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Analysis of Kinesin-2 Function in Photoreceptor Cells Using Synchronous *Cre-loxP* Knockout of *Kif3a* with *RHO-Cre*

David Jimeno^{1,2,3}, Leonard Feiner^{3,4,5}, Concepcion Lillo^{1,2}, Karen Teofilo^{1,2}, Lawrence S. B. Goldstein^{6,7}, Eric A. Pierce^{*,4}, and David S. Williams^{*,1,2}

¹Department of Pharmacology, University of California San Diego (UCSD) School of Medicine, La Jolla, California

²Department of Neuroscience, University of California San Diego (UCSD) School of Medicine, La Jolla, California

⁶Department of Cellular and Molecular Medicine, University of California San Diego (UCSD) School of Medicine, La Jolla, California

⁷Howard Hughes Medical Institute, University of California San Diego (UCSD) School of Medicine, La Jolla, California

⁴F. M. Kirby Center for Molecular Ophthalmology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

Abstract

PURPOSE—To determine the relationship between the presence of kinesin-2 and photoreceptor cell viability and opsin transport, by generating *RHO-Cre* transgenic mice and breeding them to mice with a floxed kinesin-2 motor gene.

METHODS—Different lines of *RHO-Cre* transgenic mice were generated and characterized by transgene expression, histology, and electrophysiology. Mice from one line, showing uniform transgene expression, were crossed with *Kif3a^{fllox}/Kif3a^{fllox}* mice. The time courses of photoreceptor *Cre* expression, KIF3A loss, ectopic opsin accumulation, and photoreceptor cell death were determined by Western blot analysis and microscopy.

RESULTS—One of the *RHO-Cre* lines effected synchronous expression of *Cre* and thus uniform excision of *Kif3a^{fllox}* in rod photoreceptors across the retina. After the neonatal production of CRE and the initiation of KIF3A loss, ectopic accumulation of opsin was detected by postnatal day (P)7, and ensuing photoreceptor cell death was evident after P10 and almost complete by P28. Of importance, the photoreceptor cilium formed normally, and the disc membranes of the nascent outer segment remained normal until P10.

CONCLUSIONS—The *RHO-Cre*-8 mice provide an improved tool for studying gene ablation in rod photoreceptor cells. Regarding kinesin-2 function in photoreceptor cells, the relatively precise timing of events after CRE excision of *Kif3a^{fllox}* allows us to conclude that ectopic opsin is a primary

*Each of the following is a corresponding author: Eric A. Pierce, F. M. Kirby Center for Molecular Ophthalmology, University of Pennsylvania School of Medicine, 305 Stellar-Chance Labs, 422 Curie Boulevard, Philadelphia, PA 19104; epierce@mail.med.upenn.edu. David S. Williams, Department of Pharmacology, UCSD School of Medicine, Mail code 0912, 9500 Gilman Drive, La Jolla, CA 92093-0912; dswilliams@ucsd.edu.

³Contributed equally to the work and therefore should be considered equivalent authors.

⁵Present affiliation: Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri.

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cellular lesion of KIF3A loss, consistent with the hypothesis that opsin is a cargo of kinesin-2. Moreover, it demonstrates that KIF3A loss results in very rapid photoreceptor cell degeneration.

Many genes that are important in ocular function and disease have been studied with traditional gene-targeting strategies in the mouse. However, some genes that function in the eye are also critical for normal development and homeostasis of the animal and are thus not amenable to simple knockout techniques.^{1,2} The development of conditional knockout strategies has enabled the study of these other genes. The use of CRE and other recombinases for conditional targeting permits the controlled removal or activation of genes in specific tissues and at specific times of development.^{3,4}

In a previous study, we used *Cre-loxP* mutagenesis to test for motor transport by kinesin-2 in photoreceptor cells.⁵ Vertebrate photoreceptor cells include two distal compartments: an inner segment, which contains much of the cellular machinery, and an outer segment, which is a specialized sensory cilium dedicated to phototransduction. The outer segment is linked to the inner segment by a connecting cilium, which is analogous to the transition zone of a primary cilium.⁶ Trafficking between the inner and outer segments occurs along the connecting cilium and the axoneme of the outer segment and is essential for the function and viability of the cells. Large amounts of phototransductive proteins, including the visual receptor, opsin, are transported in an anterograde direction as part of the continuous renewal of the outer segment.⁷ Moreover, at least three proteins, arrestin, transducin, and recoverin, redistribute between the inner and outer segments according to ambient lighting.⁸⁻¹⁴ Kinesin-2 is a likely candidate to provide motor transport along the connecting cilium and axoneme of photoreceptor cells, based on its role in the movement of proteins along cilia and flagella ("intraflagellar transport") in a variety of organisms, from single cell flagellates to mammals.^{15,16} Moreover, the motor subunits of kinesin-2, KIF3A, and KIF3B, have been detected in the photoreceptor connecting cilium.¹⁷⁻²⁰

In the previous study, mice were generated in which a region of the *Kif3a* gene was flanked by loxP sites and thus could be excised in the presence of CRE. CRE was introduced into the photoreceptor cells by way of an *IRBP-Cre* transgene, whose expression was restricted primarily to the photoreceptor cells.⁵ With this strategy, excision of the *Kif3a* gene occurred in photoreceptor cells, beginning after the second post-natal week. The consequential removal of KIF3A from the photoreceptor cells not only perturbed the flow of protein to the outer segment, but also killed some of the photoreceptor cells. Although this study demonstrated a requirement for kinesin-2 in photoreceptor cell protein transport and viability, gene excision was incomplete and asynchronous across each retina, and its extent varied among different animals, thus limiting the usefulness of this approach. In particular, these animals were not suitable for any type of biochemical study.

In the present study, we first set out to establish a more robust expression of *Cre*—one that would effect widespread and synchronous recombination across the retina and thus would be more useful for the study of *Kif3a* and other genes in photoreceptor cells. We settled on a line of *RHO-Cre* transgenic mice that fulfills these criteria and have characterized the expression and effects of this transgene. We have also used this line to study further the requirement of KIF3A in photoreceptor cells, and especially the time course of the change in gene expression in relation to the ensuing effects on the photoreceptor cells. Of note, we found that an abnormal accumulation of opsin is the primary cellular defect, occurring when all other aspects of cellular organization appear normal.

MATERIALS AND METHODS

Generation of RHO-Cre Transgenic Mice

A 4.5-kb *Xba*I fragment containing the human rhodopsin promoter was cloned into pBluescript NLS Cre.^{21,22} The *RHO-Cre* plasmid was digested with *Asc*I, and the 5.8-kb fragment containing the hRho promoter and Cre recombinase gene was purified. The *RHO-Cre* transgene also contains an intron and a polyadenylation signal (Fig. 1A). The eluted DNA fragment was further purified (Elutip column; Schleicher & Schuell, Keene, NH) and resuspended in injection buffer (10 mM Tris and 0.1 mM EDTA; pH 7.5). The purified DNA fragment was injected into fertilized oocytes according to standard protocols, to generate transgenic mice. The founders were screened by PCR to identify the three founders carrying the *RHO-Cre* transgene. Positive founders were crossed with *Gt(ROSA)26Sor^{tm1Sor}* reporter mice and backcrossed with C57BL/6J mice.²³

Breeding of *Kif3a^{flox}*; *RHO-Cre* mice

Kif3a^{WT}/Kif3a^{flox}, *Kif3a^{flox}/Kif3a^{flox}*, and *Kif3a^{flox}/Kif3a^{null}* mice were crossed with *Kif3a^{flox/flox};RHO-Cre* mice. Conditional knockout mice containing *RHO-Cre* and *Kif3a^{flox}/Kif3a^{flox}* or *Kif3a^{flox}/Kif3a^{null}* were thus obtained, together with control littermates lacking *RHO-Cre* and/or with a *Kif3a^{WT}* allele. Mice were kept in a 12-hour light–12-hour dark cycle under 10 to 50 lux of fluorescent lighting during the light cycle. Care was provided according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Genotyping

PCR for the *Cre* transgene was performed with tissue lysate from toe biopsy specimens as described. The *Cre* primers used were 5'-TGCC CCAA ATG TTGCTGG ATAGTTTTTA-3' and 5'-ATGCCCAAGAAGAA-GAGGAAGGTGTCCA-3', which generate a 250-bp product from Cre recombinase. A total of 30 cycles of 94°C, 45 seconds; 59°C, 45 seconds; and 72°C, 1 minute were performed with *Taq* DNA polymerase (Roche, Indianapolis, IN), and the PCR reactions were subjected to electrophoresis on 1% agarose gels. PCR primers to identify the three *Kif3a* alleles and quantitative PCR for quantification of recombination frequency were as described.^{5,24}

Electroretinography

The retinal function of F1 *RHO-Cre* and control mice was measured at 4, 7, 10, and 12 weeks of age, using established techniques.²⁵ Briefly, full-field ERGs were recorded from both eyes of anesthetized mice with differential amplifiers having a bandwidth of 0.1 Hz to 1 kHz. The filtered traces were digitized at 5 kHz and stored on a computer for further analysis. The corneal electrodes were platinum wires embedded in the contact lenses, placed on the eye on a layer of Goniosol ophthalmic solution (Ciba Vision, Duluth, GA). The reference electrode was a tungsten needle inserted subcutaneously into the forehead. The recording chamber served dually as a Faraday cage and a Ganzfeld, with appropriate ports and baffles to ensure uniform illumination. Intensities were calibrated as previously described.²⁵ Mice were dark-adapted for a minimum of 12 hours before the ERG experiments. Preparations of the animals for recordings were made under dim red light. The mice were anesthetized with an intraperitoneal injection containing (in micrograms per gram body weight): 25 ketamine, 10 xylazine, and 1000 urethane, and their pupils were dilated with 1% tropicamide solution (Alconox, New York, NY). Before recording commenced, animals were maintained in complete darkness for 15 minutes. At least three animals of each genotype were evaluated at each time point.

Western Blot Analysis

Each retina was homogenized in 100 μ L of PBS buffer with protease inhibitors (Sigma-Aldrich, St. Louis, MO) and 25 μ L of Laemmli sample buffer. Equal proportions of the retinal homogenate were loaded on a 10% highly porous sodium dodecyl sulfate polyacrylamide gel for electrophoresis (SDS-PAGE). The running gel was transblotted on to nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA) and immunolabeled with KIF3A antibodies (BD Transduction Laboratories, Lexington, KY) and alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) for staining with nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (NBT/BCIP; Roche). Quantification of the KIF3A labeling was performed with ImageJ software (available by ftp at zippy.nimh.nih.gov/ or at <http://rsb.info.nih.gov/nih-image/>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Light and Immunofluorescence Microscopy

After ERG analyses, mice were deeply anesthetized and killed by cardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). The eyes were isolated and postfixed in 4% paraformaldehyde in PBS overnight. After they were rinsed with PBS, eye cups were made, and one eye cup was processed for plastic sectioning. The eye cup for plastic sectioning was first dehydrated by placing the tissue for 1 hour in each of 70%, 95%, and 100% ethanol solutions. The dehydrated tissue was then infiltrated overnight in infiltration solution (JB-4 Plus; Polysciences Inc., Warrington, PA) and embedded in resin (JB-4 Plus). Histologic sections were cut at 3- μ m thickness and stained with Richardson's stain for 30 seconds. The slides were washed under running water for 2 minutes and mounted (Permount; Fisher Scientific, Pittsburgh, PA). Bright-field digital images were captured (model TE300; Nikon, Tokyo, Japan) with a microscope equipped with a digital camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI).

For use in X-gal staining or immunofluorescence analyses, the other eye cup was infiltrated in 30% sucrose, frozen in OCT freezing medium, and cryosectioned at 10 μ m. For immunostaining, retinal sections were blocked in PBS containing 1% or 2% normal goat serum, 1% bovine serum albumin, and 0.1% or 0.5% Triton X-100 for 1 hour and then incubated overnight with primary antibodies at 4°C. After they were rinsed with PBS, the sections were treated with fluorochrome-conjugated secondary antibodies for 1 hour (sometimes including 4',6'-diamino-2-phenylindole (DAPI), diluted 1:10,000), washed in PBS, and mounted (Fluoromount-G; Southern Biotechnology Associates, Birmingham, AL).²⁶ The primary antibodies used were monoclonal anti-Cre-recombinase (BabCO-CRP, Inc., Vienna, VA) and anti-red-/green cone opsin (JH492).²⁷ Cy2- and Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa 468- and Alexa 584-conjugated secondary antibodies were from Invitrogen-Molecular Probes (Eugene, OR). Control sections were treated with preimmune anti-C'-Rp1 or without primary antibodies. Stained sections were viewed with a confocal microscope (model LSM510; Carl Zeiss Meditec, Inc., Dublin, CA), and images were processed with the accompanying software (Meta 510; Carl Zeiss Meditec, Inc.).

Semithin sections were obtained from eye cups that were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), processed for embedding in resin (LR White; EMS, Fort Washington, PA), and immunolabeled using the PAP (peroxidase anti-peroxidase) technique. Sections (0.7 μ m) were washed in PBS and incubated overnight at room temperature with opsin pAb 01 (generated against bovine rod opsin; 1:500) in PBS plus 1% BSA and 2% goat serum, 2 hours at 37°C with a goat anti-rabbit secondary antibody (1:20; Jackson ImmunoResearch), and 2 hours at 37°C with a rabbit-PAP complex (1:10; Jackson ImmunoResearch). The sections were washed in 0.1 M Tris-HCl (pH 7.6) for 10 minutes, and

the peroxidase was detected by incubating the sections in 3,3'-diaminobenzidine (DAB) for 20 to 30 minutes. The sections were counterstained with toluidine blue, and then dehydrated and mounted with a rapid mounting medium (Entellan; EMS). For quantification of photoreceptor cell nuclei, three dorsoventral semithin sections from each retina were used. Photoreceptor nuclei were counted in the areas, located 500 μm each side of the optic nerve head.

Electron Microscopy

Ultrathin sections were obtained from resin-embedded (LR White; EMS) tissue and then incubated overnight at 4°C with opsin pAb 01 (1:300) and for 1 hour at room temperature with a secondary goat anti-rabbit IgG-10 nm gold antibody (1:30). Sections were postfixed with 2% glutaraldehyde in PB for 20 minutes at room temperature and stained with uranyl acetate (2%) for 15 minutes and with lead citrate for 10 minutes.

X-Gal Staining

β -Galactosidase activity in retinal sections or wholemounts was detected by staining with 5-bromo-4-chloro-3-indolyl- β -D-galactopyrano-side (X-Gal) as described.²⁸ For X-Gal staining, eyes were isolated as described earlier and fixed on ice for 3 hours in 4% paraformaldehyde in PBS. Eye cups were made, and the specimens, including the lens and cornea, were stained overnight at 37°C in 1 mL/mg X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl_2 in PBS. Stained samples were rinsed in PBS and postfixed at 4°C overnight in 4% paraformaldehyde in PBS buffer. For preparation of wholemounts, one eye cup was cleared overnight in 50% glycerol, the retina was removed and flatmounted in glycerol. For preparation of retinal sections from X-Gal-stained eyes, the other postfixed eye cup was rinsed in PBS, infiltrated with sucrose, frozen in OCT, and sectioned at 30 μm thickness. Retinas and sections were examined and photographed with an inverted microscope (TE300; Nikon) or a stereomicroscope (M2Bio; Carl Zeiss Meditec, Inc.) equipped with color digital cameras.

RESULTS

Expression of Cre in Rods

RHO-Cre founders were crossed with *Gt(ROSA)26Sor^{tm1Sor}* reporter mice to evaluate the pattern of *Cre*-recombinase activity.²³ As shown in Figures 1B–E, retinal wholemounts from mice of the *RHO-Cre* line 8 (*RHO-Cre-8*) demonstrated uniform β -galactosidase activity, indicating *Cre*-recombinase activity across the entire retina. This pattern of X-Gal staining was observed at all ages examined (Fig. 1D). In contrast, retinas from another line, *RHO-Cre-16*, showed staining in a stripe across the retina (Fig. 1C). These expression patterns were consistent from animal to animal within each *RHO-Cre* line.

Frozen sections of X-Gal–stained retinas showed that *RHO-Cre-8* mice expressed Cre uniformly in photoreceptor cells (Fig. 1F). Again, the *RHO-Cre-16* mice demonstrated less uniform staining, with variable staining of the outer nuclear layer (Figs. 1G, 1H). To evaluate which photoreceptor cells in *RHO-Cre-8* mice express *Cre*-recombinase, frozen sections of retinas from 1-month-old mice were double-labeled with antibodies against CRE and against cone opsin. The CRE labeling was concentrated in the nuclei of photoreceptor cells and did not overlap the cone opsin signal, indicating that *Cre* expression was restricted to rods (Figs. 1I–K).

Age-Dependent Loss of Photoreceptor Cells

To assess the health of photoreceptor cells in the *RHO-Cre-8* line of mice, retinal function and histology was evaluated at 4, 7, 10, and 12 weeks of age. Electroretinograms (ERGs) showed

that although the retinal function of 4-week-old mice was normal, rod cell function was reduced to 56% of normal by 7 weeks of age, and 20% of normal at 10 weeks of age (Fig. 2A). The observed decrease in retinal function was associated with loss of photoreceptor cells, as shown in Figure 2B. The outer nuclear layer thickness was normal at 4 weeks, but some photoreceptor nuclei had been lost by 7 weeks, and, by 12 weeks, only five rows of photoreceptor cell nuclei remained in the *RHO-Cre-8* mice.

Excision of the *Kif3a^{fllox}* gene by *RHO-Cre*

Although the *RHO-Cre-8* mouse was found to undergo retinal degeneration, there was a significant postnatal window before degeneration for conditional gene ablation studies. We mated *RHO-Cre-8* mice with *Kif3a^{fllox}* mice, in which exon 2 of the *Kif3a* allele is flanked by loxP sites.⁵ Previously, we found, using semiquantitative PCR, that the extent of the excision of photoreceptor *Kif3a^{fllox}* by *IRBP-Cre* varied greatly across the retina and from animal to animal.⁵ Using the same approach, we determined in the current study that *Kif3a^{fllox}* was also not excised in all photoreceptor cells of five of the *RHO-Cre* lines. Although we did not examine retinal wholemounts from all these lines, it is likely that the transgene was expressed in only part of the retina, as shown for the *RHO-Cre-16* line (Fig. 1C). However, the widespread, uniform expression of *Cre* in the *RHO-Cre-8* line (Figs. 1B, 1D, 1F) effected a more consistent result. No differences were detected between *Kif3a^{-/-} Kif3a^{fllox}; RHO-Cre-8* mice and *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* mice, indicating that requiring the excision of both of alleles in the latter was not limiting. *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* mice were therefore used for the analyses.

Western blot analysis of retinas from different aged *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* mice showed that CRE was weakly present just after birth and that its level increased throughout the first 2 weeks (Fig. 3A). The decrease in CRE after P14 was due to photoreceptor cell death, since CRE continued to increase up to P42 in *RHO-Cre-8* mice (Fig. 3B). Photoreceptor KIF3A levels began declining in the first post-natal week. They were estimated from Western blot analyses of retinal lysates and subtraction of the contribution by nonphotoreceptor cells (~56%). Figures 3C and 4 depict the decline, relative to levels in age-matched control mice (*Kif3a^{WT}/Kif3a^{WT}; RHO-Cre-8* and *Kif3a^{fllox}/Kif3a^{fllox}* with no *RHO-Cre-8* transgene).

Synchronous Mislocalization of Opsin after the Outer Segment Begins to Develop

As found with the *Kif3a^{-/-}/Kif3a^{fllox}; IRBP-Cre* mice,⁵ the first detectable defect in the photoreceptors of *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* mice was an abnormal accumulation of opsin within the photoreceptor inner segment (Fig. 5F). Shortly thereafter, opsin was evident throughout the plasma membrane of the entire photoreceptor cell (Figs. 5A–D, 5H), as reported for many other mouse models of retinal degeneration. This redistribution of opsin was evident before KIF3A had been lost completely from the photoreceptor cells (Fig. 4). There were two major differences in opsin mislocalization between the *Kif3a^{-/-}/Kif3a^{fllox}; IRBP-Cre* and the *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* mice. One was that it was evident much earlier in the *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* mice. Opsin was detected throughout the photoreceptor cells by P7 (Fig. 5B), whereas in the *Kif3a^{-/-}/Kif3a^{fllox}; IRBP-Cre* mice, the cells appeared normal until the third postnatal week.⁵ The second difference was that all the rod photoreceptor cells were similarly affected in the *Kif3a^{fllox}/Kif3a^{fllox}; RHO-Cre-8* retinas; in the *Kif3a^{-/-}/Kif3a^{fllox}; IRBP-Cre* retinas, there was a large and unpredictable variation from cell to cell and animal to animal.⁵

Despite the relatively early decline in KIF3A and its effects on opsin localization, the photoreceptor cilia continued to grow normally, and morphogenesis of the outer segment disc membranes was initiated. Even at P10, several days after the redistribution of opsin was first evident, the photoreceptor axonemes and nascent outer segments appeared normal (Fig. 5H).

Moreover, by electron microscopy, we detected no ultra-structural defects in longitudinal and transverse sections of the connecting cilia of remaining photoreceptor cells in the degenerating retinas of older animals (not shown).

Rapid Photoreceptor Cell Degeneration Due to KIF3A Loss

At P10, *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* retinas had a normal complement of photoreceptor cells, but by P14, 30% of the cells had been lost, and by P28, only a single row of photoreceptor nuclei were evident in the photoreceptor cell nuclear layer (Figs. 6A–D). A few of these cells are still present at P42. Most, if not all of these persisting cells were cone photoreceptors. Electron micrographs of the photoreceptor synaptic layer showed the presence of cone pedicles (Fig. 6E) and the apparent absence of rod spherules. The persistence of cone photoreceptor cells is not surprising, given that they do not express the *RHO-Cre* (Figs. 1I–K). However, it is noteworthy that they also eventually degenerate, as in other rod-initiated photoreceptor degenerations. Figure 4 summarizes the relative time courses of the appearance of CRE, the loss of KIF3A, the redistribution of opsin, and the subsequent loss of photoreceptor cells.

DISCUSSION

We have generated mice that express *Cre* recombinase in rod photoreceptor cells under control of the *RHO* promoter. In one of the lines, *RHO-Cre-8*, the expression of *Cre* was synchronous and robust throughout the retina. When used to excise a *Kif3a^{fllox}* gene, we were able to define the time course of events, from the neonatal expression of *Cre*, to the decline in photoreceptor KIF3A, the mislocalization of opsin, and, finally, the complete loss of the photoreceptor cells in the ensuing weeks.

Several photoreceptor-specific, *Cre*-expressing transgenic mouse lines have now been reported. After the study of the *Kif3a^{fllox};IRBP-Cre* mice,⁵ cone opsin *Cre* lines were generated,^{29,30} and a mouse rod opsin *Cre* line was reported recently.³¹ However, these new lines have yet to be tested in a genetic study of a photoreceptor gene. To study the *Otx2* homeobox gene in photoreceptor cell fate during development, Nishida et al.³² produced a *Crx-Cre* mouse line, in which CRE is present embryonically. With our *RHO-Cre-8* line, retinal degeneration was initiated between 4 and 7 weeks of age. It is likely that this loss of photoreceptor cells is due to overexpression of the CRE protein, as mice that express a lower level of CRE in rod photoreceptors do not demonstrate degeneration up to 8 months of age.³¹ It is unclear whether the observed toxicity is due to a specific effect of the CRE recombinase or to general protein overexpression. Because overexpression of other proteins in photoreceptor cells has been observed to lead to cell death, simple overexpression of protein in these sensitive cells appears to be the more likely cause of degeneration.³³ It is noteworthy that the level of retinal CRE continued to increase up to 6 weeks of age in the *RHO-Cre-8* mice (Fig. 3B). CRE-mediated genomic toxicity in cultured cells has been reported, although similar effects of CRE expression in trans-genic mice have not been described.^{34,35}

CRE was detected in the *RHO-Cre-8* mice just after birth. The finding that no photoreceptor cell death was observed until 7 weeks of age demonstrates that rod cells tolerate transient overexpression of *Cre*. Thus, the *RHO-Cre-8* mice will be useful for conditional gene targeting experiments during the first postnatal month. This finding also suggests that mice that express *Cre* for a limited time might be ideal for conditional gene targeting in photoreceptor cells. Limiting *Cre* expression can be effected by incorporating loxP sites into the transgene, so that it self-excises.^{35,36}

A relatively high level of expression of *Cre* is likely to be needed to achieve the synchronous excision observed in the present study. The effect of *RHO-Cre-8* on *Kif3a^{fllox}* contrasts with

that of *IRBP-Cre*, which had variable effects, spatially and temporally, across the retina and among different animals.⁵ Although the study with *Kif3a^{fllox};IRBP-Cre* mice⁵ showed that the knockout of *Kif3a* resulted in opsin mislocalization in some cells and the death of some photoreceptor cells, the extent of this effect was not determined. It was not clear whether a given normal looking photoreceptor cell in a *Kif3a^{fllox};IRBP-Cre* retina was unaffected because there had been no gene excision or because the gene excision had no effect in that cell. From the present study, with gene excision and opsin redistribution evident in every rod photoreceptor cell and occurring during well-defined and sequential intervals, we can conclude that KIF3A and the delivery of opsin to the outer segment are inextricably linked.

The clearance of opsin from the inner segment was found to be very sensitive to the presence of KIF3A. A decline in KIF3A, rather than its complete loss, was sufficient to cause the accumulation of opsin in the inner segment. Perhaps, however, the relatively abrupt change in concentration of KIF3A contributed to the defect, as well as the lower concentration itself. *Kif3a* heterozygotes, which have retinal KIF3A levels that are only 50% of wild-type levels, do not undergo retinal degeneration.³⁷ Yet deleterious effects are evident in *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* mice after the decrease in photoreceptor KIF3A to 40% of wild-type levels that occurs in the first post-natal week.

Some 3 days after the start of opsin accumulation within the inner segment, the photoreceptor connecting cilium and the disc membranes of the nascent outer segment still appeared unperturbed (Fig. 5H). At no stage, even in the photoreceptors remaining in advanced degenerate retinas, were ultrastructural abnormalities evident in the photoreceptor connecting cilium. These observations, in addition to the relatively precise timing of the events, indicate that KIF3A loss disrupts motor traffic without immediately affecting the supporting infrastructure. They thus support the hypothesis that kinesin-2 transports opsin, rather than having a less direct role in opsin delivery to the outer segment, such as by maintenance of the structural integrity of the connecting cilium. They also support the notion that the critical element leading to apoptosis is the abnormal accumulation of opsin outside of the outer segment, rather than any structural perturbation of the axoneme or outer segment.

The presence of opsin throughout the photoreceptor cell has been reported during early photoreceptor development³⁸⁻⁴⁰ and before cell death in some other inherited retinal degenerations that appear to be unrelated to opsin transport (e.g., those in the RCS rat and *rd1* and *rd5* mice⁴¹). The accumulation of opsin outside the outer segments of *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* mouse photoreceptors, as observed herein, differs from the first case, in that it occurred after this early developmental stage, when the opsin distribution was fully polarized in the control photoreceptor cells (Figs. 5A, 5C). It may differ from both cases, in that the initial accumulation is primarily *within* the inner segments (at P7, Fig. 5F). The ectopic opsin distribution during development and in other photoreceptor degenerations has been demonstrated only in the plasma membrane.³⁸⁻⁴² In *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* mouse photoreceptors, significant ectopic distribution in the plasma membrane was not evident until a slightly later stage (at P10, Fig. 5H). Opsin in the plasma membrane of the inner segment, nuclear region, and synapse may indicate leakage from the outer segment in ailing cells. By contrast, an accumulation of opsin within the inner segment is consistent with a backlog of trafficking to the outer segment. An accumulation along the anabolic pathway (from a defect in targeting rather than retention) may be a more important trigger for cell death and may be responsible for the surprisingly rapid degeneration that follows the loss of kinesin-2.

In conclusion, the *RHO-Cre-8* mice are useful for studying gene ablation in rod photoreceptor cells and clearly provide a new and improved tool for such studies. The high and widespread expression of *Cre* results in relatively synchronous excision that is necessary for many experiments, especially biochemical ones. In the present study, it has enabled us to determine

the time course of events ensuing from *Kif3a* excision and to provide a clearer depiction of the role of kinesin-2 in opsin transport and photoreceptor cell viability.

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FIGURE 1.

RHO-Cre transgene and its expression in the retina. (A) The components of the *RHO-Cre* transgene. (B–E) The distribution of Cre-recombinase activity in the retinas of *RHO-Cre-8* (B, D), *RHO-Cre-16* (C), and control (lacking *RHO-Cre*) (E) mice was evaluated by X-Gal staining of the retinas, after crossing with *Gt(ROSA)26Sor^{tm1Sor}* reporter mice. Retinal wholemounts are from the double transgenic mice at ages 1 month (B, C) and 7 days (D, E) of age. (F–H) The cellular location of Cre-recombinase activity in the retinas of *RHO-Cre-8* (F) and *RHO-Cre-16* (G, H) mice was evaluated by X-Gal staining of retinal sections of double-transgenic mice at 1 month of age. Staining of the outer nuclear layer (ONL) was uniform throughout the retina in *RHO-Cre-8* mice, but patchy in the *RHO-Cre-16* mice (G, H). (I–K) Frozen sections of retinas from 1-month-old *RHO-Cre-8* mice were immunolabeled with antibodies against CRE (red) and cone opsin (green), showing that CRE is expressed only in rod photoreceptors. The CRE signal was detected in the nuclei of rod photoreceptor cells, but it does not overlap with the cone opsin signal (e.g., arrows). The red signal in the sclera and choroid of (I) and (K) is due to autofluorescence and is present in control sections stained without primary antibody (not shown). Scale bar: (F–K) 25 μ m.

FIGURE 2.

(A) Retinal function of *RHO-Cre-8* mice. Rod and cone functions in *RHO-Cre-8* mice were evaluated by ERG at the ages indicated. Data are presented as the percentage of the normal control at each time point. At least three mice of each genotype were studied at each age. The means $\pm$ SD of each type of response are plotted. (B) Photoreceptor cell loss in *RHO-Cre-8* mice. The retinal structure of *RHO-Cre-8* mice at different ages was evaluated by histology. At 4 weeks of age, the outer nuclear layer (ONL) and outer segments (OS) were normal. At later ages, the ONL and OS layer were both reduced in thickness. Scale bar, 25 μ m.

FIGURE 3.

Western blot analysis of retinal proteins, immunolabeled with antibodies against CRE (**A, B**) and KIF3A (**C**). Each lane was loaded with 25% (**A, C**) or 20% (**B**) of lysate, containing all retinal proteins. The retinas were from *Kif3a^{flox}/Kif3a^{flox};RHO-Cre-8* mice (**A**) and *RHO-Cre-8* mice (**B**) of different ages. The decrease in CRE after P14 in *Kif3a^{flox}/Kif3a^{flox};RHO-Cre-8* mice was due to the loss of photoreceptor cells in these retinas. Note that, in *RHO-Cre-8* mice, CRE continued to increase beyond this age. (**C**) Retinas from either *Kif3a^{flox}/Kif3a^{flox}* (**C**) or *Kif3a^{flox}/Kif3a^{flox}; RHO-Cre-8* (**M**) mice of different ages, except that those shown in the two *rightmost* panels were from a 2-month-old *rd1* mouse retina (*rd1*) and its age-matched control (**C**).

FIGURE 4.

Changes in levels of photoreceptor CRE and KIF3A and number of photoreceptor cells in *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* retinas. Because CRE was present only in rod photoreceptor cells, its levels were determined directly from retinal lysates. They are shown relative to the maximum level of CRE measured in the *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* retinas, which was at P14 (CRE cf. P14). KIF3A levels were determined from measurements of retinal lysates, adjusted by subtracting the estimated contribution from nonphotoreceptor cells. This contribution (~56%) was determined from the amount of KIF3A in a 2-month-old rd1 mutant mouse retina (at this age, the rd1 retina has no photoreceptor cells) relative to that in a 2-month-old control mouse retina. KIF3A levels and photoreceptor cell numbers are shown as a proportion of that measured in *Kif3a^{fllox}/Kif3a^{fllox}* (no RHO-Cre-8) retinas of same-aged littermates. CRE and KIF3A was quantified by densitometry from immunolabeled Western blots, such as those shown in Figure 3, where each lane was loaded with the same proportion of total retinal lysate. Photoreceptor nuclei were counted in the areas 500 μ m both sides of the optic nerve head. *Arrowhead*: time point at which mislocalization of opsin was first evident (P7).

FIGURE 5.

Opsin mislocalization in *Kif3a^{lox}/Kif3a^{lox};RHO-Cre-8* retinas. (A–E) Light micrographs of semithin sections of retinas, immunolabeled with opsin antibody and peroxidase secondary antibody. Opsin was localized normally in the outer segments of the control retinas: P7 (A) and P14 (C), *Kif3a^{lox}/Kif3a^{lox}* without CRE; P28 (E), *RHO-Cre-8* only. It was distributed throughout the photoreceptor cells in the mutant P7 (B) and P14 (D) *Kif3a^{lox}/Kif3a^{lox};RHO-Cre-8* retinas. Note that at P7 the outer segments were still very small, so that, at the magnification shown in (A) and (B), immunolabeling of the outer segment was barely evident (A, arrows: labeled outer segments). The important point is that some opsin was detected throughout the photoreceptor nuclear layer in the mutant (e.g., B, arrowheads), but not in the control (A). (F–H) Electron micrographs of rod photoreceptor cells, immunogold labeled with opsin antibody. (F) Micrograph showing immunogold label accumulated in the inner segment, beneath the connecting cilium (CC) and basal body (BB) of a photoreceptor from a P7 *Kif3a^{lox}/Kif3a^{lox};RHO-Cre-8* retina. (G) Control photoreceptor cell from a P10 animal. IS, inner segment. (H) Mutant photoreceptor cell from a P10 *Kif3a^{lox}/Kif3a^{lox};RHO-Cre-8* animal. In the mutant retinas (F, H), photoreceptor cilia and the nascent outer segments had formed and were still intact at P10 (H), despite the defective distribution of opsin. Scale bars: (A–E) 25 μ m; (F), 300 nm; (G, H) 1 μ m.

FIGURE 6.

Photoreceptor degeneration due to the loss of KIF3A. Light micrographs of retinas from a control P28 mouse (A) and from P7 (B), P14 (C), and P28 (D) *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* mice. Sections were prepared from blocks embedded in resin. (E) A cone photoreceptor synaptic terminal (pedicle) from P28 *Kif3a^{fllox}/Kif3a^{fllox};RHO-Cre-8* retina. Arrowheads: synaptic ribbons; in contrast to rod synaptic terminals (spherules), cone pedicles have more than one synaptic ribbon. Scale bar: (A–D) 25 μm; (E) 500 nm.