sensitivity to an inner retinal–specific injury in the form of acute elevation of intraocular pressure (IOP). We examined how pressure-induced changes to the PhNR compare with changes to other ERG responses originating from inner retina, as well as those originating from middle and outer retina. Furthermore, we correlated functional changes after pressure elevation with retinal cell survival and cellular stress responses. Our findings suggest that the PhNR...
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can act as a single measurable end point of inner retinal health in both mouse and human studies.

MATERIALS AND METHODS

Animals

All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the requirements of our institutional Animal Research and Ethics Committee. C57BL/6J mice were born and reared in a temperature-controlled (22 ± 1°C) and light-controlled (12 hours light and 12 hours dark) environment, where food and water were available ad libitum. Experiments were performed on mice at 12 months of age. Male and female mice were used equally throughout the study.

Electroretinography

Animal Preparation. The full-field flash ERG was recorded using an Espion Diagnosys system (Diagnosys LLC, Littleton, MA). After overnight dark adaptation (minimum, 12 hours), mice (n = 25) were prepared for recording under dim red illumination. Animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Pupil dilation and corneal anesthesia were achieved by topical application of 1 drop each of tropicamide (0.5%), phenylephrine (2.5%), and proparacaine hydrochloride (0.5%). Body temperature was maintained at 37°C with a homeothermic controller and unit. Electrical signals were recorded with a 4-mm platinum wire loop electrode contacting the cornea, while a gold pellet placed in the mouth served as common reference. A subdural needle electrode inserted at the base of the tail acted as ground. Retinal responses were recorded simultaneously from both eyes over a period of 30 minutes. Electroretinograms were recorded serially in animals 1 day before (baseline) and 7 days after elevation of IOP. Values obtained at baseline were used for initial characterization of the PhNR. Responses recorded after IOP elevation were normalized to baseline values to determine pressure-induced changes.

Recording Protocols. Light stimuli were delivered via a Ganzfeld unit from white light-emitting diodes. Light energies were calibrated as luminance energy units in candela seconds per meter squared (cd/s/m²). Scotopic threshold responses (STRs) were elicited using a -5.45 log cd/s/m² stimulus. Twenty-five flashes with an interstimulus interval of 1500 ms were averaged. Scotopic a-waves and b-waves were elicited with a stimulus intensity of 2.22 log cd/s/m², with no averaging required. Based on previous literature, photopic responses to six different stimulus strengths between 0.34 and 2.22 log cd/s/m² were presented on a 40.0 cd/s/m² rod-saturating green background.2 At each intensity, 25 flashes with an interstimulus interval of 3000 ms were averaged.

Waveform Analysis. Response amplitudes of the positive STR (pSTR), a-wave, and b-wave were quantified as previously described.11 Amplitudes of the PhNR and photopic b-wave were initially measured in two ways: (1) by identifying the maximum peak and trough and obtaining the baseline trough and peak amplitude and (2) by taking the amplitude at a fixed criterion time after the stimulus onset, again with respect to baseline. Criterion times were chosen to correspond with the mean implicit time of all data from our study. Outcomes using both forms of amplitude analysis were comparable; for data presentation, we chose to use amplitudes of the maximum peak and trough.

Elevation of IOP

Intraocular pressure was elevated in mouse eyes according to published protocols.12 Briefly, animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) before resting IOP was measured using a handheld rebound tonometer (Icare Lab Tonometer; Colonial Medical Supply, Orlando, FL). The anterior chamber of the eye was cannulated with a 50-µm borosilicate needle connected in series with a pressure transducer and fluid reservoir filled with sterile-filtered endotoxin-tested saline. Intraocular pressure was raised by altering the height of the reservoir and was monitored in real time using a PowerLab (ADInstruments, Dunedin, New Zealand) data acquisition system. In each animal, pressure was elevated to 50 mm Hg for 30 minutes in one eye, while the fellow eye was cannulated and held at physiological IOP (12 mm Hg). The contralateral sham-treated eye acted as an internal control throughout the study to mitigate any variation induced by anesthesia, temperature, and other factors. The level of pressure used in our injury model is below the systolic blood pressure of mice and does not induce retinal ischemia.12

Immunohistochemistry

Mice were euthanized 1 day (n = 10) and 7 days (n = 10) after IOP elevation. Eyes were enucleated and immersion fixed in 4% paraformaldehyde for 3 hours, followed by overnight cryoprotection in 15% sucrose. Eyes were embedded in optical cutting temperature medium, and 12-µm sections were cut through the papillary–optic nerve axis. Cryosections were immunolabeled for glial fibrillary acidic protein (GFAP) (1:800; Dako, Campbellfield, Victoria, Australia), heme oxygenase 1 (HO-1) (1:400; Enzo Life Sciences, Ann Arbor, MI), and Brn-3a and β-actin. Sections were nuclear counterstained with Hoechst (1:10,000) before being coverslipped with a glycerol and gelatin medium.

Quantitative PCR

RNA was extracted from retina and optic nerve stump, and cDNA was subsequently synthesized using commercial kits (Applied Biosystems, Mulgrave, Victoria, Australia). Quantitative real-time PCR was performed using a TaqMan-based assay (Applied Biosystems) and a RotorGene Q Real-Time Cycler (Qiagen, Venlo, The Netherlands). The following probes specific for mouse mRNAs were used: Thy1 (NM_009382.3) and β-actin (NM_031144.2). Relative quantitation of gene expression was calculated using the comparative threshold cycle method,14 with values normalized to the endogenous reference gene β-actin. Results for pressure-elevated eyes are expressed relative to sham-treated contralateral eyes.

TUNEL Assay

Retinal cryosections were labeled using the TUNEL assay according to published protocols.15 Labeled sections were scanned from superior to inferior edge in 1-mm increments, and the numbers of TUNEL-positive nuclei in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) were recorded. The frequency of TUNEL-positive profiles per millimeter of retina was averaged from at least
two sections per animal, and six animals were analyzed at each time point.

**Quantification of Retinal Thickness and RGCs**

Thicknesses of the ONL, INL, and retina (inner to outer limiting membrane) were measured on digital images of Hoechst-stained cryosections as described.\(^{16}\) The ratio of ONL or INL thickness to the entire retinal thickness was used for analysis to account for obliquely cut sections. Retinal ganglion cell number was determined on retinal cryosections immunolabeled with Brn-3a, which is a reliable marker of RGCs, with previous reports showing greater than 90% correlation between the number of Brn-3a–positive cells in the GCL and the number of RGCs visualized by retrograde labeling with Fluorogold.\(^{17}\) Sections cut through the optic nerve head and ora serrate were scanned from superior to inferior edge, and Brn-3a–positive cells were counted by a masked observer (VC). Data are expressed as the number of cells per 1-mm retinal length. Measurements were averaged from at least two sections per animal, and six animals were analyzed at each time point.

**Statistical Analysis**

Pearson’s correlation and linear correlation analysis was used to correlate amplitudes of the PhNR with amplitudes of other ERG components. Additional data were analyzed using an unpaired two-tailed Student’s t-test assuming unequal variance. In all analyses, \(P < 0.05\) was considered significant.

**RESULTS**

**Characterization of the PhNR Waveform in the C57BL/6j Mouse Retina**

For initial identification of the PhNR waveform, we recorded responses of the mouse eye to a range of stimulus intensities from 0.34 to 2.22 log cd s/m\(^2\) presented on a green background. These conditions were previously used to elicit PhNRs from the rat retina.\(^2\) As shown in Figure 1A, a shallow negative potential immediately following the photopic b-wave was consistently observed at stimulus from 0.34 log cd s/m\(^2\). This negative potential was present in all eyes tested (\(n = 25\)) and is hereafter referred to as the PhNR. The PhNR increased in amplitude over a small range of stimulus intensities before saturating at 0.92 log cd s/m\(^2\) (Fig. 1B). At stimuli above 1.0 log cd s/m\(^2\), positive components dominated the response, with b-wave amplitudes and latencies continuing to increase with increasing intensities to 2.22 log cd s/m\(^2\) (Figs. 1C, 1E). The implicit time of the PhNR decreased minimally as stimulus intensity increased, from 151 ms at 0.34 log cd s/m\(^2\) to 147 ms at saturation (Fig. 1D). The implicit time of the saturated PhNR, as averaged across all animals, was 145 ms.

Absolute amplitudes of photopic b-waves recorded from mouse to mouse were highly consistent, whereas those of the PhNR varied. For this reason, baseline recordings were measured on two occasions in a subgroup of mice (\(n = 10\)) at least 2 days apart. This showed that absolute amplitudes of both b-waves and PhNRs were highly consistent on repeated measures within a single mouse (data not shown). Furthermore, b-wave and PhNR responses from two eyes of a given

**FIGURE 1.** Electretinogram responses of the C57BL/6j mouse retina to a series of increasing flash intensities presented on a rod-saturating (40.0 log cd s/m\(^2\)) green background. (A) Representative traces recorded from a single animal demonstrating the PhNR and photopic b-wave components (arrows). (B–E) Mean amplitude and latency of PhNRs and b-waves with increasing flash intensity. Data in (B) are normalized to values obtained at the lowest flash intensity (0.34 log cd s/m\(^2\)) to account for intermouse variability in absolute PhNR amplitudes. Error bars denote SEM (\(n = 25\)).
Amplitudes of the PhNR Are Correlated With Amplitudes of the Scotopic pSTR

Immediately prior to recording photopic responses, a full series of scotopic ERGs (flash stimuli ranging from −5.92 to 2.22 log cd·s/m²) was taken from each mouse eye. This allowed us to compare amplitudes of the PhNR with three other well-established ERG signals originating from inner, middle, and outer retina. Amplitudes of the pSTR, which is derived predominantly from RGCs in the mouse retina, were measured after a flash stimulus of −4.54 log cd·s/m², while amplitudes of waveforms derived from photoreceptors (a-wave) and ON-bipolar cells (b-wave) were measured in response to a stimulus of 2.22 log cd·s/m². These stimuli were of sufficient intensity to elicit saturated responses.

Figure 2 shows PhNR amplitudes plotted against amplitudes of the pSTR, a-wave, and b-wave recorded from a given eye of 25 mice. These plots show a significant positive correlation between PhNR amplitudes and a-wave and b-wave amplitudes recorded within a single ERG session, measured in response to flash stimuli of 0.92 log cd·s/m² (PhNR), −4.54 log cd·s/m² (pSTR), and 2.22 log cd·s/m² (a-wave and b-wave), with a background illumination of 40.0 log cd·s/m² for the PhNR.

Reduction of the PhNR by Elevation of IOP

We induced an inner retinal-specific injury to the mouse eye by short-term elevation of IOP. Our group and others have previously shown that a single IOP spike leads to oxidative stress, macrophage activation, and reproducible loss of inner retinal function, with minimal impact on outer retinal function in the absence of significant retinal ischemia.

In the present study, a full series of scotopic and photopic ERG responses was recorded from individual mice 1 day before (baseline) and 7 days after elevation of IOP. Scototretinogram amplitudes are presented as relative changes from baseline for each mouse and are normalized to sham-treated contralateral eyes to account for any potential confounding effects of cannulation or anesthesia. Resting eye pressures immediately prior to elevation of IOP, which were measured in anesthetized mice using a handheld rebound tonometer, were comparable across all animals (data not shown).

Confirming previous reports, elevation of IOP to 50 mm Hg for 30 minutes led to a significant reduction of pSTR amplitudes at 7 days (64% ± 10% of baseline values), while amplitudes of the scotopic b-wave and a-wave were unaffected (Fig. 3). These observations verify the specificity of this form of injury to inner retina. Herein, we add new observations that pressure elevation had no appreciable effect on the photopic b-wave but significantly reduced amplitudes of the PhNR to 71% ± 11% of baseline values. Amplitudes of the PhNR at saturation were assessed both by measuring at a fixed time after stimulus onset (145 ms) and by identifying the maximum trough. According to both analyses, PhNR amplitudes were reduced to a similar degree in response to IOP elevation.

Activation of Stress Responses in Retinal Macrogia and Neurons Following IOP Elevation

To investigate retinal responses to IOP elevation that accompanied in vivo functional changes detected by ERG, we examined protein and gene expression levels of markers of retinal injury. HO-1 and GFAP are established indicators of retinal stress that are upregulated in retinal macrogia in response to a variety of genetic and environmental insults. In sham-treated eyes, we observed very little HO-1 expression on immunolabeled retinal cross sections. However, in eyes that had been subjected to IOP elevation, HO-1 expression was prominent in radially oriented Müller cell processes, with peak expression detected at 1 day (Fig. 4A). Pressure elevation also led to marked upregulation of retinal GFAP immunoreactivity and redistribution from astrocyte cell bodies to Müller cell processes (Fig. 4A). Upregulation of GFAP expression persisted up to 7 days after elevation of IOP.

We also assessed gene expression levels of the RGC-specific protein Thy-1. Downregulation of Thy-1 serves as a marker of RGC stress (but not loss) in models of retinal damage. Expression of Thy-1 was significantly reduced 7 days after IOP elevation compared with sham-treated control eyes (Fig. 4B).
DISCUSSION

The present study demonstrates that the PhNR component of the ERG can be recorded in the mouse eye. We show that amplitudes of the PhNR correlate closely with amplitudes of the pSTR in normal eyes and that both of these ERG signals are reduced significantly in response to an inner retinal–specific insult caused by acute elevation of IOP. Furthermore, we show that loss of PhNR and pSTR amplitudes after pressure elevation is associated with activation of stress responses in retinal glia and RGCs in the absence of significant cell death.

Origin and Generation of the PhNR Signal

Our finding of a correlation between PhNR and pSTR amplitudes at baseline and the selective reduction of both signals after IOP elevation suggests that the PhNR and pSTR share a common origin that is localized to the inner retina. The best evidence to date implicates RGCs, amacrine cells, or surrounding glia as the cellular source of both the PhNR and pSTR. In studies of monkey1–3 and rat,2 the PhNR signal was selectively reduced or eliminated after administration of tetrodotoxin to vitreous. Tetrodotoxin blocks voltage-gated sodium channels, which are localized to RGC somas, RGC axons, and amacrine cells of the inner retina. Evidence that the PhNR is mediated by glial signaling comes from the demonstration that intravitreal injection of barium, which blocks inward and outward potassium currents in glia, suppressed PhNR signals in the cat.23 Similar to the PhNR, amplitudes of the pSTR were reduced or eliminated after pharmacological blockage of inner retinal signaling,18,19,24 after optic nerve transection,18 and in animal models of experimental glaucoma.11,25 While photopic and scotopic pathways converge on the same RGCs, unique contributions from other inner retinal neurons or glia are likely to account for differences between pSTR and PhNR ERG signals. In our present study of the mouse eye, the time course of the PhNR signal at baseline was slower than that of the pSTR. As glia responses are slower than those of spiking RGCs,1 this may reflect a relative greater glial cell contribution to the PhNR compared with the pSTR.

Cause of PhNR Reduction After IOP Elevation

In this study, amplitudes of the PhNR were significantly reduced in response to acute elevation of IOP, while scotopic and photopic ERG signals from outer and middle retina were unaffected. These results indicate a predominant or selective effect of elevated IOP on the inner retina, confirming previous reports.11,25 Our present results also agree with our recent clinical finding of improved PhNR amplitudes in a cohort of glaucoma and OHT patients in association with IOP lowering.10 This suggests that the PhNR signal has a degree of IOP-dependence in both mice and humans.

The loss of PhNR amplitude described herein cannot be attributed solely to elevated IOP as the reduction persisted 7 days after a single pressure spike. Reduced PhNR amplitudes have been reported in open-angle glaucoma patients but not in OHT patients,4 further indicating that non–IOP-dependent processes are at play. Decreased PhNR signals are seen in...
patients with open-angle glaucoma, anterior ischemic optic neuropathy, and autosomal dominant optic atrophy, as well as compressive optic nerve injury and after optic nerve atrophy induced by compression, trauma, or inflammation, suggesting that the PhNR is sensitive to optic nerve damage of varying etiology. The cause of reduced PhNR amplitudes may therefore be general stress in the inner retina and optic nerve, with subsequent disruption to signaling, synaptic processing, and axonal transport. We are now investigating these mechanisms.

The reduction in PhNR amplitudes reported in this study (to 71% of baseline values) was less than that seen in other animal models of optic nerve injury such as laser-induced OHT and optic nerve transection. In these alternative models, RGC damage is substantial and cell death high, while our model induces a milder injury, with no significant RGC death and no retinal ischemia. We anticipate that elevation of IOP to higher levels or for a longer duration would result in greater reduction of the PhNR signal in the mouse eye. Indeed, previous studies indicate that PhNR amplitudes decline in correlation with RGC loss and thinning of the nerve fiber layer.

### Relation Between PhNR Decline and Retinal Cell Responses

In association with reduced PhNR amplitudes, we saw activation of stress responses in retinal glia and RGCs after pressure elevation. If the mouse PhNR signal is mediated by potassium currents in glia, as has been suggested in other species, changes to the number and activation state of these cells after IOP elevation may be contributing to the observed reduction in PhNR amplitudes. In both human and animal studies, the PhNR is sensitive to optic nerve stress before clinical signs are seen and before thinning of the retinal nerve fiber layer. This is in agreement with our present finding of reduced PhNR amplitudes in the absence of RGC death and supports use of the PhNR for early detection of optic nerve damage and stress in disease or injury states.

### Application of the PhNR

The PhNR has several advantages over other ERG signals that are now used to assess inner retinal function such as the pSTR and pattern ERG (PERG). The PhNR requires shorter dark adaptation than the pSTR and is faster and easier to acquire. The pSTR of the mouse is particularly sensitive to white light; as a consequence, preparation of animals for recording must be done under very dim red light or in complete darkness using night vision equipment. Furthermore, the pSTR is poorly characterized in humans. This is in contrast to the PhNR signal, which is commonly recorded in humans and whose modulation in response to a range of retinal diseases and injuries has been reported.

The PERG is recorded under photopic conditions and measures retinal responses to contrast changes rather than luminance changes. The PERG has been described in the mouse retina, is thought to depend on functional integrity of RGCs, and is now used for glaucoma detection in the clinic. However, disadvantages of the PERG are its relatively small signals, a high signal-to-noise ratio, and the critical requirement of full refractive correction for test distance. A further advantage of the PhNR over the PERG is that a-waves and b-waves can be recorded in parallel with RGC signals, enabling the function of middle and outer retinal layers to be evaluated simultaneously.

Given its advantages, the PhNR component of the ERG stands as a useful tool for characterizing and monitoring inner retinal activity under normal conditions and in response to injury, disease, or neuroprotective treatments. Specifically, it holds promise as a common measure of inner retinal health in both mouse models and human studies.

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