Spectral sensitivity measurements reveal partial success in restoring missing rod function with gene therapy

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Restored rod visual function after gene therapy can be established unequivocally by demonstrating that, after dark adaptation, spectral sensitivity has the shape characteristic of rods and that this shape collapses to a cone-like shape before rods have recovered after an intense bleach. We used these tests to assess retinal function in eight young adults and children with early-onset severe retinal dystrophy from Phase II of a clinical gene-therapy trial for RPE65 deficiency that involved the subretinal delivery of a recombinant adeno-associated viral vector carrying *RPE65*. We found substantial improvements in rod sensitivity in two participants: dark-adapted spectral sensitivity was rod-like after treatment and was cone-like before rods had recovered

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after a bleach. After 40 min of dark adaptation, one participant showed up to 1,000-fold sensitivity improvements 4 months after treatment and the second up to 100-fold improvements 6 months after treatment. The dark-adapted spectral sensitivities of the other six participants remained cone-like and showed little improvement in sensitivity.

Introduction

Leber's congenital amaurosis (LCA) encompasses a group of recessively inherited, severe, infantile-onset, rod–cone dystrophies that typically result in severe visual impairment (Hanein et al., 2004; Perrault, Rozet, Gerber et al., 1999). One form of the disease, LCA2, is caused by mutations in the *RPE65* gene that encodes RPE65, a retinal pigment epithelium–specific 65-kDa isomerase (Gu et al., 1997; Lotery et al., 2000; Marlhens et al., 1997; Perrault, Rozet, Ghazi et al., 1999; Thompson et al., 2000). The protein catalyzes the isomerization of all-*trans*-retinyl esters to 11-*cis*-vitamin A and is thus a key component of the visual cycle—the biochemical pathway that regenerates visual pigment after exposure to light (Jin, Li, Moghrabi, Sun, & Travis, 2005; Lamb & Pugh, 2004; Mata et al., 2004; Moiseyev, Chen, Takahashi, Wu, & Ma, 2005; Redmond et al., 1998; Redmond et al., 2005; Thompson & Gal, 2003). Lack of functional RPE65 results in deficiency of 11-*cis* retinal and results in rod photoreceptor cells that are unable to respond to light (Burns & Baylor, 2001; Lamb, 1999). LCA2 patients are thus typically reported to be night blind. Cone photoreceptor cells, by contrast, have an alternative pathway that does not depend on retinal pigment epithelium–derived RPE65, thus allowing cone-mediated vision in younger patients with LCA2 (Wu et al., 2004; Znoiko, Crouch, Moiseyev, & Ma, 2002). Despite the alternative pathway, cone vision is abnormal from infancy and deteriorates with time (Hanein et al., 2004; Perrault, Rozet, Gerber et al., 1999).

We report spectral sensitivity measurements made in nine participants with an infantile onset, rod–cone dystrophy caused by *RPE65* mutations (see Table 1). All but one of the participants (P3) were enrolled in Phase II of a Phase I/II dose-escalation trial of gene therapy for RPE65 deficiency (Bainbridge et al., 2015) of which the measurements reported here formed a small part. The trial involved subretinal delivery of a recombinant adeno-associated virus (rAAV) vector expressing human *RPE65* under the control of a human *RPE65* promoter (rAAV2/2. hRPE65p.hRPE65). In Phase I of the trial, the vector was administered at a 10-fold lower dose (1×10^{11} vector particles in 1 ml) than in Phase II (1×10^{12} vector particles in 1 ml). The vector was injected in the superior retina and covered a region that included the fovea in all but two participants (P9 and P11). Further details can be found in Bainbridge et al. (2008) and Bainbridge et al. (2015).

We measured detection sensitivities as a function of wavelength with sufficiently dense spectral sampling to estimate which photoreceptors mediate detection. Here, we test specifically for restored rod visual function.

Reports of the early stages of *RPE65* gene replacement trials found mainly modest improvements in visual performance (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008). Further, Cideciyan et al. (2008, 2009b) reported improvements in sensitivity in three participants at peripheral locations near the gene-therapy treatment sites following normal periods of dark adaptation and reported further improvements after extended periods of dark adaptation of up to 8 hr in two participants. Using extended periods of dark adaptation, sensitivity was subsequently shown to improve in 15 participants (Jacobson et al., 2012). However, because only two spectrally different targets were used in these measurements, the identity of the photosensitive mechanism (or mechanisms) mediating target detection remains equivocal.

If rod function is restored by gene therapy, there should be an improvement in overall dark-adapted sensitivity plus a characteristic change in the *shape* of the measured dark-adapted spectral sensitivity function from a cone-like shape to a rod-like shape. For the shape changes to be conclusive, it is vital that measurements be made at a sufficient number of wavelengths. We found that, in general, four wavelengths (450, 500, 550, and 600 nm) were sufficient to distinguish rod from cone function in our spectral sensitivity data. As an additional control, rod visual function can be confirmed by demonstrating that the dark-adapted rod shape then collapses to a cone shape when cones, but not rods, have recovered following an intense bleach.

Methods

Subjects

Eight participants formed Phase II of the clinical trial in which the rAAV vector expressing human RPE65 was administered at 1×10^{12} vector particles in 1 ml. Extensive testing of other visual function, reported elsewhere, were performed. Here, we report only the dark-adapted and cone-plateau spectral sensitivity measurements. The participants proved to be proficient and reliable psychophysical observers with boundless patience and generous of their time.

The type of genetic mutation in each of the eight participants of the second phase of the trial (P5–P12),

Subject code	Gender	Age at treatment (years, months)	Mutation	Acuity (L/R) (Log MAR) *treated eye
P3	M	18, 0	c.[16G > T] + [499G > T] p.[Glu6X] + [Asp167Tyr]	0.50/0.76*
P5	M	23, 3	c. [1102T > C] + [1102T > C] p.[Tyr368His] + [Tyr368His]	0.31/0.36*
P6	M	17, 10	c. [1102T > C] + [1102T > C] p.[Tyr368His] + [Tyr368His]	0.53/0.68*
P7	F	10, 2	c.[11+5G > A] + [12-2A > G] p.[?] + [?]	0.46/0.44*
P8	M	10, 5	c.[271C > T] + [271C > T] p.[Arg91Trp] + [Arg91Trp]	0.69*/0.64
P9	F	6, 7	c.[11+5G > A] + [11+5G > A] p.[?] + [?]	0.82*/0.89
P10	M	6, 2	c.[11+5G > A] + [1102T > C] p.[?] + [Tyr368His]	0.80*/0.70
P11	M	13, 3	c.[370C > T]+[1590delC] p.[Arg124X]+[Phe530fs]	0.63*/0.55
P12	M	19, 0	c.[118G > A] + [118G > A] p. [Gly40Ser] + [Gly40Ser]	0.60/0.54*

Table 1. The subject code (as used in the gene therapy trial), gender, age at treatment, genetic mutation, and the left and right eye spatial acuities (in LogMAR) at time of treatment. *Notes:* The asterisks in the final column denote the treated eye.

and in the single participant of the first phase of the trial (P3), is listed in Table 1 along with the gender, age, and the spatial acuity of each eye at the time of intervention.

Dark-adapted and rod-bleach spectral sensitivities were also measured in nine normal control observers, eight of whom had normal color vision and one of whom was mildly protanomalous (his inclusion did not significantly change the rod-bleach mean). The ages of the normal observers ranged from 26 to 58 years, with a mean of 35 and a standard deviation of 8 years.

Apparatus

The psychophysical measurements were made using one channel of a standard, Maxwellian-view system illuminated by a 75-W Xe arc lamp. Radiance was controlled by the insertion of fixed neutral-density filters (Oriel, Stratford, CT) or by the rotation, under computer control, of a circular, variable neutral-density filter (Rolyn Optics, Covina, CA). Sinusoidal flicker was produced by pulse-width modulation of fast, liquid crystal light shutters (Displaytech, Carlsbad, CA) at a carrier frequency of 400 Hz. Frequencies near the 400-Hz rectangular-pulse frequency were much too high to be resolved, so that observers saw only the temporally varying stimuli produced by the sinusoidal variation of the pulse width. The experiments were under computer control. Full details of the apparatus have been given elsewhere (Stockman et al., 2008; Stockman, Sharpe et al., 2007; Stockman, Smithson et al., 2007).

The head positions of all but the two youngest participants (P9 and P10) were maintained by a dental wax impression (bite bar), and the optical system was configured as a conventional Maxwellian-view system with a 2-mm exit pupil in which the arc is imaged at the pupil and the target on the retina. The system was reconfigured for the two youngest participants (P9 and P10) to avoid the need for a bite bar. For them, we inserted a diffuser screen just after the final lens to produce a blurry image on the diffuser that could be freely and directly viewed. In this case, a chin rest was used to restrict the participants' head movements. The diffuser screen was close enough to the Maxwellian lens so that the target appeared only slightly smaller than when seen in Maxwellian view. We could not reliably control where on the screen they looked. Thus, it is possible that the lack of rod response in P9 and P10 might partially reflect foveal fixation. However, with a robust rod response, the observers would have seen rod flicker at some locations as they moved their eyes.

In the Maxwellian-view system, wavelengths were selected using a monochromator with a half-maximum bandwidth of 4 nm (H-10 Jobin Yvon, Longjumeau cedex, France). The monochromator output was too dim when the diffuser screen was used (P9 and P10), so interference filters with half-maximum bandwidths of about 10 nm (Ealing, Holliston, MA, or Melles Griot, Irvine, CA) were used instead.

For participants P5–P8, P11, and P12 in the Maxwellian-view system, the target was a disc, subtending 3.5° at the pupil. (In the earlier measurements for subject P3, the target diameter was 5.9°.) In

all cases, the target was sinusoidally flickered at 1 Hz and presented at 10° in the superior retina close to the treatment site. To aid fixation, subjects P3, P5–P8, P11, and P12 were instructed to fixate a red spot produced by a small red light-emitting diode (LED) presented 10° above the target. Subject P6 adjusted his fixation by about 6° nasal 4 months after gene therapy because, when fixating there, he reported that the stimulus viewed that way "lit up." No other participant reported visual effects remote from the fixated region. Measurements were made at four target wavelengths. At each wavelength, adult participants adjusted the radiance of the target until the flicker at 1 Hz was just visible. For younger participants (P7–P11), the experimenter adjusted the target radiance and asked, after each adjustment, whether the participant could see the flicker. If the participant indicated yes, the target was decreased in intensity, and if not, the intensity was increased. We found that young participants made consistent and sensible responses under this regime.

Stimuli

The single target was sinusoidally flickered at the system's maximum contrast of 92%. Thus, the flickering waveform, $A(t)$, is given by $\overline{R}[1 + 0.92 \sin(2\pi ft)]$, where f is the rate of flicker (Hz) and $\overline{R}$ is the mean radiance; consequently, the contrast of the waveform was 0.92 and its amplitude 0.92 $\overline{R}$. In these experiments, f was fixed at 1 Hz. The mean radiance and amplitude were varied together by adjusting $\overline{R}$ through the rotation of a variable neutral-density wedge, thus keeping the contrast at 92%.

The flickering 3.55° target for P5–P8, P11, and P12 (or flickering 5.9° target for P3) was presented at an eccentricity of 10° in the superior retina. There was no background light. Measurements were made at target wavelengths of 450, 500, 550, and 600 nm. When the diffuser was used for P9 and P10, the target wavelengths were 469, 500, 550, and 600 nm (because of lower light levels through the diffuser, a 450-nm light could not be seen, so a wavelength of 469 nm was used instead).

Procedures

Dark-adapted spectral sensitivities

Observers dark adapted for 40 min before the measurements. They then interacted with the computer that controls the apparatus by means of keypad (or in the case of the younger participants, P7–P11, by vocally or otherwise interacting with the experimenter who controlled the keypad) and received information and instructions via tones and a computer-controlled voice synthesizer (or in the case of the younger participants, questions and instructions from the experimenter). The method of adjustment was used to measure the visual responses at each wavelength. The observers adjusted the radiance of the continuously flickering target until they were satisfied that the flicker was just visible. Two buttons on the keypad increased or decreased the intensity of the target by 0.02 $\log_{10}$ unit, two additional buttons produced larger changes of 0.10 $\log_{10}$ unit, and a fifth was pressed to indicate that the flicker was at threshold (i.e., just visible).

It was important that the targets be near threshold to prevent significant light adaptation. We therefore started each set of measurements with the 500-nm target, to which rods are most sensitive, but with the target radiance set well below threshold and then asked the participant to increase the radiance to find the threshold. Once the 500-nm threshold had been found, we could easily estimate appropriate starting radiances at other target wavelengths, such that the starting radiance would also be below the rod threshold. The four target wavelengths were presented twice. In the first run through, they were presented in the order 500-550-600-450 nm for P3, P5–P8, P11, and P12; in the order 500-550-600/575-481-469 nm for P9; and 500-550-600-469 nm for P10. In the second run, they were presented in the reverse order. If the second run was significantly different from the first (rarely the case), the measurements were repeated after dark adapting once more.

Spectral sensitivity measurements were made prior to gene therapy and then, for 1 year, at approximately 2-month intervals and subsequently at 12-month intervals.

Cone plateau ("rod-bleach") spectral sensitivities

In addition to dark-adapted settings, measurements were made in the three oldest participants from Phase II during the cone plateau following an intense bleach when only cones should be signaling the target. The "cone plateau" refers to the time interval after viewing a bleaching light during which sensitivity changes very slowly and, in normal vision, has the spectral characteristics of cone vision. The plateau in normal vision occurs in the dark after a bleach because of the time interval in which cone vision, because it dark adapts rapidly, is more sensitive than rod vision. The bleaching light was a white Ganzfeld (full field) of 5.50 $\log_{10}$ scotopic trolands viewed for 30 s. This light bleaches more than 60% of the rod photopigment in normal observers (e.g., Pugh, 1988) and leads to a cone plateau that lasts between 3 and 10 min following the bleach.

Because we found no evidence for dark-adapted rod function in the younger participants, we attempted these measurements only in the three oldest partici-

pants, P5, P6, and P12, from Phase II (having already made them in P3 from Phase I).

Calibration

The radiant fluxes of the target and background fields were measured at the plane of the exit pupil using a UDT radiometer, calibrated by the manufacturer (Gamma Scientific, San Diego, CA) against a standard traceable to the U.S. National Bureau of Standards. When the diffuser was used for P9 and P10, the UDT radiometer was placed at the position of the observer's pupil and pointed at the diffuser. Because we do not know the precise retinal illuminance from such a calibration, the data for P9 and P10 are *relative* measures of quantal sensitivity. They are plotted as *estimated* absolute values in Figures A1 and A2 in the Appendix and are based on thresholds and calibrations made in control subjects who used both the Maxwellian-view system and the diffuser. The neutral-density filters (and circular neutral-density wedge) were calibrated separately for each wavelength using the radiometer in the optical system. The target radiances are reported as time-averaged values.

Approvals

The study was approved by the U.K. Gene Therapy Advisory Committee, the Medicines and Health Products Regulatory Authority, the Moorfields Research Governance Committee, and the local research ethics committee. All participants gave written informed consent. The study was conducted in compliance with Good Clinical Practice guidelines according to the European Clinical Trials Directive (Directive 2001 EU/20/EC) and the Declaration of Helsinki (www.clinicaltrials.gov NCT00643747).

Results

Figure 1 shows data for participants P6, P5, and P12 in the upper, middle, and bottom rows, respectively. P6 and P5 were the participants who showed improvement, and P12 is typical of those who showed little or no improvement. (Data in the form of Figure 1 are shown for the remaining participants in Figures A1 and A2 in the Appendix.) The left-hand panel of each row shows dark-adapted $\log_{10}$ sensitivities for detecting 1-Hz flicker at 450 nm (blue circles), 500 nm (light blue inverted triangles), 550 nm (green squares), and 600 nm (orange triangles) as a function of months relative to time of treatment, which, marked by the vertical dashed line, occurred at month 0 (note the change of scale at 12 months). Error bars, when larger than the symbols, indicate ± 1 standard error of the mean.

The data for P6 show a dramatic improvement in dark-adapted sensitivity between 2 and 4 months after treatment. Sensitivity substantially increased at all four wavelengths, with improvements ranging from about 3.0 $\log_{10}$ units at 450 nm to 1.5 $\log_{10}$ units at 600 nm. The improvements remain roughly constant for 6 to 12 months after treatment. There is a gradual decline in sensitivity at 600 nm over the next 2 years and a substantial dip at the other wavelengths at 2 years post-treatment. However, at 3 years post-treatment, there is some recovery, particularly at the shorter wavelengths.

The underlying nature of the improvements can be understood by summarizing the data as the spectral sensitivities shown in the right-hand panels, where the average of the early sensitivities (obtained between -1 and 2 months) are shown as colored diamonds and those obtained later (between 6 and 12 months) as colored circles. The error bars, again, indicate ± 1 standard error of the mean. The marked improvement in sensitivity is also clear in these data. Before gene therapy treatment, the dark-adapted spectral sensitivities for P6 are consistent with the 1-Hz flicker detection's being mediated mainly by M-cones; the data (colored diamonds) align with the M-cone spectral shape (green line). After treatment, the much-improved dark-adapted sensitivities (colored circles) are consistent with mediation by rods; the data align with rod spectral sensitivity (black line). (For the rod spectral shape, we used the Commission Internationale de l' Éclairage (CIE) scotopic luminosity function (CIE, 1951), and for the cone shapes, we used the L-, M-, and S-cone 10° spectral sensitivities of Stockman and Sharpe (2000; now also the CIE standard functions; CIE, 2006). P6's post-treatment dark-adapted sensitivity is only 1 $\log_{10}$ unit below normal rod sensitivity (see also Figure 3).

Further evidence that the dark-adapted spectral sensitivities are mediated by rods comes from measuring spectral sensitivity in a 3- to 10-min interval after an intense bleach—the "cone plateau"—when cones but not rods have recovered from the bleach. The spectral sensitivities from the cone plateau, averaged over the 6- to 12-month post-treatment interval, are also shown in the right-hand panel (dark-gray squares). The bleach, of course, produces a substantial loss of sensitivity relative to the dark-adapted condition. The crucial point is that during the cone plateau, the spectral sensitivity has an M-cone shape similar to the pre-treatment dark-adapted function, indicating the switch from rod-mediated vision after dark adaptation reverting to cone-mediated function after the bleach. Cone-mediated thresholds on the cone plateau (small gray squares) lie 0.5 $\log_{10}$ units lower than the

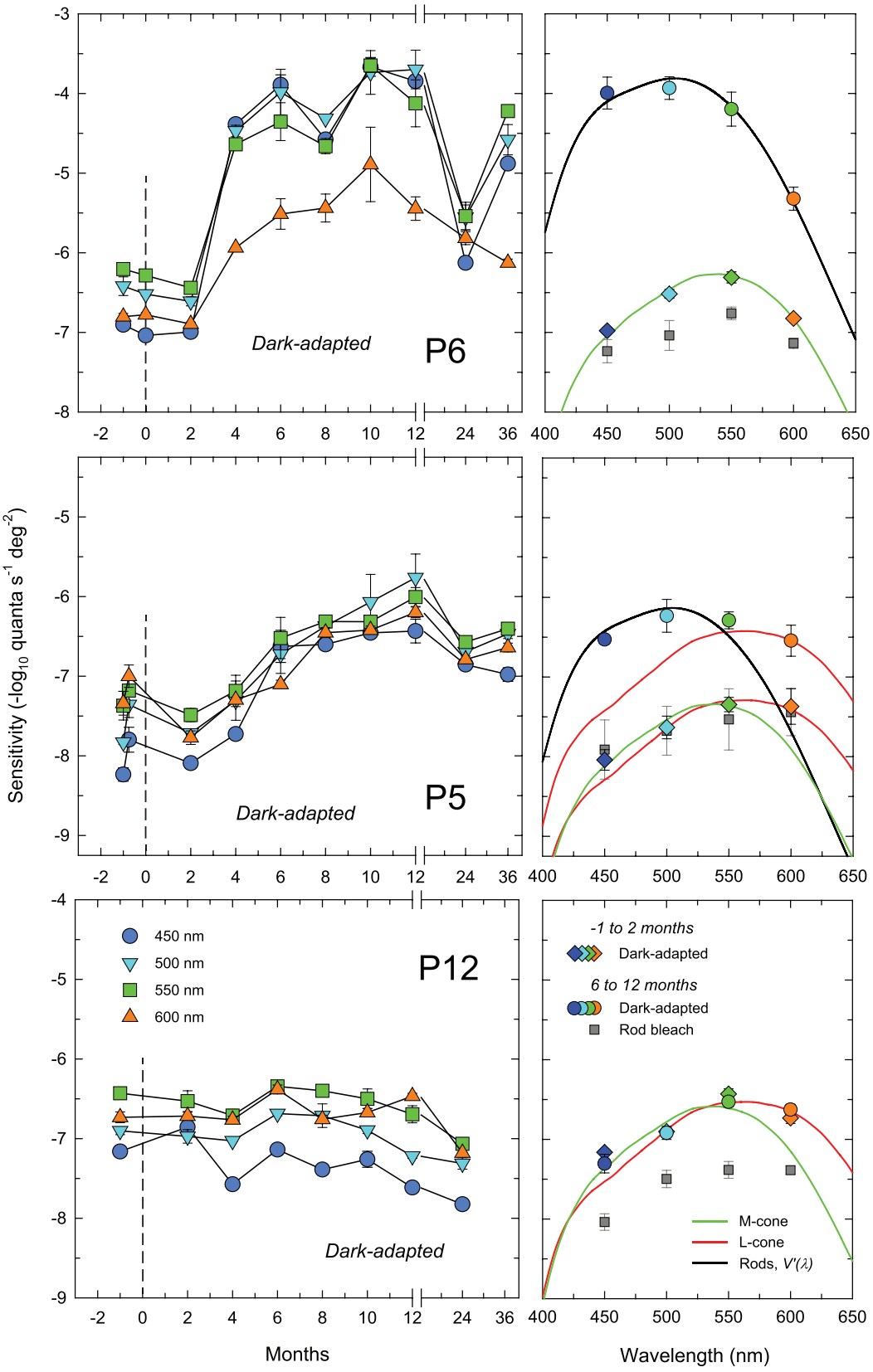

Figure 1. Sensitivity to 1-Hz flicker as a function of months relative to time of treatment for three participants: P6 (top row), P5 (middle row), and P12 (bottom row). Sensitivity is the reciprocal of the lowest radiance ($\log_{10}$ quanta s^{-1} deg^{-2}) at which the participants report seeing the high-contrast (92%) sinusoidal flicker. Left-hand panel: In all rows, the dark-adapted sensitivities for →

←

detecting 450 nm (dark blue circles), 500 nm (light blue inverted triangles), 550 nm (green squares), and 600 nm (orange triangles). The vertical dashed line marks the start of treatment at 0 months. (Note the change of scale after 12 months.) Right-hand panel: In all rows, the dark-adapted sensitivity is shown as a function of wavelength (nm). Data are for early measurements averaged over −1 and 2 months after treatment (colored diamonds) or later measurements averaged over 6 and 12 months after treatment (colored circles). Spectral sensitivities measured on the postbleach cone-plateau and averaged from measurements made between 6 and 12 months after treatment are also shown (small gray squares). The normal rod spectral sensitivity (black line) has been vertically aligned with the mean dark-adapted 6- to 12-month data (colored circles), and the standard M-cone or L-cone spectral sensitivity (green and red lines, respectively) have been vertically aligned with the mean dark-adapted −1- to 2-month data (colored diamonds), both using a least-squares fitting procedure. Error bars ±1 *SEM*.

pretreatment cone-mediated dark-adapted thresholds (colored diamonds). The difference may indicate slowed cone recovery following the bleach.

Coincident with the substantial improvement in sensitivity 4 months after treatment, P6 reported seeing the stimulus as a brightly lit flickering spot if he moved his fixation 6° nasal of the intended central treatment area of 10° superior. Once he became aware of this region, he could not ignore it, so we allowed him to change his fixation.

The middle row of Figure 1 shows data for P5 in the same format. P5's pretreatment sensitivity is about 1 log unit worse than that of P6. For this participant, there is also a sensitivity improvement in the months following treatment. Sensitivity gradually increases at all four wavelengths from 2 months after treatment rising by about 2 $\log_{10}$ units at 450 and 500 nm and by about 1 $\log_{10}$ unit at 550 and 600 nm. After 6 months, the sensitivities remained stable or increased slightly until 12 months post-treatment, when they showed nearly 2 $\log_{10}$ units improvement but remained 3 $\log_{10}$ units below normal sensitivity. The dark-adapted spectral sensitivities for P5 before treatment (colored diamonds in the right-hand panel of the middle row) are consistent with detection by M- and L-cones (green and lower red lines, respectively). After treatment, the dark-adapted sensitivities (colored circles) are consistent with detection by rods between 450 and 550 nm, as indicated by the alignment with the rod spectral shape (black line). However, at long wavelengths, detection is probably also dependent on cones, as suggested by the alignment of the L-cone shape at 600 nm (upper red line). Consistent with rod detection after dark adaptation, spectral sensitivities on the cone plateau (gray squares) show a substantial loss of sensitivity and a change in shape toward a cone-like shape similar to that of the pretreatment dark-adapted function. For P5, the cone-plateau sensitivity function is similar to the mean early (pre 2-month post-treatment) dark-adapted spectral sensitivity, which suggests that cone photopigment regeneration for P5, unlike that for P6 and P12, may be rapid. The error bars for the mean cone-plateau sensitivities for P5 (gray error bars) are larger than most other sensitivity measurements. This reflects the finding of a large improvement between 8 and 12 months after treatment in the *cone* spectral sensitivity of P5 but for no other participant. (The improvement in P5's cone spectral sensitivity can be seen in the middle panel of Figure 2, where the time course of cone-plateau sensitivities is shown.)

The results for participants P7–P11 (shown in Figures A1 and A2 in the Appendix) are exemplified by those for P12 in the bottom row of Figure 1. Data for P12 were obtained only up to 2 years post-treatment but included rod-bleach measurements. In contrast with the results for P6 and P5, there was little or no improvement in dark-adapted sensitivity, which declined over the year post-treatment. In general, the sensitivities for detecting 1-Hz flicker remained consistent with some combination of the L- and M-cone spectral sensitivity shapes (with no rod or S-cone involvement); the spectral sensitivity of P12, for example, shows a dependence on M- and L-cones similar to that of P5's pretreatment sensitivity.

Figure 2 shows cone-plateau sensitivities for P6 (upper panel), P5 (middle panel), and P12 (lower panel) as a function of months following treatment. Each panel shows the $\log_{10}$ sensitivities on the cone plateau for detecting 1-Hz flicker at 450 nm (blue circles), 500 nm (light blue inverted triangles), 550 nm (green squares), and 600 nm (orange triangles). The data for P6 and P12 show only small changes in the cone-plateau sensitivity. With the exception of P5, these changes are consistent with other measurements of cone flicker sensitivity made in all participants (not shown), which also indicate very little change in cone sensitivity. By contrast, between 8 and 12 months post-treatment, the cone-plateau sensitivities of P5 show a marked (nearly 2 $\log_{10}$ unit) improvement that is maintained until about 24 months post-treatment. The improvement is similar across target wavelengths. Other measurements of cone flicker sensitivity in P5 (not shown) also show sensitivity improvements.

P3 comes from Phase I of this trial (Bainbridge et al., 2008). In Phase I, a distinct improvement was reported in this participant, who reported much better vision in darkened rooms and darkened streets at light levels at which rods normally operate and showed improve-

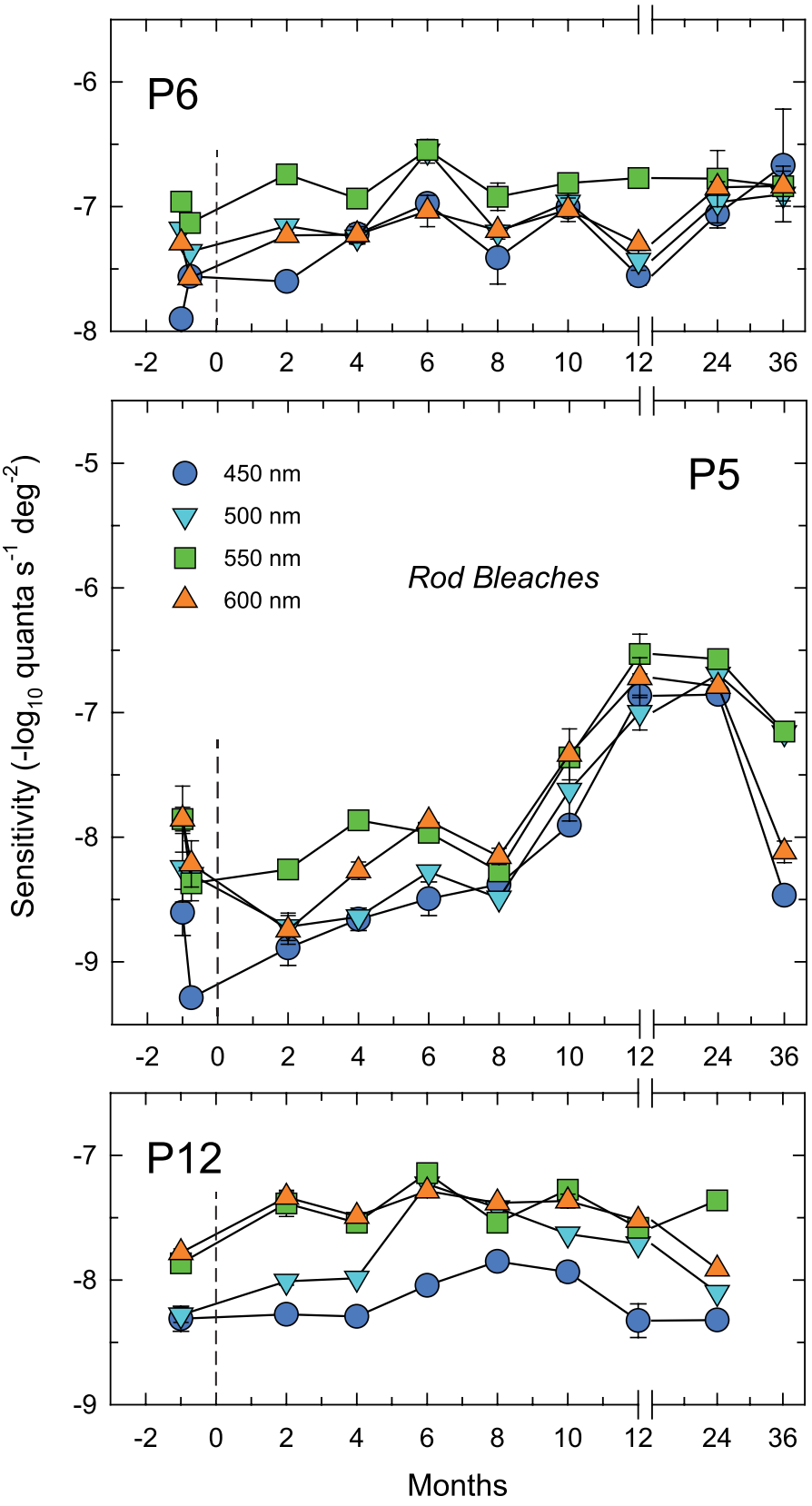

Figure 2. Detection sensitivities ($\log_{10}$ quanta s^{-1} deg^{-2}) for detecting 450 nm (blue circles), 500 nm (light blue inverted triangles), 550 nm (green squares), and 600 nm (orange triangles) 1-Hz flicker during the cone plateau following a bleach. Sensitivity is shown as a function of months relative to time of treatment for participants P6 (top panel), P5 (middle panel), and P12 (lower panel). (Note the change of scale after 12 months.)

ments in microperimetry and dark-adapted perimetry (Bainbridge et al., 2008). We investigated these improvements in P3 (post-treatment only) by measuring spectral sensitivity after 40 min of dark adaptation and found clear evidence for rod function in his treated eye (Figure 3, left panel, green diamonds). However, we also found evidence for rod function in his untreated eye, although reduced in sensitivity by about 1.15 $\log_{10}$ units compared with his treated eye (untreated eye data not shown). These results suggest that P3 most likely had rod function in his treated eye *before* the gene therapy treatment and that those rods could have benefited from the introduced RPE65 isomerase. The measurements made for P3 in Phase I established the potential importance of more detailed spectral sensitivity measurements as an indicator of treatment outcomes and led to the inclusion of spectral-sensitivity measurements in Phase II (Bainbridge et al., 2015); we include the data for P3 in Figure 3 for comparison.

The left-hand panel of Figure 3 summarizes the mean post-treatment dark-adapted spectral sensitivity data (colored symbols) for the individual participants who were measured in Maxwellian view rather than using a diffuser screen (see the Methods section) averaged between 6 and 12 months after treatment together with the mean data for nine normal observers (filled black circles). (The data for P3 [green diamonds] were averaged between 6 and 14 months post-treatment.) Among the participants, there are nearly 4 $\log_{10}$ units of variability in absolute sensitivity (i.e., in vertical position in the figure). Unsurprisingly, those in whom rod function was found (P3, P5, and P6) are more sensitive than those in whom only cone function was found, but even in those with only cone function (P7–P12), absolute sensitivity varied over 1.5 $\log_{10}$ units, which exemplifies the large variability in sensitivity loss found in this disease. As well as varying in absolute sensitivity, the cone-only dark-adapted spectral sensitivity functions also differ in shape in a way that seems to depend on the relative sensitivities of the participants' L- and M-cones. No evidence was found for S-cone function in any participant, which might suggest that the S-cones are more dependent on 11-*cis*-retinal regeneration in the RPE than the other two cone types and are therefore more susceptible to damage and loss. Also in RPE65-deficients dogs, it is mainly the S-cones that are lost, whereas L-cones are relatively well preserved (Mowat et al., 2013).

The right-hand panel of Figure 3 summarizes the mean rod-bleach spectral sensitivity data for the four participants (P3, P5, P6, and P12) in whom sensitivity during the cone plateau following a bleach was measured; comparable mean data for the nine normal controls (red circles) are also shown. The latter are consistent with contributions from the S-, M-, and L-cones, as shown by the fitted blue, green, and red lines, respectively. On the other hand, all four participants exhibit spectral sensitivities that are consistent with contributions from only M- and the L-cones and are at least 1 $\log_{10}$ unit less sensitive than the normal controls. Participant P6 is the most sensitive of the four participants, with the other three having relatively similar sensitivities.

Discussion

Our measurements of spectral sensitivity following subretinal administration of a gene-therapy vector demonstrate (a) clearly improved rod function in the treated eye of two of the eight participants (P5 and P6) from Phase II of the trial, (b) improved rod sensitivity in the treated eye of one participant (P3) from Phase I relative to his untreated eye, and (c) improved cone sensitivity in one participant (P5) from Phase II. The impact of gene therapy on rod and cone function in the area of retina tested may be limited either by the extent of established retinal degeneration and/or by insufficient restoration of RPE65.

There are a number of interesting features in the data for P5 and P6. The first is the sensitivity losses between 12 and 24 months: P6 shows a more than 2.5 $\log_{10}$ sensitivity loss (top left panel of Figure 1) and P5 a roughly 1.0 $\log_{10}$ sensitivity loss (middle left panel of Figure 1). The losses after 12 months are consistent with other measures for P5 and P6 that show a comparable decline in the effectiveness of the gene therapy treatment after 12 months (Bainbridge et al., 2015). The second is the unexpected recovery for P6 at 36 months, for which we have no easy explanation. It is possible that we somehow underestimated P6's sensitivity at 24 months, perhaps through misalignment of the participant in the apparatus or inadvertent exposure to a bright light during testing. As we noted previously, this participant seemed to have distinct retinal islands of rod function, so misalignment seems a likely cause. Nevertheless, sustained recovery would be a welcome possibility. The third is the more gradual recovery of rod function seen in P5 compared with the delayed and steeper recovery of cone function (compare the middle left panel of Figure 1 with the middle panel of Figure 2). The cone recovery may be different because it reflects an interplay between two mechanisms for the restoration of 11-*cis*-retinal following a bleach (Wu et al., 2004; Znoiko et al., 2002), one of which is restored after gene therapy.

P8 and P9, both younger participants, and although showing no rod function, may have measureable improvements in cone function between pre- and post-treatment measurements, with only small differences across the post-treatment measurements (see the middle

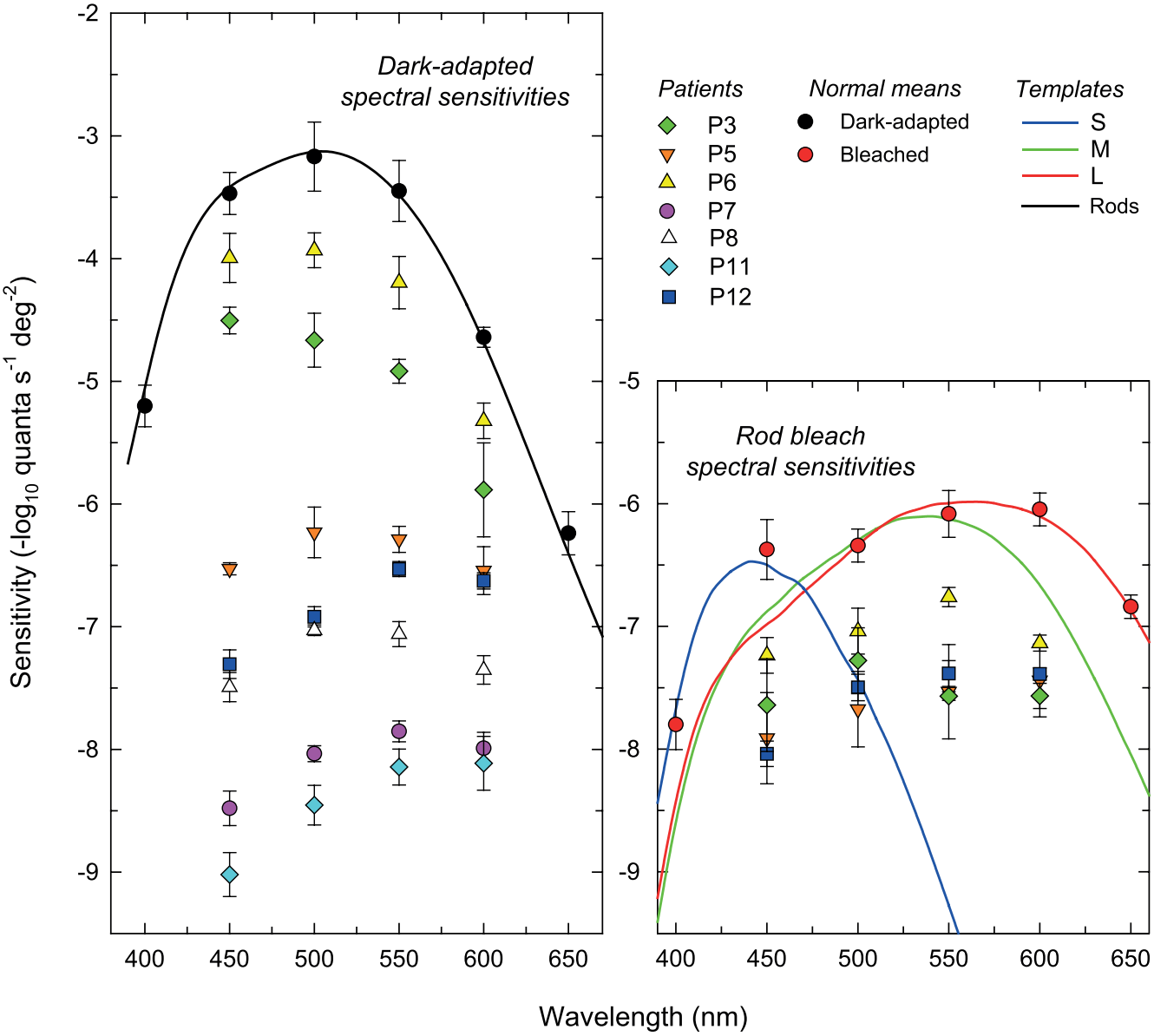

Figure 3. Left-hand panel: Dark-adapted spectral sensitivities averaged between 6 and 12 months for P5 (inverted orange triangles), P6 (yellow triangles), P7 (purple circles), P8 (open triangles), P11 (blue diamonds), and P12 (dark blue squares) compared with the mean dark-adapted spectral sensitivities for nine normal observers (filled black circles). (Data for P3 [green diamonds] measured at 13 or 14 months fare also shown.) The normal rod spectral sensitivity shape (black line) has been vertically aligned with the normal mean data. (Data for P9 and P10 measured with a diffuser screen are not included.) Right-hand panel: Cone-plateau spectral sensitivities averaged between 6 and 12 months for P5, P6, and P12 (or 14 months for P3) compared with the mean cone-plateau spectral sensitivities for nine normal observers (red circles). The normal cone-plateau data have been fitted with the S-cone shape (blue line) at 400 and 450 nm, with the M-cone shape (green line) at 500 and 550 nm and with the L-cone shape (red line) from 500 to 650 nm. Error bars ±1 *SEM* between runs for the participant data or between observers for the normal mean data.

and lower panels of Figure A1 in the Appendix). These early improvements may reflect their simply becoming familiar with the task and situation. Other cone-flicker measurements (not shown) do not show an improvement.

We are not the first to present evidence for the recovery of rod function following *RPE65* gene therapy. There is evidence for rod recovery in a series of papers from another gene therapy trial (ClinicalTrials.gov NCT00481546) mainly in the form of sensitivities and sensitivity changes at two test wavelengths (Cideciyan et al., 2008, 2009a; Hauswirth et al., 2008; Jacobson et al., 2012). However, measurements made at only two target wavelengths make the disambiguation of the sensitivities of the rods, three cone types, and, perhaps, intrinsically photosensitive retinal gan-

glion cells (ipRGCs) difficult—without relying on other assumptions or measurements.

Using 500- and 650-nm targets of 1.7° in diameter and 200-ms duration, Cideciyan et al. (2008) found improvements in dark-adapted rod sensitivity after standard periods of dark adaptation (of 1–2 hr) in three participants (their P1–P3) 30 days after treatment but more substantial improvements in two participants (their P2 and P3) after extended periods of dark adaptation (3–8 hr). The sluggish recovery of rod sensitivity in the dark was also apparent in rod-cone dark adaptation curves for their P2 and P3 measured using blue or red LEDs illuminating an opal diffuser of 1.7° in diameter following a bleach (see their figure 3B). For both participants, complete dark adaptation took longer than 8 hr, and rod detection of the short-wavelength target (evidenced by the appearance of the rod branch in the recovery data) did not begin until after at least 2 hr (see p. 1, supporting information Cideciyan et al., 2008). The improvements in sensitivity measured with the 500- and 650-nm targets were still evident 1 year after treatment (Cideciyan et al., 2009a).

Full-field sensitivity testing (FST) was also used to determine sensitivity in participants from this trial (Hauswirth et al., 2008; Jacobson et al., 2012). FST involved the use of a Ganzfeld (full-field) stimulator (ColorDome Desktop Ganzfeld, Diagnosys LLC, Littleton, MA, USA) that produced full-field flashes of 200-ms duration using LEDs with peak wavelengths of 465 and 637 nm (Román, Cideciyan, Aleman, & Jacobson, 2007). The initial FST results for P1–P3 for 30–90 days after treatment showed sensitivity improvements for detecting the 465-nm flash after 1 hr of dark adaptation (Hauswirth et al., 2008). Subsequent FST results for 15 participants up to 3 years post-treatment (Jacobson et al., 2012) showed significant improvements in the detection sensitivity for seeing blue flashes as compared with red flashes after extended periods of dark adaptation (>3 hr). Moreover, the results suggested that even before treatment, the 465-nm flash was rod detected in 7 of 15 eyes and rod/cone detected in 2 of 15 eyes (see, in particular, their efigure 2B; Jacobson et al., 2012). This is in marked contrast to our data that show rod function in only one participant (P3) before treatment.

A fundamental assumption in the series of papers (Cideciyan et al., 2008, 2009a; Hauswirth et al., 2008; Jacobson et al., 2012) is that if the sensitivity to the shorter wavelength target is sufficiently greater than the sensitivity to the longer wavelength target, then the former must be rod detected, whereas the latter must be rod and/or cone detected. For this assumption to be generally true, detection needs to be mediated primarily by rods and by some additive combination of the L- and M-cones. However, without additional evidence, there must always be a concern that other photoreceptors, such as S-cones or ipRGCs, or indeed spectrally opponent photoreceptor interactions (Sperling & Harwerth, 1971), might affect the sensitivity to one or both targets. A particular concern with the full-field targets used for the FST measurements is that ipRGCs, which peak in sensitivity near 480 nm (Hattar, Liao, Takao, Berson, & Yau, 2002; Provencio et al., 2000) might contribute to the detection of the short-wavelength flash (Brown et al., 2012; Horiguchi, Winawer, Dougherty, & Wandell, 2013; Zaidi et al., 2007). Indeed, the unusually long period of dark adaptation required for the improvement in sensitivity for seeing the 465-nm stimulus in FST measurements is consistent with melanopsin dark adaptation, which has a time constant (i.e., the time to recover to 63% of the fully dark-adapted sensitivity) of approximately 3 hr (Wong, Dunn, & Berson, 2005). But why would melanopsin, which is a bistable photopigment that can regenerate in light through photon absorption (Mure, Rieux, Hattar, & Cooper, 2007), benefit from *RPE65* gene therapy? Notably, *RPE65* mutant (*Rpe65*$^{-/-}$) mice fail to respond to diurnal light cycles (Doyle, Castrucci, McCall, Provencio, & Menaker, 2006), which suggests that the lack of RPE65 may compromise melanopsin regeneration in the dark (Doyle et al., 2006; Fu et al., 2005; Tu et al., 2006). IpRGCs may therefore benefit from *RPE65* gene therapy.

Although melanopsin could play a role in the FST measurements, it seems likely that, following gene therapy, there was significant recovery of rod function in the majority of participants measured by Hauswirth et al. (2008), Cideciyan et al. (2008, 2009a) and Jacobson et al. (2012). Why did we detect restored rod function by spectral sensitivity measurement in only a minority of subjects? One possibility is that by measuring spectral sensitivity in a discrete area of the treated part of the retina, we might have missed "hot spots" of functioning rods that could contribute to FST. Another possibility is that our vector may have mediated less effective restoration of RPE65. In addition, by using a standard dark-adaption time of 40 min, rather than periods of up to 8 hr, we might have missed residual rod responses in some participants. Dark-adapted perimetry in participant P6, however, showed that from 6 months after treatment until the end of the study, dark adaptation was nearly complete after 1 hr and thereafter improved very little (see supplementary figure 2 in Bainbridge et al., 2015), indicating that dark adaptation can be comparatively normal after intervention.

Keywords: gene therapy, RPE65, rods and cones, scotopic, flicker sensitivity, temporal processing

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Appendix

Figure A1 shows data for P7, P8, and P9, and Figure A2 shows data for P10 and P11. The data are all presented in the form of Figure 1: The left-hand panels of each figure show dark-adapted $\log_{10}$ sensitivities for detecting 1-Hz flicker at 450 nm (blue circles), 500 nm (light blue inverted triangles), 550 nm (green squares), and 600 nm (orange triangles) as a function of months relative to time of treatment. In contrast with the results for P5 and P6 (top two panels of Figure 1), but like those for P12, these five participants (P7–P11) showed relatively small changes in dark-adapted sensitivity in the area of retina tested. Although there is some suggestion of a gradual sensitivity loss for P7 with time after treatment, and slight improvements for P8 and P9, there is no evidence for rod involvement in these data.

The right-hand panels of Figures A1 and A2 show the mean 6- to 12- or 13-month post-treatment spectral sensitivities for P7–P11 as colored circles. The spectral sensitivities are consistent with some combination of the L- and M-cone spectral sensitivity shapes (with no rod or S-cone involvement). Because these participants were children, no bleaching measurements were made.

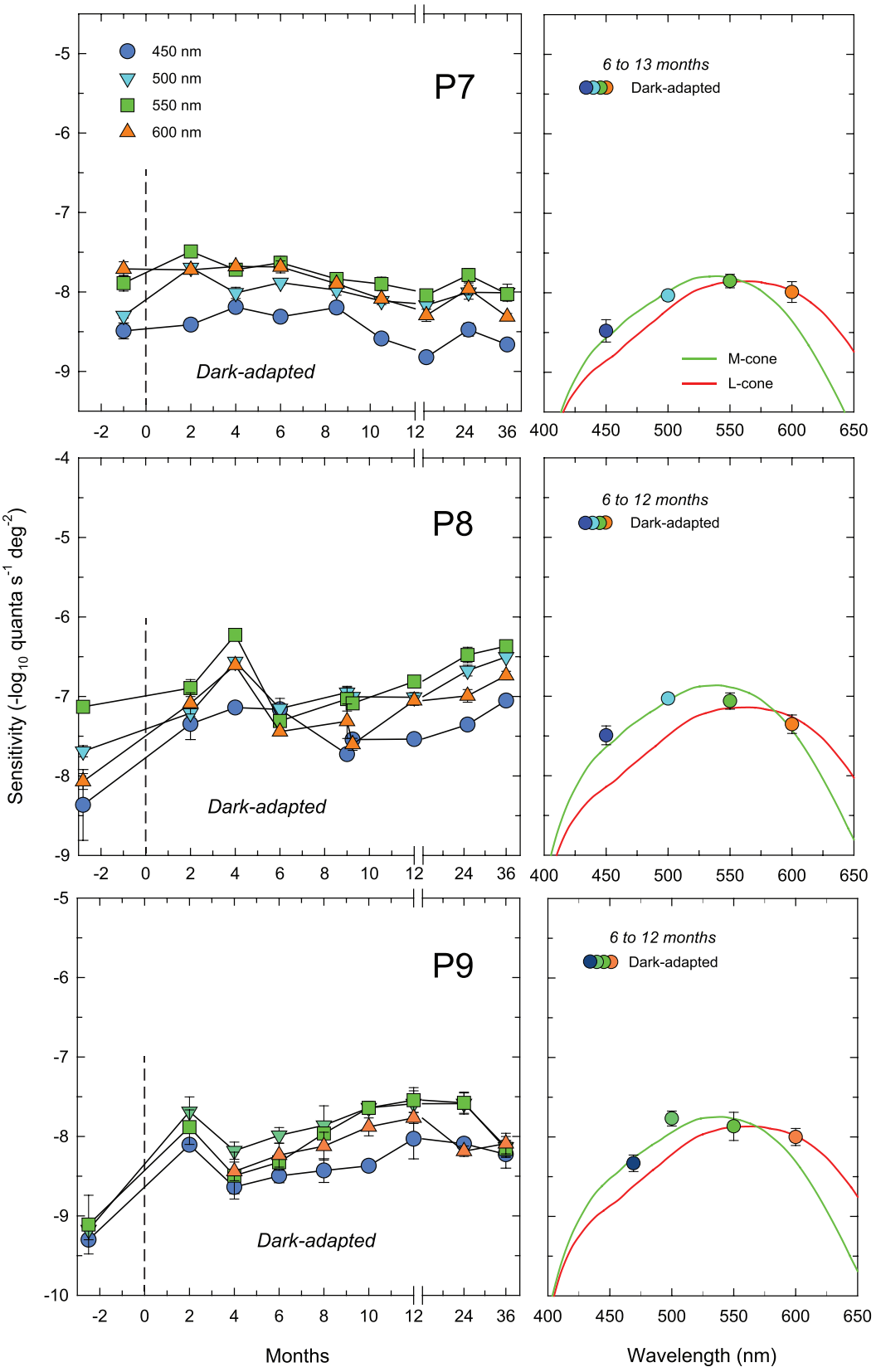

Figure A1. As for Figure 1 but for participants P7, P8, and P9 in the upper, middle, and lower panels, respectively. In the right-hand panels, the mean 6- to 12- or 6- to 13-month data (colored circles) have been fitted with the M-cone shape (green line) between 450 and 550 nm (P7) or between 450 and 600 nm (P8 and P9) and with the L-cone shape (red line) at 550 and 600 nm. Only the mean spectral sensitivity data after treatment are shown.

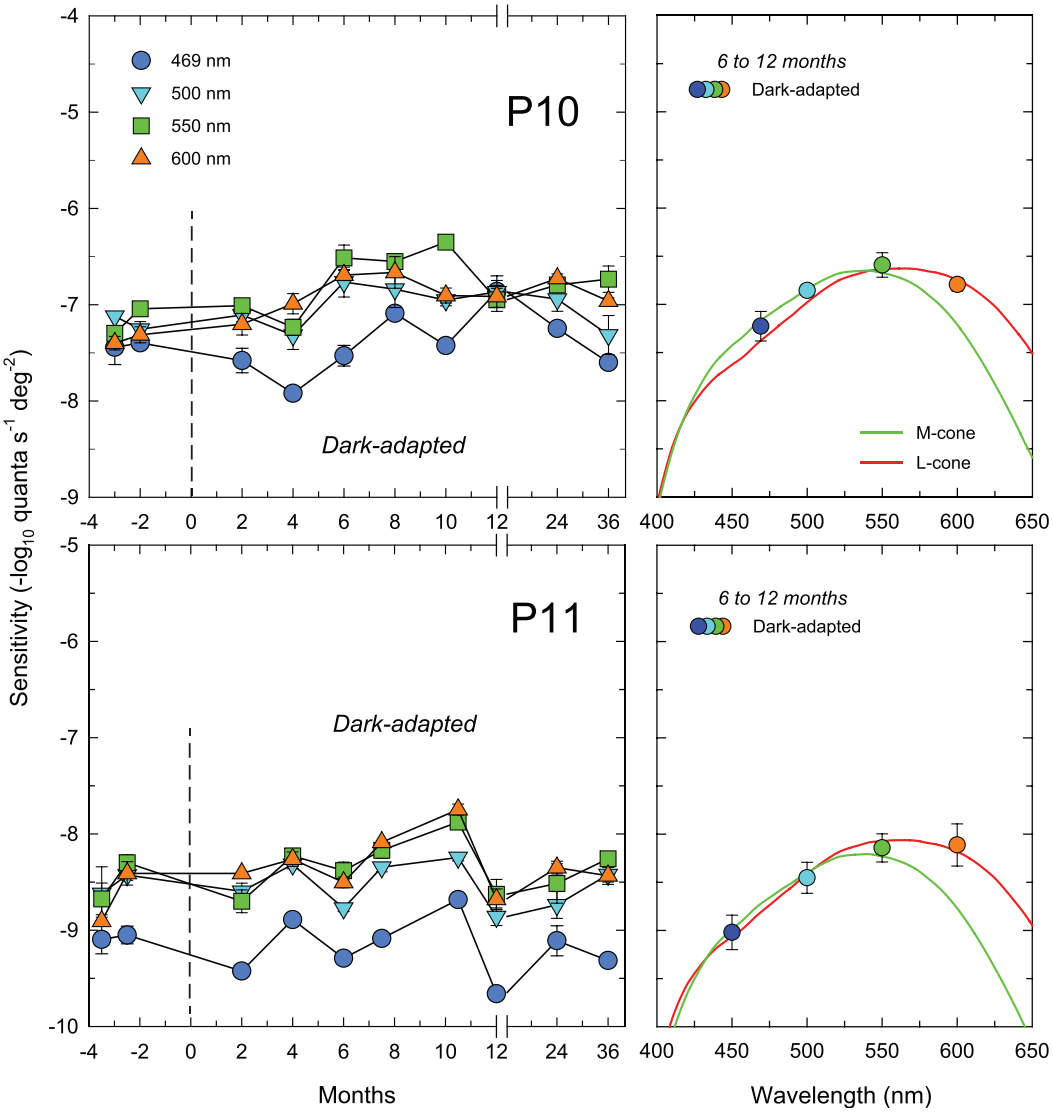

Figure A2. As for Figure A1 but for participants P10 and P11 in the upper and lower panels, respectively. In the right-hand panels, the mean 6- to 12-month data (colored circles) have been fitted with the M-cone shape (green line) between 450 and 550 nm and with the L-cone shape (red line) at 550 and 600 nm (P10) or between 450 and 600 nm (P11).