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Ultraviolet and middle wavelength sensitive cone responses in the electroretinogram (ERG) of normal and *Rpe65* −/− mice

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Abstract

Ultra-violet (UV) and middle wavelength sensitive (M) cone responses were identified in the ERG of normal and *Rpe65* −/− mice using chromatic flashes and selective chromatic adaptation. In normal mice, the UV-cone response was as large as, or larger, in the presence of a bright yellow adapting light than it is in the presence of a dim white light. The M-cone response became undetectable in the presence of the yellow adapting light. Yellow adapting light initially reduced the UV response, but it recovered in 8–10 min. The M-cone response did not recover. UV-cone responses were undetectable in *Rpe65* −/− mice. The M-cone response of young *Rpe65* −/− mice was almost as large as in normal mice. A yellow adapting light only diminished this M-cone response. With age, the M-cone response further decreased in *Rpe* −/− mice. We show a pronounced loss of UV-cone function in *Rpe65* −/− mice, which may be related to a defect UV-cones share with rods. The M-cone function is also affected already in young *Rpe65* −/− mice. The transient effect of a yellow adapting light on the UV-cone response of normal mice is suggested to be neural, because it disappears during maintained light adaptation.

Keywords: Mouse; Rpe65; Electroretinogram; Cones; Ultra-violet

1. Introduction

The *Rpe65* −/− mouse (Redmond, et al., 1998) is a model of a human form of retinal degeneration (Gu, et al., 1997; Marlhens, et al., 1988; Marlhens, et al., 1997; Morimura, et al., 1998; Perrault, et al., 1999; Lorenz, et al., 2000; Lotery, et al., 2000; Thompson, et al., 2000). The defect involves a protein, RPE65, found in retinal epithelium (Hamel et al., 1993). RPE65 is associated with the smooth endoplasmic reticulum and is necessary for the synthesis of the 11-cis isomer of vitamin A (Hamel et al., 1993; Nicoletti, et al., 1995). In the *Rpe65* −/− mice, there is an absence of rhodopsin, a selective depression of the rod electroretinogram and an accumulation of all-trans retinyl esters in the retinal epithelium (Redmond et al., 1998). There is evidence that the cone system also degenerates as these mice age (Gouras, et al., 2000), but whether this cone degeneration is due to the RPE65 defect or secondary to rod degeneration is unknown.

We have been led to examine the cone ERG of the *Rpe65* −/− mice because of experiments that we have completed on the UV-cone input to the murine superior colliculus (Ekesten & Gouras, in press). In this study, we found a strong input of UV-cones to the superior colliculus of the normal mouse where it reflects the spatial distribution of these cones in the retina. A preliminary examination of the superior colliculus of *Rpe65* −/− mice showed a profound insensitivity to UV stimuli. Accordingly, we have examined the responses of the two cone mechanisms, the UV- and M-cones, present in the ERG of normal mice (Jacobs, Neitz, & Deegan, 1991; Szel, et al., 1992) and compared them to the responses in *Rpe65* −/− mice.

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2. Material and methods

Ten *Rpe65* −/− mice, six 4-week-old mice and four mice aged 11–13 months were examined. Age-matched C57B mice served as controls. The mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The pupils were dilated with phenylephrine HCl (1%) and cyclopentolate (1%). The mouse was supported on an adjustable stage with a built-in heating device maintaining the body temperature between 36 °C and 37 °C.

A saline-moistened cotton wick electrode attached to a micropositioner was placed in direct contact with the cornea. A needle electrode placed subcutaneously on the forehead served as reference electrode. Panretinal stimulation with brief flashes was obtained from a stroboscope (Grass PS33, Grass Instruments Inc., West Warwick, RI) with a square aperture of 4 cm placed 6 cm from the eye. The stimulus light could be filtered with spectral and neutral density filters (Kodak Wratten 18A, 60 and 96, Eastman Kodak Co, Rochester, NY). The maximum stimulus intensity for the green (520 nm) stimulus was 1.7 log cds/m^2, and 1.9 log cds/m^2 for the UV (360 nm) stimulus. The ERG responses were amplified and averaged by a computerized data-acquisition system (PowerLab, AD Instruments, Mountain View, CA). The bandpass of the input amplifier was 1–250 Hz.

The spectral transmission of each chromatic filter was measured with a spectrophotometer (Beckman Instruments, Palo Alto, CA), and the wavelength of peak transmission was used to plot the action spectrum based on a constant threshold response. The flash energy at the cornea delivered through each spectral filter was measured with a digital photometer (J16, Tektronix Instruments, Beaverton, OR). Infrared energy transmitted by certain filters was eliminated by appropriate filtering. Measurements of relative sensitivity to UV light were corrected for higher absorbtion in the UV part of the spectrum by the neutral density filters than for longer wavelenghts. Energy values were converted into quanta per stimulus, and the logarithmic reciprocal of these values was used to generate spectral sensitivity functions.

When the dark-adapted ERG was studied, the mice were dark-adapted overnight prior to examination, and preparation of these animals was performed under dim red light. In experiments performed in the presence of a dim white background, the mice were subject to a diffuse white adapting light of 25 cd/m^2 for at least 30 min prior to examination. A steady, yellow adapting light (Corning glass filter passing wavelengths >490 nm), approximately 300 cd/m^2 at the cornea, was used for selective chromatic adaptation of the retina. If not stated otherwise, the yellow adapting light was turned on approximately 15 min before light-adapted ERGs were performed. The dim white background light was turned off when the yellow adapting light was employed.

Means and standard error of the mean were used as descriptive statistics. Differences between groups were tested using the Wilcoxon rank score test (JMP version 4.0.1, SAS Institute Inc., Cary, NC). Differences were considered statistically significant at $P < 0.05$.

The protocol for animal use and experimentation adhered to the Association for Research in Vision and Ophthalmology resolution for using animals in research.

3. Results

Fig. 1A, above, shows ERGs of a normal, dark-adapted mouse using an UV (360 nm) and a green (520 nm) flash. The responses to flashes of different energies extending from maximal to threshold are superimposed. The responses to these spectral stimuli are similar, although a-waves are more prominent in the responses to the green flashes. In the presence of a yellow adapting light (Fig. 1A, below), there is virtually no response to green but a conspicuous response to UV flashes.

Fig. 1B, above, shows ERGs of a normal mouse in the presence of a dim white light with the same pair of spectral flashes; again, the responses to these spectral stimuli are relatively similar. There is a greater reduction in the a- than in the b-wave response. In the presence of the yellow adapting light (Fig. 1B, below), responses to the green flashes become undetectable, but the responses to the UV flashes are larger than they are in the presence of the dim white background.

Fig. 2A, above, shows ERGs of a dark-adapted *Rpe65* −/− mouse to these two spectral flashes. There is a large response to the green but an extremely small response to the UV flash. There is no obvious a-wave in the response to the green flash. In the presence of a yellow adapting light (Fig. 2A, below), the response to the UV flash is no longer detectable, but there is an obvious response to the green flash. In fact, the response to the green flash is larger than it is under similar conditions in the normal mouse.

Fig. 2B, above, shows the ERGs of a *Rpe65* −/− mouse in the presence of a dim white background light. There is a relatively large response to the green but only a very small response to the UV flash. The amplitudes

of these responses to UV and green stimuli are comparable to those obtained in dark-adapted RPE65 $-/-$ mice, indicating that the photoreceptors are virtually unaffected by the white light, implying that they were cone-mediated. In the presence of a yellow adapting light, the response to the UV light essentially disappears, but an obvious response to the green flash remains, again larger than that detectable under the same conditions in normal mice. The tolerance to bright adapting light and the lack of a-waves suggest that the green responses are M-cone-driven.

Fig. 3 shows the relationship between the intensity of the green and UV flashes and the average amplitude of the b-wave of three normal and four *Rpe65* $-/-$ mice. Both flashes at maximum intensity produce responses of almost 800 μV in normal mice and the responses extend over about 6 logarithmic units. In the presence of the yellow adapting light, the response to the green flash is almost undetectable. In the case of the *Rpe65* $-/-$ mice, the largest responses detectable are about 300 μV and significantly larger to the green than the UV flash. In the presence of a yellow adapting light, a response to UV is almost undetectable, whereas there

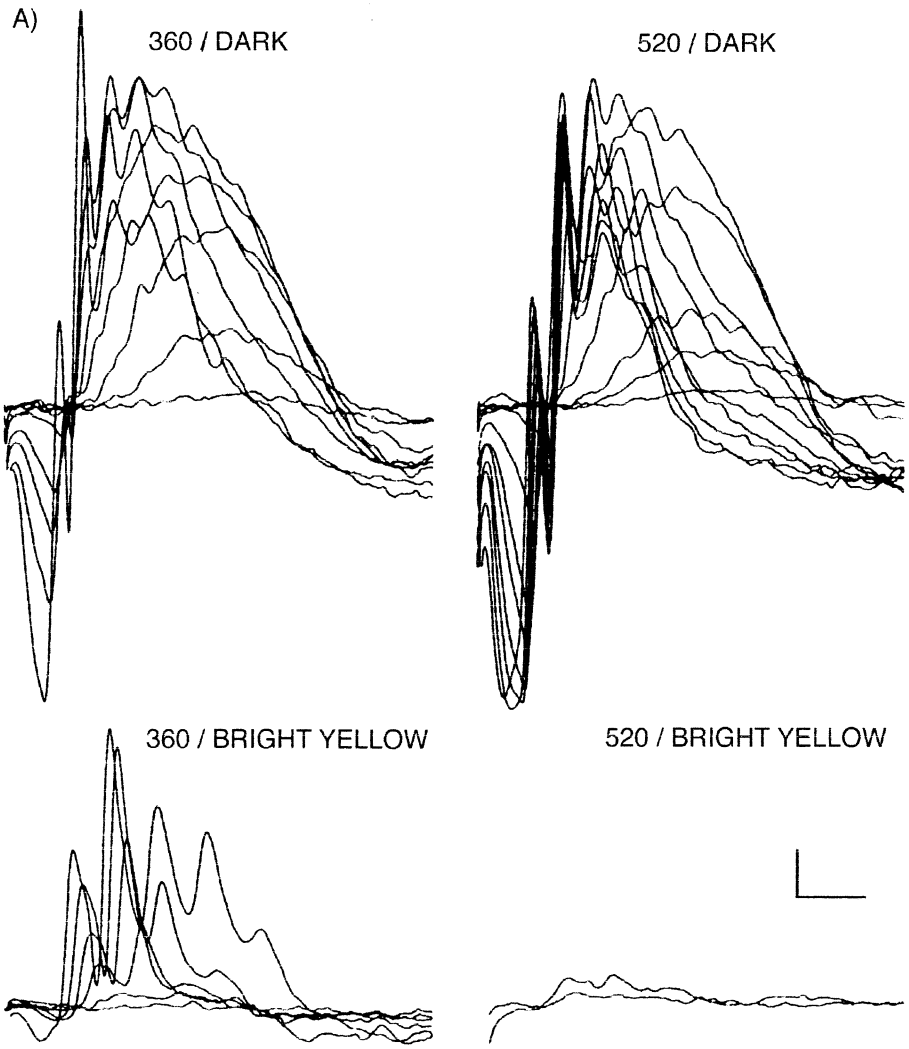

Fig. 1. ERGs of normal mice in response to 360 and 520 nm stimuli. (A) Upper graphs: bouquets of superimposed responses obtained in the dark (no background light). Responses are obtained over a 3.3 log unit range for 360 nm stimuli and over a 5.1 log unit range for 520 nm. Below: responses in presence of yellow adapting light. Stimulation with 360 nm elicits responses over a 1.2 log unit range, whereas responses driven by 520 nm stimuli tolerate less than 0.3 log units of neutral filtering. Calibration: 40 ms; 50 μV. (B) Responses to the same pair of chromatic stimuli. The upper graphs show responses in presence of a dim white background light. Responses are obtained over a 1.2 log unit range for 360 nm stimuli and over a 2.1 log unit range for 520 nm. The lower graphs are obtained in presence of the same yellow adapting field as used in the lower traces in figure (A). Calibration: 40 ms; 50 μV.

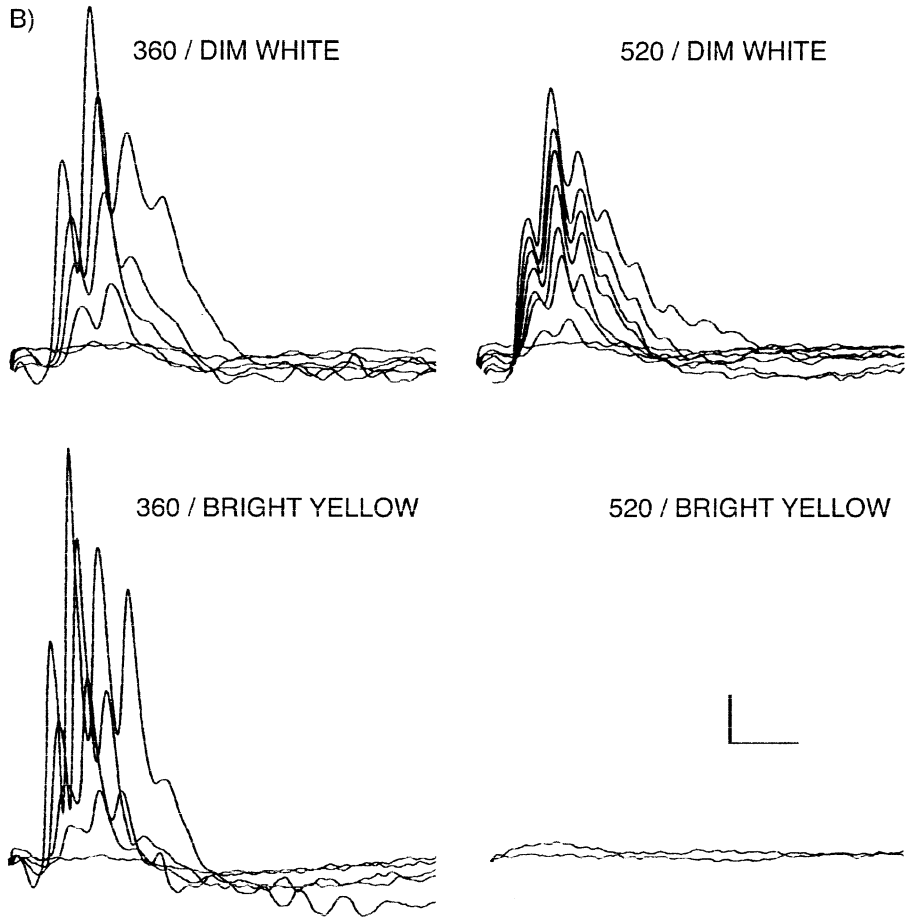

Fig. 1. (*Continued*)

is a response to the green flash over a range of about 0.5 log units.

Fig. 4 compares the maximal responses to these two spectral flashes in the presence and absence of the yellow adapting light for six normal and four *Rpe65* $-/-$ mice. In the normal mice, the responses to the green and UV flashes are about the same amplitude, but in the presence of the yellow adapting light, they become very different. The maximum response to UV in the presence of the adapting light is 394 ± 37 μV, but it is only 27.5 ± 12.5 μV to the green flash under the same conditions. In the *Rpe65* $-/-$ mice, the response to green stimuli is larger than that to the UV flash in the dark-adapted state. In the presence of the yellow adapting light, the response to UV light is undetectable, but the response to the green flash is 72 ± 12.3 μV, which is significantly larger than in the normal mouse ($P = 0.04$).

Action spectra of the dark-adapted ERG reveal a significant loss in sensitivity across the entire spectrum, but most pronounced in the UV region of the spectrum in the *Rpe65* $-/-$ mouse compared to the normal control (Fig. 5). There is an approximately 5 log unit desensitization in the UV versus about 3 log units difference in the green region of the spectrum.

These results were obtained in 4 week old *Rpe65* $-/-$ mice. Older mice were also studied, but their responses were of a lower amplitude and more difficult to analyze in the same way. In general, however, there was no qualitative difference compared to the pattern observed in the younger mice.

Thus, the only evidence for UV cone participation in the ERG of the *RPE65* $-/-$ mutant is the small sensitivity peak at 360 nm in the spectral sensitivity function in Fig. 5. However, this response to UV light could be due to the beta-band absorption of the M cone pigment.

A peculiarity in the recovery of the responses to UV stimuli during the exposure to the yellow adapting light was observed in all of the normal mice (Fig. 6). There was an enormous suppression of the response to the UV flash immediately after the adapting light was turned on. This response recovered over a period of 8–10 min to become as large as, or larger than, it was in the presence of a dim white adapting field. The response to the green flash, however, did not show any

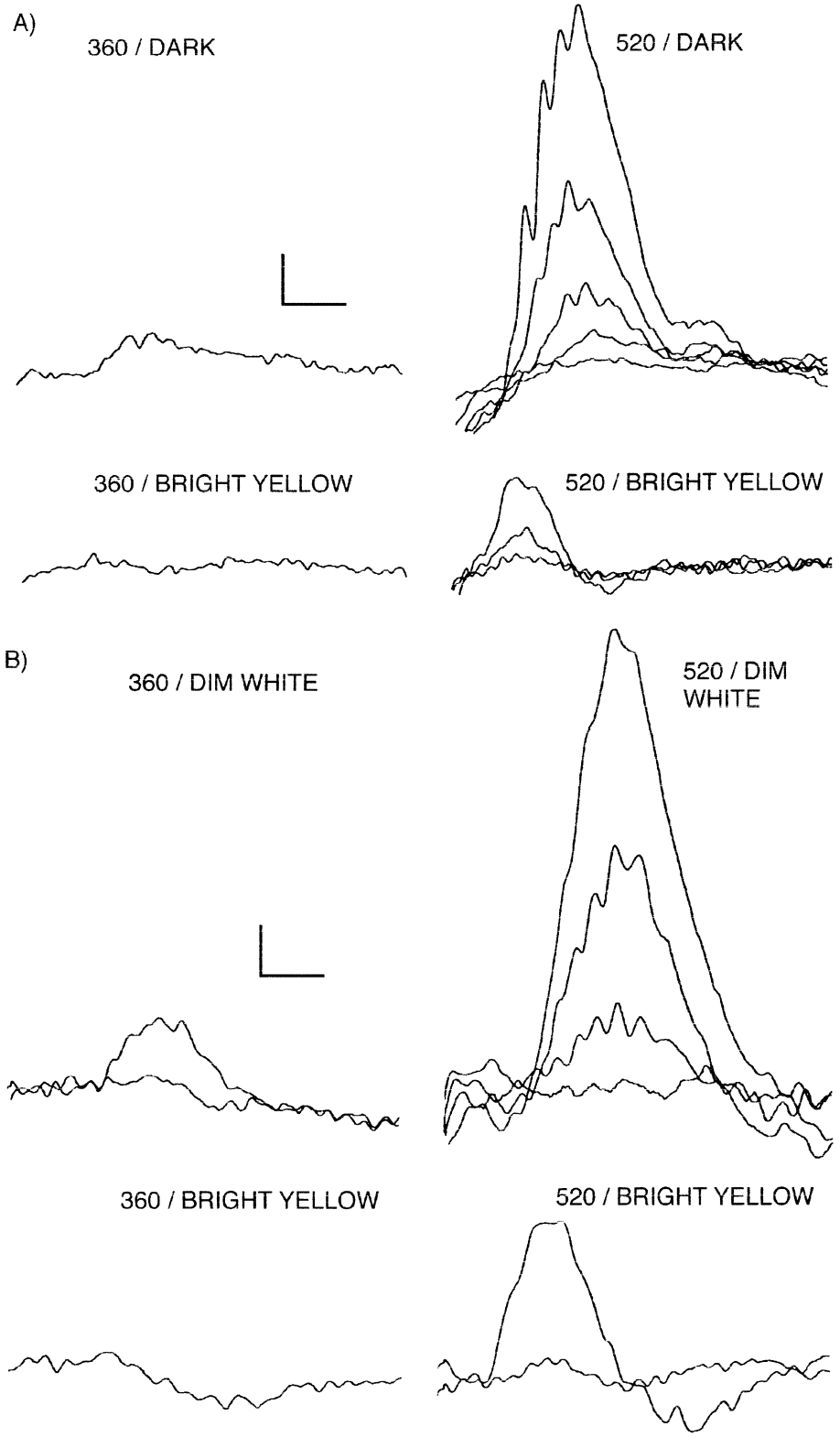

Fig. 2. ERGs of 4 week old *Rpe 65* $-/-$ mice in response to 360 and 520 nm stimuli. (A) Upper graphs: responses obtained in the dark (no background) to unattenuated 360 nm stimuli and to 520 nm stimuli over a 1.2 log unit range. Lower graphs: responses in presence of yellow adapting light. Responses to 520 nm are shown over a 0.6 log unit range, whereas 360 nm stimulation does not produce any response at all. Calibration: 40 ms; 20 μV. (B) Responses to the same pair of chromatic stimuli. The upper graphs show responses in presence of a dim white background light. Responses to 360 nm are shown over a 0.3 log unit range and over a 0.9 log unit range for 520 nm. The lower ERGs are obtained in presence of the same yellow adapting light as in the lower traces in (A). Calibration: 40 ms; 20 μV.

recovery in the presence of the continuous yellow adapting light.

4. Discussion

The results reveal a pronounced depression of the UV-cone system early in the course of the retinal dysfunction in the *RPE65* −/− mouse. In fact, we found no evidence of any UV-cone function in these mice at a stage where M-cone function is close to the normal range. In the *RPE65* −/− mouse, only a weak response to UV stimuli can be observed in the dark-adapted state, which is completely abolished by a steady yellow adapting light. This cannot be a response driven by UV-cones, because the UV-cone opsin is too insensitive to long wavelengths to be depressed by this adapting light, as shown in normal mice. It is known that both M-cones and rods respond to UV stimulation (Lyubarsky, Falsini, Pennesi, Valentini, & Pugh, 1999). The response to green stimuli in *RPE65* −/− mice is desensitized, but not eliminated, by the steady yellow adapting light. The tolerance to the bright adapting light suggests that this response is driven by M-cones and not rods. Therefore, the only responses that we have been able to detect in *RPE65* −/− are most likely to be M-cone-driven.

It is currently thought that there is a defect in the synthesis of rhodopsin that involves the RPE layer, and this selectively eliminates rod, but not cone, function

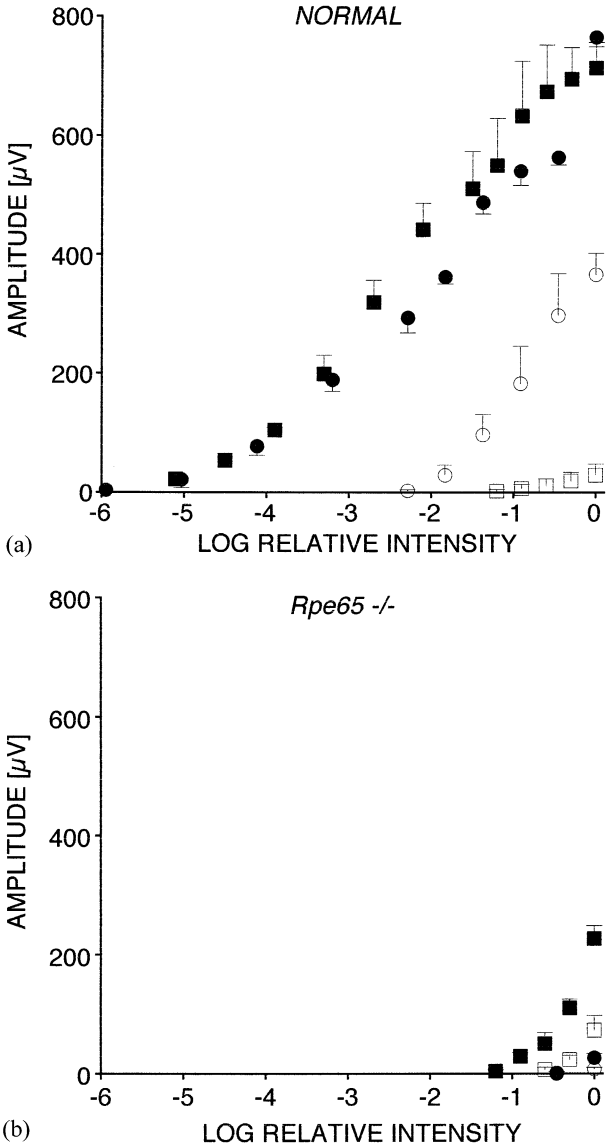

Fig. 3. Relationship between the intensity of the green and ultraviolet flashes and the mean amplitudes of the b-waves of three normal (a) and four young *Rpe65* −/− mice (b). Circles: 360 nm stimuli; squares: 520 nm stimuli; filled symbols: dark-adapted amplitudes; open symbols: amplitudes in the presence of a yellow adapting light. Error bars show the standard error of the mean.

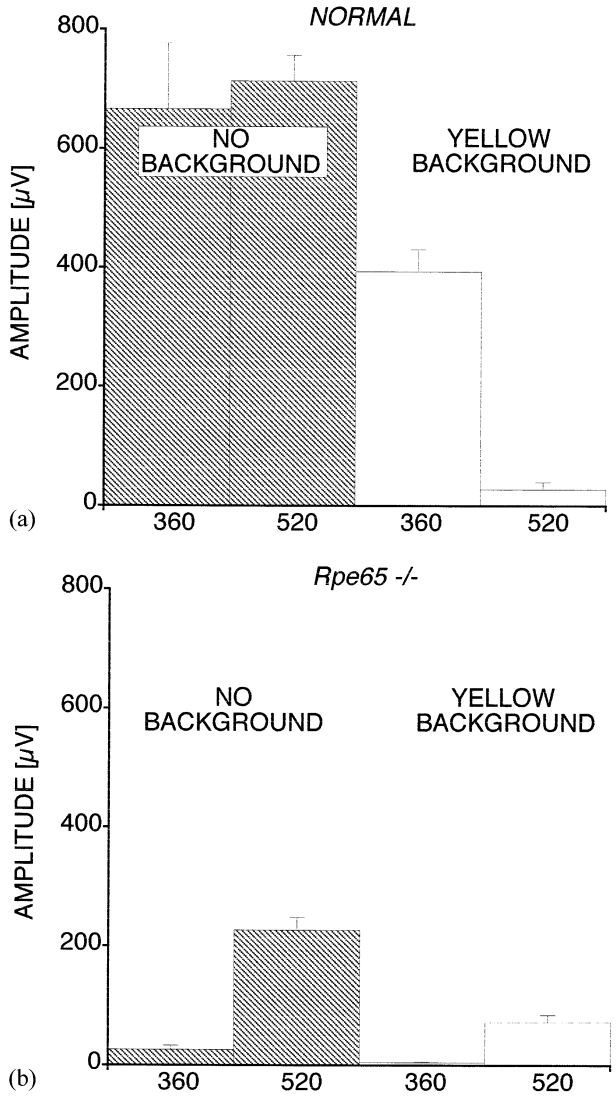

Fig. 4. Maximal responses to the 360 and 520 nm flashes in the presence and absence of the yellow adapting light for normal (a; dark-adapted $n = 3$; with yellow background $n = 6$) and four young *Rpe65* −/− mice (b).

(Redmond et al., 1998). Recently, an indication of rod a-waves was reported in *Rpe65* −/− mice receiving 9-cis retinal therapy (van Hooser, et al., 2000), but at the same time, others have reported that a cone a-wave can also be elicited in the ERG of mice (Lyubarsky, Chen, Simon, & Pugh, 2000). Therefore, it is not completely clear whether there are rod responses that are profoundly depressed or whether there are no rod and only cone responses in *Rpe65* −/− mice. However, our results are more consistent with an absence of rod responses.

The larger amplitude of the M-cone ERG in the presence of a yellow adapting light in *Rpe65* −/− mice may reflect the cone opsin density in these cones. If the density of cone opsin were lower than normal, the adapting light might be less able to desensitize these cones compared to normal cones. This would make the photoreceptors of the *Rpe65* −/− mice less light-adapted than normal mice under the same level of illumination. There is evidence that subjects with retinitis pigmentosa have a reduced cone opsin density (Francois & Verriest, 1961; Pokorny, Smith, & Ernest, 1980; Young & Fishman, 1980). The cone ERG of subjects with retinitis pigmentosa is also less influenced by light adaptation than that of normal subjects (Gouras & Mackay, 1989).

Our results suggest that there are no UV-cone responses in *Rpe65* −/− mice, and the only responses being detected are produced by M-cones. This preferential loss of UV-cone function in this *Rpe65* −/− mouse is surprising, although there is evidence that short wavelength (S) cones share certain features with rods. For example, rod and S-cone arrestin is distinctive antigenically from that of longer wavelength sensitive cones (Nir & Ransom, 1992; Nork, Mangini, & Millecchia, 1993). Carbonic anhydrase is expressed in longer wavelength sensitive cones but not in S-cones and rods (Nork, McCormack, Chao, & Odom, 1990). The S-cone

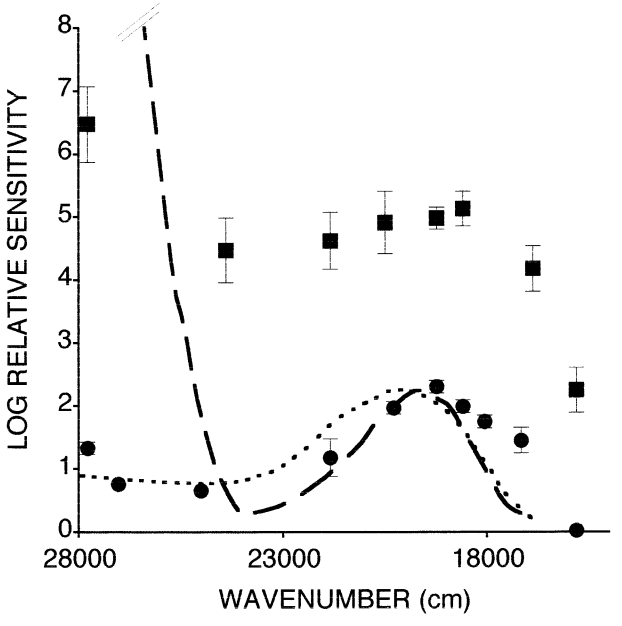

Fig. 5. Action spectra of the dark-adapted ERG in *Rpe65* −/− mice (circles) compared to the normal control (squares). The curves show the action spectra of murine rods (dotted) and cones (dashed) modified from Lyubarsky et al. (1999). Both curves have been transposed vertically to fit our data from *RPE65* −/− mice. The ordinate represents the logarithm of the relative number of quanta in the light flash, and the abscissa represents the wavenumber of the flash in centimeters.

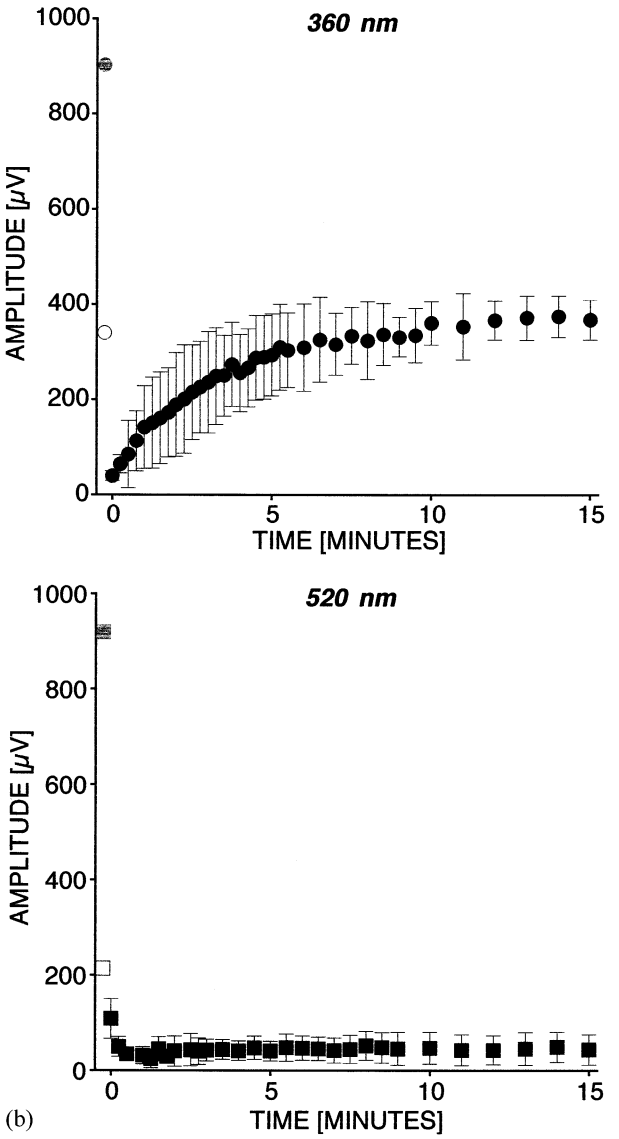

Fig. 6. ERG b-wave amplitudes of normal mice in response to 360 (a) and 520 (b) nm stimuli immediately before and during 15 min after a yellow adapting light is turned on. Circles = 360 nm stimuli; squares: 520 nm stimuli; open symbols: amplitudes in presence of the dim white light before the onset of the yellow adapting light; gray, filled symbols: amplitudes obtained in dark-adapted mice before the onset of the yellow adapting light; black filled symbols: amplitudes during adaption with the yellow adapting light. Error bars indicate the standard error of the mean.

system is also affected earlier in human retinitis pigmentosa than the longer wavelength sensitive cones, again paralleling the rods (Swanson, Birch, & Anderson, 1993; Yamamoto, Hayashi, & Takeuchi, 1999). Such subjects also show early deficiencies in blue–yellow color vision (Verriest, 1963; Pokorny, Smith, Verriest, & Pinckers, 1979; Fisherman, Young, Vasquez, & Lourenco, 1981). The features that rods and S- or UV-cones have in common, as opposed to M-cones, may also involve the way 11 cis-retinal is either synthesized or transported. In salamanders, cones have the RPE65 protein and may therefore be able to synthesize cone opsins independently of the RPE layer (Ma, Xu, Othersen, Redmond, & Crouch, 1998). It would be interesting to know if murine M- and UV-cones also contain RPE65 protein.

The initial depression of the UV-cone ERG immediately after a bright yellow adapting light is turned on parallels the effect reported by Lyubarsky et al. (1999). They found that a preceding yellow flash reduced the ERG of the UV-cones. They suggested that this could be due to the co-expression of M- and UV-cone opsins in UV-cones. Co-expression of photopigments is known to occur in a considerable number of murine cones from morphological studies (Röhlich, van Veen, & Szel, 1994; Glösmann & Ahnelt, 1998; Ahnelt & Kolb, 2000; Applebury, et al., 2000). The fact that this phenomenon gradually disappears after about 8–10 min of maintained light adaptation suggests that it may be an antagonistic neural signal rather than a cone desensitization by the bleaching of M-cone opsin. The latter should be maintained, at least if the machinery for phototransduction in each cone was shared by the M- and UV-opsins, whereas neural antagonism might be turned off by the redepolarization of the M-cones that can occur in the presence of the adapting light.

In summary, our results suggest that there is a marked, preferential loss of UV-cone function, but also an abnormal M-cone function in the *Rpe65* $-/-$ mouse. This has bearings on the diagnosis and pathogenesis of this degeneration and possible therapeutic interventions.

Acknowledgements

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