

Cone Opsin Mislocalization in *Rpe65*^{-/-} Mice: A Defect That Can Be Corrected by 11-*cis* Retinal

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PURPOSE. In retinal degenerative diseases, rod photoreceptors typically deteriorate more rapidly than cone photoreceptors. In the *Rpe65*^{-/-} mouse, a model for Leber's congenital amaurosis, cones degenerate much more rapidly than rods. In this model, the retinoid processing pathway in the retinal pigment epithelium is disrupted, and 11-*cis* retinal is not generated. This study was designed to investigate the feasibility of restoring functional cones with exogenous 11-*cis* retinal.

METHODS. *Rpe65*^{-/-}::*Rho*^{-/-} mice were used to remove any interference of rods and compared with wild-type (wt) mice. Pups were injected intraperitoneally with 11-*cis* retinal, starting at postnatal day (P)10, and were maintained in complete darkness. At P25, cone function was assessed with photopic single-flash and flicker ERGs. Cone survival was determined immunohistochemically with cone-specific antibodies, and cone opsin levels were obtained by quantitative RT-PCR.

RESULTS. At P25, cone density and transcript levels of cone opsins were drastically reduced, but a minute cone electroretinogram was detected, indicating that the cones were functional. Confocal microscopy revealed that the cone opsins were mislocalized, suggesting that their transport to the outer segments was impaired. Intraperitoneal administrations of 11-*cis* retinal before P25 led to increased transport of cone opsins to the outer segments and preserved cones anatomically and functionally.

CONCLUSIONS. The results suggest that the ligand is required during cone opsin synthesis for successful opsin trafficking and that without 11-*cis* retinal, cones may degenerate because of opsin mislocalization. These results may have important consequences for the treatment of cone dystrophies. (*Invest Ophthalmol Vis Sci.* 2005;46:3876-3882) DOI:10.1167/iov.05-0533

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RPE65 is a protein highly expressed in the RPE, where it has been shown to be essential for the generation of 11-*cis* retinal.¹ RPE65 has also been demonstrated in cone outer segments (OS) in several different species,² as well as in iris pigment epithelium.³ In the *Rpe65*^{-/-} mouse, rod photoreceptors appear to develop normally, with no apparent loss of cells until approximately 6 months of age, and rod function can be improved by the administration of exogenous chromophore up to at least 12 months of age.^{4,5} The slow degeneration of the rods observed in the mouse is controlled by both constitutive opsin activity⁶ and constitutive opsin phosphorylation.⁷ In contrast, cones degenerate quickly in this model.^{8,9} During normal aging, cones are more resilient than rods (e.g., see Ref. 10), and so it is particularly interesting that in the *Rpe65*^{-/-} mouse model, cones seem to be more vulnerable.

Leber's congenital amaurosis (LCA) is an autosomal recessive retinal dystrophy, for which mutations have been identified in approximately 50% of patients. Of these, approximately 6% were in the protein RPE65.¹¹ Because of the abundant and selective expression of the *Rpe65* gene in the RPE, this protein is a promising candidate for human gene therapy (e.g., see Refs. 12,13). It is therefore critical to understand the pathophysiology of the *Rpe65*^{-/-} phenotype, particularly with respect to the cones, as cones are the predominant photoreceptor type used for human high-acuity visual performance.

In the *Rpe65*^{-/-} mouse, a small visual response remains, even though no 11-*cis* retinal can be detected.¹ This response has been shown to correlate with the generation of a small amount of 9-*cis* retinal, forming the photosensitive isorhodopsin pigment in the rods.¹⁴ Seeliger et al.¹⁵ have reported that the remaining photoresponse is due to a rod response, based on their failure to elicit a response from a 4- to 5-week-old *Rpe65*^{-/-}::*Rho*^{-/-} mouse using normal ERG protocols.

The purpose of this study was to determine the fate of the cones in the absence of RPE-generated 11-*cis* retinal. We further sought to determine whether administration of 11-*cis* retinal can restore function to the cones as has been shown for the rod photoreceptors. The *Rpe65*^{-/-}::*Rho*^{-/-} mouse cross was used to eliminate the huge pool of rod opsin that could obscure the uptake of exogenously applied ligand. Furthermore, the cone responses in the *Rpe65*^{-/-} retina could be masked by desensitized rod responses with altered kinetics.^{7,15} In this double-knockout model, we report that cone responses are unequivocally identified, so cones are functional at early ages. We further show that cones were lost rapidly in the *Rpe65*^{-/-}::*Rho*^{-/-} mouse retina, with the remaining cones containing mislocalized opsin. Finally, treating the animals with 11-*cis* retinal starting at postnatal day (P)10 resulted in increased cone survival, properly localized cone opsin, and increased cone responses.

METHODS

Animals

Rpe65^{-/-}::*Rho*^{-/-} mice were generated and genotyped as described previously.¹⁵ Age-matched C57BL/6 (wild-type [wt]) mice were gener-

ated from breeding pairs obtained from Harlan Laboratories (Indianapolis, IN). The analysis was restricted to <1-month-old animals. Cones in the *Rbo*^{-/-} mouse have been reported to develop normally and exhibit normal or even supernormal cone responses up to 6 weeks of age,¹⁶ and no structural alterations have been observed in the inner retina at this age. To study the effect of 11-*cis* retinal treatments, littermates were randomly assigned to the experimental or the control groups and kept in the dark starting at P10. Once moved into the dark, experimental animals were injected intraperitoneally with 11-*cis* retinal (2.5 µg per dose in 150 µL vehicle [10% ethanol, 10% bovine serum albumin, and 0.9% NaCl]). A single injection of this dose has been shown to lead to the successful regeneration of ~10% of rhodopsin in the *Rpe65*^{-/-} retina.⁷ All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Medical University of South Carolina (MUSC) Animal Care and Use Committee.

ERG Analysis

Full-field ERGs were recorded as described previously,¹⁷ with minor modifications.⁷ Briefly, the setup was modified to include a dual-channel optical bench for light stimulation, providing stimulus and background light. Both optical pathways were driven by a single 250-W halogen lamp and controlled with mechanical shutters, manually operated neutral-density filters, and a 500-nm band-pass filter. The two pathways were combined and the light beam focused on the end of the light guide. Light intensity per 10 ms provided in the stimulus path was varied in steps of 0.3 log units from 2.1×10^7 to 2.2×10^{13} photons/mm², in white-light conditions, whereas the adaptation beam provided a continuous flux of 5.5×10^8 photons/mm² per second at 510 nm. Based on the characteristics of the Halogen lamp, which has a continuous spectral output from 300 to 2000 nm and the transmittance of the optical components of 350 to 2100 nm, we stimulated predominantly mouse midwavelength (MWL) cones (λ_{max} 508 nm) and, to a lesser degree, mouse short-wavelength (SWL) cones (λ_{max} 355 nm).¹⁸ Photopic electroretinograms were recorded by two protocols: (1) single-flash ERGs, and (2) 10-Hz flicker ERG of increasing light intensities, averaging three to five responses for single-flash and 10 responses for flicker ERGs. Peak b-wave amplitude was measured from the trough of the a-wave to the peak of the positive b-wave after a high-pass filter was applied, to eliminate oscillatory potentials.¹⁸ ERG recordings were stored, displayed, and analyzed with a computer interface and PClamp (Axon Instruments, Burlingame, CA) and commercial software (Origin; Microcal, Northampton, MA). ERG recordings were performed on *Rpe65*^{-/-}::*Rbo*^{-/-} mice ($n = 21$) and 11-*cis* retinal-injected *Rpe65*^{-/-}::*Rbo*^{-/-} mice ($n = 31$; ≥ 6 for each treatment paradigm). In previous experiments, such group sizes were found to be appropriate for generating statistically reliable results.⁷ Data are expressed as the mean \pm SEM and were analyzed by Student's *t*-test.

Immunohistochemistry

For immunohistochemical analysis, eyes were fixed in 4% paraformaldehyde, rinsed, cryoprotected in 30% sucrose overnight, frozen in optimal cutting temperature (OCT) compound (TissueTek; Sakura Finetek, Torrance CA) and cut into 14-µm cryostat sections. Immunohistochemistry was performed as described previously,¹⁷ with the following primary antibodies: rabbit anti-mouse SWL-cone opsin¹⁹ (a generous gift from Jeanny Chen, University of Southern California, Los Angeles, CA) and rabbit anti-human MWL-cone opsin²⁰ (JH 492; a generous gift from Jeremy Nathans, Johns Hopkins University School of Medicine, Baltimore, MD). For visualization, fluorescently labeled secondary antibodies (Alexa 488; Molecular Probes) were used. Each staining was performed on slides from at least three animals per condition. Sections were examined by confocal microscopy (Leica, Deerfield, IL), and the images were pseudo-colored (Photoshop; Adobe Systems, Mountain View, CA). To determine cone opsin distribution, images were binarized and thresholded, to set the background to zero,

and distribution profiles were analyzed in Image J (NIH; 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). In sections in which cone spacing allowed imaging of individual cones (i.e., *Rpe65*^{-/-}::*Rbo*^{-/-} mice), single cone profiles were analyzed. In all other cases, a group of photoreceptors (20–30) were measured together. Cone lengths (OS to synaptic pedicle) and distribution profiles were normalized to 100%, and data points were binned based on the smallest image of the samples to be compared (Matlab, ver. 6.5; The MathWorks, Natick, MA). The profiles of the mean \pm SD opsin distribution were plotted (Origin software; Microcal). Opsin distribution profiles were compared by averaging over the three regions of interest (0%–20%, OS; 20%–90%, inner segment cell body, and axon; and 90%–100%, synaptic pedicle relative lengths), with Student's *t*-test used to determine statistical significance.

Real-Time PCR

Real-time PCR was performed as described previously.⁹ Briefly, littermates (treated and untreated) were killed by decapitation, and retinas were quickly removed. Total RNA was isolated (TRIzol; Ambion, Austin, TX) and equal amounts of RNA were used in reverse transcription reactions (Invitrogen, Carlsbad, CA). PCR amplifications were conducted using a kit (QuantiTect SYBR Green; Qiagen, Valencia, CA), according to the manufacturer's instructions, with 0.2 µM forward and reverse primers and equal amounts of complimentary DNA (1 µL of 1:10 dilutions): β -actin, forward GCT ACA GCT TCA CCA CCA CA, reverse TCT CCA GGG AGG AAG AGG AT, 123 bp; SWL-opsin, forward TTG GGC TCT GTA GCA GGT CT, reverse CAA GTA GCC AGG ACC ACC AT, 137 bp; and MWL-opsin, forward CTC TGC TAC CTC CAA GTG TGG, reverse AAG TAT AGG GTC CCC AGC AGA, 148 bp. Reactions were treated with 0.01 U/µL Uracil N-glycosylase (AmpErase; Applied Biosystems, Inc. [ABI], Foster City, CA) to prevent carryover contamination. Real-time PCR was performed in triplicate in a sequence detection system (GeneAmp 5700; ABI) using the after cycling conditions: 50°C for 2 minutes, 94°C for 15 minutes, 40 cycles of 94°C for 15 seconds, and 58°C for 1 minute. Quantitative values were obtained from the cycle number (C_t), which is inversely proportional to the amount of a specific mRNA species in the tissue sample from which the cDNA was derived. Relative gene expression levels were calculated with the equation $y = (1 + AE)^{\Delta\Delta C_t}$, where AE is the amplification efficiency of the target gene (set at 1.0 for all calculations), and $\Delta\Delta C_t$ is the difference between the mean experimental and control ΔC_t . ΔC_t is the difference between the C_t for a retina-associated gene and the β -actin internal reference control gene.

RESULTS

Cone Survival and Function in the *Rpe65*^{-/-}::*Rbo*^{-/-} Mouse Retina

The mouse has two types of cone opsins: the UV-sensitive SWL opsin and the green MWL opsin. The cones comprise approximately 3% of the photoreceptors in the retina²¹ and many contain a mixture of the two cone pigments.²² To determine the fate of cone photoreceptors caused by *Rpe65* disruption and lack of 11-*cis* retinal in the RPE, we crossed the *Rpe65*^{-/-} with the *Rbo*^{-/-} mouse, so that cone function could be identified unequivocally in ERG recordings. ERG protocols designed to separate rod and cone responses are based on wt responses, with specific spectral, temporal, and light-intensity characteristics. However, these protocols fail in the case of the *Rpe65*^{-/-} mouse, as the rods have elevated thresholds, different response kinetics,^{6,7} and slightly altered spectral tuning properties.¹⁴

Previous results have demonstrated that cones are lost rapidly in the central portion of the *Rpe65*^{-/-} mouse retina, whereas peripheral cones are more resilient.⁹ To determine whether these remaining cones are functional, photopic ERG

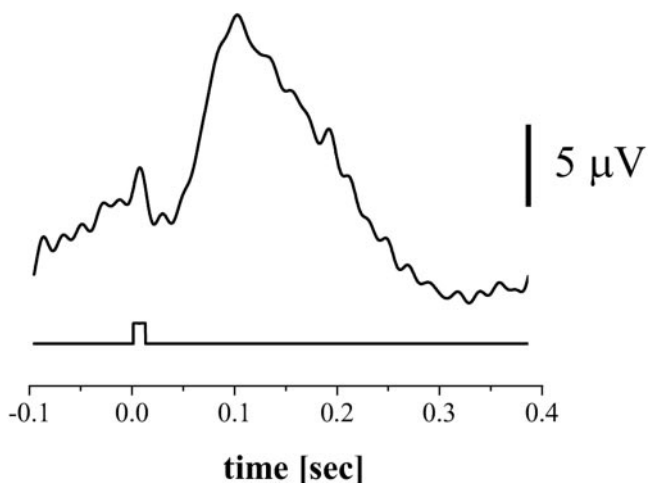


FIGURE 1. Cone ERG of *Rpe65*^{-/-}:*Rbo*^{-/-} mice. Single-flash photopic ERGs were recorded from the P25 *Rpe65*^{-/-}:*Rbo*^{-/-} mouse to isolate cone responses. At maximum light intensity (2.2×10^{13} photons/mm² using white-light stimulation), averaging 50 responses, a small ($13.7 \pm 1.87 \mu\text{V}$; $n = 9$) signal was recorded.

responses were recorded from dark-adapted P25 *Rpe65*^{-/-}:*Rbo*^{-/-} mice by using white light at maximum flash intensity ($\sim 2.2 \times 10^{13}$ photons/mm²). As reported previously,¹⁵ when normal averaging procedures (i.e., three to five times) were used, no ERG was elicited from 4- to 5-week-old animals. However, minute cone responses ($13.7 \pm 1.87 \mu\text{V}$; $n = 9$) were revealed if 50 traces were averaged in 3.5-week-old animals (Fig. 1), indicating that both the cone opsins and the cone transduction pathways are functional in the absence of RPE65. In rods, the chromophore of the small amount of active pigment has been shown to be 9-*cis* retinal.¹⁴ The ligand of the pigment(s) responsible for the minute cone response in the *Rpe65*^{-/-} mouse retina has not yet been identified.

Cone opsin levels of P25 animals were measured using quantitative RT-PCR. The cone opsin mRNAs were downregulated ~ 3.5 -4-fold when compared with those in wt mice (Fig. 2). A similar downregulation of cone opsins was observed in the P28 *Rpe65*^{-/-} mouse,⁹ although the MWL cone opsin showed a somewhat less dramatic decrease. These results show that cone opsins in a <1-month-old mouse are not adversely affected by the loss of the rhodopsin gene and thus by the lack of the rod OS.

Effect of Exogenous 11-*cis* Retinal on Opsin mRNA and Level of Cone Function

We have previously reported that intraperitoneal 11-*cis* retinal injections successfully increase rod function in young and aged *Rpe65*^{-/-} mice,^{5,7} suggesting that this would be a useful route for administration in making the 11-*cis* retinal available to the cone opsins in *Rpe65*^{-/-}:*Rbo*^{-/-} mice. Therefore, 11-*cis* retinal was injected intraperitoneally, with lipid-free BSA used as a carrier, starting at P10, followed by three additional treatments at P13, P17, and P21. ERG analysis was performed at P25, using photopic single-flash and flicker paradigms with normal averaging (i.e., 3-5 times for single-flash ERGs and 10 times for flicker ERGs). No responses were elicited in the untreated control animals (Fig. 3A), when this number of averages was used, as had been shown previously.¹⁵ However, typical single-flash cone and

flicker ERGs were recorded in the treated animals (Figs. 3B, 3C). Single-flash cone ERGs reached maximum amplitudes of $214 \pm 19 \mu\text{V}$ ($n = 12$), and flicker ERG responses reached up to 30 Hz (data not shown). These single-flash amplitudes are comparable to those obtained from age-matched *Rbo*^{-/-} mice using similar stimulus conditions.¹⁶ The flicker fusion frequency agrees well with the value of more than 30 Hz reported for mouse cone responses.²³

Animals that received 11-*cis* retinal also showed higher levels of mRNA for both cone opsins. The levels were almost restored to those of the wt mice (Fig. 2).

Effect of Chromophore on Cone Opsin Localization

Cone opsin localization was examined by confocal microscopy in radial sections by using antibodies specific to SWL¹⁹ and MWL cone opsins.²⁰ Because the remaining cones in the *Rpe65*^{-/-} and the *Rpe65*^{-/-}:*Rbo*^{-/-} mouse retinas are localized almost exclusively in the peripheral retina at P25, the analyses for all animals was restricted to those areas. As expected, both cone opsins were localized to the OS in the P25 wt mouse retina (Figs. 4A, 4D). However, in the *Rpe65*^{-/-}:*Rbo*^{-/-} cones, both the SWL and the MWL opsins appeared to be mislocalized. A significant amount of the protein was found to be localized in the cell membrane of the inner segment, cell body, axon, and synaptic pedicle (Figs. 4B, 4E). The antibody staining was found to be more pronounced for the SWL- than for the MWL-cone opsin, which may be due to differences in the binding affinity of the primary antibodies and/or differences in overall opsin levels. The wt mice have an SWL:MWL ratio of 3:1, and there was mixing of the cone opsins within single photoreceptors.²² 11-*cis* Retinal injections, which increased cone opsin transcripts (Fig. 2) and cone function (Fig. 3), resulted in the expected higher density of cones stained for cone opsins (Figs. 4C, 4F). In addition, significantly more of the generated cone opsin was localized appropriately to the OS (Figs. 4C, 4F).

Cone opsin distribution was quantified by profile analysis. Individual cones could not be analyzed in histologic sections, as they are too closely spaced in the wt and the mutant cones after 11-*cis* retinal treatment (see Figs. 4A, 4C). Thus, profiles were obtained from images of groups of cones. Cone lengths (OS to synaptic pedicle) were normalized to 100% to allow for averaging and comparison between genotypes and treatments. Cone opsin distribution analysis confirmed that cone opsins were mistargeted in the remaining cones of the *Rpe65*^{-/-}:*Rbo*^{-/-} mouse retina, whereas the profiles more

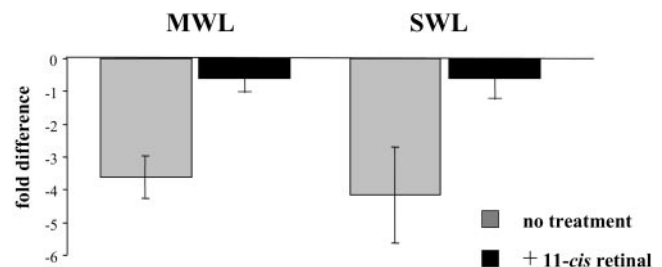


FIGURE 2. Cone opsin transcript levels of *Rpe65*^{-/-}:*Rbo*^{-/-} mice. Equal amounts of mRNA from the *Rpe65*^{-/-}:*Rbo*^{-/-} and C57BL/6 (wt) mice at P25 were used for real-time RT-PCR and normalized to β -actin levels. SWL- and MWL-cone opsin mRNA levels in *Rpe65*^{-/-}:*Rbo*^{-/-} mice (with or without 11-*cis* retinal) were expressed as α -fold change when compared with the age-matched wt mice (mean \pm SD, $n = 3$). After the application of 11-*cis* retinal, opsin levels increased almost to wt levels.

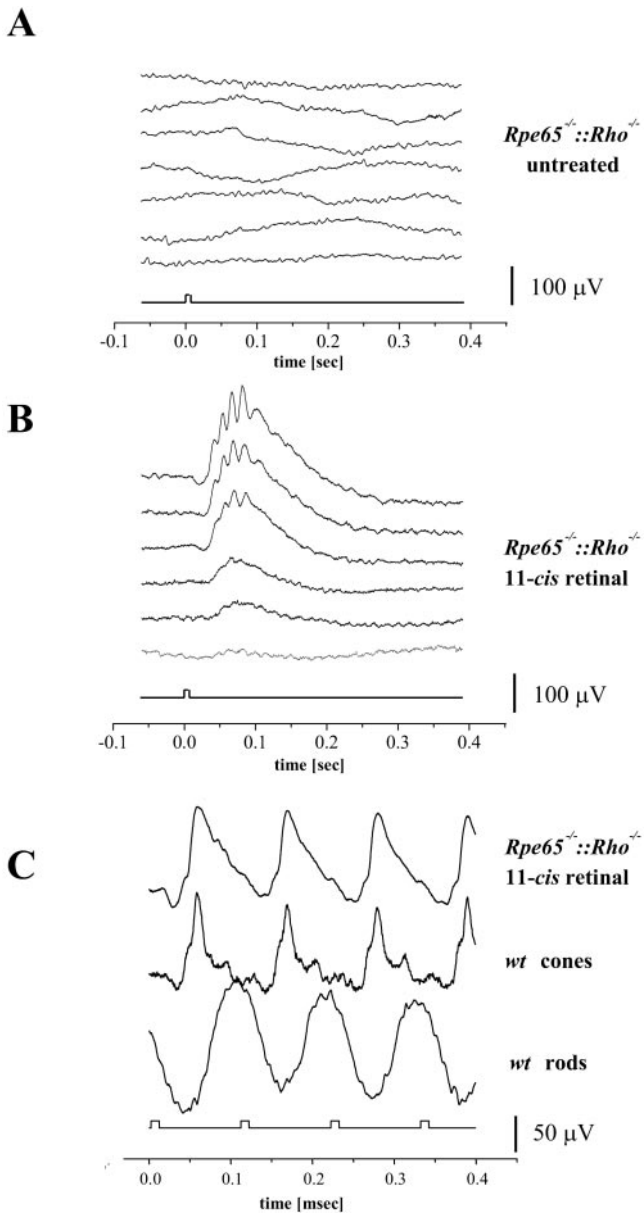


FIGURE 3. Cone ERGs in *Rpe65*^{-/-}::*Rho*^{-/-} mice after 11-*cis* retinal treatment. Families of single-flash photopic ERGs (**A**, **B**) or flicker ERGs at single-flash light intensities (**C**) were recorded from *Rpe65*^{-/-}::*Rho*^{-/-} in the absence and presence of exogenous 11-*cis* retinal. (**A**) No cone ERG response was recordable from untreated *Rpe65*^{-/-}::*Rho*^{-/-} mice when normal averaging ($n = 3-5$) was used. (**B**) However, treatment with 11-*cis* retinal elicited both a- and b-waves. (**C**) In wt mice, both typical 10-Hz rod (*bottom trace*) or cone (*middle trace*) flicker ERGs were elicited. 11-*cis* Retinal treatment of *Rpe65*^{-/-}::*Rho*^{-/-} mice revealed the saw tooth characteristics of the cone flicker ERG (*top trace*). Please note that for single-flash photopic ERGs, light intensities ranged from 2.2×10^{11} to 2.2×10^{13} photons/mm²; whereas scotopic rod flicker ERGs were elicited using 510 nm light at 3.1×10^8 photons/mm², and photopic cone flicker ERGs were recorded in the presence of steady background light at 510 nm, and white light at an intensity of 2.2×10^{12} photons/mm².

closely resemble the wt patterns in the 11-*cis* retinal-treated retinas (Fig. 5, see Table 1 for statistical analysis). It is of note that the SWL opsin distribution was not as amenable to correction by administration of 11-*cis* retinal as was the MWL opsin.

DISCUSSION

RPE65 is an essential component in the enzymatic pathway necessary for the production of 11-*cis* retinal, the chromophore for rod and cone opsins. In the *Rpe65*^{-/-} mouse, no 11-*cis* retinal is detectable in the retina or RPE,^{4,15} yet small rod responses can be recorded at least until 18 months of age.⁵ These responses have been shown to be due to the generation of a small amount of 9-*cis* retinal.¹⁴ Light sensitivity can be increased in the rods by supplying exogenous chromophore.^{4,7} Herein, we report that a small cone response was elicited from the remaining cones in a P25 *Rpe65*^{-/-}::*Rho*^{-/-} mouse. We have not identified the chromophore of these photoactive cone opsins, but it is possible that it is the 9-*cis* retinal present in the rods. This minute cone response has not been previously identified, as commonly used averaging strategies for ERG responses (2-5 times) are not sufficient to reveal the response; rather, extensive averaging (~ 50 times) is necessary to uncover the response from the background activity.

In the *Rpe65*^{-/-} mouse, the level of rod opsin remains stable for approximately 6 months, and rhodopsin can be regenerated from opsin *in vitro* from animals 18 month of age.⁵ However, the cone photoreceptors die very quickly in this mouse model.⁹ Rod opsin in the absence of chromophore has been shown to be constitutively active.²⁴⁻²⁶ Rod degeneration in the *Rpe65*^{-/-} mouse is proposed to result from opsin activity.⁶ This rod degeneration is slow, possibly because of the phosphorylation of opsin.⁷ Although constitutive activity of cone opsins may also be involved in cone degeneration in the *Rpe65*^{-/-} mouse retina, the rapid time course of cone loss suggests the involvement of additional factors. Indeed, in this model, cone opsins were found to be mislocalized, resulting in apoprotein localization to the cell membrane, in particular in the axon and the synaptic pedicle, concomitant with rapid cone loss. Cone opsin distribution resembling that in the wt retina could be produced by injection of 11-*cis* retinal, resulting in significant cone survival and almost normal levels of cone opsin mRNA. The prevention of cone loss and of opsin mislocalization by exogenous 11-*cis* retinal suggests these defects were caused by the lack of ligand, rather than indirectly by the lack of rod OS, the slow accumulation of OS debris, or the potential lack of the rod-derived cone survival factor.^{27,28}

Cone and rod opsins are similar at the genetic level, suggesting that they evolved from a common precursor. They are integral membrane proteins of the OS membrane or disc membrane and function as G-protein-coupled receptors in light. Transport of rhodopsin to the OS and correct insertion into the disc membranes appears to be dependent on protein structure and folding. It is proposed that the vesicular transport of the immature and unfolded rhodopsin from the endoplasmic reticulum (ER) through the Golgi apparatus to the OS discs is aided by two chaperones: NinaA and α -crystallin.²⁹ Wild-type rhodopsin is core glycosylated in the ER, followed by glycan group modifications in the Golgi apparatus.³⁰ These modifications appear to be sufficient for proper folding and protein stability, as transport of the apoprotein to the OS and insertion into the disc membrane appear to be normal in the *Rpe65*^{-/-} mouse retina.¹ However, in the case of the thermally unstable P23H rhodopsin mutation, chromophore binding to the opsin in the ER has been found to improve folding, posttranslational modifications of the protein, and subsequent transport to the cell membrane in HEK293 cells.³¹ Although the two mouse cone opsins have sites for potential glycosylation, glycosylation has been reported not to be required for the formation of functional bovine green cone (MWL) pigment.³²

Misfolded proteins are selectively destroyed in a ubiquitin-dependent process by the large adenosine triphosphate (ATP)-dependent proteolytic machine, the 26S proteasome.³³ How-

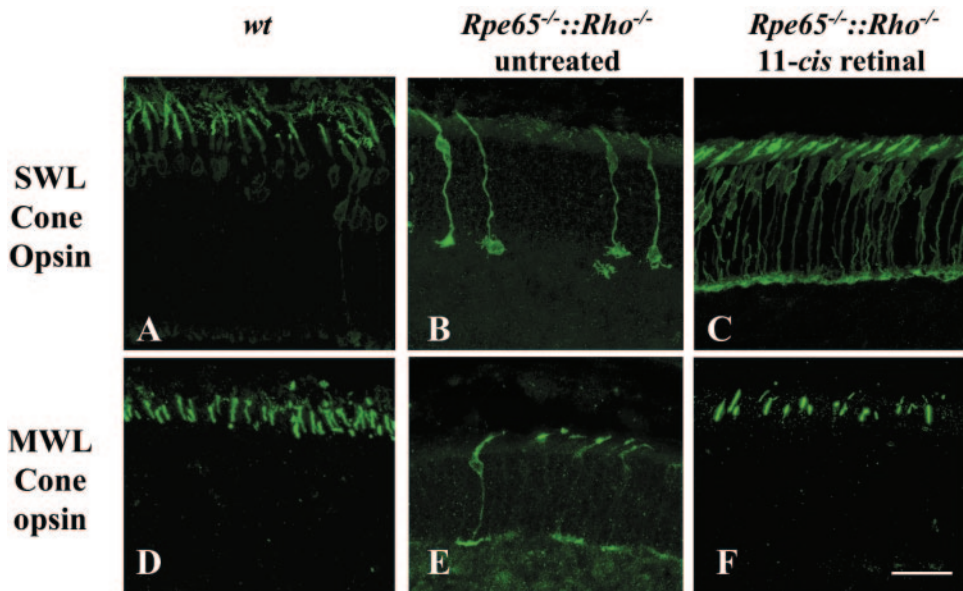


FIGURE 4. Localization of cone opsins. Retinal sections of P25 mice were stained with antibodies against SWL (A-C) and MWL-cone opsin (D-F). In C57BL/6 (wt) retina (A, D), both opsins were localized predominantly to the OS; whereas in the *Rpe65*^{-/-}:*Rho*^{-/-} mice, both SWL (B) and MWL-cone (E) opsin was mislocalized to the axon and cone pedicle. After the application of 11-*cis* retinal, most of the opsin was appropriately localized to the OS (C, F). Scale bar, 25 μm.

ever, under conditions when the normal proteolytic machinery gets saturated (i.e., in the presence of constitutive production of misfolded proteins such as in cystic fibrosis and in the major neurodegenerative diseases), aggresomes or ubiquitinated inclusions start to form.³⁴ The formation of aggresomes appears to lead to the disruption of the Golgi and intermediate filament

networks and involves the upregulation of several cellular chaperones,^{34,35} all of which may ultimately lead to cell death by apoptosis. One proposed mechanism by which photoreceptors may reduce the formation of stress-induced aggregates of protein(s) is by increasing the production of molecular chaperones such as α-crystallin.^{36,37}

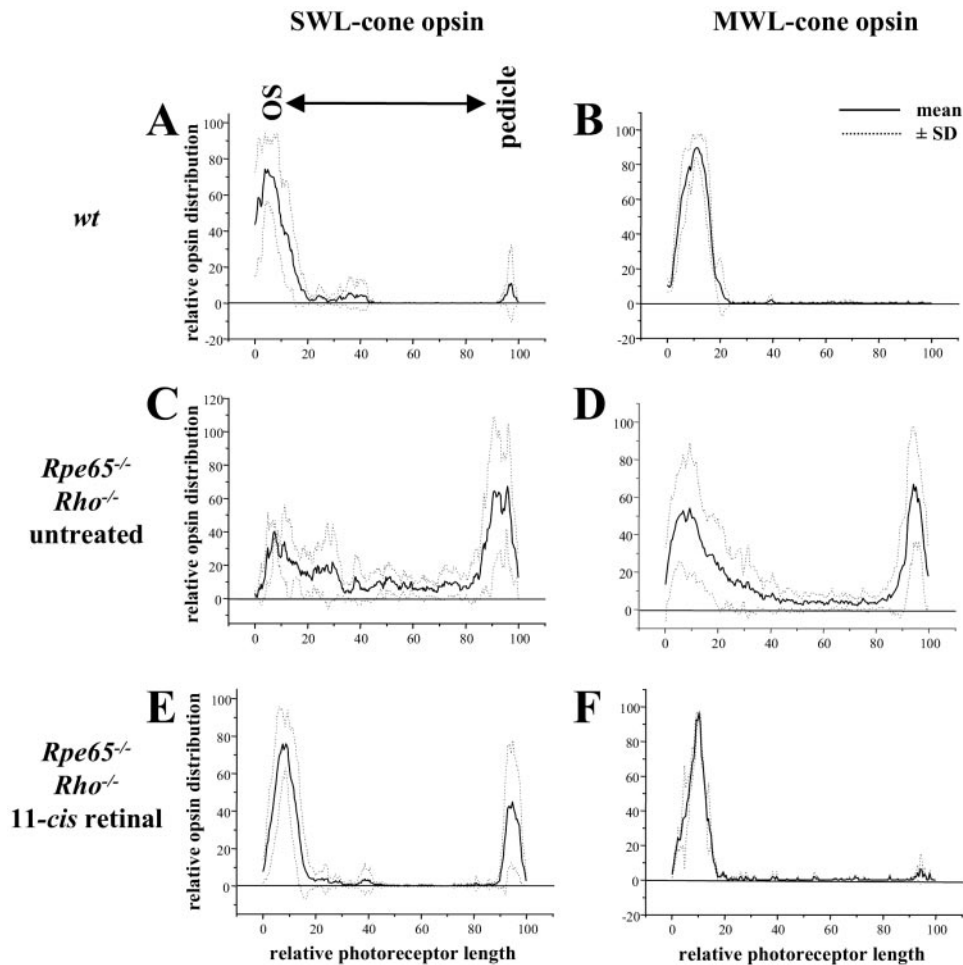


FIGURE 5. Cone opsin profiles. Cone opsin profiles were obtained from binarized and thresholded images. Intensity profiles and cone lengths (OS to synaptic pedicle) were normalized to 100%, and the mean ± SD was plotted. For statistical analysis, intensity profiles were compared by averaging over the three regions of interest (relative lengths: 0%-20%, OS; 20%-90%, inner segment, cell body and axon; and 90%-100%, synaptic pedicle). See Table 1 for statistical analysis and significance.

TABLE 1. Analysis of Cone Opsin Distribution

Animal	Opsin	OS		IS-CB-Axon		Synaptic Pedicle	
		Intensity	P	Intensity	P	Intensity	P
Wild type	SWL	47.7 ± 23.2		1.2 ± 1.8		3.4 ± 5.9	
	MWL	50.7 ± 12.5		0.4 ± 0.6		0.2 ± 0.2	
<i>Rpe65</i> ^{-/-} :: <i>Rbo</i> ^{-/-} control	SWL	21.8 ± 11.8	<0.001	11.2 ± 10.4	<0.001	50.6 ± 30.7	<0.0001
	MWL	37.8 ± 24.8	NS	6.74 ± 6.8	<0.01	33.9 ± 22.7	<0.001
<i>Rpe65</i> ^{-/-} :: <i>Rbo</i> ^{-/-} 11- <i>cis</i> retinal	SWL	41.7 ± 20.9	<0.0001	1.8 ± 2.8	<0.001	27.1 ± 22.9	<0.05
	MWL	35.7 ± 6.1	NS	0.3 ± 0.4	<0.05	1.43 ± 0.7	<0.01

Cone opsin intensity profiles and cone lengths (OS to synaptic pedicle) were all normalized to 100%. Intensity profiles were compared by averaging over three regions of interest: OS 0–20%, inner segment (IS), cell body (CB) and axon 20%–90% and synaptic pedicle 90% to 100% relative length. The mean ± SD of the average intensity is recorded. Student's *t*-test was used to test for statistical significance, and probabilities (*P*) are shown. Noted in rows 3 and 4 are the probabilities comparing wild-type versus untreated *Rpe65*^{-/-}::*Rbo*^{-/-} mice to determine the effect of the gene disruption, whereas in rows 5 and 6 the probabilities indicate the comparison between untreated versus 11-*cis* retinal treated *Rpe65*^{-/-}::*Rbo*^{-/-} mice to obtain the effect of the treatment.

NS, not significant.

Little is known about the retinoid cycling mechanisms in cones. Mata et al.³⁸ have proposed from studies in the cone-dominant ground squirrel retina that a mechanism involving the Müller cells is operational for the generation of 11-*cis* retinal for the cones. It is of interest to note the virtual absence of a cone response in a mouse that lacks *Rpe65*, suggesting that this pathway may be restricted to cone-dominant retinas or that it may involve RPE65, which we have found in cones of several species, including the mouse.² Further evidence for a variance in retinoid processing between cones and rods comes from the work of Jin et al.,³⁹ who found that whereas isolated bleached salamander rods can only incorporate retinal through the OS, bleached cones can incorporate 11-*cis* retinal through either the inner or OS, suggesting that binding to the opsin occurs in the inner segment. Although the role of the RPE65 protein in cones is unknown, its absence apparently does not prevent uptake of exogenous 11-*cis* retinal by the cones.

The results described herein provide evidence that ligand binding to opsin is necessary for protein stability and transport to the OS in cones. In its absence, both MWL- and SWL-cone opsins are mislocalized in part to the membrane of the pedicle and the axon, presumably resulting in rapid loss of cone OS and cone degeneration. On supplying 11-*cis* retinal, significantly more cone opsin is transported to the cone OS, where it can participate in light absorption and signal transduction. Concurrently, cones degenerate more slowly. Of note, the correction of this opsin mislocalization is more striking with the MWL opsin than the SWL opsin, for reasons that deserve further study. Whether the formation of aggresomes is involved in the degenerative process of the *Rpe65*^{-/-} cones is currently unclear. However, in gene array experiments, crystallins have been found to be upregulated (more than twofold) in the *Rpe65*^{-/-} retina (Rohrer B, unpublished data, 2004), whereas genes encoding for cone proteins are downregulated.⁹

In summary, our results show that if sufficient chromophore is supplied to the cones during OS development, cone opsin synthesis and transport can progress normally, preventing cone degeneration. These results need to be verified in other models, such as the Briard dog,¹³ to assure that this is not a species-specific phenomenon. However, our results suggest that if gene therapy were to be adopted for *Rpe65* LCA patient treatment with the goal of recovering both rod and cone function, an intermediate strategy should be adopted to prevent missorting of cone opsins and subsequent cone degeneration. Treatment frequency and ligand dose must be identified, and a mode of delivery that specifically targets cones must be designed for this approach to be useful for cone survival.

More generally, the systemic supplementation of 11-*cis* retinal may be useful in improving vision and ameliorating cone degeneration in a variety of disorders in which the chromophore supply to cones is primarily or secondarily impaired.

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