Assessment of Visual and Chromatic Functions in a Rodent Model of Retinal Degeneration

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Submitted: May 12, 2015
Accepted: August 18, 2015

PURPOSE. We evaluated the photoreceptor response of pigmented P23H and normal pigmented Long Evans (LE) rats over time using functional tests in variable lighting conditions.

METHODS. Pigmented P23H rats were studied by optomotor testing and electroretinogram (ERG) recordings at P30, P150, and P240. Pigmented LE rats were used as a normal wild-type control. Stimuli were modified with colored filters. Neutral density filters were used to reduce luminance.

RESULTS. Age-related decreases in visual acuity (VA) and contrast sensitivity (CS) were observed in P23H rats. Good correlations in measurements without filter and with green filter were observed between LE and P23H P30 rat values. Differences between groups were smaller with red and purple filters. A strong relationship with luminance was observed in LE rats (VA and CS) and with P23H P30 rats (CS). A decline in the ERG responses of P23H rats was consistent with the gradual loss of photoreceptors. Differences in a- and b-wave amplitudes with different colored filters were negligible with the exception of the red filter, which resulted in smaller responses.

CONCLUSIONS. Visual function parameters decreased with age in pigmented P23H rats. Irrespective of luminance, color filter, and retinal degeneration, minimum thresholds of VA and CS were found. Smaller differences than expected were found using color filters. Responses to functional tests at long wavelengths were observed, where there is very low photoreceptor spectral sensitivity. The use of filters with functional testing could minimize light-induced retinal damage in rats.

Keywords: P23H, retinitis pigmentosa, optokinetic tracking, electroretinogram

Rats are nocturnal animals with a rod-dominated retina. However, their retina also contains a small number of cones.1 Rats maintain an order of magnitude difference in the numbers of cones with maximum sensitivity in the short wavelength (S-cones, with a peak of 358 nm) compared to the middle and long wavelengths (ML-cones, peaking at approximately 509 nm).2,3 Middle and long wavelength cones constitute approximately 90% of cones; the other 10% are S-cones that are thought to be UV-sensitive elements, but they probably do not appreciably contribute to the photopic system of the rat.4 The maximum responses of the two types of cones and rods of rats have been widely studied by electrotetrogram (ERG), however the contribution of each type of cone to the visual system is not clear.5,5

Retinitis pigmentosa (RP) is the most common form of inherited photoreceptor degeneration.6,7 It comprises a group of diseases characterized by a progressive anatomical and functional loss of rod and cone photoreceptors.8 Autosomal dominant RP is responsible for most RP cases,9 and most cases are due to rhodopsin mutations. Cones are lost after the rod degeneration. The transgenic albino P23H rat is a well-studied model of autosomal dominant RP. In pigmented RP rodent models, a progressive deterioration also occurs in retinal function and anatomy. Because an important role of retinal pigmentation is to prevent light damage, we studied heterozygous pigmented P23H rats. These animals undergo slower retinal degeneration, provide a closer model to human RP, and allow straightforward evaluation by functional testing.10

Retinal damage related to exposure to intense visible light has been studied widely.11,12 The retinas of genetically inbred albino rodents have been shown to be particularly susceptible to photic injury induced by moderate and high levels of light exposure.13–15 Not all rhodopsin mutations seem to have the same susceptibility to light damage. Other genetic factors also have been reported to be involved in the light sensitivity of different rodent strains.13,14,16 There is a direct relationship between light and retinal damage, so that a longer duration of exposure or higher intensity of light results in greater retinal...
The introduction of functional devices, such as the OptoMotry system (OptoMotry, CerebralMechanics),
21,22 The device consists of a testing chamber created with four screens facing into a square. Animals were placed on a platform in the center of the square. A virtual cylinder comprised of a vertical sine wave grating was projected in 3D coordinate space and rotated around the animal. A video camera, situated above the animal, provided real-time feedback on another screen. Rats were allowed to move freely on the platform, and the spatial frequency of the grating was maintained at the animal’s viewing position by centering the cylinder on the rat’s head. The cylinder was rotated at a constant speed (12°/s).

The experimenter judged whether the rats made tracking motions with reflexive head and neck movements following the stimulus. Animals were assessed for tracking behavior for 5 seconds, and then a gray stimulus appeared, to reduce the possibility of adapting to the stimulus. Spatial frequency thresholds were calculated by systematically increasing the spatial frequency of the grating at 100% contrast until the animals no longer responded. This threshold was considered the maximum VA. A CS curve was generated by identifying the minimum contrast that generates tracking over a range of spatial frequencies.

**Methods**

**Animals**

Pigmented transgenic rats, heterozygous for the P23H rhodopsin mutation, were bred from a cross between normal pigmented LE rats and transgenic albino homozygous P23H line 1. Animals were studied by optomotor testing and ERG recordings at P30, P150, and P240. Long Evans (LE) rats over time, and to identify the relative contributions of rods and cones using functional tests in different lighting conditions.

**Visual Acuity and Contrast Sensitivity Evaluation**

To evaluate visual parameters, 8 pigmented P23H rats were measured at P50, P150, and P240. Eight LE rats were evaluated at P90. The assessment of VA and CS was performed using an OptoMotry system (OptoMotry, CerebralMechanics). The device consists of a testing chamber created with four screens facing into a square. Animals were placed on a platform in the center of the square. A virtual cylinder comprised of a vertical sine wave grating was projected in 3D coordinate space and rotated around the animal. A video camera, situated above the animal, provided real-time feedback on another screen. Rats were allowed to move freely on the platform, and the spatial frequency of the grating was maintained at the animal’s viewing position by centering the cylinder on the rat’s head. The cylinder was rotated at a constant speed (12°/s).

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**Filter Specifications**

The grating was modified with colored filters (green, red, and purple) placed in front of the screens. Transmittance of the filters from 300 to 850 nm was measured in a spectrophotometer, and curves of transmittance were plotted (Fig. 1). The total visible transmittance was 69.82%, 23.56%, and 13.76% for the green, red, and purple filter, respectively. Neutral density (ND) 12% filters were used in combination with colored filters to reduce the luminance. In that case, transmittance was 9.61%, 2.54%, and 1.46%, respectively.

Colored filters were selected as a consequence of the peaks of maximum response in S-cones (358 nm) and ML-cones (509 nm). The green filter was selected with the closest curve of transmittance to the ML-cones.27 The purple filter allowed a good transmission between 370 and 480 nm, and after 590 nm, to minimize the ML-cone response. The red filter gave a good value of transmittance only after 570 nm.

**ERG Recordings**

Pigmented P23H rats were studied by ERG recordings at P30, P150, and P240. Eight animals were studied for each time point. Eight normal LE rats at age P90 were used as wild-type controls. In both groups, four animals were tested with each colored filter and another four rats without filters.

Rats were adapted to darkness overnight and prepared for recording under a dim red light. Animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg), and maintained on a heating pad at a stable temperature. The ketamine/xylazine anesthetic shows robust ERGs with large a-wave and b-wave amplitudes, and low eye movements.28 Pupils were dilated by applying a topical drop of 1% tropicamide (Colircusi Tropicamida; Alcon, Barcelona, Spain). A topical drop of 2% Methocel (OmniVision, Puchheim, Germany) was instilled in each eye before situating the corneal electrode. Furthermore, a drop of 0.5% saline was applied occasionally to the cornea to prevent dehydration and to allow electrical contact with the recording electrode (gold wire loop). Two 25-gauge platinum needles inserted under the scalp, behind the eyes, served as the reference electrodes, with a ground electrode located in the scalp. All experiments were performed in absolute darkness. Stimulus presentation and
Mixed b-Wave

Dark-adapted b-waves show aggregate rod and cone pathway contributions. To describe them, recordings of 3 to 8 single flash presentations of 10 μs duration were displayed. Stimuli were presented at 10 increasing intensities varying from −3.70 to 2.86 log cd/m² in luminance. Interstimuli intervals (ISI) were increased to minimize the effects of bleaching on the rods, which could reduce the b-wave amplitude during successive flashes. The ISI was elevated from 10 seconds at lowest stimulus intensity (−3.70 log cd/m²) up to 120 seconds at highest stimulus intensity (2.86 log cd/m²). The amplitude of the a-wave was measured from the trough of the a-wave to the peak of the b-wave. The results of a- and b-waves were averaged for different recordings. To determine if the ERG response remained, criterion amplitudes were established at 20 μV for a- and b-waves.

Isolation of the Cone Response Using a Double Flash Protocol

The double flash protocol was similar to previous studies. A probe flash was presented 1 second after a conditioning flash. The role of the first flash is to temporarily saturate rods so that they do not respond to the probe flash. The necessary intensity of the conditioning flash for complete rod bleaching was set to 1.4 log cd/m². The probe flash intensity also was 1.4 log cd/m². The response to the probe flash, preceded by the conditioning flash, was taken as a reflection of cone-driven activity, and a rod-driven b-wave was obtained by subtracting the cone-driven response from the mixed response (obtained by the conditioning flash alone). The results were averaged for 3 recordings, with an ISI of 100 seconds to assure full recovery of rod responsiveness.

RESULTS

VA and CS Evaluation

In P23H rats, VA was progressively lost with age as shown in the measurements without filters (Fig. 2). Visual acuity values were 0.542 ± 0.011 cycles/deg for LE and 0.445 ± 0.002 cycles/deg for P23H at P30, and 0.424 ± 0.008 cycles/deg at P150 and 0.347 ± 0.019 cycles/deg at P240. In the LE group, smaller values with the green filter (0.508 ± 0.010 cycles/deg) and even smaller values with the other filters (0.458 ± 0.006 cycles/deg for red + ND 12%) were observed. Smaller differences were found in the P23H groups between measured values without a filter and the worst value from colored filters (0.042 cycles/deg at P30, 0.020 cycles/deg at P150, and 0.040 cycles/deg at P240).

A similar trend was found in CS curves (Fig. 3). In the LE rats, peaks of 52.63 ± 5.43 (with no filter) and 35.13 ± 1.23 (ND 12% filter) were obtained for a spatial frequency of 0.089 cycles/deg. The filter addition gave smaller values, between 26.99 ± 1.60 and 18.56 ± 0.64. The behavior was different with the green filter, which gave similar values to those obtained without filters, 49.29 ± 2.87. Similar results were found in the P23H groups. Peaks of 39.86 ± 0.85 and 33.87 ± 0.94 (P30), 31.11 ± 1.16 and 25.99 ± 1.21 (P150), and 31.54 ± 1.78 and 26.81 ± 1.97 (P240) were obtained for measurements without filters and with the ND 12% filter, respectively. Colored filters gave smaller values, between 24.47 ± 0.95 and 17.99 ± 0.57 (P30, excluding the values obtained with the green filter that were similar to those obtained without filters), 24.95 ± 1.09 and 18.38 ± 0.89 (P150), and 21.72 ± 1.28 and 12.17 ± 0.77 (P240). As expected, LE rats had better results in all cases. Similar results without filters and with green filters were observed between LE and P23H P30 rat values. Differences between groups were lower with red and purple filters.

Relationship Between Visual Parameters and Luminance

Luminance of the screens with different filters was measured by a luminance meter LP 471 Lum 2 (Delta Ohm, Padua, Italy). Visual acuity (Fig. 4A) and CS results (Fig. 4B) were represented as a function of luminance. The LE rats’ VA showed a strong relationship with luminance, achieving an R² value of 0.94. No relationships were found in the P23H rat groups, with R² values < 0.7. Furthermore, a relationship of CS with luminance also was observed in the LE rats (R² = 0.91).
**FIGURE 3.** Contrast sensitivity as a function of spatial frequency with colored filters and adding an ND filter (ND 12%). Measures of the wild-type control LE (black circle), P23H at P30 (white circle), P150 (triangle), and P240 (square) were done. Each point represents the mean of 8 animals. Error bars: SEM.
and the P23H rats at P30 ($R^2 = 0.85$), and a lower relationship was found at P150 ($R^2 = 0.73$).

**ERG Recordings**

In pigmented P23H rats, the scotopic a-wave (Fig. 5A) reached a maximum value of 113 ± 10 μV at P30. This value represents 37% of the same value in normal LE rats (304 ± 34 μV). Despite being affected at early ages, a-waves from P23H rats still could be evoked to P150 (25 ± 5 μV), but they were almost negligible at P240 (<20 μV), the latest age studied. At all ages of P23H rats, b-waves (Fig. 5B) were less affected than a-waves, reaching maximum values of 1092 ± 38 μV (P30), 475 ± 45 μV (P150), and 301 ± 21 μV (P240) with regard to 1178 ± 101 μV (LE at P90).

Figure 5C illustrates the results of the ERG recordings obtained by the application of the double flash protocol from control LE (P90) and P23H rats at P30, P150, and P240. The first flash elicted a mixed rod-cone response (left traces), and the second flash (one second delay) elicited a pure cone response (middle traces). The right traces were calculated by subtracting the middle traces from the left traces to obtain the...
cones. Most cone photoreceptors are ML-cones, responsible for the green photopic sensitivity of the rat. The role of the S-cones under photopic lighting conditions has been less studied. Based on the peaks of maximum absorbance of cones and rods, we selected colored filters with a specific spectral sensitivity curve (Fig. 1) for isolating the responses of different photoreceptors in vivo. Using this method, new information could be obtained about the changes in the different cone photoreceptors following the rod loss in this autosomal model of rhodopsin gene mutation. Cone evaluation and preservation should be one of the main issues after therapeutic approaches.

Figure 6 shows the ERG amplitudes as a function of luminance and the results of the double flash protocol from the LE (P90) and P23H rats (P30, P150, and P240), measured with different colored filters. In the P23H group, similar results in the a- and b-wave values were obtained by adding different filters (Figs. 6A, 6B), excluding the P30 a-wave (value changed from 65 μV with the red filter to 152 μV without the filter) and the b-wave at P150 (from 309 μV without the filter to 608 μV with the green filter). Larger differences were observed in LE rats, but we were not able to identify any pattern between a- and b-waves and colored filters (Figs. 6A, 6B).

There were differences in the a-waves of rod-driven responses in all groups (Fig. 6C). A-waves from P23H rats were negligible (<20 μV) when using red and purple filters for all ages. In LE rats, the results were smaller with the same filters (52 μV for red and 160 μV for purple, compared to 242 μV for green and 266 μV without a filter). No clear relationship between the b-wave of rod-driven responses and colored filters was found. However, there were differences in the rod-driven contribution to mixed scotopic b-waves, with a higher percentage using green filters (more than 40% in all cases) and no filters (more than 50%) compared to red and purple filters (less than 40% and, except one case, less than 33%).

**DISCUSSION**

The present study has applied functional testing (OKT and ERG) to an animal model of progressive retinal degeneration (pigmented P23H rat) and a wild-type control (LE rat) to evaluate the photoreceptor response, and the relative contributions of rods and cones in different lighting conditions. To our knowledge, there have been no examples of using these functional tests with in vivo photoreceptor responses and adding color filters to isolate the different photoreceptor responses.

It has long been known that rodents have two types of cones. Most cone photoreceptors are ML-cones, responsible for the green photopic sensitivity of the rat. The role of the S-rods-driven contribution to the ERG response. The rod-driven responses were affected by age in the P23H rats: a- and b-waves reached values of 40 and 391 μV at P30, <20 and 188 μV at P150, and 32 and 109 μV at P240, compared to 165 and 516 μV in the LE rats. The rod-driven contribution to the mixed scotopic b-waves was higher in LE rats (36%) compared to P23H rats, remaining stable for all ages (38% at P30, 40% at P150, and 37% at P240).

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The decreased ERG response with age also was clear, as already has been described (Fig. 5). The a-wave change was particularly clear, and the a-wave appears to be a better parameter than the b-wave to judge the efficacy of therapeutic manipulation in this rat model of retinal disease generated by photoreceptors. Values of 304 ± 63 μV for the LE rats and 113 ± 10 μV (P30), 25 ± 5 μV (P150), and <20 μV (no response, P240) for the P23H rats were consistent with the gradual loss of photoreceptors. So, the long-term survival of some cones in transgenic rats could not be responsible for the minimum values of VA and CS, considering the ERG results. Thus, minimum OKT thresholds are achievable in pigmented...
P23H rats without a full complement of photoreceptors, and it appears that rods and cones may not be necessary for minimum values of VA and CS, as others have concluded.\textsuperscript{37}

In P23H rats, there were no marked differences in most of the a- and b-waves with different colored filters (Figs. 6A, 6B), except in the P30 a-wave and the P150 b-wave, which could be related to the smaller measurements with the red filter and without a filter, respectively. In LE rats, a similar pattern between a- and b-waves and colored filters was not found (Figs. 6A, 6B). However, the worst results were given by red filter, which has been shown to be the most limiting in the photoreceptor response.

The results of the ERG recordings obtained by the application of the double flash protocol showed differences in all groups (Fig. 6C). A-waves of rod-driven responses from P23H rats were negligible (<20 mV) using red and purple filters for all ages. In LE rats, smaller results were found using the same filters. Moreover, there were differences in the rod-driven contribution to mixed scotopic b-waves, with larger values with green filters (>40%) and without filters (>50%) when compared to red and purple filters (<33%). These results, although not conclusive, are in agreement with the filter characteristics. Using more animals to study each filter, evaluating the possible influence of the anesthesia,\textsuperscript{28} and choosing filters with a more suitable transmittance curve could allow a more detailed analysis.

In conclusion, visual function parameters decrease with age in pigmented P23H rats. Irrespective of luminance, color filter, and retinal degeneration, minimum thresholds of visual acuity and contrast sensitivity were found. Smaller differences than expected were found using color filters, which might reflect that color differences are of minor contribution for rat vision. Responses to functional testing at long wavelengths were observed, where there is very low photoreceptor spectral sensitivity. The use of filters with functional testing could minimize light-induced retinal damage in rats.

Acknowledgments

Supported by the Spanish Ministry of Health ISCIII (PI13/01124, PS0901854), DGA Group B99, ISCIII RETICS RD12/0034/0010, and by a Zaragoza University Grant FPUZ-2011-BIO-02 (FS).

Disclosure: F. Segura, None; A. Sánchez-Cano, None; S. Jarabo, None; C. López de la Fuente, None; N. Cuenca, None; M.P. Villegas-Pérez, None; I. Pinilla, None

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