Mouse model of human RPE65 P25L hypomorph resembles wild type under normal light rearing but is fully resistant to acute light damage

Yan Li1, Shirley Yu1, Todd Duncan1, Yichao Li2, Pinghu Liu3, Erelda Gene1, Yoel Cortes-Pena1, Haohua Qian2, Lijin Dong3 and T. Michael Redmond1,*

1Laboratory of Retinal Cell and Molecular Biology, 2Visual Function Core and 3Genetic Engineering Core, National Eye Institute/NIH, Bethesda, MD, USA

Abstract

Human RPE65 mutations cause a spectrum of blinding retinal dystrophies from severe early-onset disease to milder manifestations. The RPE65 P25L missense mutation, though having <10% of wild-type (WT) activity, causes relatively mild retinal degeneration. To better understand these mild forms of RPE65-related retinal degeneration, and their effect on cone photoreceptor survival, we generated an Rpe65/P25L knock-in (KI/KI) mouse model. We found that, when subject to the low-light regime (∼100 lux) of regular mouse housing, homozygous Rpe65/P25L KI/KI mice are morphologically and functionally very similar to WT siblings. While mutant protein expression is decreased by over 80%, KI/KI mice retinae retain comparable 11-cis-retinal levels with WT. Consistently, the scotopic and photopic electroretinographic (ERG) responses to single-flash stimuli also show no difference between KI/KI and WT mice. However, the recovery of a-wave response following moderate visual pigment bleach is delayed in KI/KI mice. Importantly, KI/KI mice show significantly increased resistance to high-intensity (20 000 lux for 30 min) light-induced retinal damage (LIRD) as compared with WT, indicating impaired rhodopsin regeneration in KI/KI. Taken together, the Rpe65/P25L mutant produces sufficient chromophore under normal conditions to keep opsins replete and thus manifests a minimal phenotype. Only when exposed to intensive light is this hypomorphic mutation manifested physiologically, as its reduced expression and catalytic activity protects against the successive cycles of opsin regeneration underlying LIRD. These data also help define minimal requirements of chromophore for photoreceptor survival in vivo and may be useful in assessing a beneficial therapeutic dose for RPE65 gene therapy in humans.

Introduction

In the initial step in the visual process, the visual pigment, retinal opsin, with covalently bound 11-cis-retinal chromophore, undergoes photoactivation upon photon absorption and subsequently bleaches to opsin and all-trans-retinal. To regenerate rhodopsin and maintain normal visual sensitivity, the all-trans isomer must be released, metabolized and reisomerized to 11-cis-retinal in the retinal pigment epithelium (RPE) in a process called the visual (or retinoid) cycle (1), involving movement of retinoid between the photoreceptor and RPE. RPE65 is the key isomerase in this process, converting all-trans-retinyl ester to 11-cis-retinol (2–4). Evolutionarily conserved, RPE65 was the first RPE-specific gene identified as associated with disease, in patients with early-onset severe retinal dystrophy (EOSRD) or Leber congenital amaurosis 2 (LCA2) (5,6). In humans, >100 pathogenic mutations of RPE65 have been identified, spread over all 14 exons of the gene.
and their boundaries, about half of which are missense mutations (7) (see also http://www.retina-international.org/files/sci-news/rpe65mut.htm, and OMIM 204100). These mutations are associated with a spectrum of retinal dystrophies ranging from early-onset, severe blindness to later-onset, milder retinal degeneration (8,9). In less severe forms resembling retinitis pigmentosa, patients often have well-preserved visual function early in their lives and a slower progression of the disease (10,11).

Transgenic mouse models have been employed to understand inherited retinal degeneration caused by RPE65 mutations. The Rpe65 knock-out (KO) mouse showed that RPE65 is necessary for 11-cis retinoid production in the visual cycle (12). With no RPE65 expression, the KO mice over-accumulate the all-trans-retinyl ester substrate in the RPE, while 11-cis-retinoids are absent. Consequently, these mice have a slow retinal degeneration but abolished light sensitivity owing to lack of visual chromophore in retinas. The Rpe65 KO model has been of great utility in understanding retinal physiology and biochemistry, and their alterations caused by or related to variations in chromophore status (15–20). Rd12, a spontaneous null mutation in Rpe65 (21), and an Rpe65/R91W knock-in (KI) mouse mutant have also been reported (22). With synthesis of very low levels of 11-cis-retinal by the R91W RPE65 mutant protein, these mice show better cone function at young age than the KO mice, but their rod system is severely desensitized. Though more slowly than the KO, the RPE65 mutant mice do degenerate, consistent with the phenotype of RPE65/R91W patients diagnosed with LIRD.

Based on these observations in both human patients and transgenic mouse models, it appears that the level of impairment of the isomerase activity caused by RPE65 mutations correlates with severity of retinal dysfunction. Therefore, studies on less severe forms of RPE65-related retinal dystrophy may help us not only understand the full spectrum of RPE65-related pathology but also in assessing a beneficial therapeutic dose for RPE65 gene therapy. Recently, a homozygous P25L missense mutation of RPE65 has been associated with mild retinal pathology in a young patient (11). With a greatly reduced isomerase activity (~8% of wild type [WT] in vitro, though less severe than R91W, mutant RPE65/P25L sustained normal visual acuity in the affected patient at the age of 6 years. The retinal structure was relatively well preserved with no obvious increase in fundus autofluorescence. However, rod function was greatly impaired (night blindness), and short-wavelength cones appeared more impaired than long-wavelength cones.

To better understand the pathogenic pathway of the P25L mutation, we generated an Rpe65/P25L KI transgenic mouse model to study mild forms of RPE65-related retinal degeneration. Surprisingly, the phenotype manifested under normal light regime was quite similar to WT controls, but under damaging light intensity, like Rpe65 KO and unlike WT, it was protected against light-induced retinal damage (LIRD).

**Results**

**Normal transcription of Rpe65/P25L gene in Rpe65**

To gain further insights into the pathology of RPE65/P25L missense mutation in human disease, we generated Rpe65

"Redacted" text due to missing URL.
20-month sections). The length and compactness of the outer segments (OS) were similar to the WT control, and the thickness of the outer nuclear layer (ONL) was comparable with the WT. The well-preserved retinal structure in the Rpe65KO mice is in contrast with the progressive retinal degeneration observed in Rpe65 heterozygous mice at young age.

It has been well established that the expression of the opsins, particularly the cone opsins, is sensitive to RPE65 expression levels. In the Rpe65KO mice, drastic reductions in the expression of cone opsins were detected at Postnatal Day 25, and a significant portion of the remaining opsins were mislocalized in the cell membrane of the inner segment, cell body, axon and synaptic pedicle (data not shown) (16). In contrast, both cone opsins were correctly localized to the OS in the 7-month-old KI/KI mice.

In vivo isomerase activity of the RPE65P25L/P25L mutant is sufficient to keep opsins replete

As RPE65 is indispensable for the synthesis of 11-cis-retinal and consequent regeneration of visual pigment (12,22), we examined the retinoid composition in the 4-month-old Rpe65P25L/P25L KI/KI mice. HPLC retinoid analyses of the retinae extracts showed that homozygous KI/KI mice generate a comparable amount of 11-cis-retinal sufficient to keep photoreceptor visual pigments as replete as the WT controls (Fig. 5A), despite the greatly reduced expression levels of the mutant protein (Fig. 2). On the other hand, there was a significant excess accumulation of retinyl esters in the RPE layer of the KI/KI mice (Fig. 5B). This was an ~3-fold increase compared with the retinyl ester content in the WT littermates at 4 months. These levels, however, are not as high as the ≥20-fold increase...
in RPE retinyl ester level observed in Rpe65 KO mice (12) and Rpe65R91W/R91W KI/KI mice (22) at comparable ages.

Visual function of Rpe65P25L/KI mice is similar to WT

To evaluate rod- and cone-mediated light responses, we performed in vivo electroretinogram (ERG) recordings on KI/KI mice. Under dark-adapted (scotopic) conditions, a- and b-wave amplitudes of the KI/KI mouse retinae were similar to those observed in WT and heterozygous wt/KI retinae (Fig. 6A). Similarly, the Rpe65/P25L KI/KI mice showed no significant changes in either a- or b-wave amplitudes under light-adapted (photopic) conditions (Fig. 6B). To further isolate the sensitivities of the UV-cone- and M-cone-specific b-wave responses in the KI mice, we exposed mice to flash light stimuli of particular wavelength. As shown in Figure 6C, we found that KI/KI mice exhibited cone b-wave amplitudes similar to those in the WT controls in response to both UV (triangles) and green (circles) flash stimuli. Thus, the Rpe65/P25L mutation does not have a significant effect on the sensitivity of rod or cone responses to light stimuli when raised under the subdued light regime in standard mouse husbandry.

We next characterized the recovery of rod photoreceptors function following moderate visual pigment bleach in the KI/KI mouse retinae. After being exposed to a bleaching background light (1000 cd/m²) for 30 s [eliciting a fractional bleach of ~9.5%, as calculated from data in Pawar et al. (28)], ERG responses to a low-intensity flash stimulus (10 cd.s/m²) were continuously recorded for 1 h at 2 min intervals. The a-wave amplitude recovery was plotted against post-bleaching time; Figure 6D shows representative recovery curves for KI/KI, wt/KI and WT mouse. Each plotted recovery curve was fitted with a nonlinear regression using the published equation $A(t) = A_{max}/(1 + c_a \times \exp(-t/t_a))$ (see Supplementary Materials and Methods) (29). The three values $A_{max}$, $c_a$ and $t_a$ were calculated for each plot, and the constant values between different genotype groups were analyzed using a multiple t-test. As shown in Table 1, there were no significant differences observed among groups in the value of maximal a-wave.
amplitude following bleaching ($A_{\text{max}}$) and the degree of amplitude reduction right after bleaching ($c_a$). However, the time constant for recovery ($\tau_a$) is significantly longer in the KI/KI mice compared with the WT and heterozygous mice ($P < 0.01$), suggesting a delayed recovery rate in the KI/KI mice following a moderate visual pigment bleach.

**Rpe65P25L/P25L** KI mice are protected from LIRD

As a critical component of the visual cycle, RPE65 modulates retinal susceptibility to intensive light damage via affecting the kinetics of rhodopsin regeneration (14). It has been demonstrated that impaired chromophore synthesis owing to RPE65 mutations increases retinal resistance to intensive light damage (26,30). We examined retinal susceptibility to light damage in the Rpe65P25L/P25L KI mice using OCT and light microscopy. With dilated pupils, mouse retinas were exposed to 20 000 lux white light for 30 min. The structural integrity of the retinae was examined via OCT imaging and histology 2 weeks after the light treatment. The ONL of the WT mice was reduced, and rod outer segments were greatly shortened (Fig. 7B and C), whereas much less severe degeneration was observed in the same area of the KI/KI mice (Fig. 7F and G). Measurement of both the total retinal thickness (Fig. 7I) and the photoreceptor span (Fig. 7J) showed...
that their thicknesses after light damage were significantly reduced in the WT mice compared with the KI/KI mice, whereas the thicknesses before light damage treatment were comparable between the two genotype groups (Fig. 7I and J). Consistent with decreased sensitivity to LIRD, retinae of the KI/KI mice did not display significant cell death by apoptosis after intensive light treatment (Fig. 7H), nor did they show accumulation of A2E (Supplementary Material, Fig. S2), a derivative of free all-trans-retinal (31,32), as found in the light-treated WT retinae.

We hypothesized that a suppressed visual cycle was the underlying mechanism of protection from intense light damage in the KI/KI mice. Therefore, we examined the regeneration of 11-cis-retinal following the exposure to 20 000 lux white light for 30 min. We found that the 11-cis-retinal pool has already been fully replenished in the WT retinae 1 h after acute light treatment (Fig. 8A). In contrast, the level of 11-cis-retinal in KI/KI mice was much lower, with an observed 5-fold less 11-cis-retinal content 1 h after light treatment than WT, and it was still 3-fold less 2 h later. On the other hand, the amount of retinyl esters in the KI/KI RPE was 4-fold more than that in the WT RPE at 1 h post-light treatment, and at 2 h post-light was about 10-fold more than the completely recovered WT (Fig. 8B). As a further measure of the slowed recycling of visual cycle retinoids, we found that all-trans-retinal levels were also significantly lower in the KI/KI retinae than that in WT, both at 1 and at 2 h after light treatment (Supplementary Material, Fig. S4). These measures of retinoid product and substrate levels indicate that the reduced expression and catalytic activity of the hypomorphic RPE65/P25L enzyme dramatically slow the synthesis of visual chromophore, thereby protecting the KI/KI mice subjected to acute light stress from the successive cycles of opsin regeneration that drive the light damage outcome seen in WT animals.

**Discussion**

Herein, we present the somewhat paradoxical phenotype of the Rpe65P25L/P25L KI mouse we have generated. Paradoxical in that KI/KI mice are phenotypically very similar to WT under regular husbandry conditions but manifest an Rpe65 KO-like aspect of complete resistance to LIRD. The Rpe65P25L/P25L KI mouse phenotype parallels, in general terms, the mild phenotype of the human RPE65 homozygous P25L proband (11) for which it is a model but may differ in species-specific details. This model complements null (12,21) and near-null (22) Rpe65 models previously studied. As maintenance of an adequate visual cycle is critical for normal retinal development and function, and overwhelming evidence implicates RPE65 as playing the major role in setting the dynamic range of visual pigment regeneration, an adequate chromophore level may be crucial in preventing or limiting degeneration in patients with mutations of RPE65, or in RPE65 gene therapy, and preserving cone function is a key concern in both cases. A recent study by Jacobson et al. (33) finds that current RPE65 gene therapy fails to stop an apparently ongoing program of retinal degeneration in treated patients. Our experiments may have translational significance for the management of RPE65 gene therapy by providing a minimal estimate for an effective replacement level of chromophore that might prevent progressive photoreceptor loss.

**RPE65/P25L leads to mild phenotype in KI model but is protective against LIRD**

In line with the mild phenotype in a human patient homozygous for P25L (11), KI/KI mice display intact retinae with normal expression of cone and rod opsins. The P25L mutation has minimal
effects on visual functions under standard mouse husbandry. Only when subject to increased light intensities was a retinal phenotype manifested. When raised under the normal light intensity of $\sim 100$ lux, KI/KI mice can maintain chromophore levels comparable with WT and therefore manifest normal photopic and scotopic ERG parameters. It is noticeable that, while sufficient chromophore is generated, there is still significant retinyl ester over-accumulation in the RPE of KI/KI mice compared with WT. It seems that under dim light where minimal top-up of chromophore levels is required, the slower isomerization rate does not affect the rate-limiting step in the visual cycle and thus does not disrupt regeneration. However, this slower rate may not be able to keep up with the continuous influx of all-trans-retinol from both the circulation (34,35) and the retina, resulting in ester accumulation in the RPE. Consistent with this, the reduced isomerase activity in KI/KI mice does not replenish depletions of the 11-cis-retinal pool following bleaches as rapidly as WT, resulting in a significant delay in the recovery of rod photoresponses.

While a sustained level of chromophore is required for photoreceptor survival (12,21,36,37), it has been suggested that a burst influx of 11-cis-retinal following intense light actually triggers massive apoptosis and subsequent retinal degeneration by inducing the rapid release of all-trans-retinol from meta II opsin (38,39). Therefore, while Rpe65 KO mice display a progressive retinal degeneration and total lack of visual sensitivity (12), their lack of an active visual cycle actually protects them from acute LIRD (14,30). Just as in the Rpe65 KO mice, when exposed to acute white light ($\sim 20000$ lux for 30 min), KI/KI mice retinas are protected from LIRD. It is remarkable that while the level of chromophore regeneration supported by P25L RPE65 is sufficient

![Figure 6. Rpe65<sup>P25L/P25L</sup> KI mouse has full-field scotopic (rod), photopic (cone) ERG responses similar to WT but has delayed a-wave recovery after moderate visual pigment bleach. Dark-adapted (scotopic) ERG (A) and light-adapted (photopic) ERG (B) b-wave responses were obtained from 3-month-old homozygous KI/KI (open circle), heterozygous wt/KI (half-filled circle) and the littermate WT (filled circle) controls. Each point represents the average of six mice. Error bars show ±SD. (C) Cone signal isolation in Rpe65<sup>P25L/P25L</sup> KI mouse. B-wave amplitudes in response to a series of flashes varying intensity of UV (triangles) and green light (circles) stimuli were measured for 6-month-old homozygous KI/KI mice, heterozygous wt/KI and the littermate WT controls. Each point represents the average of four mice. Error bars show ±SD. (D) Representative a-wave recovery after moderate visual pigment bleach in KI/KI (open circle), WT (filled circle) and wt/KI (half-filled circle) mice. Continuous curves are plotted from Equation (I) fitted to the data with a nonlinear regression.]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>wt/KI</th>
<th>KI/KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{\text{max}}$</td>
<td>283.48 ± 81.60</td>
<td>297.1875 ± 62.15</td>
<td>285.44 ± 1.95</td>
</tr>
<tr>
<td>$c_a$</td>
<td>17.22 ± 10.00</td>
<td>13.32 ± 5.44</td>
<td>12.00 ± 6.45</td>
</tr>
<tr>
<td>$\tau_a$</td>
<td>6.60 ± 1.95</td>
<td>6.59 ± 1.08</td>
<td>9.10 ± 2.43</td>
</tr>
</tbody>
</table>

The calculated $c_a$ values for KI/KI mice are significantly larger than those for the WT mice and the wt/KI mice ($P < 0.01$). There are no significant differences in the values of $A_{\text{max}}$ or $c_a$ between the KI/KI group and the WT controls; $n$ indicates the number of eyes subject to the test of recovery responses in each genotype group.
to support near WT phenotype at normal light rearing, that, with as much as 8% WT activity (11), P25L is also protective against LIRD. While the overall mechanism of LIRD is far from clear, it involves formation of stressors that may include rhodopsin photointermediates, retinoid intermediates, generation of oxidative species including lipid products and calcium influx owing to excessive activation of the phototransduction cascade (40). The stressor most germane to the present experiments involves the release of all-trans-retinal from meta II opsin and the influx of 11-cis-retinal to replace it. It is likely that WT photoreceptors are bathed in toxic retinals (41) during the 30 min of light damage that are rapidly recycled back to 11-cis-retinal for repeated iteration, whereas the mutant P25L protein can destabilize dimers (27) formed with WT protein. This apparently reduced expression, however, does not appear to have a detectable physiological effect on ERGs. This is of additional interest because an apparently dominant-acting RPE65 mutation has been described in an Irish pedigree (42). However, the parents of the described P25L patient did not manifest any ophthalmologic symptoms (11). The degree of severity of RPE65 retinal phenotypes in both humans and mouse models is correlated with the expression levels and isomerase activities in the various mutants. While null/functional null RPE65 mutations lead to early-onset blindness in humans and light-insensitive mice in the KO and null models (12), the R91W missense mutation with residual protein expression (5% of WT) and isomerase activity (<1%) gives better early preservation of retinal structures and visual functions than null/functional null, in both human (23) and in mouse models (22). Nonetheless, severe retinal dystrophy at young ages and a progressive retinal degeneration are observed in both the null and R91W point mutation. The Rpe65P25L/P25L mutation also causes greatly impaired isomerase activity (~8% of WT in vitro) (11). However, in vivo, this residual activity is enough to mitigate the extent of retinal dystrophy in the homozygous human patient (11) and can successfully preserve normal retinal structures and visual functions in Rpe65P25L/P25L KI mice raised under relatively dim ‘normal’ light conditions. Similar to the RPE65/P25L, other missense mutations of RPE65, including L22P, E95Q and Y79H, may in turn lead to lower isomerase activity in the RPE of KI/KI mice. With respect to the lower than the expected level of expression in the heterozygote wt/KI, a possible explanation is that the mutant P25L protein can destabilize dimers (27) formed with WT protein. This apparently reduced expression, however, does not appear to have a detectable physiological effect on ERGs. This is of additional interest because an apparently dominant-acting RPE65 mutation has been described in an Irish pedigree (42).

**Phenotypic similarities between P25L KI model and human hypomorphic patient**

In the present study, we have demonstrated that the RPE65/P25L mutant has greatly reduced RPE65 protein levels in the transgenic mouse model, less than one-fifth of the WT RPE65. As the mutant mRNA level is comparable with that of WT, the decreased protein expression may be caused by either an inefficient mRNA translation, or more likely, destabilization of the mutant protein, which

![Figure 7. Rpe65^{P25L/P25L} KI mice are protected from light damage.](https://academic.oup.com/hmg/article-abstract/24/15/4417/2453037?display-pdf)
However, consistent with our observations, a number of mouse models with disruption/mutations of genes involved in the retinoid cycle exhibit less severe phenotypes than the clinical retinopathies in human patients. Such mouse models include mutations in Rdh11 (45), Rdh12 (46), Rbp1 (47) and Rgr (48). Similar to the Rpe65<sup>P25L/P25L</sup> KI model, phenotypic manifestation of these mutations may be largely dependent on the light intensities to which the animals are subject. Many of these mouse models have delayed dark adaptation kinetics upon bright light bleaching but regular scotopic and photopic ERG when raised under dim light conditions. Therefore, it appears that phenotypic discrepancies between human and mouse may reflect at least in part, the widely divergent photic environments occupied by these species. Indeed, the general lighting condition in vivaria is ~100 lux, whereas the light level of a sunny midday can reach 20 000 lux in shaded areas. The mildly impaired visual cycle that can sustain the visual system in the mouse models will most likely more adversely affect visual functions in humans with the orthologous mutations, as chromophore requirements are greater. It will be interesting to raise these mice under conditions of higher ambient light levels to investigate the chronic effects of the missense mutation on retinal structures and function. By in vivo titration of chromophore turnover, it might be possible to further determine effects on retinal physiology and cone survival/function and to determine a minimum level of chromophore turnover to maintain cone viability and function. In addition to better understanding of cone physiology, this may have further translational significance for the application and management of RPE65 gene therapy.

**Materials and Methods**

**Generation of Rpe65<sup>P25L/P25L</sup> knock-in mice**

Through RecE/RecT recombination as described (49), a BAC clone encompassing 5′ flanking sequence and exons 1–3 of mouse Rpe65 was used to construct a targeting vector in which a single nucleotide replacement from C to T was introduced into exon 2, changing codon 25 from proline to leucine. The targeting vector contained a PGK-driven DTA (modified diphtheria toxin A) cassette as the negative selection marker (50) for screening of the BAC clones containing the correct mutation (51). The selected construct was linearized and electroporated into ES cells (SV129). G418-resistant clones were picked, expanded and screened for homologous recombination by both long-range genomic PCR (see Supplementary Material, Table S1 for primer sequences) and Southern blotting with a 5′ probe outside of the targeting vector after NcoI digestion (Fig. 1A). Correctly targeted clones were further analyzed by karyotyping. ES cell clones carrying the targeted allele were isolated, and two clones were injected into blastocysts. The selectable marker (loxP-Neo-loxP) was removed by mating to Zp3-Cre (24) mice (Jackson Laboratories, Bar Harbor, ME, USA) expressing Cre recombinase. Successful excision of the selectable marker cassette was confirmed by PCR of a region spanning the loxP insertion site (see Supplementary Material, Table S1 for primer sequences).

**Quantitative RT–PCR (Q-PCR)**

Total RNA was isolated from the adult mouse eyecup using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA); 500 ng RNA was reverse-transcribed with a cDNA synthesis kit (Life Technologies, Grand Island, NY, USA). Q-PCR was performed with

**Phenotypic differences between mouse knock-in models and human patients**

Conversely, Rpe65<sup>P25L/P25L</sup> KI mice fail to recapitulate certain features of the human pathology, including that of night blindness, though we argue that the delayed recovery in KI/KI mice is a manifestation of this, and impaired sensitivity of blue cones. In respect of the latter, differences in cone abundance and distribution between primates and rodents may confound correlation of given aspects of the phenotype. However, the features in common may be more important than those that are not. The fact that cone survival and function in both the human P25L individual (11) and its mouse model are close to their respective WT situations suggests a common lower limit for survival in respect of chromophore supply. While the retinal visual cycle (43,44) supplements cone chromophore requirements in cone, supply from the ‘canonical’ RPE visual cycle is nonetheless crucial to cone survival.

**Figure 8.** Recovery of 11-cis-retinal levels in retinae and reduction in retinyl esters in RPE are slower in KI/KI mice. Dark-adapted mice were exposed to a light of 20 000 lux for 30 min and returned to the dark until retinae, and eyecups were collected for HPLC retinoid analysis at different time points. (A) Levels of 11-cis-retinal in retinae were measured at 1 and 2 h time points AFTER light exposure. (B) Retinyl ester levels in RPE layer were measured at 1 and 2 h time points after light exposure. Error bars indicate the SD of the mean (n = 4 for WT and n = 3 for KI/KI); *P < 0.05; **P < 0.001; LE: light exposure.
synthesized cDNA as a template and Taqman probe for mouse Rpe65 gene (Life Technologies). Gapdh and Hprt were used as reference genes for normalization. Q-PCR analysis was performed in biological triplicates for each genotype.

**Animals**

All procedures concerning animals were in accordance with institutional regulations and with the statement of the Association for Research in Vision and Ophthalmology for the use of animals in research and were carried out under an institutional Animal Study Protocol approved by the National Eye Institute (NEI), NIH Animal Care and Use Committee. The mice were raised in cyclic light (~100 lux, 12:12 h).

**Immunoblotting**

Mouse retinae or eyecups were dissected and solubilized in phosphate-buffered saline (PBS) supplemented with 0.15 mg/ml dodecyl maltoside and Complete protease inhibitor (Roche). Ten to twenty micrograms of protein extracts from mouse eyecup were analyzed by western blot using antibodies against RPE65 (PETLET, 1:2000), RDH5 (1:1000), RDH5 (1:1000, gift of J. Saari, University of Washington) and GAPDH (Cell Signaling, 1:1000). Five to ten micrograms of protein extracts from mouse retinae were analyzed by western blot using antibodies against RHODOPSIN (1D5, 1:10 000), OPN1MW (1:5000, gift of T. Li, NEI), OPN1SW (1:5000, gift of T. Li, NEI) and γ-Tubulin (Cell Signaling, 1:1000). Relative expression levels were quantified using the Image Studio Lite software package (LI-COR Biotechnology, Lincoln, NE, USA) on gel files imported from a Typhoon 9410 imaging system (GE Healthcare).

**Histology and immunofluorescence microscopy**

Enucleated eyeballs were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde and embedded in methacrylate. Serial vertical sections were cut through the pupillary-optic nerve plane, stained with toluidine and analyzed by light microscopy. For immunofluorescence, eyes were enucleated and fixed in 4% formaldehyde for 1–2 h. The anterior segments and lenses were removed. The fixed tissues were equilibrated in PBS for 3 h to overnight, snap-frozen and sectioned along the superior–inferior meridian at 10 µm thickness. Immunolabeling on frozen sections was performed using rabbit anti-RPE65 (1:500), goat anti-OPN1SW (1:100, Santa Cruz) and rabbit anti-OPN1MW (1:500, Millipore). Stained sections were imaged by confocal laser scanning microscopy (model TCS SP2; Leica, Wetzlar, Germany). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for nuclei undergoing apoptosis was performed following the manufacturers’ instructions on frozen sections (prepared as above) using the ApopTag® Red In Situ Apoptosis Detection Kit (EMD Millipore, Billerica, MA, USA).

**Retinoid analysis**

All procedures involving retinoids were conducted under dim red light. For analysis of neural retinas, a single mouse retina was homogenized in 0.5 ml of freshly made hydroxylamine buffer (50 mM MOPS, 10 mM NH₄OH, pH 6.5) using a disposable micro tissue homogenizer (BioMasher II, Warrington, PA, USA). The homogenate was transferred to a 15-ml polypropylene screw-cap tube, and an additional 0.5 ml of hydroxylamine buffer was added along with 1 ml ethanol. Samples were incubated for 30 min in the dark at room temperature. Following this, retinoids were extracted twice by addition of 4 ml hexane, vortexing and centrifugation (2000g, 8 min). The upper hexane phases were collected and pooled, and solvent was evaporated under argon at 37°C. The dried samples were redissolved in 100 µl hexane for analysis. Retinoloximes standards were prepared following Garwin and Saari (52). Standards and samples were separated on a LiChrospher Si-60 (5 µm; Merck, Darmstadt, Germany) normal-phase column using a 11.2% ethyl acetate, 2% dioxane and 1.4% octanol (v/v/v) in hexane mobile phase at a flow rate of 0.7 ml/min. Absorbance was monitored at 350 nm, and peak areas for all-trans and 11-cis-retinoloximes were integrated and quantified using external calibration curves. Data were analyzed using Empower 3 software (Waters Corp., Milford, MA, USA).

To measure retinyl esters in RPE, individual eyecups were homogenized in 1 ml ethanol using all-glass tissue grinders (Kontes Duali 21). Retinoids were extracted twice by addition of 5 ml hexane, vortex mixing and centrifugation. The upper hexane phases were combined, solvent evaporated and the remaining residue redissolved in 100 µl hexane. Retinoids were separated as mentioned earlier. Absorbance was monitored at 325 nm, and peak areas for retinyl ester were integrated and quantified using external calibration curves. Data were analyzed as mentioned earlier.

**Electroretinograms**

After overnight dark adaptation, the eyes of anesthetized mice were dilated with a drop of tropicamide and phenylephrine. Tetracaine (0.5%) drops were applied for local anesthesia of cornea. Body temperature was maintained at 37°C with a heating pad. Electroretinograms (ERGs) were recorded from both eyes using gold wire loop electrodes connected to an Espion e2 Visual Electrophysiology System (Diagnosys, Lowell, MA, USA). A gold wire loop placed in the mouth was used as a reference electrode. Dark-adapted ERG was performed using flashes with intensities ranging from 0.0001 to 10 sc cd.s/m². Light adaptation was performed with white light at 20 sc cd/m² for 2 min, and the ERG response was recorded using flashes with intensities ranging from 0.3 to 100 sc cd.s/m². A UV colordome connected to an Espion e2 system was used to provide Ganzfeld UV (365 nm) flashes, and electroretinogram recordings were performed on at least six mice per genotype.

For bleaching experiments, mice were dark-adapted overnight and then subjected to a moderate visual pigment bleach with the background light of a Ganzfeld chamber (1000 cd/m²) for 30 s. After the light was turned off, a single-flash ERG at 10 cd.s/m² was used to monitor recovery of a-wave amplitude every 2 min for 60 min. To analyze the data, the recovery of a-wave amplitude [A(t)] following bleaching is plotted against post-bleach time (t). The following equation was fitted to the raw data using Prism 6 (GraphPad Software, San Diego, CA, USA) (29):

\[ A(t) = \frac{A_{\text{max}}}{1 + C_a \times \exp (-t/\tau_a)} \]  

\[ (1) \]

where \( A_{\text{max}} \) denotes the fully recovered a-wave amplitude, \( C_a \) represents the degree of reduction immediately after the bleach and \( \tau_a \) is the time constant of recovery. These three parameters were calculated for each plot, and statistical significance among WT, heterozygous and homozygous KI mice was analyzed using one-way ANOVA.
Optical coherence tomography (OCT) imaging

Mice were anesthetized and their pupils dilated as described earlier. Artificial tears (Systane Ultra, Alcon, Fort Worth, TX, USA) were used to maintain corneal moisture and clarity. OCT images were obtained using the Bioptigen Spectral Domain Ophthalmic Imaging System (SDOIS, Bioptigen Envisu R2200, Bioptigen, Morrisville, NC, USA). Image acquisition software was provided by vendor. Blue light-induced fundus autofluorescence was recorded with Spectralis HRA-OCT (Heidelberg Engineering, Heidelberg, Germany).

Induction of retinal light damage

Mice were dark-adapted for 7 days before exposure to bright light. Acute retinal damage was induced by exposing WT and KI/KI animals with dilated pupils (as described earlier) to 20 000 lux of diffuse white fluorescent light for 30 min. Eyes were collected following euthanization 1 or 2 h after exposure for retinoid analyses, or, subsequent to OCT imaging, 2 weeks after exposure for histology or TUNEL staining.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We acknowledge the advice of Dr Robert N. Fariss and assistance of Dr Maria M. Campos (Biological Imaging Core, NEI), Dr Jianguo Fan (Molecular Structure and Functional Genomics Section, NEI) and of Dr Baerbol Rohrer (Medical University of South Carolina, Charleston, SC, USA). E.G. and Y.C.-P. were supported by the Diversity in Vision Research and Ophthalmology summer program of the NEI Office of the Director.

Conflict of Interest statement. None declared.

Funding

This research was supported by the Intramural Research Program of the National Eye Institute, National Institutes of Health (US Department of Health and Human Services).

References


