Light Prevents Exogenous 11-*cis* Retinal from Maintaining Cone Photoreceptors in Chromophore-Deficient Mice

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Purpose. To determine the effect of light/dark cycles on the cones of 11-*cis* retinal–treated RPE65/rhodopsin double knockout ($Rpe65^{-/-}Rho^{-/-}$) mice. Studies have shown that cones degenerate in chromophore-deficient mouse models for Leber Congenital Amaurosis (LCA), but exogenous supplementation of the native 11-*cis* retinal chromophore can inhibit this degeneration, suggesting that 11-*cis* retinal could be used as a therapeutic agent for preserving functional cones in patients with LCA. However, these treated mice were maintained in the dark.

Methods. 11-*cis* Retinal was introduced into $Rpe65^{-/-}Rho^{-/-}$ mice at postnatal day 10 as a single subcutaneous injection mixed with a basement membrane matrix. The mice were maintained in either normal light/dark cycles or constant dark conditions. Fluorescence microscopy was used to assess retinal morphology. Cone cell survival was determined by counting cone opsin–containing cells on flat-mounted P30 retinas. Cross-sections of P21 mouse retina were used to assess cone cell integrity by visualizing opsin localization. Cone function was determined by electroretinography (ERG).

Results. Previous studies have shown that 11-*cis* retinal–treated mice lacking RPE65 and raised in constant dark have higher cone photoreceptor cell number, improved cone opsin localization, and enhanced cone ERG signals when compared with untreated mice. However, in this study the authors show that 11-*cis* retinal–treated $Rpe65^{-/-}Rho^{-/-}$ mice raised in cyclic light did not show the improvements seen with the dark-reared mice.

Conclusions. Thus, 11-*cis* retinal by itself, as well as other agents that form photosensitive pigments, will not be good therapeutic candidates for preserving cones in LCA. (*Invest Ophthalmol Vis Sci.* 2011;52:2412–2416) DOI:10.1167/iovs.10-6437

Leber Congenital Amaurosis (LCA) is an early-onset childhood blinding disease.[1–3] Mutations to proteins involved in the visual cycle, a process wherein the chromophore (11-*cis* retinal) for visual pigments is regenerated, have been implicated in several forms of LCA. LCA2 is a predominant form and associated with defective RPE65,[4] a retinal pigment epithelium protein that is critical in the conversion of the visual chromophore from an all-*trans* to an 11-*cis* form.[5–7] Recent studies on patients with LCA2 have noted early loss of visual acuity, in particular no observed blue color vision, and thinning of the fovea, consistent with early loss of cones.[8–11] A mouse model for LCA2 in which RPE65 has been knocked out ($Rpe65^{-/-}$) has been generated by Redmond et al.[12] In this mouse, 11-*cis* retinal is not synthesized, visual pigments are not formed,[12] and cones degenerate rapidly.[13]

Rohrer et al.[14] further observed abnormal localization of both the middle- and short-wavelength sensitive (M- and S-, respectively) cone opsins within the first three weeks after birth before cone degeneration. Usually, cone opsins are predominantly localized in the cone outer segments; however, cone opsins in $Rpe65^{-/-}$ mice are distributed throughout the cone cell from the outer segments to the synapse pedicles. Interestingly, cone opsin localization and cone cell survival can be improved with early administration of 11-*cis* retinal when maintained in the dark.[14–16] Another retinal analog, 9-*cis* retinyl acetate which is converted to 9-*cis* retinal,[17] also improves cone morphology and function in similar mouse models.[18] These results suggest that receptor-ligand (cone opsin-11-*cis* retinal) interactions can help prevent cone photoreceptor cell death. They also suggest the potential of such a ligand as a useful therapeutic agent in preserving cone cell integrity and function for patients with LCA2. However, the mice were maintained in the dark after treatment; this treatment protocol may have optimized the effectiveness of 11-*cis* retinal because 11-*cis* retinal and the pigments generated with 11-*cis* retinal and cone opsins are both highly light-sensitive.[19] If the 11-*cis* form of the molecule is important, then photoisomerization might be destructive and would limit the efficacy of 11-*cis* retinal as a potential therapeutic agent for LCA2. In this study, we assessed the effectiveness of 11-*cis* retinal treatment to a mouse model for LCA, $Rpe65^{-/-}Rho^{-/-}$ mice, under cyclic light conditions. We show that there was no improvement of cone cell health and function after treatment under cyclic light conditions.

Materials and Methods

Animal Use

$Rpe65^{-/-}Rho^{-/-}$ mice were the generous gift from Mathias Seeliger (University of Tübingen, Tübingen, Germany). All experiments were designed and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Medical University of South Carolina Animal Care and Use Committee. Unless stated otherwise, mice were maintained in the MUSC core animal facilities under 12 h light/12 h dark cyclic light conditions. Light intensity at cage level varied depending on the cage location in the rack and averaged 150 ± 20 lux. Mice were killed at the ages indicated in the text by carbon dioxide exposure and cervical dislocation.

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11-*cis* Retinal Treatment

11-*cis* Retinal was introduced into postnatal day (P) 10 $Rpe65^{-/-}Rho^{-/-}$ pups (4.5–6.0 g) by injecting a 200 μL mixture of 0.9 μmol 11-*cis* retinal (44 mM stock in ethanol) and basement membrane matrix (Matrigel; BD Biosciences, Bedford, MA) subcutaneously between the shoulder blades under dim red light conditions.[20] Litter size was limited to 6 pups during the experiment. One set of 11-*cis* retinal–treated mice was maintained in constant dark as a positive control for the 11-*cis* retinal treatment; the experimental group of mice was treated with 11-*cis* retinal and returned to the core animal facilities on the same day and exposed to normal cyclic light conditions described above. As a negative control, one more group of mice was injected with ethanol (instead of an ethanolic solution containing 11-*cis* retinal) mixed with basement membrane matrix and then returned to the core animal facilities on the same day and exposed to normal cyclic light conditions.

Immunohistochemistry

Retinal Flat-mount. Animals were killed at P30. The dorsal pole of mouse eyes was first marked using a cautery pen. After enucleation and dissection, the retina-lens complexes were fixed in freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS), consisting of 10 mM Na_2HPO_4, 140 mM NaCl, 2.8 mM KCl, 1.8 mM KH_2PO_4, pH 7.4, for 2 hours on ice. Tissues were washed three times with PBS and then blocked with 5% normal donkey serum and 0.1% Triton X-100 in PBS for 1 hour at room temperature, followed by overnight incubation with primary antibody at 4°C. Tissues were rinsed with PBS and incubated with Texas Red–conjugated donkey anti-rabbit antibody (1:500, Jackson Immuno Research) for 2 hours at room temperature. After three PBS rinses, the lens was removed and the retina was mounted and flattened on a slide. The dorsal and ventral areas of the retinas were recorded by fluorescence microscopy (Axioplan II; Carl Zeiss Inc., Germany) using the 20× objective lens.

Retinal Cross-sections. Animals were killed at P21. The dorsal pole of mouse eyes was first marked using a cautery pen. Eyes were enucleated, a 1- to 2-mm hole in the cornea was made, and the eyes were fixed in freshly made 4% paraformaldehyde in PBS for 2 hours on ice. The eyes were then transferred into 15% sucrose in PBS and equilibrated for 1 hour on ice, followed by overnight incubation at 4°C in 30% sucrose in PBS. Tissues were embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Sakura Finetech, Torrance, CA) and sectioned at −26°C. The 14 μm sections were washed with PBS to remove OCT and blocked with the PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 1 hour at room temperature. S-opsin was probed with a polyclonal antibody, kindly provided by Jeannie Chen (Department of Cell & Neurobiology, Department of Ophthalmology, Zilkha Neurogenetic Institute, University of Southern California) also diluted 1:200. The sections were washed three times with PBS for 15 minutes and incubated with Texas Red–conjugated donkey anti-rabbit antibody (1:500, Jackson Immuno Research) for 2 hours at room temperature. Nuclei were stained with the DNA dye DRAQ5 (1:500, Biostatus Limited) for 10 minutes at room temperature. Images were acquired on a confocal microscope (Leica, Bannockburn, IL).

Electroretinography

Mice at P19 were dark adapted for 24 hours and anesthetized with xylazine (20 mg/kg) and ketamine (80 mg/kg). Pupils were dilated with phenylephrine hydrochloride (2.5%) and atropine sulfate (1%). Contact lens electrodes were placed on cornea with one drop of methylcellulose. Full-field electroretinograms (ERGs) were recorded on P20 mice using the universal testing and electrophysiology LKC system (UTAS, LKC Technologies, Gaithersburg, MD). ERGs were recorded in response to 10 ms single white flashes with a fixed light intensity (24.8 cd s m^{-2}) under scotopic conditions, which will not result in rod signal contributions because of the absence of rhodopsin in the mice studied. A single light intensity, rather than a family of light intensities, was chosen in order not to significantly bleach the limited number of newly formed pigment. b-Wave amplitudes are reported as a mean ± SE and analyzed by a two-tailed Student's *t*-test, accepting a significance value of $P < 0.05$.

Results

Light Reduces the Effectiveness of Improved Cone Survival with 11-*cis* Retinal

Cone cell survival was assessed by counting the number of cells containing cone opsins in flat-mounted P30 retinas from $Rpe65^{-/-}Rho^{-/-}$ mice treated with and without 11-*cis* retinal (Fig. 1). The S-cone density was very low (823 ± 63 per mm^2) in the $Rpe65^{-/-}Rho^{-/-}$ ventral region of the mouse retina at P30 (Fig. 1A), while treatment with 11-*cis* retinal at P10 followed by dark rearing for 20 more days results in higher cone density in the same region of the retina (2187 ± 188 per mm^2) (Fig. 1B). However, 11-*cis* retinal treatment to $Rpe65^{-/-}Rho^{-/-}$ mice was not as effective in improving S-cone photoreceptor density when mice were subjected to 12 hours room light each day (Fig. 1C). M-cones were also counted and found to follow a similar pattern (not shown). A limitation to these measurements is that probing the whole-mounted retina with an opsin antibody indicates the number of cells that contain the opsin

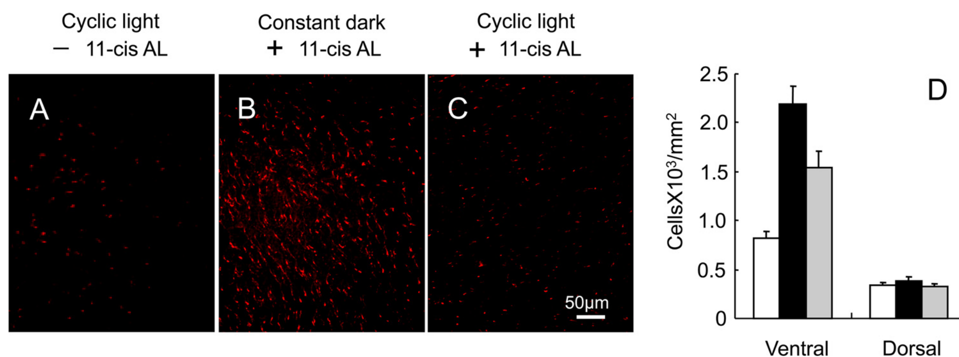

Figure 1. S-cone photoreceptor survival in $Rpe65^{-/-}Rho^{-/-}$ retinas at P30. Flat-mounted retinas were probed for S-cone opsins from mice **(A)** not treated with 11-*cis* retinal (11-cis AL), followed by cyclic light–rearing, **(B)** treated with 11-*cis* retinal, followed by dark-rearing, and **(C)** treated with 11-*cis* retinal, followed by cyclic light–rearing. Images were taken from ventral region of the retinas. Scale bar, 50 μm. **(D)** Average density of photoreceptor cells containing S-opsin in ventral and dorsal regions of $Rpe65^{-/-}Rho^{-/-}$ retinas for untreated (*white*), 11-*cis* retinal-treated mice maintained in the dark (*black*), and 11-*cis* retinal-treated mice maintained in cyclic light (*gray*). Data are presented as mean ± SE, $n = 9$.

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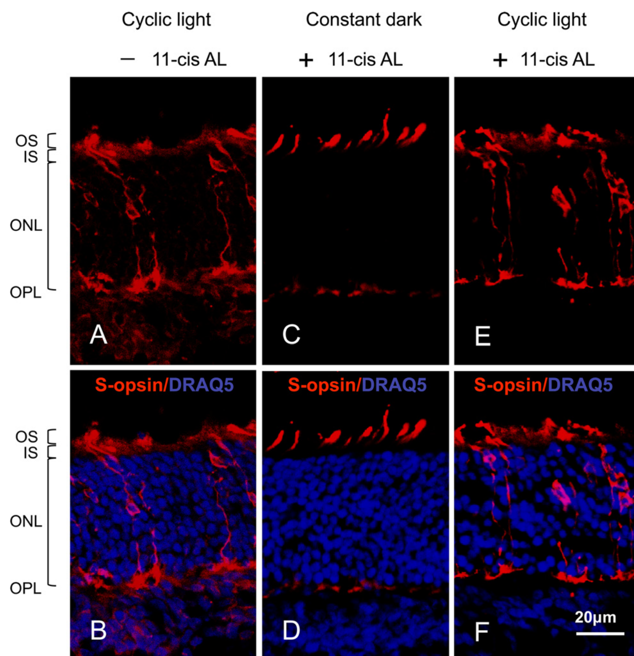

FIGURE 2. S-opsin localization in cone photoreceptors of cross-sectioned P21 *Rpe65*$^{-/-}$*Rho*$^{-/-}$ retinas. (**A**, **B**) Untreated mice; (**C**, **D**) 11-*cis* retinal-treated mice maintained in the dark; and (**E**, **F**) 11-*cis* retinal-treated mice maintained in 12 h light/12 h dark conditions. The sections were probed for S-opsin (*red*) in both the top and bottom panels. Nuclei were stained with DRAQ5 (*blue*) in the bottom panels to help orient the cross-sections and indicate outer retina integrity. The locations of the photoreceptor outer segment (OS), photoreceptor inner segment (IS), outer nuclear layer (ONL), and outer plexiform layer (OPL) are labeled beside the first image. The scale bar represents 20 μm.

and is not necessarily indicative of the viability of the cell. Thus, the treated mice exposed to light appear to have not only fewer cells but also much less S-cone opsin based on the brightness and density of the fluorescence image when compared to mice treated in the same manner but maintained in the dark. However, the absolute number of S-cone opsin containing cells is higher in the ventral area for the treated animals than in the untreated mice regardless of light conditions (Fig. 1D).

11-*cis* Retinal Fails to Improve Cone Opsin Localization under Cyclic Light Conditions

Cone opsin localization in cross-sections of three-week-old *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mouse retinas was used to assess the integrity of individual cone photoreceptor cells before their death. Consistent with previous studies,[14,15] cross-sections of a retina from an untreated P21 *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mouse that were probed for S-cone opsin illustrate the delocalized pattern of opsin throughout the cell (Fig. 2A). Figure 2B adds nuclear staining with the DNA-binding dye DRAQ5 to further illustrate morphology and orientation of the retina. Consistent with earlier work,[14,15] treating mice lacking RPE65 with 11-*cis* retinal followed by dark-rearing results in improved opsin localization in the outer segments, although there is still some visible fluorescence in the pedicles (Figs. 2C, 2D). A difference from the earlier studies, in which 11-*cis* retinal was introduced via multiple intraperitoneal injections over a 10- to 15-day period[14,15] is that in this study, 11-*cis* retinal was introduced by a single subcutaneous injection within a basement membrane matrix at P10.[20] However, the same 11-*cis* retinal treatment protocol followed by continued 12 h light/12 h dark conditions does not improve S-cone opsin; the delocalized opsin in Figures 2E and 2F appears essentially as if there had been no treatment with 11-*cis* retinal (Fig. 2A).

S-cone images shown were taken from ventral regions as S-opsin-containing cones are more dominant in the ventral than in the dorsal region of mouse retinas while M/L opsin are dominant in the dorsal region.[21] At P21, M/L opsin appears only in dorsal regions of the *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mouse retinas. Although the improvement of M/L opsin localization is not as dramatic as S-opsin, the density of M/L opsin in outer segments is higher in dark-reared 11-*cis* retinal–treated mouse groups (data not shown). These results are slightly different from those of Rohrer et al.[14] but consistent with those from Zhang et al.[15] There is no apparent difference in the localization of either opsin between the untreated and cyclic light–reared, 11-*cis* retinal–treated mouse groups.

11-*cis* Retinal Fails to Improve Cone Function under Cyclic Light Conditions

Previous studies showed marked improvement in light-induced ERG signals in mice lacking RPE65 treated with 11-*cis* or 9-*cis* retinal and maintained in the dark.[20,22] We repeated these measurements with *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice under our treatment protocol, followed by either constant dark or cyclic light–rearing conditions. Because the rod opsin is absent in *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice, any photoresponse arises from cones. The b-wave amplitudes from dark-reared 11-*cis* retinal–treated *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice were significantly enhanced (40 ± 3 μV) over those from untreated *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice (9 ± 3 μV) (Fig. 3). This is generally consistent with previously published results[14,20] although with different treatment and ERG protocols, the absolute amplitudes do differ. However, such

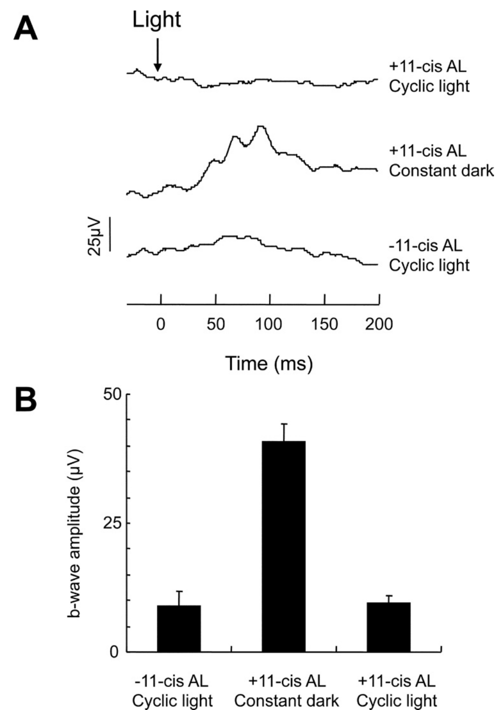

FIGURE 3. ERG recordings from *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice at P20. (**A**) Representative single-flash ERG in response to 24.8 cd s m^{-2} white light from mice treated in the following manner (*bottom to top traces*): untreated and maintained in cyclic light, treated with 11-*cis* retinal and maintained thereafter in the dark, and treated with 11-*cis* retinal and maintained in cyclic light. (**B**) Averages of the ERG b-wave amplitudes from the untreated and treated *Rpe65*$^{-/-}$*Rho*$^{-/-}$ mice. Data are presented as mean ± SE, n = 10.

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increases in b-waves are not detected in the 11-*cis* retinal–treated cyclic light–reared group (9 ± 2 μV).

Discussion

In healthy wild-type cone photoreceptor cells, opsins are primarily localized in the outer segments. However, in mice lacking the ability to produce 11-*cis* retinal, cone cells are lost approximately 1 month after birth.[13] Earlier studies demonstrated that multiple injections of 11-*cis* retinal into mouse models for LCA2 could impede these events, suggesting a potentially useful therapeutic agent for LCA2 patients, but the mice were maintained in the dark once treatment with 11-*cis* retinal commenced.[13,14,16] Two other mouse models for LCA2 (*Gnat1$^{-/-}$Lrat$^{-/-}$* and *Gnat1$^{-/-}$Rpe65$^{-/-}$*) also showed improved cone morphology and function when treated with 9-*cis* retinyl acetate and maintained in the dark.[18] Maeda et al.[17] had previously reported that 9-*cis* retinyl acetate treatment results in increased levels of 9-*cis* retinal in the retina, which can form visual pigments, but that the *cis* retinal disappeared with light. If 11-*cis* or 9-*cis* retinal were to be useful for preserving cone photoreceptor cell integrity and function in LCA patients, we reasoned that the improved effects of exogenously added 11-*cis* retinal needs to occur under cyclic light living conditions. However, we find that the presence of light eliminates the benefits of treating RPE65-deficient mice with 11-*cis* retinal.

In this study, we have used the *Rpe65$^{-/-}$Rho$^{-/-}$* mouse because 11-*cis* retinal is not produced and rod opsins would not compete for exogenously added 11-*cis* retinal nor obscure light-driven ERG signals as a mixture of rod and cone signals.[23] Although the lack of rhodopsin in mice results in degeneration of the retina,[24–26] its absence in the double knockout for this study does not appear to interfere with our results. Cones have been reported to appear quite normal within the first month in *Rho$^{-/-}$* mice[26]; morphologic properties of treated and untreated double knockout mice are similar to the single *Rpe65$^{-/-}$* mice.[15]

Delocalized cone opsins followed by cone cell death has been observed in the retinas of other knockout mouse strains.[15,16,27] Furthermore, other membrane proteins normally associated with the outer segment were also found to be mislocalized in the different mouse models.[15,27,28] Similar to the targeting of rhodopsin to rod outer segments as proposed by Deretic et al.,[29] Karan et al.[30] have proposed cone opsins as part of a post-Golgi transport vesicle targeting the cilium, but unlike in rods, trafficking in cones involves vesicles comprised of a complex of multiple signaling proteins such that their transport to the outer segment is tightly coupled. Thus, one could argue that the role 11-*cis* retinal plays is to induce a conformation of the opsin that favors it being packaged into this transport vesicle complex; whereas, the apoprotein is in an unfavorable conformation. As 11-*cis* retinal is an inverse agonist of cone opsins,[31,32] it maintains the protein in a conformation that can differ from the apoprotein. Furthermore, cone pigment formation within the inner segment appears possible, especially with recent studies implicating Müller cells as a source of 11-*cis* retinoids[33–36] and a previous study indicating that cones can take up 11-*cis* retinoids from the inner segment.[37] As to why bleaching is not a problem for wild-type retinas, we can only speculate it might be due to the constant availability of 11-*cis* retinal in wild-type mice compared with limited supply in the 11-*cis* retinal–treated knockout mice. Perhaps there is a threshold of number of pigments necessary to form a transport vesicle complex.

Finally, we would like to address why a therapeutic agent would be useful for LCA2. Gene therapy replacing missing or mutant RPE65 gene has shown great promise.[38–41] In human trials, the time at which gene therapy was administered has likely limited the degree of restoration[42–44] as a significant amount of cones could have already irreversibly degenerated. Jacobson et al.[10] reported early thinning of the fovea as well as diminished visual acuity and no observed blue color vision—all consistent with loss of cones. Results from an age-dependent study of RPE65 gene therapy on humans are consistent with this notion.[45] Thus cone photoreceptor preservation and overall integrity of the retina are important components in the eventual overall rescue of vision in LCA. Our results indicate that there might be great success in accomplishing this with an agent that is a ligand for cone opsins but does not form a photosensitive pigment.

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