Using the NAFX to Measure the Effectiveness over Time of Gene Therapy in Canine LCA

Jonathan B. Jacobs,[1,2] *Louis F. Dell'Osso,*[1,2,3] *Zhong I. Wang,*[1,3] *Gregory M. Acland,*[4] *and Jean Bennett*[5]

Purpose. To use ocular motility recordings to determine the changes over time of infantile nystagmus syndrome (INS) in RPE65-deficient canines with Leber Congenital Amaurosis (LCA) and assess the time course of the recalibration of the ocular motor system (OMS).

Methods. Nine dogs were treated bilaterally with AAV-*RPE65*. A second cohort of four dogs was treated with AAV2.RPE65, an optimized vector. Their fixation eye movements were recorded before treatment and at 4-week intervals for 3 months, by using high-speed (500 Hz) digital videography. The dogs were suspended in a sling and encouraged to fixate on distant (57 inches) targets at gaze angles varying between ±15° horizontally and ±10° vertically. The records for each eye were examined for qualitative changes in waveform and for quantitative changes in centralisation with the expanded nystagmus acuity function (NAFX) and compared with ERG results for restoration of receptor function.

Results. First group: Before treatment, five of the dogs had clinically apparent INS with jerk, pendular, or both waveforms and with peak-to-peak amplitudes as great as 15°. One dog had intermittent nystagmus. At the 1- and 2-month examinations, no change in nystagmus waveform or NAFX was observed in any of the initial dogs, while at 10 weeks, one dog treated bilaterally with the standard dosage showed reduced nystagmus in only one eye. The other eye did not respond to treatment, as confirmed by ERG. This result was unexpected since it was previously documented that unilateral treatment leads to bilateral reduction of INS. The other dog treated with the standard dosage showed no reduction of its small-amplitude, high-frequency pendular nystagmus despite positive ERG responses. Second group: Only one dog of the four had clinically detectable INS, similar in characteristics to that seen in the affected dogs of the first group. Unlike any previous dog studied, this one showed a damping of the nystagmus within the first 4 weeks after treatment.

Conclusions. In all but one of the cases in which OMS recalibration occurred, as measured by the clinical appearance of nystagmus and by quantitative measurement using the NAFX, the improvement was apparent no sooner than 10 weeks after treatment. Longer term, dose-related studies are needed to determine the minimum necessary degree of restored receptor functionality, the duration after rescue for recalibration of the OMS, and the conditions under which recalibration information can successfully affect the contralateral eye. (*Invest Ophthalmol Vis Sci.* 2009;50:4685–4692) DOI:10.1167/iovs.09-3387

The developing ocular motor system (OMS) requires good visual input for it to calibrate properly. There are myriad reports of interruptions of this process due to afferent visual deficits such as unilateral and bilateral congenital cataracts[1–3]; deliberate visual deprivation[4]; the "searching" eye movements of the blind that are often mistaken for nystagmus[5]; and retinal dystrophies that lead, usually in short order, to instabilities or poor calibration of the OMS, often resulting in some form of nystagmus, most commonly fusion maldevelopment nystagmus syndrome (FMNS)—formerly known as latent/manifest latent nystagmus (LMLN) in the case of monocular deprivation—and infantile nystagmus syndrome (INS), formerly known as congenital nystagmus (CN).[6]

In all forms of true nystagmus, attempts to hold the fovea on a target are disrupted by a slow eye movement (slow-phase) that drives the eye off target. This movement is periodically interrupted by a corrective saccade (fast-phase) that attempts (in the case of INS) either to bring the eye back to the target (foveating saccade) or to act in opposition to the runaway slow-phase (braking saccade).[7,8] This nearly continuous motion of the target onto and off the fovea, except during brief ~20- to 300-ms foveation periods each cycle after a foveation saccade, degrades visual acuity[9] and, when combined with an afferent deficit, can severely reduce a patient's ability to function normally.

Leber Congenital Amaurosis (LCA) is a retinal dystrophy that is the most common genetic cause of visual impairment in children. Although the optic disc may look normal,[10] retinal degeneration begins shortly after birth and can lead to total blindness within a few years. There are at least nine genes that are implicated in LCA, of which *RPE65* accounts for between 10% and 20% of all cases. This gene is necessary for the photoisomerization of all-*trans* retinal to 11-*cis* retinal, part of a cycle that regenerates the pigment rhodopsin in photoreceptors.[11,12] There are currently two animal models of *RPE65*-deficient LCA, the *Rpe65*$^{-/-}$ mouse[13] and the Briard dog,[14,15] that have served as the basis for the development of novel genetic therapy treatments that involve the use of a serotype 2 adenoassociated virus (AAV) to deliver the missing *RPE65* gene to the retinal pigment epithelium where it can reestablish local production of photopigment[16] and in short order restore receptor function and rescue vision. In this study, we did not attempt to determine the effectiveness of different vectors or

From the [1]Daroff-Dell'Osso Ocular Motility Laboratory, Veterans Affairs Medical Center; and the Departments of [2]Neurology and [3]Biomedical Engineering, Case Western Reserve University and University Hospitals of Cleveland; Cleveland, Ohio; the [4]James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York; and the [5]F. M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia, Pennsylvania.

Supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, National Institutes of Health (NIH) Grants EY06855, EY10820, U10UF01109, NIH Training Grant EY07157, the Center for Cellular and Molecular Therapeutics at The Children's Hospital of Philadelphia, Research to Prevent Blindness, Foundation Fighting Blindness, the F. M. Kirby Foundation, and Paul and Evanina Mackall Foundation Trust.

Submitted for publication January 8, 2009; revised March 18, 2009; accepted July 29, 2009.

Disclosure: **J.B. Jacobs**, None; **L.F. Dell'Osso**, None; **Z.I. Wang**, None; **G.M. Acland**, None; and **J. Bennett**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jonathan B. Jacobs, Daroff-Dell'Osso Ocular Motility Laboratory, Louis Stokes Cleveland Veterans Affairs Medical Center, 10701 East Boulevard, Cleveland, OH 44106; jxj24@case.edu.

their dosages. We restricted our analyses to the ocular motor recalibration that results.

In previous work with *RPE65*-deficient dogs that had clinically apparent INS,[17] we presented the novel finding that it was possible under some conditions for the OMS to recalibrate and stabilize after the repair of the afferent deficit. In that study we looked only at the long-term (~10 month) posttreatment results, believing them to be representative of a steady state plateau, based on historical observations of the long-term effectiveness of traditional nystagmus surgeries[18] that have been borne out by analysis of the more recently developed tenotomy procedure.[19,20] It has been observed that once this plateau is reached, it remains stable: Patients report stable results decades after Kestenbaum surgery, and the first tenotomy procedures performed on humans have maintained improved vision nearly a decade later. We also showed that treatment and restoration of vision in only one eye could result in noticeably damped nystagmus in both eyes, often to subclinical levels, although the foveation characteristics (duration and repeatable accuracy) were not as good in the untreated eye, which is wholly understandable, as there is little visual impetus to drive such a degree of fixation. We have repeatedly seen this occur in humans with strabismus who fix with one eye at a time.

To analyze the improvement in nystagmus waveform, we used the expanded nystagmus acuity function (NAFX),[21] an objective measurement of best *potential* visual acuity, based on the above-mentioned foveation characteristics. We developed this function because previous metrics for nystagmus analysis (e.g., peak-to-peak amplitude or frequency), or their product *intensity,* are poor indicators of actual visual performance, as they do not take foveation criteria into account. The NAFX has proven its utility as an objective measure of INS waveforms and their improvement with therapy and is the direct descendant of the NAF, a function developed for evaluating the data of subjects who had sufficient ocular motor control that their fixations usually fell within one foveal radius of each other, and the eye was sufficiently slowed during these periods to under 4 deg/s. Unfortunately, many subjects with INS do not have this level of control, and so it was necessary to redesign the algorithm to allow for greater position and velocity variability in subject foveation, while still allowing for its use with subjects who could meet the more stringent criteria of the original function.

In this study, we again worked with the *RPE65*$^{-/-}$ line of dogs to further examine the time course of the stabilization of the OMS, as afferent visual information becomes available after successful receptor rescue. We paid particular attention to the first 3 months after treatment, noting the time when, according to ERGs, receptor function was detected.

Methods

We performed ocular motility recordings on two separate groups of dogs for this study. The first cohort of nine dogs was recorded before treatment, and every 4 weeks over the space of 4 months after treatment. The second cohort of four dogs was recorded before treatment and again at four weeks after treatment. All animal work followed the guidelines set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Canines

The *RPE65*-mutant strain of dog is an autosomal recessive model of Leber Congenital Amaurosis and displays no extraocular abnormalities. Its disease originated as a naturally occurring disorder affecting the Briard breed of dog in several countries.[14,15] Although the disease was initially[15] classified as a congenital stationary night blindness, affected dogs have variably severe abnormalities of cone-mediated vision as well, show slow progression of symptoms with age, and have very slow development of degenerative retinal morphologic changes.[22] The *RPE65*-mutant strain used in this study is a crossbred strain derived by breeding a single affected Briard dog into a research colony strain of normal Beagle-crossbred dogs. This breeding has created a dog much more suitable for research studies and colony housing than are purebred Briards. The natural course of the disease in dogs means that there is an extremely long window of time in which therapeutic trials can be undertaken. The eye-movement data recorded for this study were from canines that were undergoing separate studies to examine minimum critical dosage and safety and efficacy of different vectors. Thus, we had no control over their availability for study or the decisions regarding the time when they would be used for post mortem studies. Although we would have preferred to examine these dogs in a more detailed manner, we were fortunate to have access to them at all.

Clinical Examination

Before any recordings, each dog's eye movements were examined for qualitative appearance with either an ophthalmoscope or a high-positive magnifying glass and penlight. Nystagmus, if present, was roughly characterized for amplitude, frequency and plane(s). Ocular alignment was noted as well. Visual behavior was tested by allowing the dog to wander freely in the examination room (14 × 12 feet) and observing whether it reacted to movements made by the people in the room and how well it could avoid obstacles during its explorations. This behavior was videotaped. If the dog was able to successfully navigate the room in normal light, the light level was then reduced to the lowest level that would still permit videotaping, and the dog's performance was again observed as an indicator of scotopic visual function.

After treatment, in the weeks between recording sessions, the animal caretaking staff undertook periodic visual inspections of the eyes of the dogs with nystagmus, to note if any clinically evident changes developed, as an adjunct to the objective ocular motor recordings described in later.

Treatment

The first group of dogs received bilateral injections of AAV-RPE65 at one of four dosages. The standard dosage (SD) of 2×10^{12} particles/150 μL (i.e., total dose of 3×10^{11} particles) was given to two dogs; attenuated injections of 0.01 × SD were given to two dogs; one dog received 0.001 × SD; two dogs received 0.001 × SD; and one control dog received only saline. Further details on the gene therapy and ERGs can be found elsewhere.[23] The dogs of the second cohort were treated with a different vector, AAV2.hRPE65v2, abbreviated as AAV2.RPE65, whose transgene cassette was modified for greater expression. In addition, a surfactant (0.001% PF68) added to the infusion solution resulted in a higher proportion of particles not being lost to the inert surfaces of the delivery system (syringes, tubing). The greater delivery of the vector to the retina was determined to result in a faster and more widespread (in area) rescue of receptor function than the vector used for the first cohort. The dogs received 150 μL containing a total of 8.25×10^{10} vg (vector genome). Further details for this cohort have been published elsewhere.[24]

Recording

Eye-movement data were recorded with a high-speed video system (EyeLink II; SR Research Ltd., Osgoode, ON, Canada) capable of measuring horizontal and vertical movement simultaneously at a sampling frequency of 500 Hz and resolution of 16 bits. Although the video system was designed for use in human subjects, we were able to modify it for use in dogs by repositioning the cameras more laterally to compensate for the greater interpupillary distance in dogs, whose eyes are not as forward-pointing as humans. Because the video cameras are mounted on a headband (specifically designed for human head-fixed operation) that does not fit a dog's head, we mounted the cameras to an adjustable and lockable earth-fixed frame.

Dogs with long hair that could potentially cover their eyes, or otherwise interfere with the sensors or cameras were shaved the day before recording. Their eyes were irrigated to remove any stray hairs, and they were examined with an ophthalmoscope for signs of irritation. They were then returned to their kennels after a brief session of socialization.

In previous work,[25] we described the techniques necessary to record eye movements using head-mounted IR from comfortably restrained, alert, relaxed, co-operating, but untrained dogs. For this study, the only modification necessary was to better assure that, during recording, the dogs did not make head movements, which—if not detected during experiments—would lead to motion artifacts. Firm but gentle hand pressure behind the dog's occiput accomplished this. Such contact served three purposes: In addition to restraint, it allowed the investigator to report on any movement so that corrupted data could be discarded. Furthermore, the constant contact helped to relax the dogs. As an additional safeguard against poor data quality due to the dog's movement or inattention, sessions were video recorded, with a live video feed also present at the data acquisition operator's station.

Before recording, each dog's eye movements were calibrated against known targets in the horizontal and vertical planes. Special effort was made to increase accuracy during calibration. Each target was presented multiple times within the dog's binocular field to ensure sufficient accurate conjugate saccades to the target. These techniques allowed for a "ballpark" calibration of the video system, using its native routines (which are suitable for behaving subjects, such as humans or lower primates that have been trained to reliably fix on a target). A final, more accurate calibration was applied post hoc to the data by using custom software developed in a commercial package (MATLAB; The MathWorks, Natick, MA), to examine where each dog looked during the repeated target presentations. Finally, to ensure accuracy over the course of an experiment, the calibration presentation was repeated during each trial.

Recording Protocol

All ocular motility recordings were performed in accordance with the Institutional Animal Use and Care Committees' (IACUC) guidelines regarding animal experimentation. A trained veterinarian was present or could be summoned at all times. An experiment consisted of between two and seven trials, determined by how co-operative the investigators felt the dog to be. If a dog refused to allow its head to be held still, or ceased to attend to targets, it was allowed to rest in the sling apparatus for several minutes before further attempts were made. If it still refused to co-operate, it was immediately released for that session. No more than two sessions were attempted for any dog per day.

Each trial lasted from 30 to 90 seconds, again depending on the dog's ability and willingness to co-operate. During each trial the dog was required to fix on an object that was determined to hold its interest, such as an electronic toy that made noise and had a flashing light. One examiner stood 57 in. from the dog's eyes and, holding the fixation target near his own eyes, moved repeatedly between the points of 0°, ±15° horizontally and ±10° vertically, holding the target for approximately 5 seconds at each point. Because he was looking directly into the dog's eyes, the examiner was able to verify when the dog looked at each target and report if the dog failed to do so. The data acquisition operator also monitored the dog's performance using both a live feed from the video camera recording the session and the on-screen feedback provided by the video system. Recordings made when a dog ceased to pay attention to the target or moved its head (also monitored by the investigator stabilizing the dog's head) were marked accordingly.

Analysis

Eye-movement recordings by the video system were exported using routines provided by SR Research into a format that could be read and analyzed with the custom-written software. Only position data were measured directly; velocity was calculated by means of a two-point central differentiator algorithm that also acted as a low-order, low-pass filter with a cutoff frequency that decreased as the separation between the difference points increased. More information on this algorithm and its consequences may be found elsewhere (Jacobs JB, et al. *IOVS* 2003;44:ARVO E-Abstract 4249). Further filtering was performed using a fourth-order, low-pass filter with a cutoff frequency of 20 Hz, sufficient to reduce noise while retaining the eye movement data, which follows a 1/f spectrum. Effects on saccadic velocity were no more than a 5% reduction in peak velocity, which was not a factor in this study.

Foveation quality and its potential effect (not accounting for afferent deficit) on human visual acuity are calculated using the NAFX, which returns a single value that ranges linearly between 0.0 and 1.0, corresponding to no vision at the low end, to a Snellen acuity of 20/15 (1.33) at the high end for young adults. It is important to note that these values represent human vision. Most dog breeds have a lower maximum acuity, approximately 20/70 (e.g., several breeds of working dogs, especially hunting dogs), owing to the less developed nature of the canine *area centralis* compared with the human fovea. The NAFX calculates this value based on the duration and repeatability (i.e., the standard deviations of fixation positions and velocities) of *centralisation* (foveation in humans) periods, defined as the data points that simultaneously satisfy particular position and velocity limits, defined as the *centralisation window.* Although details for application of the NAFX have been described previously,[21] it is instructive to summarize the most important rules. Most crucial is to ensure that the data are accurately calibrated to allow identification of the fixating eye. Then, only segments of data should be selected where the subject is known to have been attending to the target, because without attention, foveation does not have a physical meaning. It is imperative to avoid long stretches of data (e.g., minutes), when the subject (patient with nystagmus, normal human, or canine) does not always maintain concentration or, in many cases, may switch which eye is fixing the target. As a consequence of this failure to maintain fixation throughout such records, they are too noisy (including blinks) to accurately measure the quality of fixation, the nystagmus mechanisms, or to compare pre- and posttherapy nystagmus.[26] The second important point is to select the position and velocity criteria for the foveation window as small as necessary to suit the fixation ability of the subject being measured, *but no smaller than that.* In the case of the dogs, the centralisation window boundaries were ±3.0° horizontally and ±1.5° vertically, reflecting the extent of the area centralis. The velocity limits were set between ±4 and ±10 deg/s, as for humans. We limited our analyses to data segments that were no longer than 10 seconds and that showed no changes (or loss) in fixation during that time. Records where the dogs made head movements, or failed to attend to the targets were not analyzed.

Results

Pretreatment

Five of the dogs in the first cohort (BR248, BR251, BR246, BR235, and EMB28) had continuous, clinically apparent INS, with waveform components that were pendular (P), jerk (J) or both. Only one dog from the second cohort, BR334, had nystagmus detectable either clinically or with recordings. All dogs had poor ocular motor control, resulting in large excursions of eye position beyond that of the nystagmus amplitude. Table 1 lists the dogs examined in the ocular motor portion of the study and summarizes their nystagmus characteristics, the treatment that they received, and their visual and ocular motor outcomes.

Figure 1 shows waveforms representative of the pretreatment nystagmus typically seen in these dogs. Both pendular and jerk waveforms were commonly seen. In EMB28 a dual-jerk waveform was recorded. Compared with human INS, there were fewer foveating and braking saccades, probably due to

TABLE 1. Clinical and Ocular Motor Data from Canines with Nystagmus

Dogs with Nystagmus	Age at Treatment	Nystagmus (Pre)	Dosage	ERG (Post) (OS, OD)	Nystagmus (Post)
BR248	11 wk	P & J = 5–8 Hz ~2° p-p	AAV, SD	−, +	Uniocular improvement
BR251	11 wk	P = 10 Hz ~1° p-p	AAV, 0.01 SD	−, −	Unchanged
BR246	11 wk	Intermittent P = 7–10 Hz ~2° p-p	Saline	−, −	Unchanged
BR235	7 mo	P = 8–10 Hz ~1–1.5° p-p	AAV, SD	+, +	No clinical improvement
EMB28	7.25 mo	DJ (J = 2 Hz, P = 8 Hz) ~1–2° p-p	AAV, 0.01 SD (OD)*	−, −	Unchanged
BR334	16 wk	P = 8 Hz ~1–2° p-p	AAV2, (OD)*	+, −	Ampl: LE≪RE Calm periods: LE≫RE

SD, standard dosage of 2×10^{12} particles/150 μL; P, pendular; J, jerk; DJ, dual jerk; p-p, peak-to-peak amplitude; +, detectable ERG signal; −, undetectable ERG signal.

* Major portion of dose delivered into the intravitreous space.

the ($\pm 3° \times \pm 1.5°$) area centralis, which is larger than the ($\pm 0.5°$) fovea of humans. The amplitudes were on the low end of human INS, and the frequencies were slightly higher; as a result, the velocities were also higher.

After Treatment

Electroretinograms. ERGs were performed on the first cohort no sooner than 1 month after treatment. Restored receptor function was detected in three of the four eyes that received the AAV-RPE65 SD. The eyes that received attenuated dosages showed greatly reduced results, with rescue of none of the four eyes treated with 0.01 × SD, only one of the two eyes at 0.001 SD, none of the four eyes treated with 0.0001 × SD, and, unsurprisingly, none of the four control eyes. For this group of dogs, ERGs were measured only as present (detectable signal) or not present.

ERGs for the dogs of the second cohort were performed at 5 weeks and 3 months after injection. Unlike with the first group, quantitative measurements were obtained. Restored a-waves were detected in all eyes that received subretinal injection of the AAV2.RPE65, a direct indication of photoreceptor activity. At 5 weeks, the peak amplitude of 55 μV measured in BR334's left eye was greater than 60% of that in an unaffected dog. By 3 months, the peak had declined to less than 20%, but was still significantly greater than before treatment.

Ocular Motility Recordings. We performed recordings on the dogs of the first cohort before treatment and then at 4, 8, and 10 weeks after injection. Until the session at 10 weeks there were no noticeable changes in the dogs' nystagmus, either qualitatively or quantitatively, which was consistent with the results we had reported previously.[17]

BR246, which received only normal saline, and BR251 and EMB28, which each received 0.01 × SD, showed no recovery of receptor function according to ERG. Similarly, no changes were measured in their nystagmus.

Although BR235 was treated binocularly with the SD and showed restoration of receptor function, this dog also had no apparent improvement in its nystagmus during any of the recording sessions, nor at the time of any follow-up clinical examination. By inspection, before and after treatment, though the high-frequency (~10 Hz) pendular nystagmus appeared to

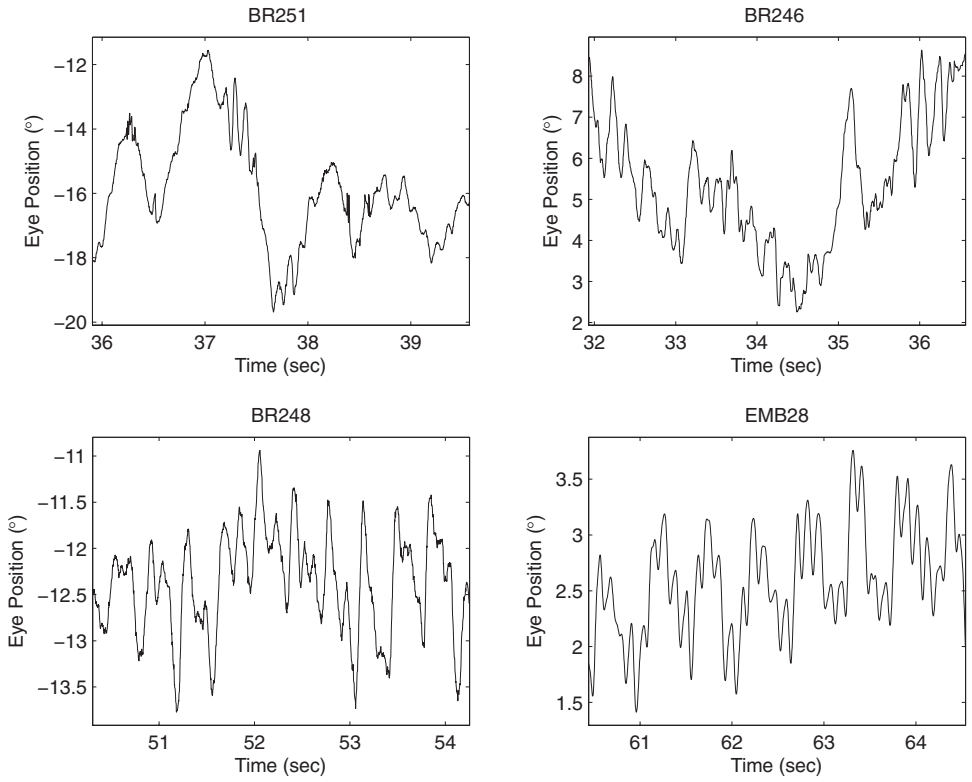

FIGURE 1. Representative pretreatment waveforms from four of the dogs. Note the presence of large drifts of eye position, in addition to the oscillations due to the nystagmus and the dual-jerk waveforms of EMB28 (*bottom right*).

be well aligned from cycle to cycle, detailed analysis detected little or no actual centralisation (i.e., time-per-cycle within the centralisation window), as shown by the waveforms in Figure 2 and phase plane in Movie S1, http://www.iovs.org/cgi/content/full/50/10/4685/DC1, resulting in a very low NAFX in each case.

More unusual was BR248, also treated binocularly at the standard dose. In this case, treatment was effective in only one eye. ERG performed on the right eye showed recovery, whereas there was minimal response in the left eye. Until the 10th week, no improvement was quantitatively recorded; however, sometime between the week 8 and week 10 recording sessions, we observed that the nystagmus in the right eye decreased to a clinically undetectable level most of the time, though it usually remained noticeable in the left eye, confirmed by quantitative recording as shown in Figure 3, top, and Movie S2, although it would occasionally appear at pretreatment levels (Fig. 3, middle) or be clinically undetectable in either eye (Fig. 3, bottom). On rare occasions, the amplitudes would invert from the most common case, resulting in a clinically detectable oscillation in the normally quiescent right eye, whereas the usually florid left eye would damp below clinical levels. This mostly uniocular response is puzzling, directly contrasting with our previous finding of binocular recalibration resulting from uniocular treatment.

Close examination of BR248's posttreatment waveforms (Fig. 4) showed the beginnings of well-developed centralising saccades and the extension of centralisation periods, reminiscent of the development of foveation in infants.[27] These better periods led directly to an increase in the NAFX for this posttreatment waveform. Contrast this with the lack of centralisation demonstrated by BR235 in Figure 2.

BR334, from the second cohort, was treated with the more effective AAV2.RPE65 vector at a dose of 8.25×10^{10} vg. The dosage to the left eye was injected subretinally and lacked the surfactant, whereas the right eye injection contained the surfactant and was delivered 10% subretinally and 90% delivered intravitreously—a far less efficient method. Clinical examination showed increased pupillary response, increased visual behavior, and decreased nystagmus within 15 days, although it was not possible to make quantitative OMS recordings until 4 weeks. Even though receptor function was restored in only the left eye, the nystagmus was damped in both eyes. In contrast to BR248, the other dog that was successfully treated in only one eye, this result is more in line with the previous finding[17] that successful restoration of vision to one eye could lead to decreased nystagmus in both eyes.

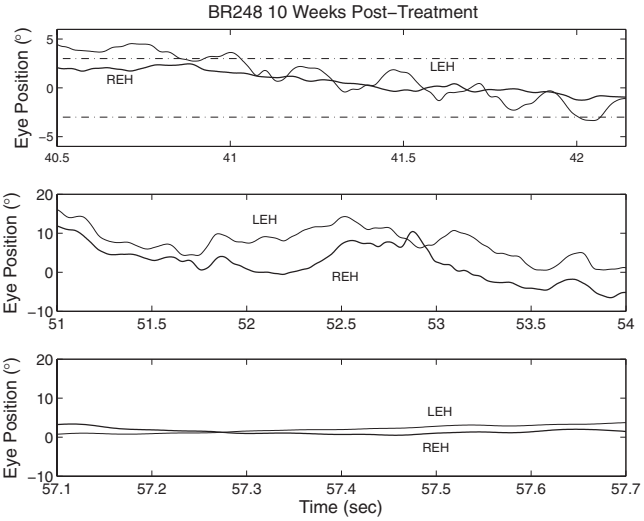

FIGURE 3. BR248, 10 weeks after treatment. Three successive segments of data demonstrate the variability in nystagmus improvement. *Top*: most commonly, the right eye showed improvement in damping, whereas the left eye did not. *Middle*: although considerably less common than before treatment, the nystagmus could appear at its pretreatment levels. *Bottom*: damping was apparent in both eyes. *Dashed-dotted lines*: area centralis. Abbreviations as in Figure 2.

Before treatment, there were frequent periods when one eye (or both eyes) was not stable enough to allow calculation of an NAFX because of insufficient centralisation duration. Posttreatment, these episodes were greatly reduced in number and duration. Figure 5 shows the contrast in nystagmus before and after treatment. It should be noted that, even before treatment, there were occasional brief periods during which the nystagmus was nearly quiescent, leading to high NAFX values. However, these periods were much more common and of much greater duration after treatment. These greatly contrasting conditions could appear one right after the other, as

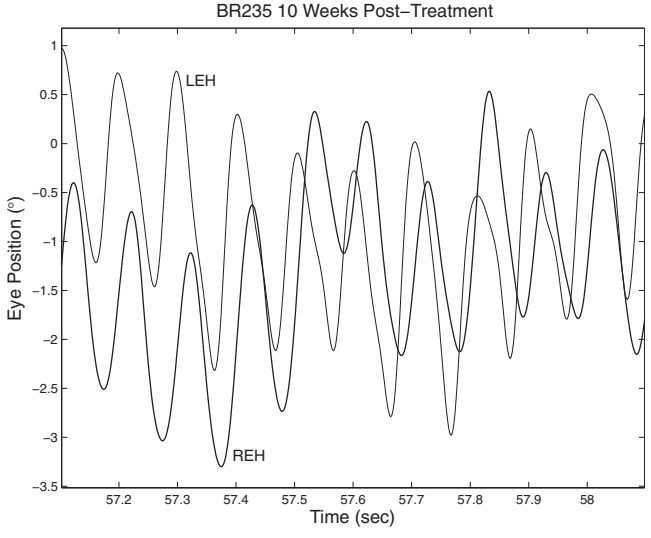

FIGURE 2. Posttreatment waveforms from BR235. The underlying pendular waveform was not diminished in amplitude, nor did any centralising saccades develop. REH, right eye horizontal; LEH, left eye horizontal.

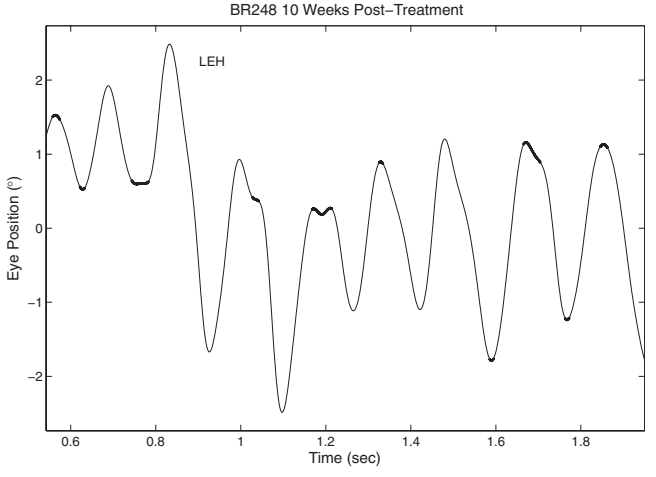

FIGURE 4. Detailed examination of the posttreatment waveforms for BR248. Note the appearance of distinct centralisation periods, marked in *bold*. The presence of these periods almost every cycle has a direct correlation to increased visual acuity. Abbreviations as in Figure 2.

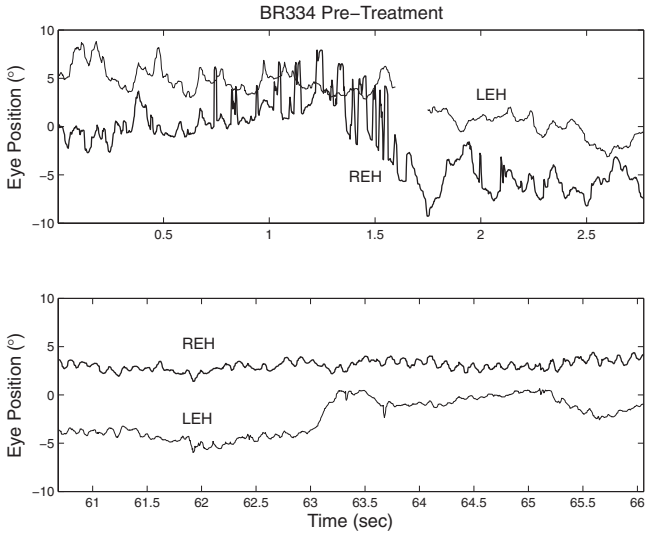

FIGURE 5. BR334's horizontal nystagmus before and after treatment. Waveforms are plotted on the same scale to facilitate comparison. In addition to the amplitude damping, ocular motor control appears to be improved as indicated by reduction in drifts off target. Abbreviations as in Figure 2.

shown in Figure 6, top, leading to a great range in NAFX—in this figure from 0.07 to 0.32 in the space of just a few cycles—even though the right eye had little or no vision. Part of this variability is most likely dependent on the dog's state of arousal, just as is commonly seen in human INS. The left eye's horizontal waveform has a decreased peak-to-peak amplitude, which is especially notable when compared with that of the right eye, as shown in Figure 6, bottom.

We performed NAFX analysis only in the dogs that initially had nystagmus and that, after treatment, had a measurable improvement in retinal function. For BR235, the analysis yielded such low and widely spread NAFX values (average, <0.1; standard deviation ~ 0.1) that comparison between the pre- and posttreatment data was not meaningful. The other two dogs, BR248 and BR334, had valid NAFX results, which are shown in Figure 7.

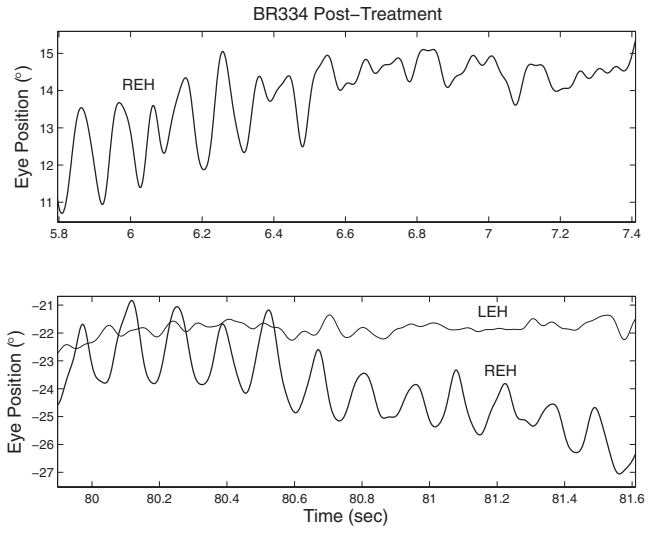

FIGURE 6. BR334, after treatment. *Top*: the right eye, which did not respond to treatment, still demonstrated brief periods when the nystagmus was damped. *Bottom*: comparison of damping in the left eye, which did respond to treatment, and the right eye. Note the difference in peak-to-peak amplitudes between the two eyes. Abbreviations as in Figure 2.

DISCUSSION

We used ocular motility recordings to determine the time changes of INS in RPE65-deficient LCA canines and assessed the time course of the recalibration of the ocular motor system. As stated in the Methods section, these dogs were part of separate studies to examine minimum critical dosage and safety and efficacy of different vectors. Therefore, access to them was limited.

The study of canine eye movements and vision is another area where the NAFX has proven its value. The foveation or centralisation improvements of nystagmus waveforms are directly related to the potential visual acuity of humans and canines, respectively, as the same basic mechanisms of vision and their physical limitations apply. Two dogs, BR248 and BR251, received gene therapy at a young age with the SD and 0.01 SD AAV, respectively. As a control, BR246 received saline at a young age. Two dogs, BR235 and EMB28, received gene therapy at an older age with the SD and 0.01 SD AAV, respectively. Finally, BR334 received gene therapy at a young age with AAV2. BR248 showed improved receptor function and NAFX; the older dog, BR235, also showed improved receptor function but not NAFX. The young dog, BR334, showed improved receptor function and NAFX. Based on these data, there appears to be a time window of less than 7 months during which improved vision should allow for ocular motor recalibration. As a rule, at the low dosages and control used in this study, the NAFX did not show improvement after treatment. This result was as expected, since receptor function did not improve.

An additional consideration regarding why BR235 showed no improvement is that vision was so poor that fixation was minimal and the NAFX may be less meaningful for values that low. Another complicating factor is the variability in the dogs' fixation behavior, which is comparable to children and some adult patients. We are limited by their willingness to fixate a target that is not inherently interesting and by their desire to co-operate. However, for dogs as for human patients, our experimental paradigm allows for analysis of repetitive, short periods of target fixation.

Dogs showed improvement only after 10 weeks after treatment. Re-examination of data from our previous study also

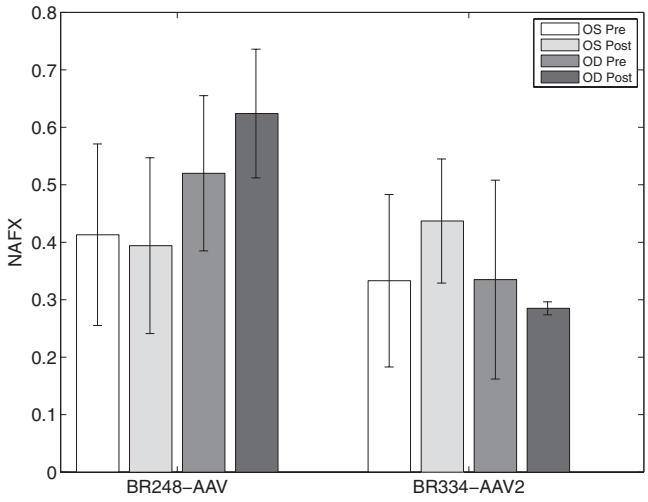

FIGURE 7. Pre- and posttreatment NAFX values in both eyes of BR248 and BR334.

showed no improvement in nystagmus before 10 weeks after treatment.[17] We previously reported littermates treated several years apart in which the window had definitely closed for the adult (Jacobs JB, et al. *IOVS* 2005;46:ARVO E-Abstract 2401). However, it is possible for recalibration to occur relatively quickly (i.e., within a month). This occurrence is not without precedent, in that other forms of recalibration (e.g., myasthenia gravis or paretic patching)[28,29] occur relatively quickly. Why did one dog (BR334) recalibrate so quickly (15 days), whereas others have taken many weeks longer? We hypothesize that this is because of how quickly the receptors regained function due to the new vector/delivery system. This recovery of function suggests that the quantity or quality of new visual information plays a crucial role in ocular motor recalibration. In BR235, the NAFX pretreatment was very poor compared with that in other dogs in the study. While seemingly allowing ample room for improvement, the age at treatment was possibly a factor, even though it is possible for some older dogs to show improvement. Also, there may just have been too high a deficit to overcome; taken together, these results suggest highly idiosyncratic therapeutic outcomes.

The question arises as to whether there is an all-or-nothing response or a quantifiable progression over the first 10 weeks. To answer this question, it would be necessary to examine ERGs at a finer time scale to observe the recovery and to relate the results to OMS recalibration. Unfortunately, because of the need to anesthetize the dogs, canine ERG testing is so stressful and potentially dangerous that the rate of progression may not be discoverable until human clinical trials can be done, as these tests can be performed on awake subjects.

It is critical to avoid confusing the interval between restoration of afferent signal and recalibration of the OMS with the sometimes almost-immediate decrease in nystagmus that occurs after extraocular muscle surgery. In the former case, the change is effected centrally,[30] whereas in the latter it is the result of a rapid modification of peripheral response (i.e., the tenotomy and reattachment of the muscle and the resulting proprioceptively modified small-signal response).[31,32]

In BR334, treated binocularly with AAV2, successful restoration of vision to one eye led to clinically decreased nystagmus in both eyes, albeit unequally, with an improved NAFX only in the eye with improved retinal function. In BR248, treated binocularly with SD AAV, the treatment was effective in only one eye. An ERG performed on the right eye showed recovery, whereas there was minimal response in the left eye; the NAFX values reflected this disparity. These mostly uniocular responses are puzzling, directly contrasting with our previous finding of binocular ocular motor recalibration resulting from uniocular treatment.[17] Even though receptor function was restored in only the left eye, the nystagmus was damped in both eyes. The current results suggest variability of "rubber-band yoking."[25] Although there is probably an emotional or motivational input to the nystagmus, just as in humans, it is unlikely to be the cause here, as the dog showed no change in the other eye.

These differences suggest that, as previously noted,[25] the yoking between eyes is highly variable, ranging from very tightly coupled, where both eyes make nearly identical version movements, differing only slightly in their foveation periods, to very loosely coupled, as presented herein, where not only may the amplitudes and even the phases differ between eyes, but also the underlying frequency of the nystagmus. We observed this in BR334, with one eye oscillating at 8 Hz and the other at 10 Hz.

Finally, in a previous study[17] we had examined two littermates that had nearly identical nystagmus. One dog, BR58, was treated at 10 months of age, and BR57 was originally left untreated as a control. As documented, BR58 showed restored receptor function and also greatly reduced nystagmus. Several years later, BR57 also received treatment at the age of 52 months with the same dosage of AAV.*RPE65*, but although it too showed improved receptor at a level comparable to BR58 and there were isolated periods where its nystagmus damped to the same level as measured in its sibling, for the majority of the time, the NAFX remained unchanged (Jacobs JB, et al. *IOVS* 2006;47:ARVO E-Abstract 2513).

In summary, if OMS recalibration occurred (as determined by the clinical appearance of nystagmus and quantified by NAFX improvement), the improvement was apparent no sooner than 10 weeks after treatment in all but one case. This finding applies to the results of this study as well as to the 11 dogs documented previously. Longer term, dose-related studies would be needed to determine the minimum necessary degree of restored receptor functionality, the duration after rescue for recalibration of the OMS, and the conditions under which recalibration information can successfully affect the contralateral eye. Recently, human trials have begun. One of these includes nystagmus as an outcome measure, and the early results are consistent with those reported for the canine model of LCA.[33]

Acknowledgments

The authors thank the individuals who performed the core afferent system studies in the *RPE65* mutant dogs that were essential for the present study to be conducted: Gustavo D. Aguirre characterized the pathogenetic and molecular basis of the disease in the *RPE65* mutant dogs; Samuel G. Jacobson, Artur V. Cideciyan, and Tomas S. Aleman performed the psychophysical and ERG studies evaluating effects of gene delivery on visual function; Nadine S. Dejneka cloned the cDNA encoding human RPE65 that was used to generate both of the AAVs used in the study; William W. Hauswirth generated and purified the AAV.*RPE65* vector; Jeannette Bennicelli generated the plasmid used to produce the AAV2-hRPE65v2 vector, and Fraser Wright purified this AAV; Albert M. Maguire surgically delivered the AAV.*RPE65* and AAV2-hRPE65v2 to the mutant dogs; and Andras Komaromy assisted with clinical/surgical procedures in the affected dogs. The authors thank all the staff at the University of Pennsylvania's New Bolton Animal Facility—Amanda Nichols, Gerri Antonini, Shannon Edwards, Tracy Greiner, Alice Eidson, Siobhan Spears, and Jill Wells—for their invaluable assistance working with the dogs to gain their trust and to collect the data.

References

1. Abadi RV, Forster JE, Lloyd IC. Ocular motor outcomes after bilateral and unilateral infantile cataracts. *Vision Res.* 2006;46(6-7):940–952.
2. Lundvall A, Kugelberg U. Outcome after treatment of congenital bilateral cataract. *Acta Ophthalmol Scand.* 2002;80(6):593–597.
3. Lundvall A, Kugelberg U. Outcome after treatment of congenital unilateral cataract. *Acta Ophthalmol Scand.* 2002;80(6):588–592.
4. Tusa RJ, Mustari MJ, Das VE, Boothe RG. Animal models for visual deprivation-induced strabismus and nystagmus. *Ann N Y Acad Sci.* 2002;956:46–360.
5. Leigh RJ, Zee DS. Eye movements of the blind. *Invest Ophthalmol Vis Sci.* 1980;19:328–330.
6. CEMAS_Working_Group. *A National Eye Institute Sponsored Workshop and Publication on The Classification of Eye Movement Abnormalities and Strabismus* (*CEMAS*). Bethesda, MD: National Eye Institute Publications, National Institutes of Health, National Eye Institute: 2001,
7. Dell'Osso LF, Daroff RB. Braking saccade: a new fast eye movement. *Aviat Space Environ Med.* 1976;47:435–437.
8. Jacobs JB, Dell'Osso LF, Erchul DM. Generation of braking saccades in congenital nystagmus. *Neuro Ophthalmol.* 1999;21:83–95.

9. Bedell HE, Loshin DS. Interrelations between measures of visual acuity and parameters of eye movement in congenital nystagmus. *Invest Ophthalmol Vis Sci.* 1991;32:416–421.
10. Sullivan TJ, Lambert SR, Buncic JR, Musarella MA. The optic disc in Leber congenital amaurosis. *J Pediatr Ophthalmol Strabismus.* 1992;29(4):246–249.
11. Saari JC. The sights along route 65. *Nat Genet.* 2001;29(1):8–9.
12. Seeliger MW, Grimm C, Stahlberg F, et al. New views on RPE65 deficiency: the rod system is the source of vision in a mouse model of Leber congenital amaurosis. *Nat Genet.* 2001;29(1):70–74.
13. Redmond TM, Yu S, Lee E, et al. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet.* 1998; 20(4):344–351.
14. Aguirre GD, Baldwin V, Pearce-Kelling S, Narfstrom K, Ray K, Acland GM. Congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect. *Mol Vis.* 1998;4:23.
15. Narfstrom K, Wrigstad A, Nilsson SE. The Briard dog: a new animal model of congenital stationary night blindness. *Br J Ophthalmol.* 1989;73(9):750–756.
16. Acland GM, Aguirre GD, Ray J, et al. Gene therapy restores vision in a canine model of childhood blindness. *Nat Genet.* 2001;28(1):92–95.
17. Jacobs JB, Dell'Osso LF, Hertle RW, Acland GM, Bennett J. Eye movement recordings as an effectiveness indicator of gene therapy in RPE65-deficient canines: Implications for the ocular motor system. *Invest Ophthalmol Vis Sci.* 2006;47:2865–2875.
18. Flynn JT, Dell'Osso LF. Congenital nystagmus surgery. *Irish Fac Ophthalmol Yearbook.* 1980;1980:11–20.
19. Dell'Osso LF, Tomsak RL, Wang Z, Leigh RJ, Rucker JC, Jacobs JB. Combining peripheral-surgical (tenotomy) with either central-pharmacological (memantine) or other peripheral-surgical (anderson) therapies to damp acquired pendular or downbeat nystagmus and oscillopsia. In: Callaos N, ed. *Proceedings of the World Multiconference on Systemics, Cybernetics, and Informatics* (WMSCI). Orlando, FL: International institute of Informatics and Systemics; 2006:34–38.
20. Hertle RW, Dell'Osso LF, FitzGibbon EJ, Yang D, Mellow SD. Horizontal rectus muscle tenotomy in patients with infantile nystagmus syndrome: a pilot study. *J AAPOS.* 2004;8:539–548.
21. Dell'Osso LF, Jacobs JB. An expanded nystagmus acuity function: intra- and intersubject prediction of best-corrected visual acuity. *Doc Ophthalmol.* 2002;104:249–276.
22. Wrigstad A, Nilsson SE, Narfstrom K. Ultrastructural changes of the retina and the retinal pigment epithelium in Briard dogs with hereditary congenital night blindness and partial day blindness. *Exp Eye Res.* 1992;55(6):805–818.
23. Acland GM, Aguirre GD, Bennett J, et al. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol Ther.* 2005;12(6):1072–1082.
24. Bennicelli J, Wright JF, Komaromy A, et al. Reversal of blindness in animal models of Leber Congenital Amaurosis using optimized AAV2-mediated gene transfer. *Mol Ther.* 2008;16(3):458–465.
25. Dell'Osso LF, Williams RW, Jacobs JB, Erchul DM. The congenital and see-saw nystagmus in the prototypical achiasma of canines: comparison to the human achiasmatic prototype. *Vision Res.* 1998;38:1629–1641.
26. Dell'Osso LF. Tenotomy and congenital nystagmus: a failure to answer the wrong question. *Vision Res.* 2004;44:3091–3094.
27. Hertle RW, Maldanado VK, Maybodi M, Yang D. Clinical and ocular motor analysis of the infantile nystagmus syndrome in the first 6 months of life. *Br J Ophthalmol.* 2002;86(6):670–675.
28. Schmidt D, Dell'Osso LF, Abel LA, Daroff RB. Myasthenia gravis: dynamic changes in saccadic waveform, gain and velocity. *Exp Neurol.* 1980;68:365–377.
29. Abel LA, Schmidt D, Dell'Osso LF, Daroff RB. Saccadic system plasticity in humans. *Ann Neurol.* 1978;4:313–318.
30. Dell'Osso LF. New treatments for infantile and other forms of nystagmus. In: Leigh RJ, Devereaux MW, eds. *Understanding Mechanisms and Treatment of Congenital Forms of Nystagmus*. New York: Oxford University Press; 2008:87–98.
31. Dell'Osso LF, Wang ZI. Extraocular proprioception and new treatments for infantile nystagmus syndrome. *Prog Brain Res*. 2008; 171:67–75.
32. Wang Z, Dell'Osso LF, Zhang Z, Leigh RJ, Jacobs JB. Tenotomy does not affect saccadic velocities: support for the "small-signal" gain hypothesis. *Vision Res.* 2006;46:2259–2267.
33. Maguire AM, et al. Safety and efficacy of gene transfer for Leber's Congenital Amaurosis. *N Engl J Med.* 2008;358:2240–2248.