Evaluation of 9-*cis*-Retinyl Acetate Therapy in *Rpe65*$^{-/-}$ Mice

Tadao Maeda,[1,2] *Akiko Maeda,*[1] *Gemma Casadesus,*[3] *Krzysztof Palczewski,*[1] *and Philippe Margaron*[4]

Purpose. Mice lacking retinal pigment epithelium–specific 65-kDa protein (RPE65) develop retinopathy and blindness resembling Leber congenital amaurosis. Effects of 9-*cis*-retinyl acetate (9-*cis*-R-Ac) on visual function and retinopathy progression were tested in *Rpe65*$^{-/-}$ mice.

Methods. Young C57Bl/6 mice were given 9-*cis*-R-Ac in each of four different oil-based vehicle solutions by gastric gavage to identify the vehicle most suitable for drug delivery by measuring retinoid levels in plasma. Then doses of 9-*cis*-R-Ac ranging from 1 to 100 mg/kg were administered to 5- to 12-week-old *Rpe65*$^{-/-}$ mice by different treatment regimens, including single doses and either intermittent or daily doses for various periods up to 8 weeks. Retinoid effects on visual function were evaluated by electroretinography, retinoid analyses, histologic methods, and vision-dependent behavioral testing.

Results. Soybean oil vehicle provided the highest 9-*cis*-R-Ac metabolite levels in plasma. Single doses of 9-*cis*-R-Ac (6.25–50 mg/kg) provided significant dose-dependent improvement in electroretinographic responses. Well-tolerated daily doses (1–12.5 mg/kg) for 2 weeks induced remarkable improvement of retinal function. Significant dose-dependent improvement of electroretinographic responses was observed 6 days after administration of 9-*cis*-R-Ac daily for 3 days at 1 to 12.5 mg/kg. Mice given either daily or intermittent 9-*cis*-R-Ac treatment at 1 and 4 mg/kg and evaluated 8 weeks later displayed dose-dependent improvement of retinal function and morphology, whereas retinal function deteriorated in control animals. Treated mice also performed better than control animals in vision-dependent behavioral tests.

Conclusions. Treatment with 9-*cis*-R-Ac improves visual function and preserves retinal morphology in *Rpe65*$^{-/-}$ mice. (*Invest Ophthalmol Vis Sci.* 2009;50:4368–4378) DOI: 10.1167/iovs.09-3700

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Visual perception results from the biological conversion of light energy to electrical signaling by retinal photoreceptors in the eye, a process called phototransduction. Visual pigments, consisting of the chromophore 11-*cis*-retinal bound to apoprotein G protein-coupled receptor opsins,[1] initiate this process on the absorption of a photon that triggers photoisomerization of the chromophore into its *trans* form.[1,2] The isomerized chromophore, all-*trans*-retinal, then is reduced to all-*trans*-retinol, transported to the retinal pigment epithelium (RPE), and converted to fatty acid all-*trans*-retinyl esters by lecithin/retinol acyltransferase (LRAT). Regeneration of 11-*cis*-retinal completes this retinoid (visual) cycle and is critical for maintaining vision.[3] Defects in 11-*cis*-retinal production are associated with a number of inherited degenerative retinopathies.[4] Two examples are Leber congenital amaurosis (LCA), a childhood-onset retinal disease causing severe visual impairment, and retinitis pigmentosa (RP), another retinopathy with a more variable age of onset. At or soon after birth, LCA patients characteristically exhibit severe visual impairment exhibited by wandering nystagmus, amaurotic pupils, a pigmentary retinopathy with loss of cone and rod sensitivity, absent or greatly attenuated electroretinographic (ERG) responses, and an approximately 100-fold decrease in cone flicker amplitudes.[5–7]

RPE65, a 65-kDa protein specific to and abundant in the RPE[8] that catalyzes the isomerization of fatty acid all-*trans*-retinyl esters to 11-*cis*-retinol, has been recently described as the retinoid isomerase involved in the regeneration of 11-*cis*-retinal.[9–11] Mutations in the *RPE65* gene account for up to 16% of LCA cases and 2% of autosomal recessive RP cases.[4,12–15] Spontaneous or engineered deletions of *Rpe65* in mice and dogs result in 11-*cis*-retinal deficiency, an early-onset and slowly progressive retinal degeneration with dramatically reduced ERG responses and typical LCA pathology[16–19] accompanied by an accumulation of fatty acid all-*trans*-retinyl esters in the RPE.[16,20]

LCA is incurable, but several possible therapies are being investigated. *RPE65* gene augmentation therapy and retinal prostheses have shown preliminary encouraging signs of visual rescue in early-stage clinical evaluations.[21–23] Recently, visual chromophore replacement therapy with 9-*cis*-retinal has been proposed as a novel pharmacologic approach to bypass the defective retinoid cycle.[24–27] 9-*cis*-Retinal binds to opsin to form the rod cell pigment, *iso*-rhodopsin, which initiates phototransduction similar to that of rhodopsin.[1] Oral administration of 9-*cis*-retinal or its precursors have regenerated opsin as *iso*-rhodopsin in the eyes, improved retinal function as assessed by ERG responses, and ameliorated the pupillary light reflex in *Rpe65* and *Lrat* knockout mice, two genetic models of LCA.[24–27] These observations have led to the development of synthetic 9-*cis*-retinoids as orally administered, prodrug forms of 9-*cis*-retinal for the treatment of various forms of inherited retinal degeneration caused by defects in the retinoid cycle.

Here we report pharmacokinetic and pharmacodynamic effects of the prodrug 9-*cis*-R-Ac that is converted to another prodrug in the liver (i.e., mostly to 9-*cis*-retinyl palmitate) in

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the *Rpe65*$^{-/-}$ mouse model. We describe the selection of soybean oil as an appropriate vehicle for administering the 9-*cis*-R-Ac prodrug by gastric gavage, based on postabsorptive levels of its pharmacologically active metabolites in plasma. We assessed 9-*cis*-R-Ac efficacy with the use of electroretinography and vision-dependent behavioral tests in single, intermittent, and daily dosing studies that also included biochemical quantification of retinoids in the eye. Dose-dependent improvement of the level and duration of retinal function was observed in these knockout animals. Importantly, pharmacologic activity was sustained for sufficiently long periods after dosing to enable the formulation of a flexible, intermittent dosing schedule.

Materials and Methods

Animals

All mice used in this study were housed in the animal facility at the School of Medicine, Case Western Reserve University, where they were maintained on a standard diet in a 12-hour light (<10 lux)/12-hour dark cycle. All manipulations were made under dim red light transmitted through a safelight filter (transmittance > 560 nm; No. 1; Kodak, Rochester, NY). *Rpe65*-deficient mice (background, 129SV/C57Bl/6) were obtained from Michael Redmond (National Eye Institute, National Institutes of Health, Bethesda, MD) and genotyped as described previously.[16] Pharmacokinetic studies were carried out in C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME), and 5- to 12-week-old animals of both sexes were used for all experiments. All animal procedures and experiments were approved by the Case Western Reserve University Animal Care Committee and conformed to recommendations of both the American Veterinary Medical Association Panel on Euthanasia and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Synthesis of 9-*cis*-R-Ac

9-*cis*-R-Ac was synthesized in QLT facilities by acetylation of 9-*cis*-retinol formed after catalytic palladium isomerization and alkaline hydrolysis of all-*trans*-retinyl acetate (all-*trans*-R-Ac); 9-*cis*-retinol was purified by selective crystallization from a mixture of hydrolyzed 9-*cis*-R-Ac and all-*trans*-R-Ac. Industrial production of this compound will be published separately. The synthetic method for this compound was also reported in our previous work.[27]

Administration of 9-*cis*-R-Ac and Light Exposure

9-*cis*-R-Ac was administered to mice by oral gavage, as previously described.[24–28] Briefly, all doses of 9-*cis*-R-Ac were dissolved in soybean oil (United State Pharmacopeia; Spectrum Chemicals, Gardena, CA), so that 180 μL of the resultant solution could be given to each animal through a gavage needle (20-gauge straight, no. 7902; Popper & Sons) by the various experimental treatment protocols (see Figs. 3, 5, 7). Mice in control groups were treated with soybean oil in the same way. Mice were maintained in cages from 8 am to 4 pm under laboratory lighting conditions with fluorescent light (luminance range, 500-1500 lux in the cage, F1818-WW, 18 W; General Electric, Cleveland, OH) followed by 16 hours in the dark unless otherwise specified.

Electroretinography

Electroretinograms were recorded from anesthetized mice.[29,30] Briefly, mice first were dark-adapted overnight before recording. Then under a safety light, they were anesthetized by intraperitoneal injection of 20 μL/g body weight of 6 mg/mL ketamine and 0.44 mg/mL xylazine diluted with 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl. Pupils were dilated with 1% tropicamide. A contact lens electrode was placed on the eye, and a reference electrode and ground electrode were positioned on the ear and tail, respectively. Electroretinograms were recorded with a universal testing and electrophysiological system (E-3000; LKC Technologies, Inc., Gaithersburg, MD).

Single-Flash Recording

White light flash stimuli were used with a range of intensities (from -3.7 to 1.6 log cd/s $\cdot$ m^{-2}), and flash durations were adjusted according to intensity (from 20 μs to 1 ms). Two to five recordings were made at sufficient intervals between flash stimuli (from 10 seconds to 10 minutes) to allow mice time to recover. Typically, four to eight animals were used for recording each point. One-way ANOVA test was used for pairwise comparisons.

Histology and Immunohistochemistry

Histologic procedures used for retinal analyses were previously described.[29]

Analyses of Retinoic Acid and Nonpolar Retinoids

All experimental procedures related to extraction, retinoid derivatization, and separation of retinoids were conducted under dim red light provided by a safelight filter (transmittance >560 nm; No. 1; Eastman Kodak). Polar retinoids in plasma, eye, and liver were analyzed[27] with an HPLC (Agilent 1100; Agilent Technologies, Palo Alto, CA) and two tandem normal-phase columns (Microsorb Silica, 3 μm, 4.6 $\times$ 100 mm [Varian, Palo Alto, CA]; Ultrasphere-Si, 5 μm, 4.6 $\times$ 250 mm [Beckman, Fullerton, CA]).[26] An isocratic normal-phase system of hexane/2-propanol/glacial acetic acid (1000:4.3:0.675; vol/vol/vol) was used for elution at a flow rate of 1 mL/min at 20°C with detection at 355 nm. Nonpolar retinoids in plasma, eye, and liver were analyzed with normal-phase HPLC (Ultrasphere-Si, 5 μm, 4.6 $\times$ 250 mm; Beckman) with 10% ethyl acetate and 90% hexane at a flow rate of 1.4 mL/min with detection at 325 nm HPLC (HP1100; Hewlett Packard, Palo Alto, CA) with a diode array detector and HP software (Chemstation A.03.03; Hewlett Packard), as described previously.[24,25,29,31]

Vision-Dependent Behavioral Testing

Three different vision-dependent behavioral tests were used to evaluate higher order brain function in 12-week-old *Rpe65*$^{-/-}$ mice given 9-*cis*-R-Ac by different treatment regimens. Mice underwent gavage with 9-*cis*-R-Ac or soybean oil and were dark adapted for 3 days before these tests.

Dark/Light Preference Test

The experimental dark/light box was composed of dark gray plastic and consisted of two chambers. Both were square, and the light chamber was slightly larger (20 $\times$ 20 cm) than the dark chamber (15 $\times$ 15 cm). A small rectangular opening entrance (4 cm high by 6 cm wide) connected the two chambers. The dark chamber was entirely enclosed with a solid black plastic top, and the light chamber was open with a 100-W light bulb placed 40 cm above its floor. Mice were placed in a brightly lit open area of the light chamber, facing away from the dark side, and their exploration pattern was tracked for 5 minutes. Their time delay in crossing over into the dark side, total stay in the light side, and number of reentries into the light side after having entered the dark side were recorded.

Visual Placement Test

The visual placement test took advantage of the forepaw-reaching reflex used to test the visual acuity of a mouse when it reflexively extends its paws to find the edge of a surface.[32] For this test a mouse was lifted by the base of its tail 20 cm away from a counter edge and approximately 20 cm above its surface and then was moved forward in a diagonal fashion toward the counter edge at a steady speed. This speed was decreased as the mouse approached the edge. The forepaw-reaching reflex was scored five times as it neared the counter edge, with 30 seconds between each trial. Data were quantified as the

percentage of forepaw-reaching episodes that did not involve the whiskers touching the surface edge.

Water Escape Task

The water escape task test was a variant of a visual acuity task used previously in rats.[33] A cylindrical pool (1 m in diameter) colored with white nontoxic paint was filled with water at 23°C. The pool was split into four virtual quadrants, and a 40-W light overhung the middle of each quadrant approximately 10 cm from the edge of the pool. All trials were conducted in a dimly lit room to minimize distal visual cues and to maximize the proximal visual cue (light). For each trial, a randomly assigned light was turned on, and the mouse was placed in the center of the pool facing the opposite unlit quadrant and given 60 seconds to find a hidden platform. After finding the platform, the mouse was left on it for 15 seconds and then placed in a holding cage behind a curtain, and the light was turned off before room light was restored. Each mouse was given a platform-finding daily training session over 3 days, each consisting of eight trials divided into two to four sessions with an intertrial interval of approximately 15 minutes. If the animal did not find the platform, it was gently guided to it and left there for 15 seconds. To determine whether animals had used visual cues to reach the platform, a dark probe test was performed on all trained mice on the fourth day, during which the testing room was kept dark and all light cues were turned off. Other conditions were identical with those of previous training sessions. All trials were tracked with a video tracking system (EthoVision XT 5.0; Noldus, Leesburg, VA), and the swimming distance, swimming velocity, and time to find the platform were recorded during all training and dark probe trials.

Results

Choice of Vehicle for 9-*cis*-Retinoid Administration

Several different oil-based preparations were tested as vehicles for 9-*cis*-R-Ac administration to 5-week-old C57Bl/6 mice by gastric gavage. Despite large variations in experimental results, 9-*cis*-R-Ac solution in either soybean oil or sunflower oil compared with canola and rapeseed oils appeared to provide the best absorption of this prodrug, as exhibited by the highest plasma levels of fatty acid 9-*cis*-retinyl esters and 9-*cis*-retinol, both active metabolites of 9-*cis*-R-Ac (Figs. 1A, 1C; Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/50/9/4368/DC1). The highest plasma levels of these 9-*cis*-retinoids were noted at approximately 3 hours. Plasma levels of all-*trans*-retinol and fatty acid all-*trans*-retinyl esters did not differ significantly, either among the test vehicles or during the 23-hour test period, suggesting that *cis*-retinoids were not converted to all-*trans*-retinoids (Figs. 1B, 1D). Soybean oil was chosen as the suspending vehicle for 9-*cis*-R-Ac in subsequent experiments because it also stabilized this prodrug (data not shown).

Effects of Single Doses of 9-*cis*-R-Ac on Retinal Function of *Rpe65*$^{-/-}$ Mice

To test whether 9-*cis*-R-Ac might deliver artificial chromophore to the eye, we treated 5-week-old *Rpe65*$^{-/-}$ mice with single doses (2–50 mg/kg) of 9-*cis*-R-Ac in soybean oil. After dark adaption for 3 days, post-gavage, scotopic, single-flash electroretinograms were recorded, and eyes were collected to assess 9-*cis*-retinal levels. Mice tolerated the treatment without loss of weight or gross changes in behavior, even after receiving the highest 50-mg/kg dose. Scotopic electroretinograms of treated mice showed a dose-dependent increase in a-wave and b-wave amplitudes (Figs. 2A, 2B); the lowest tested dose that provided significant improvement after high intensity stimuli was 6.25 mg/kg. Similarly, a dose-dependent accumulation of 9-*cis*-retinal was found in the eyes of treated mice that correlated with improvement in retinal function (Fig. 2C). No fatty acid 9-*cis*-retinyl esters were detected in any of the analyzed eyes, whereas levels of fatty acid all-*trans*-retinyl esters ranged from approximately 1.0 to 1.6 nmol/eye and did not differ significantly among the four treatment groups. Moreover, fatty acid all-*trans*-retinyl ester levels were similar to the 1.2 nmol/eye reported for untreated 5-week-old *Rpe65*$^{-/-}$ mice.[24–26]

9-*cis*-Retinol (43 pmol/eye) was detected only in the eyes of mice dosed with 50 mg/kg, whereas all-*trans*-retinol levels, varying from 14 to 22 pmol/eye, did not differ significantly among the four treatment groups. No 11-*cis*-retinoids were detected in any of the eyes, in agreement with previous reports about this model.[24,25]

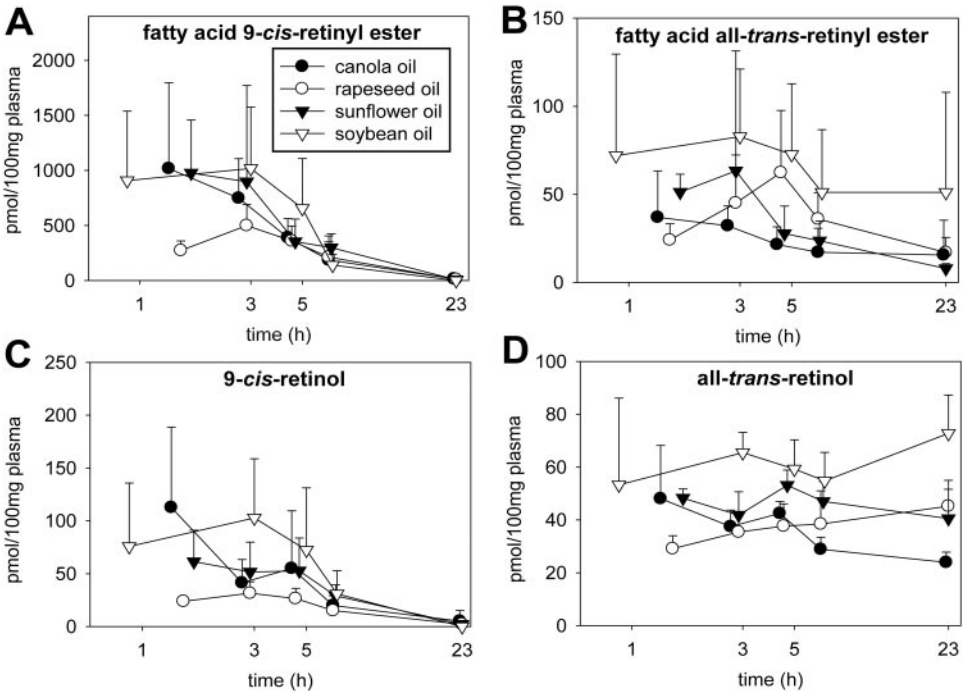

Figure 1. Solubilizing vehicle effects on plasma retinoid levels in mice gavaged with 9-*cis*-R-Ac. A single 50-mg/kg dose of 9-*cis*-retinyl acetate (9-*cis*-R-Ac) suspended in four different vehicle oils was administrated by gastric gavage to 5-week-old C57/Bl6 mice, and retinoid levels were determined in the plasma thereafter ($n = 5$ for each time point per group). (**A**) Fatty acid 9-*cis*-retinyl esters. (**B**) Fatty acid all-*trans*-retinyl esters. (**C**) 9-*cis*-retinol. (**D**) All-*trans*-retinol levels were determined at five sequential time points. 9-*cis*-Retinoid levels in plasma were significantly higher and were maintained for 3 hours after administration, when 9-*cis*-R-Ac was given in either soybean or sunflower oil. Bars indicate SD.

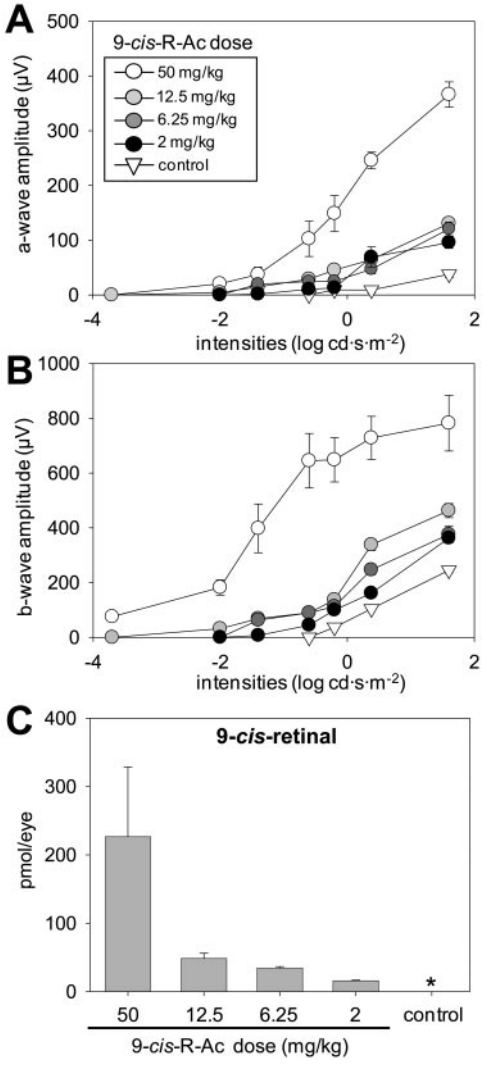

FIGURE 2. Improvement of retinal function in *Rpe65*$^{-/-}$ mice given single doses of 9-*cis*-R-Ac. Five-week-old *Rpe65*$^{-/-}$ mice underwent gavage with single doses of 9-*cis*-R-Ac ranging from 2 to 50 mg/kg and then were maintained for 3 days in the dark ($n = 2$ at 50 mg/kg; $n = 3$–4 for other groups). Scotopic single-flash ERGs were recorded from these mice (**A**, **B**; bars indicate SE), and 9-*cis*-retinal levels were quantified in their eyes (**C**; bars indicate SD). ERGs showed a dose-dependent positive response for a- and b-wave amplitudes in mice given 9-*cis*-R-Ac, and 9-*cis*-retinal accumulated in their eyes in a similar manner. *Undetectable amounts.

Effects of 9-*cis*-R-Ac Given Daily for 14 Days

We next investigated retinal function of *Rpe65*$^{-/-}$ and C57Bl/6 mice after repeated dosing of 9-*cis*-R-Ac. A daily dose of 12.5 mg/kg was well tolerated by 5-week-old C57Bl/6 and *Rpe65*$^{-/-}$ mice without loss of weight or gross changes in behavior, posture, or coat condition in this 14-day study. 9-*cis*-Retinal was readily detected in the eyes of knockout animals, but neither fatty acid 9-*cis*-retinyl esters nor 9-*cis*-retinol was present (Supplementary Figs. S2A–S2C). However, fatty acid 9-*cis*-retinyl esters did accumulate in a dose-dependent manner in the livers of C57Bl/6 and *Rpe65*$^{-/-}$ mice (Supplementary Fig. S2D). The presence of 9-*cis*-retinal in the eyes of these mice suggested improvement in retinal function as observed in single-dose studies of *Rpe65*$^{-/-}$ mice. To test this directly, we subjected 5-week-old *Rpe65*$^{-/-}$ mice to gavage daily with 9-*cis*-R-Ac in soybean oil at doses of 1, 4, or 12.5 mg/kg for 14 days and exposed them to an alternating dark and fluorescent light (luminance range, 500-1500 lux in the cage) environment for the last 11 days of treatment. Then we recorded scotopic single-flash electroretinograms and measured retinoid levels in the eyes (Fig. 3). Again, mice tolerated this regimen well. Electroretinograms showed a dose-dependent increase in the amplitudes of a-and b-waves in treated compared with baseline control *Rpe65*$^{-/-}$ mice (Figs. 4A, 4B). Even the lowest daily test dose of 1 mg/kg evoked significant improvement in retinal function compared with the control group. There also was a corresponding dose-dependent accumulation of 9-*cis*-retinal in the eyes of treated mice (Fig. 4C). No 9-*cis*-retinal was detected in eyes of the baseline and 1-mg/kg treated groups, whereas 38 ± 4 and 95 ± 14 pmol were measured in the daily 4- and 12.5-mg/kg groups, respectively. Levels of fatty acid 9-*cis*-retinyl esters were low (1 pmol/eye) in the 4- and 12.5-mg/kg/d groups and were undetectable in eyes from other groups (Fig. 4D). Neither all-*trans*-retinol nor 9-*cis*-retinol was found in any group. Levels of fatty acid all-*trans*-retinyl esters (essentially palmitate, stearate, and oleate) in the eyes of mice exposed to 9-*cis*-R-Ac ranged from 1.2 to 1.4 nmol/eye and were not significantly different from those in control eyes (1.2 nmol/eye).

Duration of Improved Retinal Function after 3 Daily Doses of 9-*cis*-R-Ac

9-*cis*-Retinol in the form of fatty acid 9-*cis*-retinyl esters accumulated in the livers of *Rpe65*$^{-/-}$ mice given repeated doses of 9-*cis*-R-Ac of at least 12.5 mg/kg for 2 weeks (Supplementary Fig. S2D). To assess the capability of mice to store 9-*cis*-retinoids and use them later in the retinoid cycle, 9-*cis*-R-Ac in soybean oil was gavaged once daily for 3 consecutive days at a dose of 1, 4, or 12.5 mg/kg/d into 5-week-old *Rpe*65$^{-/-}$ mice kept in the dark. Mice then were exposed to cycles of 8 hours of fluorescent light with luminance ranges of 500 to 1500 lux, followed by 16 hours in the dark. Electroretinography and retinoid analyses were performed at the end of the first (day 4), second (day 5), fourth (day 7), and sixth (day 9) days of light exposure (Fig. 5). At each time point, a- and b-wave amplitudes of ERG responses recorded up to day 9 (Figs. 6A–F) were dose dependent and declined with the number of light exposures. The highest tested dose (12.5 mg/kg/d) significantly improved a- and b-wave amplitudes up to day 9 (Figs. 6A, 6B) at high-intensity stimuli, whereas doses of 4 mg/kg and 1 mg/kg

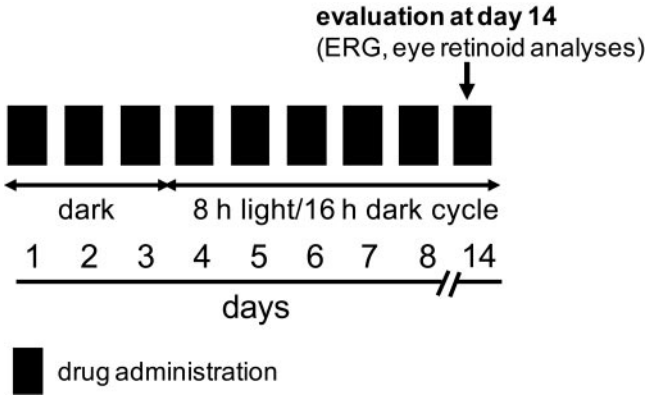

FIGURE 3. Experimental protocol for 14-day daily treatment of *Rpe65*$^{-/-}$ mice with 9-*cis*-R-Ac. Groups of 5-week-old *Rpe65*$^{-/-}$ mice underwent gavage daily for 14 days with different doses of 9-*cis*-R-Ac (1, 4, or 12.5 mg/kg; $n = 4$–6 per group). Mice were kept in the dark from day 1 to day 3 and then were maintained in an 8-hour light/16-hour dark cycle environment from day 4 to day 14, when ERGs were recorded and eyes were analyzed for retinoids.

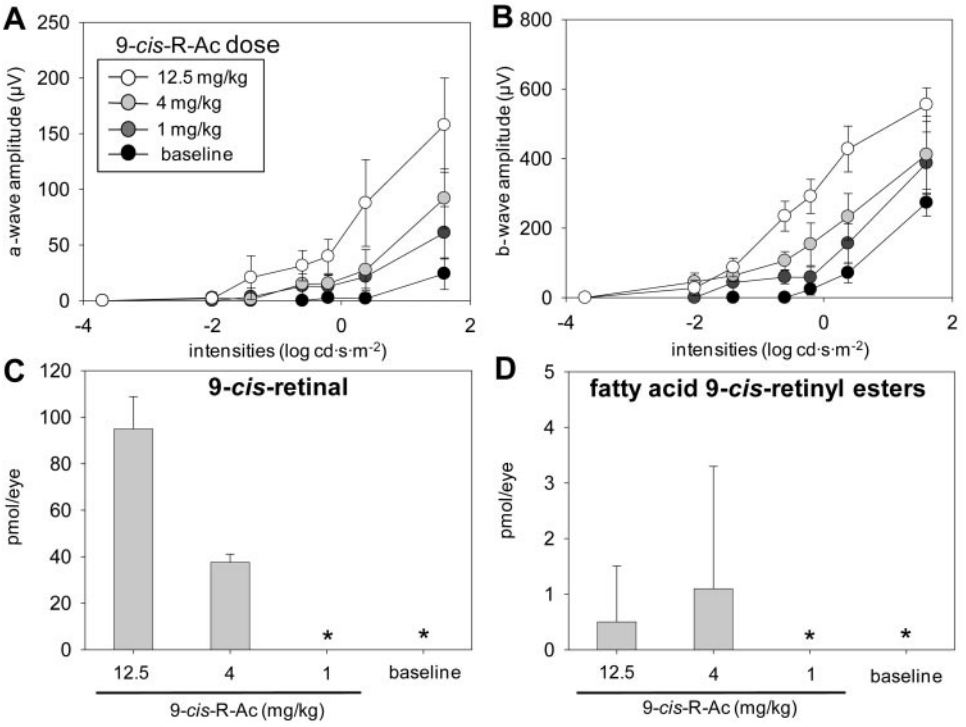

FIGURE 4. Improvement of retinal function in 5-week-old *Rpe65*$^{-/-}$ mice after gavage with daily doses of 9-*cis*-R-Ac for 14 days. Scotopic single-flash ERGs from these mice were obtained on day 14, and functional a- and b-wave amplitude plots are shown (**A**, **B**). ERG responses improved in a dose-dependent manner. Bars indicate SE (n = 4–6 per group except baseline; baseline, n = 2). Eye levels of 9-*cis*-retinoids also are shown for day 14 (**C**, **D**). 9-*cis*-Retinoids accumulated in a dose-dependent fashion in the eyes of mice treated with 4 and 12.5 mg/kg 9-*cis*-R-Ac, but these compounds were not detectable (*) in mice treated with the lowest dose (1 mg/kg) or left untreated (baseline). Bars indicate SD (n = 4–6 per group except baseline; baseline, n = 2). ERG wave amplitudes and retinoid levels in the eyes of treated mice were compared with similar data obtained from baseline mice. The key (**A**) indicates doses of 9-*cis*-R-Ac, as do numbers under the bars (**C**, **D**).

showed improvement in a-wave amplitudes up to day 9 and day 7, respectively, and in b-wave amplitudes up to day 9 (Figs. 6C–F). Levels of 9-*cis*-retinal in the eye also were dose dependent and decreased over time (Supplementary Fig. S3). This compound was detected in the retinas of all treated mice at day 4 (Supplementary Fig. S3) but was detected in the group exposed to 4 and 12.5 mg/kg only at day 5 and in the group exposed to 12.5 mg/kg only at day 7. No 9-*cis*-retinal was found in the retinas of treated or control mice by day 9. Thus, daily administration of 9-*cis*-R-Ac was not needed to deliver 9-*cis*-R-Ac to the eye and to sustain improvement in retinal function of *Rpe*65$^{-/-}$ mice.

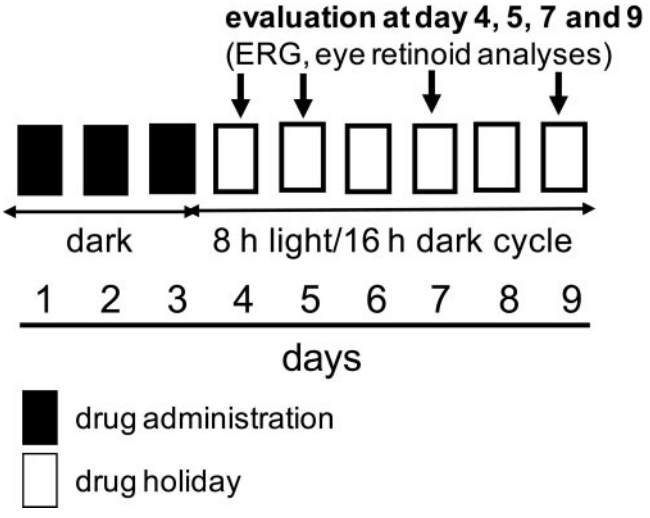

FIGURE 5. Experimental protocol for assessment of retinal function after daily administration of 9-*cis*-R-Ac for 3 days. This experiment was designed to test the duration of visual improvement after the completion of brief treatment with 9-*cis*-R-Ac. Groups of 5-week-old *Rpe65*$^{-/-}$ mice were given 9-*cis*-R-Ac by oral gavage (12.5, 4, or 1 mg/kg) or soybean oil for 3 days (days 1–3) and were exposed daily to fluorescent light with a luminance range of a 500 to 1500 lux in the cage for 8 hours before they were returned to the dark. Retinal function was evaluated by ERGs, and eye retinoid level analyses were conducted on the first (day 4), second (day 5), fourth (day 7), and sixth (day 9) days after the completion of 9-*cis*-R-Ac therapy, as shown.

Retinal Function of *Rpe65*$^{-/-}$ Mice after Intermittent and Daily Administration of 9-*cis*-R-Ac for 8 Weeks

Because three low daily doses of 9-*cis*-R-Ac improved ERG responses after 6 days of light exposure (Figs. 6A–F), we tested whether a prolonged intermittent dosing regimen might improve retinal function as well. *Rpe65* $^{-/-}$ mice were split into two groups, each treated for a total of 8 weeks with 1 or 4 mg/kg of 9-*cis*-R-Ac. One group was dosed daily for 3 days followed by a 4-day drug holiday during each week of the 8-week regimen (intermittent group), and the other was dosed daily for the full 8-week period (daily group; Fig. 7). Mice were exposed to a daily cycle of 8 hours of fluorescent light with a luminance range of 500 to 1500 lux followed by 16 hours of darkness. Electroretinograms were recorded at day 28 and again at day 56, after which tissues were collected for retinoid analyses of the eye and liver and for histologic examination of the eye. Intermittent dosing and daily dosing regimens evoked dose-dependent increases in the amplitudes of a- and b-waves on days 28 and 56 (Figs. 8A–H). Responses were more pronounced in the daily dosed than in the intermittently dosed group. The lower dose (1 mg/kg) was sufficient to cause significant improvement in ERG responses over the control group at high-intensity stimuli, irrespective of the treatment schedule. In addition, a- and b-wave amplitudes were similar at days 28 and 56, suggesting that equilibrium might have been achieved between the intake and storage of 9-*cis*-retinol on one hand and its mobilization in the retina to support the retinoid cycle on the other. In agreement with these ERG results, 9-*cis*-retinal was detected in a dose-dependent manner in the eyes in which levels were higher in mice dosed daily (Supplementary Fig. S4A). Fatty acid 9-*cis*-retinyl esters at low variable

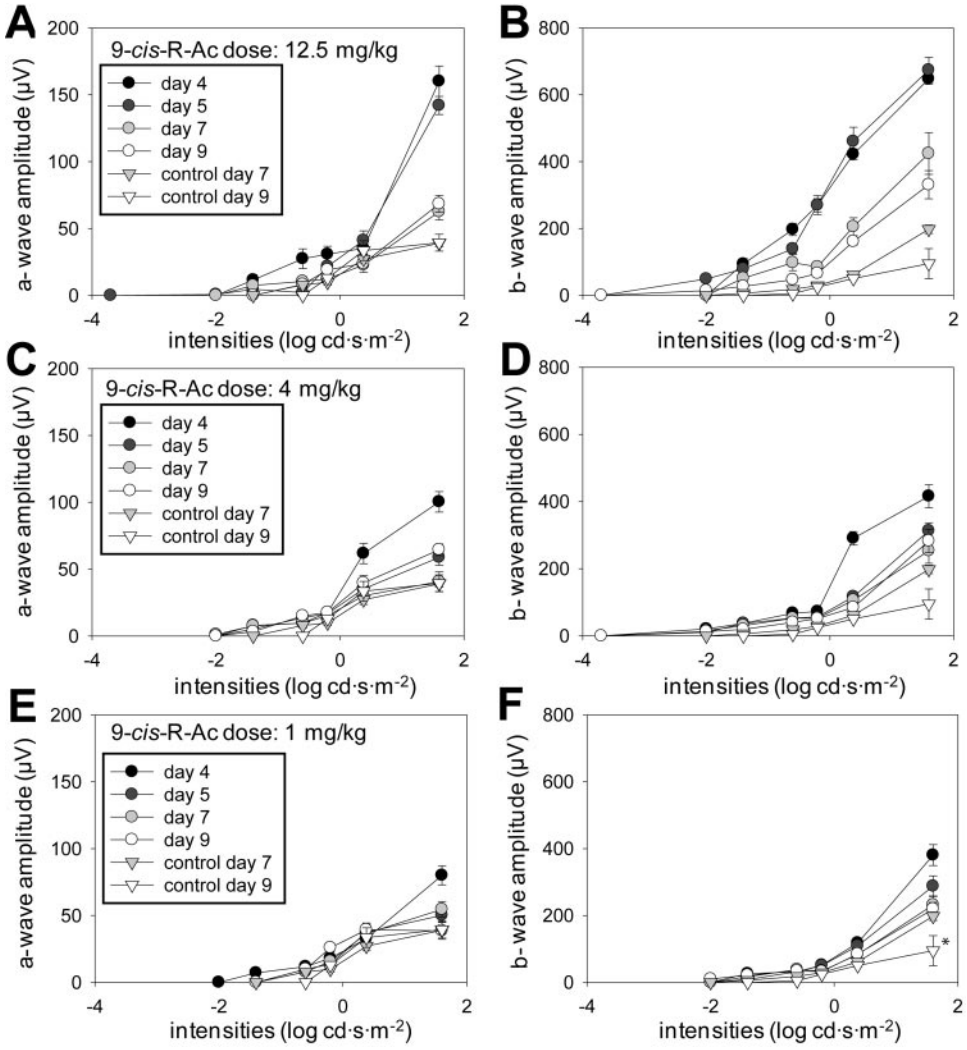

FIGURE 6. Duration of improvement in retinal function after 3-day administration of 9-*cis*-R-Ac. When 8-hour fluorescent light exposure ended at 4 pm, single-flash scotopic ERGs were recorded at four sequential time points to evaluate the duration of improvement in retinal function after treatment with 9-*cis*-R-Ac (Fig. 5; $n = 3–4$ per group except for day 9, at a dose of 12.5 mg/kg, and control day 9, at $n = 2$ each). Bars indicate SE. ERG responses of 9-*cis*-R-Ac treated and control mice were plotted, and functional a- and b-wave amplitudes are shown: (**A**, **B**), 12.5 mg/kg; (**C**, **D**), 4 mg/kg; (**E**, **F**), 1 mg/kg. Even the lowest dose (1 mg/kg) significantly improved b-wave amplitudes of ERG responses at high-intensity stimuli on day 9 compared with responses of control mice. $P < 0.05$.

levels also were found in the eyes of both sets of treated animals (Supplementary Fig. S4B). A dose-dependent slight increase in fatty acid all-*trans*-retinyl esters also was noted in the eyes of treated mice regardless of the regimen. In the liver, 9-*cis*-retinol was essentially stored in the form of fatty acid 9-*cis*-retinyl esters in a dose- and regimen-dependent manner

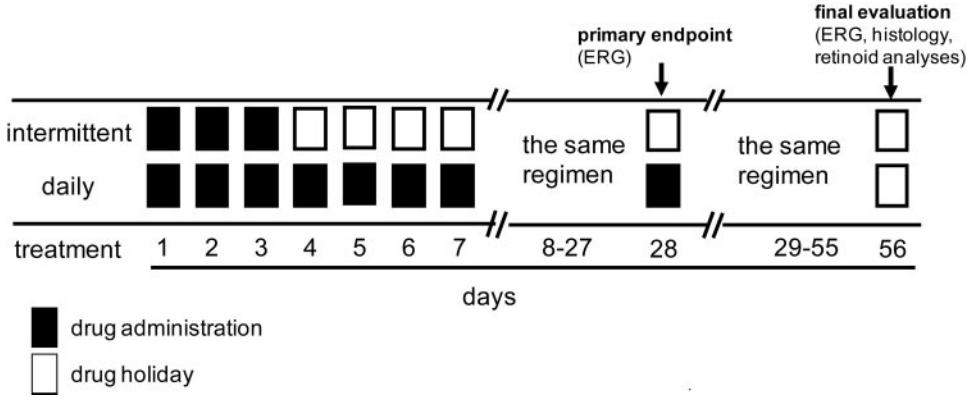

FIGURE 7. Experimental protocol for examining the effects on retinal function of intermittent and daily administration of 9-*cis*-R-Ac to 5-week-old *Rpe65*$^{-/-}$ mice for 8 weeks. Groups of *Rpe65*$^{-/-}$ mice were treated for 8 weeks with either 1- or 4-mg/kg daily doses of 9-*cis*-R-Ac for 3 days followed by 4-day weekly drug holiday periods (intermittent group, $n = 6–7$ per group), or the same two doses were given daily (daily group, $n = 6–7$ per group). Mice were maintained under an 8-hour fluorescent light (luminance range, 500-1500 lux in the cage)/16-hour dark cycle during the 8-week experiment. ERGs were recorded on days 28 and 56, and the tissues were removed for histology and retinoid analyses of eyes and liver on day 56.

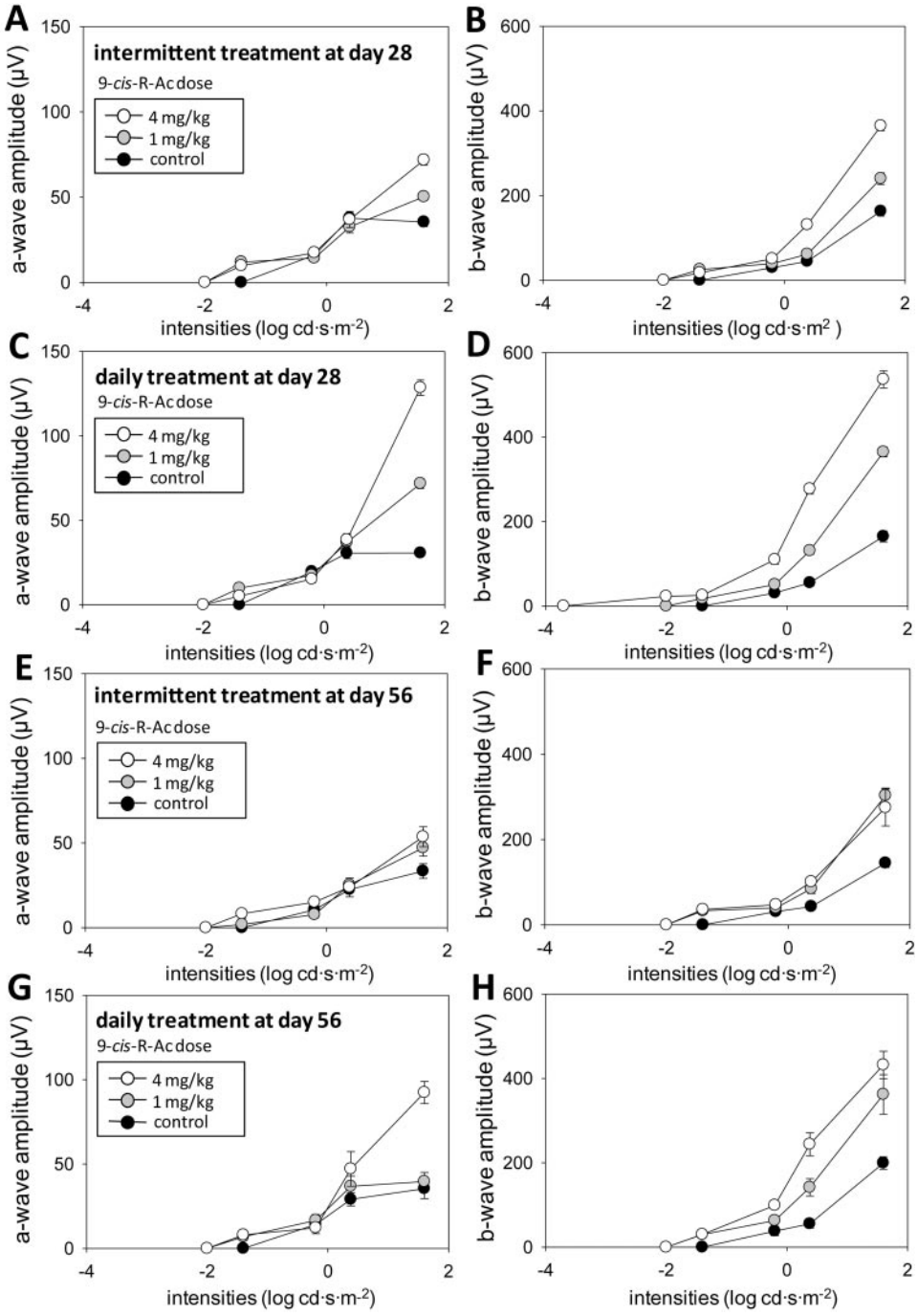

FIGURE 8. Effects of intermittent and daily administration of 9-*cis*-R-Ac on ERG responses of *Rpe65*$^{-/-}$ mice recorded on days 28 and 56. Dose-dependent improvement in the amplitude of a- and b-waves is shown with both treatment regimens at day 28: (**A**, **B**), intermittent treatment; (**C**, **D**), daily treatment; n = 5–7 per group; and day 56: (**E**, **F**), intermittent treatment; (**G**, **H**), daily treatment; n = 5–7 per group. ERG responses of both groups of treated mice were superior to those from controls. Bars indicate SE.

(Supplementary Figs. S5A, S5B). Levels of fatty acid all-*trans*-retinyl esters were not significantly affected by these regimens, though there might have been a slight increase in mice receiving 4 mg/kg 9-*cis*-R-Ac. Long-term administration of 9-*cis*-R-Ac had a dose-dependent protective effect on the retina, as assessed by the lengths of the photoreceptor outer segments (Figs. 9A, 9C) and the number of nuclei in the outer nuclear layer (Figs. 9B, 9D); however, >50% less ROS length and >30% less nuclei numbers were observed in mice receiving 4 mg/kg 9-*cis*-R-Ac daily compared with wild-type mice of similar ages in our previous study.[34] These effects were more pronounced in the superior than the inferior retinas of mice treated at the 4-mg/kg dose. Highly magnified images of retinal cross-sections showed improvement in rod outer segment (ROS) morphology and fewer oil droplet-like structures in parts of the superior and inferior portions of retinas from mice treated with the 4-mg/kg daily or the 4-mg/kg intermittent regimens (Figs. 9E, 9F). However, no significant change was observed in retinas of mice receiving the 1-mg/kg 9-*cis*-R-Ac dose by either schedule (Figs. 9G, 9H) compared with retinas of control mice (Fig. 9I). These results are consistent with those of our previous study.[25]

Improvement in Vision-Dependent Behavior in *Rpe65*$^{-/-}$ Mice Treated with 9-*cis*-R-Ac

To evaluate the effects of 9-*cis*-R-Ac treatment on higher order vision-based brain function, three different vision-dependent behavioral tests were conducted with groups of *Rpe65*$^{-/-}$ mice after they received two different 9-*cis*-R-Ac treatment regimens (single 100-mg/kg dose or three daily 12.5-mg/kg

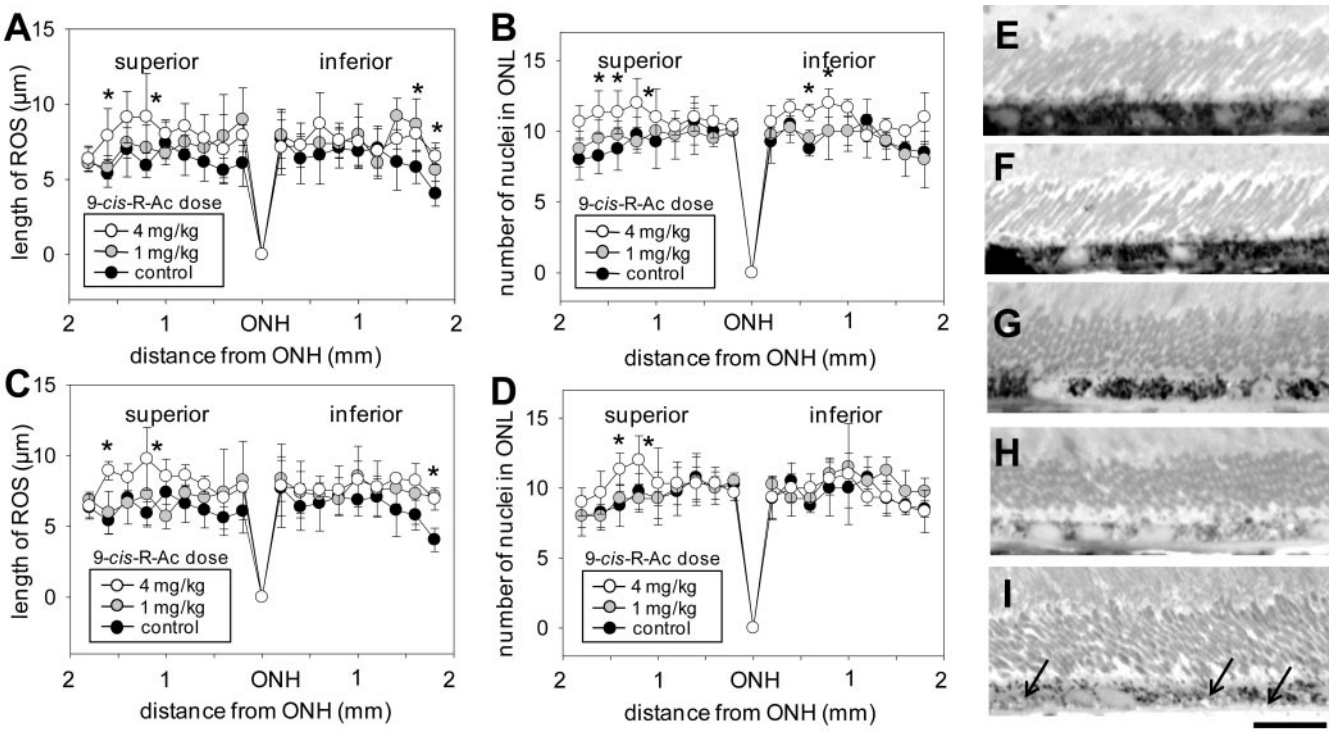

FIGURE 9. Retinal morphology of 9-*cis*-R-Ac–treated mice. ROS lengths and numbers of nuclei in the outer nuclear layer (ONL) of treated and control mice are shown at day 56. Data points are plotted as a function of the distance from the optic nerve head (ONH). Lengths of the ROS (**A**, intermittent treatment; **C**, daily treatment) and the number of nuclei in the ONL (**B**, intermittent treatment; **D**, daily treatment) in the superior retina were significantly greater in a dose-dependent manner for 9-*cis*-R-Ac–treated mice compared with control groups. These positive effects were more prominent in mice treated at the 4-mg/kg dose, whereas positive effects were observed only in limited areas in mice treated with the 1-mg/kg dose (n = 3–4 per group). Bars indicate SD. Images of the ROS and RPE interface in control and treated mice at day 56 are shown: (**E**), 4 mg/kg intermittent treatment; (**F**), 4 mg/kg daily treatment; (**G**), 1 mg/kg intermittent treatment; (**H**), 1 mg/kg daily treatment; (**I**), control. Improved ROS structures and fewer oil droplet-like structures were present in mice treated with 4 mg/kg 9-*cis*-R-Ac compared with control mice *Arrows*: oil droplet-like structures in (**I**). Scale bar, 5 μm.

doses of 9-*cis*-R-Ac); soybean oil vehicle was used as the control. The dark/light preference test showed that 9-*cis*-R-Ac–treated mice left the illuminated area for the dark area significantly faster than did control animals (Fig. 10A). As expected, the visual placement test also indicated a success rate that was significantly higher for treated compared with control mice in reflexively extending their forearms in response to observing a counter edge (Fig. 10B). Treated mice in the water escape task took significantly less time (32% less at 100 mg/kg, 25% less at 12.5 mg/kg) to complete the task during the day 1- to day 3-test period (Fig. 10C) but could not complete the dark-probe test (data not shown). Improvement was not observed in control animals for the dark-probe tests. These results suggest that treatment with 9-*cis*-R-Ac improved retinal function and morphology in *Rpe65*$^{-/-}$ mice and enabled them to execute various vision-dependent, high-order brain functioning tasks more efficiently.

Discussion

To develop pharmacologic therapies for retinal degeneration in humans, it is important first to evaluate the efficacy and safety of candidate compounds in appropriate animal models of human disease. We have reported that 9-*cis*-retinoids are effective prophylactic agents for the treatment of retinal degeneration in mouse models of LCA and other human retinopathies.[24,25,27,34–36] After oral administration, vitamin A (all-*trans*-retinol) is stored in the liver as its retinyl-esters. These esters are hydrolyzed to all-*trans*-retinol and delivered to RPE cells where they are stored as all-*trans*-retinyl-esters. Finally, these esters are converted to visual chromophore, 11-*cis*-retinal, by the visual cycle to regenerate visual pigments in photoreceptor cells.[37] Nine orally administered *cis*-retinoids are delivered to RPE cells by a pathway similar to that of all-*trans*-retinol, where they regenerate visual pigments as *iso*-rhodopsin by bypassing chemical reactions in the visual cycle. This process restores and compensates for retinal dysfunction because of lack or depletion of 11-*cis*-retinal in animal models.[27,34,38] In our previous study wherein various 9-*cis*-retinoid derivatives were tested for their bioavailability, both 9-*cis*-R-Ac and 9-*cis*-retinyl-succinate showed greater efficacy than 9-*cis*-retinal.[27] Thus, 9-*cis*-retinyl acetate was chosen for this study because of the ease of its preparation and analyses compared with other active retinoid derivatives.

Vision-dependent behavioral testing is a powerful method for characterizing retinal dysfunction and evaluating therapeutic effects of pharmacologic agents in mice. After designing and performing various experiments to address these issues in a mouse model of human LCA, we now present the first evidence that administration of the prodrug 9-*cis*-R-Ac can preserve vision-dependent behavior without apparent toxicity in *Rpe65*$^{-/-}$ mice.

Effects of a Single Dose of 9-*cis*-R-Ac on the Vision of *Rpe65*$^{-/-}$ Mice

Soybean oil was used to suspend the 9-*cis*-R-Ac administered by gastric gavage in all studies described herein because, of several agents tested, this stabilizing vehicle provided the best relative absorption of 9-*cis*-R-Ac and plasma retention of its active metabolites, fatty acid 9-*cis*-retinyl esters and 9-*cis*-retinol

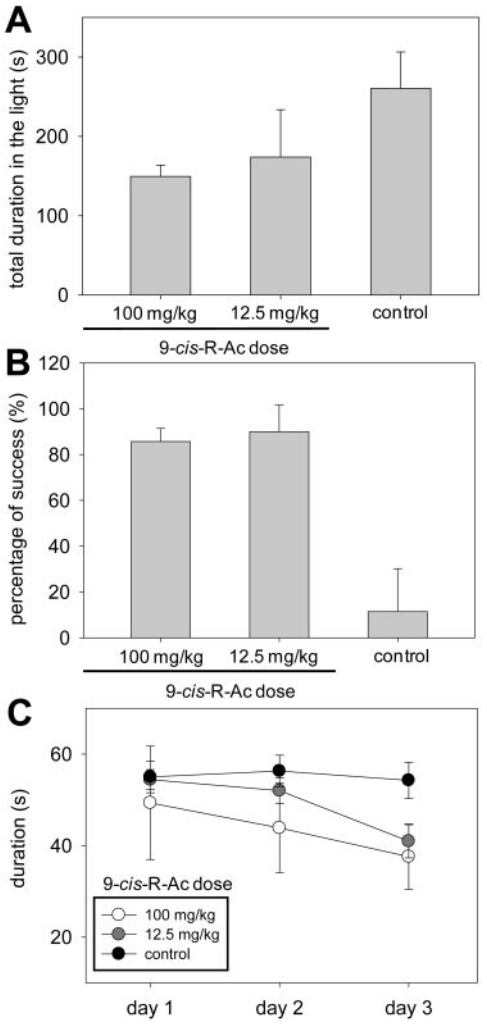

FIGURE 10. Improvement of vision-dependent behavior in *Rpe65*$^{-/-}$ mice treated with 9-*cis*-R-Ac. Twelve-week-old *Rpe65*$^{-/-}$ mice were subjected to three different vision-dependent behavioral tests—a dark/light preference test, a visual placement test, and a water escape task—after they had received 9-*cis*-R-Ac either as a single 100-mg/kg dose or as 12.5 mg/kg daily for 3 days (n = 3 per group). In the dark/light preference test, treated mice moved to a dark space from an illuminated area significantly faster than control mice (**A**). The visual placement test also demonstrated a significantly higher success rate of reflexive arm extension in response to visualization of a counter edge in treated compared with control mice (**B**). In the water escape task, treated mice took significantly less time (average 32% less at 100 mg/kg, 25% less at 12.5 mg/kg) to find the hidden platform by using a light as a cue in a dose-dependent manner, whereas improvement in escape latencies was not observed in control mice (**C**). Bars indicate SD.

(Fig. 1). Dose-dependent improvement in ERG responses was documented along with increasing levels of 9-*cis*-retinal in the eye, results consistent with previous studies[25,27] suggesting that 9-*cis*-retinal recombined with opsin to form *iso*-rhodopsin. Importantly, lower doses of 9-*cis*-R-Ac (2 and 4 mg/kg) induced positive ERG effects even though only trace levels of 9-*cis*-retinal were detected in the eyes (Fig. 2). These results encouraged us to evaluate the tolerance of these knockout mice to daily administration of 9-*cis*-R-Ac.

Daily Administration of 9-*cis*-R-Ac for 14 Days

The safety of 9-*cis*-R-Ac was tested by administering doses of 12.5 and 50 mg/kg/d by gastric gavage for 14 days to groups of *Rpe65*$^{-/-}$ and C57Bl/6 control mice. The physical condition of these animals was carefully evaluated daily before and after drug administration, and retinoid levels in eyes and liver were determined after 14 days (Supplementary Fig. S2). With the exception of body weight loss at the higher 50-mg/kg dose, these doses appeared to be well tolerated. Based on these results, groups of 5-week-old *Rpe65*$^{-/-}$ mice then underwent gavage daily with 9-*cis*-R-Ac at three different doses (1, 4, and 12.5 mg/kg), and the efficacy and kinetics of this retinoid were evaluated 2 weeks later. Doses appeared well tolerated, and ERG responses again improved in a dose-dependent manner. Interestingly, the lowest dose (1 mg/kg) significantly improved ERG responses compared with baseline in control *Rpe65*$^{-/-}$ mice, even though 9-*cis*-retinal and fatty acid 9-*cis*-retinyl esters were not detected in the eye (Fig. 4). This suggests that 9-*cis*-retinal disappears with light exposure (8 hours light/16 hours dark) and that fatty acid 9-*cis*-retinyl esters are used to regenerate *iso*-rhodopsin instead. Indeed, accumulation of fatty acid 9-*cis*-retinyl esters was detected in liver samples in a dose-dependent manner. The last observation suggests that hepatic stores of fatty acid 9-*cis*-retinyl esters can serve as a reservoir to generate 9-*cis*-retinal and *iso*-rhodopsin in the eye, thereby explaining the efficacy of low doses of 9-*cis*-R-Ac.

Duration of Retinal Function Improvement in *Rpe65*$^{-/-}$ Mice Given 9-*cis*-R-Ac

We recorded electroretinograms and tested levels of 9-*cis*-R-Ac in the eyes of *Rpe65*$^{-/-}$ mice that had received either 9-*cis*-R-Ac or soybean oil for 3 days to determine the duration of this compound's beneficial effects (Fig. 5). As shown in Figure 6, ERG amplitudes improved in a generally dose-dependent manner, and this positive effect was maintained for up to 4 to 6 days after treatment. Moreover, a similar pattern was noted for 9-*cis*-retinal levels found in the eyes of these animals (Supplementary Fig. S3). Importantly, improvement of ERG responses at the 4-mg/kg dose level lasted for 4 to 6 days after the cessation of treatment, when 9-*cis*-retinal could no longer be found in the eyes. These results may indicate that the positive effects of 9-*cis*-R-Ac therapy are retained by trace levels of 9-*cis*-retinal in the retina that stabilize the ROS as observed in previous studies,[27,25] whereas ERG responses in the control groups had deteriorated. Retinoid kinetics were then examined during dark adaptation after light exposure. Restoration of fatty acid 9-*cis*-retinyl esters and 9-*cis*-retinal in the eyes occurred during dark adaptation (data not shown). From these observations, we suggest that 9-*cis*-retinoids are delivered to the retina in two ways, primarily and promptly from the circulating blood and secondarily and more slowly from 9-*cis*-retinoids stored in the liver.

Effects of Intermittent and Daily Administration of 9-*cis*-R-Ac for 8 Weeks on Retinal Function in *Rpe65*$^{-/-}$ Mice

To investigate longer term effects of 9-*cis*-R-Ac administration on safety and retinal function in *Rpe65*$^{-/-}$ mice, groups of 5-week-old animals underwent gavage for 8 weeks with 1 or 4 mg/kg of 9-*cis*-R-Ac according to an intermittent or a daily dosing schedule; efficacy was evaluated by electroretinography on day 28 and by electroretinography, retinoid analyses, and retinal histology on day 56 (Fig. 7). As expected, ERG responses of treated mice were significantly better than those of control mice at day 28 and day 56, whereas mild tapering of amplitudes between day 28 and day 56 was noted in the 9-*cis*-R-Ac–treated and control mice. 9-*cis*-Retinal in the eyes and the storage form of 9-*cis*-retinal in the liver, fatty acid 9-*cis*-retinyl esters, were detectable in a dose-dependent manner, and levels of both 9-*cis*-retinoids were comparable to those

observed in the dose efficacy duration study. Abnormal accumulation of fatty acid all-*trans*-retinyl esters was not found in treated mice. ERG responses of mice treated intermittently with 9-*cis*-R-Ac exhibited no significant difference between the 1- and 4-mg/kg dose groups at day 56, suggesting that the lower 1-mg/kg dose might have similar efficacy if given continuously. As shown in Figure 9, morphologic preservation of ROS was observed such that ROS lengths were significantly longer in the superior retina of mice treated with 4 mg/kg, whereas this therapeutic effect was less in animals given the 1-mg/kg dose. From these observations, it is strongly suggested that prolonged treatment regimens with 1 and 4 mg/kg maintained retinal function in *Rpe65*$^{-/-}$ mice without significant clinical toxicity or abnormal retinoid accumulation in the eyes and liver.

Vision-Dependent Behavioral Testing

Behavioral testing is one of the most powerful ways to evaluate vision-dependent higher brain functions such as pupillary responses to light.[27,39] For example, it has been reported that neural function is disrupted in the LCA mouse model.[27,40,41] In this study, a battery of vision-dependent behavioral tests, including a dark/light preference test, visual placement test, and water escape task, was used to assess the efficacy of 9-*cis*-R-Ac treatment.

First, by taking advantage of the innate preference of mice for dark enclosures, we carried out a light/dark preference test in which mice were given a choice of leaving a highly illuminated area for darker surroundings.[42] In this test, the time for 9-*cis*-R-Ac–treated mice to vacate to the dark place was significantly shorter than for control mice, suggesting that mice could still perceive light in a darkened environment. Second, the visual placement test, a test that takes advantage of the forelimb extension reflex when a suspended animal is faced with a shelf edge, was performed to evaluate the visual resolution of these mice. Again, treated mutant mice reflexively extended their forelimbs significantly more than the control group, supporting the idea that their visual resolution was improved by 9-*cis*-R-Ac. Third, a cued-learning version of the water escape task was used by which a mouse learned to associate a bright light with an escape platform under water. These tests were conducted with male mice older than 12 weeks of age (after 12 weeks, they lose more than 80% of their retinal cone cells).[36] Such treated mice successfully completed the water escape task progressively faster, and retained rod function was the most likely explanation for this result. Together these results of vision-dependent behavior testing strongly suggest that 9-*cis*-R-Ac treatment helps to maintain connections from the retina to the visual and connected frontal lobe centers of the brain.

Possible Use of 9-*cis*-R-Ac to Treat Patients with LCA

LCA is an inherited, severe, and incurable form of retinal degeneration that is a leading cause of blindness during childhood. Extensive efforts have been made to identify its causative genes and to characterize its pathophysiology. Animal model studies have accelerated the development of promising therapies through two radically different approaches. Gene therapy trials with recombinant adenoassociated virus were performed in LCA patients with *RPE*65 mutations.[21,22,43] The results seem encouraging, so the same approach may apply to patients with different LCA gene mutations and forms of retinal degeneration. The pharmacologic approach involves the use of artificial retinoids to bypass critical blockades in the retinoid cycle and thereby generate an artificial *cis*-retinoid chromophore that can functionally combine with opsin.[24,25,27]

In this study, the possibility of using 9-*cis*-R-Ac to treat human patients with LCA was assessed by using several different regimens in *Rpe65*$^{-/-}$ mice to evaluate drug efficacy and safety. Dose- and administration period-dependent retention of visual function were observed, even at the lowest 1- and 4-mg/kg doses tested (Figs. 2, 4, 6, 8). Significantly, a dose-dependent prolongation of efficacy was documented for this prodrug, boding well for the application of this synthetic retinoid for the treatment of LCA.

Conclusions

9-*cis*-R-Ac is a pharmaceutical prodrug that is converted by the liver to another metabolic prodrug form, namely fatty acid 9-*cis*-retinyl esters, stored there in hepatic lipid droplets. Fatty acid 9-*cis*-retinyl esters and 9-*cis*-retinol are mobilized from the liver into the circulation, where they travel to the eye and RPE. There, they are converted to 9-*cis*-retinal, which ultimately combines with photoreceptor opsins to form active visual pigments. In this study, low doses (1 and 4 mg/kg) of the prodrug 9-*cis*-R-Ac were found to be clinically safe and effective in maintaining visual function in *Rpe65*$^{-/-}$ mice as assessed by ERG recordings, retinoid levels in the eyes, retinal histology, and vision-dependent behavioral studies. This compound may ultimately prove useful in treating patients with retinopathies stemming from inadequate retinoid chromophore generation.

Acknowledgments

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