Mutations of the Opsin Gene (Y102H and I307N) Lead to Light-induced Degeneration of Photoreceptors and Constitutive Activation of Phototransduction in Mice*

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Mutations in the Rhodopsin (Rho) gene can lead to autosomal dominant retinitis pigmentosa (RP) in humans. Transgenic mouse models with mutations in Rho have been developed to study the disease. However, it is difficult to know the source of the photoreceptor (PR) degeneration in these transgenic models because overexpression of wild type (WT) Rho alone can lead to PR degeneration. Here, we report two chemically mutagenized mouse models carrying point mutations in Rho (Tvrm1 with an Y102H mutation and Tvrm4 with an I307N mutation). Both mutants express normal levels of rhodopsin that localize to the PR outer segments and do not exhibit PR degeneration when raised in ambient mouse room lighting; however, severe PR degeneration is observed after short exposures to bright light. Both mutations also cause a delay in recovery following bleaching. This defect might be due to a slower rate of chromophore binding by the mutant opsins compared with the WT form, and an increased rate of transducin activation by the unbound mutant opsins, which leads to a constitutive activation of the phototransduction cascade as revealed by in vitro biochemical assays. The mutant-free opsins produced by the respective mutant Rho genes appear to be more toxic to PRs, as Tvrm1 and Tvrm4 mutants lacking the 11-cis chromophore degenerate faster than mice expressing WT opsin that also lack the chromophore. Because of their phenotypic similarity to humans with B1 Rho mutations, these mutants will be important tools in examining mechanisms underlying Rho-induced RP and for testing therapeutic strategies.

Rhodopsin is a light sensitive G-protein-coupled receptor composed of a membrane-bound opsin, encoded by the rhodopsin gene (Rho), and a covalently bound, light-sensitive chromophore, 11-cis-retinal. Upon light exposure, 11-cis-retinal is isomerized to all-trans-retinal, which induces a conformational change in rhodopsin to yield its active form, metarhodopsin II (R*) (2). R* is deactivated via phosphorylation by rhodopsin kinase and binding to arrestin. In parallel, all-trans-retinal is released from R* and recycled through the visual cycle, to form 11-cis-retinal, which regenerates rhodopsin in the rod outer segment. The release of chromophore from R* can also lead to high levels of free opsin in the retina. Free opsin can activate the phototransduction cascade, albeit at a lower rate than R*, and can potentially lead to constitutive activation of transduction after it is phosphorylated and forms a complex with arrestin (1, 2), a phenomena associated with photoreceptor degeneration (1).

The maintenance of rod photoreceptors is critically dependent on normal levels of rhodopsin. Rod degeneration is observed in Rho−/− mice (3–5) and the human disorder retinitis pigmentosa (RP) caused by Rho mutations is characterized by progressive rod degeneration (6). More than 100 point mutations in rhodopsin collectively account for ~25% of autosomal dominant RP as well as some forms of autosomal recessive RP (7, 8). These observations have spurred the development of transgenic lines expressing comparable rhodopsin point mutations (9–13). Although these models are a valuable resource, rod degeneration is also observed in mice overexpressing wild type (WT) rhodopsin (14). It is, therefore, difficult to know with certainty whether rod degeneration observed in transgenic lines reflects the effects of the introduction of rhodopsin point mutations or the overexpression of rhodopsin.

Phenotypically, mutations in Rho are divided into two classes (8, 15). Class A mutations cause rapid rod degeneration, leading to early onset night blindness in patients (15). Patients with class B mutations display a slower disease progression, and may be further subdivided into class B1 and B2 (15), differentiated

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* This work was supported, in whole or in part, by National Institutes of Health Grant CA-34196 from the NCI, grants from the Maine Institute of Human Genetics & Health (to P. M. N.), Foundation Fighting Blindness (to P. M. N.), National Institutes of Health Grant EO16510 to (P. M. N.), Research to Prevent Blindness (to N. S. P.), Prevent Blindness Ohio (to M. S.), the Medical Research Service Department of Veterans Affairs (to N. S. P.), Hope for Vision (to N. S. P.), EyeSight Foundation of Alabama (to A. K. G.), the Karl Kirchgessner Foundation (to A. K. G.), and National Institutes of Health Grant EY07981 to T. G. W. J.

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2 The abbreviations used are: R*, metarhodopsin II; TVRM, translational vision research models; TUNEL, terminal uridine deoxynucleotidyl transferase dUTP nick end labeling; ERG, electroretinogram; ISI, inter-stimulus interval; Bistris propanoate, 1,3-bis[(tris(hydroxymethyl)methylamino)propane; ANOVA, analysis of variance; RP, retinitis pigmentosa; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid; HPLC, high pressure liquid chromatography; GTPγS, guanosine 5′-3-O-(thio)triphosphate.
by whether rod degeneration is focal (class B1) or panretinal (class B2). The class B1 patients also exhibit impaired deactivation of phototransduction after exposure to high intensity light flashes (15, 16).

Despite the importance of Rho mutations in human RP, the different classes of disease causing mutations and intense efforts to identify useful models of retinal disease in mice (17), only two models expressing alleles of Rho mutations under endogenous control have been identified. The Noerg1 mouse model harbors a C110Y point mutation that results in early onset and rapidly progressing rod degeneration (18). The T4R canine model develops a phenotype that closely resembles RP patients with class B1 Rho mutations (19). From this model, we have learned that light exposure plays a significant role in accelerating the disease phenotype (16). An allelic series of models with the different rhodopsin mutations implicated in human disease would be extremely useful in gaining insights into the many rhodopsin residues that are required for rhodopsin signaling or are modified post-translationally to modulate its function.

In this study, we report two new mouse models identified through screening of cohorts of chemically mutagenized mice by indirect ophthalmoscopy in the Translational Vision Research Models (TVRM) program sited at The Jackson Laboratory. The mice carry either the Y102H (Tvrm1) or I307N (Tvrm4) point mutations in the Rho gene. Under standard housing conditions, neither model develops significant rod degeneration. Interestingly, however, in both Tvrm1 and Tvrm4 mutants, even brief exposure to high intensity light leads to rapid rod degeneration, providing a phenotypic link to humans with class B1 Rho mutations. These light-inducible Rho models will be important not only for elucidating the functional domains within rhodopsin, but also for examining the mechanisms underlying the pathological changes observed and for testing therapeutic strategies to delay or prevent visual impairment.

**Materials and Methods**

*Animals*—All procedures used in animal experiments were approved by Institutional Animal Care and Use Committees of the institutions involved and were in accordance with the procedures of the Association for Research in Vision and Ophthalmology. Tvrm1 and Tvrm4 mice were identified in cohorts of mice chemically mutagenized by the Neuroscience Mutagenesis Facility or the embryonic stem cell mutagenesis program of Dr. John Schimenti. For Tvrm1, male C57BL/6J (B6) mice were mutagenized with ethynitrosourea administered in 3 weekly injections of 80 mg/kg by the Neuroscience Mutagenesis Facility program. G3 offspring, generated using a three-generation backcross mating scheme (20), which were previously screened for neurological phenotypes, were re-screened by indirect ophthalmoscopy in the TVRM program. Tvrm4, generated by chemical mutagenesis of F1 hybrid 129/SvJae × C57BL/6J embryonic stem cells and a subsequent two generation backcross mating scheme (21), was moved onto the B6 background for a minimum of 5 backcross generations, the point at which ~3.75% of unlinked genomic regions are from the 129 strain. Rd12 mice (MGI 005379) were acquired from the Eye Mutant Resource, The Jackson Laboratory. A.B6-Tyr<sup>+</sup> and C3A.BLiA-Pde6b(+/+) mice that do not carry the rd1 mutation) were used for Tvrm1 and Tvrm4 mapping crosses, respectively. The F1 progeny were backcrossed to the WT parental strain and the resultant backcross progeny were used for mapping purposes. All mice were bred and maintained under standard conditions in the Research Animal Facility at The Jackson Laboratory with a 12:12 h dark:light cycle (0–35 lux, and once a week the mice were exposed to 440 lux during box changes).

*Mapping*—Mice generated from the backcrosses were phenotyped by indirect ophthalmoscopy ~24 h following light exposure to 12,000 lux for 5 min. DNA was prepared from tail snips using proteinase K digestion and isopropyl alcohol extraction. DNA isolated from 252 Tvrm1 and 150 Tvrm4 backcross progeny were genotyped by microsatellite markers to develop fine structure maps of the regions encompassing these mutations.

*Sequencing*—Total RNA was isolated from whole eyes of Tvrm1, Tvrm4, C57BL/6J, and 129/1 mice using TRIzol Reagent (Invitrogen) per the manufacturer’s protocol. Total RNA was treated with RNase-free DNase I (Ambion) and quantified with a NanoDrop (ND-1000) spectrophotometer. RNA quality was evaluated using an Agilent Technologies 2100 Bioanalyzer. cDNA was made using the Reversekit script (Ambion). Two overlapping primer sets were used to amplify and sequence the entire coding region of the rhodopsin gene, amino-terminal portion: Rho-F1, GTC AGT GGC TGA TGT GGT CAT CTT CTT CCT GAT CTG CTG GC; carboxy-terminal portion: Rho-F2, GCC CAT CTC AGG GTA CCT GGA; carboxyl-terminal portion: Rho-F3, GCC TGT GGG CCC AAA GAC GAA GCA, and Rho-F4, GCC GCC TGC ATG ACC TCA TCC CAA. Reverse transcription-PCR was done using eye cDNA in a 24-μl PCR containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 250 μM each of dATP, dCTP, dGTP, 0.2 μM forward and reverse primer, 1.5 mM MgCl<sub>2</sub>, and 0.6 units of Taq polymerase.

PCR products were electrophoresed on a 1% agarose gel and visualized using ethidium bromide staining. DNA fragments were sequenced on an Applied Biosystems 3730XL Sequencer (using a 50-cm array and POP7 polymer).

*Genotyping*—Allele-specific genotyping protocols were developed for both mutations. The following primer sets are used for PCR amplification of Tvrm1: Tvrm1, forward, AAC TTC CTC ACG CTC TAC GTG ACC GT; Tvrm1, reverse 1, ACA GCC TGT GGG CCC AAA GAC GAA GCA, and Tvrm1, reverse 2, CAA AGA AGC CCT GAT GTG ACC CTG TGG GCC CAA AGA CGA GGT G; and Tvrm4, reverse, GGT CAT CTT CCT CCT GAT CGT CGC TGG GCC CAA AGA CGA GGT G; and Tvrm4, forward, GGT CAT CTT CCT CCT GAT CGT CGC TGG GCC CAA AGA CGA GGT G; and Tvrm4, reverse, GCC CAG GCA CCT GCT TGT TCA ACA TCA, and Tvrm4, reverse 2, CTC CAC ACG CCC TGC CTC A; CCA GCC ACC TGC TTG TTC AAC TTT T. The following PCR cycling programs were used: 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension of 72 °C for 2 min. PCR products were electrophoresed on 4% Metaphor agarose gels and visualized using ethidium bromide staining.

*Ophthalmic Examination and Fundus Photography*—Eyes of dark-adapted mice were dilated with one drop of 1% atropine and examined with an indirect ophthalmoscope with a 78 or 90 diopter aspheric lens. Fundus photographs were taken with a
The membrane was stained with a nitrocellulose membrane. The membrane was electrophoresed on a 12% SDS-PAGE gel and transferred to a protocol (ApopTag TUNEL staining kit, Chemicon). deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used on paraffin sections according to the manufacturer's protocol (ApopTag TUNEL staining kit, Chemicon). For detection of cells undergoing apoptosis, terminal uridine lar Probes, 1:200) and visualized by fluorescence microscopy. with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, 1:100, anti-goat) and subsequently labeled mouse) and subsequently labeled with Cy3-conjugated secondary antibody (Leinco Technologies, 1:500, anti-rhodopsin antibody (Leinco Technologies, 1:100, anti-goat) and subsequently labeled with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, 1:200) and visualized by fluorescence microscopy. For detection of cells undergoing apoptosis, terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used on paraffin sections according to the manufacturer's protocol (ApopTag TUNEL staining kit, Chemicon).

**Western Blot Analysis**—Total protein from WT, Rho<sup>Term</sup>, and Rho<sup>Term+</sup> mice was isolated by standard methods in RIPA buffer (1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate in phosphate-buffered saline) containing a protease inhibitor (Roche Applied Science). Twenty μg of total protein was electrophoresed on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was stained with anti-rhodopsin antibody (Sigma) and anti-β-actin (Sigma) for loading control. All bands were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies followed by the ECL detection system (PerkinElmer Life Sciences). The Western blots were imaged using a LAS-1000 (Fuji) and the intensity of each band was quantified using ImageGauge (Fuji).

**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 pupils (1% tropicamide, 2.5% phenylephrine HCl, 1% cyclopentolate HCl). Mice remained on a temperature-regulated heating pad throughout the recording session. ERGs were recorded with a stainless steel loop that made contact with the corneal surface through a thin layer of 0.7% methylcellulose. Needle electrodes placed in the cheek and the tail served as reference and ground leads, respectively. Responses were differentially amplified (0.05–1500 Hz), averaged, and stored using a signal averaging system (UTAS E-3000; LKC Technologies, Gaithersburg, MD).

To examine different aspects of retinal function, three types of recording sessions were conducted. The first examined standard ERG intensity-response functions (23). Dark-adapted responses were recorded first using flash intensity that ranged from −3.6 to 2.1 log candles s/m². Stimuli were presented in order of increasing intensity, and the number of successive responses averaged together decreased from 20 for low-intensity flashes to 2 for the highest intensity stimuli. The duration of the inter-stimulus interval (ISI) increased from 4 s for low-intensity flashes to 90 s for the highest intensity stimuli. A steady rod-desensitizing adapting field (1.5 log cd/m²) was then presented within the Ganzfeld bowl. After allowing a 7-min period of light adaptation, cone ERGs were recorded to flashes superimposed on an adapting field. Flash intensity ranged from −0.8 to 1.9 log cd s/m², and responses to 100 flashes presented at 2.1 Hz were averaged at each intensity level.

The second recording session was used to measure rod photoreceptor response recovery by varying the ISI between pairs of high-intensity (2.1 log cd s/m²) flashes that were presented in darkness (24). Six trials were run for each mouse, in which the ISI between the first (conditioning) flash and the second (probe) flash was decreased from 64 to 2 s in octave steps. Mice were dark-adapted between stimulus pairs for 5 min.

The third recording session was used to monitor bleaching recovery, using the protocol developed by Kim et al. (25). After a dark-adapted (baseline) response was measured, mice were exposed for 3 min to a high intensity (500 cd/m²) bleaching field. After the bleaching field was extinguished, an initial response was measured at 10 s and responses were then measured at 5-min intervals thereafter.

The amplitude of the a-wave was measured 8 ms after flash onset from the prestimulus baseline. Amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if no a-wave was present, from the prestimulus baseline.

**Retinoid Analysis**—Whole mouse eyes were grouped according to treatment and genotype (12 per group), but the identities of groups were masked during analysis. Retinoids were extracted from whole mouse eyes in pools of three eyes (four separate samples for each genotype and condition) under dim red light as described (26). Briefly, the mouse eyes were placed in a solution of 0.43 ml of MOPS buffer containing 50 μM MOPS, pH 6.8, 40 μM NH<sub>2</sub>OH, and 1 ml of methanol and homogenized with a Tekmar Tissumizer homogenizer for 20–30 s. Each sample was incubated at room temperature for 30 min to form the oximes. After the addition of 0.57 ml of 50 mM MOPS, pH 6.8, 40 mM NH<sub>2</sub>OH, the sample was extracted from whole mouse eyes in pools of three eyes (four separate samples for each genotype and condition) under dim red light as described (26). Briefly, the mouse eyes were placed in a solution of 0.43 ml of MOPS buffer containing 50 μM MOPS, pH 6.8, 40 μM NH<sub>2</sub>OH, and 1 ml of methanol and homogenized with a Tekmar Tissumizer homogenizer for 20–30 s. Each sample was incubated at room temperature for 30 min to form the oximes. After the addition of 0.57 ml of 50 mM MOPS, pH 6.8, 40 mM NH<sub>2</sub>OH, the sample was extracted with 2 ml of dichloromethane and vortexed and subsequent centrifugation at 3,000 × g for 5 min at 4 °C. The organic phase was collected and the aqueous phase was extracted three more times with dichloromethane. The extracts were pooled and dried under an argon stream. The samples were dissolved with 300 μl of hexanedione (100:6, v/v) and filtered through an Acrodisc 0.45-μm syringe filter.

Retinoid standards, retinyl palmitate, all-trans-retinal, and all-trans-retinol were purchased from Sigma and 11-cis-retinal was provided from Dr. Rosalie K. Crouch (Medical University of South Carolina, Charleston, SC) and the National Eye Institute of the National Institutes of Health. Oxime standards were synthesized as described (27). The retinoid concentrations were determined by the absorbance spectra and extinction coefficients in hexane (28) (in units of M<sup>−1</sup> cm<sup>−1</sup>): retinyl palmi-
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tate $e = 49,256$ at 326 nm; all-trans-retinal $e = 47,996$ at 368 nm; 11-cis-retinal $e = 26,355$ at 362 nm; all-trans-retinal oxime $e = 55,500$ at 357 nm; 11-cis-retinal oxime $e = 35,900$ at 347 nm; and all-trans-retinol $e = 51,800$ at 325 nm.

The samples were analyzed with a Shimadzu dual-pump (LC-20AD) gradient HPLC system and a system of hexane (solvent A) versus 1,4-dioxane (solvent B) with a flow rate of 1.0 ml/min. A 100-$\mu$L volume of each sample was injected into a normal phase column (Supelco, Ascentis Si, 15 cm $\times$ 4.6 mm, 3 $\mu$m) pre-equilibrated with hexane by an automatic sampler (SIL-20A, Shimadzu). Retinoids were eluted with a linear gradient of 0–25% dioxane in 20 min. Retinyl ester and retinol were monitored by the absorbance at 325 nm using a UV-visible photodiode array detector (RF-10AXL, Shimadzu) both interfaced to a computer (LCsolution software; Shimadzu). 11-cis Retinal, all-trans-retinal, all-trans-retinol, 11-cis-retinal oxime and all-trans-retinol oxime were monitored by the absorbance at 362, 368, and 350 nm, respectively, using the UV-visible photodiode array detector. The area of the retinoid peak was calculated and compared with the area versus mass curve for the standard. Amounts were determined from the integrated peaks by comparison to the standard curves, and results for each group were averaged.

In Vitro DNA Mutagenesis, Expression, and Spectroscopy of Opsins—All opsins variants were expressed using modified forms of a plasmid containing a synthetic bovine opsin gene cDNA in a pMT3-based vector (29, 30). The use of the bovine gene facilitates mutagenesis as multiple unique restriction sites are available, and direct comparison to a large body of work on previous rhodopsin mutations is possible. Mutations were introduced using the QuikChange™ mutagenesis kit (Stratagene). Expression in COS-1 cells, membrane isolation, reconstitution with 11-cis-retinal, and protein purification using 1D4 antibody affinity chromatography were carried out as described previously (31, 32). UV-visible absorption spectra of purified receptors were recorded with the use of a Varian Cary50 spectrophotometer modified for dark room use. All readings were of 1.0-cm path length samples taken at 23–25°C.

Determination of Metarhodopsin II Lifetimes—The rate of MII decay was measured in a manner similar to that previously published (32), with small modifications. Specifically, the decay rate was determined utilizing the fact that 11-cis-retinal binds to opsin at a faster rate than MII decays. First, the spectrum of the purified pigment in 2 mM sodium phosphate, pH 6.7, containing 0.1% (w/v) dodecyl maltoside (Anatrace, Maumee, OH) was recorded in the dark. Second, $\sim 2$ eq of 11-cis-retinal were added to the sample, and the resulting spectrum taken. The sample was subjected to light from a 300-watt tungsten bulb passed through a 455-nm long-pass filter for 1 s to selectively activate rhodopsin in the sample, without photosomerizing the excess 11-cis-retinal. Absorbance at 500 nm was measured post-flash until no further increase in absorbance was detected. Half-lives for the reactions were determined as previously reported (32), averaged over at least four trials, and plotted. Error bars represent the S.D. To prevent denaturation of opsin in detergent, these experiments were performed on proteins that had N2C and D282C mutations of rhodopsin that have been shown to stabilize WT rhodopsin without affecting its functional properties (33).

Retinal Binding to Opsins—Rhodopsin formation was monitored with UV-visible absorbance spectrophotometry as an increase in absorbance at 500 nm over time after addition of a 3-fold molar excess of 11-cis-retinal. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) and fit to the equation: $Y = Y_0 + A_1(1 - \exp(-k_1 \times t)) + A_2(1 - \exp(-k_2 \times t))$.

COS Cell Membrane Preparation—Membranes were prepared as previously described (31). In brief, 72 h after transfection with DNA, COS cells from five 150-mm culture plates were harvested with a cell scraper and washed with 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. The cells were then hypotonically lysed with 15 ml of 10 mM Tris, pH 7.4, containing a saturating amount of phenylmethylsulfonyl fluoride. The cells were passed through a 25-gauge needle four times and membranes were separated by layering the lysate onto 20 ml of 37% (w/v) sucrose in 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 0.1 mM EDTA, and centrifugation at 33,000 g at 4°C. Membranes were collected from the interface with a transfer pipette, diluted 10-fold with lysis buffer, and membranes were collected via centrifugation at 100,000 $\times$ g. The membrane pellet was resuspended in 1 ml of Tris, pH 7.4, 150 mM NaCl, 1 mM each of MgCl$_2$ and CaCl$_2$, and 0.1 mM EDTA. Rhodopsin concentration was estimated from immunoblots using the rhodopsin carboxyl-terminal 1D4 monoclonal antibody.

Transducin Activation Assays—The ability of receptors to catalyze the exchange of GDP for radiolabeled [$^{35}$S]GTPyS in transducin was examined using a filter binding assay as previously described (32), with the following exceptions. Time points were taken every minute, and light was introduced at $t = 6$ min 10 s. All assays were performed with rhodopsin in COS cell membranes diluted 10-fold with transducin assay buffer (10 mM Bistris propane, pH 6.7, 150 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 0.1 mM EDTA). Reaction mixtures contained transducin assay buffer supplemented with 1 mM dithiothreitol, 2 $\mu$m transducin, and 3 $\mu$m [$^{35}$S]GTPyS (5 Ci/mmol). Reactions were initiated by addition of GTPyS. For light-dependent reactions, membranes were incubated with 100 $\mu$m 11-cis-retinal for at least 1 h on ice prior to use in assays. The final reaction volume was 150 $\mu$L, and 10-$\mu$L aliquots were withdrawn and assayed at the times indicated. Due to inherent opsin stability in membranes, the thermally stabilizing mutations N2C and D282C were not used in this assay. Assay temperature was maintained at 23–25°C. Under these conditions, the reaction rate was directly proportional to the rhodopsin concentration. Although COS cell membranes have a different lipid composition than rod outer segments, this disadvantage is balanced by the benefit that hydroxylamine is not needed in these assays, thus avoiding activation of opsin by retinal oxime. Data were analyzed using KaleidaGraph (Synergy Software, Inc., Reading, PA).

RESULTS

Light-induced Degeneration of Photoreceptors in Tvrm4 and Tvrm1 Mouse Mutants Is Caused by Missense Mutations in Rho—Abnormal fundus pigmentation was observed by indirect ophthalmoscopy in a family of G2 B6/129 hybrid mice generated by

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chemical mutagenesis of embryonic stem cells. In an outcross of affected Tvrn4 mice to WT B6 mice, 50% of F1 offspring were affected, indicating a dominant inheritance pattern. As multiple indirect ophthalmoscopic exposures were necessary to observe the phenotype, independent of the age of the mice, this suggested that the examinations were inducing the loss of photoreceptors adjacent to the optic disc (Fig. 1A,left).

The Tvrn4 mutant mice were outcrossed to C3A.BLiA-Pde6b+/J, and affected F1 mice were backcrossed to the C3A.BLiA-Pde6b+/J parental line, to generate a backcross population for mapping purposes. To standardize the phenotyping protocol, the backcrossed animals were exposed to 12,000 lux of light for 5 min, which in the mutants led to visible bleaching of the entire retina for 1 h following exposure (Fig. 1A, right panel). Retinas of WT mice remained unaffected by the light exposure (Fig. 1A, middle panel). The Tvrn4 mutation was mapped to chromosome 6 between D6Mit62a (115.7 Mb) and D6Jmp8 (120.3 Mb) (data not shown).

The rhodopsin gene (Rho) mapped within the critical region, located about 115.9 Mb. The light-dependent nature of the Tvrn4 phenotype suggested that Rho was an excellent candidate gene. Sequence comparisons of Rho cDNA from Tvrn4 mutant and WT mice revealed a single nucleotide change. The missense mutation was predicted to lead to a change of amino acid 307, isoleucine (ATC), to asparagine (AAC) (Fig. 1B). Ile-307 is located in the 7th transmembrane region of RHO and is conserved across species, including human, primate, cat, dog, and rat (Fig. 1C). The Tvrn4 mutation was introgressed onto the C57BL/6 (B6) background by sequential backcrossing to B6 for 5 to 7 generations to remove potential influences of a segregating B6/129 genetic background on the disease phenotype.

During the course of screening ethynitrosourea-mutagenized G3 C57BL/6 mice, a litter of two mice with depigmented retinas was identified. This was the only litter of mice with a phenotype similar to Tvrn4 mutants observed in screening 10,000 G3s. Heritability testing of this mutation, named Tvrn1, indicated a dominant inheritance pattern, and similar to Tvrn4 mutants multiple examinations by indirect ophthalmoscopy were necessary to induce the disease phenotype. Heterozygous C57BL/6-Tvrn1 mice (hereafter referred to as Tvrn1) were outcrossed to A.B6-Tyr+ mice and affected F1 mutants were backcrossed to A.B6- Tyr-. Backcross progeny exposed to 12,000 lux of bright light were used to map the mutation to chromosome 6 between markers D6Jmp1 (115.5 Mb) and D6Jmp7 (119.3 Mb). A single base pair change was found in the Rho gene of Tvrn1 mutants. The missense mutation is predicted to lead to a change of amino acid 102, tyrosine (TAC), to histidine (CAC) (Fig. 1B). Tyr-102 is in the first extracellular loop of Rho and is also conserved among species (Fig. 1C).

**FIGURE 1.** Light-induced degeneration of photoreceptors in Tvrn4 and Tvrn1 mutants are caused by missense mutations in the Rhodopsin gene (Rho). A, fundus photographs of Tvrn4 mutant mice and controls. After light exposure from indirect ophthalmoscopy (left), the central retina becomes hyperpigmented, indicating photoreceptor degeneration, shown by a white arrow. Within 24 h after exposure to bright light (center and right panels) the mutant retina becomes hyper-reflective (right), whereas the WT retina does not (center). B, schematic of the Rho sequences depicting the Tvrn4 and Tvrn1 mutations. Single base pair substitutions (arrow) were found and predicted to lead to a change of amino acid 307, isoleucine to asparagine (I307N) in Tvrn4, and tyrosine to histidine (Y102H) in Tvrn1, n = 3 animals. C, the amino acid residues, Ile-307 and Tyr-102, are conserved among species. Protein sequences are from different species containing Ile-307 and Tyr-102 amino acid residues. The sites of mutations are indicated in the rhodopsin crystal structure (PDB coordinates 1U19) using the molecular modeling program UCSF Chimera.
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Rho Mutants Raised in Ambient Light Do Not Exhibit Loss of Photoreceptor Cell Bodies or Changes in Photoreceptor Function—To determine whether the missense mutations identified affected either the level or localization of rhodopsin, Western analysis and immunohistochemical studies were carried out, respectively. No significant differences in the level of rhodopsin were noted between 1- or 5-min exposures to bright light. Histological changes in the retinas of RhoTvrn4/mutants were observed within 1 h (top) following exposure to bright light. At 24 h (middle) following the exposure to bright light, the outer and inner segments of the RhoTvrn1/mutants were disorganized. At 1 week (bottom) following exposure, the outer nuclear layer is reduced to two rows of cell bodies. B, rhodopsin (red), ezrin (yellow), and 4',6-diamidino-2-phenylindole (blue) staining of the retinas from WT and RhoTvrn4/mutants 1 or 24 h after exposure to bright light. Photoreceptor outer segments are engulfed within the RPE cells of mutant eyes at 1 h after exposure to bright light, n = 2 animals. C, photoreceptors of Rho mutants are apoptotic 24 h following exposure to bright light. TUNEL staining of the retina was 24 h following bright light exposure, n = 3 animals. D, the Tvrn1 mutation makes the retina more vulnerable to light damage than the Tvrn4 mutation. Light microscopy of the central retina at different times following different durations of exposure to bright light. Red arrows indicate chromatin aggregates and blue arrows indicate macrophages, n = 3 mice for all groups except for RhoTvrn1/mutants, where n = 6.

Rhodopsin levels, localization, and photoreceptor morphology of RhoTvrn4/mutants were comparable with that of RhoTvrn1/mutants and WT controls (data not shown).

To evaluate overall retinal function, ERGs were recorded to full-field stimulus flashes presented under dark-adapted conditions, to isolate rod-driven activity, or superimposed upon a steady adapting field, to monitor activity of the cone pathway. At both 0.1 and 12 months (Fig. 2E) of age, there was no reduction in the amplitude of a- or b-waves recorded from RhoTvrn1/mutant or RhoTvrn4/mutant mice, as compared with those obtained from WT littermates. Because the leading edge of the dark-adapted ERG a-wave reflects the closure of cyclic nucleotide-gated channels along the rod photoreceptor (34), these results indicate that the normal complement of rod photoreceptors retained by RhoTvrn1/mutant and RhoTvrn4/mutant mice functions normally when stimulated by light flashes. Similar results were obtained for responses recorded under light-adapted conditions. In comparison to responses obtained from WT littermates, cone ERGs were not reduced in RhoTvrn1/mutant or RhoTvrn4/mutant mice at either of the ages tested (Fig. 2, E and G).

RhoTvrn1/mutant Mutants Are More Vulnerable to Light than RhoTvrn4/mutant Mutants—The time course over which light-induced changes developed in Tvrn1 mutants was examined by sacrificing RhoTvrn4/mutants and WT mice at different times following a 5-min exposure to 12,000 lux of bright light. Fig. 3A presents a series of representative retinal cross-sections taken at different durations of time following light exposure. Within 1 h, the photoreceptor outer segments of RhoTvrn1/mutant, but not WT, mice appeared disorganized and some areas were devoid of outer segments, whereas others appeared to contain outer segment aggregates. In addition, the apical processes of retinal pigment epithelium that normally ensheath the outer segments appeared to extend further along the outer segments in RhoTvrn4/mutant mice than in WT controls (Fig. 3B). Twenty-four hours following light exposure, it was not possible to discern
the border between the outer and inner segments of the *RhoTvrm4/+* retina, and both compartments were highly disorganized. The outer nuclear layer also appeared disorganized, although cell loss was not apparent. At this time, many *RhoTvrm4/+* photoreceptors were TUNEL-positive indicating that they were undergoing apoptosis; TUNEL-positive staining was not detected in WT retinas (Fig. 3C). Thereafter, photoreceptor degeneration proceeded rapidly, with two or fewer layers of photoreceptor nuclei remaining at 1 week following light exposure. At this late stage of the degenerative process, multiple aggregates of chromatin and macrophages were also observed (Fig. 3, A and D).

To determine the duration of light exposure required to induce photoreceptor degeneration in *RhoTvrm4/+* mutants, the duration of exposure to a 12,000 lux stimulus was varied and the retinas were harvested at varying times following exposure to a light stimulus. In 2 of 3 animals tested, a 2-min exposure was sufficient to induce photoreceptor degeneration, whereas a 1-min exposure produced no discernible changes (Fig. 3D).

The retinas of *RhoTvrm1/+* animals were more sensitive to light-induced damage than the retinas of *RhoTvrm4/+* animals. In *RhoTvrm1/+* mice, photoreceptor degeneration was observed in 4 of 6 mice exposed to 12,000 lux for only 30 s (Fig. 3C).

**Recovery from High Intensity Bleach Is Impaired in the Mutants, but the Deactivation of Phototransduction Is Normal**—Our observations of extreme sensitivity to light-induced photoreceptor degeneration in *RhoTvrm1/+* and *RhoTvrm4/+* mice raised the possibility that these mutations interfered with phototransduction deactivation and/or bleaching recovery. We examined these possibilities using two ERG paradigms. To monitor phototransduction deactivation, we used a two-flash protocol in which a probe stimulus flash follows a conditioning stimulus (35). By using high intensity stimuli that saturates the photoreceptors, and a series of trials with different ISIs, the recovery of a-wave amplitude to the second probe flash monitors the deactivation process following the initial conditioning stimulus (36). This approach has been used to characterize the effect of other rhodopsin mutants on rod phototransduction deactivation (24, 37).

Fig. 4A summarizes the results obtained in *RhoTvrm1/+* and *RhoTvrm4/+* mice by plotting the ratio of the a-wave amplitude obtained to the probe flash (R2) to that obtained to the conditioning stimulus (R1). In comparison to results obtained from WT littermates, there was no difference in phototransduction deactivation in either *RhoTvrm1/+* or *RhoTvrm4/+* mice.

To monitor bleaching recovery, we used a standard protocol in which recovery toward a dark-adapted baseline was monitored at intervals following exposure to a bleaching stimulus (25). Fig. 4B summarizes the results obtained from WT, *RhoTvrm1/+*, and *RhoTvrm4/+* mice. In WT mice, the amplitude of the a-wave recovered with a time constant of ~6.5 min, achieving the dark-adapted baseline within ~20 min. *RhoTvrm1/+* mice recovered more slowly, with a time constant of ~11.5 min, and the asymptotic level fell short of the dark-adapted value. *RhoTvrm4/+* mice recovered with a time constant of ~9.5 min, but achieved an asymptote that was only ~60% of the dark-adapted value. Thus, both *RhoTvrm1/+* and *RhoTvrm4/+* mice display deficits in bleaching recovery when assayed with an outcome measure related to photoreceptor function.

**Retinoid Metabolism Is Transiently Affected in the Mutants—**HPLC using both fluorescence and multiwavelength absorbance detection allowed separation and quantification of the major retinoid species (Fig. 5A). The levels of the major retinoid species present in extracts from whole dark-adapted eyes did not differ significantly for *RhoTvrm1/+* or *RhoTvrm4/+* mice as compared with WT controls (Fig. 5B). However, *RhoTvrm4/+* eyes collected immediately after exposure to 12,000 lux for 5 min (Fig. 5C) contained significantly lower levels of all-trans-retinol than did WT eyes (p < 0.05). Levels of these retinoids were also lower in *RhoTvrm1/+* eyes, although the differences were not statistically significant (p > 0.05). After 1 h of recovery following light exposure (Fig. 5D), there were no statistically significant differences in levels of any retinoids between the mutant and WT animals. The differences in retinoid levels...
observed in the mutants are thus transient, and do not seem to involve significant differences in the availability of 11-cis-retinal following a bleach.

The Mutant Opsins Are Constitutively Active and Bind Chromophore More Slowly than Does WT Opsin—To better understand the relationship between the observed disease phenotype and the effects of the mutations on rhodopsin function, we expressed Y102H (Tvrm1) opsin and I307N (Tvrm4) opsin in COS cells and analyzed the biochemical outcome of the respective mutations. We first examined the ability of the receptors to signal effectively. When 11-cis-retinal was added, both Y102H and I307N rhodopsin formed pigments with visible absorbance spectra characteristic of WT rhodopsin (Fig. 6B). Similar to WT rhodopsin, both mutant rhodopsins activate transducin in a light-dependent manner (Fig. 6A), however, unlike WT opsin, Y102H and I307N opsins are constitutively active (Fig. 6A).

The toxicity of a constitutively active opsin mutant may depend upon how long it stays in the free opsin form before binding to 11-cis-retinal to regenerate rhodopsin. To determine the effect of the rhodopsin mutations on regeneration kinetics, we introduced them in the context of a N2C,D282C mutant background that allows WT and mutant opsins to be purified in detergent without denaturation in the absence of chromophore (33). When we measured the kinetics of regeneration (Fig. 6C), we observed that the mutants bound retinal more slowly than WT bound retinal; the mean lifetimes measured were as follows: 0.655 min (±0.014 S.E.) for WT, 4.32 min (±0.44 S.E.) for Y102H, and 5.94 (±0.29 S.E.) for I307N (p < 0.0001, one-way ANOVA). These results reveal that not only are the mutant opsins constitutively active, but their binding to the inverse agonist 11-cis-retinal is slower.

However, whereas the mutant opsins are slow to bind 11-cis-retinal and are constitutively active, the lifetimes of the active form of the mutant receptors (metarhodopsin II) are similar to that of WT metarhodopsin II. Fig. 6D shows that the mean half-life of MII decay is only 48% longer for Y102H than that of WT, and 53% longer for I307N. ANOVA or a Dunnett test of results for all three proteins taken together yields a p < 0.05; therefore, although we cannot rule out that there may be small differences in the kinetics of MII decay, there is no evidence for such differences contributing to the observed retinal degeneration.

Mutant Opin Leads to Photoreceptor Degeneration without Exposure to Bright Light—We hypothesized that free opsin might be toxic to the retina because the mutant opsins were constitutively active and the photoreceptors degenerated after exposure to bright light, which would lead to increased production of free opsin not bound to its chromophore. To test this
the central retina. The morphological changes in the photoreceptors of the double mutants were different from those observed for RhoTvrm1/+ or RhoTvrm4/+ single mutants at 1 week following bright light exposure (Fig. 2B). Neither double mutant developed chromatin aggregates nor disruption of the laminar organization of the retina.

**DISCUSSION**

Described herein are two new mouse models harboring point mutations in the rhodopsin gene. Although the affected amino acid residues involved lie in different domains of the rhodopsin molecule, these models share several important similarities. First, both lines originally identified by a hypopigmented fundus appearance are highly sensitive to light-induced photoreceptor degeneration. Second, despite this increased susceptibility to light damage, when reared under standard animal housing conditions, both lines retain a normal complement of photoreceptors up to 1 year of age, the latest time point examined. Third, whereas the activation and deactivation kinetics of rod phototransduction appear normal upon ERG analysis, both Y102H and I307N mutations cause a delay in the response recovery following bleaching. When examined at the biochemical level, this delay appears to be the net result of a decrease in the rate at which the mutant opsins bind the chromophore 11-cis-retinal and a higher than normal activation of transducin by unbound opsin. Finally, unlike transgenic lines that are currently available, the mutant rhodopsin gene is expressed under control of the endogenous promoter in both RhoTvrm1/+ and RhoTvrm4/+ mice.

**Exposure to Bright Light Is Necessary for Degeneration of the RhoTvrm1/+ and RhoTvrm4/+ Photoreceptors**—Unlike other Rho models in which photoreceptor degeneration is accelerated by light exposure and occurs even when mice are dark-reared (16, 39–42), RhoTvrm1/+ and RhoTvrm4/+ mice maintained under standard animal housing conditions have normal photoreceptor morphology and function. A rapid and severe degeneration of photoreceptors only occurs upon exposure to bright light. Our results show that the rate of this degeneration is dependent upon the duration of light exposure. It seems likely that the threshold at which degeneration is induced reflects the bleach-
ing level at which the capacity of the visual cycle is surpassed, thereby allowing free opsin to remain in an active state for a damaging period of time. This feature of inducible photoreceptor degeneration may make these mice valuable models to study mechanisms underlying light-induced retinal damage and for testing pharmacological and environmental therapeutic strategies as the initiation of disease by light exposure can be reproducibly controlled.

**RhoTvrm1+/+ and RhoTvrm4+/+ Mutants Recapitulate Features of the RHO Class B1 Phenotype**—The disease phenotypes of RhoTvrm1+/+ and RhoTvrm4+/+ mice includes focal areas of photoreceptor degeneration and normal photoreceptor functionality with delayed dark adaptation. These features closely resemble the phenotype of patients with RHO class B1 mutations (15, 16). In fact, the residue altered in RHO class B1 mutations (Y102H) lies close to a G106Y missense mutation found in humans with class B1 RP (43). The altered residue in RhoTvrm4+/+ mutants (I307N) is close to the Q312ter mutation that leads to class B RP (15) and an A295V mutation associated with stationary night blindness (44).

Although a causal role for light in the progression of photoreceptor degeneration in humans with RHO mutations has not been established, light has been shown to induce photoreceptor degeneration in animal models of class B1 RP (45). It is difficult to establish that any known human rhodopsin mutation is light-inducible, as humans with RP are typically exposed to sunlight that is much brighter than standard animal housing lighting conditions. Although mice are different from humans, it is possible that limiting exposure to bright light may not only help to slow the progression of photoreceptor loss in patients with some RHO mutations, but as shown in the mutants studied here, may actually prevent the onset of degeneration. Our data suggest that patients bearing rhodopsin mutations that cause high opsin activity or slow regeneration might be well advised to avoid exposure to bright light.

**Mutant Opsin Leads to Prolonged Dark Adaptation and Constitutive Activation**—Aside from light-induced photoreceptor degeneration, the only functional defect detectable in the Rho mutants is a prolonged dark adaptation recovery following bleaching light levels. Dark adaptation delays have been previously observed in RP patients bearing RHO mutations (46) and in the T4R dog model (19). Dark adaptation is a function of phototransduction deactivation and chromophore release, recycling, and regeneration with opsin (47). We hypothesized that only those processes directly involving the opsin molecule might be affected in the RhoTvrm1+/+ and RhoTvrm4+/+ and that recycling of the chromophore itself would not be altered by the observed mutations. Our *in vitro* studies implicate several features of rhodopsin biochemistry in the dark adaptation delays noted functionally. For example, rhodopsin regeneration is delayed simply because Y102H and I307N opsins bind to 11-cis-retinal at a slower rate than WT opsin. In addition, immediately following bright light exposure, the total levels of retinoids were decreased in the retinas of mutant animals compared with the WT. As a consequence, the pool of retinoids necessary for rhodopsin regeneration are significantly reduced during the initial stages of dark adaptation. Finally, we noted that the unbound Y102H and I307N opsins increase transducin activation at higher levels than WT opsin. Although free WT opsin is capable of activating transducin, the activation levels are far below those for R* (48). In the case of the Y102H and I307N mutants, constitutive transducin activation by the free mutant opsin molecules also slows recovery following exposure to bleaching levels of light.

**Mutant Free Opsin May Lead to Photoreceptor Degeneration in RhoTvrm1+/+ and RhoTvrm4+/+ Mutants**—Exposure to bright light leads to production of high levels of retinoid metabolites and intermediates of phototransduction, as well as to higher levels of free opsin. In the mutants, delayed rhodopsin regeneration serves to increase the level of free opsin following bright light exposure. This excess of mutant-free opsin is likely to have a greater propensity to activate transducin. To test the hypothesis that mutant-free opsin is an important contributor to the photoreceptor degeneration in RhoTvrm1+/+ and RhoTvrm4+/+ mutants, both Rho lines were crossed to Rpe65rd12/rd12 mice lacking functional RPE65 (38), in which the retinal pigment epithelium is unable to regenerate 11-cis-retinal and therefore, leads to an accumulation of free opsin. Although Rpe65rd12/rd12 mice expressing WT rhodopsin retain a near-normal complement of photoreceptors at 4 weeks of age, photoreceptors of double mutants: Rpe65rd12/rd12/RhoTvrm1+/+ and RhoTvrm4+/+ mice degenerated without exposure to bright light and at a much faster rate than unexposed RhoTvrm1+/+, RhoTvrm4+/+, or Rpe65rd12/rd12 Rho+/+ mice. This indicates that the Y102H and I307N opsins may be more toxic than WT opsin or alternatively, higher levels of constitutive activation may accelerate the photoreceptor degeneration observed in the Rho mutants. Constitutive opsin activation is thought to underlie the loss of visual sensitivity in forms of congenital stationary night blindness caused by rhodopsin mutations in which photoreceptor degeneration does not occur (31, 49, 50), or occurs at a slow rate (44). Taken together, we interpret the results of the double mutant experiment to indicate that the Y102H and I307N rhodopsin mutations result in a longer-lived opsin with dominant cell-toxic features. Under normal housing conditions, the levels of free opsin generated are below the threshold for inducing photoreceptor degeneration. Only when exposed to bright light, or deprived of 11-cis-retinal, do the opsin levels exceed the threshold for initiation of apoptotic cell death pathways.

**Allelic Differences between Tvrn1 and Tvrn4—The RhoTvrn1+/+ and RhoTvrn4+/+ mutations are located in the 1st extracellular loop and the 7th transmembrane region, respectively. Despite these differences in locations, they cause similar disease phenotypes. The regions in which the mutations occur do not have similar reported functions. However, it is interesting to note that they are both located near regions of the molecule that play a role in rhodopsin dimer formation (51). RhoTvrn1+/+ (Y102H) lies near Tyr-96 and His-100 from helix II, which form a secondary cluster of side chains connecting the two rhodopsin subunits. RhoTvrn4+/+ (I307N) lies close to Cys-322 and Cys-323 of H-8 helix that have associated palmitoyl groups that may also play a role in dimer stabilization. Although we do not have any evidence that the mutations do indeed affect the stability of the rhodopsin dimers, the availability of these Rho models will allow for further *in vivo* exploration of this observation.
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Finally, whereas both RhoTvrm1/+ and RhoTvrm4/+ mutants share similar disease phenotypes, their rate of disease progression and the level of light exposure necessary to induce photoreceptor degeneration differ. The RhoTvrm1/+ mutation is more susceptible to light-induced damage than RhoTvrm4/+ mutants and a shorter duration of light exposure is necessary to induce photoreceptor degeneration. However, the RhoTvrm4/+ opsin appears to be more cytotoxic, as the retinas of RhoTvrm4/+ mutants degenerate faster when expressed in the absence of Rpe65. This suggests that mutant opsin toxicity and the functionality of the mutant opsin upon exposure to bright light are not completely equivalent and that bright light exposure not only increases the level of free opsin in the retina but possibly affects other factors that lead to photoreceptor degeneration. The models described here will allow for further exploration of the in vivo structure and function of the rhodopsin molecule.

Acknowledgments—We thank Ananth Badrinanth for genetic mapping of the mutation in the backcross progeny, the animal technicians, and the scientific services at The Jackson Laboratory. The Jackson Laboratory core services were supported by institutional Grant CA-24190.

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