Increased Sensitivity to Light-Induced Damage in a Mouse Model of Autosomal Dominant Retinal Disease

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PURPOSE. To describe a sensitivity to light-induced damage associated with expression of a T17M mutant human rhodopsin (hT17M) transgene in mice, with the goal of minimizing retinal injury during the subretinal delivery of rAAV-mediated gene therapy.

METHODS. Mice were bred to express the hT17M rhodopsin transgene in a line that was hemizygous null for wild-type mouse rhodopsin (mrho+/−), and the eyes of transgenic mice and nontransgenic littermates were exposed for 2.5 minutes to unilateral illumination with fiber-optic light ranging from 5,000 to 10,000 lux. Funduscapic images were made with a handheld camera (Genesis; Kowa Company, Ltd., Tokyo, Japan). Full-field scotopic electroretinographic analysis (ERG) was performed to measure loss of retinal function. Morphometry in the light microscope was used to measure loss of rod photoreceptors. TUNEL staining and a nucleosome release assay were used to measure levels of apoptosis in retinal specimens.

RESULTS. mrho+/−;hT17M mice exhibited a sensitivity to light-induced damage that caused severe loss of a- and b-wave ERG responses. hT17M transgenic mice on the mrho+/− background were equally sensitive to light-induced damage. Histologic analysis showed a concomitant loss of photoreceptors and TUNEL labeling of fragmented DNA in rod photoreceptor cells, demonstrating that the damage occurred via an apoptotic pathway. Nontransgenic littermate mice were not affected by this exposure to light. Mice expressing an hP23H mutant human rhodopsin transgene were minimally sensitive to light-induced damage at these intensities, in comparison to hT17M mice. Treating the hT17M mice with an equivalent regimen of exposure to red light was less damaging to the retina, as measured by ERG and histology.

CONCLUSIONS. Expression of a human hT17M mutant rhodopsin transgene in mice is associated with photoreceptor apoptosis in response to moderate exposure to light. This phenotype was not observed in nontransgenic littermates or in mice expressing an hP23H mutant human rhodopsin transgene. The results suggest that elimination of the glycosylation site at N15 is associated with increased sensitivity to light-induced damage.

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Retinitis pigmentosa (RP) is the common name for a group of retinal disorders characterized by a progressive photoreceptor degeneration that culminates in loss of vision.1–3 The disease affects from 50,000 to 100,000 people in the United States and approximately 1.5 million people worldwide. Initial symptoms include night blindness and loss of peripheral vision, usually occurring during the late teens or early twenties. As the photoreceptors continue to degrade, the visual impairment progresses toward the center of the retina, eventually affecting the cone photoreceptors that are responsible for central vision in bright light conditions and resulting in the manifestation of tunnel vision, in which the visual fields of the patient constrict to <20°. The loss of vision is accompanied by visual pigment depositions in the retina for which the disease is named.4,5 In most patients, the visual fields continue to constrict until the person becomes completely blind.6

In 1990, the first ADRP-causing rhodopsin mutation was reported, consisting of a DNA mutation (CCC to CAC) that caused histidine to be substituted for proline (hp23H) at the 23rd amino acid of the protein.7 Rhodopsin has subsequently become the most extensively characterized gene associated with retinitis pigmentosa. Mutations in the rhodopsin gene account for ~10% of all reported cases of RP.8 Since the identification of the hp23H mutation, more than 130 different mutations of rhodopsin have been shown to cause the disease. Most of these mutations lead to autosomal dominant RP, and most of them are thought to cause retinal degeneration by a toxic gain-of-function mechanism.9–10

The development of animal models has been vital to the study of retinitis pigmentosa. With the prevalence of rhodopsin mutations causing RP, it is not surprising that a large number of mouse models involving targeted rhodopsin deletions and mutations have been designed. In 1998, Li et al.11 reported creation of a mouse model of ADRP expressing a human rhodopsin transgene with a threonine-to-methionine mutation at the 17th residue of the protein (hT17M). This mutation abolishes the glycosylation site at Asn15 and results in a class I RP phenotype, as characterized by Kaur and Khorana,12 that could be partially rescued by dietary vitamin A supplementation.13

We obtained the hT17M mouse line with the goal of treating the associated retinal disease with AAV-delivered ribozyme gene therapy, a strategy that led to substantial rescue in an hp23H rat model of ADRP.14–15 However, this approach proved ineffective at treating the hT17M mice because of retinal damage caused by intense fiber-optic illumination of the retina during the subretinal injection of our gene therapy vectors. Herein, we describe the physiological and histologic characteristics of light-induced retinal damage in the hT17M mouse line and contrast these findings with the results of light exposures in an hp23H mouse line, which did not show equivalent sensitivity to light-induced damage.

METHODS

Animals

The hT17M mouse line was the kind gift of Tiansen Li (Harvard University, Boston, MA). The hp23H line was generated at the University of Florida using a human genomic clone that was the gift of...
Thaddeus Dryja of Harvard University (Lewin AS, et al. IOVS 2004;45: ARVO E-Abstract 5075). Both the hT17M and hP23H mouse lines were bred to express the mutant human rhodopsin transgene on a hemizygous knockout mouse rhodopsin (mrho+/−) background.16 Some experiments were also performed on mice in which the hT17M transgene was expressed on a wild-type mouse rhodopsin (mrho+/+) background. All animal care and use adhered to the guidelines detailed in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of Genomic DNA

Genomic DNA was isolated from candidate mice (DNeasy kit; Qiagen Inc., Valencia, CA). Primer oligonucleotides used for PCR genotyping were ordered from Invitrogen (Carlsbad, CA) and were desalted but otherwise unpurified by the manufacturer. The sequences were as follows: hExon 2 sense primer: 5′-GAGTGCAACCTCTTCTAGGCA-3′, hExon 2 antisense primer: 5′-TCTCTAGTCTGGAGCCTCT-3′; mrho Exon 1 sense primer: 5′-CCAAACAGCTTGTCTCTGTCTA-3′, mrho Exon 1 antisense primer: 5′-TCTTGGCGAGCTTCTGTGACT-3′; Neo sense primer: 5′-AGAGCTCTGCTCCTGAAGCTCTGCT-3′, Neo antisense primer: 5′-AAGACTCGTCAAGAGGCGATAGAAGGCG-3′; RPE65 L450 sense primer: 5′-TCTAAAGCAGCTTCTGTAAAGC-3′, and RPE65 antisense primer: 5′-TGATCTACCTTTGTTCTTCGAC-3′.

PCR Analysis of Genomic DNA

Endogenous mouse rhodopsin genotypes were determined using mrho Exon 1 primers and primers complementary to the G418 resistance marker (Neo) present in the disrupted rhodopsin gene. Genomic DNA from mice that are wild-type at the mouse rhodopsin locus (mrho+/−) produce products with the mrho Exon 1 primers, which amplify a 270-bp fragment, whereas the Neo primers produce no product, as there is no Neo gene to amplify. Conversely, DNA from mice that are homozygous for a knockout at the mouse rhodopsin locus (mrho−/−) produce a 490-bp fragment with the Neo primers, while producing no product when amplified with the mrho Exon 1 primers. DNA from mice that are heterozygous null at the mouse rhodopsin locus (mrho+/−) produces both fragments. The presence of the human hT17M or hP23H rhodopsin transgenes was determined by PCR amplification of genomic DNA with the hExon 2 primers, which amplify a 290-bp fragment in the presence of the transgene.

The polymorphism at position 450 of the RPE65 gene was characterized by performing PCR reactions using a common downstream primer (RPE65 antisense) in combination with sense primers that differ in their 3′ nucleotide, allowing them to amplify specific sequences containing either ATG (using the RPE65 M450 sense primer) or CTG (using the RPE65 L450 sense primer) at codon 450. Tail DNA isolated from a C57BL/6 mouse was used as a control for the presence of the M450 RPE65 variant and the absence of the L450 RPE65 variant. DNA isolated from the spleen of a BALB/c mouse was used as a control for the presence of the L450 RPE65 variant and the absence of the M450 variant. A positive signal using both sets of primers indicated that the animal in question was heterozygous for the polymorphism.

Retinal Illumination

At weaning age (21–24 days), mice were removed from their parents, and their right eyes were diluted with 1% atropine (Akorn, Buffalo Grove, IL). The next morning, and again an hour before the illumination procedure, the right eyes were diluted with 1% atropine. The right eye also received a drop of 2.5% phenylephrine and 0.5% proparacaine HCl (Akorn) an hour before illumination. Immediately before exposure to light, the animals were anesthetized and again treated with a drop each of 1% atropine, 2.5% phenylephrine, and 0.5% proparacaine HCl. The right eyes of these animals then received a drop of 2.5% hypromellose (Akorn) and were illuminated with a 150-W fiber-optic light source (Southern Micro Instruments, Marietta, GA) with fiber optic arms (Schott-Fostec, Auburn, NY) at an intensity of 10,000 or 5,000 lux for a period of 2.5 minutes. Left eyes were manually covered. Light intensities were measured with a data-logging light meter (Extech, Waltham, MA).

Electoretinography

Mice were dark adapted overnight. All subsequent ERG procedures were performed under dim red light (wavelength, >600 nm), which does not activate rhodopsin. Mice were anesthetized with IP injections of xylazine (13 mg/kg) and ketamine (87 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO). The mouse corneas were anesthetized with a drop of 0.5% proparacaine HCl and diluted with a drop of 2.5% phenylephrine HCl. Measurement electrodes tipped with gold wire loops were placed on both corneas with a drop of 2.5% hypromellose to maintain electrode contact and corneal hydration. A reference electrode was placed subcutaneously (SC) in the center of the lower scalp of the mouse, and a ground electrode was placed SC in the hind leg. The mouse rested on a homemade sliding platform that kept the animal at a constant temperature of 37°C. The animal was positioned so that its entire head rested inside the Ganzfeld (full-field) illumination dome of a visual electrodiagnostic system (UTAS-E 2000; LKC Technologies, Inc., Gaithersburg, MD). Full-field scotopic ERGs were measured by 10-ms flashes at an intensity of 0.9 log cd·s·m−2 at 1-minute intervals. Responses were amplified at a gain of 4000, filtered between 0.3 to 500 Hz, and digitized at a rate of 2000 Hz on two channels. Five responses were averaged, after which the electrodes were switched to the opposite eyes for a second set of flashes; responses from these two sets were then averaged. Wave traces were analyzed using the system’s software package (LKC Technologies, Inc.). a-Waves were measured from the baseline to the peak in the cornea-negative direction; b-waves were measured from the cornea-negative peak to the major cornea-positive peak.

Funduscopy

Mice were anesthetized, and their corneas anesthetized and diluted as described earlier. Fundus photography was performed with a handheld fundus camera (Genesis; Kowa Company, Ltd., Tokyo, Japan) focused through a stereo fundus lens (Volk Super 66; Keeler, Berkshire, UK). Two photographs of each eye were generally taken to ensure a properly focused image.

Histology

Mice were euthanatized by an overdose of isoflurane (Abbot, North Chicago, IL) followed by cervical dislocation. Eyes were quickly removed, fixed overnight at 4°C in freshly made 4% paraformaldehyde, and transferred to a solution of phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM PO4, and 2.7 mM KCl [pH 7.4]). To obtain the sections used to determine the thickness of the outer nuclear layer (ONL), histologic sectioning and subsequent hematoxylin and eosin (H&E) staining were performed by the University of Florida Histology Core. This program of sectioning resulted in 12 serial sections through the entire eye. Sections that contained the optic nerve were then photographed at 20× power (Axioskop Z microscope; Carl Zeiss Meditec, Inc., Dublin, CA) equipped with a color video camera (DXC-970MD 3CCD; Sony) and a stage (MCID Elite; Imaging Research, Inc., St. Catharines, Ontario, Canada), using analysis software ( Imaging Research, Inc.) that stitched individual images together to create a tile-field composite image of the entire retina. The images were viewed (Photostar; Adobe, San Jose, CA), and a radial template overlay was used to define six equivalent and equally spaced regions of the retina. From each of these areas, the mean value from three separate ONL counts was determined, and these regional measurements were then averaged to generate a value that represented the ONL thickness of the retina, reported as rows of ONL nuclei. Statistical comparisons between the transgenic and nontransgenic values were performed to generate probabilities using the paired, one-tailed Student’s t-test feature of spreadsheet software (Excel; Microsoft, Redmond, WA).

Sensitivity to Light-Induced Damage
TUNEL Visualization of Apoptosis

Animals were killed by overdose of isoflurane, followed by cervical dislocation. Eyes were enucleated, and a small hole was placed in the cornea with a 27-gauge needle. They were then fixed overnight at 4°C in freshly made 4% paraformaldehyde. The next day, they were incubated in solutions of sucrose diluted in phosphate buffer (pH 7.4) at concentrations of 7% (2 hours at 4°C), 15% (2 hours at 4°C), and 30% (overnight at 4°C), for cryoprotection. After the final incubation, the eyes were suspended in optimal cutting temperature (OCT) compound embedding medium (Tissue Tek; Sakura Finetek, Torrance, CA) such that the cornea and optic nerve formed an axis parallel to the bottom of the mold, with the cornea to the front. The blocks were then frozen in isopentane at a temperature of −80°C. Frozen eyes were stored at −80°C. Twelve to 14 μm retinal sections were then obtained from these frozen eyes using a cryostat (Microm H550; Microm, Walldorf, Germany), with particular care taken to obtain sections around the optic nerve. Microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) were used for these sections. DNA fragmenting, a characteristic of apoptosis, was detected with a terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay. For these experiments, a cell viability kit (In Situ Cell Death Detection Kit, TMR red; Roche Applied Sciences, Mannheim, Germany) was used according to the manufacturer’s instructions.

ELISA Quantification of Apoptosis

The characteristic DNA fragmentation resulting from apoptosis can be quantified by a sandwich enzyme immunoassay using mouse monoclonal antibodies directed against DNA and histones. For this purpose, we used an ELISA kit (Cell Death Detection ELISA; Roche Diagnostics). Twenty-four hours after retinal illumination, animals were killed by overdose of isoflurane, followed by cervical dislocation. Retinas were harvested and placed into 400 μL of kit lysis buffer, kept on ice. The retinas were then homogenized for 3 seconds with a tissue homogenizer (Polytron PT 1200; Kinematica AG, Littau-Lucerne, Switzerland). The homogenate was centrifuged at 200g for 10 minutes, and 10 μL of the resultant supernatant was further diluted into 990 μL of kit lysis buffer. Twenty microliters of this final dilution was used in the assay according to the kit’s instructions.

RESULTS

Natural History of hT17M-Mediated Retinal Degeneration

Serial ERG measurements were made to determine the severity of the retinal degeneration seen in mice bred to be heterozygous null at the mouse rhodopsin locus containing the T17M mutant human rhodopsin transgene. Two litters were genotyped for this study, producing eight mrho+/− animals and eight mrho+/-; hT17M siblings. Compared with their nontransgenic littersmates, the hT17M transgenic animals exhibited a lower a- and b-wave response at every age tested (Fig. 1). The a-wave response showed particularly early erosion, as the response in transgenic mice was only half that of the nontransgenic siblings at 1 month of age. Mutant a-wave responses remained at around 50% of those of the nontransgenic animals for the next 2.5 months, when they again underwent a significant decline relative to their nonmutant siblings, to around 30% of nonmutant a-wave amplitude. The b-wave amplitudes of the transgenic animals were less severely affected, starting out at 70% to 80% percent of those in nonmutant siblings, to around 30% of nonmutant a-wave amplitude. The b-wave amplitudes of the transgenic animals were less severely affected, starting out at 70% to 80% percent of those in nonmutant siblings, to around 30% of nonmutant a-wave amplitude. The b-wave amplitudes of the transgenic animals were less severely affected, starting out at 70% to 80% percent of those in nonmutant siblings, to around 30% of nonmutant a-wave amplitude. The b-wave amplitudes of the transgenic animals were less severely affected, starting out at 70% to 80% percent of those in nonmutant siblings, to around 30% of nonmutant a-wave amplitude.
when compared with their non-hT17M littermates killed at the 6.5-month time point. hT17M mice killed and analyzed at the 4-month time point also showed a reduction in the ONL thickness.

**Sensitivity of hT17M+ Mice to Light-Induced Retinal Damage**

Repeated attempts were made to treat hT17M-mediated retinal degeneration in mice with subretinal injection of rAAV expressing either human rhodopsin-specific ribozymes or wild-type mouse rhodopsin transgenes. Analysis of treated animals showed severely reduced ERG responses in the injected eyes. At first, we suspected this reduction to be the result of injection damage, but it was observed that the ERG reduction was only seen in mice carrying the hT17M mutant rhodopsin transgene. We hypothesized that the mechanism of injection itself was detrimental to the visual response of the hT17M mutant animals and not to their nontransgenic siblings. The subretinal injection technique had two components that seemed likely candidates for the damage: the introduction of a virus-containing solution into the subretinal space and the use of bright fiber-optic light to illuminate the extremely dilated eyes of these animals during the actual procedure. Because dogs bearing a mutation that eliminates the glycosylation site at Asn2 of rhodopsin are unusually sensitive to light-induced damage, we decided to examine light as the causative agent.

To test this hypothesis, the right eyes of an experimental group consisting of nine mrho/H11001/H11002 and eight mrho/H11001/H11002;hT17M mice, aged 21 to 23 days, were illuminated as described with light of 5000 lux for 2.5 minutes. These parameters were chosen to mimic the retinal light exposure of an animal during a typical subretinal injection procedure. Scotopic electroretinographic analysis was then performed on the mice at several intervals. The results (Fig. 2A) demonstrate a significant decrease of 15% to 40% in both a- and b-wave responses at each time point in the illuminated eyes of animals containing the hT17M transgene, compared with the nonilluminated eyes (P < 0.05). Retinal illumination of this duration and intensity had no effect on nontransgenic siblings (Fig. 2A). Additional experiments using light of 10,000-lux intensity resulted in a similar loss of ERG responses in the illuminated eyes of hT17M mice (data not shown).

**TABLE 1. ONL Thickness of mrho++/hT17M Mice and Nontransgenic Littermates**

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<tr>
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<th>mrbo++ Mice</th>
<th>mrbo++/hT17M Mice</th>
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<tr>
<td>ONL thickness at 4 mo (n = 1)</td>
<td>8.8 rows</td>
<td>5.9 rows</td>
</tr>
<tr>
<td>ONL thickness at 6.5 mo (n = 7)</td>
<td>7.7 ± 0.6 rows</td>
<td>2.4 ± 0.7 rows</td>
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* P < 0.001.

**FIGURE 2.** Functional and structural effects of light-induced damage in hT17M mice. (A) Right eyes of hT17M mice (n = 8) and their non-transgenic littermates (n = 9) were exposed to 2.5 minutes of illumination with 5000-lux white light. Full-field scotopic ERG was performed at 1, 3, and 5 weeks after the exposure. Illuminated eyes of hT17M mice showed significantly reduced a- and b-wave ERG responses at all time points, compared with nonilluminated eyes from the same animals. Nontransgenic (mrho++) littermates, were not affected. (B) Tile field-mapped image of an illuminated hT17M retina shows panretinal apoptotic cell death. Arrows: TUNEL-positive ONL nuclei (*P < 0.05).
Apoptosis in hT17M+ Retinas Damaged by 5000 Lux Illumination

Light-induced damage to the retina has been extensively studied, and is associated with a cascade of events culminating in apoptosis of photoreceptor cells. It is cell death, rather than loss of function, that is responsible for the depressed ERG responses observed in light-damaged animals. To test whether apoptotic cell death was the cause of the ERG reduction that was seen in the retinal illumination experiments, two mrho−/− and three mrho−/−:hT17M mice were subjected to 5000-lux illumination for 2.5 minutes, as described previously. The animals were killed after 24 hours, and their eyes were fixed and sectioned. Sections containing the optic nerve were then TUNEL stained for apoptotic cells, with DAPI (4′,6-diamino-2-phenylindole) counterstain to reveal retinal morphology. The results show evidence of considerable photoreceptor cell death in the illuminated retinas of the animals containing the hT17M transgene, but not in the retinas of their nontransgenic littermates (Fig. 3A).

To document the extent of photoreceptor death, panretinal images were obtained from the TUNEL-stained sections using tile field mapping of 20× images of a section through the entire retina. The results showed that the photoreceptor damage (TUNEL-positive nuclei) was widespread, rather than tightly localized (Fig. 2B).

Sensitivity of hT17M+ Mice to Retinal Damage from Fundus Photography

Funduscopic examination is one of the most common ophthalmic procedures, often performed as part of a complete eye examination, to detect and evaluate symptoms of eye disease. The characteristic bone spicule deposits associated with retinitis pigmentosa are among the funduscopic indicators that a patient presenting with reduced visual fields and impaired night vision has RP. During this procedure, the patient’s iris is dilated, and the back of the retina is visualized with a bright white light. Photographs of the retina must be taken with intense flashes of light to record the images. It has been demonstrated that funduscopic examination damages the retinas of dogs containing a T4R rhodopsin mutation.

To determine whether funduscopic photography of the retinas of mice carrying the hT17M human mutant rhodopsin transgene is harmful, eight mrho+/+ mice, four with the hT17M transgene and four that were nontransgenic, had two fundus photographs taken of their right eyes at 3 and 6 weeks of age. One week after each set of photographs, electroretinography was performed as described and the results averaged and plotted. These ERG recordings showed clearly depressed a- and b-wave amplitudes (a-waves were depressed by 35% and b-waves by 15%; P < 0.05) after the first set of photographs) in the hT17M transgenic mice after both sets of funduscopic photography, whereas their nontransgenic littermates were unaffected (Fig. 4).

It seemed reasonable to assume that the depression of ERG response in hT17M transgenic mice after fundus photography would be accompanied by photoreceptor apoptosis, as was noted with the animals damaged by low-intensity fiber-optic illumination. To confirm this, two mrho+/− littermate mice, one containing the hT17M mutant rhodopsin transgene and one that was nontransgenic, were subjected to fundus photog.
were not affected (\( *P < 0.05 \)).

**FIGURE 4.** Functional effects of light-induced damage from fundus photography in hT17M mice. Right eyes of hT17M mice \((n = 8)\) and their nontransgenic littermates \((n = 4)\) received two fundus photographs at 5-week intervals. Left eyes were not photographed. Full-field scotopic ERG was performed 24 hours after each photograph. Photographed eyes of hT17M mice showed reduced a- and b-wave amplitudes after photography when compared with nonphotographed eyes from the same animals. Nontransgenic \((\text{mrho}^{+/+})\) littermates were not affected (\( *P < 0.05 \)).

**FIGURE 5.** hP23H mice exhibit minimal sensitivity to light-mediated retinal damage. Right eyes of hP23H mice \((n = 8)\) and their nontransgenic littermates \((n = 5)\) were exposed to 2.5-minute illumination with white light of 10,000-lux intensity. Full-field scotopic ERG was performed at 1, 3, and 5 weeks after illumination to determine the effect of the light exposure on their visual response. These data are summarized in Figure 5.

Although there were significant depressions of a- and b-wave ERG amplitudes in the hP23H transgenic mice after illumination \((P < 0.05 \) at 1 and 3 weeks after illumination\), the reduction of a- and b-wave responses was approximately 15% and 8%, respectively, at 1 week after illumination, compared with \(~39\%\) and \(~30\%\) reduction of a- and b-waves, respectively, in the hT17M transgenic mice. Furthermore, the hT17M mice continued to exhibit reduction in a- and b-wave ERG responses at 5 weeks after illumination, whereas the hP23H transgenic mice showed contralateral a- and b-wave amplitudes that were virtually identical at this time point. These results suggest that the hP23H mice are at most minimally sensitive to light-induced damage at this light intensity, though, as noted, a different hP23H transgenic line shows increased sensitivity to a more extreme light-exposure regimen.

To confirm this result, histology and TUNEL staining were performed as described previously. The right eyes of four \(\text{mrho}^{+/+};\text{hP23H}\) mice and one \(\text{mrho}^{+/+}\) littermate were illuminated with 10,000 lux fiber-optic light for 2.5 minutes. hP23H transgenic mice exhibited no increase in apoptosis in their illuminated eyes, either compared with their own nonluminated left eyes, or the illuminated right eye of a nontransgenic littermate (Fig. 3C). To confirm that the TUNEL assay was appropriately sensitive, a control \(\text{mrho}^{+/+}\) retina was treated with DNase before TUNEL staining (Fig. 3C, +DNase). These results support the conclusion that hP23H transgenic mice are not as sensitive to retinal light-induced damage as the hT17M transgenic line.

**Red Filters Reduce Light-Induced Retinal Damage**

To protect hT17M transgenic mice from light-induced damage during injections, we placed a red photographic filter over the light source. To ensure that the filters actually filtered out light below 600 nm, the transmittance spectrum was determined.
The results indicated that these filters efficiently excluded light with wavelengths below 600 nm (data not shown). In addition, the transmittance cutoff was a sharp, almost vertical line, as opposed to a gradual curve up to a 600-nm cutoff, demonstrating that these filters only pass a small amount of light with wavelengths immediately below 600 nm that can overlap with the rhodopsin absorbance spectrum (and thus activate rhodopsin).

The intensity of light transmitted through the filters when the source was turned to full power was 5000 lux. Therefore, using the red filters at the full-power setting created illumination conditions that were similar to the unfiltered illumination that led to retinal damage (Figs. 2, 3), with the exception that the light contained only wavelengths greater than 600 nm. A large number of animals were used in these experiments to ensure that any protective effect seen from light filtering was reproducible and statistically significant. Thirty-four mice were exposed to 5000-lux light passed through the 600-nm filters. The animals underwent ERG analysis at 1, 3, and 5 weeks after illumination, and the average a- and b-wave maximum amplitudes at the hT17M and non-hT17M sets were plotted and compared.

The results, shown in Figure 6, demonstrate that the 600-nm filters provided substantial protection against light-induced damage in the hT17M transgenic mice. Although there was a statistically significant reduction in a- and b-wave amplitudes at 1 and 3 weeks after illumination (P < 0.05), which averaged 13% to 17% for the a-waves and 6% to 9% for the b-waves, these effects were not as severe as those previously observed after 5000-lux illumination with unfiltered light in the same line (Fig. 2), which averaged greater than a 30% reduction in a-wave responses and around a 20% reduction in b-wave responses. The differences between treated and untreated eyes were also transient: at the 5-week ERG measurement, the right and the left eyes of the hT17M transgenic animals showed virtually identical a- and b-wave ERG responses.

To ensure that the protection seen at the ERG level was mirrored by a corresponding lack of apoptosis in rod photoreceptors, we performed histologic sectioning and TUNEL labeling. Five mrmho+/−:hT17M mice and 1 mrmho+/− littermate underwent 2.5-minute right eye illumination with 5000 lux of 600-nm filtered light, as just described. A day later, the animals were killed and their eyes fixed and sectioned, and sections containing the optic nerve were TUNEL labeled with DAPI counterstain, as described, to identify photoretinal photoreceptors. The results of this experiment, illustrated in Figure 3D, demonstrate that 600-nm illumination resulted in a less-substantial induction of photoreceptor cell apoptosis in the illuminated right eyes of hT17M transgenic mice.

Quantification of Light-Induced Apoptosis
To quantify the protective effect of the red filter and to measure the extent of light-induced apoptosis in the entire retina, we used a sandwich ELISA assay that measures nucleosome release, a hallmark of cell death via apoptosis. The right eyes of mrmho+/−:hT17M (6 transgenic, 10 nontransgenic) and mrmho+/−:hP23H (8 transgenic, 4 nontransgenic) mouse cohorts were exposed to 2.5 minutes of white fiber-optic light at 5000 lux. Left eyes were not illuminated. Twenty-four hours later, the animals were killed and their retinas removed and assayed for nucleosome release. The results, which are summarized in Table 1 and illustrated in Figure 6, show that this illumination resulted in an eight-fold increase in apoptosis in the mrmho+/−:hT17M animals compared with their nontransgenic (mrmho+/−) littermates, whereas mrmho+/−:hP23H mice were only mildly affected. This experiment was also performed on an hT17M line that was bred to be wild-type at the mouse rhodopsin locus (mrmho+/+). Five transgenic and two nontransgenic mice were used. The results, also illustrated in Figure 7, indicate that mrmho+/−; hT17M animals exhibited a sensitivity to light-induced damage that was similar to that of the mrmho+/−:hT17M mice. In contrast, the mrmho+/−:hP23H mice exhibited only a 1.5-fold increase in apoptosis compared with the nontransgenic littermates. In addition, this assay was used to quantify the rescue from light-induced damage provided by red filtration. The right eyes of a cohort of six hT17M transgenic and four nontransgenic littermate mice were exposed to red-filtered light of 5000 lux for 2.5 minutes. Red-filtered light caused a threefold increase of apoptosis in the retinas of hT17M animals, in comparison with their nontransgenic littermates (Fig. 7). Although this does not show total protection from light-induced damage, it is an improvement compared with unfiltered light exposure.

Contribution of the Rpe65 L450M Polymorphism to Light-Mediated Damage
The L450M variant of the Rpe65 gene is known to confer increased resistance to light-induced photoreceptor damage in mice. To ensure that the damage we observed is not related to the Rpe65 genotype, we developed a PCR assay to determine which Rpe65 variant was present in the various lines used for the nucleosome release assay described earlier (data not shown). The mrmho+/−:hT17M line was homozygous for the light-sensitive, L450 Rpe65 variant, whereas the mrmho+/−; hT17M and mrmho+/−:hP23H lines were heterozygous for the polymorphism, containing one L450 and one M450 Rpe65 allele. Within all three lines, both transgenic mice and their nontransgenic littermates were identical with respect to this polymorphism. Since neither the nontransgenic mice from either of the hT17M lines, nor any of the mice from the hP23H lines exhibited significant retinal damage from our light-exposure regimen, we conclude that the intensity and duration of
light used for these experiments was below the threshold required for damage associated with the L450 variant of the Rpe65 gene.

**DISCUSSION**

Transgenic mice carrying the human T17M rhodopsin gene showed significant reduction in both a- and b-wave amplitudes of eyes that were illuminated with either 5,000 or 10,000 lux of white light for a period of 2.5 minutes. This reduction was seen as early as 1 week and persisted for at least 5 weeks after illumination. Nontransgenic mrho+/− littermate mice were not affected by this light exposure, and mice expressing a rhodopsin transgene containing an hP23H mutation were only minimally affected. Light exposure due to fundus photography of the hT17M mutant mice also led to a reduction of ERG amplitude in the photographed eyes, whereas, again, the nontransgenic mice were unaffected. Both TUNEL analysis of retinal sections and ELISA analysis of dissected retinas from mutant mice exposed to retinal illumination revealed that there was significant photoreceptor apoptosis as early as 24 hours after illumination. These assays revealed almost no apoptosis in nontransgenic littermates or in mice expressing an hP23H mutant rhodopsin transgene that were subjected to identical retinal illumination. Our observations support the conclusion that the hT17M mutant rhodopsin transgene confers significant light sensitivity to mice expressing it.

Light-mediated retinal damage (LMD) has been studied extensively. Although there are many different theories as to the exact mechanism of LMD, all share two central points: first, rhodopsin is the initial mediator of the damage,21−23 and, second, apoptotic cell death is the ultimate fate of the affected photoreceptors.24 Apoptotic cell death is also a central event in the retinal degeneration in patients who have ADRP arising from mutations in the rhodopsin gene. This knowledge has led researchers to study the pathways involved in light-mediated photoreceptor apoptosis, with the goal of achieving a better understanding of apoptotic photoreceptor death in patients who have rhodopsin-mediated ADRP.

Hao et al.18 reported evidence of at least two apoptotic pathways involved in light-mediated retinal degeneration. One, termed the “acute” pathway, was induced by 10-minute illumination of BALB/c mice with white light at an intensity of 5000 lux and was shown to be independent of transducin activity, meaning that the phototransduction cascade was not necessary for the induction of apoptosis observed in photoreceptor layers of these mice. This result, coupled with the fact that mice deficient in both rhodopsin kinase and arrestin, which are involved in the inactivation of rhodopsin, are extremely sensitive to acute light exposure, led Hao et al. to reason that the “acute” pathway is caused by activated rhodopsin or its photobleached products. This type of apoptosis was also found to be dependent on expression of the transcription factor AP-1. A second light-induced damage pathway, termed the “low-intensity” pathway, was noted in animals with defects in either arrestin or rhodopsin kinase. These mice were shown to undergo retinal degeneration after prolonged exposure to normal room light. In contrast to the “acute” pathway, transducin activity was central to the “low-intensity” apoptotic pathway, and mice lacking a functional transducin gene were protected from this form of LMD. In the low-intensity pathway, photoreceptor cell apoptosis did not require the induction of AP-1.

The mature form of rhodopsin is glycosylated at Asn2 and Asn15. It has been demonstrated in isolated frog retinas that inhibition of rhodopsin glycosylation with tunicamycin leads to defects in the morphogenesis of photoreceptor outer segments.25 Similar studies suggest that rhodopsin must be glycosylated, for it to be properly incorporated into nascent disc membranes.26 More recently, a naturally occurring dog model of RP that contains a T4R rhodopsin mutation has been described. This mutation disrupts glycosylation at Asn2 but does not affect the site at Asn15.27 These dogs are extremely sensitive to light-mediated retinal damage and exhibit a fourfold increase in AP-1 after acute exposure to light from a fundus...
camera. Fundus photography in this model resulted in rapid and severe retinal degeneration restricted to the illuminated area of the retina. The T4R the mutant protein is less stable, and releases all retinal faster after bleaching than normal rhodopsin. Light exposures that lead to bleaching also cause retinal degeneration in T4R dogs. With this in mind, it is important to note that the T17M rhodopsin mutation abolishes glycosylation at the Asn15 site of rhodopsin. It seems reasonable to postulate that inhibition of rhodopsin glycosylation by the T4R and T17M mutations results in a protein that is uniquely affected by exposure to light, leading to photoreceptor cell death. Mutations at both of these residues cause retinal degeneration predominantly in the lower hemisphere, consistent with light-induced damage from overhead sources. It is possible that exposure to light increases the rate of aberrant disc morphogenesis in these animals, which might be expected to result in photoreceptor cell death. In any event, the link between the loss of rhodopsin glycosylation and sensitivity to light-mediated photoreceptor cell death is worthy of further investigation.

Numerous attempts have been made to overcome light-induced apoptosis with pharmacological agents (for an excellent review, see Wenzel et al.). Dexamethasone treatment has been shown to lower AP-1 levels and protect against light-induced damage in the “acute” model of Hao et al. and Wenzel et al. Administration of halothane anesthesia, which has been shown to limit rhodopsin regeneration after phototoxication, is also protective against light-mediated damage in albino mice and rats. In 2001, Gao et al. reported the protective effect of intravitreal injection of pigment epithelium-derived growth factor and basic fibroblast growth factor in albino Sprague-Dawley rats. Although many agents are known to prevent the retinal degeneration induced by light-induced damage, only PEDF (pigment epithelium-derived factor) has been shown also to be of benefit in inherited retinal degenerations of the rat and mouse. Discovering a pharmacological intervention that is capable of preventing light-induced damage in the T17M mouse line could therefore not only provide a greater understanding of the underlying mechanism of damage in this specific case, but could also lead to the identification of novel drug therapies for RP in general. Mutation-specific, light-induced damage should be of concern to clinicians advising and treating patients with retinitis pigmentosa. Funduscopic examination is one of the simplest and most common of all ophthalmic techniques, and the data reported herein indicate that it is likely that this type of examination could result in retinal illumination with intensities of white light that could damage the retinas of RP patients with certain rhodopsin mutations. Ophthalmic surgery can also involve ocular exposure to intense white light of the type shown in this study to cause retinal damage and exacerbation of RP-associated photoreceptor degeneration. Because of the heterogeneous nature of RP and the various rates with which it affects even different patients with the same rhodopsin mutations, it would be easy to conclude that a large decline in visual acuity reported by an person with RP after ophthalmic procedures involving intense retinal illumination is the result of the patient’s having a rapid-onset form of the disease, rather than damage incurred from the procedure. Future studies designed to determine whether patients with RP caused by certain extremely light-sensitive rhodopsin mutations sustain greater retinal damage from such procedures than patients with other rhodopsin mutations seem warranted.

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References


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