The Pupillary Response to Color and Luminance Variant Multifocal Stimuli

Corinne F. Carle, Andrew C. James, and Ted Maddess

PURPOSE. We are developing multifocal pupillographic objective perimetry (mfPOP) to assess localized changes in function within visual pathways. In this study, we investigate novel mfPOP stimuli designed to target neural components from either or both the sub-cortical pupillary luminance response and the cortically driven color response.

METHODS. Pupillary responses of 12 subjects were recorded to eight mfPOP stimulus variants (protocols). Forty-eight visual field test-regions (24/eye) were stimulated concurrently with uncorrelated sequences of either high or low luminance-contrast, luminance- plus color-contrast, or equiluminant color-exchange stimuli. Stimulus pulses were of 50 ms duration and were presented at mean intervals of 4 seconds/region. Test durations were 4 or 8 minutes; therefore, estimated responses were derived from 60 or 120 stimulus presentations to each test region.

RESULTS. Pupillary response amplitudes were more influenced by luminance-contrast than the color-contrast of stimuli; response delays, however, were more closely linked to the proportion of color- versus luminance-contrast in each protocol. Significant differences ($P < 0.05$) in amplitudes but not delays were present between all three high luminance-contrast protocols and a low luminance-contrast luminance protocol, regardless of color content. The reverse pattern was observed between the equiluminant color exchange protocol and this same low luminance-contrast luminance protocol. Only the low luminance-contrast plus color exchange protocol differed significantly from the low luminance-contrast luminance protocol in both measures.

CONCLUSIONS. Two protocols, utilizing low and high luminance-contrast plus color exchange, were identified as likely to incorporate both cortical and subcortical response components, and were deemed potential candidates for further investigation in clinical studies. (*Invest Ophthalmol Vis Sci*. 2013;54:467-475) DOI:10.1167/iops.12-10829

We have recently developed multifocal pupillographic objective perimetry (mfPOP), with which retinotopic loss or dysfunction of the visual system can be mapped through differences in the magnitude or time-course of dynamic pupil responses.¹⁻⁵ As well as clinical applications, the ability to measure pupillary sensitivity at many locations in the visual field provides a unique opportunity to explore the processing of visual stimuli that influence pupil responses. Midbrain projecting *melanopsin retinal ganglion cells* (mRGCs) form the afferent arm of the subcortical pupillary pathway,⁶ the vast majority of other classes of RGCs being involved in conveying retinal responses to the thalamus for transmission to the visual cortex and the subsequent processing of a conscious visual percept. MfPOP stimuli generally involve transient pulses of increased luminance²; mRGCs exhibit an ON response to luminance⁷⁻⁹; therefore, the subcortical processing of pupil responses to these stimuli is highly likely.

Changes in pupil diameter are not, however, effected only by changes in luminance. Pupils also respond to variation in the spatial or temporal characteristics of stimuli,¹⁰⁻¹⁷ as well as to isoluminant color exchange.¹⁸⁻²⁰ The extrastriate visual cortex is strongly implicated in the generation of pupil responses elicited by these types of stimuli.^{18,21,22} Evidence for this comes partly from patients with homonymous hemianopia, whose pupils are unresponsive to grating or color stimuli in their damaged hemifields, but exhibit reduced but otherwise normal pupillary *luminance* responses.^{23,24} Pupil responses to equiluminant changes in color have been proposed to be mediated by the inferior temporal cortex, since lesions in this area abolish pupillary color responses in primates.²⁵ Connections between this region of the temporal lobe and the pretectum have been identified,²⁶ providing a possible conduit for this cortically processed information to the pupillary system.

In general, responses mediated by mRGCs are bidirectional (i.e., an increase in total luminance will result in pupillary constriction, a luminance decrease in dilation).^{7,14,19} In contrast, pupillary responses to isoluminant color exchanges are independent of the direction of the exchange.²⁷ We have observed mfPOP responses of both types in preliminary studies: stimuli shown at lower luminance than the background result in dilations, isoluminant red/green color exchanges produce constrictions whether using red stimuli on green backgrounds or the reverse.

Color exchange stimuli, therefore, have the potential to provide a means of objectively assessing function in cortical visual pathways using the pupillary response. A stimulus that elicits pupil responses not just from subcortically projecting mRGCs, but also from RGCs that are involved in conscious visual perception, could have increased utility for assessing visual function. This study, therefore, aims to assess the contribution of cortical and subcortical components to mfPOP responses.

From the ARC Centre of Excellence in Vision Science, The John Curtin School of Medical Research, The Australian National University, Canberra City, Australia.

Supported by the Australian Research Council through the ARC Centre of Excellence in Vision Science (CE0561903), and Seeing Machines Ltd.

Submitted for publication August 23, 2012; revised October 20, 2012; accepted November 19, 2012.

Disclosure: C.F. Carle, None; A.C. James, Seeing Machines (F), P; T. Maddess, Seeing Machines (F), P

Corresponding author: Corinne F. Carle, Eccles Institute of Neuroscience, The John Curtin School of Medical Research, The Australian National University, Canberra ACT 0200, Australia; corinne.carle@anu.edu.au.

TABLE 1. Luminance and Color Characteristics of the mfPOP Stimulus Protocols

Protocol	Stimulus		Background		Description*
	cd/m ²	Color	cd/m ²	Color	
Session 1: 4 protocols of 8-min duration					
Y-hi/Y-mid	290	Yellow	115	Yellow	low luminance-contrast only: yellow→ yellow →yellow
G-hi/R-mid	290	Green	115	Red	low luminance-contrast + color exchange: red→ green →red
GRG-hi/R-mid	290	Green/red	115	Red	low luminance-contrast + color exchange + 30 Hz flicker: red→ green → red → green →red
G-mid/R-mid	115	Green	115	Red	zero luminance-contrast color exchange: red→ green →red
Session 2: 4 protocols of 4-min duration					
Y-hi/Y-lo	290	Yellow	10	Yellow	high luminance-contrast only: yellow→ yellow →yellow
Y-hi/R-lo	290	Yellow	10	Red	high luminance-contrast + color addition: red→ yellow →red
G-hi/R-lo	290	Green	10	Red	high luminance-contrast + color exchange: red→ green →red
G-hi/Y-mid	290	Green	115	Yellow	low luminance-contrast + color subtraction: yellow→ green →yellow

MfPOP stimuli consisted of 50-ms duration deviations from the background within each visual field test region, in either luminance-contrast, color-contrast, or a combination of both.

* Stimulus color shown in *bold*, background in *standard text*, i.e., the sequence describes the changes from background (*nonbold*) to stimulus (*bold*) and return to background condition (*nonbold*). The protocol names (*left column*) represent the protocol stimulus attributes/background attributes. Uppercase letters, therefore, refer to the stimulus or background color (Y = yellow, G = green, or R = red); lowercase text refers to the luminance (hi = 290 cd/m², mid = 115 cd/m², or lo = 10 cd/m²).

METHODS

Subjects

Twelve subjects (six males) aged 33.0 ± 7.2 years were each tested with eight mfPOP variants. Subjects' visual fields were assessed using

frequency doubling technology C-20 perimetry (Carl Zeiss Meditec, Inc., Dublin, CA), and color discrimination using the Farnsworth 100-Hue Test (Luneau Ophthalmology, Chartres, France). Exclusion criteria included the presence of ocular pathology or previous ocular surgery, refractive errors greater than ±6 spherical diopters (D) or >2 D of

FIGURE 1. Stimulus protocol characteristics. (A) Proportional cone photoreceptor activations for long-wavelength sensitive (L), medium-wavelength sensitive (M), and short-wavelength sensitive (S) cones for each of the stimulus colors used. (B) CIE coordinates for each color and luminance used in this experiment. The two most central symbols (+) represent the two lowest luminances of 10 cd/m², used in protocol backgrounds. (C) Layout of 24-region stimulus shown as if all regions were active at the same time. (D) Single frame of a 24-region mfPOP protocol: spatially adjacent stimuli were uncommon (spatial sparseness).

FIGURE 2. Response variables across all subjects for the stimulus protocols of the main experiment, ordered by increasing color contribution and decreasing luminance contribution (i.e., from luminance-only protocols at *left*, to color exchange only at *right*). (A) Mean constriction amplitudes. (B) Signal to noise ratios. (C) Times to peak. The inputs to these models are the median values for each parameter across pupils, eyes and then test regions. The data-point border color reflects the background luminance: *black* representing 10cd/m² and *gray* 115 cd/m² (lo and mid in the abscissa labels). *Shading* of data points similarly reflects the *stimulus* luminance: *gray* representing 115 cd/m² and *white* representing 290 cd/m² (mid and hi). The shape of each datapoint additionally indicates color and luminance characteristics (refer to legend). Note that amplitudes (A) vary more substantially with luminance-contrast, whereas times to peak (C) are more dependent on relative color contribution. *Error bars* represent the standard errors of these means. Each panel is from a separate multivariate model with df = 11. Also plotted in the *upper panel* (A), *gray dashed line* is the first-order kernel from a more complex fit undertaken to examine the second-order kernel (see “Methods”).

cylinder, best corrected far acuity worse than 6/9, color vision abnormalities, or systemic disease or medication that might impair vision or pupillary responses. Informed written consent was given by all participants after explanation of the nature and possible consequences of the study, according to the Australian National University Human Experimentation Ethics approval 238/04. All research adhered to the tenets of the Declaration of Helsinki.

Multifocal Pupillographic Objective Perimetry

Presentation of stimuli and pupillary response measurement was carried out using a prototype of the Truefield Analyzer (Seeing Machines Ltd., Braddon, Australia). This device utilizes dichoptic presentation of temporally and spatially sparse multifocal stimuli at 60 frames/s.^{1,3,4,28} Each of the two experimental sessions comprised a

randomly ordered series of four stimulus variants or protocols (Table 1). These protocols consisted of either eight or 16 30-second segments with resultant test durations of 4 or 8 minutes. The stimulus layout extended $\pm 30^\circ$ radius from fixation and consisted of 24 visual field test regions/eye (Fig. 1).¹ Subjects fixated a small cross in the center of the viewing field whilst mfPOP stimuli were presented, as concurrent uncorrelated sequences of pulses to each of the test regions, the mean rate being one presentation/4 seconds/test region. The mean number of stimuli active at any given point in a test was 0.60 (± 0.68 SD) for 4-minute protocols and 0.60 (± 0.73 SD) for 8-minute protocols.

The mfPOP onset stimuli consisted of 50 ms deviations from the background in various combinations of luminance-contrast (0, 1.5, or 28) and/or color (red, green, or yellow). In addition to these onset stimuli, one protocol utilized red/green equiluminant flicker at 30 Hz (i.e., each 50 ms stimulus comprised one frame each of red, green, and then red again). Responses to these stimuli show no indication of adaptation during a stimulus run, with no significant differences seen in amplitudes or times to peak between the first and second halves of any of the protocols in this experiment. The naming convention for stimulus protocols depicts the stimulus attributes on the background attributes: uppercase letters refer to the stimulus or background color (Y = yellow, G = green, or R = red); lowercase text refers to the luminance (hi = 290 cd/m², mid = 115 cd/m², or lo = 10 cd/m², Table 1). Thus, a low luminance-contrast color exchange protocol with green 290 cd/m² stimuli on a 115 cd/m² red background is designated G-hi/R-mid. Proportional cone activations, estimated using human cone sensitivity functions,²⁹ and CIE coordinates for each color and luminance level are plotted in Figure 1.

Response Estimation

Each subject's individual test-region responses were extracted from the continuous records of the raw pupillary diameters using least-squares multiple linear regression as previously described.^{30,31} Two different methods provided individual sets of 96 response estimates for each subject, each set comprising direct and consensual responses from stimulation of right and left eyes for each of the 24 test regions per eye. Both methods fitted each compound pupil diameter signal as being a baseline diameter with superimposed 2-second contraction waveforms for each of the 48 test regions, estimating the contraction waveform that would be due to one pulse presentation in a region. The first method modeled the waveforms as being an estimated waveform basis function, with 48 separate coefficients estimating amplitudes for the 48 test regions of the two eyes. This provided a relatively model-independent estimate of waveform shape for each recording. The second method modeled each of the 48 elementary response waveforms as having the parametric form of a lognormal function, $f(t)$, below, with separate amplitudes, A , and time-to-peak parameters, t_p , for each of the 48 regions, and a common waveform shape parameter, s .

$$f(t) = A * \exp \left[-\frac{1}{2} * \left(\log(t/t_p)/s \right)^2 \right] \quad (1)$$

This produced estimates of amplitude and time to peak, with standard errors, more suited to quantitative analysis. In addition to these standard fitting methods, a more complex fit was carried out to examine the first to third off-diagonals of the second-order kernel (representing 2-second, 4-second, and 6-second time steps, respectively) for temporal interactions. The mean of the first-order kernel from this fit is shown in panel A of Figure 2 (*gray dashed line*). This kernel dominated the response with medians of the second-order terms not approaching significance in any protocol.

All reported pupil constriction amplitudes from lognormal fits have been normalized relative to the fitted baseline pupil diameter, by scaling to produce amplitudes that would correspond to a baseline pupil diameter of 3500 μ m.^{1,3,4}

FIGURE 3. Region-wise amplitude and time to peak effects from two multivariate linear models. Effects are plotted for the difference (i.e., additive effect) in test regions between protocols with decreasing luminance influence and increasing color influence, and the low luminance-contrast only protocol, Yhi/Y-mid, the constant for these models. From left to right, the additive effects are due to: (A, E) higher luminance-contrast only; (B, F) higher luminance-contrast plus color exchange; (C, G) color exchange only; (D, H) color exchange plus reduced luminance-contrast. The background of each set of plots is the grayscale shade representing a value of 0 (no difference from the reference conditions), positive values in each plot are represented by *lighter shades* than this, negative values in *darker shades*. A *black* or *white asterisk* (*) placed within a test region indicates that the effect for that individual region is significant at $P < 0.05$. Inputs to these models were the median values across direct and consensual responses, and across eyes.

Multiple Linear Regression

Multiple linear regression was used to quantify differences in times to peak and normalized amplitudes between stimulus protocols. All response estimates where time to peak values were less than 300 ms or greater than 675 ms were removed from analyses—these constituted <1% of the total data. These spurious estimates are occasionally produced where the noise inherent in the response exceeds the response's magnitude.

In models reporting regional means, an assumption of complete correlation between direct and consensual responses, and eyes, was made. Therefore, regionwise linear models utilized the median of measurements firstly across left and right pupils, then across left and right eyes, rather than both sets of responses separately. Where comparisons were between protocols, median values were taken across pupils, eyes, then test regions.

Performing linear regression on data that does not meet certain criteria may lead to misleading results. In order to meet these assumptions of linearity, constant variance, and normally distributed residuals, the median amplitude measurements for all regressions on data from the main experiment were subjected to a nonlinear transformation in the form of a generalized logarithm. This corresponds

to decibels for large values, but tends toward a straight-line for small values:

$$\text{glogdB}_\lambda(x) = 10 * \log_{10} \left[\frac{(x + \sqrt{x^2 + \lambda^2})}{2} \right], x > 0 \quad (2)$$

Straight-line extrapolation was used for values below 0. Generalized logarithm transformations produce values with approximately constant variance when the raw data variance has both an additive (variability increases incrementally), and a multiplicative (variability increases logarithmically) component.³² The parameter lambda (λ) is the value of the raw data at which additive and multiplicative variance contribute equally. A λ of 5 produced the most normally distributed residuals, as well as the best model r^2 and linearity between variables for this dataset. Where units for amplitude are reported in dB, this always refers to generalized log dB with $\lambda = 5$. Time to peak values had stable variance and so were not transformed. The significance level for all analyses is 5%.

Linear model results are reported as *contrasts* relative to a *constant* reference. In the region-wise models (Figs. 3, 4), these additive effects represent the difference between the mean across pupils, eyes, and subjects of each region of a protocol of interest (the

FIGURE 4. Linear model contrasts for the region-wise effects of the flickered stimulus protocol GRG-hi/R-mid relative to two constants—in this case, two sets of reference conditions. (A, C) Low-contrast color exchange protocols. (B, D) Low-contrast luminance only protocols. As in Figure 3, the background of each set of plots is the *grayscale shade* representing a value of 0 (no difference from the *reference conditions*), positive values in each plot are represented by lighter shades than this, negative values in darker shades. A *black or white asterisk* (*) placed within a test-region indicates that the effect for that region is significant at $P < 0.05$. Inputs to these models were the median values across direct and consensual responses, and across eyes.

contrast), and the means of corresponding regions of a second reference protocol (the constant). In Table 2, the constant term refers to the responses of the subject with the amplitude closest to the median across all subjects, to the low-contrast luminance-only protocol.

RESULTS

Amplitudes and Times to Peak

Protocols with high luminance-contrast produced the largest mean pupillary constriction amplitudes (Fig. 2, *upper*). Mean times to peak were not directly related to the large differences in amplitude between protocols, but instead varied with the proportional influence of color versus luminance of stimuli (Fig. 2, *lower*): as the proportion of color exchange relative to luminance-contrast increased, so did the latency of the peak of

the constriction. These observations were quantified using two multivariate linear models, effects were estimated for between-protocol and between-subject variation. The measures of time to peak and amplitude for each protocol were compared against those of the low luminance-contrast only Y-hi/Y-mid protocol (Table 2). The amplitudes of each of the three high luminance-contrast protocols, Y-hi/Y-lo, Y-hi/R-lo, and G-hi/R-lo, were significantly larger than those of the Y-hi/Y-mid protocol. Although these differences in amplitude were substantial, no significant differences in times to peak were observed between any of these protocols and the Y-hi/Y-mid protocol. The reverse pattern of significantly longer times to peak accompanied by no significant difference in amplitudes was observed in the comparisons contrasting the zero-contrast color exchange protocol G-mid/R-mid and the flickered protocol GRG-hi/R-mid against Y-hi/Y-mid. The low luminance-contrast plus color exchange protocol G-hi/R-mid was the only protocol that differed significantly from Y-hi/Y-mid in both measures.

Regional effects were contrasted against the mean responses by region of the low-contrast luminance only Y-hi/Y-mid protocol in the same manner as the overall means of the previous model. A subset of protocols were ordered according to their stimulus attributes starting with high luminance-contrast only through to zero luminance-contrast color exchange (Fig. 3). Thus, Figure 3 shows the regional differences for four protocols relative to each of the regional amplitudes or times to peak of the Y-hi/Y-mid protocol, the reference conditions. Regions with significant differences ($P < 0.05$) are marked by an *asterisk*. Almost identical topography of the differences in amplitude from the reference conditions can be seen for both the high-contrast luminance only and high-contrast color exchange protocols (Figs. 3A, 3B). Times to peak in these two protocols—although not significantly different from the constant—appeared to differ, however, with Y-hi/Y-lo latencies tending to be somewhat shorter than (Fig. 3E), and G-hi/R-lo being very similar to (Fig. 3F), the reference values. There was little difference between the regional constriction amplitudes of the zero-contrast color protocol, G-mid/R-mid, and the low-contrast Y-hi/Y-mid (Fig. 3D). This comparison, however, also produced the largest differences in the times to peak (Fig. 3H). Overall, clear and opposing gradients in amplitude and time to peak effects can be seen as protocols become less dominated by luminance-contrast and more by color exchange.

The regional effects for the red-green flickered protocol GRG-hi/R-mid showed that this protocol produced similar times to peak, but smaller amplitudes than the equivalent low-contrast non-flickered color exchange protocol G-hi/R-mid (Figs. 4A, 4C). Compared with the equivalent low-contrast luminance only protocol Y-hi/Y-mid (Fig. 4B), amplitudes were similar, which might suggest that the rapid red-green flicker was treated as a red + green mixture (i.e., a luminance signal). Complicating this, however, is that the times to peak were in general much longer than the luminance-dominated cases, although variability caused these differences not to reach significance in the majority of regions (Fig. 4D).

Pupillary Response Morphology

No difference was observed in the constriction wave-forms between stimuli utilizing color, luminance, or combinations of both. Unconstrained waveform estimates instead appeared to vary according to the amplitude of the constriction: smaller constrictions to stimuli of all types closely followed the general form of the G-mid/R-mid protocol, moderate and larger amplitude constrictions exhibiting the same form as those of protocols G-hi/R-mid and G-hi/R-lo (Fig. 5A). An additional delay of approximately 30 ms in the onset of responses to the

TABLE 2. Additive Effects of Stimulus Protocols Obtained Using Multivariate Linear Regression

Protocol or Subject	Amplitude Model df = 77, $r^2 = 0.82$			Time to Peak Model df = 77, $r^2 = 0.66$		
	b (dB)	t	P*	b (ms)	t	P*
Constant: Yhi/Y-mid (Subject 7)	8.61			426.67		
Yhi/Y-lo	4.77	8.80	*	-2.74	-0.22	0.83
Yhi/R-lo	4.96	9.15	*	1.35	0.11	0.92
G-hi/Y-mid	0.36	0.67	0.51	15.34	1.21	0.23
G-hi/R-lo	5.07	9.37	*	11.34	0.89	0.37
G-hi/R-mid	2.68	4.94	*	30.02	2.37	*
GRG-hi/R-mid	0.03	0.05	0.96	29.15	2.30	*
G-mid/R-mid	-0.02	-0.03	0.98	50.31	3.96	*
Subject 1	-1.55	-2.34	*	17.72	1.14	0.26
Subject 2	-0.86	-1.29	0.20	18.40	1.18	0.24
Subject 3	-0.66	-1.00	0.32	5.84	0.38	0.71
Subject 4	-0.17	-0.26	0.80	-18.46	-1.19	0.24
Subject 5	-0.19	-0.28	0.78	49.27	3.17	*
Subject 6	-0.16	-0.24	0.81	46.41	2.99	*
Subject 8	0.40	0.60	0.55	100.69	6.48	*
Subject 9	1.11	1.67	0.10	-11.93	-0.77	0.45
Subject 10	1.31	1.97	0.05	12.21	0.79	0.43
Subject 11	1.94	2.92	*	40.07	2.58	*
Subject 12	2.13	3.21	*	-31.58	-2.03	*

Effects are reported relative to a reference constant describing the mean amplitudes and times to peak of the low-contrast luminance only protocol, Yhi/Y-mid for the subject with constriction amplitudes closest to the median. Under these two additive models (one for amplitude and one for time to peak) the fitted effects (b, columns 2 and 5), *t* statistics, and *P* values refer to the difference of each factor from the reference condition. Inputs to these models were the median values across direct and consensual responses, eyes, and test-regions thus circumventing the effects of correlation at those levels.

* $P < 0.05$.

zero-contrast color exchange protocol G-mid/R-mid is apparent in both nonscaled and scaled plots. All other protocols produced similar onset latencies.

Peak constriction velocities were estimated by calculating the slope at the steepest region of the constriction phase using both nonscaled and scaled mean waveforms for each protocol (Fig. 5B). The G-mid/R-mid and Yhi/Y-mid protocols produced the smallest slopes; the high luminance-contrast protocols Yhi/Y-lo and G-hi/R-lo the largest, these values representing the slowest and fastest constriction velocities observed. The peak velocities of responses to the color exchange protocols increased in conjunction with increases in luminance-contrast from zero through to high.

Linear combinations of the mean response waveforms for luminance-contrast only and zero luminance-contrast color exchange protocols were compared with actual responses of the combined protocols (Fig. 5C). The prediction from the low luminance-contrast Yhi/Y-mid and color exchange G-mid/R-mid protocols (Fig. 5C, *top*) was smaller in amplitude and longer in time to peak than the G-hi/R-mid response. In contrast, the equivalent comparison using high luminance-contrast protocols (Fig. 5C, *bottom*) produced an overprediction of amplitude, but accurate time to peak.

DISCUSSION

The impetus for this research was the development of a mfPOP stimulus protocol in which a substantial proportion of the pupillary response can be attributed to cortically projecting visual pathways. A protocol of this type could potentially provide information regarding function not only in afferent subcortical pupillary pathways, but also from pathways more directly involved in conscious visual perception. To this end, we investigated the response characteristics and topography of pupillary responses to mfPOP stimuli, which differed from the

stimulus background in various combinations of luminance, color, and flicker.

Isoluminance and Melanopsin Retinal Ganglion Cells

Perceptually isoluminant stimuli have been utilized in past research in attempts to isolate pupillary responses to changes in color, believed to be cortical in origin, from those due to changes in luminance.^{12,18–20,22,24,25,27} The supposition, however, that the absence of subjective luminance information in a stimulus means that any resultant change in pupil diameter is due solely to chromatic factors, is now known to be inaccurate. This is because much of the pupillary response is derived from a subcortical pathway, with quite different dynamics to that of the main visual pathway, in which neural activity is not generally able to be consciously perceived.^{7,33}

A large proportion of the rapid pupillary response to transient changes in luminance, is probably mediated by mRGCs.^{6,7,9} These cells receive input via bipolar cells from rod and cone photoreceptors in the form of short-wavelength sensitive (S-) cone OFF, and rod and medium-wavelength sensitive (M-) + long-wavelength sensitive (L-) cone ON inputs. In addition to this synaptic input, mRGCs express the photopigment, melanopsin. The action spectrum of melanopsin peaks at 482 nm and overlaps that of all three cone types. The sluggish time-course of melanopsin photo-transduction in mRGCs results in pupillary constrictions that are comparatively slow at both onset and offset.^{6,7} This sets them aside from the faster, more transient mRGC responses that at photopic levels are believed to result from cone bipolar-cell inputs.³⁴

Pupillary responses to unidirectional color exchanges or discrete but long-duration color stimuli are therefore subject to variable influence from melanopsin. For example, a high-luminance long-duration green stimulus presented on a red background of the same luminance, with equivalent inputs to

FIGURE 5. Mean pupillary response waveforms estimated across the entire dataset by protocol, for the zero, low, and high luminance-contrast color exchange protocols (G-mid/R-mid, G-hi/R-mid, and G-hi/R-lo) and low and high luminance-contrast luminance only protocols (Y-hi/Y-mid and Y-hi/Y-lo). These estimates use raw pupillary diameters, not AmpStd as other analyses. (A) *Fine black lines* represent averaged response waveforms from three representative stimulus protocols, means estimated across the entire dataset by protocol. *From top:* color exchange only (G-mid/R-mid); color exchange plus low luminance-contrast (G-hi/R-mid); color exchange plus high luminance-contrast (G-hi/R-lo). Also shown in *grey (thicker lines)* are equivalent lognormal response functions for these waveforms. (B) The slope of the steepest portion of the constriction phase of the waveform (i.e., at the point at which the second derivative is closest to 0) has been estimated for each protocol. These values, reported in the legend, provide an approximation of the maximum constriction velocity. The *upper panel* shows the raw waveforms with no scaling (^areported in $\mu\text{m} [100 \text{ ms}]^{-1}$). Traces in the lower panel have been scaled along the ordinate so that each point on the waveform is relative to the peak of the mean response for that protocol. This enables comparison of waveforms and relative velocities (^breported in percentage proportion of peak response $[100 \text{ ms}]^{-1}$). The *grey dashed line* at $y = -0.2$, therefore, denotes the 20% maximal constriction. (C) The response waveforms for low luminance-contrast only (Y-hi/Y-mid, *top*) and high luminance-contrast only (Y-hi/Y-lo, *bottom*) protocols are shown along with that of the zero luminance-contrast color exchange protocol (G-mid/R-mid) and the linear sum of each pair (*solid grey line*). In addition, the appropriate contrast level color exchange protocol response is plotted for comparison against the summed prediction (*dashed and dotted lines*, no markers: G-hi/R-mid, G-hi/R-lo).

these cells from M- and L-cone photoreceptors, can elicit a pupillary constriction independently of any cortical influence, because a green light will generate a stronger melanopsin response than red. This influence, providing no conscious percept, cannot be adjusted for using psychophysical methods to determine isoluminance. For this reason, we chose to utilize transient 50 ms onset stimuli of longer wavelengths, which should be minimally influenced by the slow melanopsin response. It was hoped that the use of red and green, which both provide ON input from cones to mRGCs, would reduce the subcortical pupillary luminance signal in zero luminance-contrast color exchange protocols, and minimize input from S-cone OFF bipolar cells. This curtailing of mRGC response components should have facilitated the observation of components resulting from the processing of color-opponent information carried by cortically-projecting neurons.

Data from a preliminary experiment utilizing zero luminance-contrast color exchange stimuli is shown in Figure 6. The unidirectional nature of these responses to green on red and red on green stimuli indicates that luminance was not their primary driver. The larger amplitudes of responses to green on red, than to the reverse, is possibly due to chromatic

adaptation of cone photoreceptors³⁵ to the stimulus background. This is feasible given that the proportional activation, and therefore adaptation, of L-cones to the green channel is much greater than that of M-cones to red (Fig. 1). This greater adaptation of L-cones to the green background would therefore reduce responses to red stimuli by more than green stimuli on red. That being said, the ability of all the zero-contrast stimuli to elicit constrictions indicates that at least a portion of these responses was due solely to color exchange in the absence of a luminance signal, the proof of principle needed to continue with the main experiment.

The M+L ON cone input to mRGCs makes them the most likely mediator of the pupillary response to the transient luminance stimuli used in this study. Responses to luminance signals, therefore, are at least in part processed subcortically. In addition, this means that mRGCs are insensitive to differences between red and green input, so the unidirectional responses observed to red/green and green/red exchange are most likely mediated by a different population of RGCs. This is crucial to this study, since our aim is to develop a protocol that produces pupil responses involving two different populations of retinal ganglion cells.

FIGURE 6. Mean constriction amplitudes (*top*) and times to peak (*bottom*) from a preliminary experiment investigating pupillary responses to zero luminance-contrast color exchange stimuli. Three runs of six different protocols were tested using a single subject (female, aged 46). These comprised three pairs of 8-minute duration, red on green and green on red protocols with the same temporal and spatial characteristics of those of the main experiment. The background and stimulus luminances of each pair were 90, 115, or 140 cd/m². As with earlier models, inputs are the median values for each parameter across pupils, eyes, and then test regions. Beta values (means) and standard errors are presented in μm for clarity.

Summation of Color and Luminance

One of the principal observations in this experiment was that the latencies of the peak of pupillary constrictions were increasingly delayed as the proportion of color over luminance influence increased in protocols. Longer latencies to the onset of pupillary responses have been previously observed when comparing luminance stimuli against those involving more complex processing of information, and are taken to indicate cortical involvement.^{15,18,25,27,36} In this study, the slope and amplitude of the Yhi/Y-mid and G-mid/R-mid protocols were almost identical; however, the mean time to peak of the latter was around 50 ms later. Although onset time was not included in the study parameters, the constriction velocities (Fig. 5B, *top*) and amplitudes (Table 2) would suggest that the onset delays differ by a similar amount to the times to peak. Although difficult to discern from the raw waveforms, this can be seen clearly at the point of 20% maximal constriction (Fig. 5B, *bottom*). A delay in onset latency of 30 to 40 ms as seen here is consistent with the range reported in the literature.^{15,27,36} This delay is not apparent in the combined color and luminance protocols, likely due to masking by the earlier onset of the luminance component.

The influence of both luminance and color components is apparent also in the normalized constriction velocities (Fig. 5B, *bottom*). A consistent increase in velocity as a proportion of amplitude can be seen with increasing contrast; similar increases in constriction velocity have been observed with increases in intensity of luminance stimuli in macaques.³⁷ The lower values seen in the color plus luminance-contrast protocols relative to their luminance-contrast only equivalents

is likely due to the summation of the early onset, fast luminance component with the later onset, slower color component.

Further evidence of summation of these components is provided by the larger amplitudes of the G-hi/R-lo and G-hi/R-mid luminance and color protocols compared with their luminance only equivalents. At high contrast, the luminance component appears to dominate with changes in color having minimal effect. Color appears to have influenced responses to low luminance-contrast stimuli to a larger degree. Transient cone-initiated and sustained melanopsin responses both increase linearly with the log of the stimulus luminance,^{2,37,38} and have been reported to sum in a linear fashion.²¹ This does not, however, appear to be the case for the summation of luminance and color response components here (Fig. 3B). Where luminance responses were small due to low luminance-contrast, the addition of color exchange produced a response that was somewhat larger and faster than the sum of these components predicted. The addition of color exchange to high luminance-contrast, however, resulted in responses that were somewhat smaller than predicted. This apparently nonlinear summation provides further evidence of the separate processing of these two response components.

CONCLUSIONS

Substantial differences were observed between responses to the luminance and color exchange mfPOP protocols in this study. The amplitudes of pupillary responses were greatly influenced by the luminance-contrast of stimuli, whereas times to peak appeared more dependent on the proportional influence of color. The delay in the onset latency of responses to color exchange, compared with luminance, is consistent with observations in the literature and has previously been taken as an indication of higher-level cortical processing of these stimuli. This observation is extended to multiple visual-field locations here.

The effect on pupillary responses of the interaction between color and luminance stimulus components is complex, with further studies needed to fully understand these hypothesized mechanisms. From our results, however, the latency of the times to peak in this experiment may be taken as a rough estimate of the relative contribution of color to the responses obtained using a particular protocol. Thus, it appears likely that the G-hi/R-lo and G-hi/R-mid protocols, being influenced by both color and luminance, would make suitable candidates for perimetric stimuli aimed at assessing the combined function of cortically and subcortically projecting retinofugal pathways. The topography of responses to these protocols does not differ greatly from those of luminance-contrast only protocols. It is hoped, therefore, that this assessment over a wider pool of retinal ganglion cells and their recipient neurons will enable more accurate evaluation of dysfunction in visual pathways.

References

- Maddess T, Bedford SM, Goh XL, James AC. Multifocal pupillographic visual field testing in glaucoma. *Clin Exp Ophthalmol*. 2009;37:678–686.
- Maddess T, Ho YL, Wong SS, et al. Multifocal pupillographic perimetry with white and colored stimuli. *J Glaucoma*. 2011; 20:336–343.
- Bell A, James AC, Kolic M, Essex RW, Maddess T. Dichoptic multifocal pupillography reveals afferent visual field defects in early type 2 diabetes. *Invest Ophthalmol Vis Sci*. 2010;51:602–608.

4. Carle CE, James AC, Kolic M, Loh YW, Maddess T. High-resolution multifocal pupillographic objective perimetry in glaucoma. *Invest Ophthalmol Vis Sci*. 2011;52:604-610.
5. Sabeti F, James AC, Maddess T. Spatial and temporal stimulus variants for multifocal pupillography of the central visual field. *Vision Res*. 2011;51:303-310.
6. Dacey DM, Liao HW, Peterson BB, et al. Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature*. 2005;433:749-754.
7. Gamlin PD, McDougal DH, Pokorny J, Smith VC, Yau KW, Dacey DM. Human and macaque pupil responses driven by melanopsin-containing retinal ganglion cells. *Vision Res*. 2007;47:946-954.
8. 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;424:76-81.
9. Young RS, Kimura E. Pupillary correlates of light-evoked melanopsin activity in humans. *Vision Res*. 2008;48:862-871.
10. Slooter J, van Norren D. Visual acuity measured with pupil responses to checkerboard stimuli. *Invest Ophthalmol Vis Sci*. 1980;19:105-108.
11. Ukai K. Spatial pattern as a stimulus to the pupillary system. *J Opt Soc Am A*. 1985;2:1094-1100.
12. Barbur JL. A study of pupil response components in human vision. In: Robbins JG, Djamgoz MBA, Anthony T, eds. *Basic and Clinical Perspectives in Vision Research*. New York: Plenum Press; 1995:3-18.
13. Tsujimura S, Wolffsohn JS, Gilmartin B. Pupil response to color signals in cone-contrast space. *Curr Eye Res*. 2006;31:401-408.
14. Young RS, Kennish J. Transient and sustained components of the pupil response evoked by achromatic spatial patterns. *Vision Res*. 1993;33:2239-2252.
15. Barbur JL, Harlow AJ, Sahaie A. Pupillary responses to stimulus structure, colour and movement. *Ophthalmic and Physiol Opt*. 1992;12:137-141.
16. Nowak W, Nakayama M. Preliminary examination of chromatic flicker light stimulation on EEG and pupil size for observing human activity levels. *MCBC'09: Proceedings of the 10th Wseas International Conference on Mathematics and Computers in Biology and Chemistry*. Stevens Point, WI: World Scientific and Engineering Academy and Society (WSEAS); 2009:48-52.
17. Drew P, Sayres R, Watanabe K, Shimojo S. Pupillary response to chromatic flicker. *Exp Brain Res*. 2001;136:256-262.
18. Young RS, Alpern M. Pupil responses to foveal exchange of monochromatic lights. *J Opt Soc Am*. 1980;70:697-706.
19. Kohn M, Clynes M. Color dynamics of the pupil. *Annals N Y Acad Sci*. 1969;156:931-950.
20. Kimura E, Young RS. A chromatic-cancellation property of human pupillary responses. *Vision Res*. 1996;36:1543-1550.
21. Kimura E, Young RS. Nature of the pupillary responses evoked by chromatic flashes on a white background. *Vision Res*. 1995;35:897-906.
22. Kimura E, Young RS. S-cone contribution to pupillary responses evoked by chromatic flash offset. *Vision Res*. 1999;39:1189-1197.
23. Keenleyside MS, Barbur JL, Pinney HD. Stimulus-specific pupillary responses in normal and hemianopic subjects. *Perception*. 1988;17:347-347.
24. Barbur JL, Keenleyside MS, Thomson WD. Investigation of central visual processing by means of pupillometry. In: Kulikowski JJ, Dickinson CM, Murray JJ, eds. *Seeing Contour and Colour*. Oxford: Pergamon; 1987:431-451.
25. Heywood CA, Nicholas JJ, LeMare C, Cowey A. The effect of lesions to cortical areas V4 or AIT on pupillary responses to chromatic and achromatic stimuli in monkeys. *Exp Brain Res*. 1998;122:475-480.
26. Steele GE, Weller RE. Subcortical connections of subdivisions of inferior temporal cortex in squirrel-monkeys. *Vis Neurosci*. 1993;10:563-583.
27. Gamlin PD, Zhang H, Harlow A, Barbur JL. Pupil responses to stimulus color, structure and light flux increments in the rhesus monkey. *Vision Res*. 1998;38:3353-3358.
28. Maddess T, James AC, Bowman EA. Contrast response of temporally sparse dichoptic multifocal visual evoked potentials. *Vis Neurosci*. 2005;22:153-162.
29. Stockman A, MacLeod DI, Johnson NE. Spectral sensitivities of the human cones. *J Opt Soc Am A Opt Image Sci Vis*. 1993;10:2491-2521.
30. James AC. The pattern-pulse multifocal visual evoked potential. *Invest Ophthalmol Vis Sci*. 2003;44:879-890.
31. James AC, Ruseckaite R, Maddess T. Effect of temporal sparseness and dichoptic presentation on multifocal visual evoked potentials. *Vis Neurosci*. 2005;22:45-54.
32. Berson DM, Dunn FA, Takao M. Phototransduction by retinal ganglion cells that set the circadian clock. *Science*. 2002;295:1070-1073.
33. Hattar S, Kumar M, Park A, et al. Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *J Comp Neurol*. 2006;497:326-349.
34. 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.
35. Kremers J, Stepien MW, Scholl HP, Saito C. Cone selective adaptation influences L- and M-cone driven signals in electroretinography and psychophysics. *J Vis*. 2003;3:146-160.
36. Barbur JL, Wolf J, Lennie P. Visual processing levels revealed by response latencies to changes in different visual attributes. *Proc Biol Sci*. 1998;265:2321-2325.
37. Pong M, Fuchs AF. Characteristics of the pupillary light reflex in the macaque monkey: metrics. *J Neurophysiol*. 2000;84:953-963.
38. Gamlin PD, Zhang H, Clarke RJ. Luminance neurons in the pretectal olivary nucleus mediate the pupillary light reflex in the rhesus monkey. *Exp Brain Res*. 1995;106:169-176.