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***Nrl*-Knockout Mice Deficient in Rpe65 Fail to Synthesize 11-*cis* Retinal and Cone Outer Segments**

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Abstract

Purpose—To define rod and cone function further in terms of visual cycle mechanism, the retinal phenotype resulting from Rpe65 (retinoid isomerase I) deficiency in *Nrl*^{−/−} mice having a single class of photoreceptors resembling wild-type cones was characterized and outcomes of retinoid supplementation evaluated.

Methods—*Rpe65*^{−/−}/*Nrl*^{−/−} mice were generated by breeding *Rpe65*^{−/−} and *Nrl*^{−/−} strains. Retinal histology, protein expression, retinoid content, and electroretinographic (ERG) responses were evaluated before and after treatment with 11-*cis* retinal by intraperitoneal injection.

Results—Retinas of young *Rpe65*^{−/−}/*Nrl*^{−/−} mice exhibited normal lamination, but lacked intact photoreceptor outer segments at all ages examined. Rpe65, Nrl, and rhodopsin were not detected, and S-opsin and M/L-opsin levels were reduced. Retinyl esters were the only retinoids present. In contrast, *Nrl*^{−/−} mice exhibited decreased levels of retinaldehydes and retinyl esters, and elevated levels of retinols. ERG responses were elicited from *Rpe65*^{−/−}/*Nrl*^{−/−} mice only at the two highest intensities over a 4-log-unit range. Significant retinal thinning and outer nuclear layer loss occurred in *Rpe65*^{−/−}/*Nrl*^{−/−} mice with aging. Administration of exogenous 11-*cis* retinal did not rescue retinal morphology or markedly improve ERG responses.

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Conclusions—The findings provide clarification of reported cone loss of function in *Rpe65*^{-/-}/*Nrl*^{-/-} mice, now showing that chromophore absence results in destabilized cone outer segments and rapid retinal degeneration. The data support the view that rod-dominant retinas do not have a cone-specific mechanism for 11-*cis* retinal synthesis and have potential significance for therapeutic strategies for rescue of cone-rich retinal regions affected by disease in the aging human population.

The basic mechanisms used by rod and cone photoreceptor cells to detect and respond to light are similar in many respects; however, the molecular bases underlying differences in kinetics and sensitivity, as well as disease involvement and effects of aging are not well understood. Such issues critically relate to understanding cone disease in macular and cone-rod dystrophies and age-related macular degeneration.^{1,2} Furthermore, successful therapeutic intervention relative to visual cycle defects is likely to depend on accommodating differential responses of rod and cone cells to various treatment paradigms.

RPE65 encodes an abundant protein present in the retinal pigment epithelium (RPE). Studies in mice have shown that Rpe65 is necessary for 11-*cis* retinal synthesis needed for generation of the rod photopigment rhodopsin, with Rpe65 deficiency resulting in massive accumulation of its substrate, all-*trans* retinyl ester, in the RPE.³ Recombinant RPE65 exhibits retinoid isomerase activity, generating 11-*cis* retinal from added all-*trans* retinol when coexpressed with lecithin retinol acyl transferase (LRAT).⁴⁻⁶ Mutations in human *RPE65* cause severe early-onset retinal degeneration, including Leber congenital amaurosis.^{7,8} Constitutive opsin activity and cone pigment instability likely contribute to associated pathogenesis, with cone cell death preceding that of rods.^{9,10}

A body of evidence indicates that there are fundamental differences in the mechanisms of photopigment regeneration in rod-dominant versus cone-dominant retinas (reviewed in Ref. 11). It has been further questioned whether 11-*cis* retinal supplied by the RPE is sufficient to sustain daylight vision of cones.^{12,13} An RPE65-independent visual cycle has been proposed to operate in the cone-dominant retinas of ground squirrels and chickens involving novel enzyme activities, including an all-*trans* retinol isomerase and a retinyl ester synthase located in cone and Müller cells.^{13,14} These enzyme activities have not yet been detected in the retinas of rod-dominant species, including humans and mice.

Mice lacking the transcription factor *Nrl* experience a block in the differentiation of rod precursor cells, resulting in retinas containing a single class of photoreceptors that are indistinguishable from authentic cones on the basis of number of criteria.¹⁵⁻¹⁸ Outer segments are short, contain a large proportion of discs not enclosed by plasma membrane, and are covered with a cone sheath. The nuclei contain condensed chromatin and mitochondria that are typical of mouse cones. The cells express several cone-specific proteins, synapse with rod and cone bipolar cells, and exhibit the spectral sensitivity and time constants of photoresponses typical of cones.^{17,18} Although the retinas of *Nrl*-knockout mice experience age-related changes after 3 months of age, studies in young animals provide a powerful approach to understanding cone cell function in a normally rod-dominant retina.

With an interest in further defining the role of RPE65 in visual cycle function and cone cell viability, we generated *Nrl*^{-/-} mice deficient in Rpe65 by mating the two null strains and characterizing the resulting retinal phenotype. Our studies expanded on recent analysis of visual cycle function in *Rpe65*^{-/-}/*Nrl*^{-/-} mice,¹⁹ now showing that loss of 11-*cis* retinal synthesis is accompanied by defects in outer segment formation and retinal degeneration that cannot be readily reversed by retinoid replacement therapy.

Materials and Methods

Breeding and Genotyping

Rpe65^{-/-} mice³ and *Nrl*^{-/-} mice¹⁵ were mated, *Rpe65*^{+/-}/*Nrl*^{+/-} offspring were identified by genotyping and then crossbred to generate animals homozygous null for both genes. *Rpe65*^{-/-} mice were maintained on an Sv129;C57BL/6J mixed background. The *Nrl*^{-/-} mice were on a C57BL/6J (*Rpe65*-Met450) background. Wild-type control animals were C57BL/6J. Mice were reared in a 12-/12-hour light-dark cycle (~800 lux room light), or in total darkness and were euthanatized by CO₂ inhalation. All experimental procedures complied with the regulations of the University of Michigan Committee on Use and Care of Animals, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histology

For phase-contrast microscopy, anesthetized mice were perfused with 4% paraformaldehyde in PBS, the eyes were embedded in JB-4 plastic, and sections (5 μm) were stained with Lee's stain and imaged on a microscope (Eclipse E800; Nikon, Tokyo, Japan) with a digital camera. For electron microscopy, mice were perfused with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), the eyes were postfixed in 1% OsO₄, dehydrated, and embedded in Epon 812. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate for analysis by electron microscopy.^{20,21}

Immunohistochemical and Western Analysis

Eyes from mice perfused with 4% paraformaldehyde were washed in PBS, transitioned to sucrose/OCT, and flash frozen. Sections (10 μm) were blocked with 20% sheep serum and 0.2% Triton X-100 and incubated with primary antibodies for 2 hours, then with fluoro-chrome-conjugated secondary antibody for 1 hour. Species and dilutions of primary antibodies were as follows: rabbit anti-S-opsin (1:500) and anti-M/L-opsin (1:500; Chemicon, Temecula, CA), mouse anti-rhodopsin (1D4; 1:500 of 1 mg/mL stock), mouse anti-RPE65 (8B11; 1:250 of 1 mg/mL stock), Alexa Fluor 555-conjugated anti-mouse IgG (H+L; 1:500) and Alexa Fluor 488-conjugated anti-rabbit IgG (H+L; 1:500; Invitrogen, Carlsbad CA). Images were obtained by fluorescence microscopy (Eclipse E800; Nikon). For Western analysis, total mouse eye proteins were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 5% milk and 0.1% Tween 20, and incubated with primary antibody overnight at 4°C and then with alkaline phosphatase-conjugated secondary antibody for 1 hour at RT. The immunoblots were developed by using standard methods.²²

Bleaching and Regeneration

The *Nrl*^{-/-} mice were dark adapted overnight. The pupils were dilated with topical 1% phenylephrine and 1% atropine, and the mice were placed in a plastic box lined with aluminum foil and illuminated with UV light produced by four black-light UV tubes (cat no. 0014687; Bulbtronics, Farmingdale, NY) for 1 minute. The mice were returned to complete darkness for the times indicated in the figures, and the retinoid content was assayed.

Retinoid Analysis

All procedures were performed in dim red or infrared light using a modification of the lipid-extraction protocol of Bligh and Dyer.²³ One to two frozen mouse eyes were placed in a centrifuge tube on dry ice, 150 to 200 μ L dissection medium was added (vol/vol: 60% MeOH, 30% chloroform, 10% 1 M NH₂OH; pH 7), the eyes were minced, and medium was added to 0.6 mL final volume. Samples were cooled on dry ice for 3 to 5 minutes, homogenized (Pellet Pestle; Kontes, Vineland, NJ), and sonicated with a microtip probe (30 pulses at 0.3 power and 0.1 duty cycle; Branson, Danbury, CT) with temperature maintained at 15 to 20°C (BAT-10 microthermocouple; Physitemp, Clifton, NJ). Chloroform (200 μ L) was added, samples were vortexed for 5 seconds, 250 μ L water was added, and samples were vortexed and then centrifuged at 14,000 rpm for 5 minutes. The chloroform phase was collected, dried under nitrogen, and dissolved in 200 μ L HPLC mobile phase. HPLC was performed with a column (Hypersil column; 5.0 μ m, 4.6 $\times$ 100 mm; Thermo Fisher Scientific, Waltham, MA) developed with *n*-hexane/ethanol 99.5:0.5 (A) or *n*-hexane/ethanol 99.75:0.25 (B) on an HPLC system (model 1100; Agilent, Palo Alto, CA) equipped with multidiode array detector and software (Chemstation; Thermo Fisher Scientific). Retinyl esters were collected and saponified (as described in Ref. 24), and head groups were evaluated by using the same run conditions. All-*trans* retinal, 9-*cis* retinal, retinyl palmitate, and all-*trans* retinol reference compounds were from Sigma-Aldrich (St. Louis, MO), and 11-*cis* retinal was the kind gift of Rosalie Crouch (Medical University of South Carolina, Charleston). Retinal oximes were obtained by reaction with NH₂OH. 11-*cis* retinol was obtained by reduction of 11-*cis* retinal with sodium borohydride. Peak identification was by comparison to retention times and spectra for reference samples, with quantification calculated by scaling peak integrals to reference compounds. Yields of retinyl esters were 20% to 30% higher, and yields of retinal oximes and retinols were comparable to those obtained for extractions using NH₂OH in methanol or ethanol.^{24,25} The limit of sensitivity of was ~2 pmol retinoid/eye.

11-*cis* Retinal Supplementation

Mice reared in complete darkness received intraperitoneal injections of 11-*cis* retinal in 10% ethanol, 10% BSA, and 0.9% NaCl (30 μ L) or injections of vehicle alone.²⁶ Single injections were given to the mice at 4 weeks of age (10 or 100 μ g 11-*cis* retinal/dose), or multiple injections (three to six) were given from 1 to 4 weeks of age (1.25-50 μ g 11-*cis* retinal/dose). ERG recordings were performed 24 hours after the final injection, with subsequent processing of the eyes for Western, retinoid, and TEM analyses.

Electroretinogram Recording and Analysis

Mice were dark adapted for 12 hours and prepared in dim red illumination. Animals were anesthetized with a loading dose of ketamine (93 mg/kg, IP) and xylazine (8 mg/kg, IP). Pupils were dilated with topical 1% atropine and 0.5% tropicamide. Body temperature was maintained at 37°C with a heating pad. Corneal ERGs were recorded from both eyes using gold wire loops with 0.5% tetracaine topical anesthesia and a drop of 2% methylcellulose for corneal hydration. A gold wire loop placed in the mouth served as the reference, and a ground electrode was placed on the tail. ERGs were recorded in a Ganzfeld configuration with brief xenon white flashes, with flash intensity varied using combinations of neutral density filters and photostimulator intensity settings (-0.5 - $3.5 \log \text{cd} \cdot \text{s/m}^2$ per flash measured with a IL1700 Research Radiometer; International Light, Newburyport, MA). Responses were amplified (model CP5111 AC amplifier; Grass-Telefactor, West Warwick, RI) at 10,000 gain at 0.1 to 1000 Hz, and digitized (PCI-1200 I/O board; National Instruments, Austin, TX). A notch filter was used to remove 60-Hz line noise. Dark-adapted ERGs were recorded at 3- to 60-second intervals depending on the stimulus intensity. Animals were then light adapted for 10 minutes by exposure to a white 32 cd/m^2 rod-saturating background, and photopic ERGs were recorded for single flash white stimuli over a 4-log-unit range. Responses were computer-averaged at all intensities with at least 25 records averaged for the weakest signals. Flash stimuli had a duration of 10 μs . ANOVA was performed to determine statistical significance at $P = 0.05$.

Results

Alterations in Retinoid Metabolism in Eyes of *Nrl*^{-/-} Mice

In baseline studies, we evaluated retinoid content and processing in eyes from *Nrl*^{-/-} mice using HPLC. In dark-adapted animals, we found that total retinoid levels in the *Nrl*^{-/-} mice were three to four times less than in the wild-type mice, because of decreases in 11-*cis* retinal, all-*trans* retinal, and (total) retinyl esters (Figs. 1A, 1B). The decrease in 11-*cis* retinal content ($\sim 106 \text{ pmol/eye}$ in *Nrl*^{-/-} versus $\sim 528 \text{ pmol/eye}$ in wild type) is consistent with earlier studies showing that opsin levels are also decreased in *Nrl*^{-/-} mice ($\sim 90 \text{ pmol cone opsin/eye}$ versus $\sim 520 \text{ pmol rhodopsin/eye}$ in wild type¹⁷), due to the smaller outer segment size of cones than rods. Thus, *Nrl*^{-/-} mice retain a chromophore-to-opsin ratio that is close to unity, suggesting that nearly all the 11-*cis* retinal present in the retina is bound in visual pigment, as in wild-type mice.²⁷ After exposure to high-intensity illumination that isomerizes more than 90% of the 11-*cis* retinal, and return to the dark, the *Nrl*^{-/-} mice rapidly converted all-*trans* retinal to all-*trans* retinol, followed by a recovery of 11-*cis* retinal (Fig. 1C). Although absolute rates of 11-*cis* retinal synthesis were slower in the *Nrl*^{-/-} mice than in the wild type (1.77 vs. $3.06 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{eye}^{-1}$), relative rates (calculated as the percentage from dark value) were faster in the *Nrl*^{-/-} mice ($1.77\% \text{ min}^{-1}$ versus $0.58\% \text{ min}^{-1}$; Fig. 1D). The *Nrl*^{-/-} mice also exhibited four- and sixfold increases in percentage of total 11-*cis* retinol and all-*trans* retinol, retinoids normally present in the retina in minor amounts.

Photoreceptor Layer Changes in *Rpe65*^{-/-}/*Nrl*^{-/-} Mice

The mice homozygous null for both *Rpe65* and *Nrl* were viable and could not be distinguished from wild-type littermates by simple observation. Their eyes were unremarkable in overall appearance, with sections of retina/RPE/choroid showing normal cellular lamination in light micrographs. However, the layer normally containing photoreceptor inner and outer segments was extremely thin in the double-knockout mice, even when compared with that in the *Nrl*^{-/-} mice, which had shorter outer segments typical of cones (Fig. 2).

Ultrastructural analysis using transmission electron microscopy showed a widespread lack of outer segment disc membranes in retinas of *Rpe65*^{-/-}/*Nrl*^{-/-} mice at all ages examined (1-5 weeks old). In mice at 2 weeks of age, there were some cells with disc membranes, but nothing like a complete outer segment (Fig. 3). The short stacks of membranes were evident among the apical processes of the RPE (Fig. 3A). Many cilia that did not bear an outer segment at all were observed (Figs. 3B, 3C). At 5 weeks of age, disc membranes were not seen, but some cilia were still evident (not shown). Thus, it appears that many photoreceptor cells form at least part of an outer segment by 2 weeks of age, but most of these cells are lost due to cell death by 5 weeks of age. The remaining photoreceptor cells have only a few or no discs—although they all appear to have a cilium. Oil droplets similar to those present in *Rpe65*^{-/-} mice³ were occasionally observed in the RPE of the *Rpe65*^{-/-}/*Nrl*^{-/-} mice (data not shown), with the RPE otherwise normal in appearance, with its apical processes seen as long parallel membranes adjacent to the inner segments.

Decreased Expression of Outer Segment Proteins in *Rpe65*^{-/-}/*Nrl*^{-/-} Mice

In animals at 4 weeks of age, Western analysis of whole-eye lysates showed the expected absence of *Nrl* and rhodopsin in the *Nrl*^{-/-} and *Rpe65*^{-/-}/*Nrl*^{-/-} mice and the absence of *Rpe65* in the *Rpe65*^{-/-} and *Rpe65*^{-/-}/*Nrl*^{-/-} mice (Fig. 4A). In addition, rhodopsin levels were markedly decreased in the *Rpe65*^{-/-} mice, whereas slight reductions in *Nrl* and *Rpe65* were present in the *Rpe65*^{-/-} and *Nrl*^{-/-} mice, respectively. The expression of *Rpe65* in *Nrl*^{-/-} mice was relatively stable, showing little reduction from 1 to 7.5 months of age (Fig. 4B). Expression of *Rpe65* was not observed in the rosette formations that were present in the ONL of *Nrl*^{-/-} retinas (Fig. 4C). Expression of *Cralbp*, *arrestin*, *Rdh5*, and *Gapdh* was observed in all four genotypes.

Immunohistochemical analysis showed S-opsin and M/Lopsin were decreased in the *Rpe65*^{-/-} and *Rpe65*^{-/-}/*Nrl*^{-/-} mice relative to wild type, and relative to *Nrl*^{-/-} mice that have elevated levels of both opsins (Fig. 5). In the double knockout animals, diffuse, low-level S-opsin reactivity was apparent in most photoreceptor cells, with distinct M/L-opsin labeling present in a subset of the cells. In both cases, opsin reactivity was mislocalized and detected throughout the photoreceptor inner segment, as previously reported for *Rpe65*^{-/-} mice.²⁶ The downregulation and mislocalization of opsins and failed outer segment formation in the *Rpe65*^{-/-}/*Nrl*^{-/-} mice suggests that little or no light-responsive signal transduction machinery remained intact in the double-knockout animals.

Absence of 11-*cis* Retinoids in Eyes of *Rpe65*^{-/-}/*Nrl*^{-/-}

HPLC analysis of retinoid content showed that only retinyl esters were present in significant amounts in the eyes of *Rpe65*^{-/-}/*Nrl*^{-/-} mice, at levels approximately one-third those in *Rpe65*^{-/-} mice (Fig. 6). In both *Rpe65*-deficient genotypes, more than 98% of the total retinoid content was in the form of retinyl esters, with 11-*cis* retinal and all-*trans* retinal below the level of detection, and levels of all-*trans* retinol intermediate between those in wild-type and *Nrl*^{-/-} mice. Analysis of the ester fraction from *Rpe65*^{-/-}/*Nrl*^{-/-} mice by saponification and head group identification showed that only all-*trans* retinyl esters were present, with complete absence of 11-*cis* isomers in the double-knockout mice.

Absence of ERG Responses in *Rpe65*^{-/-}/*Nrl*^{-/-} Mice

Previous studies of *Nrl*^{-/-} mice showed ERG responses typical of cones, with markedly reduced responses under scotopic conditions, and supranormal b-wave amplitudes under photopic conditions.^{15,17,18} In contrast, *Rpe65*^{-/-}/*Nrl*^{-/-} mice exhibited no recordable ERG responses when subjected to a series of flashes in dark-adapted conditions (-5.81-1.09 log cd · s/m² per flash) or light-adapted conditions (-0.91-1.09 log cd · s/m² per flash) that elicited robust responses from *Nrl*^{-/-} and wild-type mice and reduced responses from *Rpe65*^{-/-} mice (Figs. 7A, 7B). However, when ERGs were recorded from the *Rpe65*^{-/-}/*Nrl*^{-/-} mice over an extended intensity range of 4.0 log units (-0.5-3.5 log cd · s/m² xenon flashes delivered from a photographic flash unit), significant b-wave responses were evoked in two of five animals, in both dark-adapted (data not shown) and light-adapted conditions (Fig. 7C). There were no recordable responses for flashes less than 2.5 cd · s/m² in intensity under dark- or light-adapted conditions. The a-wave was absent except for a very small signal seen with the highest intensity flash (3.5 log cd · s/m²), with previous studies showing recordable a-waves in *Nrl*^{-/-} mice.^{15,17,18} As the a-wave arises primarily from the suppression of the photoreceptors' light-sensitive current, the near complete absence of a-waves in the double-knockout mice indicates that the photoreceptor current has declined to undetectable levels, or that its sensitivity to light has become so low that it can be only minimally activated. In contrast, the b-wave arises from inner retinal neurons (primarily, ON-bipolar cells), and its presence indicates that some photoreceptor function remains, whereas its low amplitude reflects insensitivity and/or decline in inner retinal integrity.

Retinal Pathology in Eyes of *Rpe65*^{-/-}/*Nrl*^{-/-} Mice

At ages older than 4 weeks, the retinas of the *Rpe65*^{-/-}/*Nrl*^{-/-} mice exhibited striking pathology that increased over time. In sections of central retina inferior to the optic nerve head, ONL thickness was approximately equivalent in mice of all four genotypes, consisting of 9 to 10 rows of nuclei at 4 weeks of age (Figs. 2, 8A). At 7 weeks of age, the *Rpe65*^{-/-}/*Nrl*^{-/-} mice had lost at least two rows of photoreceptor nuclei, and overall thinning of the retina was evident. At 12 weeks of age, the total retinal thickness of the *Rpe65*^{-/-}/*Nrl*^{-/-} mice was approximately half that of *Nrl*^{-/-} mice, with further losses in the ONL that were more severe in the superior than in the inferior central retina. Rosette formations present in most sections from the *Nrl*^{-/-} mice at 12 weeks of age were seen only occasionally in the *Rpe65*^{-/-}/*Nrl*^{-/-} mice, perhaps because the conditions needed for rosette formation are not maintained as the number of cells in the ONL of the double-knockout

mouse decreases with aging. Morphometric analysis of ONL thickness in the *Rpe65*^{-/-}/*Nrl*^{-/-} and wild-type mice at 4, 7, and 12 weeks of age clearly demonstrated the progressive cell loss in the double-knockout animals, with the superior and temporal regions affected most severely (Fig. 8B).

Failure of Exogenous 11-*cis* Retinal to Rescue the Phenotype of the *Rpe65*^{-/-}/*Nrl*^{-/-} Mice

Dark-reared *Rpe65*^{-/-}/*Nrl*^{-/-} and *Rpe65*^{-/-} mice were dosed with 11-*cis* retinal by intraperitoneal injection, an approach previously shown to rescue the retinal phenotype of *Rpe65*^{-/-} mice.^{26,28,29} Protocols involving single and multiple doses were used, starting at 1 week to 4 weeks of age. Approximately 24 hours after the final injection, ERGs were recorded under dark-adapted and light-adapted conditions, over a range of flash intensities from -0.5 to 3.5 log cd · s/m². In control experiments, *Rpe65*^{-/-} mice that received either single or multiple doses of 11-*cis* retinal exhibited improved ERG responses in both dark-adapted and light-adapted states. Figure 9A summarizes data for *Rpe65*^{-/-} mice given six injections of 11-*cis* retinal (1.25-5.0 μg) at postnatal day (PN)10 to PN30. In the treated animals, maximum responses significantly increased for the dark-adapted b-wave (*P* = 0.042) and appeared to be increased for the light-adapted b-wave (although statistical significance was not reached due to the small sample size). In contrast, fewer than half of the *Rpe65*^{-/-}/*Nrl*^{-/-} mice that received exogenous 11-*cis* retinal showed any improvement in ERG recordings, and only when multiple injections were given. Figure 9B summarizes the data for the *Rpe65*^{-/-}/*Nrl*^{-/-} mice injected with 11-*cis* retinal. No significant difference in dark-adapted b-wave responses recorded at the brightest flash intensity was seen between the treated and untreated animals. Under light-adapted conditions, an apparent doubling of the b-wave amplitude was evident in treated *Rpe65*^{-/-}/*Nrl*^{-/-} mice (*P* = 0.036). However, the maximum response obtained in the treated double-knockout animals (~75 μV) was less than half that of untreated *Rpe65*^{-/-} mice (~165 μV), and a fraction of that elicited from untreated *Nrl*^{-/-} mice using stimulus intensities 2 log units lower. Intraperitoneal injection of 11-*cis* retinal did not increase expression of S-opsin and M/L-opsin in *Rpe65*^{-/-}/*Nrl*^{-/-} mice or increase chromophore accumulation in the retina (data not shown). Neither was there improvement in photoreceptor morphology, with continued absence of intact outer segment structures in mice given either single or multiple injections of 11-*cis* retinal beginning as early as 1 week of age. A representative image is shown in Figure 10.

Discussion

Our characterization of the phenotype resulting from *Rpe65* deficiency in the all-cone retina of *Nrl*^{-/-} mice represents an important extension of findings in a previous study,¹⁹ with our studies now showing that the absence of chromophore results in decreased opsin content, failed outer segment morphogenesis, and severe retinal degeneration in *Rpe65*^{-/-}/*Nrl*^{-/-} mice. As the cones of *Nrl*^{-/-} mice appear to resemble those of the wild type in both structure and function,¹⁵⁻¹⁸ these combined observations suggest that normally rod-dominant mice do not have an independent mechanism for cone photopigment regeneration. Our studies also suggest that cone-rich regions of the retina affected by visual cycle defects may be difficult to rescue with retinoid therapy.

In mice deficient for only *Nrl*, we found that retinoid processing in the retina remained intact, consistent with previous measures of the ERG responses in these animals.^{15,17} During recovery after bleaching, reduction of all-*trans* retinal occurred rapidly, with relatively, but not absolutely, higher rates of 11-*cis* retinal synthesis compared with wild type. Dark-adapted levels of 11-*cis* retinal in *Nrl*^{-/-} mice were decreased; however, cone opsin levels were also decreased relative to rhodopsin levels in wild-type mice¹⁷ (reflecting the smaller outer segments of cones), suggesting that virtually all the 11-*cis* retinal present in the retina is bound to opsin protein, as in wild type.²⁷ A distinct feature of *Nrl*^{-/-} mice was relatively increased levels of all-*trans* and 11-*cis* retinol, present in minor amounts in wild-type mice. Thus, it may be interesting to consider whether cone-specific stores of retinoids exist in the retina. We did not observe increased retinyl ester content, increased levels of Rpe65 protein, or localization of Rpe65 in ONL rosettes, as reported by Wenzel et al.¹⁹ Similarly, no increase in Rpe65 transcript levels in *Nrl*^{-/-} mice was detected using microarray analysis (Mears A, personal communication, September 2005). As the *Nrl*^{-/-} mice in all three studies were derived from the same source,¹⁵ and carry the same *Rpe65*-Met450 polymorphism,³⁰ other genetic background effects may underlie the observed differences, as the breeders used were mixed with respect to Sv129 and C57BL/6 backgrounds. Previous studies have shown that photoreceptor survival in *Rho*^{-/-} mice is increased on the C57BL/6 genetic background relative to that on Sv129.³¹

In mice deficient for both Rpe65 and *Nrl*, we found that retinoid processing was blocked after uptake and storage of vitamin A in the RPE, in agreement with the findings of Wenzel et al.¹⁹ Only all-*trans* retinyl esters were detected in significant amounts, with levels of 11-*cis* retinal below the limit of detection, and no ERG responses were elicited at flash intensities that produce supranormal responses in *Nrl*^{-/-} mice.^{15,17} However, our studies also showed that *Rpe65*^{-/-}/*Nrl*^{-/-} mice had dramatically reduced levels of S-opsin and M/L opsin, as well as profound loss of stable photoreceptor outer segment structures. Although intact ciliary structures were visible by transmission electron microscopy, stacks of disc membranes were found to be associated with only a fraction of photoreceptor cells and only in mice at very young ages (2 weeks). It is not yet clear whether the absence of disc membranes in older animals simply results from wide-spread photoreceptor cell death, or whether the outer segment structures that initially form are unstable and degenerate over time.

A previous study of mice deficient in only Rpe65 showed that cone opsin stability is decreased in the absence of chromophore, resulting in subcellular mislocalization of cone opsin and early cone cell death in the central retina.³² However, at young ages, cone structures in the peripheral retina remain intact in *Rpe65*^{-/-} mice, and administration of exogenous 9-*cis* or 11-*cis* retinal effectively corrects opsin expression and revives cone ERG responses.^{26,28,29,33,34} In addition, gene-replacement therapy has been shown to rescue the function of peripheral cone photoreceptors remaining in the rodless retinas of *Rho*^{-/-} mice that are also deficient in Rpe65.²⁶ In contrast, in *Rpe65*^{-/-}/*Nrl*^{-/-} mice, we found that administration of 11-*cis* retinal (beginning as young as 1 week of age) did not significantly increase opsin expression, correct opsin mislocalization, improve outer segment morphogenesis, or increase retinoid accumulation in the retina. In addition, the ERG

responses obtained in treated double-knockout mice were much lower than those obtained in treated *Rpe65*^{-/-} mice. Thus, our findings add to the body of evidence suggesting that the efficacy of therapeutic intervention in the visual cycle is likely to differ in rod- versus cone-rich regions of the retina.

In some respects, the retinal phenotype of *Rpe65*^{-/-}/*Nrl*^{-/-} mice appears more similar to that resulting from rhodopsin deficiency³⁵ than that due to *Rpe65* deficiency alone, both in terms of defective photoreceptor outer segment formation and early-onset retinal degeneration. In *Rho*^{-/-} mice, stable rod outer segments fail to form due to loss of a major structural component of the disc membrane that may also influence the trafficking of additional proteins from the inner to outer segment.^{36,37} In contrast, in *Rpe65*^{-/-} mice, chromophore loss has minor effects on rod structure, retinal degeneration is slow, and rescue by administration of exogenous 11-*cis* retinal is effective until advanced ages.²⁹ Differences in the stability of rod versus cone outer segments and photopigments are likely to reside in several factors, including affinity for the chromophore and signals involved in outer segment morphogenesis. Additional factors, including rod-derived cone viability factor (RdCVF)³⁸ may also be important. In this regard, it is interesting to note that many patients with mutations in visual cycle genes show signs of macular degeneration and scarring consistent with increased damage occurring in cone-rich regions of the retina.³⁹⁻⁴¹

In summary, our data support the view that the RPE-based visual cycle is the only significant source of chromophore in the rod-dominant retina, as our data indicate that *Rpe65* expression is restricted to the RPE of both rod- and cone-dominant retinas,⁴² and others who report *Rpe65* expression in cones find only minute levels.⁴³ A role for *Rpe65* in facilitating entry into a cone-centric pathway of 11-*cis* retinal synthesis cannot be excluded; however, there is no experimental evidence of such a mechanism. Although both rods and cones in the mouse retina appear dependent on *Rpe65* for 11-*cis* retinal synthesis, for cone cells, loss of chromophore results in devastating photoreceptor instability and rapid degeneration. Recent studies of individuals with *RPE65*-mutations also suggest that RPE65-based mechanisms are critical for 11-*cis* retinal production necessary for cone function and maintenance of normal numbers of cone cells.⁴⁴ Thus, special considerations may be needed to achieve rescue of the cone-rich central retina in patients with mutations in visual cycle genes resulting in decreased chromophore synthesis, or in other conditions resulting in decreased cone stability. We further conclude that alternative mechanisms of 11-*cis* retinal synthesis associated with cone photoreceptors described in chicken and ground squirrel^{13,14} are likely to represent specializations of cone-dominant retinas.

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Figure 1.

Retinoid content and processing in *Nrl*^{-/-} and wild-type mice at 5 weeks of age. (**A, B**) Quantitative analysis of retinoid compounds present in whole-eye extracts. *Nrl*^{-/-} (□); wild-type (●). (**C**) Time course of retinoid interconversion during recovery from >90% bleaching, shown as a percentage of the total. 11-*cis* retinal (○); all-*trans* retinal (●); all-*trans* retinol (□); retinyl esters (●). (**D**) Time course of 11-*cis* retinal recovery after an exposure to intense light, shown as a percentage of dark value.

Figure 2.

Retinal histology of *Rpe65*^{-/-}/*Nrl*^{-/-}, single-knockout, and wild-type mice. Phase-contrast micrographs of toluidine blue-stained sections from animals at 4 weeks of age. OS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 3.

Transmission electron micrographs of the RPE/photoreceptor layer of *Rpe65*^{-/-}/*Nrl*^{-/-} mice at 2 weeks of age. (A-C) Images at different levels of magnification. *Arrows*: basal bodies of photoreceptor cilia. N, RPE nuclei; M, RPE melanosomes; V, RPE apical villi; OS, outer segment membranes. Scale bars, 1 μ m.

Figure 4.

Analysis of protein expression in *Rpe65^{-/-}/Nrl^{-/-}*, *Rpe65^{-/-}*, *Nrl^{-/-}*, and wild-type mice. (A) Immunoreactivity in whole-eye extracts from mice at 4 weeks of age probed with antibodies specific for the proteins indicated. (B) Rpe65 immunoreactivity in whole-eye extracts from *Nrl^{-/-}* mice at the ages indicated. (C) Rpe65 immunoreactivity in RPE/retina cryosections from *Nrl^{-/-}* mice. P, phase contrast; D, DAPI (4',6-diamidino-2-phenylindole)-stained nuclei; R, immunoreactivity with mAb 8B11 specific for Rpe65.

Figure 5. Immunohistochemistry of S-opsin and M/L opsin in *Rpe65*^{-/-}/*Nrl*^{-/-}, *Nrl*^{-/-}, *Rpe65*^{-/-}, and wild-type mice in RPE/retina cryosections. *Green*: opsin immunoreactivity (AlexaFluor-conjugated secondary antibody). *Blue*: DAPI (4',6-diamidino-2-phenylindole)-stained nuclei.

Analysis was performed on mice 34-38 days old

Figure 6.

HPLC analysis of the retinoid content of eyes of *Rpe65*^{-/-}/*Nrl*^{-/-} mice. **(A)** HPLC traces of total retinoid extracts from two eyes of a *Rpe65*^{-/-}/*Nrl*^{-/-} mouse. According to retention times and absorbance spectra, the peaks were assigned as follows (arrows): 1, retinyl esters; 2, *syn* 11-*cis* retinal oxime; 3, *syn* all-*trans* retinal oxime; 4, anti-11-*cis* retinal oxime; 5, 11-*cis* retinol; 6, all-*trans* retinol. **(B)** HPLC traces of the retinyl ester fraction collected (as denoted with a bar over the peak on trace a), and after saponification and rechromatography (trace b). *Inset*: Peak absorbance spectra for the saponification product on trace b and the standard compounds 11-*cis* retinol (trace c, peak 1) and all-*trans* retinol (trace c, peak 2). **(C)** Retinoid content of eyes from *Rpe65*^{-/-}/*Nrl*^{-/-}, *Nrl*^{-/-}, *Rpe65*^{-/-}, and wild-type mice.

Figure 7. ERG waveform series of *Rpe65*^{-/-}/*Nr1*^{-/-}, single-knockout, and wild-type mice recorded over a range of stimulus intensities. (A) Dark-adapted flash ERG responses (scotopic). (B) Light-adapted flash ERG responses (photopic). (C) Light-adapted flash ERG responses (photopic) for an extended range of stimulus intensity, maximum 3.5 log cd · s/m². Representative responses are shown. The number of traces averaged differed for different genotypes, with approximately 25 traces averaged for the *Rpe65*^{-/-}/*Nr1*^{-/-} mice.

Figure 8.

Retinal histology and thickness measurements in *Rpe65*^{-/-}/*Nr1*^{-/-}, single-knockout, and wild-type mice at 4, 7, and 12 weeks of age. **(A)** Phase-contrast micrographs of toluidine-stained RPE/retina sections. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. CI, central retina inferior to optic nerve head; CS, central retina superior to optic nerve head. **(B, C)** Plots of ONL thickness in *Rpe65*^{-/-}/*Nr1*^{-/-} and wild-type mice, with measurements made on sections parallel (superior, inferior) and orthogonal (nasal, temporal) to the vertical meridian of the eye with standard deviations shown: 4 weeks (▲); 7 weeks (●); 12 weeks (○).

Figure 9.

ERG responses to maximum flash intensity of $3.5 \log \text{cd} \cdot \text{s/m}^2$ for mice treated with 11-*cis* retinal by intraperitoneal injection. (A) *Rpe65^{-/-}* and (B) *Rpe65^{-/-}/Nrl^{-/-}* mice housed in complete darkness received injections of 11-*cis* retinal as follows: postnatal day (PN)10 and PN14, 1.25 μg ; PN17, 2.5 μg ; PN21, 3.75 μg ; PN27 and PN33, 5.0 μg . ERG analysis was performed 24 hours after the final injection. (•) 11-*cis* retinal injected ($n = 4$ *Rpe65^{-/-}*, $n = 2$ *Rpe65^{-/-}/Nrl^{-/-}*); (▨) vehicle injected ($n = 3$ *Rpe65^{-/-}*, $n = 3$ *Rpe65^{-/-}/Nrl^{-/-}*). Error bars, mean $\pm$ SEM.

Figure 10.

Transmission electron micrographs of the RPE/photoreceptor layer of a 37-day-old *Rpe65*^{-/-}/*Nrl*^{-/-} mouse, treated by intraperitoneal injection of 11-*cis* retinal, twice weekly, beginning at 7 days of age. *Arrows*: basal bodies of photoreceptor cilia. N, RPE nucleus; M, RPE melanosome; V, RPE apical microvilli. Scale bar, 1 μ m.