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Gene therapy in the second eye of RPE65-deficient dogs improves retinal function

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Abstract

The purpose of this study was to evaluate whether immune responses interfered with gene therapy rescue using subretinally delivered recombinant adeno-associated viral vector serotype 2 carrying the *RPE65* cDNA gene driven by the human RPE65 promoter (*rAAV2.hRPE65p.hRPE65*) in the second eye of *RPE65*^{-/-} dogs that had previously been treated in a similar manner in the other eye. Bilateral subretinal injection was performed in nine dogs with the second eye treated 85–180 days after the first. Electroretinography (ERG) and vision testing showed rescue in 16 of 18 treated eyes, with no significant difference between first and second treated eyes. A serum neutralizing antibody (NAb) response to rAAV2 was detected in all treated animals, but this did not prevent or reduce the effectiveness of rescue in the second treated eye. We conclude that successful rescue using subretinal *rAAV2.hRPE65p.hRPE65* gene therapy in the second eye is not precluded by prior gene therapy in the contralateral eye of the *RPE65*^{-/-} dog. This finding has important implications for the treatment of human LCA type II patients.

Keywords

rAAV2; Leber congenital amaurosis; RPE65; canine model; immune response; repeated injection

INTRODUCTION

Leber congenital amaurosis is a severe early-onset inherited form of retinal degeneration that shows genetic heterogeneity. The estimated prevalence of LCA in the North American population is 1: 81 000.¹ The condition is characterized by severe visual impairment in dim light, typically progressing to complete blindness in the second decade of life. LCA type II results from mutations in the *RPE65* gene, and accounts for 10–15% of LCA cases.^{2,3} *RPE65* encodes a protein that forms an essential component of the visual cycle and is expressed within the retinal pigment epithelium.³ The visual cycle is responsible for the supply of the chromophore, 11-*cis*-retinal, to the photoreceptor cells for combination with the rod and cone opsins to form the visual pigments. *RPE65* is an isomerohydrolase that

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

converts esters of vitamin A to 11-*cis*-retinol for subsequent oxidation to 11-*cis*-retinal prior to transport to the photoreceptors. A spontaneous 4 basepair deletion in *RPE65* in the Briard breed of dog results in a premature stop codon and an absence of *RPE65* gene product, resulting in a very similar phenotype to LCA type II.⁴ Affected dogs have markedly reduced vision and an abnormal electroretinogram with greatly elevated threshold of responses.^{4,5} The similarities between the human and canine disease resulting from *RPE65* mutations, make the *RPE65*^{-/-} Briard a valuable large animal model for LCA type II.

Dramatic restoration of vision with gene therapy was first reported in the canine *RPE65*^{-/-} model of LCA.⁶ A number of rod studies have shown and cone photoreceptor rescue using rAAV vectors to deliver a normal copy of the *RPE65* gene via a subretinal injection in the *RPE65* mutant Briard.⁶⁻¹³ On the basis of the great success of the canine trials, phase I/II clinical trials of rAAV-*RPE65* gene replacement therapy in human LCA patients have started with the first reported results showing great promise.¹⁴⁻¹⁶ Thus far in all human patients only one eye has been treated. A critical aspect of the management of LCA type II individuals will be the ability to achieve rescue in the second eye. There are concerns that immune responses to the viral capsid and transgene may limit rescue achieved by repeated administration.

Immune responses following rAAV-mediated gene delivery have been analyzed in several detailed studies in animal models, but have generated some contradictory reports and remain inconclusive, with immune responses appearing to depend on the route of administration, vector dose and species differences.¹⁷ There are conflicting reports on the success of repeated gene therapy in nonocular tissues. In some studies, readministration of rAAV at later time points was less successful than the initial administration because of neutralizing antibodies (NAb) to the viral capsid proteins.¹⁸⁻²¹ Serotype switching and transient immunosuppression have been used to try and overcome this obstacle.^{19,20} Other studies have reported additional transduction events and successful transgene expression after readministration of rAAV, despite the presence of serum NAb to the vector.^{22,23} In ocular tissues, the site of vector administration is reported to impact on the degree of immune interference with subsequent rAAV administration.²⁴ A study evaluating bilateral intravitreal rAAV2 injections 1 month apart in C57BL/6J mice found that the second eye had very poor reporter gene expression compared with the first treated eye.²⁵ In contrast, the immune privilege of the subretinal space appears to allow for successful readministration. Subretinal injection of rAAV to the contralateral eyes of two previously treated monkeys achieved reporter gene expression at an equivalent level to that seen in the first treated eye.²⁶ Similarly in the *rd12* mouse, administration of rAAV-*RPE65* to the subretinal space of the second eye also achieved rescue similar to that obtained in the first eye.²⁷ In addition, prior intravitreal injection of the first eye did not interfere with the degree of reporter gene expression achieved by subretinal injection in the second eye of C57BL/6J mice.²⁸

Short duration of rAAV-mediated transgene expression in a number of clinical trials has been linked to the very high level of pre-exposure to wild type AAV in the human population.²⁹ However, a much more limited systemic response to vector has been observed when rAAV was administered to the brain, which is an immune privileged site, with no anti-vector antibodies detected in the CNS itself and only a minority of patients developing circulating NAb.³⁰ The eye is also a site of immune privilege, and thus is a good target for gene delivery. Three clinical trials for LCA II have been initiated, in which one eye of each patient has been treated with rAAV2. All three trials have shown the vector to be well tolerated, with no evidence of antibody responses against the *RPE65* transgene product in any patient, and only limited transient NAb responses against the rAAV2 capsid in a minority of patients.^{14,15}

Establishing if repeat subretinal injection of *rAAV-RPE65* can achieve rescue in the second eye of *RPE65*^{-/-} dogs is required prior to including treatment of the second eye in human clinical trial protocols. Previous studies have reported bilateral subretinal injections in *RPE65*^{-/-} dogs but these have mostly been performed concurrently. Acland *et al.*⁸ reported bilateral subretinal rAAV2-RPE65 injections in nine dogs, of these only one dog (BR29 in Table 1) had the second eye injected at a later date and outcome for this eye was not shown. Bennicelli *et al.*¹¹ reported bilateral subretinal injections of *rAAV-RPE65* in two dogs, but both eyes were treated at the same time. In this study, we sought to investigate the rescue achieved in the second eye by gene replacement therapy in *RPE65*^{-/-} dogs that had previously had the same vector construct administered by subretinal injection in the contralateral eye. We report that successful rescue in the second treated eye of *RPE65*^{-/-} dogs occurs at a level comparable with that achieved in the first eye. This finding has important implications for the treatment of human LCA II patients.

RESULTS

Subretinal injection of RAAV2/hRPE65 in *RPE65*^{-/-} crossbred dogs

Both eyes of nine *RPE65*^{-/-} crossbred dogs were subretinally injected with *rAAV2.hRPE65p.hRPE65*, with the second eye treated 85–180 days after the first eye (Table 1). The age of the dogs at the time of treatment ranged from 137 to 755 days. The injections were made in the superior fundus of both eyes and the mean proportion of the subretinal bleb in relation to tapetal area was 40% (range 6–62%). The retinal detachments created by the subretinal injections typically resolved over the subsequent few days. All eyes had complete retinal reattachment on indirect ophthalmoscopic examination by 1 week after injection, with the exception of eye 6a that still had a small subretinal bleb remaining at that time point (Figure 1c). The small retinotomy created by the cannula sealed in all cases with complete reattachment of the retina at the injection site. The injection site was often visible as a small-pigmented scar (Figures 1c and g). A ‘high-water’ mark indicating the edge of the detachment could be detected on careful fundoscopic examination.

Evaluation for ocular inflammation

Eyes were examined for ocular inflammatory responses throughout the study. As anticipated following the subretinal injection, mild inflammation, as indicated by slightly lowered intraocular pressure (compared with pre-injection levels) and very mild aqueous flare (ranging from trace flare to 1 on a scale of 1–4), was appreciated on biomicroscopic examination for up to 7 days. The tract of the cannula through the vitreous could be observed. However, no vitreal changes that would indicate an inflammatory reaction were detected in any of the eyes. There was no difference in the post-surgical inflammatory response between first and second injected eyes and in all cases this was a very mild response, which in our experience is typical for that seen in dogs following this sort of surgical intervention. Ophthalmoscopically detectable fundic changes over the period of the study were minimal in 14 of 18 treated eyes. Four eyes showed changes in tapetal reflectivity. In three eyes (3b, 5a, 7a) small foci of tapetal hyper-reflectivity were scattered across the area of the created subretinal bleb. In one eye (2b), a larger area of tapetal hyper-reflectivity developed, covering the majority of the treated fundus, consistent with retinal thinning as described in a previous report.¹⁰

Evaluation of retinal function

Retinal function was assessed with full-field flash electroretinography (ERG). Rod and cone photoreceptor rescue was observed for all treated eyes with the exception of eyes 4a (very small retinal bleb with majority of vector administered choroidally because of poor injection) and 2b (as anticipated because of aforementioned changes). Representative dark-

adapted ERG tracings and light-adapted cone (33 Hz) flicker responses measured before and after treatment are shown in Figures 2 and 3. Dark-adapted a- and b-wave intensity–response curves were generated. These showed that there was an increased a- and b-wave amplitude and lower response threshold for post-injection ERGs relative to pre-treatment recordings in 16 of the 18 eyes. These improvements in ERG waveforms were comparable between first and second injected eyes (Figure 4). As a measure of predominately rod rescue, the amplitude of the dark-adapted b-wave at a flash intensity of $0.0 \log \text{cdSm}^{-2}$ was selected for further analysis. This flash intensity was below the response threshold for all untreated *RPE65*^{-/-} dogs. Cone function was assessed by the amplitude of the 33 Hz flicker response. This response was either unrecordable or of a very low-amplitude response for all eyes before injection. Using these parameters, recovery of rod and cone photoreceptor function was observed at all time points post-injection for all eyes except 2b and 4a. There was no significant difference in the degree of rod or cone rescue between first and second treated eyes at any time point (Tables 2 and 3). A power analysis was performed to indicate the magnitude of difference in the mean b-wave amplitude at $0.0 \log \text{cdSm}^{-2}$ and the mean cone flicker amplitudes that given the sample size and variability would have reached significance (at $P < 0.05$). These values were 13.3 and 2.5 μV , respectively.

The mean post-injection amplitudes of rod and cone responses did not change significantly over the period of the study (Table 3). However, there was a trend towards an increase in b-wave amplitude between the 2- and 4-month ERGs, and a decline between the 9–12- and 20–24-month ERGs. This observation was consistent between the two eyes. When the effect of age at injection, total viral dose, injection volume, vector concentration, subretinal bleb size and NAb response on rod and cone rescue was assessed, only the subretinal bleb size correlated significantly with the amplitude of rod and cone ERG responses (Table 4).

Evaluation of vision

A vision-testing apparatus that assessed the dog's ability to see an open exit tunnel was used to quantitatively measure visual function under varying light levels as previously described.³¹ Visual function was evaluated at normal room light and two lower light intensities, by recording the time to exit the device and the first exit tunnel entered. A significant improvement in visual function relative to pre-treatment values was seen at the two lower light intensities for both parameters evaluated (Figure 5). Importantly, there was no significant difference in visual function between first and second treated eyes (Table 5).

Immunology

Evaluation for serum NAb to rAAV2 capsid showed that prior to gene delivery, 1 out of 9 dogs (dog 5) had detectable circulating NAb against rAAV2. Four weeks after vector delivery, all dogs had substantially increased titers of NAb that declined by 12 months, but remained above pre-treatment levels. The NAb titer increased again in 7 out of 9 dogs after the second delivery of vector and peaked at a higher level after the second injection compared with the first injection. A large variation was observed in the NAb titers between dogs; 4 weeks after the first injection the NAb titer ranged from 1/100 to 1/32 000 and 4 weeks after the second injection titers ranged from 1/125 to 1/35 000 (Table 6). Aqueous humor collected from the second injected eye at the time of injection did not have detectable levels of NAb (data not shown). Notably, higher serum NAb titers to rAAV2 capsid were observed in dog 4 after the first injection and again after the second injection relative to the other dogs. The response after the first injection correlated with choroidal administration of vector construct in this eye (eye 4a). Enzyme-linked immunosorbent assay performed on serum to evaluate for potential humoral immune responses directed to *RPE65* showed no detectable change in antibody response specific to *RPE65* in any dog after first or second subretinal injection of rAAV2 (data not shown). An analysis was performed on the ERG

results of the second injected eyes to see if there was a correlation in ERG outcome and the NAb level at the time of the second injection. There was no correlation between the second eye b-wave at $0.0 \log \text{cdSm}^{-2}$ and the NAb at the time of injection ($P=0.718$), nor between cone flicker outcome at 4 months and NAb at the time of injection ($P=0.909$). The analysis was repeated following adjustment of the ERG amplitudes for the size of the bleb and again there was no significant correlation.

DISCUSSION

In this study, we sought to establish whether successful rescue could be achieved in the second eye of *RPE65*^{-/-} dogs following previous gene therapy treatment of the fellow eye. We found that rescue achieved in the second eye was comparable to that in the first treated eye, with no evidence of interference of RPE transduction by immune reaction to the first treatment. These findings have significant implications for treatment of human Leber congenital amaurosis type II patients.

Consistent with other studies evaluating immune responses to rAAV, we saw elevated serum NAb titers after all injections. After injection of the first eye, the maximum titer observed was 1/32 000 in the dog 4. In eye 4a, the majority of vector was injected choroidally resulting in a very small subretinal bleb (6% of tapetal fundus). The NAb titers in this animal were much higher than all other animals and as the choroid is not protected by the blood retinal barrier this finding is not surprising. The highest NAb titer after exclusively subretinal administration was 1/5000. The titers declined by 12 months post-injection but remained higher than baseline pre-injection NAb levels. The serum NAb response was higher after the second injection than after the first injection, similar to a prime-boost effect, but this did not appear to affect the rescue of the second injected eye compared with the first. NAb were not detected in the aqueous of the second eye at the time of injection, indicating that the serum NAb did not cross the blood–aqueous barrier in the untreated eye. The NAb response showed no significant correlation with the degree of mean rod or cone rescue achieved. It is of interest that eye 4a, which received vector choroidally, had a lack of detectable ERG and vision rescue. The lack of rescue in this eye may be accounted for by the very small subretinal bleb that was achieved, but we cannot rule out an effect of the presence of a high NAb titer. Although both rod and cone photoreceptor rescue was seen in the second injected eye of this dog (4b), it was observed that the amplitude and threshold of ERG responses for eye 4b were lower than anticipated by the size of the subretinal bleb achieved in this eye. It is possible that the lower than anticipated rescue seen in eye 4b is a function of the higher systemic NAb titer seen after the first injection. However, with only one dog with this complication any correlation between the high systemic antibody level at the time of injection of the second eye and outcome cannot be proven.

In addition to the importance of route of administration, trials of repeated AAV gene therapy in other organ systems have shown that the vector dose is important in determining the degree of immune response and success of transgene expression. Consistent with this, we have previously observed transgene expression in both eyes of *Rd12* mice when a lower concentration ($1 \times 10^{11} \text{ vg ml}^{-1}$) of vector construct was used, whereas a higher concentration ($5 \times 10^{11} \text{ vg ml}^{-1}$) resulted in higher NAb titers and variable transgene expression in the second eye.²⁷ In the larger canine eye, we have achieved successful photoreceptor rescue at both high and low concentrations (1×10^{11} and $1 \times 10^{12} \text{ vg ml}^{-1}$) in both first and second treated eyes without evidence of any significant variation in NAb response. Similarly, administration of a larger total dose of vector did not have a major effect on immune responses or degree of rescue in the first or second treated eyes. This variation may reflect the higher relative dose of viral particles in the smaller murine eye or species-specific immune responses.

Importantly, we observed no ocular or systemic adverse effects after successful subretinal gene delivery. The mild post-injection inflammatory response seen in all injected eyes was typical for that seen after this sort of intervention in dogs. Aqueocentesis itself in the dog induces a mild inflammatory response and has been used in canine studies to test efficacy of anti-inflammatory medications.³² The mild inflammation resolved completely, and there was no indication of any ongoing inflammatory reaction in any eye. Despite choroidal administration of vector and subsequently elevated serum NAb titers, eye 4a did not show evidence of an inflammatory response greater or more persistent than that in other eyes. In this eye, an anticipated degree of post-operative aqueous flare was noted, comparable with that observed in other treated eyes. Small foci of tapetal hyper-reflectivity were observed in 3 of 18 injected eyes, consistent with focal retinal thinning as described in a previous report.¹⁰ The presence of the tapetum in the dog allows for detection of retinal thinning by ophthalmoscopic examination more readily than in species with no tapetum. Previous studies have documented the histologic appearance of lesions resulting from subretinal injections in dogs and include damage at the injection site, retinal thinning and focal defects in reattachment resulting in retinal 'ripples'.^{10,11,33} The small areas of tapetal hyper-reflectivity that we detected in 3 of the 18 eyes did not preclude successful recovery of rod and cone function in these eyes. Reasons for the more diffuse tapetal hyper-reflectivity and presumed retinal thinning in the injected area of eye 2b are not clear but may reflect the potential detrimental effects of the creation of a retinal detachment. Certainly, this finding correlated with failed recovery of rod and cone ERG responses in this eye.

Improved rod and cone function was seen in 16 of 18 injected eyes, this success rate is comparable with that reported in previous studies evaluating unilateral injections.⁶⁻¹³ However, the amplitudes of rescued cone responses were somewhat variable between eyes. Consistent with this finding Acland *et al.*⁸ describe rod rescue in 23 of 26 eyes, but saw cone rescue in only 8 of these 23 eyes using more stringent criteria for rescue. In considering these outcomes, previous biochemical and immunochemistry data show that only the area of the subretinal bleb regains functional *RPE65* expression and subsequent 11-*cis*-retinal production.⁸ Similarly, PCR results have shown that viral DNA only persists in the neural retina, RPE and choroid of the injected area.⁶ Concordant with these reports, we saw a significant effect of subretinal bleb size on the amplitude and threshold of dark-adapted rod ERG responses, and on the amplitude of cone (33 Hz) flicker responses. In fact, subretinal bleb size explained the majority of variability observed in ERG responses between treated eyes in this study. Although we sought to create a consistently sized subretinal bleb located over the area centralis, the formed blebs varied somewhat in size and location, consistent with a previous description.¹⁰ The area centralis has the highest cone density and corresponds to the human macula, although it has a lower cone density and does not have a rod-free region.³⁴ The location and size of the subretinal bleb likely has a significant role in determining measurable cone photoreceptor rescue.

Improved rod and cone ERG responses were observed for the duration of the study. However, although the mean rod responses at 4 months and those at 20–24 months were not statistically different, there was a statistically significant decline in cone flicker responses between the two time points. These findings were consistent between the first and second injected eyes. These results are similar to those reported by Narfstrom *et al.*,¹² who describe a peak rod ERG response at 3–6 months post-injection and a peak cone ERG response at 18 months post-injection, both rod and cone responses declining thereafter. In contrast, Acland *et al.*⁸ followed 2 eyes out to 3 years and did not report a significant decline in rod or cone function. Bennicelli *et al.*¹¹ report a decline in response from initial ERG at 5 weeks to the second ERG at 3 months, but also described one treated eye followed out to 3 years whose rod ERG varied by <10%. The cause for this apparent variability in the maintenance of ERG

responses in the longer term is not clear and its potential significance to human clinical trials of gene replacement therapy for LCA type II remains to be determined.

We have demonstrated functional rescue of the ERG and improved visual outcomes in the *RPE65*^{-/-} dog injected subretinally with rAAV2.*hRPE65p.hRPE65* into both eyes, 85–180 days apart. This demonstrates that vector of the same serotype and expressing the same transgene can be effectively readministered to treat the contralateral eye of previously treated *RPE65*^{-/-} dogs without evidence of an untoward effect of the therapy. These results support the inclusion of treatment of the second eye in current clinical trials of gene therapy in LCA type II patients.

MATERIALS AND METHODS

Subjects

Nine *RPE65*^{-/-} dogs were used (Table 1), from a colony maintained at Michigan State University Comparative Ophthalmology Laboratory. All animals were housed under 12:12 h light–dark cycles and cared for in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Procedures performed were approved by Michigan State University’s Institutional Animal Care and Use Committee.

rAAV2/2 construct and subretinal injection

A recombinant rAAV2/2 vector was used, containing the human *RPE65* cDNA coding sequence driven by the human *RPE65* promoter (rAAV.hR-PE65p.hRPE65), flanked by AAV2 ITRs encapsidated in an AAV2 shell. Viral vector was produced by Targeted Genetics (Seattle, WA, USA), with the use of a B50 packaging cell line.³⁵ The rAAV titers were determined by dot blot, generating vector concentrations of 1×10^{11} and 1×10^{12} vg ml⁻¹. Subretinal injections were performed as previously described.³⁶ An aqueous paracentesis of 100 μ l was performed immediately before the injection, and the sample was stored at -80°C for the immunological studies. Two different volumes of injection were administered 250 and 500 μ l (Table 1). Dogs were treated with oral prednisone 0.5 mg kg⁻¹ daily 1 week before vector administration. Immediately after subretinal injection, all dogs received a subconjunctival injection of 2 mg dexamethasone solution and 8 mg gentamicin. Oral prednisone was administered post-operatively at 1 mg kg⁻¹ daily for week 1, 0.5 mg kg⁻¹ for week 2, 0.25 mg kg⁻¹ for week 3 and 0.125 mg kg⁻¹ for week 4.

Ophthalmic evaluation and fundic imaging

To monitor for any resultant ocular inflammation, complete ophthalmic examination was performed, including slit lamp biomicroscopy (model SL14; Kowa, Tokyo, Japan), indirect ophthalmoscopy (Welch Allyn, Skaneateles Falls, NY, USA) and fundus photography (RetCam II, Clarity Medical Systems, Pleasanton, CA, USA). Examination was performed pre-injection and then after injection every other day for the first week, twice weekly for the first 2 months, then monthly. Wide-angle digital fundus images captured immediately post-subretinal injection were used to calculate the proportion of the tapetal fundus injected, with measurements performed using Photoshop CS4 (Adobe, San Jose, CA, USA). The extent of the tapetum was similar between all dogs; therefore this method of measuring the subretinal bleb was comparable between eyes.

Electroretinography

ERG recordings were performed pre-injection, and 2, 4 and then between 9–12 months after injection for all treated eyes. A final ERG was recorded at 20–24 months post-injection for 15 of the 18 eyes. ERGs were recorded under inhalant isoflurane anesthesia as previously

described, except ERG-Jet corneal contact lens electrodes were used.³⁷ Briefly, globes were positioned in primary gaze using stay sutures of 4–0 silk (Ethicon, Inc., Piscataway, NJ, USA), and the pupils were dilated with 1% tropicamide (Mydracil, Alcon Laboratories, Honolulu, HI, USA) and 10% phenylephrine hydrochloride (AK-Dilate, Akorn Inc., Buffalo Grove, IL, USA). Full-field flash ERGs were recorded using ERG-Jet lenses (Microponent, Le Cret-du-Lochie, Switzerland) and the UTAS-E 3000 electrophysiology unit with a Ganzfeld (LKC Technologies Inc.; Gaithersburg, MD, USA). Band pass filter cutoff was set at 0.5–500 Hz. Dark-adapted ERG responses were recorded following 1 h of dark adaptation, from a series of 16 white flash stimuli (−3.6 −3.18, −2.79, −2.41, −2.0, −1.6, −1.19, −0.79, −0.39, 0.00, 0.39, 0.85, 1.36, 1.9, 2.38 and 2.82 log cdSm^{−2}). Interstimulus and intervals were increased from 1 s at low intensities to 360 s at the highest intensity to avoid light adapting the rods.³⁷ Following exposure to a background light of 30 cd m^{−2} for 10 min, cone-mediated flicker responses were recorded at 33 Hz (0.39 log cdSm^{−2}).

For the assessment of rod responses, the dark-adapted b-wave amplitude at 0.0 log cdSm^{−2} was used. This flash intensity was below the response threshold for all pre-treatment *RPE65*^{−/−} eyes. Dark-adapted ERGs were assessed for threshold of response and shape of waveform. Dark-adapted b-wave intensity:response curves were plotted. The a- and b-wave amplitudes were measured for each averaged response. The a-wave amplitude was measured from the onset of light stimulus to the trough of the first-negative wave; b-wave amplitude from the trough of the first-negative wave to the peak of the first-positive wave. To evaluate cone responses, the amplitudes of light-adapted 33 Hz flicker responses were analyzed. Flicker responses were chosen rather than single flash light-adapted responses because of the concern that rods not supplied with 11-*cis* retinal have markedly reduced sensitivities and may still retain some recordable function even in the presence of a background light that saturates normal canine rods. The origin of the sometimes relatively large amplitude response to bright flash stimuli in untreated *RPE65*^{−/−} dogs is not established. For the 33 Hz flicker responses, amplitude (trough to peak) was measured.

Vision testing

Vision testing was performed using a vision-testing device as previously described.³¹ Evaluation was performed twice, before injection and 70–255 days post-injection for all dogs. Each injected eye was individually assessed by placement of an eye mask over the contralateral eye. Vision was tested by seven repeated trials at three different light intensities (0.02–0.04, 1.0–1.5 and 35–45 cd m^{−2}). Average time to exit the device and the number of first correct choices of exit tunnel were recorded.

Detection of neutralizing antibodies to rAAV

Serum was collected pre-injection, every 2 weeks until 28 weeks post-injection, then at 9 and 12 months after injection of each eye. Aqueous humor was collected from all eyes immediately after the first injection. Aqueous and serum were stored at −80°C. To determine NAb titers, serial dilutions of serum were prepared in triplicate and 1 × 10⁸ vg rAAV.CMV.GFP was added to each sample. Plates were incubated at 37°C for 1 h, then the contents of the wells were added to 96-well plates of 293T cells containing 2.5 × 10⁴ cells per well. Plates were incubated for 48 h, and then the number of GFP-positive cells per well was counted using an inverted fluorescence microscope. The titer of NAb was defined as the highest dilution that produced 50% fluorescence compared with the rAAV2-positive media only control.

Detection of total IgG and IgM to recombinant RPE65

Aqueous and serum was collected and stored as for detection of NAb to rAAV. In all, 96-well Maxisorp microtitre plates (NUNC, Roskilde, Denmark) were coated recombinant

human *RPE65* (gift from Professor Martin Warren, University of Kent, UK) (1 in 5000 dilution in 100 μ l phosphate-buffered saline (PBS)) overnight at room temperature, then washed with PBS+0.05% Tween-20 (PBS-T). Plates were blocked with 1% BSA in PBS for 1 h at room temperature, then washed and samples (1:100 or 1:200 dilution) were applied. Plates were incubated at room temperature for 1.5 h and washed 3 \times with PBS-T. Bound Canine IgG or IgM was detected with sheep-anti-canine IgG-HRP or goat-anti-canine IgM-HRP (AdB Serotec, Kidlington, UK) for 1.5 h at room temperature, then washed 3 \times with PBS-T and color was developed with TMB substrate (Pharmingen, Oxford, UK) and quenched with 1 N HCL. Absorbance at 450 nm was quantified using a plate reader (E Max, Molecular Devices, Wokingham, UK).

Statistical analysis

The ERG results and vision-testing outcomes data were analyzed using PASW Statistics 17.0 (SPSS Inc., Chicago, IL, USA). Independent samples *t*-tests were used to test for differences between average pre- and post-injection outcomes as well as to assess for any difference between first and second eye injected at all time points. Analysis was performed on 33 Hz flicker responses and b-wave amplitudes at 0 log cdSm⁻². Power analysis calculations were performed using the program G*Power, version 3.1.2 (Dusseldorf, Germany). For correlation between age, bleb size, viral dose and ERG outcomes, two-tailed Pearson analysis was performed to assess the relationship between the variables. For vision-testing outcomes, mean time to exit, and the mean number of correct exits, independent samples *t*-tests were again used to test for differences between pre- and post-injection outcomes as well as to assess for any difference between first and second eye injected. Independent samples *t*-tests were chosen above more complex tests because of the relatively low sample size of this study. Data were considered significant at *P*<0.05.

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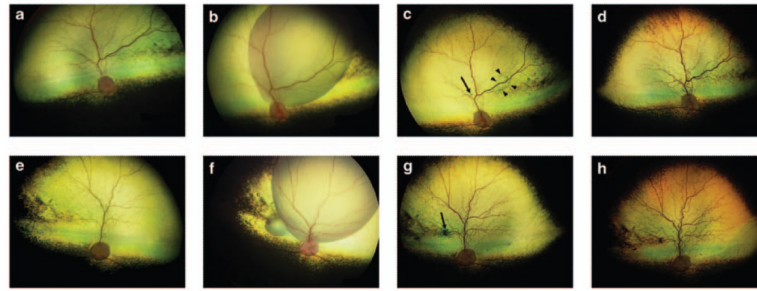


Figure 1.

Wide-angle fundus images (RetCam II, Clarity Medical Systems) of first and second subretinally injected eyes of a *RPE65*^{-/-} dog (dog 6). The right eye (OD) was injected first and the left eye (OS) was injected 90 days later. (a) OD pre-injection, (b) OD immediately after subretinal injection, (c) OD 1-week post-injection. Note that in this eye, a small region of subretinal fluid remains (indicated by arrowheads in c), (d) OD 1-year post-injection, (e) OS pre-injection, (f) OS immediately after subretinal injection, (g) OS 1-week post-injection, (h) OS 1-year post-injection. The scars resulting at the injection site are indicated with arrows in (c, g).

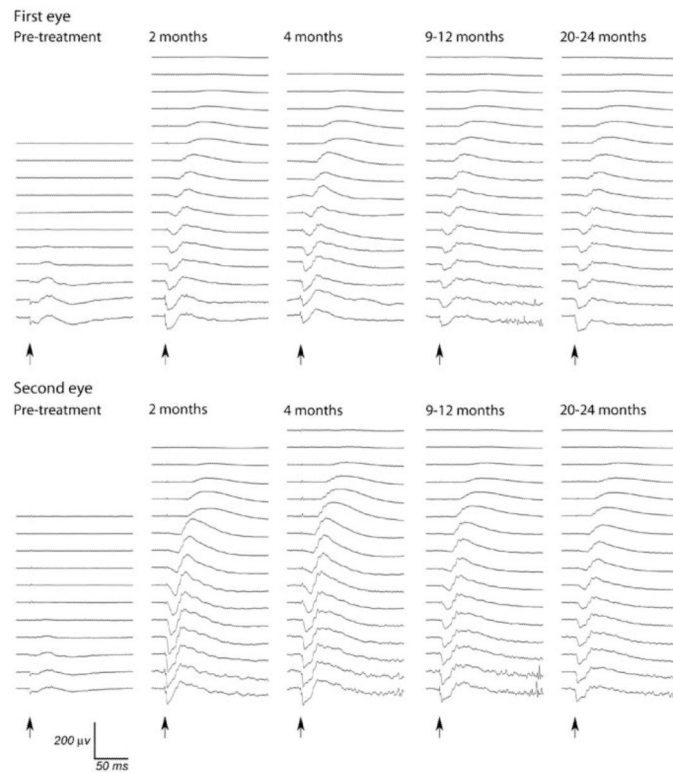


Figure 2. Dark-adapted ERGs from first and second subretinally injected eyes of dog 6. The second injected eye was treated 90 days after the first eye. Flash intensities from bottom to top were 2.82, 2.38, 1.9, 1.36, 0.85, 0.39, 0.0, -0.39 , -0.79 , -1.19 , -1.6 , -2.0 , -2.41 , -2.79 , -3.18 , -3.6 $\log \text{cdSm}^{-2}$. Arrows indicate timing of flashes.

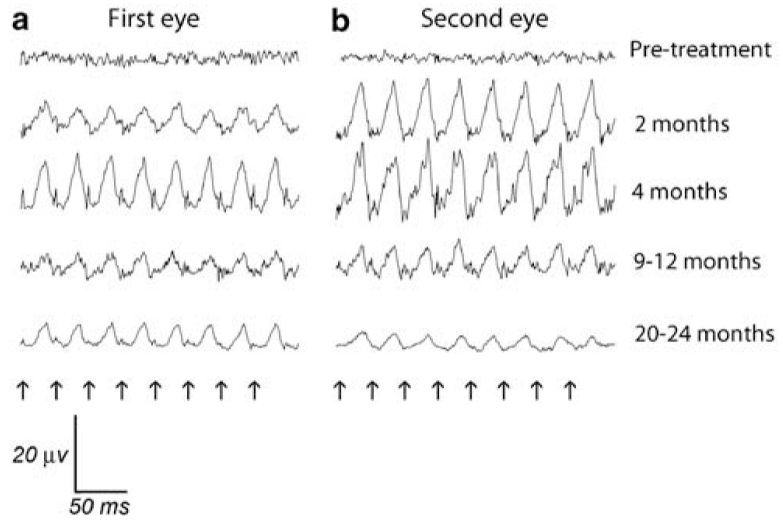


Figure 3. Cone flicker responses from first and second injected eyes of dog 6. Flicker frequency was 33 Hz, at a light intensity of $0.39 \log \text{cd m}^{-2}$. (a) First injected eye, (b) Second injected eye, treated 90 days after the first eye. Note that there was a marked reduction in flicker amplitudes from the peak response by the 20–24 months time point. Arrows indicate timing of flashes.

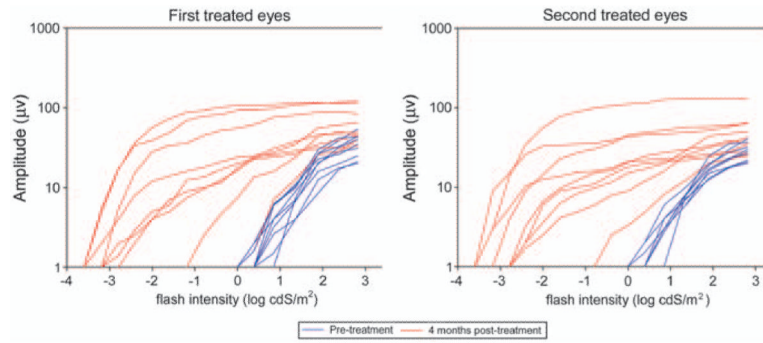


Figure 4. Montage of dark-adapted b-wave log intensity response curves for all treated eyes. Pre-injection intensity: response curves are shown in blue, 4 months post-injection are shown in red.

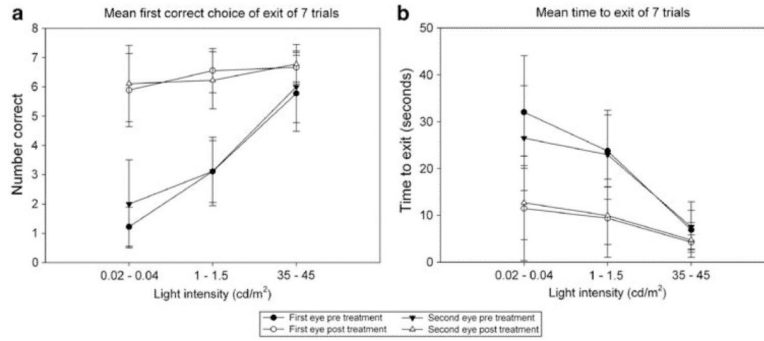


Figure 5. Graphical representation of mean vision-testing outcomes for both outcomes measured with the vision-testing device. Pre-injection values are compared with post-injection for first and second subretinally injected eyes. Testing was performed at three light intensities: 0.02–0.04, 1.0–1.5 and 35–45 cd m^{-2} for all trials. (a) Mean time to exiting the vision-testing apparatus is shown, averaged from seven trials per eye (b) mean outcome for the first correct choice of four exit tunnels is displayed as number correct of seven trials. Error bars=s.d.

Table 1
Overview of all dogs treated and details of subretinal injections

Dog	Sex	Eye injected	Age at injection (days)	Titer (vgml ⁻¹)	Volume (μl)	Total dose (vg)	Proportion of tapetal fundus injected	Post-injection ocular changes
1	F	First (a)	179	10 ¹¹	250	2.5×10 ¹⁰	0.11	None
		Second (b)	271	10 ¹¹	500	5×10 ¹⁰	0.43	None
2	F	First (a)	172	10 ¹¹	250	2.5×10 ¹⁰	0.44	None
		Second (b)	263	10 ¹¹	500	5×10 ¹⁰	0.37	Tapetal hyper-reflectivity
3	M	First (a)	179	10 ¹¹	250	2.5×10 ¹⁰	0.45	None
		Second (b)	271	10 ¹¹	500	5×10 ¹⁰	0.48	Mild tapetal hyper-reflectivity
4	F	First (a)	172	10 ¹¹	250	2.5×10 ¹⁰	0.06	Majority choroidal injection
		Second (b)	263	10 ¹¹	500	5×10 ¹⁰	0.43	None
5	M	First (a)	137	10 ¹¹	250	2.5×10 ¹⁰	0.53	Mild tapetal hyper-reflectivity
		Second (b)	221	10 ¹¹	500	5×10 ¹⁰	0.49	None
6	M	First (a)	137	10 ¹¹	250	2.5×10 ¹⁰	0.55	None
		Second (b)	221	10 ¹¹	500	5×10 ¹⁰	0.62	None
7	M	First (a)	574	10 ¹²	500	5×10 ¹¹	0.52	Mild tapetal hyper-reflectivity
		Second (b)	755	10 ¹²	250	2.5×10 ¹¹	0.33	None
8	M	First (a)	176	10 ¹²	500	5×10 ¹¹	0.17	None
		Second (b)	359	10 ¹²	250	2.5×10 ¹¹	0.24	Cataract
9	M	First (a)	176	10 ¹²	500	5×10 ¹¹	0.46	None
		Second (b)	357	10 ¹²	250	2.5×10 ¹¹	0.44	None

Table 2
ERG results for individual dogs comparing pre-treatment and 4 months post-treatment for first and second injected eyes

Dog	Eye	Rod a-wave pre-injection (μV)	Rod a-wave post-injection 4 months (μV)	Rod b-wave pre-injection (μV)	Rod b-wave post-injection 4 months (μV)	Cone flicker pre-injection (μV)	Cone flicker post-injection 4 months (μV)
1	First (a)	0	2	0	7	0	4
	Second (b)	0	4	0	21	0	4
2	First (a)	0	28	0	94	0	9
	Second (b)	0	1	0	3	0	0
3	First (a)	0	4	0	20	0	5
	Second (b)	0	3	0	15	0	2
4	First (a)	0	0	0	0	0	0
	Second (b)	0	4	0	9	0	3
5	First (a)	0	5	0	16	2	8
	Second (b)	0	19	0	45	0	7
6	First (a)	0	22	0	56	0	4
	Second (b)	0	44	0	111	0	14
7	First (a)	0	10	0	24	0	3
	Second (b)	0	11	0	21	0	3
8	First (a)	0	8	0	18	0	4
	Second (b)	0	5	0	18	0	2
9	First (a)	0	50	0	107	0	15
	Second (b)	0	24	0	43	0	5
Normals ^a			46 (± 18)		124 (± 38)		21 (± 9)

Abbreviation: ERG, electroretinography.

^aERG results from 18 *RPE65*^{+/+} eyes of dogs of similar breeding (mean \pm s.d.). Rod responses recorded at 0.0 log cdSm⁻², cone responses recorded from 33Hz flicker.

Table 3

Mean (a) dark-adapted b-wave amplitude (μV) at 0 log cdSm⁻² and (b) light-adapted 33Hz flicker results for all dogs pre- and post-injection, with *P*-values comparing amplitude of response for first and second treated eyes

Eye	Pre-injection	2 months	4 months	9-12 months	20-24 months	<i>P</i> -value (pre vs 4months)	<i>P</i> -value (2months vs 4months)	<i>P</i> -value (9-12months vs 20-24months)	<i>P</i> -value (4months vs 20-24months)
<i>(a)</i>									
First (a)	0	25	38	36	27	<0.001*	0.122	0.633	0.235
Second (b)	0	30	32	30	25	<0.001*	0.573	0.364	0.348
<i>P</i> -value (1st vs 2nd)		0.748	0.722	0.814	0.887				
<i>(b)</i>									
First (a)	0	4.6	5.8	5.9	3.0	<0.001*	0.214	0.519	0.10
Second (b)	0	4.5	4.4	4.0	1.9	<0.001*	0.322	0.157	0.041
<i>P</i> -value (1st vs 2nd)		0.954	0.474	0.303	0.350				

* Significant difference.

Table 4
Impact of variables affecting mean rod and cone rescue at 4 months (*P*-values displayed)

<i>Variable (P-value)</i>	<i>Rod b (0 log cdSm⁻²)</i>	<i>Cone (33Hz) flicker</i>
Age at injection	0.438	0.229
Total dose of vector for both first and second injected eyes	0.564	0.601
Total dose of vector—first injected eyes	0.42	0.56
Total dose of vector—second injected eyes	0.69	0.64
Volume of injection	0.653	0.843
Concentration (vpml ⁻¹)	0.511	0.565
Subretinal bleb size	0.024*	0.0034*
Peak NAb response	0.161	0.123
Change in NAb levels (before and after injection)	0.146	0.102
NAb level at time of second injection with ERG outcome in second eye	0.718	0.909

Abbreviations: ERG, electroretinography; NAb, neutralizing antibody.

* Significant correlation.

Table 5

***P*-values for the vision testing assessments**

<i>P</i>-values	<i>Time to exit (7 trials)</i>		<i>First correct choice of exit (7 trials)</i>			
Light intensity (cdm ⁻²)	35–45	1.0–1.5	0.02–0.04	35–45	1.0–1.5	0.02–0.04
<i>P</i> —pre vs post (1st eye)	0.063	<0.001*	0.002*	0.052	<0.001*	<0.001*
<i>P</i> —pre vs post (2nd eye)	0.025*	0.001*	<0.001*	0.043*	<0.001*	<0.001*
<i>P</i> —1st vs 2nd eye (pre)	0.797	0.848	0.327	0.714	1.0	0.174
<i>P</i> —1st vs 2nd eye (post)	0.739	0.982	0.877	0.694	0.525	0.716

* Significant difference.

Table 6
rAAV2 serum NAb titers pre- and post-injection for first and second injected eyes

<i>Dog</i>	<i>Eye injected</i>	<i>Titer pre-injection</i>	<i>Titer 4 weeks post-injection</i>
1	First (a)	1 in 2	1 in 300
	Second (b)	1 in 150	1 in 125
2	First (a)	1 in 2	1 in 100
	Second (b)	1 in 150	1 in 8500
3	First (a)	1 in 2	1 in 2000
	Second (b)	1 in 800	1 in 800
4	First (a)	1 in 2	1 in 32 000
	Second (b)	1 in 8500	1 in 35 000
5	First (a)	1 in 150	1 in 5000
	Second (b)	1 in 2000	1 in 8000
6	First (a)	1 in 3	1 in 3000
	Second (b)	1 in 1000	1 in 5000
7	First (a)	1 in 2	1 in 200
	Second (b)	1 in 25	1 in 25 000
8	First (a)	1 in 2	1 in 3500
	Second (b)	1 in 200	1 in 3500
9	First (a)	1 in 2	1 in 750
	Second (b)	1 in 1000	1 in 7500

Abbreviation: NAb, neutralizing antibody.