Light-Evoked Responses of the Retinal Pigment Epithelium: Changes Accompanying Photoreceptor Loss in the Mouse

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Submitted 20 January 2010; accepted in final form 16 May 2010

Samuels IS, Sturgill GM, Grossman GH, Rayborn ME, Hollyfield JG, Peachey NS. Light-evoked responses of the retinal pigment epithelium: changes accompanying photoreceptor loss in the mouse. J Neurophysiol 104: 391–402, 2010. First published May 19, 2010; doi:10.1152/jn.00088.2010. Mutations in genes expressed in the retinal pigment epithelium (RPE) underlie a number of human inherited retinal disorders that manifest with photoreceptor degeneration. Because light-evoked responses of the RPE are generated secondary to rod photoreceptor activity, RPE response reductions observed in human patients or animal models may simply reflect decreased photoreceptor input. The purpose of this study was to define how the electrophysiological characteristics of the RPE change when the complement of rod photoreceptors is decreased. To measure RPE function, we used an electroretinogram (dc-ERG)-based technique. We studied a slowly progressive mouse model of photoreceptor degeneration (PrphRd2/H11001), which was crossed onto a Nycet® background to eliminate the b-wave and most other postreceptoral ERG components. On this background, PrphRd2/H11001 mice display characteristic reductions in a-wave amplitude, which parallel those in slow PIII and the loss of rod photoreceptors. At 2 and 4 mo of age, the amplitude of each dc-ERG component (c-wave, fast oscillation, light peak, and off response) was larger in the amplitude of each dc-ERG component (c-wave, fast oscillation, light peak, and off response) was larger in

INTRODUCTION

The retinal pigment epithelium (RPE) is in close physical proximity with the neural retina and supports its normal functions. Rod photoreceptor outer segments (OSs) interdigitate with the apical processes of the RPE where nutrient transport between the cells, phagocytosis of shed outer segments, regeneration of rhodopsin, and removal of metabolic end products by the RPE facilitate the health and function of the photoreceptors (Bok 1993; Marmorstein 2001; Strauss 2005). Mutations in RPE genes underlie some forms of retinitis pigmentosa (Maw et al. 1997), Leber’s congenital amaurosis (Marlheens et al. 1997), Malattia Leventinese/Doyne Honeycomb retinal dystrophy (Marmorstein et al. 2002; Stone et al. 1999), and Sorsby’s fundus dystrophy (Weber et al. 1994). These diseases are collectively characterized by photoreceptor degeneration, despite the restricted expression of the mutated genes to the RPE. This scenario, in which RPE gene defects initiate structural/functional disruption of photoreceptors, underscores the importance of understanding the relationship between RPE abnormalities and photoreceptor degeneration.

RPE function can be noninvasively measured in mice using a modified electroretinogram technique (dc-ERG). The light-evoked responses of the RPE are characterized by four relatively slow components identified as the c-wave, fast oscillation (FO), light peak (LP), and off response (OR). These components allow different aspects of RPE function to be monitored (Steinberg et al. 1985) and provide a means for the identification of RPE defects in mouse models of human photoreceptor degeneration (Strauss 2005). Despite being evoked by light stimuli, none of the dc-ERG components reflects a direct response of the RPE to light. Instead, each is generated secondary to rod photoreceptor activity (Steinberg 1985; Wu et al. 2004b). The initial c-wave reflects the interaction of two signals. A positive polarity component reflects the light-evoked hyperpolarization of the apical membrane of the RPE, generated in response to the decrease in subretinal [K+] induced by rod photoreceptor activity (Schmidt and Steinberg 1971; Steinberg et al. 1970). A negative polarity component (slow PIII) reflects Kir4.1 channel activity in Müller glial cells (Kofuji et al. 2000; Oakley and Green 1976; Steinberg and Miller 1973; Witkovsky et al. 1975). These components combine to define the c-wave recorded at the corneal surface (Wu et al. 2004a).

The negative polarity FO follows the c-wave and reflects the recovery of the c-wave and slow PIII as [K+] is restored in the subretinal space (SRS) and a delayed hyperpolarization of the basal RPE membrane from a [Cl−] conductance (Griff and Steinberg 1984; Linsenmeier and Steinberg 1982). The identity of the Cl− channel is not yet known, although the retention of the FO in mice lacking different Cl− channels (Cftr, Best1, Clcn2) argue against their playing a major role in FO generation (Edwards et al. 2010; Marmorstein et al. 2006; Wu et al. 2006).

The slow forming LP follows the FO and reflects the depolarization of the RPE basal membrane by a Cl−–based conductance (Fuji et al. 1992; Gallemore and Steinberg 1989, 1993; Linsenmeier and Steinberg 1982). Although it is possible to evoke a c-wave and FO from an isolated RPE preparation by reducing apical [K+]2, the LP is not generated (Gallemore et al. 1988; Steinberg et al. 1985). This observation led to the concept of a “light peak substance,” a ligand that is required for LP generation and is released by the neural retina in response
to light that binds to a receptor located on the RPE. The identity of the light peak substance is not known, although a number of candidates have been examined (Dawis and Niemeyer 1986; Gallemore and Steinberg 1990; Joseph and Miller 1992; Nao-i et al. 1989; Quinn et al. 2001; Wu et al. 2004). Altered LPs in rodents treated with the voltage-dependent calcium channel (VDCC) blocker nifedipine or in mice lacking VDCC subunits (α1D, β1) implicate a role for calcium-sensitive Cl− channels in LP generation/regulation (Marmorstein et al. 2006; Wu et al. 2007). LPs are reduced in mice expressing a single Clcn2 allele (Edwards et al. 2010).

The OR is generated when light stimulii are extinguished. Unlike all other ERG components, the polarity of the mouse OR depends on stimulus intensity, indicating that this is a complex response with more than one underlying generator.

Because each dc-ERG component is generated secondarily to rod photoreceptor activation (Wu et al. 2004b), interpretation of dc-ERG components is most straightforward when the activity of rod photoreceptors (reflected in the ERG a-wave) is maintained at a normal level (Marmorstein et al. 2006; Wu et al. 2006, 2007). As noted earlier, however, many mouse models of RPE gene defects are associated with rod photoreceptor degeneration, which will alter the rod photoreceptor-derived signal delivered to the RPE and thus the generation of each dc-ERG component. To understand the relationship between a decrease in rod photoreceptor activity and the response properties of the dc-ERG, we used a well-studied model of photoreceptor degeneration, PrphRd2+/− mice (Cheng et al. 1997; Hawkins et al. 1985; Sanyal and Hawkins 1989). Originally named rds (retinal degeneration slow), PrphRd2+/− mice express a single wild-type peripherin/rds allele and show altered OS elaboration, resulting in short disorganized OSs with irregular membrane whorls (Hawkins et al. 1985). As these mice age, the outer retina exhibits a progressive loss of photoreceptors accompanied by thinning of the outer nuclear layer (ONL). Functionally, PrphRd2+/− mice display progressive reductions in a-wave amplitude, which coincide with photoreceptor degeneration (Cheng et al. 1997). We recorded light-evoked activity from the retina and RPE from these mice and control littermates over time to define the relationship between RPE function and loss of photoreceptor activity. We found that the dc-ERG components were maintained at larger amplitudes than predicted by measures of rod photoreceptor activity or structure. The data presented here indicate that the RPE can undergo extensive loss of input as well as damage to the RPE cells themselves without displaying a significant decrease in light-evoked activity, as demonstrated by the amplitude of dc-ERG components. Moreover, this study demonstrates that specific aspects of RPE function can be meaningfully evaluated despite a profound reduction in photoreceptor activity.

**METHODS**

**Mice**

PrphRd2+ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and mated with Nxy+/+ mice, which lack the ERG b-wave component (Pardue et al. 1998) and in which anatomical defects have not been observed (Ball et al. 2003; Gregg 2007; Pardue et al. 1998). The resulting offspring were crossed to generate mice that were homozygous for the Nxy+/+ and carried one PrphRd2+ allele or two Prph−/− alleles. Throughout this study, Nxy−/− mice that carry a single Prph−/− allele will be referred to as Prph−/−, whereas Nxy+/+ mice that carry two Prph−/− alleles will be referred to as controls or Prph−/−. Mice were screened for the Prph−/− or PrphRd2−/− allele by polymerase chain reaction amplification using two sets of primers for each.

**For Prph−/− allele:** sense (5′-CCGAAATAGGTCGCTCGCC-3′) antisense (5′-GACAGCTAGGCTATGCGG-3′) cytoplase sense (5′-ATGACGAGCGCTTGGCC-3′) cytoplase antisense (5′-CTGAGGACCTTGGGATC-3′) For PrphRd2−/− allele: sense (5′-ACGGTCCCGTGGGACGAG-3′) antisense (5′-TGACGCAACAGCAGCCTGGG-3′) actin sense (5′-GAGAAACCGTGGCCATGGGCTC-3′)

**Electroretinography**

After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). Eye drops were used to anesthetize the cornea (1% proparacaine HCl) and to dilate the pupil (2.5% phenylephrine HCl, 1% tropicamide, and 1% cyclopentolate HCl). Mice were placed on a temperature-regulated heating pad within the recording session. All procedures involving animals were approved by the local Institutional Animal Care and Use Committee and were in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.

Two stimulation-recording systems and protocols were used for this study. Strobe flash ERGs were recorded using a stainless steel electrode in contact with the corneal surface via 1% methylcellulose. Needle electrodes were placed in the cheek and the tail for reference. Two stimulation-recording systems and protocols were used for this study. Strobe flash ERGs were recorded using a stainless steel electrode in contact with the corneal surface. Needle electrodes were placed in the cheek and the tail for reference. Two stimulation-recording systems and protocols were used for this study. Strobe flash ERGs were recorded using a stainless steel electrode in contact with the corneal surface via 1% methylcellulose. Needle electrodes were placed in the cheek and the tail for reference. Two stimulation-recording systems and protocols were used for this study. Strobe flash ERGs were recorded using a stainless steel electrode in contact with the corneal surface via 1% methylcellulose. Needle electrodes were placed in the cheek and the tail for reference.
Ramsey, NJ) focused on the output of the fiber-optic bundle. A Uniblitz shutter system was used to control stimulus duration at 7 min. Mice were tested at 2 and 4 mo of age. Intensity–response functions for the strobe-flash ERG were obtained in a single session. Intensity–response functions for the dc-ERG were defined in several recording sessions, separated by ≥2 days with each day using a single stimulus intensity. An individual mouse underwent no more than four dc-ERG recording sessions.

**ERG analysis**

The amplitude of the a-wave was measured at 6 ms after flash presentation from the prestimulus baseline (see Fig. 1A). The leading edge of the a-waves obtained in response to high-intensity stimuli was analyzed with Eq. 1, a modified form of the Lamb–Pugh model of rod phototransduction (Hood and Birch 1994; Lamb and Pugh 1992; Pugh and Lamb 1993)

$$P_3 = \frac{1 - \exp\left[-S(t - t_0)^2\right]}{R_{mP3}} \ (1)$$

where $P_3$ represents the massed response of the rod photoreceptors and is analogous to the PIII component of Granit (1933). The amplitude of $P_3$ is expressed as a function of flash energy ($t$) and time ($t_0$) after flash onset. $S$ is the gain of phototransduction, $R_{mP3}$ is the maximum response, and $t_0$ is a brief delay.

Amplitude of the slow PIII was measured from the prestimulus baseline to the value of the trough at 150 ms (see Fig. 1A) and fitted with the Naka–Rushton equation

$$\frac{R}{R_{max}} = \frac{I^v}{(I^v + K^v)} \ (2)$$

where $I$ is the stimulus luminance of the flash; $R$ is the ERG amplitude at $I$ luminance; $R_{max}$ is the asymptotic ERG amplitude; $K$ is the half-saturation constant, corresponding to retinal sensitivity; and $v$ is a dimensionless constant controlling the slope of the function.

Amplitudes of the dc-ERG components were calculated as previously described (see Fig. 1B; Wu et al. 2004b). The amplitude of the c-wave was measured from the prestimulus baseline to the maximum of the peak. The FO was measured from the peak of the c-wave to the minimum value of the trough. The OR amplitude was determined from the difference between the value of the asymptote and the value of the minimum of the FO trough. The OR amplitude was calculated by the difference between the maximum/minimum value of the waveform on light offset and the value of the LP asymptote.

**Histology and immunohistochemistry**

After mice were killed, the superior cornea was marked before enucleation. For immunohistochemistry, eyes were fixed in 0.1 M sodium phosphate buffer (pH 7.4) containing 4% paraformaldehyde. After removal of the cornea and lens, the posterior pole was immersed through a graded series of sucrose solutions as follows: 10% for 1 h, 20% for 1 h, and 30% overnight. Eyes were embedded in OCT freezing medium, flash frozen on powderized dry ice, and immediately transferred to −80°C. Tissue was sectioned at 10 μm thickness with a cryostat (Leica, Wetzlar, Germany) at −30°C, mounted on superfrost slides, and stored at −80°C until processed. Sections were blocked in 0.1% Triton X-100, 1% bovine serum albumin, and 5% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature (RT) and then washed three times with PBS for 5 min each time. The sections were incubated overnight at 4°C with the primary antibody. Sections were rinsed with PBS three times for 10 min each time and incubated with secondary antibody (Alexa 488 or Alexa 594, 1:500; Molecular Probes) for 1 h at RT. After rinsing sections three times for 10 min each time with PBS, sections were mounted with DAPI (Vectorshield; Vector Laboratories, Burlingame, CA) and coverslipped. Primary antibodies used were rabbit anti-peripherin/rds (1:500, a kind gift of Andy Goldberg, Oakland University) and mouse anti-ezrin (1:50; Neomarkers; Freemont, CA).

For light microscopy, eyes were fixed in 0.1 M phosphate buffer (pH 7.4) containing 2% formaldehyde and 2.5% glutaraldehyde. The tissues were then osmicated, dehydrated through a graded ethanol series, embedded in epoxy resin (Epon), and processed for evaluation. Sections (1 μm thick) were cut approximately along the horizontal meridian and through the optic nerve. Single eyes from at least three individual mice, taken from separate litters, were used for each time point.

**Histological analysis and statistics**

Sections were imaged with a fluorescence/differential interference contrast microscope (BX-61; Olympus, Tokyo), equipped with a charge-coupled device monochrome camera (Hamamatsu Photonics, Bridgewater, NJ). Images were digitally captured (SlideBook software, version 4.2; Intelligent Imaging Innovations, Denver, CO), using either a ×40 or ×100 (oil) objective and exported for analysis with Image ProPlus software (Image-Pro PLUS, version 6.2; Media Cybernetics, Bethesda, MD). Thickness of the RPE, OS, inner segment layer (IS), outer plexiform layer (OPL), outer nuclear layer (ONL), and inner plexiform layer (IPL) were measured in light microscopic fields that spanned nearly 200 μm and were centered 300 μm from the edge of the optic nerve head or 300 μm from the edge of the peripheral retina. The average number of photoreceptor nuclei spanning the ONL was also determined for each microscopic field. These measurements were performed on both sides of the optic nerve head and no differences were found between the regions. Hypertrophic RPE cells were counted in retinal sections from at least four mice per genotype.

For all graphs, error bars represent the SE.
RESULTS

Peripherin/rds is not found in retinal pigment epithelial cells

Peripherin/rds is an integral transmembrane glycoprotein responsible for proper membrane folding of the photoreceptor disk array. As a semidominant mutation, expression of a single PrphRD2 allele prevents normal development of the photoreceptor outer segment, ultimately leading to progressive photoreceptor degeneration (Hawkins et al. 1985). Because we are investigating the response of the RPE to a loss of photoreceptor input using the PrphRD2/+ model, we began by assessing the localization of peripherin/rds in the retina and RPE. Figure 2 depicts an adult PrphRD2/+ retina stained for peripherin/rds (green) and ezrin (red) to identify the RPE microvilli. Ezrin is an epithelial cytoskeletal marker, which serves as a bridge between actin filaments and plasma membrane proteins and localizes to RPE microvilli (Bonilha et al. 2006). Sections were counterstained with DAPI (blue) and imaged to illustrate the localization of peripherin/rds in the retina and RPE. In agreement with previous reports, peripherin/rds was found to be limited to the retina, specifically to photoreceptor OSs (Fig. 2B; Arikawa et al. 1992; Travis et al. 1989). Therefore changes seen in dc-ERG components of PrphRD2/+ mice reflect changes in rod photoreceptor activity, not an intrinsic insult to the RPE itself.

Strobe-flash electroretinogram components (a-wave, slow PIII) are progressively reduced in PrphRD2/+ mice with age

The ERG measures the summed potentials emitted by the retina/RPE in response to light. The hyperpolarization of rod photoreceptors underlies the a-wave (Penn and Hagins 1969), which is normally followed by the b-wave. The use of the Nyxrob genetic background eliminates the b-wave (Pardue et al. 1998), allowing slow PIII, which is generated by Müller cells (Witkovsky et al. 1975), to be measured directly (Wu et al. 1998), allowing slow PIII, which is generated by Müller cells (Witkovsky et al. 1975), to be measured directly (Wu et al. 1998), allowing slow PIII, which is generated by Müller cells (Witkovsky et al. 1975), to be measured directly (Wu et al. 1998), allowing slow PIII, which is generated by Müller cells (Witkovsky et al. 1975), to be measured directly (Wu et al. 1998). Figure 3A represents the grand average of strobe-flash responses from Prph+/+ (n = 7) and PrphRD2/+ mice (n = 12) at 2 mo of age. Compared with Prph+/+ littermates at this age, PrphRD2/+ mice exhibit a 45% reduction in the Rmax amplitude of slow PIII and a 60% reduction in the maximum amplitude of the a-wave (RmP3) (Fig. 3, C and D, respectively). Consistent with a progressive photoreceptor degeneration, at 4 mo of age (Fig. 3B, n = 15 for each group), PrphRD2/+ mice exhibit a 70% reduction in RmP3 amplitude (Fig. 3C). Slow PIII is also further decreased at 4 mo, with Rmax reduced by 60% compared with Prph+/+ mice (Fig. 3D). Table 1 presents the values of RmP3, A, and t4 derived when Eq. 1 was fit to individual responses evoked by a 1 log cd s/m2 flash. In contrast to the significant reductions in RmP3, values of A obtained from PrphRD2/+ mice were not different from those from Prph+/+ mice at either 2 or 4 mo. This is consistent with previous findings (Birch et al. 1997; Cheng et al. 1997) and indicates that the amplification characteristics of the phototransduction cascade are operating normally. The amplitude of t4 was slightly larger in PrphRD2/+ mice than that in Prph+/+ mice at 2 mo. This may reflect an increased latency in the time rod photoreceptors take to respond, but a similar difference was not seen at 4 mo. Table 2 presents the values of Rmax, n, and K derived when Eq. 2 was fit to each individual response generated by a 1 log cd s/m2 flash. Although values of Rmax were significantly decreased, values for the slope parameter n were not different between genotypes at either age, whereas the sensitivity parameter K was significantly increased in PrphRD2/+ mice at both time points.

a-Wave amplitude reductions reflect rod photoreceptor degeneration in PrphRD2/+ mice

To define the relationship between rod photoreceptor integrity and a-wave amplitude we assessed the anatomical state of the retina at the time points studied electrophysiologically. Figure 4 depicts sections from Prph+/+ and PrphRD2/+ eyecups taken at 2 (Fig. 4, A and B) and 4 (Fig. 4, C and D) mo of age. Analysis was performed on both central (300 μm from the optic nerve head) and peripheral (300 μm from the distal edge

![Fig. 2. Peripherin/retinal degeneration slow (rds) is not found in the retinal pigment epithelium (RPE). A: photomicrograph of an adult Prph+/+ retina section stained with anti-peripherin/rds (green). Sections were counterstained with DAPI (blue). Each layer is identified. OS, outer segment layer; IS, inner segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Images were taken at ×40; scale bar = 20 μm. B: higher magnification (×100) photomicrograph of the denoted area from A; scale bar = 20 μm.](http://jn.physiology.org/).
of the retina) regions of the retina in sections passing through the optic nerve head. PrphRd2/−/− mice displayed abnormalities in photoreceptor outer segments at 2 mo. Rod OSs were short and vacuoles were readily seen within this layer (Fig. 4, A and B). The thickness of the OS layer was reduced by roughly 40% in both central and peripheral regions. Additionally, photoreceptor death was under way because the thickness of the ONL in central regions of PrphRd2/−/− retinas was 8.4 ± 1.03 cells per column compared with 10.75 ± 0.51 cells in Prph+/+/+ mice (Fig. 4, A, B). At 4 mo, the abnormalities seen in PrphRd2/−/− mice were more pronounced (Fig. 4, C, D, F). Both the OS and IS layers of PrphRd2/−/− mice were shorter and the remaining OSs were arranged in whorls. Compared with Prph+/+/+, OS length was reduced by roughly 50% in the PrphRd2/−/− retina.
n.s., not significant.

4-mo PrphRd2+/ mice (Fig. 5A, red tracings) for PrphRd2+/ mice developed anatomical abnormalities in the RPE well after the onset of photoreceptor degeneration. These anatomical presentations allow us to examine RPE function following photoreceptor degeneration alone (2 mo) and when photoreceptor degeneration is coupled with structural defects in the RPE as well (4 mo).

**Light-evoked responses of the RPE are reduced in PrphRd2+/ mice**

Figure 6 presents averaged responses obtained from 4-mo PrphRd2+/ mice (black tracings) and PrphRd2+/ mice at 2 mo (Fig. 6A, blue tracings) and 4 mo (Fig. 6B, red tracings) for each stimulus intensity presented. At both ages, all dc-ERG components were present but reduced in PrphRd2+/ mice compared with those in Prph+/+ littermates. Figure 6, C–F illustrates intensity–response functions for each dc-ERG component. At 2 mo, the amplitude of the PrphRd2+/ c-wave was about 50% that of Prph+/+, a factor that was consistent across all stimulus intensities (Fig. 6C). A similar relationship was seen at 4 mo, with a further amplitude decrease. The changes noted in the FO were more complex. Although overall amplitude was decreased, at 2 mo there was an intensity-dependent shift to the right, indicating that a greater light intensity is required to elicit the same amplitude response as in Prph+/+ mice. At 4 mo, the shift was to the left (Fig. 6D). At both ages examined, the LP component was reduced in amplitude at all stimulus intensities. At 4 mo, however, the amplitude of the LP was no longer modulated by stimulus intensity and, instead, was relatively stable at 0.5–0.75 mV (Fig. 6E). Finally, the amplitude of the OR was attenuated at all light intensities. In addition, the intensity at which the OR reverses polarity was shifted to the right in PrphRd2+/ compared with that in Prph+/+ mice (Fig. 6F). The magnitude of this shift was similar at 2 and 4 mo of age and to the shift of the slow PI1 intensity–response function (Table 2). Although differences in ocular pigmentation may induce a similar shift (Wu et al. 2004b), all mice studied here were littermates and similarly pigmented. Therefore we attribute the shift of the OR intensity–response function in PrphRd2+/ mice to a photoreceptor degeneration-associated decreased input to the OR generators.

**TABLE 2. Naka–Rushton parameters fit to the slow PI1 intensity–response functions**

<table>
<thead>
<tr>
<th></th>
<th>R_{max}</th>
<th>n</th>
<th>K</th>
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<tr>
<td>2-mo Prph+/+</td>
<td>569.6 ± 40.3</td>
<td>0.57 ± 0.04</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>2-mo PrphRd2+/</td>
<td>309.0 ± 25.3</td>
<td>0.47 ± 0.05</td>
<td>0.90 ± 0.14</td>
</tr>
<tr>
<td>t-test</td>
<td>P &lt; 0.0005</td>
<td>n.s.</td>
<td>P = 0.01</td>
</tr>
<tr>
<td>4-mo Prph+/+</td>
<td>610.0 ± 37.0</td>
<td>0.61 ± 0.05</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>4-mo PrphRd2+/</td>
<td>248.0 ± 12.3</td>
<td>0.53 ± 0.04</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>t-test</td>
<td>P &lt; 0.0005</td>
<td>n.s.</td>
<td>P &lt; 0.0005</td>
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Values are means ± SE, n = 8–12/group at 2 mo; n = 15/group at 4 mo. n.s., not significant.

whereas IS length was reduced by roughly 30%. OPL length was decreased by about 25% (Fig. 4F). Furthermore, ONL thickness was greatly reduced because PrphRd2+/ mice had an average of 5.9 ± 0.22 cells per column \( n = 3 \) in central regions of the ONL compared with 10.6 ± 0.42 cells spanning the central ONL in Prph+/+ mice \( n = 4 \). At both ages examined, central and peripheral regions were equivalently affected in PrphRd2+/ mice. These data demonstrate the expected photoreceptor degeneration characteristic of PrphRd2+/ mice.

**The amplitude of the ERG a-wave reflects the mass response of rod photoreceptor OSs.** As a consequence, a-wave amplitude will decrease when the total area of OSs are lost, due to OS shortening or a loss of rod photoreceptors. Both of these changes occur in PrphRd2+/ mice and we compared the extent of a-wave amplitude reduction with anatomical measures of the mutant retina. The thickness of the averaged central and peripheral ONL and OS length were reduced to 60 and 85% of Prph+/+, respectively. When these anatomical measures are combined, they predict that the PrphRd2+/ a-wave will be reduced to 51% of Prph+/+, which is somewhat lower than predicted by the reduction in \( R_{m3} \) (62%). At 4 mo, \( R_{m3} \) values of PrphRd2+/ mice were roughly 30% of Prph+/+ littermates. At this age, the averaged central and peripheral ONL thickness was 67% of Prph+/+ and OS length was 50% of Prph+/+ thickness, indicating that a-wave generators are reduced to about 33.5%. These data demonstrate that ERG component amplitudes in PrphRd2+/ mice correlate closely with the underlying anatomical structure at 4 mo, confirming that the a-wave can be used to quantify the number of functional OSs in the PrphRd2+/ retina and thus the effective stimulus to the RPE. Despite the disorganization of the OSs within the PrphRd2+/ mice, the basic structure of the retina is preserved and our data demonstrate that a-wave amplitude is a reliable measure of photoreceptor responsiveness.

Hawkins et al. (1985) reported that 2- to 3-mo-old PrphRd2+/ mice have abnormally large phagosomes within the RPE and slow turnover of shed OS disks. Although these findings likely reflect the massive amount of rod photoreceptor material presented to the RPE in the PrphRd2+/ mutant, they also point toward a possible defect in RPE phagocytosis. Although rod photoreceptor degeneration was clearly evident in 2-mo-old mutants (Fig. 4), we found no changes in thickness or structure of the RPE cell layer at this time (Fig. 5A; Hawkins et al. 1985). At 4 mo, however, numerous vacuoles were observed in the OS layer and we also observed vacuolization in RPE cells (dimpled arrowheads, Fig. 5A). Furthermore, swollen, hypertrophic RPE cells, with lightly stained cytoplasm and reduced apical membranes were present throughout the epithelial layer (arrows, Fig. 4A). RPE layer thickness was also slightly greater in PrphRd2+/ mice compared with that in Prph+/+ animals (Fig. 5B). The density of hypertrophic cells (Fig. 5C) was greater in PrphRd2+/ mice \( 7.25 ± 1.65/\text{section} \) compared with that in Prph+/+ \( 1.0 ± 0.41/\text{section} \). Our findings demonstrate that PrphRd2+/ mice develop anatomical abnormalities in the RPE well after the onset of photoreceptor degeneration. These anatomical presentations allow us to examine RPE function following photoreceptor degeneration alone (2 mo) and when photoreceptor degeneration is coupled with structural defects in the RPE as well (4 mo).

**TABLE 1. Lamb and Pugh parameters fit to the light-evoked response elicited by a 1 log cd s/m² stimulus**

<table>
<thead>
<tr>
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<th>( R_{m3} )</th>
<th>( A )</th>
<th>( t_{a} )</th>
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<tbody>
<tr>
<td>2-mo Prph+/+</td>
<td>−441.9 ± 30.4</td>
<td>0.007 ± 0.001</td>
<td>1.36 ± 0.16</td>
</tr>
<tr>
<td>2-mo PrphRd2+/</td>
<td>−167.8 ± 22.8</td>
<td>0.008 ± 0.001</td>
<td>2.23 ± 0.31</td>
</tr>
<tr>
<td>t-test</td>
<td>( P &lt; 0.0005 )</td>
<td>n.s.</td>
<td>( P = 0.042 )</td>
</tr>
<tr>
<td>4-mo Prph+/+</td>
<td>−485.8 ± 20.7</td>
<td>0.018 ± 0.002</td>
<td>1.22 ± 0.14</td>
</tr>
<tr>
<td>4-mo PrphRd2+/</td>
<td>−144.7 ± 10.8</td>
<td>0.016 ± 0.003</td>
<td>1.48 ± 0.26</td>
</tr>
<tr>
<td>t-test</td>
<td>( P &lt; 0.0005 )</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
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</table>

Values are means ± SE, \( n = 8–12/\text{group at 2 mo; } n = 15/\text{group at 4 mo. } \)
RPE function is conserved despite reduced rod photoreceptor input

The nonneuronal light-evoked responses, recorded as the dc-ERG and slow PIII, are generated secondary to rod photoreceptor activity. To assess how these components are altered following photoreceptor degeneration in PrphRd2+/+ mice, we compared the relative changes in dc-ERG components and slow PIII to those of the a-wave. In Fig. 7, A–E, the diagonal lines indicate where the two measurements are reduced by similar factors. If the slow PIII/dc-ERG component is reduced to a greater or lesser extent than the a-wave, points will fall below or above the diagonal line, respectively. Figure 7 plots the amplitude of R_{max} (Fig. 7A) and of each dc-ERG component (Fig. 7, B–E) as a function of a-wave maximum amplitude (RmP3), with all measures expressed as a proportion of the corresponding Prph^{+/+} responses. To determine the relationship between the photoreceptor response and the Müller cell response, we plotted relative R_{max} as a function of relative RmP3 at 2 (n = 12; blue symbols) and 4 (n = 15; red symbols) mo of age. Our data reveal that slow PIII is conserved relative to the photoreceptor response, given that all points fall above the line (Fig. 7A). A similar result was obtained for each dc-ERG component (Fig. 7, B–E). Despite the overall conservation of the dc-ERG responses, the components were affected differently as the mice aged. Both the c-wave and LP were affected to a greater extent at 4 mo than at 2 mo of age, suggesting that the RPE was less resilient to the decline in a-wave amplitude. These findings could reflect the increasing

FIG. 4. Slow degeneration of rod photoreceptors in PrphRd2+/+ mice induces a progressive thinning of all inner retinal layers. Retinal cross sections obtained from 2 (A and B) and 4 (C and D) mo Prph^{+/+} and Prph^{Rd2+/+} mice. Light micrographs were taken from central (300 μm from the ONH [optic nerve head]) and peripheral (300 μm from the edge of the retina) areas of the retina. Each layer is identified. RPE, retinal pigment epithelium; OS, outer segment layer; IS, inner segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer. All images were taken at ×40. Scale bar = 20 μm. Thickness of each layer was measured at both time points (E and F, respectively). Compared with Prph^{+/+} mice, Prph^{Rd2+/+} mice have disorganized OSs and fewer photoreceptors (B). These changes become more severe at 4 mo, when vacuoles are apparent throughout the OS and each layer has become thinner (D).
age of the mice, the further loss in photoreceptor activity, or the RPE damage found at this later age. The FO was affected to the same extent at both time points and the OR was spared to a greater extent at 4 mo than at 2 mo. The greater conservation of the OR at 4 mo suggests that the maximum impact of the \( PrphRd2 \) mutation on this response component was achieved by 2 mo. Collectively, these data indicate that the amplitude of ERG components generated by Müller and RPE cells are retained to a greater extent than predicted by a-wave amplitude.

**DISCUSSION**

Light-evoked rod photoreceptor responses induce the generation of a series of electrical potentials by nonneuronal cell types. These responses can be recorded at the corneal surface as components of the ERG. The RPE generates four discernable potentials (c-wave, FO, LP, OR), whereas slow PIII reflects activity of retinal Müller cells. These response components are readily recorded from mice and can be used to characterize functional changes in mouse mutants involving genes expressed in Müller or RPE cells. However, the interpretation of any abnormality in these responses is complicated by the possibility that a mouse mutant may also develop rod photoreceptor degeneration or dysfunction, which will alter the amplitude and/or timing of the nonneuronal response. In the present study, we have established a basis for interpreting functional changes in mutant mouse models. We used the \( PrphRd2 \) mouse because this model of photoreceptor degeneration has been extensively characterized (Arikawa et al. 1992; Cheng et al. 1997; Hawkins et al. 1985; Jansen and Sanyal 1984; Sanyal and Hawkins 1989) and because peripherin/rds is thought to be restricted to rod OSs and not to RPE or Müller cells. We confirmed this localization using immunohistochemistry and demonstrated the exclusive localization of peripherin/rds to rod outer segments.

At 2 mo of age, a-waves of \( PrphRd2 \) mice were reduced by roughly 50%. A similar reduction was found in slow PIII and all dc-ERG components, although there was a general tendency for all components to be preserved above the level predicted by our a-wave and anatomical analyses. This “sparing” was seen at both 2 and 4 mo of age. There is no doubt that the dc-ERG components and slow PIII are initiated by rod photoreceptor activity. However, several of these components are known to be evoked by changes in [K⁺]. As a consequence, the disrupted architecture of the
**PrphRd2**/mice might contribute to their relative preservation. For example, if the SRS were significantly smaller in **PrphRd2**/ than that in **Prph**+/mice, an effective change in $[K^+]$ could be induced despite a decrease in photoreceptor number and outer segment length.

In addition to demonstrating that RPE and Müller cell function can be characterized in mouse models of photoreceptor degeneration, our results begin to define the relationship between rod photoreceptor degeneration and the electrical responses induced in nonneuronal cell types by photoreceptor degeneration.

*FIG. 6.* **PrphRd2**/+ mice exhibit progressive reductions in dc-ERG components. dc-ERG recordings were performed on **Prph**+/+ and **PrphRd2**/+ mice at 2 (A) and 4 (B) mo of age in response to a 7-min stimulus at a series of intensities (1.4–4.4 log cd/m²). The averaged **Prph**+/+ waveforms are shown in black ($n = 4–14$ at 2 mo; $n = 5–11$ for 4 mo) and the averaged **PrphRd2**/+ waveforms are in blue ($n = 5–7$, 2 mo) and red ($n = 4–6$, 4 mo). C–F: the amplitude of each dc-ERG component was measured and graphed as a function of stimulus intensity at both time points.
activity. Although a number of mouse models involving genes expressed in Müller or RPE cells do not develop rod photoreceptor degeneration (Edwards et al. 2010; Marmorstein et al. 2006; Wu et al. 2004a,b, 2006, 2007), photoreceptor degeneration is observed in others (e.g., Duncan et al. 2003; Won et al. 2008). In these latter models, our results indicate that a reduction in Müller or RPE cell response amplitude beyond that seen at the level of the a-wave would be required to provide strong evidence of Müller or RPE cell-specific dysfunction.

It is not clear why Müller and RPE cell responses are spared in the PrphRd2+/ mice. However, it has been reported that disorders that include severe RPE abnormalities (such as rubella retinopathy and diffuse drusen) are not associated with changes in RPE electrophysiology, as evidenced by normal or mildly affected electrooculogram and nonphotic responses (Gupta and Marmor 1994; Marmor 1991). Each dc-ERG component reflects the activity of ion channels that undoubtedly play many roles in maintaining retinal homeostasis. Although several ion channels (e.g., Kir4.1, Clcn2) have been implicated in the generation of the dc-ERG components studied here, it is not yet possible to explain the generation of a particular ERG component in terms of the activity of a specific ion channel(s). As the identification of additional ion channels involved in the generation of each component continues, it will be interesting to determine whether their density and/or location are altered in the PrphRd2+/ retina, which may contribute to the pattern of results we report. In this regard, Takeuchi et al. (2008) demonstrated that systemic administration of the calcium channel blocker, nilvadipine, to PrphRd2+/ mice (with a wildtype Nystnob background) restores a- and b-wave amplitudes. Electron microscopy demonstrated that the treatment was associated with a partial restoration of photoreceptor OS disc arrays (Takeuchi et al. 2008). It would be interesting to analyze RPE function in these mice and to determine the relative impact of nilvadipine on the a-wave and dc-ERG components.

Slow PIII is known to reflect a cornea-negative potential generated by Müller cells (Kofuji et al. 2000; Wu et al. 2004a), which is normally masked by the larger amplitude and opposite polarity b-wave component. Slow PIII can be unmasked pharmacologically, using glutamate agonists that block b-wave generation (Malchow and Yazulla 1988; Oakley and Green 1976; Steinberg and Miller 1973; Witkovsky et al. 1975), but these agents have a short effective period (Smith et al. 1989), may increase excitotoxic photoreceptor degeneration (Olney 1982), and have not been used over a timeframe comparable to that examined here. In the present study, we used a genetic approach to isolate slow PIII. For mouse-based studies focused on the outer retina, crossing the mutation of interest to a mouse model that lacks the ERG b-wave such as Gm6nrob (Maddox et al. 2008; Masu et al. 1995) or Trpm1−/−.
(Morgens et al. 2009), will allow slow PIII to be measured. In this study, we used Nyx<sup>med</sup> to unmask slow PIII and were able to demonstrate that it was relatively conserved compared with a-wave amplitude in Prph<sup>Rd2</sup>/+ mutants. Because slow PIII is generated by Kir4.1 activity (Kofuji et al. 2000), a potential explanation for its modest reduction involves up-regulation of Kir4.1 in Müller cells. However, Landieva et al. (2006) reported that Kir4.1 levels are not altered in Prph<sup>Rd2</sup>/+ mice, despite the Müller cell hypertrophy seen in this model (Ekstrom et al. 1988). Therefore further investigation into the mechanism of slow PIII action in the face of photoreceptor degeneration is warranted.

Proper RPE function is indispensable for photoreceptor health and retinal homeostasis. As such, mutations in genes expressed in the RPE underlie a wide range of human maculopathies and retinal dystrophies. Electrophysiological studies of mouse models for these genes have historically been restricted to protocols that focus on the functional properties of rod and cone photoreceptors and inner retinal neurons. The data presented here demonstrate the ability to measure and meaningfully analyze RPE physiology and provide a useful diagnostic tool for mouse models of these inherited retinal disorders. By demonstrating that RPE function is retained at fairly advanced disease levels and by defining how the response properties of the dc-ERG change with photoreceptor degeneration, it is hoped that the functional assays used here will be more broadly applied, especially to mouse models involving genes expressed in the RPE.

**Acknowledgments**

We thank V. Bonilha and Y. Li for technical assistance.

**Grants**

This work was supported by the Veterans Administration Medical Research Service, Foundation Fighting Blindness Center Grant, Research to Prevent Blindness, and National Eye Institute Grant R24-EY-15638.

**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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