The influence of stimulus size on heterochromatic modulation electroretinograms

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When combined with the electroretinogram (ERG), the heterochromatic flicker photometry procedure allows an objective in vivo assessment of postreceptoral activity. Responses evoked at intermediate (approximately 12 Hz) and high (>30 Hz) temporal frequencies reflect the red–green cone opponent (possibly parvocellular) and the luminance (possibly magnocellular) responses, respectively. Previously, we found that cone-isolating stimuli at intermediate temporal frequencies elicited ERG responses with similar amplitudes and phases for different spatial arrangements of the stimuli, whereas response amplitudes at high temporal frequencies were positively correlated with stimulus size. The purpose of this study was to investigate whether the influence of stimulus size was confined to cone-isolating stimuli or whether it was a general feature of heterochromatic

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stimulation. Furthermore, we aimed to determine the smallest spatial extent for a significant response in the two postreceptoral mechanisms. Monocular ERGs were recorded to red–green counterphase modulated sinusoidal stimuli (mean luminance of 200 cd/m^2) presented at 12 and 36 Hz at different stimulus sizes. At each stimulus condition, a series of ERGs were recorded with the red-contrast fraction (F_R) [$F_R = C_R/(C_R + C_R)$] of the stimulus varying between 0.0 and 1.0. Response amplitudes at 36 Hz changed with F_R for all subjects, exhibiting a V-shaped amplitude profile with a minimum close to the psychophysics-based isoluminance, where the ERG phase changed by 180°. As stimulus size decreased, the amplitudes to 36 Hz also decreased. In contrast, amplitudes and phases at 12 Hz generally were constant for all values of F_R. These amplitudes were invariant to stimulus sizes larger than 10° but decreased with decreasing stimulus size below 10°. Phase also changed in this range. Thus, luminance pathway ERG responses (36 Hz) show direct dependency on stimulus size, whereas chromatic pathway responses (12 Hz) are independent of the stimulus size above 10°.

Introduction

In the human visual system, signals from the different photoreceptors are distributed in different pathways for parallel processing. Cone signals are processed in several retinogeniculate pathways that are important for visual perception, of which the magnocellular (luminance) and parvocellular (red–green color opponent) pathways are the main streams because the majority of retinal ganglion cells and lateral geniculate nucleus cells belong to these pathways (Dacey, 2000; Gouras & Zrenner, 1979; Kaplan, Lee, & Shapley, 1990; Lee, Martin, & Grunert, 2010).

Electroretinography (ERG) provides a noninvasive electrophysiological assay of retinal function. The technique has been used extensively for clinical assessment of the integrity of the human retina because it provides information about light-triggered electrical activity of the different neurons in the eye (Armington, 1974). Recently, the ERG technique was also found to reflect activity in the cone opponent and luminance pathways, expanding the scope of the technique to processing in retinogeniculate pathways (Kremers & Link, 2008; Kremers, Rodrigues, Silveira, & da Silva-Filho, 2010; Parry et al., 2012).

Heterochromatic flicker photometry is a psychophysical method used to determine the luminance of a stimulus. Typically, two different colored stimuli are modulated in counterphase (i.e., heterochromatically) at high temporal frequency. The modulation in the stimuli is maximal (i.e., at 100% Michelson contrast), and the mean luminance of one of the two lights is changed by the observer. By definition, the two lights are isoluminant when the perceived flicker is minimal. The work of Jacobs and colleagues (Jacobs & Deegan, 1997; Jacobs & Neitz, 1993; Jacobs, Neitz, & Krogh, 1996) has shown that this stimulus paradigm can also be employed to perform photometry based on ERG signals; ERG response amplitudes are minimal at isoluminance. However, a disadvantage of the heterochromatic flicker photometry technique is that changing the mean luminance of one of the two lights also changes the state of adaptation in the eye.

A related method that evades this problem is heterochromatic modulation photometry (Huchzermeyer et al., 2014; Pokorny, Smith, & Lutze, 1989). In this method, the mean luminance of the two stimuli is fixed but the ratio of their contrasts is varied. The modulations in the two stimuli are isoluminant when perceived flicker is minimal. Because the mean luminance is not changed in heterochromatic modulation photometry, the state of adaptation remains constant. It was found that ERG signals were also minimal for contrast ratios that resulted in subjective isoluminance (as in the psychophysical experiments), provided that the measurements were performed at high temporal frequencies (>30 Hz; Kremers et al., 2010). At the ERG amplitude minimum, the phases shifted by about 180°. At frequencies of about 12 Hz, no ERG minimum was found. In fact, response amplitudes and phases were relatively stable for all contrast ratios, indicating that the ERGs reflected a cone opponent mechanism. These results were in agreement with those of other measurements (Kremers & Link, 2008; Parry et al., 2012) showing the possibility of measuring L- and M-cone opponency–based (red–green chromatic) signals at 12 Hz and luminance-based (luminance) signals at frequencies above 30 Hz.

Recently, work in our lab has shown that the two mechanisms also have different dependencies on the spatial configuration of the stimuli (Jacob et al., 2015). The high-frequency ERGs depended strongly on the stimulus area, whereas the stimulus area had hardly any influence on the 12-Hz ERGs (neither on their amplitudes nor on their phases). These findings were obtained with L- and M-cone isolating circular stimuli between 10° in diameter and full field (FF) as well as for annular stimuli with 70° outer diameter and varying inner diameter. Obviously, the responses should change and ultimately disappear if the stimulus area is approaching zero. The purpose of the present study was to explore a larger range of stimulus areas in order to study the spatial dependency of the two ERG mechanisms in more detail. For this, responses with better signal-to-noise ratios than those obtained with the cone-isolating stimuli were required. We therefore used the above-mentioned heterochromatic modulation photometry stimulus paradigm, with which larger

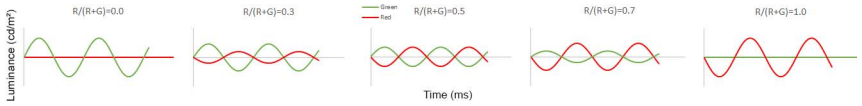

Figure 1. Representation of five different stimulus conditions: F_R 0.0, 0.2, 0.5, 0.8, and 1.0. In condition 0.0 (left), the red light-emitting diode (LED) was constant and the green LED was modulated with 100% contrast. In condition 0.5 (middle), both the red and green LEDs were modulated with 50% contrast in counterphase. In condition 1.0 (right), the red LED was modulated with 100% contrast and the green LED was constant.

contrasts can be reached. The second purpose of the present study was to compare the results of the two stimulus paradigms (cone-isolating stimuli vs. heterochromatic modulation) in order to establish whether the two spatial characteristics genuinely can be attributed to two different retinal pathways.

Method

Subjects

Five healthy volunteers (three males and two females; aged 26–54 years) participated in this study. The study was approved by the ethics committee of the Medical Hospital Erlangen, Friedrich-Alexander University (329_12B), and adhered to the tenets of the Declaration of Helsinki. Subjects were informed and signed the consent prior to the experiments. All subjects had normal color vision as established with the anomaloscope (Oculus Optikgeräte GmbH, Wetzlar, Germany).

Visual stimulation

The stimuli were provided by a Ganzfeld (Q450SC; Roland Consult Stasche & Finger GmbH, Brandenburg, Germany) stimulator equipped with six different colored light-emitting diodes and controlled by RETIport software (Roland Consult). In this study, only the red (638 nm; CIE1931: $x = 0.6957$, $y = 0.2966$) and green (523 nm; CIE1931: $x = 0.2016$, $y = 0.7371$) light-emitting diodes were used. Both the red and green stimuli had mean luminances of 100 cd/m^2. Mean hue was yellow (CIE coordinates: $x = 0.5813$, $y = 0.4030$). The stimuli were 12- and 36-Hz sine wave modulations of the red (with Michelson contrast R) and green (with Michelson contrast G) lights in counterphase (Barboni, Pangeni, Ventura, Horn, & Kremers, 2011; Barboni, Ventura, & Kremers, 2010; Kremers et al., 2010). At each stimulus condition, a series of ERGs were recorded, varying in their fraction of red modulation contrast of the total modulation contrast [$F_R = C_R/(C_R + C_R)$] from 0.0 (i.e., green stimulus was modulated with 100% Michelson contrast and red stimulus was constant) to 1.0 (i.e., red stimulus was modulated with 100% Michelson contrast and green stimulus was constant) in steps of 0.1 (Figure 1). The total amount of R and G contrast was constant at 100% ($C_R + C_G = 100\%$). The 0.5 condition was measured twice at different instances during the measurements to check for stability in the recording conditions.

The measurements were repeated for different stimulus sizes: FF and circular stimuli with 70°, 50°, 30°, and 10° diameter. Three additional stimulus sizes were measured for the 12-Hz responses: 7.5°, 5°, and 2.5° diameter. These were custom made with black cardboard and positioned at a 3-cm distance from the subject, with the central point aligned with the circular opening of the stimulator.

ERG recordings

ERGs were recorded monocularly from a dilated eye (0.5% tropicamide; Pharma Stulln GmbH, Stulln, Germany). The subjects' heads were stabilized by a chin rest. Three electrodes were used: Gold cup electrodes filled with electrode paste (DO Weaver & Co., Aurora, CO) were placed on the forehead (ground electrode) and on the ipsilateral temple (reference electrode) after cleaning the skin with Nuprep abrasive gel (DO Weaver & Co.), and a corneal DTL fiber (active electrode) was placed over the lower conjunctiva from the outer to inner canthus of the eye. Impedance of the reference and active electrodes was below 5 kΩ.

ERG signals were recorded using the RETIport system, were amplified 100,000 times, bandpass filtered between 1 and 300 Hz, and digitized at a rate of 1024 Hz. The intrusion of 50-Hz signals from the mains was small so that no notch filter was needed.

For FF and 70° stimulation, ERG responses were averages of at least 40 sweeps at 12 Hz and 20 sweeps at 36 Hz, with each sweep lasting 1 s. The number of sweeps per average response was increased to 120 for 36-Hz recordings with smaller stimulus sizes between 50° and 10° and to at least 160 for 12-Hz recordings with stimulus sizes between 7.5° and 2.5°.

Data analysis

All signals were Fourier analyzed with a self-written MATLAB program (R2013b, MathWorks, Natick, MA). The amplitudes and phases of the first harmonic (fundamental) component were used to describe the responses. A response was accepted only when the signal-to-noise ratio (quantified by the ratio of the fundamental component and the average of the component amplitudes at adjacent frequencies; e.g., values at 11 and 13 Hz for 12-Hz responses) was ≥ 2. SigmaPlot (version 12.0; Systat Software GmbH, Erkrath, Germany) was used for linear regression analyses and graph presentation.

Results

Figure 2 shows individual response amplitudes (left) and phases (right) to the 36-Hz stimuli for the different stimulus sizes as a function of F_R for the five subjects. A model that assumed that the responses were vector averages of L- and M-cone driven responses was fitted to the data. Similar models were used previously to describe interactions of responses driven by different photoreceptor types (Kremers, Usui, Scholl, & Sharpe, 1999; McAnany, Park, & Cao, 2015; Park, Cao, Collison, Fishman, & McAnany, 2015) or different postreceptoral mechanisms (Kondo & Sieving, 2001; Kremers et al., 2010). The model was fitted to response data in vector space in which responses are represented by vectors with lengths determined by the response amplitudes and the angles with the positive abscissa reflecting the response phases. The fits were performed by minimizing the squared distances between model and measured responses. The model contained four free parameters: the amplitudes and phases of L- and M-cone driven responses. The fits were performed on all subjects and conditions. All fits were well constrained by the data.

From these fits we obtained estimates of L-response fractions: $[G_L/(G_L + G_M]$, in which G_L and G_M are L- and M-cone driven ERG response amplitudes or gains. These fractions are given in Table 1. The data suggest a decrease in L-response fraction with a decrease in stimulus size from FF to 70°. A further decrease in stimulus size did not result in a systematic change of L-response fraction.

The grouped mean (and standard deviation) of these response amplitude and phase data is given in Figure 3. It can be seen from both individual and grouped data that the response amplitudes strongly depended on F_R. For all stimulus sizes tested, the amplitudes were the largest at F_R 0.0 and 1.0, while the amplitudes were minimal at intermediate conditions between 0.3 and 0.5. At the F_R condition where the amplitude was minimum, response phases changed by about 180°. These results are in agreement with earlier studies that involved only FF stimulation (Barboni et al., 2010, 2011; Kremers et al., 2010).

In addition, the response amplitudes were positively correlated with stimulus size. They decreased by a factor of about 50 when stimulus size decreased from FF to 10° diameter. The response phases did not change strongly with stimulus size. The phase change at the amplitude minimum appeared to be more abrupt for FF and for the 10° stimuli. These results are in agreement with previous data on ERG recordings to high-frequency L- and M-cone isolating stimuli (Jacob et al., 2015).

Figure 4 shows 12-Hz data in the same format as Figure 2 for every subject. However, the measurements were performed with three additional stimulus sizes down to 2.5° in diameter. Grouped results (and standard deviation) are displayed in Figure 5. In contrast to 36-Hz data, the response amplitudes and phases to the intermediate flicker were relatively constant for all conditions. This was found to be the case for all subjects, which suggests that the responses reflect cone opponent chromatic mechanisms because the red–green chromatic content in the stimulus did not change in either amplitude or phase. However, there were some small but systematic changes at intermediate stimulus sizes, where a decrease in response amplitude and phase can be observed with increasing values of F_R. This trend was not found for the FF and 2.5° diameter stimuli.

Stimulus size between FF and 10° did not influence the response amplitudes. Below 10°, however, amplitude responses decreased with decreasing stimulus size for all subjects. With a 2.5° stimulus, the responses were about a factor of 10 smaller than those measured with stimuli larger than 10° in diameter. The response phases did not change dramatically for stimuli between FF and 30° diameter either, but a systematic phase change was noted also for smaller stimuli. The data for stimuli of 10° and larger are in agreement with the data from Jacob et al. (2015), where the responses at 12 Hz reflect a chromatic mechanism that were constant for stimuli larger than 10° in diameter. The data extend the previous data in that the response amplitudes and phases strongly change for stimuli smaller than 10° in diameter. However, it was surprising that measurements could be performed with stimuli with diameters as small as 2.5°.

To quantify the dependency of response amplitude on stimulus size, the mean amplitudes for three stimulus conditions ($F_R = 0$, 0.5, and 1) are plotted as a function of the stimulus diameter in Figure 6. For the 36-Hz data we also used the amplitudes at the stimulus condition that resulted in a minimal response, thereby

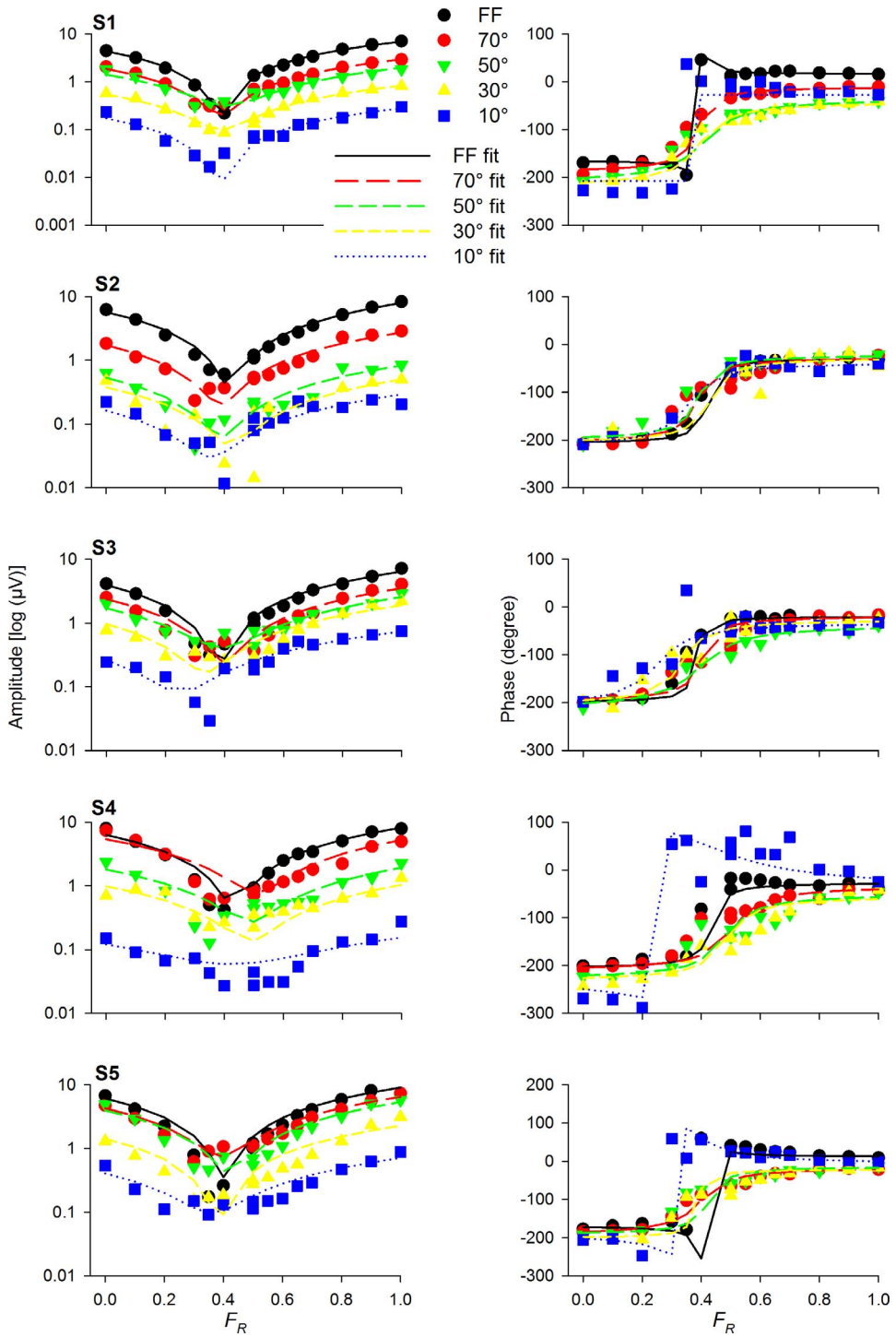

Figure 2. Response amplitudes (left; logarithmic scale) and phases (right) of ERGs to 36-Hz stimulation versus F_R. The plots are color coded for the different stimulus sizes used (see legend) and shown separately for five different observers (S1–S5). The curves are fits of a vector addition of L- and M-cone driven responses to the data.

taking into account that this minimum, probably corresponding to the individual isoluminance point, may depend on individual differences and on stimulus size. In this double logarithmic plot, the slope of the linear regression quantifies the eccentricity-dependent inhomogeneity of the retinal responses. If retinal responses were homogeneous, then the response amplitudes would be proportional with the stimulus area ($R = k \cdot S^2$, where R is response amplitude and S is stimulus diameter) and a slope of 2 would be expected: $\log(R) = \log(k) + 2\log(S)$. Figure 6 shows a linear regression at 36 Hz (upper graph). The slopes were

Stimulus size	S1	S2	S3	S4	S5
Full field	0.97	0.89	0.96	0.85	0.92
70°	0.91	0.90	0.90	0.67	0.85
50°	0.80	0.90	0.81	0.71	0.86
30°	0.85	0.84	0.86	0.71	0.96
10°	1.00	0.86	0.78	0.65	0.83

Table 1. Fraction of the L-cone driven responses $[G_L/(G_L + G_M)]$ obtained from model fits to ERG data for different subjects and stimulus sizes.

between 1.1 and 1.6, similar to those found for cone isolating stimuli (Jacob et al., 2015).

The lower graph shows that for 10° stimuli (log diameter 1.0) and larger, the response amplitudes at 12 Hz did not change. For smaller stimuli, the linear regressions had slopes between 0.95 and 1.5, indicating that the response amplitudes were more proportional with stimulus diameter than with its area. Figure 7 shows the phases of the 36-Hz (upper graph) and 12-Hz (lower graph) responses as a function of stimulus diameter and for the three stimulus conditions, confirming the above observation that they were invariant at 36 Hz but changed for stimuli smaller than 30° at 12 Hz.

Discussion

The purpose of the present study was twofold. First, we wanted to compare the results of measurements with heterochromatic modulation of red and green lights with those using L- and M-cone isolating stimuli in a previous study with similar stimulus parameters (Jacob et al., 2015). Second, we wanted to study the dependency of the responses, particularly those reflecting activity of cone opponent mechanisms, on the size of circular stimuli in more detail by including smaller stimuli than used with the cone isolating stimuli. In this range of stimuli, a dependency of response amplitude on stimulus size was expected.

Generally, the results presented here are in agreement with earlier studies (Jacob et al., 2015) showing that amplitudes, but not the phases, of the high-frequency ERGs strongly depend on stimulus diameter. There is also agreement in that the amplitude and stimulus diameter have a linear relationship in a double logarithmic plot (see Figure 6) with a slope between 1.1 and 1.6. Very similar slopes have been found by Jacob et al. (2015) for 36-Hz stimuli. This slope is substantially more shallow than the slope that is expected for a completely homogeneous retinal mechanism. The data therefore indicate that the retinal periphery contributes less to the ERG amplitude than the central retina.

Jacob et al. (2015) found that the L:M ratio in high-frequency responses is much larger with FF than with smaller stimuli, indicating that the responses that originate in the periphery are strongly L-cone dominated. This is confirmed in other ERG recordings (Challa et al., 2010; Kuchenbecker, Sahay, Tait, Neitz, & Neitz, 2008; Tsai et al., 2016) and messenger ribonucleic acid analyses (Hagstrom, Neitz, & Neitz, 1997, 2000). The L:M ratio determines the stimulus conditions for isoluminance: The larger the L:M ratio, the smaller the values for F_R at isoluminance (because less red modulation is needed to counteract the response to the green light). Therefore, a shift of the isoluminance conditions was expected from small values at FF to larger values with smaller stimuli. This, however, was not obvious from our data. However, the model fits show a tendency for the L-response fraction, and thus the L:M ratio, to decrease when stimulus size was decreased from FF to 70°. The reason why we do not have stronger correlations between isoluminance and stimulus size is unclear. Possibly, the different

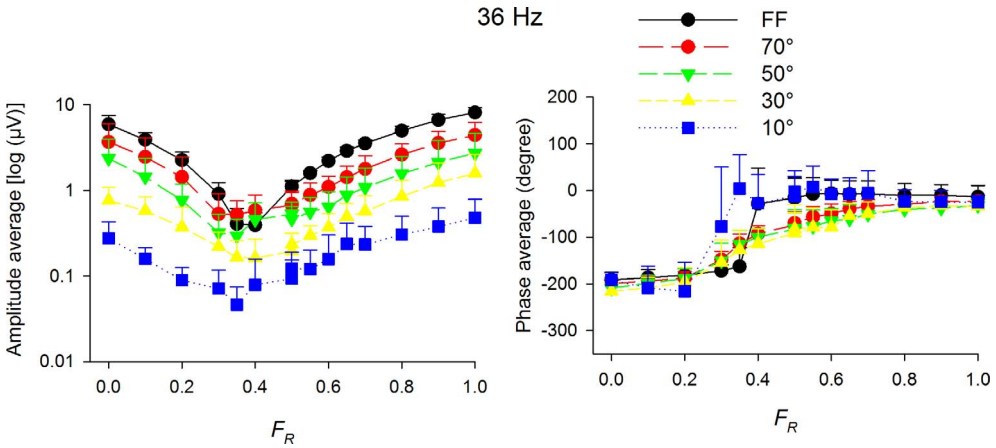

Figure 3. Group-averaged (and standard deviation) F_R response amplitudes (left; logarithmic scale) and phases (right) to 36-Hz stimulation for the different stimulus sizes (color coded as in Figure 2).

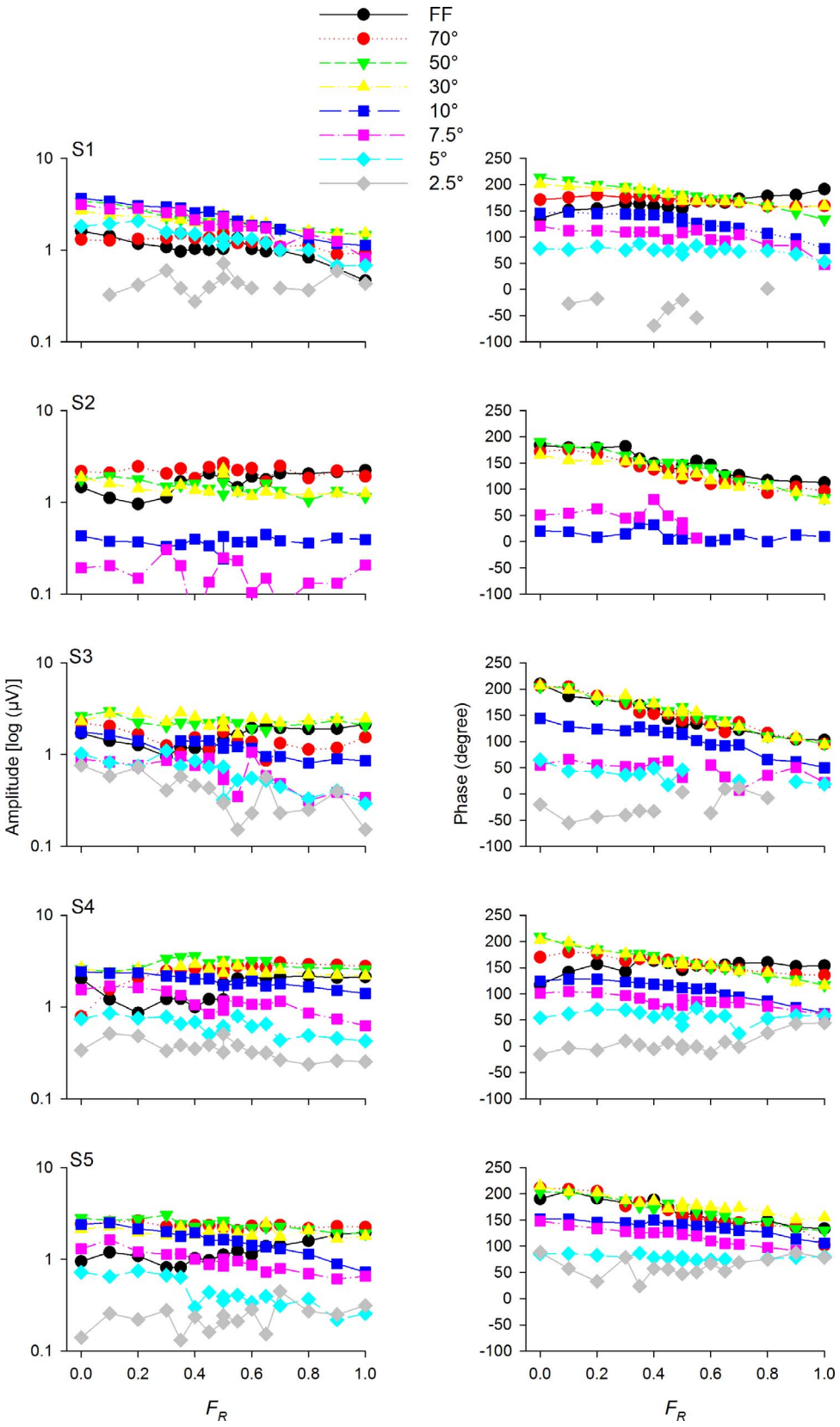

Figure 4. Response amplitudes (left plots) and phases (right plots) as a function of F_R with 12-Hz stimulation for each observer (S1–S5). The plots are color coded for the different stimulus sizes used (see legend).

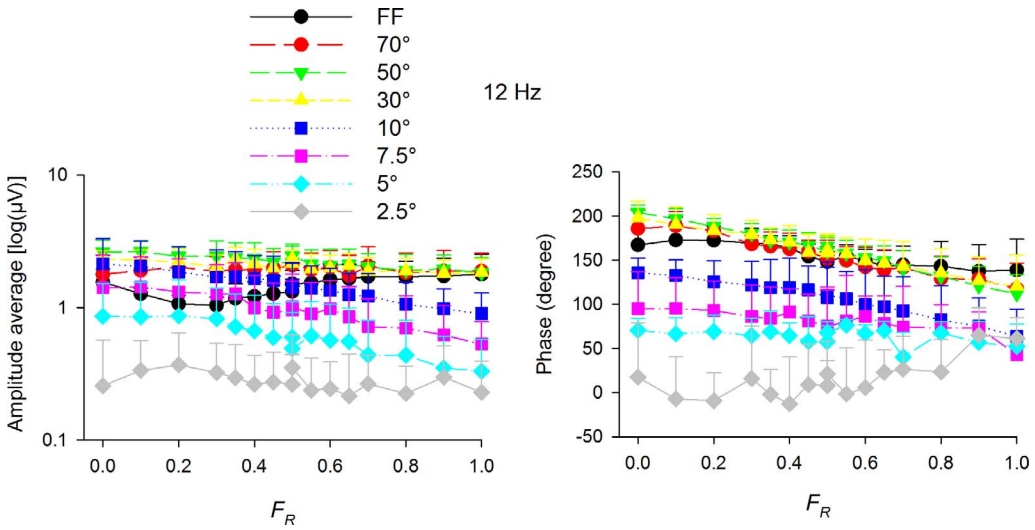

Figure 5. Group averaged (and standard deviation) amplitude (left) and phase (right) responses at 12 Hz as a function of F_R. Data recorded using different stimulus sizes are color coded as in Figure 4.

mean chromaticity in the present study is an explanation for this.

The present 12-Hz data and the results of Jacob et al. (2015) also agree in that for stimuli larger than 10° in diameter, the response amplitudes and phases are relatively invariant. Our data further provide evidence for the notion that these responses reflect cone opponent processes. However, we find small but consistent amplitude and phase decreases for increasing values of F_R (see Figure 5). We interpret this trend as an indication of intrusion of rod and possibly S-cone driven responses. These are not silenced in the present experiments as with the L- and M-cone isolating stimuli used by Jacob et al. (2015). We calculated that the silent substitution conditions for rods and the S-cones are both reached for F_R values between 0.9 and 1.0, thereby providing a possible explanation for an approximately monotonous relationship between amplitude and phase on the one hand and F_R on the other hand. Further evidence for this notion comes from the finding that the trend is not visible for FF stimuli, in which rods are completely desensitized (whereas with spatially restricted stimuli, rods in parts of the retina that are dark adapted may be stimulated by stray light; Aher and Kremers [2016]). The trend is neither visible for small stimuli with 2.5°, which possibly mainly stimulates retinal areas free of rods and S-cones.

Our 12-Hz data complements previous findings in that the responses were also recorded for smaller stimuli (<10° in diameter). Not surprisingly, the invariance of the response amplitude for different stimulus sizes could not be pushed to very small stimulus sizes approaching a diameter of 0°. We found instead that the response amplitudes depended on stimulus size for stimuli smaller than 10° (Figure 6). It was, however, still surprising that 12-Hz responses

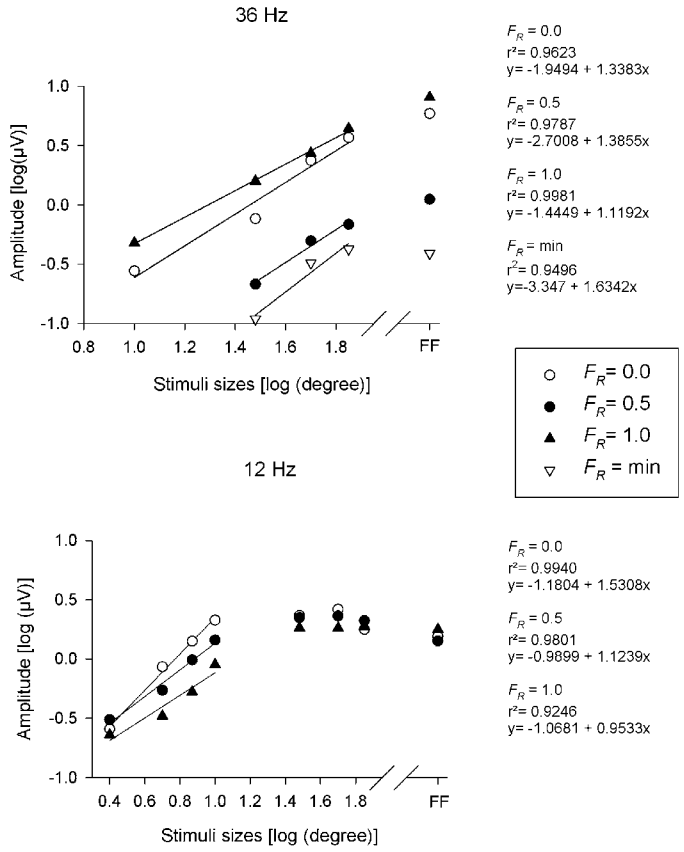

Figure 6. Double logarithmic plots of response amplitudes as a function of stimulus diameter at 36-Hz (upper plot) and 12-Hz (lower plot) temporal frequencies, plotted separately for three values of F_R. For the 36-Hz data, the minimal amplitudes are also plotted. These occur at slightly different values of F_R. The linear regressions show positive correlations between response amplitude and stimulus size at 36 Hz and for stimuli smaller than 10° at 12 Hz.

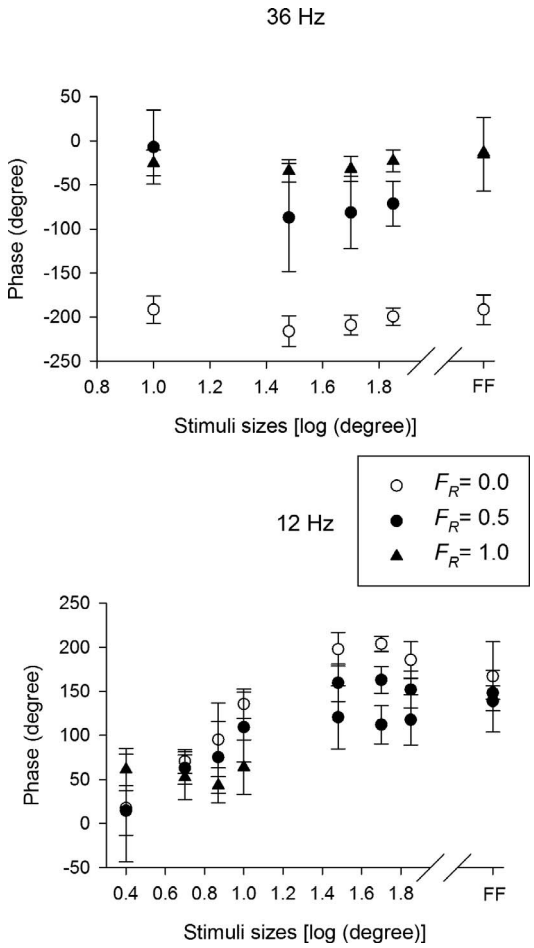

Figure 7. Averaged phases of 36-Hz (upper plot) and 12-Hz (lower plot) responses at stimulus conditions with F_R equal to 0.0, 0.5, and 1.0 versus stimulus size. The phases at 36 Hz are relatively constant for the different stimulus sizes, whereas at 12 Hz the phases decrease for stimuli smaller than 30°.

could still be obtained with a 2.5° diameter stimuli. The 36-Hz responses were hardly measurable with 10° diameter stimuli (i.e., with a stimulus that has an area that was a factor of 16 larger). This finding is even more surprising because the signal-to-noise ratio is generally substantially smaller for 12-Hz stimulation (Kremers & Pangeni, 2012).

In contrast, stimuli larger than 10° in diameter elicited responses with similar amplitudes. This finding can be interpreted as that the 12-Hz (putative cone opponent) ERG mechanism strongly saturates for large stimuli and might indicate that this mechanism is restricted to the central retina. However, previous data (Jacob et al., 2015) showed that similar response amplitudes can also be obtained with annular stimuli, in which the central retina was not stimulated. Even with annular stimuli with a 70° outer diameter and 60° inner diameter, large 12-Hz responses were found. This argues against the possibility that the generating chromatic mechanism is restricted to the central retina. The area of this stimulus equals the area of a circular stimulus with about 36° diameter. Thus, a saturating mechanism may still explain the large response with annular stimuli, even if the stimuli are not restricted to the central retina.

Another surprising feature of the 12-Hz responses with small stimuli was that their phases strongly changed with stimulus size. A stimulus size dependent phase change was not observed for larger stimuli at 12 Hz. The origin of the phase change is still unclear. However, this finding is another indication of how the retinal circuitries that generate the 12-Hz ERGs are fundamentally different from those on which the 36-Hz responses are based.

Conclusions

Heterochromatic modulation ERGs reveal different mechanisms: (a) a high-frequency mechanism that putatively reflects a luminance driven response, the amplitude of which depends on the stimulus size, and (b) a 12-Hz mechanism that displays constant responses for stimuli larger than 10° in diameter, but the amplitudes and the phases strongly depend on stimulus size for stimuli smaller than 10°.

Keywords: electroretinography, magnocellular, parvocellular, stimulus size, temporal frequency

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