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The Role of the Human Visual Cortex in Assessment of the Long-Term Durability of Retinal Gene Therapy in Follow-on *RPE65* Clinical Trial Patients

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Purpose: Gene therapy (GT) has offered immense hope to individuals who are visually impaired because of *RPE65* mutations. Although GT has shown great success in clinical trials enrolling these individuals, evidence for stability and durability of this treatment over time is still unknown. Herein we explored the value of functional magnetic resonance imaging (fMRI) as an objective measure to assess independently the longevity of retinal GT.

Design: Individuals with *RPE65* mutations who underwent GT in their worse-seeing eye in a phase 1 clinical trial received a second subretinal injection in their contralateral eye in a follow-on clinical trial. Functional magnetic resonance imaging (MRI) was performed longitudinally to assess brain responses of patients with *RPE65* mutations after stimulation of their most recently treated eye before and 1 to 3 years after GT.

Participants: Seven participants with *RPE65* mutations who were part of the follow-on clinical trial gave informed consent to participate in a longitudinal neuroimaging fMRI study.

Methods: All participants underwent fMRI using a 3-Tesla MRI system and a 32-channel head coil. Participants' cortical activations were assessed using a block design paradigm of contrast reversing checkerboard stimuli delivered using an MRI-compatible video system.

Main Outcome Measures: The primary parameters being measured in this study were the qualitative and quantitative fMRI cortical activations produced by our population in response to the visual task.

Results: Functional MRI results showed minimal or no cortical responses before GT. Significant increase in cortical activation lasting at least 3 years after GT was observed for all participants. Repeated measures analysis showed significant associations between cortical activations and clinical measures such as full-field light sensitivity threshold for white, red, and blue colors; visual field; and pupillary light reflex.

Conclusions: Participants with *RPE65* mutations showed intact visual pathways, which became responsive and strengthened after treatment. Functional MRI results independently revealed the efficacy and durability of a 1-time subretinal injection. The fMRI results paralleled those recently reported during the long-term clinical evaluations of the same patients. Results from this study demonstrated that fMRI may play an important role in providing complementary information to patients' ophthalmic clinical evaluation and has usefulness as an outcome measure for future retinal intervention studies. *Ophthalmology 2017;■:1–11* © *2017 by the American Academy of Ophthalmology*

Supplemental material is available at www.aaojournal.org.

Leber's congenital amaurosis (LCA) is a rare blinding disease, usually inherited in an autosomal recessive fashion.[1] It is symptomatic at birth or in the first few months of life and affects approximate 1 in 81 000 people.[2] Leber's congenital amaurosis has been associated with at least 18 different genes.[3,4] The gene encoding retinal pigment epithelium-specific protein 65 kDa (*RPE65*) is involved in one of the more common forms of LCA called LCA2. *RPE65* mutations can also cause retinitis pigmentosa and other early-onset autosomal recessive retinal degenerations.[5,6] Individuals with *RPE65* mutations are good candidates for gene transfer therapy because the degeneration of retinal cells is slow, providing an extended potential time window for intervention. Recent studies in both animal models[7–11] of LCA and in humans[12–17] have demonstrated success in restoring retinal and visual function using measures such as visual acuity (VA), visual fields (VFs), light sensitivity, pupillary light reflex (PLR), mobility, or a combination thereof. There are several clinical trials that have carried out gene therapy for individuals with *RPE65*-mediated disease (see www.clinicaltrials.gov).[18] The program at the Children's Hospital of Philadelphia and the University of Pennsylvania is the first to carry out administration of AAV2-hRPE65v2 to the contralateral eye.

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Until now, it was not known whether severe impairment of the visual pathway resulting from congenital or early-onset inherited retinal degeneration would limit the responsiveness of vision processing neurons in the occipital cortex. Recently, we showed that in humans with LCA resulting from *RPE65* mutations, the visual cortex can be made responsive to visual input through unilateral ocular gene therapy, even after prolonged visual deprivation of up to 35 years.[19] In our previous studies, we used dim light stimuli because it is known that young individuals with *RPE65* mutations have some ability to see and navigate under brightly lit conditions.[20–22] Also, to account for variability in the disease stage among study participants and to correlate functional magnetic resonance imaging (fMRI) results with each participant's psychophysical measures, functional analyses were carried out separately for each individual participant.

In our initial report, treated and untreated eyes within the same *RPE65* participants were compared to assess the efficacy of gene therapy. Although there is a high degree of symmetry in disease progression between the 2 eyes, lack of baseline fMRI data for the initial injection made it difficult to reach a definitive conclusion on the magnitude and timing of the reported functional improvements. In the follow-on phase 1 clinical trial, the same participants who originally received a subretinal injection to their worse-seeing eye were candidates to receive administration of the AAV2-hRPE65v2 vector to their previously untreated contralateral eye. Neuroimaging results from 3 adults with *RPE65* mutations in the follow-on study subsequently were reported comparing the baseline cortical response of the contralateral eye with short-term effects of retinal gene therapy on the human visual cortex.[23] This report demonstrated that the visual cortex is extremely responsive to the stimulation of the photoreceptors via retinal gene therapy. As compared with baseline, participants with *RPE65* mutations showed significant cortical activations at 1 and 3 months after gene therapy administration. The follow-on study also demonstrated that prior exposure to the AAV2 vector did not result in any adverse effects to the second administration of AAV2-hRPE65v2 because of potential immunologic complications.[23] The current study went beyond examining the short-term effects of retinal gene therapy and evaluated the human brain responses in a large population of participants with *RPE65* mutations over a 3-year period. We hypothesized that fMRI results would be similar to those recently reported for the *RPE65* follow-on clinical trial[1] and that the fMRI results would demonstrate independently the long-lasting effects of a 1-time retinal gene therapy.

Methods

Study Participants

Participants were enrolled and evaluated as described (see ClinicalTrials.gov identifier NCT01208389; http://www.med.upenn.edu/carot/) at baseline and 1 to 3 years after surgical administration of AAV-hRPE65v2 to the fellow eye in the follow-on study. Although participants with *RPE65* mutations in the initial phase 1 clinical trial had received different doses and volumes of AAV2-hRPE65v2 in their first eye,[17,24] all participants received the high dose of 1.5E11 vector genomes in 300 μl for the follow-on study (Table 1). (The approximate location of the subretinal injection for the contralateral eye of all participants with *RPE65* mutations is presented in a recent report outlining a 3-year longitudinal clinical outcome of the follow-on clinical trial).[1] Overall, all participants with *RPE65* mutations except for 1 (patient CH10) received their subretinal injection as close as possible to the superior macula location.[1]

Seven of the original 10 participants who participated in the phase 1 neuroimaging study[19] were evaluated in the follow-on second eye study (Table 1). From the 10 original participants, patient CH13 was not eligible for intervention because of glaucoma in the contralateral, uninjected eye. Patient CH06 elected not to continue with the neuroimaging study. Longitudinal fMRI results from patient NP01 also are not included in the current report because patient NP01 had a history of smoking, and chronic smoking is known to abate cortical blood flow and has a dramatic effect on the fMRI cortical activations.[25] Patients NP03 and NP04 did not participate in any neuroimaging studies. After providing a complete description of the study, written informed consent (and when necessary, parental consent and child assent) was obtained from all participants for the longitudinal neuroimaging study. The Institutional Review Board of the Children's Hospital of Philadelphia approved all study procedures. All participants were assessed clinically as part of their qualification to enter the clinical trial for retinal gene therapy.[16,17,24] This study complied with the Health Insurance Portability and Accountability Act.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) scans were obtained at Children's Hospital of Philadelphia on a research-dedicated 3-Tesla Siemens Verio system using a 32-channel head coil (Siemens Medical Systems, Erlangen, Germany). All scans were carried out by a single operator and were monitored to be free of artifacts at the time of acquisition.

Functional Magnetic Resonance Imaging Sequence. Functional data were acquired using blood oxygenation level-dependent imaging, acquiring 3-mm isotropic resolution (matrix, 64×64; repetition time/echo time, 3000/30 ms) with a total acquisition time of 4:39 minutes. To permit T1 saturation, 3 additional volumes were acquired at the beginning of the fMRI experiment, but were not used in image analysis. A transistor-transistor logic pulse was used to start the stimuli automatically in sync with the start of fMRI acquisition. An MRI-compatible response device (a button that the participant pushed when recognizing the stimulus) was used to record participant responses. Participants were instructed to press the button once when the checkerboard first appeared.

Functional Magnetic Resonance Imaging Paradigm. In the past, while using simple contrast reversing checkerboard stimuli, we have been successful in showing the efficacy of gene therapy in this participant population.[19,23] Similar to our earlier study,[19,23] the current report used dim stimuli that went unperceived by most of the participants at baseline before retinal intervention. The purpose of this dim stimuli presentation was to assess the ability of participants in perceiving dim light after gene therapy. The fMRI paradigm consisted of 15-second blocks of flickering (8-Hz) black-and-white checkerboards, which consisted of 3 contrasts of high, medium, and low, interleaved with 15 seconds of blank (black) screens.[19,23] Participants were asked to fixate on a yellow cross in the center of the checkerboard patterns or, if they could not see the cross, to look straight ahead to their central vision. Participants additionally were asked to press the response button immediately after presentation of the visual stimuli and to hold the response

Table 1. Characteristics of Study Participants

Patient Identification	Age (yrs) at Readministration and Baseline Functional Magnetic Resonance Imaging Scan	Readministered Eye	*RPE65* Mutation(s)
NP02	30	Left	E102K/E102K
CH08	12	Left	F530fs/F530fs
CH09	11	Right	R124X/K297del1aggA
CH10	14	Left	IVS1+5g→a/F530del1ttc
CH11	26	Left	V473D/V473D
CH12	46	Left	K303X/W431C
NP15	14	Left	D167W/H313R

button if they experienced phosphenes.[26] Resonance Technology VisuaStim[27] goggles (Northridge, CA) featuring a digital display and a 30° horizontal field of view were used to present the fMRI stimuli. The visual paradigm was programmed in E-Prime (Psychology Software Tools, Inc., Pittsburgh, PA).[28]

Functional Magnetic Resonance Imaging Processing. All fMRI analyses were performed using the general linear model and the contrast of active blocks (checkerboard stimuli) minus the rest blocks (blank, black screen) as implemented in BrainVoyagerQX (Brain Innovation, Maastricht, The Netherlands).[29] To account for variability in the disease stage among study participants, fMRI data were analyzed individually (see Supplemental Information, available at www.aaojournal.org) for each participant (and not grouped as is done in most fMRI studies). A single participant approach is appropriate because participants with *RPE65* mutations differed by age and disease progression, and thus in the area of the retina in which there was evidence of sufficient (albeit unhealthy) retinal cells. Therefore, it is reasonable to expect that each individual participant will have a unique response to gene therapy and accordingly a unique fMRI cortical activation pattern. However, correlational analyses with clinical measures were carried out on a group level.

Real-Time Functional Magnetic Resonance Imaging. The research MRI system at Children's Hospital of Philadelphia is equipped with Siemens fMRI software that allows real-time monitoring of the participants' performances during fMRI experiments as well as their translational and rotational head movements.[23,26] Using the real-time feature, fMRI acquisition with 0.6 mm or more translational movement or 0.6° or more rotational movement was terminated, the participant was informed to stay still, and then the experiment was restarted.

Three-Dimensional T1-Weighted (Magnetization-Prepared Rapid Gradient Echo [MPRAGE]) Imaging. A 3-dimensional isotropic structural high-resolution T1 sequence was acquired with inversion preparation pulse (repetition time, 2080 ms; echo time, 2.54 ms; bandwidth (BW), 180 Hz/Px; matrix size, 320×320; field of view (FOV), 256×256 mm^2; 192 axial slices; slice thickness, 0.8 mm; inversion time, 1200 ms; with flip angle, 8°; number of extractions (NEX), 1; echo spacing, 7.8; integrated parallel acquisition technique (iPAT), 2; and scan time, 7:04 minutes). This sequence was obtained for visual activation localization and generation of inflated hemispheres.[29]

Vision Testing and Ocular Examination. Participants with *RPE65* mutations underwent a comprehensive clinical evaluation as part of an approved clinical trial protocol. In addition to other measures, participants' clinical evaluations included multiple clinical tests of visual function including evaluation of VA, VF, pupillometry light reflex (PLR) evaluating for difference in responses between the 2 eyes, and full sensitivity testing (FST) for white, red, and blue light sources.[1,17,24] Spark Therapeutics, Inc. (Philadelphia, PA), carried out measures of VA and VF, FSTs, and other testing under sponsorship. For the current study, amplitudes of the pupillary light reflex were measured in each eye individually at baseline to the second eye injection and then in follow-up testing. The PLR results were measured independently from the follow-on clinical trial retinal and visual function data carried out by Spark Therapeutics. Results from the above-mentioned clinical tests were obtained for the follow-on study at baseline and 1 to 3 years after gene therapy evaluation to assess possible correlations between these clinical measures and fMRI results.

Results

Functional Magnetic Resonance Imaging Results

Functional MRI results presented here are the sum of high- and medium-contrast stimuli in response to stimulation of the newly treated eye using a flickering checkerboard paradigm.[19,23] We did not include the results from the low-contrast stimuli because almost no participant with *RPE65* mutations was able to see the lowest contrast (dimmest stimulus), and fMRI analysis of low-contrast stimulus demonstrated minor or no cortical activations. We also do not discuss the results from the eyes injected before the follow-on study because there was no baseline fMRI assessment performed before initial administration of gene therapy. Detailed fMRI results for individual patients are presented in the Supplemental Information (available at www.aaojournal.org). Overall, fMRI after gene therapy administration to the contralateral eye showed significant cortical activation in and around the visual cortex for all participants with *RPE65* mutations for full-field contrast-reversing (8-Hz) checkerboard stimuli at high and medium contrasts. Presentation of the same stimuli at baseline (before gene therapy) showed minimal cortical activations (except in patient NP15) for high- and medium-contrast stimuli. As shown in Figure 1, compared with baseline, all participants with *RPE65* mutations showed significantly elevated levels of cortical activations at 1 year and continued this trend for their fMRI evaluations at years 2 and 3. As expected, patterns of activations varied in each individual depending on their age, the progression of disease, and location of the subretinal injection. Generally, participants showed compensatory activations primarily in and around the extrastriatal cortex outside the primary visual cortex at the follow-on baseline time point (see the first column of Fig 1, except patient NP15).

This pattern of activation changed dramatically after retinal gene therapy, showing a significant increase in the amount of activation within the primary visual areas for all participants except patient CH12, who is the oldest study participant with advanced retinal degeneration (47 years of age; see columns 2–4 of Fig 1). Annual fMRI evaluation for a few participants could not be obtained for some of their time points because of participant

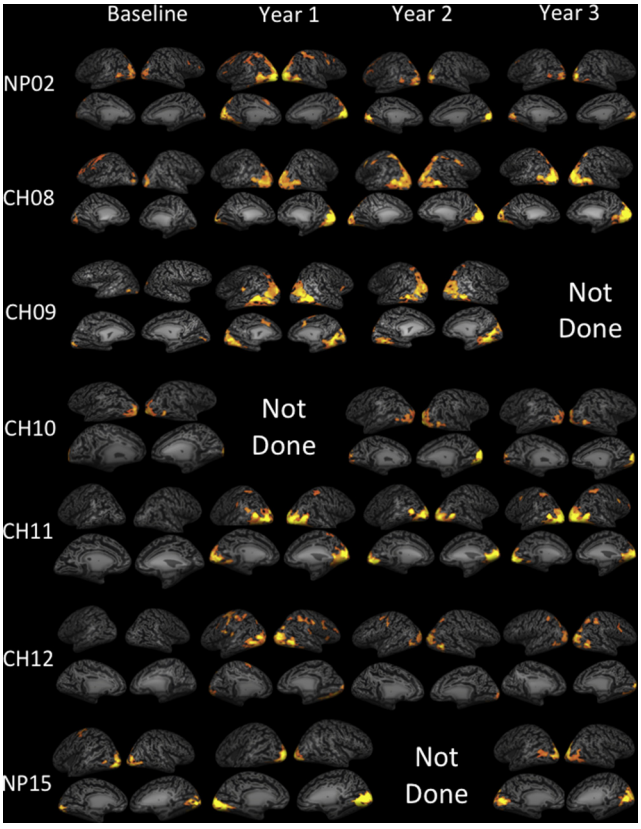

Figure 1. Longitudinal functional magnetic resonance imaging (fMRI) results for 7 individual participants with *RPE65* mutations at baseline, 1 year, 2 years, and 3 years after retinal gene augmentation therapy superimposed on their inflated cortex. Cortical activations are assessed in response to the stimulation of the newly treated eye for both the high- and medium-contrast stimuli.[19] Results are presented for the left eye for all but 1 participant (patient CH09) who received readministration to his right eye. The fMRI results for all participants were corrected for multiple comparisons using a false discover rate (<5%) and a corrected $P < 0.004$, with a continuous connected area threshold ($\geq$100 mm^2) that further controls for multiple comparison type 1 error.[19,23] To show cortical activations at baseline, fMRI results required considerably relaxed statistical threshold of an uncorrected $P < 0.05$ and continuous connected area threshold of 50 or more, except for patients NP15 and NP02. Using the above statistics, statistically significant areas of cortical activations are shown as yellow and orange clusters overlaid onto the medial and lateral representations of inflated cortex for each individual participants. Overall comparison of the baseline and 3-year follow-up fMRI results shows a notable increase in widespread bilateral activations in all areas of visual cortex extending from medial to lateral and posterior to anterior aspects of the occipital cortex 1 year after gene therapy for most participants. Activation patterns vary for individual participants depending on age, disease progression, or other reasons. Changes in cortical activations over time for baseline and 1 year after retinal gene therapy using responses to only the high-contrast stimuli (here we used responses to the high and medium contrasts) were reported in a recent report by Bennett et al (2016).[1]

unavailability or a change in their status preventing fMRI examination (e.g., having dental braces). Three of the participants (patients NP02, CH08, and CH11) intermittently experienced phosphenes.[26] To control for effects of this phenomenon during the course of fMRI, participants were directed to press a button if they were experiencing phosphenes. The experiment was repeated if participants reported seeing phosphenes during the stimulus presentation. However, some of the longitudinal results, particularly from these 3 participants, may be attributed to unreported phosphene events. All fMRI analyses were performed using the same statistical threshold of false discovery rate (fdr) (5%)-corrected P value less than 0.004 and an extent threshold (continuous connected area) of 100 or more continuous voxels. The following is a detailed explanation of the results for each participant with *RPE65* mutations before and after gene therapy.

Changes of the Visual Cortex Activation over Time

The mean and standard error for the cortical activations for baseline and 1 to 3 years after the delivery of subretinal injection are presented in Supplemental Table S1 (available at www.aaojournal.org). The graphic representation of the changes in the mean value of cortical activations over time for the entire visual cortex and activation of its medial surface are presented in Figure 2A and B, respectively. Changes in cortical activations over time for baseline and 1 year after retinal gene therapy using responses to only the high-contrast stimuli (here we used responses to the high and medium contrasts) were reported in a recent report by Bennett et al.[1]

Association between Total Visual Cortex Activations and Clinical Measures

Longitudinal regression analysis using the linear mixed effect models (using random intercept) was performed with image parameters as predictors and clinical measures as outcome variables to assess the association between the activations across the entire surface area of the visual cortex (Fig S1, available at www.aaojournal.org), the medial surface of the visual cortex (Fig S2, available at www.aaojournal.org), and each of the clinical measures over time. The results of the mixed effects models that evaluate the association between the entire areas of the right hemisphere (RH), left hemisphere (LH), and total hemisphere (TH) activations over time and all available clinical measures are presented in Table 2. As shown in Table 2, all clinical measures were correlated significantly with the longitudinal fMRI results except for the VF and VA.

The FST measures for the white light were associated with the LH (regression coefficient [rc], 74.24; $P < 0.003$), RH (rc, 72.61; $P < 0.009$), and TH (rc, 146.85; $P < 0.005$), and it also correlated for the red light (LH: rc, 101.97; $P < 0.02$; RH: rc, 96.02; $P < 0.05$; and TH: rc, 197.24; $P < 0.03$) and blue light (LH: rc, 75.63; $P < 0.002$; RH: rc, 73.65; $P < 0.008$; and TH: rc, 149.28; $P < 0.004$). Total occipital cortical activation areas were associated strongly with temporal changes in the PLR of the participants with *RPE65* mutations (LH: rc, 39.99; $P < 0.02$; RH: rc, 53.71; $P < 0.004$; and TH: rc, 93.93; $P < 0.007$). The graphic representation of the association between the area of activations for TH and the FST measures of the white, red, and blue lights for all their longitudinal measurements are presented in the top section of Figure 3. The graphical results for the association between the TH area of activations and PLR, VF, and VA measures are presented in the top section of Figure 4. All other graphs depicting the association between the LH and RH areas of activations and FST, PLR, VF, and VA measures are presented in Supplemental Figures S3–S6 (available at www.aaojournal.org).

Association between the Activations of the Medial Visual Cortex and Clinical Measures

The association between the clinical measures and the volume of activations located mainly within the medial surface of the visual

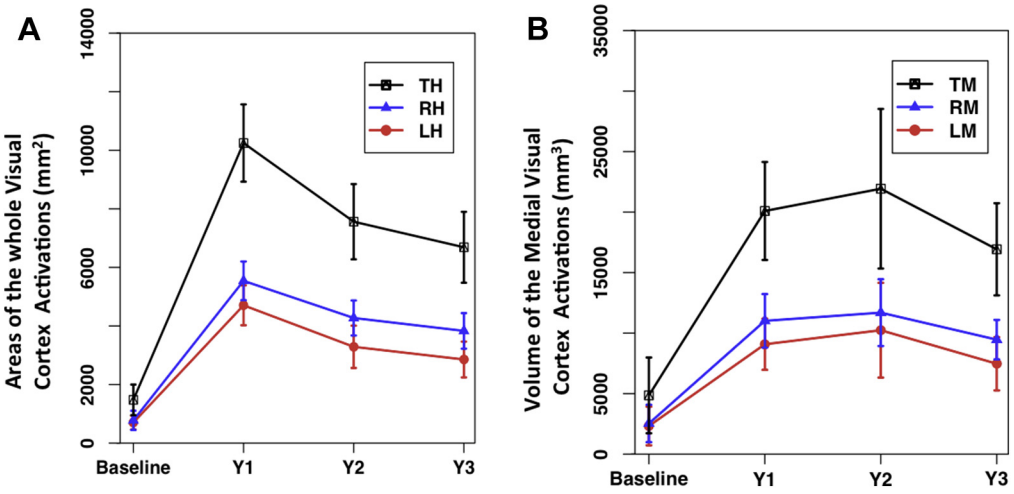

Figure 2. Graphs showing longitudinal cortical activation results (mean and standard error of the left, right, and total visual cortical activations) in response to a contrast-reversing checkerboard stimulus for patients with *RPE65* mutations at baseline and 1 to 3 years (Y) after receiving gene therapy in their contralateral eyes. **A**, Mean ± standard error (SE) of the area of cortical activations for the entire area of the left hemisphere (LH), right hemisphere (RH), and total visual cortex (TH) over time. **B**, Mean ± SE of the volume of cortical activations within the medial surface of the visual cortex for the left medial activations (LM), right medial activations (RM), and total medial activations (TM). As compared with baseline, cortical activations at years 1 through 3 for both the medial and total visual cortex activations were all statistically significant ($P < 0.05$).

cortex (in and around the primary visual cortex; Fig S2, available at www.aaojournal.org) is presented in Table 3. As shown in Table 3, stronger associations were observed with the volume of the medial activations and participants' clinical measures as compared with regression coefficients and significance observed with the total visual cortex surface activations and participants' clinical measures (compare with values in Table 2). This is particularly evident for the association of the medial cortical activation volume with the participants' VF (left medial [LM]: rc, 5.05; $P < 0.21$; right medial [RM]: rc, 6.38; $P < 0.07$; total medial [TM]: rc, 11.62; $P < 0.11$). The changes in the volume of the medial cortical activations were associated strongly with the changes observed in participants' FST for white light (LM: rc, 227.65; $P < 0.01$; RM: rc, 264.84; $P < 0.001$; TM: rc, 495.08; $P < 0.002$), red light (LM: rc, 294.97; $P < 0.06$; RM: rc, 392.00; $P < 0.005$; TM: rc, 689.58; $P < 0.02$), and blue light (LM: rc, 212.80; $P < 0.02$; RM: rc, 262.79; $P < 0.001$; TM: rc, 475.63; $P < 0.005$). The strongest and the most significant association was observed between the volume of activations of the medial visual cortex and the PLR results of participants with *RPE65* mutations (LM: rc, 119.57; $P < 0.001$; RM, rc, 139.71; $P < 0.001$; TM: rc, 261.36; $P < 0.001$). Similar to the total visual cortex surface activation, the volume of activations of the medial surface did not show any association with the VA measurements of participants with *RPE65* mutations over time. The graphic representation of the association between the volume of activations for TM and for FST measures of the white, red, and blue lights over time are presented in the bottom section of Figure 3. The graphic results for the association between the TM volume of activations and PLR, VF, and VA measures are presented in the bottom section of Figure 4. All other graphs depicting the association between the LM and RM volumes of activations and FST, PLR, VF, and VA measures are presented in Supplemental Figures S3–S6 (available at www.aaojournal.org).

Table 2. Regression Analysis Results between Overall Cortical Activations and Clinical Measures

	Area of Cortical Activation (mm²)					
	Left Hemisphere		*Right Hemisphere*		*Total Hemisphere (Left Hemisphere + Right Hemisphere)*	
Clinical Measures	*Regression Coefficient (Standard Error)**	P *Value*	*Regression Coefficient (Standard Error)**	P *Value*	*Regression Coefficient (Standard Error)**	P *Value*
Visual field (degrees)	0.75 (1.08)	0.50	0.78 (1.14)	0.50	1.45 (2.14)	0.51
Full-field stimulus threshold (dB)						
White light	74.24 (21.13)	0.003	72.61 (24.58)	0.009	146.85 (44.91)	0.005
Red light	101.97 (40.15)	0.02	96.02 (45.48)	0.0499	197.24 (84.03)	0.03
Blue light	75.63 (20.86)	0.002	73.65 (24.38)	0.008	149.28 (44.44)	0.004
Pupillary light reflex (mm × 100)	39.99 (14.52)	0.02	53.71 (14.90)	0.004	93.93 (29.10)	0.007
Visual acuity (logMAR × 10)	−3.14 (73.69)	0.97	28.72 (80.01)	0.72	25.58 (151.68)	0.87

logMAR = logarithm of the minimum angle of resolution.
The linear regression analysis results for the association between the entire visual cortex activation areas of the left hemisphere, right hemisphere, and total hemisphere and each of the *RPE65* clinical measures.
*From linear mixed effects model with each of measure of area of cortical activation as dependent variables and each of clinical measures as independent variables.

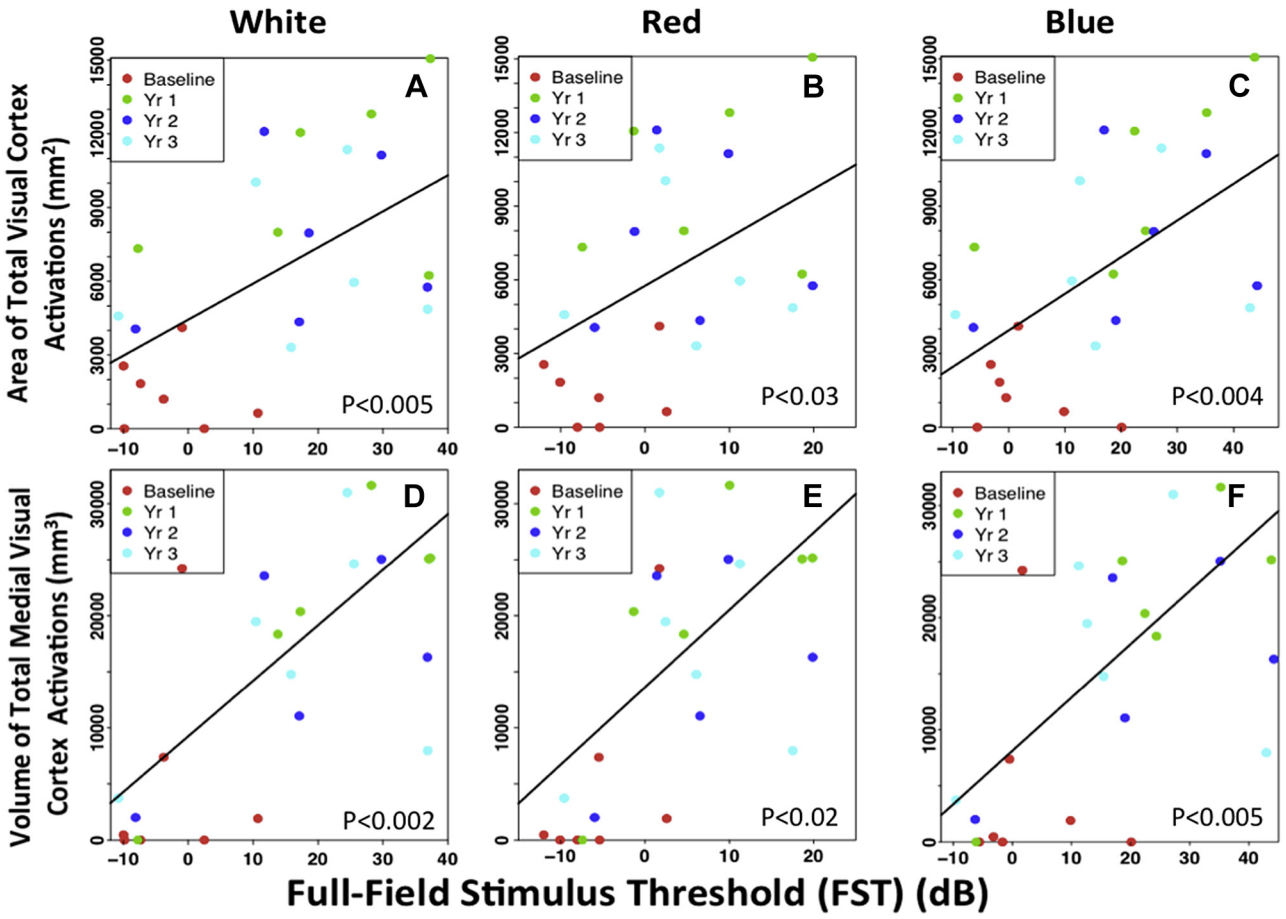

Figure 3. Scatterplots with linear regression lines from the mixed effect model showing the association of cortical activations and full-field light sensitivity threshold (FST) measures using white, red, and blue light sources. The top portion presents the association between the FST measures of (**A**) white, (**B**) red, and (**C**) blue light stimuli and the area of the total visual cortex activations. The bottom section shows the association between the FST measures of (**D**) white, (**E**) red, and (**F**) blue lights and the volume of the total medial visual cortex activations. Both values of the cortical activations are associated significantly with the increase in light sensitivity in *RPE65* patients over time. However, the activations of the medial cortex are associated more strongly with the FST light sensitivity measures (see "Discussion"). Yr = year.

Discussion

This study was carried out in a population of participants with *RPE65* mutations who received administration of AAV2-hRPE6v2 (1.5×10^{11} vector genomes) to the contralateral eye as a follow-on study of an *RPE65* gene therapy phase 1 clinical trial. Previously, the same participant group had received unilateral AAV2 subretinal injections in their worse-seeing eye[17,24] in a dose-escalation study, with doses ranging from 1.5×10^{10} to 0.5×10^{11} vector genomes. Gene therapy administration to the contralateral eye occurred an average of 2 years after phase 1 unilateral treatment administration. As part of the follow-on clinical trial, participants with *RPE65* mutations received follow-on evaluations for 3 years after administration of gene therapy to their contralateral eyes. Their long-term clinical evaluations demonstrated not only a high degree of safety and efficacy despite having a previous subretinal injection, but also a long-lasting effect of up to 3 years for a 1-time delivery of gene augmentation therapy.[1] Separate from the main clinical trial, a longitudinal neuroimaging study was conducted to characterize independently the efficacy of this 1-time subretinal injection in a subgroup of the participants with *RPE65* mutations who participated in the follow-on clinical trial (Table 1). The neuroimaging study compared the spatiotemporal patterns of this subgroup's brain activations using fMRI at baseline and annually for 3 years after receiving gene therapy. Functional MRI comparisons were used to assess and quantify the association between cortical activations and the participants' clinical measures over time. As we have described previously,[19] the fMRI paradigm used in our experiments was selected to be composed of stimuli with dim light because it is known that young participants with *RPE65* mutations have some ability to see and navigate their surroundings under high-intensity lighting conditions.[20–22] Also, to account for variability in disease progression among the different participants with *RPE65* mutations and to assess the association of fMRI results with a participant's clinical measures, functional analyses were carried out separately for each individual participant.

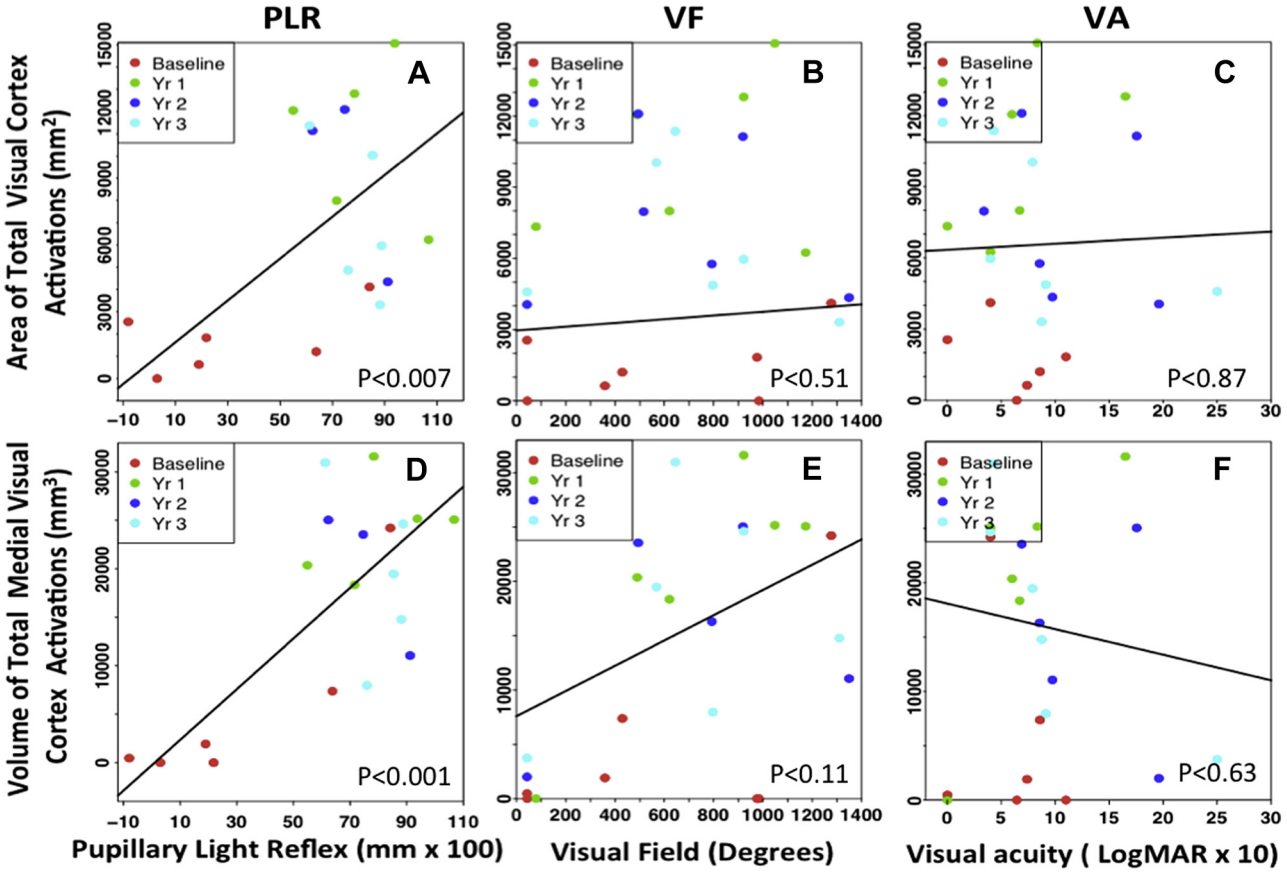

Figure 4. Scatterplots with linear regression lines from the mixed effect model showing the association of cortical activations with the pupillary light reflex (PLR), visual field (VF), and visual acuity (VA) measures. The top portion shows the association of the (**A**) PLR, (**B**) VF, and (**C**) VA measures with the area of the total visual cortex activations. The bottom portion shows the association between the (**D**) PLR, (**E**) VF, and (**F**) VA measures with the volume of the total medial visual cortex activations. Both measures of the cortical activations are significantly associated with the PLR of the patients with *RPE65* mutations over time. However, the medial activations are associated more strongly with the VF over time. No association was observed with the VA for either the area of the total visual cortex or the volume of the medial activations. logMAR = logarithm of the minimum angle of resolution; Yr = year.

In our previous report,[19] treated and untreated eyes within individual participants with *RPE65* mutations were compared to assess the efficacy of gene therapy. However, lack of baseline data on cortical responses from an untreated eye (for the initial injection and study) prevented a true presentation of the magnitude and pattern of improvement induced by gene therapy. In the present report, we provide functional brain responses that clearly

Table 3. Regression Analysis Results between Medial Cortical Activations and Clinical Measures

	Volume of Medial Activation (mm^3)					
	Left Medial		*Right Medial*		*Total (Right Medial + Left Medial)*	
Clinical Measures	*Regression Coefficient (Standard Error)**	*P Value*	*Regression Coefficient (Standard Error)**	*P Value*	*Regression Coefficient (Standard Error)**	*P Value*
Visual field (degrees)	5.05 (3.86)	0.21	6.38 (3.27)	0.07	11.62 (6.95)	0.11
Full-field stimulus threshold (dB)						
White light	227.65 (78.11)	0.01	264.84 (62.77)	<0.001	495.08 (137.00)	0.002
Red light	294.97 (145.13)	0.06	392.00 (120.41)	0.005	689.58 (260.44)	0.02
Blue light	212.80 (84.41)	0.02	262.79 (68.78)	0.001	475.63 (149.19)	0.005
Pupillary light reflex (mm × 100)	119.57 (26.98)	<0.001	139.71 (26.96)	<0.001	261.36 (51.39)	<0.001
Visual acuity (logMAR × 10)	−142.21 (255.63)	0.59	−102.75 (228.04)	0.66	−235.33 (477.05)	0.63

logMAR = logarithm of the minimum angle of resolution.
The linear regression analysis results for the association between the volumes of the visual cortex medial activations with each of the *RPE65* clinical measures.
*From linear mixed effects model with each measure of medial activations as dependent variables and each of clinical measures as independent variables.

show the efficacy of retinal gene therapy and its durable effect for at least 3 years after a subretinal injection. Comparison of presurgical and postsurgical fMRI data provides further evidence for the effectiveness of gene therapy for participants with *RPE65* mutations and shows that a therapeutic effect is observed even after prior exposure to AAV2-hRPE65v2.

Consistent with our previous reports,[19,23] the longitudinal fMRI results from the second eye showed that when presented with the same visual stimuli,[19] participants with *RPE65* mutations responded minimally at baseline as compared with their postintervention results. Thus, before receiving the subretinal injection, participants with *RPE65* mutations had minimal compensatory or no response to the checkerboard stimuli, whereas all participants became dramatically responsive after treatment and their initial postsurgical responsiveness to the treatment was maintained through the duration of this fMRI study. The results from the longitudinal fMRI study, which shows lasting cortical activations over 3 years with a 1-time subretinal injection, parallels the 3-year longitudinal clinical evaluation results recently reported.[1] These lasting cortical activations likely are related to structural improvements observed in these participants, which in turn are strengthened further through visual experience.[30–32]

The fMRI results clearly show that the activation of the visual cortex closely corresponds to the AAV-exposed portion of the retinas. As depicted in Figure 1, fMRI results from the participants with *RPE65* mutations after gene therapy showed bilateral occipital cortex activations that correspond to the superior macula subretinal injection site that has spread to the entire nasotemporal retinal area for all participants except patient CH10 (see column 1 of Fig 1 in Bennett et al[1]). Activation for patient CH12, the oldest participant at 46 years of age, resulted in increased bilateral compensatory activations in the extrastriatal cortex, although there was no change in the activation of the primary visual cortex (medial surface of the occipital lobe). The location of the subretinal injection is expected to generate bilateral fMRI activation in the cortical distribution, as was observed in most participants with *RPE65* mutations, with lesser extent in patient CH10. However, as shown in Figure 2A and B, cortical activations were consistently higher for the right hemisphere at all time points. This asymmetric activation pattern can be attributed to the fact that most participants with *RPE65* mutations (6/7) received the first subretinal injection in the right eye in the superior temporal aspect of their retina. Injections administered in this area of the retina cause preferential right visual pathway strengthening because the right temporal projections from the retina stay on the right visual cortex and do not cross to the left hemisphere.[32]

In general, visual processing starts at the retina, where the light rays that enter the eye are converted by the photoreceptors (rods and cones) to electrical signals. These signals then are transmitted through the retina to the retinal ganglion cells (RGCs), which transfer this information through the optic nerve to the lateral geniculate nucleus of the brain. The lateral geniculate nucleus then proceeds to send signals to the primary visual areas of the occipital lobe.[33] Information from these areas then is projected to other areas of the cerebral cortex (extrastriate) that are involved in higher complex visual perceptions. Thus, these primary visual areas (which are located along the medial surfaces of the visual cortex) receive the main visual signals from the photoreceptors in the retina and are responsible for our first sense of visual perception.[33] In participants with *RPE65* mutations whose photoreceptors are highly nonfunctional,[3] the process of light-to-electrical signal conversion is greatly interrupted. As such, depending on the disease progression, few or no light rays are converted by the photoreceptors to be transmitted to RGCs, resulting in diminished visual signal transmission to the primary visual areas. On subretinal administration of the AAV2-hRPE65v2 vector (gene therapy), the missing isomerohydrolase (*RPE65*) is restored, and portions of the revived photoreceptors regain their processing ability of transferring electrical activities to the RGCs and eventually to the primary visual areas.

We believe the fMRI results from participants with *RPE65* mutations, presented in Figure 1, accurately reflect the timeline of changes in the stimulation of the photoreceptor populations from their nonfunctioning state at baseline to when they reconnect with RGCs and reinstate vision after gene therapy. As shown in the first column of Figure 1, at baseline (before retinal intervention and when photoreceptors are nonfunctional), there are no or small amounts of activation in and around primary visual areas for all participants with *RPE65* mutations except for patient NP15, who showed the highest visual functions and cortical activations at baseline. As depicted in columns 2, 3, and 4 of Figure 1, subsequent to receiving retinal gene therapy (when portion of photoreceptors are reinstated by gene therapy), with the exception of patient CH12 (the oldest participant at 46 years), the levels of activation within the primary visual areas (medial surface of visual cortex) of participants with *RPE65* mutations increased considerably as compared with baseline and stayed at an elevated level through years 2 and 3 of fMRI examination. Although levels of activations within the extrastriate cortex also increased, it is important to note that the primary visual areas in fact mediate much of the information to the extrastriate cortex.[33,34] In addition to receiving information from the primary visual areas, the extrastriate cortex also is known to receive direct inputs from the retina through the pulvinar and superior colliculus.[35–38] This component is particularly dominant in the absence of visual signals to the primary visual areas.[39–42] This may explain the reason for higher levels of extrastriate activations before gene therapy (see first column in Fig 1), when few or no visual signals were transmitted to the primary visual areas because of photoreceptor malfunction. Thus, when comparing column 1 of Figure 1 (baseline, before gene therapy) with other columns (after gene therapy), the change in the pattern of cortical activations closely follows the process of gene therapy. Our results show minimal activations when photoreceptors are nonfunctional and significant amount of

activations after the rescue or revival of the photoreceptors, particularly in the primary visual cortex.

Because of the primary visual cortex's key role in the hierarchy of the visual system, the fMRI correlations with patients' clinical measures were performed separately for the total cortical activations distributed across the entire surface of the visual cortex (Fig S1, available at www.aaojournal.org) and the activations limited to the medial surface of the visual cortex (Fig S2, available at www.aaojournal.org). Results for the association of the clinical measures and fMRI cortical activations over time are presented in Figures 3 and 4 for the entire visual cortex activations and the activations restricted to the medial surface of the visual cortex for each hemisphere, respectively. As expected, values for the primary visual cortex activations were stronger predictors of the change in the participants' clinical measures over time, particularly for the VF measure (Tables 2 and 3 and Figs 3 and 4). We observed similar associations for the change in cortical activations over time and the participants' clinical measures for the left and the right visual cortex separately (Supplemental Figs S3–S6, available at www.aaojournal.org). However, comparing the results from the left and right hemispheres, the right hemisphere's medial surface activation values showed higher significance in association and predictive power for the participants' clinical outcomes (Tables 2 and 3; and Figs S3–S6, available at www.aaojournal.org). We hypothesize that the observed left–right asymmetry in the correlation results may be because 6 of 7 participants received gene therapy to their right eyes with a subretinal injection in the superior aspect of their temporal retina, which has cortical projections to the brain that remain on the ipsilateral (right) cortex.[32]

Neither of the 2 cortical activation measures (medial surface or entire visual cortex) showed significant association with the changes in the VA of participants with *RPE65* mutations. Most importantly, this lack of association could be because the 3-year follow-up results from the clinical assessment of these participants with *RPE65* mutations showed no improvement in VA over time.[1] This also may be because the cortical activation areas of the most posterior pole of the visual cortex—to where the foveal region of the retina projects—were not measured separately for corrections with VA. Smaller checkerboard patterns than the one used in our experiments are reported to stimulate the foveal region responsible for VA preferentially (Kothari et al, 2014)[43]; however, larger checkerboard size is used because most individuals with retinal disease lack the VA to detect smaller checkerboard stimuli (Donnell, 2015).[44] Additionally, only 5 of the participants with *RPE65* mutations received full foveal exposure, and 3 of them did not participate in the neuroimaging study. Furthermore, the foveal cones may have degenerated severely by the time the vector was administered, and it may not have been possible to recover them successfully (unless treatment is applied earlier in life).

The mechanism by which vision changes affect neuronal circuitry is complex. Much of our knowledge of visual system plasticity after visual restoration comes from animal studies. We studied this phenomenon in vivo, through noninvasive brain imaging before and longitudinally after retinal intervention in a group of participants with autosomal recessive mutations in *RPE65* who experienced improved vision after retinal gene therapy. To our knowledge, there is no prior demonstration of long-term temporal/spatial changes in retinal/cortical activations in humans, as reflected by the response of the visual cortex. The longitudinal fMRI results unequivocally show the efficacy of 1-time gene therapy by demonstrating the absence of cortical responses from affected retinal cells before gene therapy and significant and widespread cortical activation after gene therapy, with durability of up to 3 years and ongoing observation. More importantly, the long-lasting visual cortex activations, particularly those restricted to the primary visual areas, showed significant association with the changes in the participants' clinical measures, such as FST response to the white, blue, and red lights; PLR; and, to a lesser extent, the changes in the participants' VFs. As such, fMRI may have the potential to provide quantitative neuroimaging biomarkers to serve as an outcome or predictive measure potentially to augment clinical evaluations of future gene and cell therapy participants before and after retinal intervention.

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Abbreviations and Acronyms:
fMRI = functional magnetic resonance imaging; **FST** = full-field light sensitivity threshold; **GT** = gene therapy; **LCA** = Leber's congenital amaurosis; **LH** = left hemisphere; **LM** = left medial; **MRI** = magnetic resonance imaging; **PLR** = pupillary light reflex; **RGC** = retinal ganglion cell; **RH** = right hemisphere; **RM** = right medial; **TH** = total hemisphere; **TM** = total medial; **VA** = visual acuity; **VF** = visual field.

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