Assessing Rod, Cone, and Melanopsin Contributions to Human Pupil Flicker Responses

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PURPOSE. We determined the relative contributions of rods, cones, and melanopsin to pupil responses in humans using temporal sinusoidal stimulation for light levels spanning the low mesopic to photopic range.

METHODS. A four-primary Ganzfeld photostimulator controlled flicker stimulations at seven light levels (−2.7 to 2 log cd/m²) and five frequencies (0.5–8 Hz). Pupil diameter was measured using a high-resolution eye tracker. Three kinds of sinusoidal photoreceptor modulations were generated using silent substitution: rod modulation, cone modulation, and combined rod and cone modulation in phase (experiment 1) or cone phase shifted (experiment 2) from a fixed rod phase. The melanopsin excitation was computed for each condition. A vector sum model was used to estimate the relative contribution of rods, cones, and melanopsin to the pupil response.

RESULTS. From experiment 1, the pupil frequency response peaked at 1 Hz at two mesopic light levels for the three modulation conditions. Analyzing the rod–cone phase difference for the combined modulations (experiment 2) identified a V-shaped response amplitude with a minimum between 135° and 180°. The pupil response phases increased as cone modulation phase increased. The pupil amplitude increased with increasing light level for cone, and combined (in-phase rod and cone) modulation, but not for the rod modulation.

CONCLUSIONS. These results demonstrate that cone- and rod-pathway contributions are more predominant than melanopsin contribution to the phasic pupil response. The combined rod, cone, and melanopsin inputs to the phasic state of the pupil light reflex follow linear summation.

Keywords: melanopsin, flicker, silent-substitution, pupil light reflex

In this study, we determined the relative contributions of rods, cones, and melanopsin to phasic pupil responses in humans using temporal sinusoidal stimulation over a large light adaptation range spanning mesopic and photopic light levels to alter differentially the sensitivity of the inner and outer retinal photoreceptors. The use of sinusoidal stimulation allowed us to investigate the phasic component of the pupillary responses with a constant adaptation level that produces specific rod, cone, and melanopsin excitations. Experiment 1 measured the temporal frequency response function of the retinal rod and cone inputs to identify the optimal temporal frequency that produced robust pupil responses. Experiment 2 used the optimal temporal frequency for isolating the phasic pupil response to estimate the relative contributions of rods, cones, and melanopsin, and the differential delays of these photoreceptor signals. We showed that a linear summation, rather than a “winner-takes-all” mechanism, describes the inner and outer retinal contributions to phasic pupil responses for light levels spanning the low mesopic to photopic range.
TABLE. Light Levels Used in Both Experiments

<table>
<thead>
<tr>
<th>Light Levels Units</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logphotopic cd/m²</td>
<td>-0.9 0</td>
<td>-2.7 -1.8 -0.9 0 0.2 1 1.3 2 2.7</td>
</tr>
<tr>
<td>Log photopic Td</td>
<td>0.2 1</td>
<td>11.1</td>
</tr>
<tr>
<td>Logquanta/cm²/s</td>
<td>11.9 12.1</td>
<td>11.9 12.1</td>
</tr>
</tbody>
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The values in Td were computed with the average baseline value of the pupil size during the stimulation. The values in log quanta/cm²/s are expressed at the cornea.

MATERIALS AND METHODS

General Methods

Observers. Three of the investigators participated in the study: DC (43 years), NN (22 years), and PAB (32 years). All observers had normal or corrected-to-normal visual acuity, and normal color vision assessed through the Farnsworth-Munsell 100 Hue test and the Nagel anomaloscope. Written consent was obtained for each subject. The study protocols were approved by the Institutional Review Board at The University of Illinois at Chicago, and were in compliance with the Declaration of Helsinki.

Apparatus. A ColorDome Ganzfeld in an Espion E3 Electrophysiology System (Diagnosys LLC, St. Lowell, MA) was used for stimuli generation. The Ganzfeld contains three rings of light-emitting diodes (LEDs; a “bright,” “dim,” and “low dim” ring) with each operating in different light range to achieve a large dynamic range that spans scotopic to photopic light levels. The “dim” ring consisted of four types of LEDs with dominant wavelengths of 466, 514, 590, and 634 nm, respectively. The “bright” ring also consisted of four types of LEDs with dominant wavelengths of 442, 516, 594, and 634 nm, respectively. Therefore, the “dim” or “bright” rings of LEDs were programmed to serve as a four-primary photostimulator to control independently the stimulations of rods and three types of cones (S-, M-, and L-cones). The theoretical basis for achieving independent control of the activities of four types of photoreceptors (S-cones, M-cones, L-cones, and rods) in the human retina is silent substitution.18,19

A small red LED located in the center of the Ganzfeld was used as the fixation point. The low mesopic light between -2.7 and 0 log cd/m² was achieved in the “dim” ring of LEDs and the high mesopic to photopic range between 0.2 and 2 log cd/m² was achieved in the “bright” ring of LEDs. Calibrated neutral density filters were used to attenuate the primary lights further. The values of the light levels used in both experiments appear in the Table. The spectral distribution of each LED was measured with a PhotoResearch PR-670 spectroradiometer (Photo Research, Inc., Chatsworth, CA). The built-in linearization function was verified by luminance measurement at various digital levels to confirm linearity.

Pupil diameters were measured binocularly using an Eyelink II eyetracker (SR Research Ltd., Kanata, Ontario, Canada) at a sampling rate of 250 Hz and with a spatial resolution of 0.1% of the baseline pupil diameter. A trigger signal from the computer synchronized the stimulus presentation and pupil recording. The device recorded the pupil sizes in arbitrary units. Therefore, at the end of each session, we determined the scaling factor between the recorded pupil size in arbitrary units and millimeters by recording a 6 mm black circular dot positioned in the plane of the pupil without moving the position of the headband or eye tracker cameras. The observer placed his head on a chinrest to fix his head position, leading to the stimuli presented in the ColorDome having a horizontal visual angle of 54°. Although both pupils were measured, we analyzed conservatively pupil data from the right eye only.

Stimuli. Three kinds of sinusoidal photoreceptor modulations were generated using silent substitution: rod stimuli with rod excitation modulated while maintaining constant cone excitations; cone stimuli with in-phase temporal modulation of the L-, M-, and S-cone excitations at the same contrast, so only cone luminance was modulated with a constant cone chromaticity while maintaining constant rod excitation at zero contrast; and the combined rod and cone stimuli with temporal modulation of rod and cone luminance signals, where cone luminance signals were modulated in various phase differences from a fixed rod phase. The cone excitations were computed based on the Smith-Pokorny cone fundamentals for the CIE 1964 10° Standard Observer as opposed to the CIE 1931 2° Standard Observer to minimize the effect of macular pigment. The time-averaged cone chromaticity was L/(L + M) = 0.77 and S/(L + M) = 0.2 in a cone Troland space. Macular pigment could affect color matching for smaller stimuli (e.g., 4° diameter or less). Therefore, individual differences in macular pigment optical density will be negligible with the 10° colorimetric data applied to our 5° extended field. In addition, it was shown that using the Standard Observer function without observer calibration produced a very small isolation error for rod or cone stimulations generated in the ColorDome for observers under 45 years old. Therefore, we used the same Standard Observer function for all observers. The experimental data for the three observers in this study showed no difference in the patterns between the youngest (22 years) and oldest (43 years). Therefore, any effect of difference in lens or macular pigment optical density is insignificant. Note that for each of these conditions melanopsin excitation was not constant, and this will be discussed in experiments 1 and 2.

General Procedures. A session consisted of sequential presentations of various sinusoidal stimulus types separated by interstimulus intervals (ISIs; 45 seconds for Experiment 1 or 60 seconds for Experiment 2) measured at one steady adapting background light level (Fig. 1A). Each session started with a 15-minute dark adaptation, followed by a 2-minute light adaptation to the background light before the first stimulus presentation. During the sinusoidal stimulus presentation, the observers were asked to focus on the fixation point without blinking. Observers could blink during the ISIs. A brief sound was signaled 10 seconds before the presentation of each stimulus (16 seconds for Experiment 1 or 30 seconds for Experiment 2). Two to four light levels were tested each day starting with the lowest light level, then increasing the light level sequentially. All of the conditions were repeated three times on different days by each observer. Each observer collected the data at similar times to minimize the effect of circadian variation on ipRGC function.

Data Analysis. Raw pupil diameter data were sampled at 4 ms intervals and converted into millimeters based on the scaling factor determined at the end of each session. For each condition, a Discrete Fourier Transform (DFT) derived the amplitude and phase at the first harmonic of the stimulus frequency using the Fast Fourier Transform algorithm in MATLAB (Mathworks, Inc., Natick, MA). Figure 1B depicts a...
To estimate the relative rod, cone, and melanopsin contributions to pupil control pathway in response to sinusoidal stimulation, we first identified the optimal temporal frequency that produced robust pupil responses, so that the amplitude and phase relationships between the photoreceptor inputs to the phasic pupil response could be studied.

**Stimuli.** The rod and/or cone excitations were modulated at temporal frequencies from 0.5 to 8 Hz at two light levels, −0.9 log photopic cd/m² and 0 log photopic cd/m² (see Table). Note that the melanopsin activation threshold is approximately 11 log quanta/cm²/s for primate retina. A computation of the background light levels in the plane of the retina for the three observers after correction for the optical density of the ocular media showed that the retinal quanta is below melanopsin threshold for −0.9 log cd/m², and above melanopsin threshold for 0 log cd/m². The Michelson contrast of the rod or cone excitation was set to 25%. The silent-substitution method with a four-primary photostimulator generated rod and/or cone isolating stimuli, but could not set the melanopsin excitation constant for each stimulus type. The melanopsin excitation contrast was computed using melanopsin spectral sensitivity function proposed by Enezi et al. and was 29.3% for the rod stimuli, 4.3% for the cone stimuli, and 25.0% for the combined stimuli.

A computation of the pupil response amplitude and phase simultaneously (Fig. 2C). We expect that for light levels below melanopsin threshold, only rods and cones contribute to the pupil response. If rod signals dominate cone signals at low mesopic light levels, the pupil amplitude should be independent of the phase of the cone signal and a flat amplitude response curve would be observed due to minimal cone contribution (Fig. 2C). With increasing light level, cone contributions will increase and we predict a V-shape amplitude response curve would be observed due to minimal cone contribution.
response as a function of cone modulation phase with the minimum value indicating the phase of the rod-cone cancelation. For light levels above melanopsin threshold (0 log cd/m²), melanopsin contributions will alter this predicted pupil response pattern. Previous studies have suggested that with pulsed stimuli, a linear summation mechanism can explain the M- and L-cone interaction in pupil responses. However, a ‘’winner-takes-all’’ mechanism was postulated to explain the relative contributions of rods, cones, and melanopsin to the PLR tonic state. In this case, the data should follow the rod, cone, or melanopsin prediction according to the light level (Fig. 2C).

Stimuli. Pupil responses were measured with the three stimulus types at 1 Hz between -2.7 and 2 log photopic cd/m² (see Table). Due to the difference in the gamut for the different sets of four primary light, the rod or cone contrast was 25% for stimuli at light levels of ≤0 log cd/m² (the melanopsin contrast was the same as for experiment 1) and 21% for stimuli at light levels of >0 log cd/m² (the melanopsin contrast was 24.6% for the rod photoreceptor modulation and 3.6% for the cone photoreceptor modulation). For the combined rod and cone stimuli, the rod modulation phase was fixed at 0°, while cone modulation was varied between 0° and 360° in 45° steps, leading to a melanopsin contrast signal between 25.0% and 33.6% for stimuli at light levels of >0 log cd/m² or between 21.0% and 28.2% for stimuli at >0 log cd/m². For the cone modulation phases in the combined rod and cone stimuli, the melanopsin phase ranged from the minimum phase of 0° (cone phase = 0°) to a maximum phase of 16° (cone phase = 180°) independent of the light level. Since baseline pupil sizes were different for different light levels, we also computed the pupil amplitude relative to the pupil size measured with the steady adapting background at each light level to compare the modulations across light levels. To allow comparison of the responses to the different stimulus contrasts (21% and 25%), the pupil amplitudes for light levels of >0 log cd/m² were linearly extrapolated by a factor of 1.19.

RESULTS

Experiment 1

The amplitudes and phases of pupil responses as a function of temporal frequency for the rod, cone, and combined (rod and cone) stimuli are shown in Figure 3. Overall, the pupil response amplitude peaked at 1 Hz at both light levels and was attenuated at higher frequencies, with the amplitude at 4 or 8 Hz being 2 or 3 log units lower than the amplitude at 1 Hz. In addition, the peak response amplitudes were decreased at the lower light level (~0.2 log units) for all three stimulus types. The amplitudes at 1 Hz were different among stimulus types, with higher or similar amplitude for the combined rod and cone stimuli compared to the amplitudes for the cone or rod stimuli. The pupil response phases at both light levels did not differ substantially, especially at 1 Hz, and the phases decreased monotonically between 1 and 8 Hz. In other words, for both light levels, 1 Hz stimuli generated to the largest response amplitudes. Since our measurements were recorded under binocular conditions, we determined the effect of physiologic feedback on the amplitude and phase response,
and the analyses showed that it was negligible (see Supplementary material SA).

**Experiment 2**

Figure 4 shows the relative pupil response amplitude and phase as a function of light levels for the three stimulus types. First, we considered the pupil responses for the rod, cone, and combined rod and cone stimuli with rod and cone modulation in phase.

For each observer (Fig. 4) the relative pupil amplitudes for the combined rod and cone stimulus modulation (circles) increased monotonically with increasing light level and, similar to other visual processes, it shows saturation. The saturation value for the 548 stimulus was approximately 1.1 log cd/m². The pupil amplitudes for the cone modulation (squares) increased with increasing light level, but were reduced at the highest light level (2 log cd/m²) when compared to the combined rod and cone modulation condition (circles). The pupil amplitudes for the rod stimulus modulation (triangles) increased for the lower light levels (<0.9 log cd/m²), and had an initial peak between −0.9 and 0 log cd/m², followed by an amplitude reduction at 0.2 log cd/m², then a monotonic increase. The cross point of the rod and cone curves between −0.9 and 0 log cd/m² indicated a similar contribution to the pupil amplitude. The response phases for the cone modulation, and combined rod and cone modulation increased monotonically with increasing light levels, consistent with previous observations using nonphotoreceptor-specific sinusoidal stimulation. However, for the rod modulation condition, there was an abrupt phase shift between zero and 0.2 log cd/m² that was common to the change in the pupil amplitude response. Note that the photoreceptor contributions cannot be determined by the 0° phase results (Fig. 4), because our four-primary system can only silence the stimulation of three types of photopigments. Our stimuli were designed to modulate only rods or cones and produced a calculable level of melanopsin excitation with each of these rod-isolating or cone-isolating stimuli. For this reason, we used the phase data to determine the relative contributions of the three photoreponses.

The pupil response amplitudes and phases for the combined photoreceptor modulations measured as a function of the cone modulation phase are shown in Figure 5 for light levels of ≤0 log cd/m². Figure 6 shows the pupil response for light levels of >0 log cd/m². At the lowest light level (~2.7 log cd/m²), the pupil response was weak, and the response amplitude and phase did not vary with cone modulation phase.
At levels of $\geq -1.8 \text{ log cd/m}^2$, a V-shaped pattern for the response amplitudes (upper panels in Figs. 5, 6) appeared for all observers, with a minimum occurring between 135° and 180° of cone modulation phase. The pupil response phases at levels of $\geq -1.8 \text{ log cd/m}^2$ (lower panels in Figs. 5, 6), increased as cone modulation phase increased, followed by an abrupt phase shift at a cone modulation phase between 135° and 180°. Note that for higher light levels, this abrupt change can be approximated as a monotonic function if 360° are added to the pupil phase for cone phases higher than 135°.

Comparing the predictions of Figure 2 to the data of experiment 2, it was evident that a "winner-takes-all" mechanism could not explain amplitudes and phases simultaneously for sinusoidal stimulation. Therefore, we implemented the vector sum model to estimate the relative contributions of rods, cones, and melanopsin to the pupil responses (Fig. 2C; see Supplementary material SB for mathematical equations). A total of five free parameters was optimized: relative rod-cone delay (ms), relative melanopsin-cone delay (ms), rod contribution amplitude, cone contribution amplitude, and melanopsin.
contribution amplitude. The initial values of the delays, and rod and cone contribution amplitudes were set based on the results for the rod and cone stimuli (Fig. 4). The melanopsin contribution was set as a linear function of the melanopsin excitation contrast produced by the stimuli.

The solid lines in Figures 5 and 6 represent the model fits from simultaneous optimization of pupil response amplitudes and phases. For all of the conditions, the fits were reasonably good (the average adjusted $R^2$ was 0.95 ± 0.03), especially for the phase data. At light levels below melanopsin threshold ($\leq -0.9 \log \text{cd/m}^2$), the models with a weighted rod and cone contributions were adequate to account for the data. At higher light level, the rod, cone, and melanopsin contributions were estimated. The estimated relative photoreceptor contributions and differential delays derived from the vectorial summation model are shown in Figures 7A and 7B, respectively. Clearly, rod contribution (relative to cone contribution) decreased monotonically with increasing light level, and became insignificant at 1.1 and 2.0 log cd/m$^2$. The melanopsin contribution (relative to cone contribution) was negligible from 0 to 0.2 log cd/m$^2$, then increased with increasing light level. The rod-cone delays were approximately 80 ms at light levels of $\leq -0.9 \log \text{cd/m}^2$, which reduced to approximately 10 ms at 0 and 0.2 log cd/m$^2$ (Fig. 7B, rod-cone delay at 1.1 and 2.0 log cd/m$^2$ were not shown due to insignificant rod contributions), consistent with the involvement of two rod-pathways$^{35,36}$ in the pupil control pathway. On the other hand, the melanopsin-cone delay decreased with increasing light level.

**DISCUSSION**

The relative contributions of rods, cones, and melanopsin to human phasic pupil response were measured using sinusoidal stimulation for light levels spanning the low mesopic to photopic range. The results showed that the contributions of rods, cones, and melanopsin were combined as a vector sum of their inputs (Figs. 5, 6).

According to the fitted vector summation model output (Fig. 7), the relative rod, cone, and/or melanopsin input to the pupil control pathway depends on the light level. For lower light levels, rods and their postreceptoral circuitry dominate the response with no activation of melanopsin. For higher light...
levels, cones, and their postreceptorial cuitry dominate the response with a significant melanopsin contribution and insignificant rod contribution. For a small range of intermediate light levels (0–0.2 log cd/m² in the present conditions), contributions from the three photoreceptor types coexisted. A previous study suggested high-pass filtering mediating cone inputs to the ipRGCs. Therefore, favoring cone participation in phasic responses. The data in Figure 4 evaluated this proposal and are consistent with the predominance of cone participation above 0 log cd/m² for the combined rod and cone photoreceptor modulation condition. These results may have clinical implications. For instance, at −0.9 log cd/m², there is no melanopsin contribution, and rod and cone contributions are similar in magnitude. Therefore, deficits in rod- and cone-mediated function could be assessed using pupil measurements at this light level. On the other hand, at 2 log cd/m², rod contribution was minimal and, therefore, pupil measurement at this level can be used to assess the cone and melanopsin function. With respect to the phase differences, physiologic in vivo studies with a similar methodologic approach reported shorter rod-cone delays with increasing light level. Furthermore, the latencies of melanopsin-isolated response were found to be approximately 30 times longer than cone-isolated response latencies in primate in vitro ipRGC recordings. These physiologic reports are consistent with the delays estimated from the summation model.

Vectorial Summation Versus Winner-Takes-All
To examine how rod, cone, and melanopsin signals are combined, we tested the hypothesis that photoreceptor inputs to the pupil control pathway were combined by vectorial summation. The summation of cones and ipRGC photoreponses initially was suggested by Dacey et al. Previous investigations of tonic pupil responses, including the constriction amplitude during exposure to continuous light simulation of varying duration, explained the combination of inner (melanopsin) and outer (rods and cones) ipRGC signals using a “winner-takes-all” mechanism. It has been suggested that tonic and phasic behaviors are controlled independently in human physiology. In this study, a linear summation process could explain the phasic behavior, as suggested by previous studies of L- and M-cone interactions in pupil responses with pulsed stimuli. Therefore, both proposed mechanisms could coexist to control the pupil size, depending on the temporal properties of the input signal and the pupillary light response studied.

Melanopsin Temporal Dynamics
Previous works have reported that the postillumination pupil response (PIPR) is caused by the melanopsin activation, whereas it has been suggested that outer retinal photoreceptors control pupil responses to intermittent (square-wave) stimulation due to the slow temporal response of melanopsin signaling. This latter observation is contrary to our findings. The sluggish nature of the pupil response is related to the type of stimulation. Those previous studies used square stimuli with large contrast changes. On the other hand, the contrast in our experiments is low (21% and 25%) and we focused on the response to 1 Hz sinusoidal stimulation, which means the pupil responded predominantly to the pure frequency component (1 Hz), whereas square-wave stimulation produced a strong steady component and important harmonics at higher frequencies. Electroretinographic (ERG) measurements also showed evidence that the melanopsin response is significant in 1 Hz consistent with the present study. Moreover, sinusoidal stimulation was shown to be a good method to observe the melanopsin contribution to the pupil response. The melanopsin relative contribution is based on the model results and it is supported by previous physiologic data as has been shown. However, it is possible that the photopic pupil response includes some rod contribution, since they might not be completely saturated at high light levels. McDougal and Gamlin found, for irradiance levels above melanopsin threshold and for their shortest pulse stimulation (1.78 seconds), an important contribution of the melanopsin photoreponse. This duration is related more with the phasic nature of the PLR, suggesting that the melanopsin is active during short stimulation period. This finding is consistent with our measurements. Also, Park et al. considered, besides the tonic state, the phasic state of the PLR. In a closed loop paradigm, those investigators showed clear rod and cone contributions to the phasic state of the PLR depending on the light preadaptation state, and melanopsin contribution to the PIPR with stimulations as short as 100 ms. These findings agree with our model results in which, besides rods and cones, melanopsin photoreponses contribute to Hz stimulation signals during the stimulus presentation. Furthermore, Markwell et al. and Park et al. have shown that rods dominate the phasic response for light levels lower than 0 log cd/m², which is consistent with the findings of the present work.

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References


