Journal of Heredity 2003:94(1):31–37
DOI: 10.1093/jhered/esg015

In Vivo Gene Therapy in Young and Adult *RPE65*−/− Dogs Produces Long-Term Visual Improvement

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Abstract

Defects in the *RPE65* gene, which is selectively expressed in the retinal pigment epithelium (RPE), result in blindness and gradual photoreceptor cell degeneration. Experiments were conducted to assess the efficacy of gene replacement therapy in restoring retinal function in *RPE65*−/− dogs. Long-term effects of *RPE65* gene therapy were assessed using visual behavioral testing and electroretinographic (ERG) recordings at 10–12 weeks and 6–9 months after surgery in five affected dogs. Subretinal injections of similar dosages of two constructs were performed in affected dogs at the ages of 4–30 months: rAAV.RPE65 into one eye and, in four of five dogs, rAAV.GFP contralaterally. Before surgery all *RPE65*−/− dogs were behaviorally blind with either no or very low-amplitude ERG responses to light stimuli. Marked improvements in visual behavior and ERG responses were observed as early as 4 weeks after surgery in affected animals. Except for light-adapted 50 Hz ERG flicker responses, all ERG parameters tested increased significantly in the eyes treated with the rAAV.RPE65 construct at the early follow-up. Gradual progressive improvements in ERG responses were observed in the RPE65-treated eyes over time. An unexpected finding was that on long-term follow-up, marked improvement of photopic ERG responses were also observed in the contralateral control eye in both young and older dogs. These results are promising for future clinical trials of human patients with retinal degenerative diseases, such as Leber congenital amaurosis, that result from *RPE65* gene defects.

A line of briard dogs has been identified that is affected by an autosomal recessively inherited retinal disease resulting in severe, early onset visual impairment (Narfström et al. 1994). Clinical studies of affected dogs have shown normal external structures of the eye, except for, in most cases, a rapid quivering movement of the eyes (nystagmus) and a wider resting pupillary diameter than in normal dogs. The internal structures of the eye are normal appearing as seen by ophthalmoscopy up to the age of 3–4 years, then vascular attenuation and slight color changes of the fundus are often seen. Electroretinography (ERG) in affected dogs is diagnostic at the age of 5 weeks; dark-adapted responses are below the limit of detection and light-adapted and flicker responses are either barely or nonrecordable. Ultrastructural studies of the disease have shown changes in the outer retina; large lipoid-like inclusions are seen in the retinal pigment epithelium (RPE) and there is disorganization and later degeneration of photoreceptor outer and inner segments. The disease is slowly progressive as the buildup of inclusions in the RPE increases and the photoreceptors continue to degenerate. Through molecular genetic studies, using a strain of Swedish briard-beagle dogs, a 4-bp deletion in the *RPE65* gene was found, causing a frameshift and a null mutation in homozygous dogs (Veske et al. 1999).

RPE65 is a protein that is preferentially and abundantly expressed in the RPE (Hamel et al. 1993a,b) and was recently

Table 1. Dogs included in the study, age at surgery, treatment chosen, and time from surgery at follow-up

Dog	Age at surgery	Genotype	AAV.RPE65	AAV.GFP	BSS	Early follow-up ERG	Late follow-up ERG
Milly	4 months	−/−	OD			12 weeks	9 months
Rex	4 months	−/−	OD	OS		12 weeks	6 months
Candy	4 months	−/−	OD	OS		11 weeks	6 months
Perdita	1 year	−/−	OD	OS		12 weeks	9 months
Bonus	2.5 years	−/−	OD	OS		11 weeks	6 months
Jonna	2 years	+/−		OD	OS	10 weeks	11 months
Alonzo	2 years	+/+		OD/iv OS		11 weeks	nd

BSS, balanced salt solution; iv, intravitreal; nd, not done; OD, right eye; OS, left eye.

also found to be expressed in cone photoreceptors (Znoiko et al. 2002). Mutations in the gene that encodes this protein result in severe forms of early onset retinal dystrophy in humans, including some forms of Leber congenital amaurosis (Thompson et al. 2000). Although the precise function of this protein still remains to be determined, it is clear that the protein is required for formation of 11-*cis* retinal from all-*trans* vitamin A. In eyes lacking functional RPE65 protein, such as in the *Rpe65*−/− mouse, almost no photoreceptor visual pigment is synthesized, and all-*trans* vitamin A esters accumulate in the RPE (Redmond et al. 1998).

Gene therapy is a promising tool for treatment of inherited genetic disorders in which the specific gene defect is known, particularly if the gene defect affects only a single tissue or organ. Many clinical gene transfer therapy trials have been performed using an ex vivo approach, that is, cells are extracted from the patients, treated with insertion of the desired gene, and then the gene-corrected cells are returned to the patient. For ophthalmic hereditary disease, in vivo gene therapy appears promising since the eye is an easily accessible organ and there is a specific immune privilege for its internal structures, such as the retina. The most severe forms of human hereditary retinal degeneration are primarily disorders of photoreceptors, although the RPE may be the initial focus in some diseases. Between the photoreceptor outer segments and the RPE is the subretinal space, which displays two important immune privilege features. It accommodates and protects tissue grafts from immune rejection and it promotes the acquisition of systemic immune deviation to antigens placed within it (Streilein et al. 2002).

Since the effects of the *RPE65* gene defect have been shown to be restricted to the retina, treatment with localized gene therapy appeared promising. Further, it was recently reported that gene therapy in three 4-month-old dogs with the same mutation resulted in visual improvement (Acland et al. 2001). Thus experiments were carried out to assess the efficacy of adeno-associated virus (AAV)-mediated gene therapy in reversing the effects of the *RPE65* defect in a larger group of *RPE65*−/− dogs (Narfström et al. in press). The goal of the present study was to investigate the long-term effects of *RPE65* gene therapy in affected dogs of various ages using visual behavioral testing and ERG follow-up recordings in animals treated with a similar dosage of the gene transfer construct.

Materials and Methods

Normal RPE65 dog cDNA was cloned into a pCl vector (Promega, Madison, WI) carrying the human cytomegalovirus (CMV) promoter and the late SV40 polyadenylation signal (poly A). Following transfection into Cos-7 cells (ATCC, Rockville, MD), the expression of RPE65 protein was analyzed by Western blotting. Subsequently the cassette was cloned into pSSVG (Samulski et al. 1989). A control vector with green fluorescent protein (GFP) cDNA in place of the RPE cDNA was also constructed. Human embryonic kidney (HEK 293) cells (ATCC, Rockville, MD) were transfected with pAAV.CMV.GFP or pAAV.CMV.RPE65 and assessed for the expression of the transgenes by fluorescence microscopy and Western blot analysis, respectively. The excision and replication of the rAAV.CMV.RPE65 was assessed by Hirt analysis (Skulimowski and Samulski 1995). The verified DNA from the same batches was used for the production of large-scale recombinant viruses at the Vector Core Facility at the University of North Carolina Gene Therapy Center (Chapel Hill, NC) (Zolotukhin et al. 1999). The titers of the rAAV.CMV.GFP and rAAV.CMV.RPE65 were 2×10^{10} transducing units/ml and 2×10^{12} particles/ml, respectively.

Five homozygous affected dogs, ages 4 months to 2.5 years, were included in the present study, as were two controls: one heterozygous and one genetically normal dog (Table 1). The seven dogs all underwent intraocular surgery and were followed for 6–11 months after treatment. All affected dogs received doses of between 70 and 100 µl of rAAV.RPE65 into the right eye and, in four of the affected dogs, the same volume of rAAV.GFP contralaterally. In one of the affected dogs the contralateral eye was not treated due to pupillary constriction at the time of surgery. The two normal control dogs were treated bilaterally according to Table 1.

Surgical procedures were performed using aseptic techniques under an operating microscope by trained vitreoretinal surgeons. A transvitreal approach to the subretinal space was applied. A two-port entry through the sclera was used in order to enhance visualization during the intraocular procedure: the light guide was guided through one port and a custom-made glass micropipette, containing the construct, was guided into the other. The retina was perforated with the tip of the micropipette and the construct

injected into the subretinal space, resulting in the formation of a bleb encompassing 25%–30% of the central inferior fundus.

Prior to surgery, all dogs underwent general and ophthalmic clinical examinations. To establish a baseline for comparison, visual capacity was evaluated at 6–7 weeks of age in a dimly lit room and then in ordinary room light. Visual maze testing was performed 6–11 months after surgery on the two control animals and the five affected treated animals. A series of obstacles of various sizes and shapes was placed within the path of a maze. Animals were walked with a handler through this maze in dim light and then in bright light conditions with the arrangement of obstacles changing between tests. For subjective vision assessment, the behavior of the animal was observed while negotiating a maze in dim and bright light conditions and scored (0–2) by degree of hesitation or ease, such that hesitation or collision was graded as a zero, some hesitation or delayed yielding to objects was graded as a 1, and no hesitation with object avoidance was graded as a 2. Objective visual testing was measured as the number of times an animal bumped into an object while negotiating the maze in bright and dim light conditions. The investigator counting the number of collisions was blinded to the treatment of the dogs. Ophthalmic examinations were performed using indirect ophthalmoscopy and slit lamp biomicroscopy.

Objective retinal function was tested with ERG prior to surgery using bilateral, simultaneous full-field stimulation. The dogs were dark adapted for 2 h prior to the ERG recordings, which were performed under general anesthesia using a commercial computerized Ganzfeld ERG system (ERG System Tor, Scanditronix, Uppsala, Sweden). Scotopic ERGs were recorded at low-intensity stimuli (−2.0 log cd sec/m^2) and at high-intensity stimuli (0.6 log cd sec/m^2). After 10 min of light adaptation, using white background light at 37 cd/m^2, photopic recordings were obtained with white light stimuli at 0.0 log cd sec/m^2. Single flash responses, as well as flicker responses at 30 and 50 Hz were recorded. When impossible to differentiate electrical responses from background noise, amplitude values were set at 2 μV. The mean ERG amplitude values were obtained and paired *t*-tests used to calculate the significance in amplitude changes and in amplitude differences between eyes.

Results

On the basis of behavioral evaluations, all of the affected dogs were deemed clinically blind prior to gene therapy treatment. Four weeks after surgery there was a marked change in the visual behavior of the previously blind dogs. They appeared more alert and did not rely as much on auditory and olfactory stimuli as had previously been observed. Ophthalmic examination revealed that the rapid quivering movement of the eyes disappeared bilaterally in all rAAV.RPE65-treated affected dogs approximately 10 weeks after the gene transfer surgery.

Subjectively, no significant difference in visual testing could be identified between control and rAAV.RPE65-treated animals in dim and bright light conditions. The subjective behavioral testing also showed that bright light maze negotiation was significantly better in the RPE65-treated group when compared to dim light maze testing. Objective visual testing clearly demonstrated that the entire group of affected *RPE65−/−* dogs treated by the presently described dosage of the transgene had improved vision over presurgical observations. Bright light vision was better than dim light vision in these animals, as evidenced by a significantly ($P = .003$) greater number of collisions occurring under dim light conditions while negotiating the maze. Vision in the control dogs was not significantly different between dim and bright light conditions. Furthermore, while a significantly ($P = .018$) higher number of collisions were noted between the treated and control animals in dim light conditions, no difference was noted between the treated and control animals in bright light conditions.

Preoperative ERG amplitudes were very low to non-recordable in all affected animals. Figures 1 and 2 show scotopic and photopic amplitude values for all ERG parameters measured for each dog. Table 2 shows the mean ERG amplitudes of the affected dogs prior to treatment and at 10–12 weeks and 6–9 months follow-up. Normal values for ERGs in our laboratory in this strain of dog are given for comparison.

All ERG parameters increased significantly in the eyes treated with the rAAV.RPE65 construct in the early follow-up except for 50 Hz flicker responses (scotopic b-wave, low intensity, $P = .016$; scotopic a-wave, high intensity, $P = .005$; scotopic b-wave, high intensity, $P = .013$; photopic a-wave, $P = .006$; photopic b-wave, $P = .039$; 30 Hz flicker, $P = .032$; 50 Hz flicker, $P = .457$). When statistical analysis was performed on the differences in amplitudes between the eyes, differences in 50 Hz amplitudes were also statistically significant ($P = .024$). Most of the amplitude values in the fellow control eye, treated with rAAV.GFP ($n = 4$) or not treated ($n = 1$), were similar to baseline parameters at the early follow-up except that the photopic a-wave amplitude was increased ($P = .006$). At follow-up at 6–9 months, photopic b-wave amplitudes and 30 Hz flicker amplitudes had also increased significantly in the eyes treated with the control vector or left untreated ($P = .018$ and $P = .038$, respectively).

The photopic b-wave response in the rAAV.RPE65-treated eyes showed a remarkable recovery at 10–12 weeks (47% of normal b-wave amplitude). At the follow-up at 6–9 months the photopic b-wave amplitudes were reduced in the treated eye but still increased above the baseline level (Table 2, Figure 3). On the other hand, the photopic b-wave amplitude in the contralateral control eyes had increased considerably at 6–9 months after treatment (39% of normal b-wave amplitude), resulting in a statistically significant negative difference between the rAAV.RPE65-treated and the contralateral control eye ($P = .046$).

When ERG parameters at the 6- to 9-month follow-up were compared to baseline measurements there was a

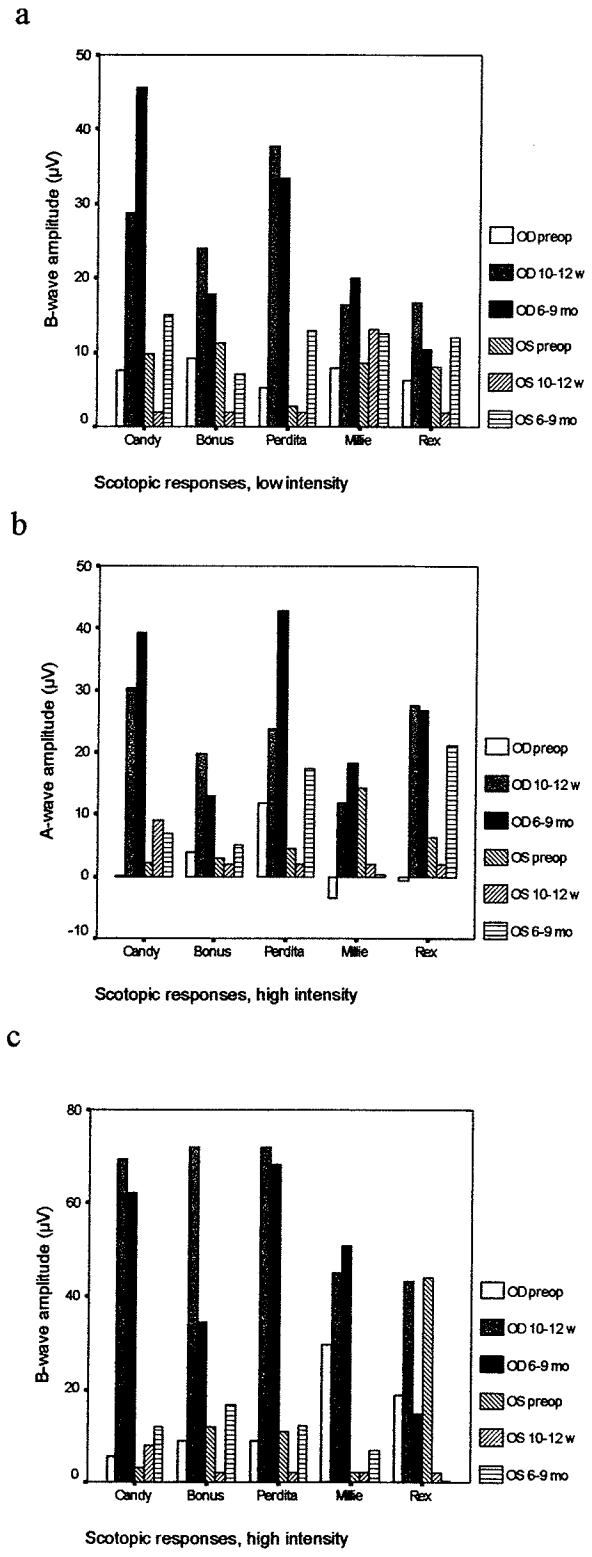

Figure 1. (a–c) Mean of the dark-adapted (scotopic) ERG a- and b-wave amplitude responses for five affected dogs at the preoperative, 10- to 12-week, and 6- to 9-month follow-up recordings for the right rAAV.RPE65-treated eyes (OD) and left (OS) control eyes, respectively.

significant increase in the scotopic low-intensity b-wave, scotopic high-intensity a-wave, and 30 Hz flicker responses in the rAAV.RPE65-treated eyes ($P = .047$, $P = .007$, $P = .004$, respectively). In the control eyes of the affected dogs, no changes in the scotopic ERG amplitudes were observed between the baseline and the 6- to 11-month posttreatment studies.

No complications directly related to the surgical procedures were incurred by any of the animals. Low-grade uveitis (intraocular inflammation) was identified in four of the rAAV.RPE65-treated eyes 2–6 days postoperatively. Systemic and topical anti-inflammatory therapy was instituted and the inflammatory signs resolved within 3–6 days. Topical anti-inflammatory therapy was continued for several weeks in the uveitis-affected eye only. In the control eyes there were no inflammatory reactions.

Discussion

Gene transfer in the *RPE65−/−* dogs using an rAAV.RPE65 construct results in improvement of retinal function, as demonstrated by postoperative ERG recordings and visual behavior testing. We have shown that retinal function improves as early as 4 weeks after treatment and appears to persist for at least 9 months. In addition, for the first time, an improvement in ERG responses of both the rAAV.RPE65-treated eye and the contralateral control eye following unilateral subretinal injection of the lacking RPE65 DNA has been demonstrated.

Transgene expression in the retina after AAV-mediated gene transfer appears to persist long term, if not permanently. In a study by Dudus et al. (1999), it was reported that GFP gene expression in the dog retina persisted for at least 6 months after intravitreal delivery of rAAV-GFP. Likewise, in our studies we have detected both GFP and RPE65 expression in the retina and RPE up to 6 months after treatment with the corresponding AAV vectors (Narfström et al. in press).

In normal dogs there is considerable variation in ERG parameters. The ERGs were recorded simultaneously from both eyes in order to reduce the risk of variations in amplitude parameters due to external and other causes. Also, we wanted to record the RPE65-treated eye together with the rAAV.GFP-treated or untouched eye for more reliable comparison of the results.

An unexpected finding was the increase in ERG amplitudes in the control eyes at the long-term follow-up. The photopic b-wave response showed a remarkable recovery at 10–12 weeks. Furthermore, the photopic amplitude at the long-term follow-up declined in the treated eye, but increased considerably in the control eye, so that the relative changes between the two eyes were statistically significant. This phenomenon needs further investigation.

The recovery of ERG responses in the control eyes does not appear to be result of the treatments with the rAAV.GFP vector. One of the dogs in this study (Millie) was treated with the rAAV.RPE65 vector in one eye, and the contralateral eye

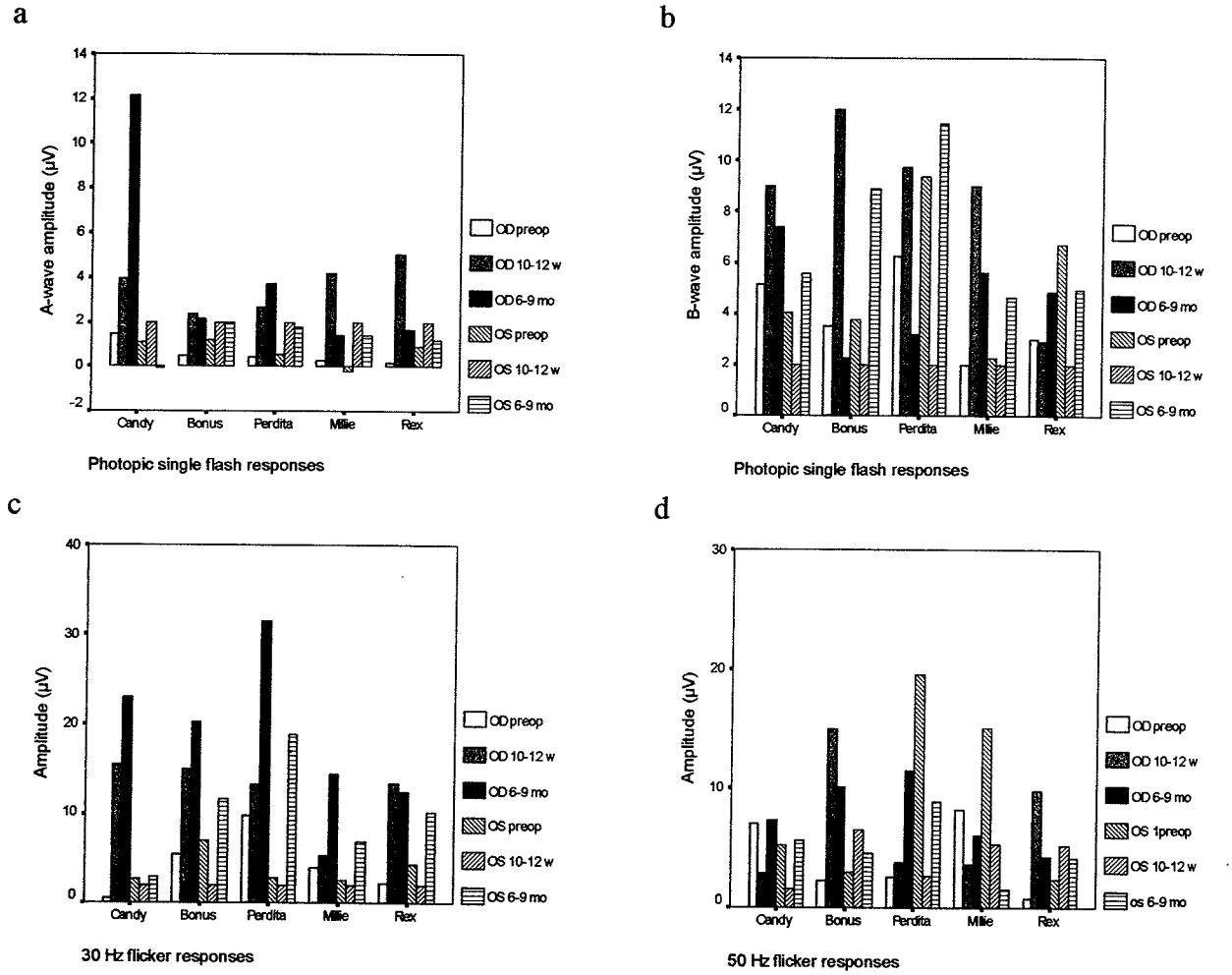

Figure 2. (a–d) Mean of the light-adapted (photopic) ERG a- and b-wave amplitude responses for five affected dogs at the preoperative, 6- to 10-week, and 6- to 9-month follow-up recordings for the right rAAV.RPE65-treated eyes (OD) and the left (OS) control eyes, respectively.

received no treatment at all. The improvement in ERG response in the untreated eye was similar to that observed in eyes that were treated with the control rAAV.GFP vector (see Figure 2, single flash and 30 Hz flicker photopic responses). Thus the improvement in the control eyes was most likely due to transfer of the gene therapy benefit from the eye treated with rAAV.RPE65 to the opposite eye. The most likely mechanism for this effect is via transport of RPE65 protein from cells in the eye treated with the gene therapy vector to the untreated eye. Dudus et al. (1999) gave

Table 2. Mean ERG amplitudes of the *RPE65−/−* dogs prior to treatment and at the 10- to 12-weeks and 6- to 9-months follow-up

	ERG amplitude values ± SEM (μV)						
	Preoperative		10–12 weeks follow-up		6–9 months follow-up		
	OD	OS	OD	OS	OD	OS	Normal
Scotopic b-wave (low intensity)	7.3 ± 0.7	8.1 ± 1.4	24.7 ± 4.0	4.2 ± 2.2	25.5 ± 6.3	12.0 ± 1.3	93 ± 39
Scotopic a-wave (high intensity)	2.4 ± 2.7	6.0 ± 2.1	22.7 ± 3.2	3.4 ± 1.4	28.0 ± 5.8	10.2 ± 3.9	85 ± 21
Scotopic b-wave (high intensity)	14.5 ± 4.4	14.4 ± 4.4	60.4 ± 6.7	3.2 ± 1.2	46.1 ± 9.7	9.6 ± 2.8	149 ± 26
Photopic a-wave	4.2 ± 0.5	0.7 ± 0.5	3.6 ± 0.5	2.0 ± 0.0	4.2 ± 2.0	1.3 ± 0.8	14 ± 2
Photopic b-wave	4.0 ± 1.7	5.2 ± 1.3	8.5 ± 1.5	2.0 ± 0.0	4.7 ± 0.9	7.1 ± 1.3	18 ± 4
30 Hz flicker	4.4 ± 1.6	3.9 ± 0.9	12.5 ± 1.9	2.0 ± 0.0	20.4 ± 3.4	10.1 ± 2.6	26 ± 6
50 Hz flicker	4.2 ± 1.4	9.0 ± 3.5	7.0 ± 2.4	4.3 ± 0.9	7.8 ± 1.3	5.0 ± 1.2	13 ± 3

Values for normal dogs in our laboratory are given for comparison.

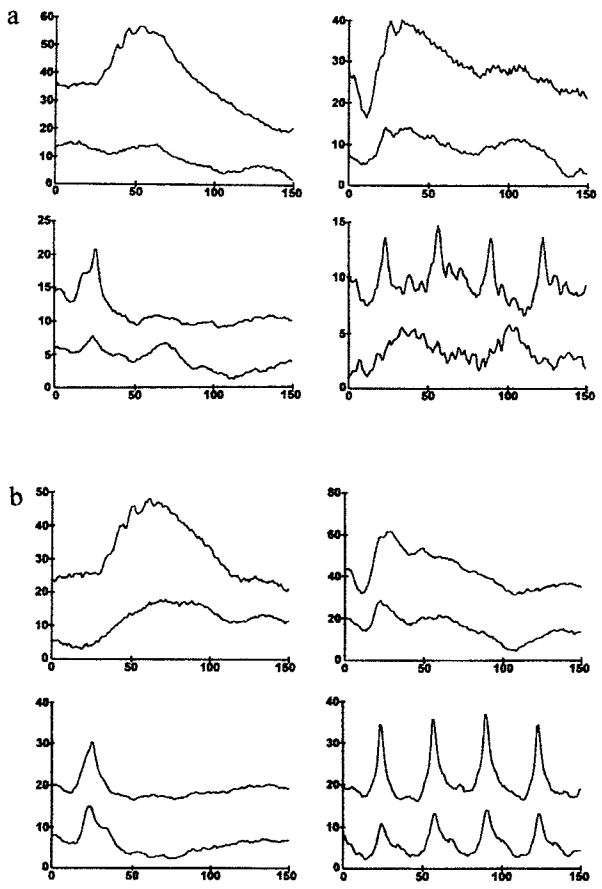

Figure 3. Actual, bilateral full-field ERG recordings in a 2.5-year-old *RPE65*−/− dog (Bonus) at **(a)** 11-weeks and **(b)** 6-months follow-up. Upper two sets show dark-adapted responses (low- and high-intensity scotopic stimuli, respectively) and the lower two sets in each, the light-adapted responses (single flash and 30 Hz flicker responses, respectively). In each set of recordings the upper curve is the response from the right rAAV.RPE65-treated eye and the lower curve is from the left (control) eye. For each recording session, the abscissa indicates time in milliseconds and the ordinate the amplitude in microvolts.

mice and dogs intraocular injections of rAAV.GFP. Transduction of retinal cells resulted in the appearance of GFP fluorescence in brain tissues known to be innervated by retinal ganglion cells. There was no transgene expression beyond the first synapses of the transduced cells, indicating that it was the GFP protein that was exchanged between cells, and not the transgene. In the present experiment, some of the RPE65 protein produced by transduced retinal cells in the rAAV.RPE65-treated eye may have been transported via the optic nerve to the brain, and then via retrograde transport to the retina and RPE of the contralateral eye. If this mechanism is correct, it may be possible to use immunochemical methods to detect RPE65 protein in the optic nerves and in the control retina and RPE of dogs treated with rAAV.RPE65 in one eye. Another explanation for the effect on the contralateral eye is that there is low-level release into the bloodstream of 11-*cis* retinoids from the rAAV.RPE65-treated eye that are taken up by the contralateral eye. This possibility is consistent with recent experiments that reversed the electrophysiological phenotype of *Rpe65*−/− mice by dosing with 9-*cis* retinal by gavage (van Hooser et al. 2000) or 11-*cis* retinal by injection (Ablonczy et al. 2002). In a similar fashion, the ERG changes in serum retinol binding protein-deficient (*Rbp*−/−) mice were reversed, in the absence of any intervention, over a period of 6 months by slow uptake by the RPE of postprandial all-*trans* retinol from the bloodstream (Quadro et al. 1999). An alternative possibility could be that the transfected RPE cells of the rAAV.RPE65-treated eye may cause an up-regulation of the RPE65 protein that is partially secreted, possibly into the neuroretina of the RPE65-treated eye, but also into the bloodstream. The retina of the contralateral eye may thus be provided with some of the lacking RPE65 protein. Contradicting this possibility is the lack of any evidence suggesting that RPE65 can be secreted. In future postmortem studies the therapeutic vector sequence will be analyzed in the control eye.

Results of subjective and objective behavioral testing revealed that visual function in the treated rAAV.RPE65 −/− dogs was similar to that of control animals in bright light. In the treated affected group, dim light visual behavior was also improved, but to a lesser extent. These findings provide clear evidence that subretinal injection of the *RPE65* gene construct results in improved vision.

Subretinal treatment with the transgene construct had a positive influence on ERG recordings, as evidenced by the significant increase in ERG amplitudes when compared to those obtained prior to surgery. The increase in postsurgical ERG responses in the affected group is in line with the subjective and objective visual behavioral test findings. The observation that vision in rAAV.RPE65-treated affected dogs is better in daylight conditions than in dim light conditions suggests that there is a greater return of cone function than rod function. In most retinal degenerative disorders, rods degenerate earlier than cones. Thus the greater relative recovery in bright light sensitivity of the retina may be due to the fact that there was a greater fraction of the original cone population than of the rod population that survived to the time of treatment.

Owing to the risk of complications associated with vitreoretinal surgery and subretinal injections, unilateral gene therapy resulting in bilateral improvement in vision can only have a positive impact on the use of this treatment modality in human patients. Further, the finding that this treatment is effective not only in young dogs but also in older animals, up to 2.5 years, in which there is already significant retinal degeneration, may allow for application of this novel treatment to older individuals with more advanced retinal disease.

References

Ablonczy Z, Crouch RK, Goletz PW, Redmond TM, Knapp DR, and Rohrer B, 2002. 11-*cis* retinal reduces constitutive opsin phosphorylation

and improves quantum catch in retinoid deficient mouse rod photoreceptors. J Biol Chen 25:40491–40498.

Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, and Bennett J, 2001. Gene therapy restores vision in a canine model of childhood blindness. Nat Genet 28:92–95.

Dudus L, Anand V, Acland GM, Chen S, Wilson JM, Fisher KJ, Maguire AM, and Bennett J, 1999. Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. Vision Res 39:2545–2553.

Hamel CP, Tsilou E, Harris F, Pfeffer BA, Hooks JJ, Detrick B, and Redmond TM, 1993a. A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. J Neurosci Res 34:414–425.

Hamel CP, Tsilou E, Pfeffer BA, Hooks JJ, Detrick B, and Redmond TM, 1993b. Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. J Biol Chem 268:15751–15757.

Narfström K, Katz ML, Bragadottir R, Seeliger M, Boulanger A, Redmond TM, Caro L, Lai C, and Rakoczy E, in press. Functional and structural recovery of the retina after gene therapy in the RPE65 null mutation dog. Invest Ophthalmol Vis Sci.

Narfström K, Wrigstad A, Ekesten B, and Nilsson SEG, 1994. Hereditary retinal dystrophy in the briard dog: clinical and hereditary characteristics. Prog Vet Comp Ophthalmol 4:85–92.

Quadro L, Blaner WS, Salchow DJ, Vogel S, Piantedosi R, Gouras P, Freeman S, Cosma MP, Colantuoni V, and Gottesman ME, 1999. Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein. Embo J 18:4633–4644.

Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, and Pfeifer K, 1998. *Rpe65* is necessary for production of 11-*cis*-vitamin A in the retinal visual cycle. Nat Genet 20:344–351.

Samulski RJ, Chang LS, and Shenk T, 1989. Helper free stocks of recombinant adeno-associated viruses: Normal integration does not require viral gene expression. J Virol 63:3822–3828.

Skulimowski AW and Samulski RJ, 1995. Adeno-associated virus: integrating vectors for human gene therapy. Meth Mol Genet 7:3–12.

Streilein JW, Ma N, Wenkel H, Ng TF, and Zamiri P, 2002. Immunobiology and privilege of neuronal retina and pigment epithelium transplants. Vision Res 42:487–495.

Thompson DA, Gyurus P, Fleischer LL, Bingham EL, McHenry CL, Apfelstedt-Sylla E, Zrenner E, Lorenz B, Richards JE, Jacobson SG, Sieving PA, and Gal A, 2000. Genetics and phenotypes of *RPE65* mutations in inherited retinal degeneration. Invest Ophthalmol Vis Sci 41:4293–4299.

van Hooser JP, Aleman TS, He YG, Cideciyan AV, Kuksa V, Pittler SJ, Stone EM, Jacobson SG, and Palczewski K, 2000. Rapid restoration of visual pigment and function with oral retinoid in a mouse model of childhood blindness. Proc Natl Acad Sci USA 97:8623–8628.

Veske A, Nilsson SE, Narfström K, and Gal A, 1999. Retinal dystrophy of Swedish briard/briard-beagle dogs is due to a 4-bp deletion in *RPE65*. Genomics 57:57–61.

Znoiko SL, Crouch RK, Moiseyev G, and Ma J, 2002. Identification of the RPE65 protein in mammalian cone photoreceptors. Invest Ophthalmol Vis Sci 43:1604–1609.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, and Muzyczka N, 1999. Recombinant adeno-associated virus purification using novel methods improves infection titer and yield. Gene Ther 6:973–985.

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