Determination of Rod and Cone Influence to the Early and Late Dynamic of the Pupillary Light Response

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Purpose. This study aims to identify which aspects of the pupil light reflex are most influenced by rods and cones independently by analyzing pupil recordings from different mouse models of photoreceptor deficiency.

Methods. One-month-old wild type (WT), rodless (*Rho*$^{-/-}$), coneless (*Cnga3*$^{-/-}$), or photoreceptor less (*Cnga3*$^{-/-}$; *Rho*$^{-/-}$ or *Gnat1*$^{-/-}$) mice were subjected to brief red and blue light stimuli of increasing intensity. To describe the initial dynamic response to light, the maximal pupillary constriction amplitudes and the derivative curve of the first 3 seconds were determined. To estimate the postillumination phase, the constriction amplitude at 9.5 seconds after light termination was related to the maximal constriction amplitude.

Results. *Rho*$^{-/-}$ mice showed decreased constriction amplitude but more prolonged pupilloconstriction to all blue and red light stimuli compared to wild type mice. *Cnga3*$^{-/-}$ mice had constriction amplitudes similar to WT however following maximal constriction, the early and rapid dilation to low intensity blue light was decreased. To high intensity blue light, the *Cnga3*$^{-/-}$ mice demonstrated marked prolongation of the pupillary constriction. *Cnga3*$^{-/-}$*; Rho*$^{-/-}$ mice had no pupil response to red light of low and medium intensity.

Conclusions. From specific gene defective mouse models which selectively voided the rod or cone function, we determined that mouse rod photoreceptors are highly contributing to the pupil response to blue light stimuli but also to low and medium red stimuli. We also observed that cone cells mainly drive the partial rapid dilation of the initial response to low blue light stimuli. Thus photoreceptor dysfunction can be derived from chromatic pupillometry in mouse models.

Keywords: pupillary response, photoreceptors, mouse model, retinal dystrophy

Identification of melanopsin expressing retinal ganglion cells as nonrod, noncone photoreceptors has promoted studies to better understand the relative contributions of melanopsin, rods, and cones to pupil responses to light. It has been shown that melanopsin alone can evoke pupillary constriction to light under photopic conditions but with a higher threshold compared to rods and cones.[1–3] Separating rod and cone inputs to the pupil light reflex in mice is hampered due to the considerable overlap of the spectral sensitivity of M-cones and rods. However, by replacing the murine cone opsin (peak sensitivity at 511 nm) by the human long-wavelength cone opsin (556 nm), Lall et al.[4] demonstrated that cones can independently contribute to the murine pupil response to light.

Such studies have used static photographs under steady-state conditions of illumination to assess the photoreceptor input, whereas studies of rod, cone, and melanopsin input on the transient and sustained characteristics of the pupil light reflex utilizing dynamic recordings are scarce. Two early studies continuously recorded pupillary movement over 10 to 18 seconds following a light stimulus in mouse models of bipolar cell deficiency.[5,6] These studies demonstrated that bipolar cells are important intermediates in retinal signaling of the pupil light reflex and their absence affects amplitude, latency, and recovery rate. Neither study, however, characterized or quantified the postillumination dynamics of the pupil response.

Aleman et al.[7] more carefully described the pupillary waveform in chromophore deficient mice. Pupil responses from dark adapted, anesthetized RPE65 knockout mice were recorded to brief white, green, or orange light stimuli at increasing intensities to determine if an alternative nonrhodopsin, nonmelanopsin pigment could be responsible for the residual visual function noted in these mice.[7] In addition to the maximal constriction amplitude and latency, these authors measured the postillumination constriction amplitude (2.5 seconds after stimulus onset) as a measure of the early dilation phase. These authors found that pupil responses of *Rpe65*$^{-/-}$ mice are similar to wild type (WT) but with lower sensitivity. Absence of differences between green and orange light suggested that there was no iso-rhodopsin[8] driven response. Kostic et al.[9] noted a similar decrease in the sensitivity in the missense *Rpe65*$^{R91W/R91W}$ mouse model using short white

stimuli but dynamic evaluation of the postillumination pupil response was also limited (5 seconds after stimulus onset).

A clear understanding of the relative contributions of rod, cone, and melanopsin to the dynamics of the pupil light reflex in normal and in photoreceptor degenerations is lacking. This study was aimed at narrowing this knowledge gap by taking advantage of different genetic mutations in mouse photoreceptor degeneration models, combined with the use of narrow bandwidth light stimuli, to study the dynamics of the pupil light reflex. To this end, we aimed to better characterize the pupil light response of mice selectively deficient in rods, cones, or both photoreceptors in order to identify their independent and combined input to the pupil light reflex.

Methods

Animals

The animals were handled in accordance with the statement of the "Animals in Research Committee" of the Association for Research in Vision and Ophthalmology, and protocols were approved by the local institutional committee. The mice were kept at 20°C under a 12-hour light/12-hour dark cycle with light on at 7 AM and were fed ad libitum. *Cnga3*$^{-/-}$ were obtained from Martin Biel.[10] *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ mice were obtained from breeding *Cnga3*$^{-/-}$ mice to *Gnat1*$^{-/-}$ mouse strain[11] and *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ were obtained from breeding *Cnga3*$^{-/-}$ mice to *Rho*$^{-/-}$.[12,13] Sv129 mice were used as WT controls. All mice were tested at 1 month of age.

Light Stimuli and Pupillary Recording

The A2000 pupillometer for small rodents (Neuroptics, Inc., Irvine, CA, USA) was used to stimulate the retina by four banks of different wavelength LEDs in combination with a diffuser lens with a separate optical pathway for a single infrared camera for each eye.[14] The pupil was imaged through a telecentric lens so that small changes in distance between the lens and the eye would not affect magnification when the pupil border was in focus. The animal was maintained at a defined and constant distance from the camera to ensure a reproducible illumination between animals. The light stimulus was a 500-ms red (622 ± 8 nm) or blue (463 ± 8 nm) light of increasing intensities over almost a four-log unit range. Unilateral stimulation was performed and the pupil of the stimulated eye (direct response) was continuously recorded at 31 Hz. The pupil diameter was determined automatically by the Neuroptics, Inc. software. Unless stated, mice were dark adapted overnight and tested under mesopic (<5 lux) red light. The mice were not anesthetized to avoid medication effect on the pupillary movement but were manually and gently restrained in front of the camera. Calmed in this manner, they generally looked straight ahead with minimal head movements. Baseline pupil diameters were reproducible for each animal, but a potential effect of handling stress on the pupil light reflex cannot be ruled out.

The following light sequence was used: red 1.2 log lux (−1.2 log W/m^2, 0.065 W/m^2), blue 0.6 log lux (−1.1 log W/m^2, 0.074 W/m^2), red 2 log lux (−0.4 log W/m^2, 0.408 W/m^2), blue 1.2 log lux (−0.5 log W/m^2, 0.3 W/m^2), red 4.5 log lux (2.1 log W/m^2, 129.018 W/m^2), blue 2 log lux (0.3 log W/m^2, 1.893 W/m^2). For convenience in the following text, we qualified 1.2 log lux red and 0.6 log lux blue as low intensity red and low intensity blue, respectively; 2 log lux red and 1.2 log lux blue as medium intensity red or blue, respectively; and 4.5 log lux red and 2 log lux blue as high intensity red and blue, respectively. Blue light was not increased to the equivalent intensity of red (129.018 W/m^2) because the light was so bright that the mice had an aversive head turn and eye closure response and pupils could not be recorded. It has to be noted that the radiance for 0.3 log W/m^2 blue light (the brightest blue light used in this study, 1.893 W/m^2) is around 100-fold less than the brightest red (129.018 W/m^2). The pupil recording started 500 ms before the light stimulus and continued for 29 seconds after the light stimulus. The light stimuli were given independently in sequence without a fixed interstimulus interval as the mice were occasionally freed and permitted to move, which facilitated their calming before resuming the next light stimulus and pupil recording. The interval was generally less than a minute between stimuli. We assumed only a limited bias due to handling stress because reproducible baseline pupil diameters were obtained before each stimulus and between individuals of similar genotype.

Pupillary Light Reflex Analysis

The pupil response data were exported to a worksheet. The baseline pupil diameter was determined from the mean pupil size during 500 ms before the light stimulus. All pupil sizes thereafter were converted to relative pupil diameter against the baseline (=100%). For each light stimulus, the averaged response from 6 to 15 pupil recordings (unilateral recordings of different animals) was calculated and plotted using the GraphPad Prism 5.01 software (GraphPad Software, Inc., San Diego, CA, USA). The pupil response was divided in two phases: (1) the initial response during the first 2.5 seconds following the stimulus onset and (2) the postillumination phase from 2.5 seconds to 9.5 seconds following stimuli onset.

Three different parameters were selected to describe the initial pupil response. The first parameter was the maximal constriction amplitude (percent change from baseline) during the first 1.8 seconds following stimulus onset. The second was a first derivative (velocity) curve analysis of the first 3 seconds from light stimulus onset. The 3-second time window was selected to be comparable to the 2.5-second poststimulus analysis by Aleman et al.[7] who described two types of dilation speed depending on stimuli intensity. To obtain the derivative curve, the first derivative (six orders, 12th neighbors, GraphPad Prism 5.01 software) of the relative pupil size was determined and plotted as a function of time. The derivative curve permitted us to describe the velocity of constriction and dilation during the initial 3 seconds (transient phase) of the pupillary light reflex. When the pupil is in a steady state, for example during the first 500 ms prior to light stimulation, the derivative values are essentially zero. A change toward a smaller pupil size is indicated by negative values, and the rate of change is indicated by the magnitude of these negative values. The peak negative value is the point of maximum velocity of constriction, which becomes slower as constriction continues toward maximum amplitude. When the constriction stabilizes briefly at minimum pupil size, the derivative value returns to zero. Thereafter, if, the derivative values become positive, this indicates a change toward increasing pupil size which corresponds to a dilation movement. When these positive values increase rapidly, a peak is distinguishable, indicating a rapid and early dilation whose maximal velocity (the positive peak) is reached within 3 seconds of the light stimulus. Alternatively, if the derivative stays around zero without reaching a peak for positive values, it indicates the absence of a rapid dilation within this 3-second period. The third parameter was the time to peak, defined as the time following the light onset to the maximum pupil constriction amplitude, when the pupil diameter was the smallest.

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In order to quantify the extended postillumination phase, we calculated the ratio of the sustained constriction amplitude (% constriction at 9.5 seconds following stimulus offset) to the maximal constriction amplitude. This sust/max ratio reflects how much the pupil tends to stay contracted after the light is terminated, relative to the maximum constriction in response to the light stimulus onset. The 9.5-second time point was selected for the sustained postillumination pupil response by determining the time point with the most significant *P* values as a function of time after stimulus offset for this ratio at each time frame of 31 Hz for the *Rho*$^{-/-}$, *Cnga3*$^{-/-}$, *Cnga3*$^{-/-}$; *Rho*$^{-/-}$, and *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ mice compared to WT mice.

Statistics

Statistical analyses to compare maximal amplitudes and postillumination ratios were performed by two-way ANOVA, and Bonferroni posttests determined the statistical significance between the different groups (WT, *Rho*$^{-/-}$, *Cnga3*$^{-/-}$, *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$, *Cnga3*$^{-/-}$; *Rho*$^{-/-}$). One-way ANOVA analysis was applied to determine the influence of stimuli (blue and red of different intensities) for WT animals and the influence of the genotype on the baseline pupil size. Full statistical data are available in Supplementary Table S1. To define the time point with maximal ratio difference between groups, *t*-tests were performed to compare *Rho*$^{-/-}$ or *Cnga3*$^{-/-}$ or *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$, *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ amplitude with WT amplitude at different time points (every 0.33 seconds) during the first 15 seconds of the protocol.

Results

Variations of the Color and Intensity of the Stimuli Modulate the Early Dynamic of the Pupil Light Response (PLR) in WT Mice

We first characterized the pupil response to blue and red light stimuli in dark-adapted, nonanesthetized WT mice. The relative pupil diameter was plotted against time to obtain pupil response curves to red light and blue light stimuli, as shown in Figures 1A and 1B. The time to peak constriction is equivalent for the low blue stimulus and for low and medium red stimuli, respectively (1 ± 0.03 seconds, 0.9 ± 0.02 seconds, and 0.94 ± 0.02 seconds). For higher intensities of blue and red stimuli, the time to peak constriction is significantly increased to 2.62 ± 0.44 seconds, 2.73 ± 0.24 seconds, and 2.66 ± 0.43 seconds, respectively. This prolongation of peak time seen with higher intensities is similar to that described with longer duration stimuli[5,6,15] and also similar to responses from mice devoid of classical photoreceptors.[2]

At the lowest intensity, there was no difference between constriction amplitude in response to low intensity blue and low intensity red lights (Figs. 1A, 1B). However, when responses to similar irradiance values of medium blue and red were compared,[16] the maximal constriction amplitude was larger for the blue light ($F(5, 41) = 17.72$, $P < 0.05$). These results indicate a shift of sensitivity at medium light levels, where the blue wavelength is more efficient to drive pupil constriction than red.

From the pupil response curves, we observed two patterns of the dilation dynamics. A pattern showing early and rapid dilation was found for both the low intensity blue and the low and medium intensity red stimuli. A second pattern showing a more sustained constriction with delayed and slow dilation was observed for medium and high intensity blue, and high intensity red light. From the first derivative curves, we also identified these two patterns, or "shapes," of the pupil dilation (Figs. 2A, 2B). For low blue (Fig. 2A), and for low and medium red (Fig. 2B), following the maximal velocity of the constriction (peak negative value), the derivative increased toward positive value before 3 seconds after light onset corresponding to the rapid dilation of the pupil immediately following maximal constriction. However, for brighter blue stimuli (–0.5 and 0.3 log W/m^2, Fig. 2A) and for the brightest red stimulus (2.1 log W/m^2, Fig. 2B), the derivatives values after the negative peak tended to reach zero velocity but never crossed the *x*-axis into positive values indicative of a prolongation of the initial constriction.

For the extended postillumination phase, we found that the sustained constriction varied from 30% to 60% of the maximal constriction amplitude and this was dependent on the stimulus intensity (Table). This result is consistent with previous studies in which higher stimuli intensities produced, a greater ratio due to a more prolonged pupil constriction.[7,17]

Rodless and Coneless Mice

To better understand the rod and cone contributions to the pupil response described above in WT mice, we recorded the pupil response in mice selectively void of rod or cone function using rodless (*Rho*$^{-/-}$) and coneless (*Cnga3*$^{-/-}$) mice. One-month-old animals were examined to avoid a secondary effect of degeneration on the nonaffected photoreceptor. The baseline pupil size of *Cnga3*$^{-/-}$mice (1.41 ± 0.03 mm) was 17% smaller than WT (1.61 ± 0.04 mm) without statistical significance, whereas *Rho*$^{-/-}$ mice (1.93 ± 0.04 mm) had 20% larger baseline pupil diameter than WT ($F(4, 41) = 29.8$, $P < 0.01$). Despite the smaller baseline size of the *Cnga3*$^{-/-}$ mice, none of the evoked pupil constrictions of any groups recorded in this study reached the mechanical limit of the pupil size as reported previously using topical carbachol application[3] or high light exposure (0.36 mm diameter[18]). Thus differences in the pupil response are not biased by a difference in the baseline diameter. However, baseline size in dark-adapted animals does appear to be influenced by the classical photoreceptors.

In the rodless mouse (*Rho*$^{-/-}$), the maximal constriction amplitude to blue stimuli was 25% to 50% less than WT (Fig. 1C; Table) at all intensities. A significant decrease in the maximal constriction to red stimuli was also noted compared to WT ($F(4, 127) = 146.8$, $P < 0.001$; Fig. 1D; Table). Surprisingly, we noted as much as 80% loss of the constriction amplitude in response to the lowest red stimuli indicating that rods have an important contribution to pupil response to red stimuli. Concerning the early dynamics of the response, rodless mice still reached positive derivative values between 1 and 1.5 seconds indicating no real modification in the pattern of the derivative curve compared to WT (Figs. 2C, 2D; Table). In other words, rodless mice maintain a rapid and early partial dilation that, we hypothesize, is due to the remaining cone function. However, in the later postillumination phase, a more prolonged constriction at 9.5 seconds following stimuli onset was revealed by an increased postillumination phase ratio (Table). The sust/max ratio for all red and blue lights was increased in the *Rho*$^{-/-}$ mice compared to WT but the difference was significant only for blue lights. To summarize, despite a larger baseline pupil size, *Rho*$^{-/-}$ have a reduced and prolonged pupillary constriction in response to both blue and red stimuli, indicating that rods contribute to both the initial pupil constriction to light as well as postillumination phase response. However, the pattern of the early pupillary dynamics of the *Rho*$^{-/-}$ mice is similar to WT.

In the coneless *Cnga3*$^{-/-}$ mice, the maximal constriction amplitude was not significantly reduced compared to WT, for

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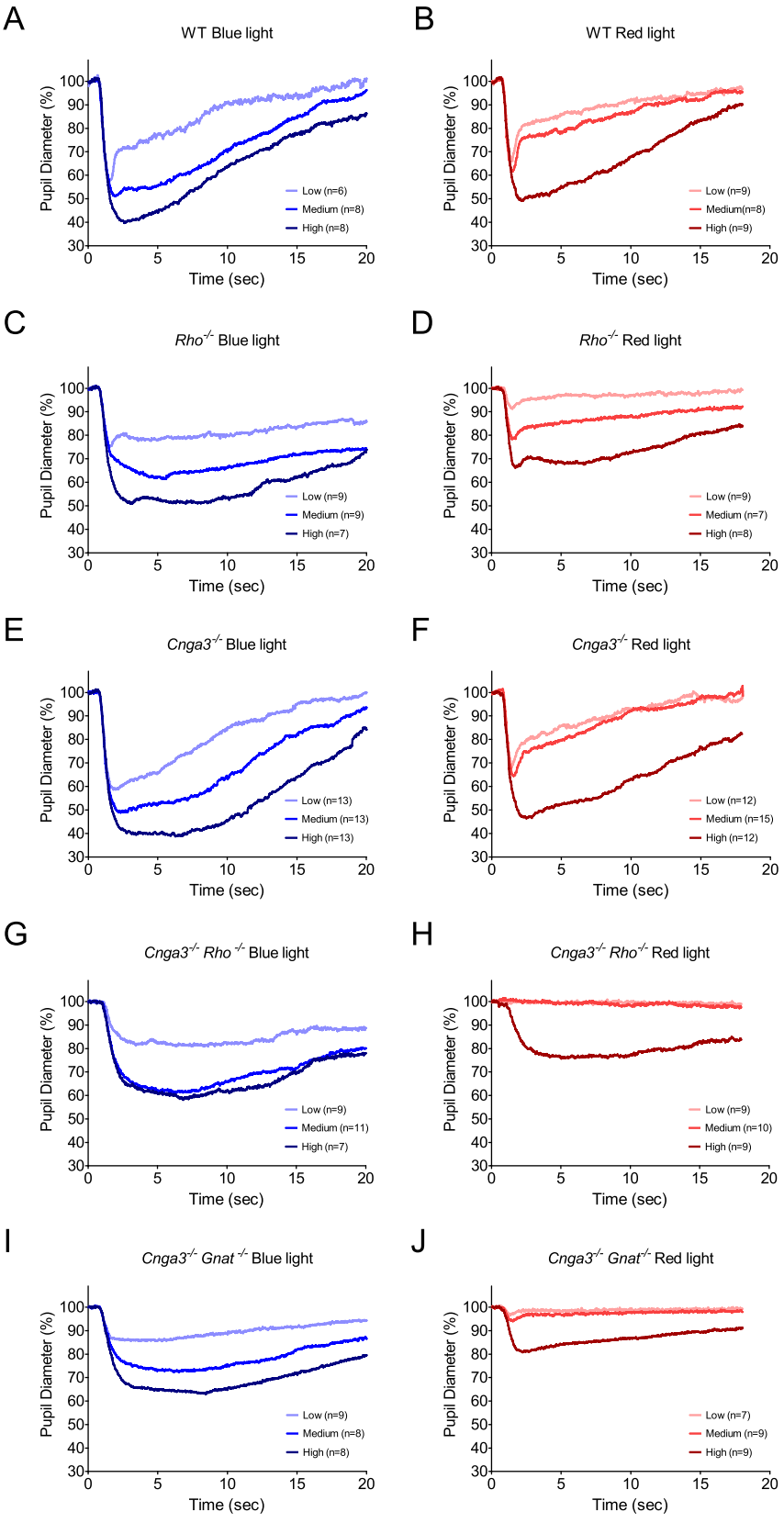

Figure 1. Mean pupil responses of normal and knockout mice to chromatic light stimulation (500 ms red or blue light) of varying intensity. (**A**, **B**) Wild type mice. (**C**, **D**) *Rho*$^{-/-}$ mice have rhodopsin mutation (no functional rods). (**E**, **F**) *Cnga3*$^{-/-}$ mice have a cone channel deficiency (no functional cones). (**G**, **H**) *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ mice have a cone channel deficiency and rhodopsin mutation (both cone and rod loss). (**I**, **J**) *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ mice have a cone channel deficiency and rod transducin deletion (no functional cones and severe impairment of rod phototransduction). *X*-axis: time in seconds; *Y*-axis: pupil diameter (in %) relative to baseline diameter determined from 500 ms before each stimulus. Low: 0.6 log lux blue, 1.2 log lux red; Medium: 1.2 log lux blue, 2.0 log lux red; High: 2.0 log lux blue, 4.5 log lux red.

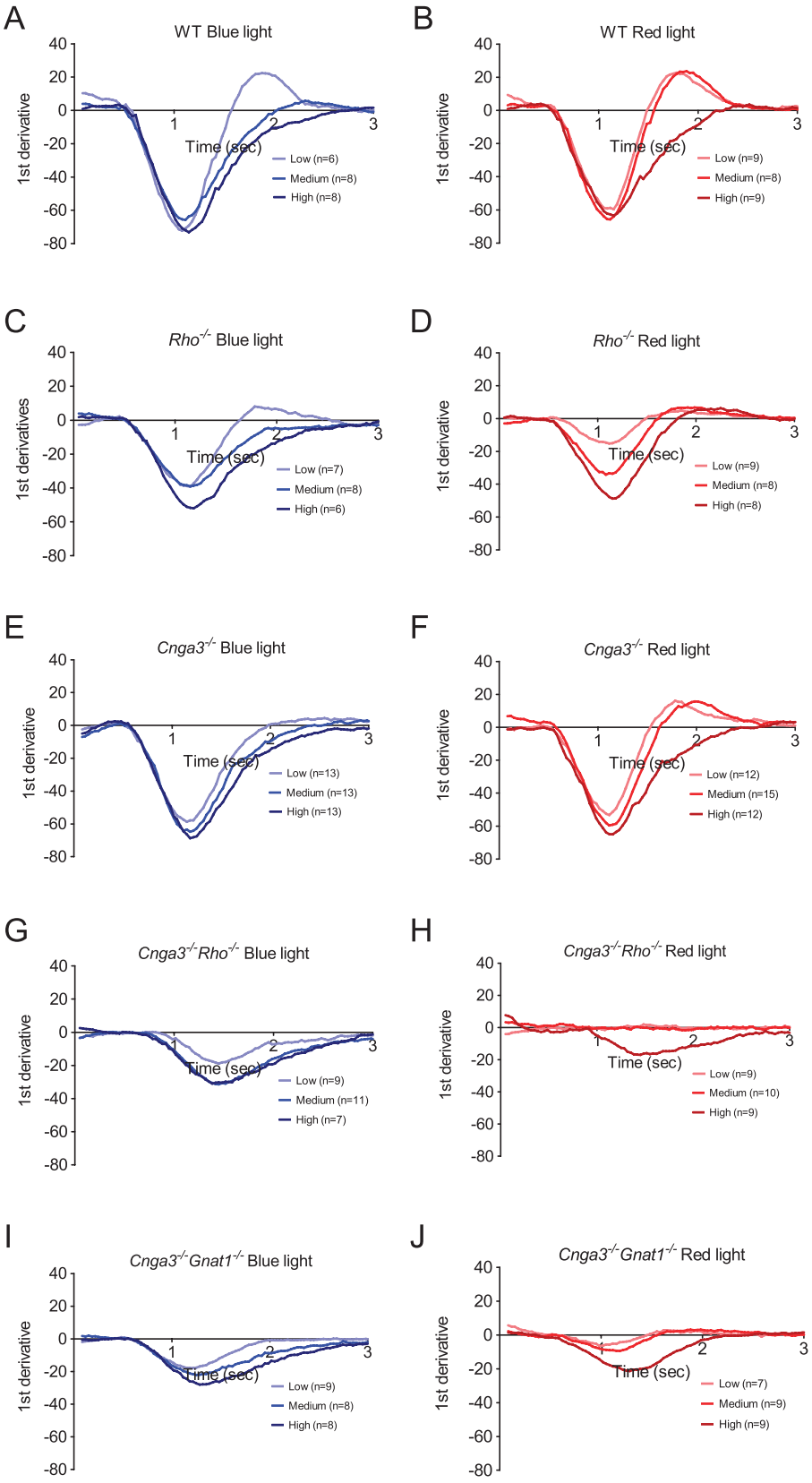

Figure 2. First derivatives of the 3 first seconds of the pupil response to red and blue light stimulation reveals two patterns of the early dynamics. See Figure 1 legend for explanation of abbreviation of mouse model and light intensities used. Wild type responses are shown in **A** and **B**. Negative derivative values indicate a decreasing pupil size (constriction) and positive derivative values indicate an increasing pupil size (dilation). Positive values between 1.5 and 2 seconds indicate that dilation occurred immediately following maximal constriction. This particular pattern was clear for WT at low blue and low medium red intensities, as well as for *Cnga3*$^{-/-}$ at low and medium red. Despite being very small, positive values were still noted for *Rho*$^{-/-}$ at low blue and low and medium red as well as for *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ low and medium red. In the *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ model, no positive values were detected in the first 3 seconds.

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TABLE. Comparison of the Different Pupil Light Response (PLR) Parameters for the Different Mouse Model

	Blue (Log Lux)									Red (Log Lux)								
	Low			Medium			High			Low			Medium			High		
Strain	Max*	*f(x)* >0	Ratio†	Max	*f(x)* >0	Ratio	Max	*f(x)* >0	Ratio	Max	*f(x)* >0	Ratio	Max	*f(x)* >0	Ratio	Max	*f(x)* >0	Ratio
WT	43.22 ± 1.99	+‡	0.21	50.82 ± 2.88	–§	0.53	60.53 ± 1.83	–	0.55	34.65 ± 2.47	+	0.23	39.17 ± 3.12	+	0.27	52.04 ± 1.89	–	0.57
Rho$^{-/-}$	23.03 ± 2.28	+	0.76	31.14 ± 2.64	–	1.02	44.86 ± 4.61	–	0.99	6.49 ± 1.6	+	0.30	18.04 ± 3.46	+	0.55	35.10 ± 2.34	+	0.73
Cnga3$^{-/-}$	43.55 ± 1.25	–	0.33	52.42 ± 1.53	–	0.62	58.49 ± 1.77	–	0.89	32.96 ± 2.13	+	0.24	37.12 ± 1.96	+	0.22	53.88 ± 1.84	–	0.63
Cnga3$^{-/-}$; *Rho*$^{-/-}$	14.24 ± 2.51	–	1.08	27.52 ± 4.16	–	1.07	28.21 ± 6.39	–	1.20	2.45 ± 0.54	–	ND	1.47 ± 0.38	–	ND	17.00 ± 5.32	–	1.24
Cnga3$^{-/-}$; *Gnat1*$^{-/-}$	15.39 ± 3.34	–	0.59	22.73 ± 3.92	–	0.93	29.81 ± 3.5	–	1.07	4.31 ± 1.58	+	0.29	6.39 ± 1.31	+	0.38	19.80 ± 3.86	–	0.69

ND, not determined.

* Maximum constriction amplitude expressed as percentage of the baseline and expressed as the mean ± SEM.

† Sustained constriction amplitude/maximum constriction amplitude.

‡ Indicates positive value between 1.5 and 2 seconds.

§ Indicates no positive values between 1.5 and 2 seconds.

either red or blue stimuli (Figs. 1E, 1F; Table). From the derivative analysis, we observed no difference in the pattern of the early dynamics to red lights. However, there was a notable change in pattern after low intensity blue (Fig. 2E; Table). Likewise, the sust/max ratio was significantly increased compared to WT at high intensity blue (Table) (compare Fig. 1A with Fig. 1E). These results suggest a major contribution of cones to the early dynamic of pupil response to low intensity blue and to the postillumination phase at 9.5 seconds in response to high intensity blue light stimulation. While saturation effects might influence individual pupil responses to the high intensity stimuli, they would not confound the results of genotype comparisons.

Chromatic Protocol in Photoreceptor-Deficient Mice

In order to demonstrate the classical photoreceptor component of the pupil response to our particular protocol, we examined *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ mice that, in addition to lacking cone function, have no rod function. The pupil response of this model is thus the result of the melanopsin activity only. Compared to *Cnga3*$^{-/-}$, the double knockout mice demonstrated no pupil response to low and medium red lights and confirm the rod origin of these responses to red light in the coneless *Cnga3*$^{-/-}$ mice (Figs. 1G, 1H). The pupil responses to other stimuli revealed the melanopsin-mediated contribution but a 40% to 60% reduction in the maximal constriction amplitude was observed, indicating that both rod and cone photoreceptors and melanopsin contribute to the maximum pupil constriction in WT mice. On the derivative curves, the positive values were conspicuously absent and consistent with an absence of the early rapid dilation observed *Cnga3*$^{-/-}$ mice to blue stimuli (Figs. 2E, 2G). This observation confirms that the photoreceptor components are required for the early dynamics of the pupil response that is to say, for both the initial constriction and the immediately following partial dilation. Similar to single photoreceptor knockout mice (*Rho*$^{-/-}$ and *Cnga3*$^{-/-}$ mice), in both coneless and rodless mice, the sust/max ratio in response to blue stimuli is increased compared to WT (Table) showing a significant delay in the pupil dilation pattern in the later postillumination phase in absence of classic photoreceptor function.

We then examined another double knockout model, *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ mice, which lacks cone function and have severely impaired rod phototransduction but not complete ablation of rod function. There were significant differences in the mean baseline pupil size of these two double knockout strains ($F(4, 41) = 29.8$, $P < 0.001$). The baseline diameter of *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ (1.89 ± 0.03 mm) and *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ (2.29 ± 0.01 mm) was larger than WT (14% and 42%, respectively). The most notable difference to WT was a decreased maximal constriction amplitude (Figs. 1I, 1J; Table) and abolishment of positive values in the derivative analysis after blue stimuli. Interestingly, after red illumination, a residual constriction was still detectable at low and medium red lights and positive derivative values were observed in red responses (Fig. 2J). This remaining pupil response well illustrates the incomplete rod loss described in the literature for this model.[15]

DISCUSSION

Our study revealed that blue and red light illumination of varying intensity can elicit different types of pupil response dynamics during the initial 3 seconds from light onset and during a more extended postillumination phase. In dark-

adapted WT mice, when we compared the response to medium light intensities having relatively similar irradiance values,[16] we noted that the pupillary constriction to blue light is greater than to red light. However, this difference is not seen at low light intensities. This observation could indicate a recruitment of additional photosensitive cells with increasing blue light intensity, which might be either the melanopsin or M-opsin cells that have peak sensitivities close to rods.[4] These results demonstrate the high sensitivity of the rodent system to blue light.

The time to peak constriction is also dependent on stimulus intensity and wavelength and may also reflect the origin of the photoreceptor input. Consistent with the pupil response to low white stimuli described by Aleman et al.[7], the short time to peak (1 second) of low blue and low and medium red indicates a photoreceptor input. We believe the late time to peak (2.6 seconds) of higher intensities with this protocol indicates a strong melanopsin input and not greater recruitment of rods and cones. This is because, compared to WT, the *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ mice had pupil responses having low constriction amplitude with longer time to peak.

From the *Rho*$^{-/-}$ mouse model, we observed a significant decrease in the maximal constriction amplitude and also a more prolonged constriction for the blue stimuli, indicating the important rod input to the initial and postillumination response. Given a peak wavelength sensitivity of rods at 498 nm, the observed decrease in constriction amplitude in the *Rho*$^{-/-}$ mouse to 622 nm red light indicated that rods are sufficiently activated with red light to contribute to the pupil response in WT mice. From this knockout model, we observed that lack of rod function was insufficient to abolish the early dilation, which by default can be now partly attributed to M-opsin cone modulation of the pupil dilation.

Unlike the *Rho*$^{-/-}$ mice, *Cnga3*$^{-/-}$ mice did not demonstrate a significant loss of maximal constriction amplitude to red light stimuli, suggesting M-cones have minimal input to the parameter under conditions of this protocol. One reason may be that heterogeneity in the response (intermouse variability) may mask real but small differences in the measured response when mean values are calculated. Another possible explanation is that the large rod input simply overrides the relatively smaller cone input at red wavelengths. In addition, since direct functional connections between rods and cones have been shown to exist in other studies,[19–21] we cannot exclude the possibility that genetic models develop alternate circuitry of the retinal cells.

The dynamic analysis of the initial response proved to be the superior parameter for assessing cone influence. Following low intensity blue light, we observed a change in the derivative curve pattern, which showed the disappearance of the positive derivative value in *Cnga3*$^{-/-}$. This is the first objective evidence of the important contribution of cones to the early and rapid dilation phase of the pupil response in mice. Our results also demonstrate that there is cone contribution to the postillumination phase after bright blue light (more prolonged constriction at 9.5 seconds compared to WT). The response to low intensity blue supports the notion that cones impact the pupil recovery after the initial constriction, whereas the prolonged response to high blue could be the consequence of saturation of the system in the *Cnga3*$^{-/-}$ devoid of cones. It has been recently shown that, at least in mice, cones pedicles directly contact the dendrites of the M1 intrinsically photosensitive retinal ganglion cells (ipRGCs) during development and some connections persist in the adulthood.[22] Whether this anatomic link translates to a direct functional cone modulation on the pupil response remains to be determined.

Having a defect in cone and rod function, *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ and *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ were models for isolating the melanopsin-driven pupil response. To all blue stimuli, we observed a pupil response that did not show any rapid dilation following initial constriction and in fact, remained strongly contracted following light termination for the entire duration of the recording. This was consistent with other reports that have described the isolated melanopsin-mediated pupil response.[1–3] Compared to *Rho*$^{-/-}$ mice, the *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ mice lost the small constriction seen to low and medium red stimuli, which is presumably cone-mediated. In the *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ a weak rod activity persists in this model, due to compensation by the GNAT2 gene found to be residually expressed in rods.[15] In these mice, very bright stimuli are needed to record an electroretinographic response from the residual rods. We were, however, able to record rod activity in the pupil response to low and medium red light stimuli.

For the two double knockout models, we observed a pupillary response with prolonged constriction to high intensity red light. While this response is presumably the result of melanopsin activation, we recognize a potential contamination by residual rod activity in the *Cnga3*$^{-/-}$; *Gnat1*$^{-/-}$ model, which may explain the shorter time to peak constriction amplitude. Thus the isolated melanopsin-driven component to this bright red stimuli is largely confirmed by the pupil response of *Cnga3*$^{-/-}$; *Rho*$^{-/-}$ that have no rod or cone function. This is consistent with the reported spectral characteristics of melanopsin that extends to longer wavelengths[16,23] but as with rods and cones, spectral sensitivity measurements will be needed to confirm the individual contributions to the pupil light reflex.

In summary, our protocol has better specified the aspects of the pupil response that are predominantly influenced by rods and cones. We found that rods, unexpectedly, are a major input to the pupil constriction to red (long wavelength) stimuli at low to moderate intensities. The cones play a more critical role for the early and rapid dilation that follows the initial pupil constriction. While rods are generally implicated in the late postillumination recovery to blue light, cones assume a greater importance when the blue light is bright.

Despite species-related anatomical differences, the use of knockout murine models helps our understanding of the participation of the different photosensitive retinal cells to the pupil response in humans. For example, we observed that in healthy mice, the rod contribution overwhelms and masks the cone input and as such, the *Rho*$^{-/-}$ model was critical for revealing the cone contribution to the pupil response. In clinical studies using human eyes, the available means for separating photoreceptor contributions has been to vary light adaptation, light intensity and light wavelength.[17,24–26] The presentation of very dim blue light stimuli under dark-adapted condition may permit isolation of rod activity, but the broad range of cone sensitivities is problematic for using an approach of preselected light stimuli. Pupilloconstriction evoked by photopic red lights are presumed to represent cone activity, but such pupil responses have not been convincingly shown to represent isolated cone activity.[17] In our analysis using the first derivative curves, we found that the better parameter for observing cone contribution was the early dilation following pupilloconstriction. It will be interesting to see if this aspect analyzed from first derivative curves may better define the cone activity in human pupil responses as well. A preliminary review of human pupil recordings from other published studies suggests this may be so. Park et al.[17] recorded the pupil responses to 1 second red and blue light stimuli at various intensities to bias for rod and cone activity. In their figures, we note similarities of the waveform features of the early phase of pupil dilation response from healthy control subjects to the early dynamic in WT mice. Higher intensities of blue stimuli, which aimed to bias for the melanopsin input,

decreased the rapid dilation in favor of a sustained and prolonged constriction, also observed in WT mice.

We noted two patterns of the early dilation component of the pupil response in mice and likewise, the human pupil responses described by Lei et al.[27] also show two patterns depending on the stimulus conditions. The pupil responses to 1 second stimuli of red (0.1 to 400 cd/m^2) or low blue light (<1 cd/m^2) have a rapid dilation while the pupil response to 1 second blue stimuli higher than 1 cd/m^2 show a more prolonged constriction. In addition, the authors demonstrated that by varying the stimuli duration between 1 ms and 1000 ms for two intensities (100 and 400 cd/m^2), the shortest duration of light stimulation to reach the maximal sustained phase was 400 ms. Our study used a 500-ms stimulus and this permitted distinction of differences in the early dynamic of the pupil response, depending on stimuli intensity and wavelength. Thus the stimulus conditions of our study appear adequate for detailed examination of the recovery phase of the pupil light reflex in both human and rodents. Ultimately, mouse models of photoreceptor degeneration and knockout as we have employed will allow more precise quantification of individual photoreceptor contribution, which may be an important tool for human clinical studies and anticipated gene therapy trials.

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References

1. Hattar S, Lucas RJ, Mrosovsky N, et al. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature*. 2003;424:75–81.
2. Lucas RJ, Douglas RH, Foster RG. Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nature Neurosci*. 2001;4:621–626.
3. Lucas RJ, Hattar S, Takao M, Berson DM, Foster RG, Yau KW. Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. *Science*. 2003;299:245–247.
4. Lall GS, Revell VL, Momiji H, et al. Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. *Neuron*. 2010;66:417–428.
5. Iwakabe H, Katsuura G, Ishibashi C, Nakanishi S. Impairment of pupillary responses and optokinetic nystagmus in the mGluR6-deficient mouse. *Neuropharmacology*. 1997;36:135–143.
6. Thompson S, Stasheff SF, Hernandez J, et al. Different inner retinal pathways mediate rod-cone input in irradiance detection for the pupillary light reflex and regulation of behavioral state in mice. *Invest Ophthalmol Vis Sci*. 2011;52:618–623.
7. Aleman TS, Jacobson SG, Chico JD, et al. Impairment of the transient pupillary light reflex in Rpe65(-/-) mice and humans with leber congenital amaurosis. *Invest Ophthalmol Vis Sci*. 2004;45:1259–1271.
8. Fan J, Rohrer B, Moiseyev G, Ma JX, Crouch RK. Isorhodopsin rather than rhodopsin mediates rod function in RPE65 knockout mice. *Proc Natl Acad Sci USA*. 2003;100:13662–13667.
9. Kostic C, Crippa SV, Pignat V, et al. Gene therapy regenerates protein expression in cone photoreceptors in Rpe65(R91W/R91W) mice. *PLoS One*. 2011;6:e16588.
10. Biel M, Seeliger M, Pfeifer A, et al. Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. *Proc Natl Acad Sci USA*. 1999;96:7553–7557.
11. Calvert PD, Krasnoperova NV, Lyubarsky AL, et al. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha -subunit. *Proc Natl Acad Sci USA*. 2000;97:13913–13918.
12. Lem J, Krasnoperova NV, Calvert PD, et al. Morphological, physiological, and biochemical changes in rhodopsin knockout mice. *Proc Natl Acad Sci USA*. 1999;96:736–741.
13. Jaissle GB, May CA, Reinhard J, et al. Evaluation of the rhodopsin knockout mouse as a model of pure cone function. *Invest Ophthalmol Vis Sci*. 2001;42:506–513.
14. Kuburas A, Thompson S, Artemyev NO, Kardon RH, Russo AF. Photophobia and abnormally sustained pupil responses in a mouse model of bradyopsia. *Invest Ophthalmol Vis Sci*. 2014;55:6878–6885.
15. Allen AE, Cameron MA, Brown TM, Vugler AA, Lucas RJ. Visual responses in mice lacking critical components of all known retinal phototransduction cascades. *PLoS One*. 2010;5:e15063.
16. Lucas RJ, Peirson SN, Berson DM, et al. Measuring and using light in the melanopsin age. *Trends Neurosci*. 2014;37:1–9.
17. Park JC, Moura AL, Raza AS, Rhee DW, Kardon RH, Hood DC. Toward a clinical protocol for assessing rod, cone, and melanopsin contributions to the human pupil response. *Invest Ophthalmol Vis Sci*. 2011;52:6624–6635.
18. Pennesi ME, Lyubarsky AL, Pugh EN Jr. Extreme responsiveness of the pupil of the dark-adapted mouse to steady retinal illumination. *Invest Ophthalmol Vis Sci*. 1998;39:2148–2156.
19. Nelson R. Cat cones have rod input: a comparison of the response properties of cones and horizontal cell bodies in the retina of the cat. *J Compar Neurol*. 1977;172:109–135.
20. Raviola E, Gilula NB. Intramembrane organization of specialized contacts in the outer plexiform layer of the retina. A freeze-fracture study in monkeys and rabbits. *J Cell Biol*. 1975;65:192–222.
21. Schneeweis DM, Schnapf JL. Photovoltage of rods and cones in the macaque retina. *Science*. 1995;268:1053–1056.
22. Renna JM, Chellappa DK, Ross CL, Stabio ME, Berson DM. Melanopsin ganglion cells extend dendrites into the outer retina during early postnatal development. *Developmental Neurobiol*. 2015;75:935–946.
23. Bailes HJ, Lucas RJ. Human melanopsin forms a pigment maximally sensitive to blue light (lambdamax approximately 479 nm) supporting activation of G(q/11) and G(i/o) signalling cascades. *Proc Biol Sci*. 2013;280:20122987.
24. Kardon R, Anderson SC, Damarjian TG, Grace EM, Stone E, Kawasaki A. Chromatic pupil responses: preferential activation of the melanopsin-mediated versus outer photoreceptor-mediated pupil light reflex. *Ophthalmology*. 2009;116:1564–1573.
25. Kardon R, Anderson SC, Damarjian TG, Grace EM, Stone E, Kawasaki A. Chromatic pupillometry in patients with retinitis pigmentosa. *Ophthalmology*. 2011;118:376–381.
26. Joyce DS, Feigl B, Cao D, Zele AJ. Temporal characteristics of melanopsin inputs to the human pupil light reflex. *Vision Res*. 2015;107:58–66.
27. Lei S, Goltz HC, Chandrakumar M, Wong AM. Full-field chromatic pupillometry for the assessment of the postillumination pupil response driven by melanopsin-containing retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2014;55:4496–4503.