Responses of Postreceptoral Pathways Elicited by L- and M-Cone Isolating ON- and OFF-Electroretinograms in Glaucoma Patients

Avinash J. Aher,[1] Folkert K. Horn,[2] Cord Huchzermeyer,[2] Robert Lämmer,[2] and Jan Kremers[1,3,4]

[1]Section for Retinal Physiology, University Hospital Erlangen, Erlangen, Germany
[2]Department of Ophthalmology, University Hospital Erlangen, Erlangen, Germany
[3]School of Optometry and Vision Science, University of Bradford, Bradford, West Yorkshire, United Kingdom
[4]Department of Anatomy II, University of Erlangen-Nürnberg, Erlangen, Germany

PURPOSE. To compare the electroretinographical (ERG) responses elicited by L- and M-cone isolating ON- and OFF-sawtooth stimuli in normal subjects and glaucoma patients.

METHODS. Twenty-one normal subjects and 44 primary open-angle glaucoma patients participated in the study. L- and M-cone isolating (18% cone contrast; 284 cd/m^2) rapid ON- and rapid OFF-sawtooth (4 Hz) stimuli with two stimulus sizes (full-field (FF) and central 70° diameter) were generated using the triple silent substitution technique. ON- and OFF-response asymmetries were studied by adding the two (to obtain L-add and M-add responses). The initial positive (P) and subsequent late negative (LN) components of the L-add and M-add ERGs were compared between the subject groups and correlated with retinal nerve fiber layer thickness (RNFLT) and pattern ERG responses.

RESULTS. The responses to L-ON and to M-OFF stimuli and vice versa resembled each other particularly with 70° stimuli. The P_{L-add} amplitudes were not significantly different between the normal subjects and glaucoma patients, whereas the LN_{L-add} amplitude was significantly ($P < 0.01$) smaller in the glaucoma patients. Both P_{M-add} and LN_{M-add} were not significantly different between the subject groups. The PERG amplitude with 0.8° check sizes and the 0.8°/16° amplitude ratio (PERG ratio) were significantly ($P < 0.05$) different between the subject groups. The 70° LN_{L-add} amplitude and the 0.8° PERG amplitude were significantly correlated with RNFLT.

CONCLUSIONS. The ERGs to 70° cone isolating sawtooth stimuli reflect cone opponency. The cone opponent ERG responses were not significantly different between glaucoma patients and normal subjects. Luminance driven L-add responses were significantly different, indicating that central luminance signals are mainly affected in glaucoma.

Keywords: ON- and OFF electroretinogram, silent substitution, glaucoma, sawtooth stimuli, stimulus size, parvocellular, magnocellular

Several lines of evidence suggest that glaucoma preferentially affects retinal ganglion cells which project to the magnocellular layers of the lateral geniculate nucleus. Silverman et al.[1] demonstrated magnocellularly based motion perception deficits in glaucoma patients.[2–4] Anderson et al.[5] reported significant losses of resolution acuity when stimulated with 30 Hz phase reversal gratings, which are thought to stimulate a higher proportion of magnocellular ganglion cells. Furthermore, sinusoidal achromatic gratings with low spatial frequencies are perceived at twice its spatial frequency when flickered in counterphase at 20–30 Hz temporal frequency.[6] This frequency doubling phenomenon possibly has magnocellular (MC) origin.[7] Maddess et al. reported a method for clinical testing for glaucoma based on the spatial frequency doubling illusion and found significantly reduced sensitivity in the glaucoma patients.[7] However, White et al.[8] argued for the involvement of cortical loss of temporal phase discrimination, which may be the principal cause of the illusion.

The pattern electroretinogram (PERG) response reflects the integrity of retinal ganglion cells and hence has been used clinically for many years, particularly in the assessment of glaucomatous neuropathy.[9–11] Lingley et al.[12] demonstrated that the N_{95} component of transient PERG resembles an average of the photopic negative responses (PhNRs) of ON- and OFF- electroretinograms elicited by luminance increments and decrements of the uniform fields. We have reported previously that the ON- and OFF-ERG responses elicited by full-field luminance incremental and decremental sawtooth (4 Hz) stimuli (with white mean chromaticity) were significantly altered in glaucoma patients.[13] Particularly the late negative (LN; PhNR-like) components of ON- and OFF-ERG responses, and the LN of the summed responses (LN_{add} analog to the N_{95} of the PERG) were significantly altered.

The alterations were significantly correlated with alterations of the retinal nerve fiber layer thickness (RNFLT) and of the PhNR in the flash ERG. It is not known if the cone-opponent mechanism is equally or differently altered by glaucoma as the luminance mechanism.

The electroretinogram (ERG) elicited by photoreceptor type specific stimulation allows the study of the functional characteristics of each photoreceptor type and the postreceptoral visual pathways to which they impart their signals. With the silent substitution technique, based on spectral compensation,[14–18] each photoreceptor type (L-cone, M-cone, S-cone, and rod) can be stimulated with known strength (expressed in terms of cone and rod contrast) and at constant states of adaptation. The isolation and comparison of ERGs driven by single photoreceptor types are important to understand the fundamental visual processes in parvocellularly based red-green chromatic and in magnocellularly based luminance retinal pathways. There is evidence that ERGs elicited by cone and rod specific stimulation using the silent substitution technique can be useful to study the retinal abnormalities which affect vision.[19–23]

Electroretinograms elicited by photoreceptor excitation increments (ON-ERG) and decrements (OFF-ERG) have components with distinct properties.[13,24] By employing periodic sawtooth stimuli of moderate frequency (4 Hz), ON- and OFF-ERG responses can be reliably recorded. The ON-ERG response shows an early negative potential similar to the a-wave of flash ERGs, which originates in photoreceptors and OFF-bipolar cells,[25] followed by a positive deflection similar to the b-wave, which is mainly generated by ON-bipolar cell activity.[26] The OFF-ERG response shows an initial positive d-wave, which is mainly the activity of OFF-bipolar cells and cone photoreceptors.[26,27] Similar to the photopic negative response (PhNR) in the flash ERG, both ON- and OFF-ERG responses show a late negative deflection after the positive b- and d-waves, respectively. The summation of ON- and OFF-ERG responses to stimuli with relatively low temporal frequencies (<4 Hz) generate a pattern ERG-like response[12,13,28,29] with an initial positive wave (P_{add}) followed by a late negative wave (LN_{add}) that can be considered to be analogous to the P_{50} and N_{95} components of the transient PERG, respectively.

Recently, we found that the ERG responses elicited by opposite polarities of L- and M-cone isolating sawtooth stimuli (i.e., the L-ON and M-OFF responses on the one hand, and the L-OFF and M-ON on the other hand) of a moderate temporal frequency (4–8 Hz) resembled each other,[24] indicating the presence of cone opponency in the responses. These similarities were particularly clear with spatially restricted stimuli (spanning the central 35° of the retina). It has yet to be shown how glaucoma affects the ON- and OFF-ERG responses and their asymmetries elicited by cone isolating sawtooth stimuli. Alterations in the additions of the L- and M-cone driven sawtooth responses may therefore give information about the involvement of cone-opponent mechanisms in glaucoma. The cone isolating sawtooth stimuli could give additional information about how glaucoma affects the postreceptoral pathways in the retina. Therefore, in the present study, we sought to investigate the ON- and OFF-ERG responses and their asymmetries elicited by full-field and spatially restricted L- and M-cone isolating sawtooth stimuli in normal subjects and glaucoma patients.

METHODS

Subjects

Twenty-one normal subjects (10 males and 11 females, mean age ± SD: 63.1 ± 11.6, age range: 45–80 years), and 44 primary open-angle glaucoma patients (26 males and 18 females, mean age ± SD: 65 ± 9.06, age range: 43–80 years) participated in the present study. There was no significant age difference between the groups. The glaucoma patients were recruited from the Erlangen Glaucoma Registry, Department of Ophthalmology, University Hospital Erlangen. All participants were informed about the protocol and the purpose of the experiment. Informed written consent was obtained from each subject. The experiments were conducted in accordance with the tenets of the Declaration of Helsinki and the protocol was approved by the local institutional ethics committee (medical faculty of the University Erlangen-Nürnberg).

All participants underwent a comprehensive ophthalmological investigation including best-corrected visual acuity, slit-lamp examination, funduscopy, gonioscopy, standard automated perimetry, and pupillometry before the ERG recordings. The ERG recordings were performed on the same day as the ophthalmological investigation. The subjects who had a visual acuity of 0.3 logMAR or higher and a myopic refractive error better than −8.5 diopters were included in the study. Subjects with mild and severe cataracts, as established in slit-lamp examinations, were excluded. Criteria for the diagnosis of glaucoma were an open anterior chamber angle and glaucomatous damage of the optic nerve head, along with abnormally small neuroretinal rim area of the optic disc and cup-to-disc ratios larger in the vertical than in the horizontal direction assessed by funduscopy and optical coherence tomography (OCT).[30,31] As mentioned above, visual field measurements were performed but these data were not used for the diagnosis of glaucoma. Preperimetric patients (i.e., without a visual field defect) were also in the patient group.

Visual Stimulation

L- and M-cone isolating photopic stimuli were generated using a Ganzfeld bowl (Q450SC, Roland Consult, Brandenburg an der Havel, Germany) equipped with six differently colored arrays of light-emitting diodes (LEDs). The Retiport software (Roland Consult) allows independent control of mean luminance, Michelson contrast, phase, temporal frequency, and stimulus profile of each LED array. To generate the L- and M-cone isolating stimuli using triple silent substitution,[15,16] the red (peak wavelength ± half bandwidth at half height: 638 ± 9 nm), green (523 ± 19 nm), blue (469 ± 11 nm), and amber (594 ± 8 nm) LEDs were activated. The emission spectra of the LEDs were measured using a CAS 140 spectroradiometer (Instrument Systems, München, Germany). The emission spectra and photoreceptor fundamentals [32] were used to calculate mean photoreceptor excitation and excitation modulation (expressed as cone or rod Michelson contrast) by a linear transformation. This procedure is described in detail elsewhere.[14,33] Briefly, the outputs of the LEDs were modulated with rapid-ON or rapid-OFF sawtooth temporal profiles. All four LEDs were modulated simultaneously. The linear transformation converting LED contrasts into

rod- or cone contrasts can be described by a 4×4 matrix:

$$\begin{pmatrix} S_{L\text{-}c,R} & S_{L\text{-}c,A} & S_{L\text{-}c,G} & S_{L\text{-}c,B} \\ S_{M\text{-}c,R} & S_{M\text{-}c,A} & S_{M\text{-}c,G} & S_{M\text{-}c,B} \\ S_{S\text{-}c,R} & S_{S\text{-}c,A} & S_{S\text{-}c,G} & S_{S\text{-}c,B} \\ S_{Rod,R} & S_{Rod,A} & S_{Rod,G} & S_{Rod,B} \end{pmatrix} \begin{pmatrix} R \\ A \\ G \\ B \end{pmatrix} = M \begin{pmatrix} R \\ A \\ G \\ B \end{pmatrix} = \begin{pmatrix} L\text{-}c \\ M\text{-}c \\ S\text{-}c \\ Rod \end{pmatrix}$$

In which *R, A, G, B* are the Michelson contrasts in the red, amber, green, and blue LEDs, respectively. *L-c, M-c, S-c,* and *Rod* are the (Michelson) contrasts of excitation modulation in the four photoreceptor types. The values *S* in the 4×4 matrix (*M*) are the conversion factors for each LED and each photoreceptor type. They were obtained by multiplying the emission spectra of the LEDs at the chosen mean luminance with the cone fundamentals and with V_λ' (for rods) and by integrating over wavelength. For instance, the sensitivity of the L-cones to the red LED: $S_{L\text{-}c,R} = \int E_{Red} \cdot F_{L\text{-}c} \cdot d\lambda$. Where E_{Red} is the emission spectrum of the red LED and $F_{L\text{-}c}$ is the L-cone fundamental.

All contrasts (*R, A, G, B, L-c, M-c, S-c,* and *Rod*) could obtain positive (meaning by definition rapid-ON sawtooth) or negative values (meaning rapid-OFF sawtooth).

The inverse matrix M^{-1} was used to calculate the contrast and polarity in each LED necessary to obtain a particular condition. For instance, the stimulus condition for a 10% rapid-ON L-cone isolating stimulus (i.e., 0% contrast in the M- and S-cones and in the rods, so that their excitation was not modulated) was:

$$M^{-1} \begin{pmatrix} 10 \\ 0 \\ 0 \\ 0 \end{pmatrix} = \begin{pmatrix} R \\ A \\ G \\ B \end{pmatrix}$$

To create this stimulus, not all LEDs were modulated with rapid-ON profiles. To silence the other photoreceptor types, some had to modulate with rapid-OFF profile (indicated by negative contrast values).

To obtain the different stimuli, only contrast and polarity were varied. Mean luminance of each LED was not varied. This ensured that the overall mean luminance and chromaticity (and thus the state of adaptation) was the same for all conditions.

In the present study, either L- or M-cone contrast was 18% with the other cone type silenced. In all conditions, the rods and S-cones were silenced (i.e., 0% contrast for rod and S-cone) resulting in triple silent substitutions. The mean luminances of the red, green, blue, and amber LED arrays were set to 80, 40, 4, and 160 cd/m², respectively. This resulted in a reddish mean chromaticity with the CIE 1931 coordinates x = 0.5686 and y = 0.3716 and a total mean luminance of 284 cd/m² (about 14,200 photopic trolands retinal illuminance, assuming an 8 mm pupil diameter). Four hertz (4 Hz) sawtooth temporal excitation profiles for the L- and M-cone isolating stimuli were employed. A sudden cone excitation increment (ON) followed by a ramping decrement elicited ON-ERG responses whereas a nearly instantaneous decrement (OFF) in cone excitation followed by an incremental ramp was used to elicit OFF-responses. Accordingly, we generated L-ON, L-OFF, M-ON, and M-OFF stimuli (see Fig. 1). The ERG responses elicited by these L- and M-cone isolating incremental and decremental stimuli were recorded each at two stimulus sizes: full field (FF) and 70°

diameter. The 70° diameter spatial stimulus configuration was created with a cardboard field stop placed in front of the standard Ganzfeld aperture at a distance of 3 cm from the observer's eye.

The steady-state pattern ERG recordings were performed using the RetiPort system (Roland Consult), which also controlled an LCD monitor (AOC 919Vz) for PERG recordings. The stimulus frequency was 7.5 Hz (15 reversals/s). Two check sizes (0.8° and 16°) were employed. The total stimulus was rectangular with 32° side size at a viewing distance of 50 cm. The temporal frequency was 7.5 Hz, that is, 15 reversals per second. The mean luminance of the bright checks was 240 cd/m². The dark checks had a luminance of 0.8 cd/m². As a result, the luminance contrast was 99 %, and the time-averaged luminance about 120 cd/m². The CIE coordinates of the white checks were x = 0.31 and y = 0.34.

ERG Recording and Data Analysis

A fiber electrode, placed over the lower limbus and attached at the inner and outer canthus, served as active electrode. Gold cup surface electrodes filled with electrode paste (Ten20 conductive, Weaver and Company, Aurora, Colorado, USA) were placed at the ipsilateral temple and on the forehead and served as reference and ground electrodes, respectively. To minimize the impedance, the ipsilateral temple and the forehead skin were scrubbed with Nuprep abrasive skin preparing gel (Weaver and Company) before placing the electrodes with medical-grade tape. The impedance was maintained below 5 kΩ during the measurements.

Full-Field and 70° ERG

Monocular electroretinograms were recorded from 65 eyes (of 21 normal subjects and 44 glaucoma patients). The fellow eye was covered by an eye patch. The pupil of the examined eye was dilated with a drop of 0.5% tropicamide (Pharma Stulln, Pointe-Claire, QC, Canada). To minimize eye movements, the subjects were asked to fixate a central red LED in the Ganzfeld stimulator. The experiment was performed in a dark room.

Eighty epochs of ERG measurements, each lasting for one second, were averaged. To avoid onset artifacts, the first two seconds after stimulus onset were not recorded. The recorded potentials were amplified by a factor of 10⁶, band-pass filtered between 1 and 300 Hz cutoff frequencies and digitized at 2048 Hz sampling rate.

ERGs elicited by the incremental and decremental L- and M-cone isolating sawtooth stimuli were summed (i.e., L_{ON} + L_{OFF} = L-add; M_{ON} + M_{OFF} = M-add) to construct spatially homogeneous L- and M-cone driven responses as analogs of pattern ERGs. The nomenclature of respective ERG components is given in Figure 1. The amplitudes of $P_{L\text{-add}}$ (in a time window between 33 and 54 ms) and $P_{M\text{-add}}$ (25–50 ms) were measured from their preceding minimum, whereas $LN_{L\text{-add}}$ (55–162 ms) and $LN_{M\text{-add}}$ (51–140 ms) were measured from the baseline-to-trough (baseline was the average of the amplitudes of the first six data points in the recordings after the sudden change in cone excitation).

Noise was measured for each subject by using 0% cone contrast modulation stimuli under the full-field condition. Amplitudes were taken for further analysis when the signal-to-noise ratio (SNR) was above 2. An SNR of 2.85 was recommended previously [34]. However, that estimation was only based on response amplitudes. We found
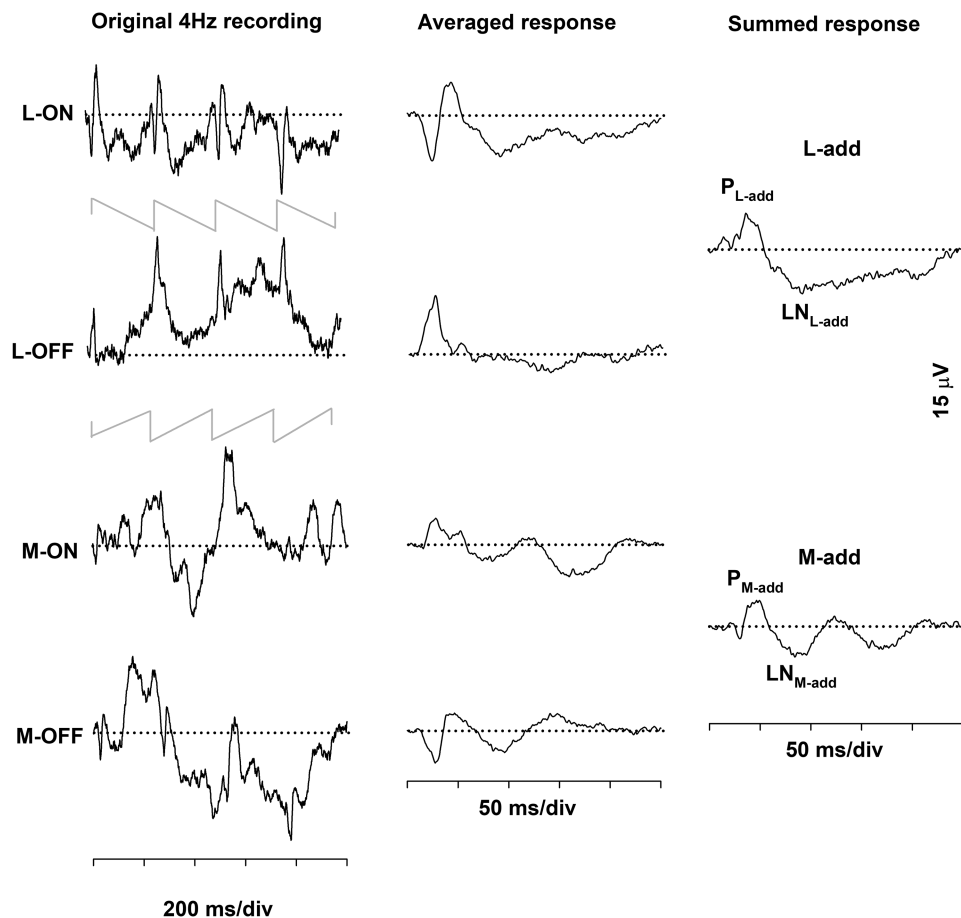
FIGURE 1. The left column shows the ERGs elicited by 70° diameter 4 Hz sawtooth L-cone (upper two signals) and M-cone (lower two signals) isolating rapid ON and rapid OFF stimuli from a representative normal subject. The rapid-ON and rapid-OFF stimulus profiles are depicted below the L-ON and L-OFF original 4 Hz recording respectively in *grey*. The original 4 Hz ERG responses given in the left column were further averaged and a single wave of 250 ms length for L-cone (upper two signals) and M-cone (lower two signals) were constructed as shown in the middle column. Observe the resemblance between L-ON and M-OFF responses and between L-OFF and M-ON responses. The averaged ON- and OFF-ERG responses were added for L-cone (upper signal, $L_{ON} + L_{OFF}$ = L-add) and M-cone (lower signal, M_{ON} + M_{OFF} = M-add) isolating stimuli, respectively. The *dotted lines* in all the traces indicate a baseline defined as an average of amplitudes of the first six data points.

that the latencies of the response components were in the expected range for SNRs down to about 2, indicating that these responses were still reliable. Otherwise, amplitudes and corresponding latencies were discarded.

Pattern ERG

The pattern ERGs were recorded with the same fiber electrode from a subpopulation of 15 normal subjects and 18 glaucoma patients with an undilated pupil before recordings to the cone-isolating stimuli. PERGs were averaged for 80 epochs, amplified 100,000 times, bandpass filtered between 1 Hz and 50 Hz, and sampled at 2048 Hz. Fourier analysis was performed on the recorded PERG responses, and amplitudes and phases at 15 Hz were extracted. The PERG recordings were also performed in the darkroom.

Statistical Analysis

SPSS version 18 (SPSS Inc., Chicago, IL, USA) software was used for the statistical analysis. Amplitudes of the ERG components were compared between the normal and glau-coma patients and between the FF and 70° diameter stimuli. A Mann-Whitney U test was used to compare amplitudes between the subject groups. Within the subject group, the FF versus 70° amplitude difference was compared using Wilcoxon tests. The comparisons were corrected for multiple testing. Spearman's correlation coefficients were used to verify correlations between the ERG components, RNFLT, and PERG components.

RESULTS

The normal subjects showed no ocular abnormalities which could affect vision during the ophthalmological investigation. They also had normal visual fields. The visual acuity (VA) in normal subjects was (mean ± SD) 0.92 ± 0.11 (range: 0.7–1.0). Intraocular pressure (IOP) was (mean ± SD) 17.8 ± 2.8 mm/Hg (range: 12–22 mm/Hg). The myopic refractive error in the normal subjects was (mean ± SD) -0.768 ± 2.59 diopters (range: -6.38 to $+3$ diopters). In the patient's group, the VA 0.86 ± 0.15 (range: 0.3–1.0) and the IOP was 26.68 ± 8.8 mm/Hg (range: 11–58 mm/Hg). The myopic refractive error was (mean ± SD) -1.20 ± 2.65
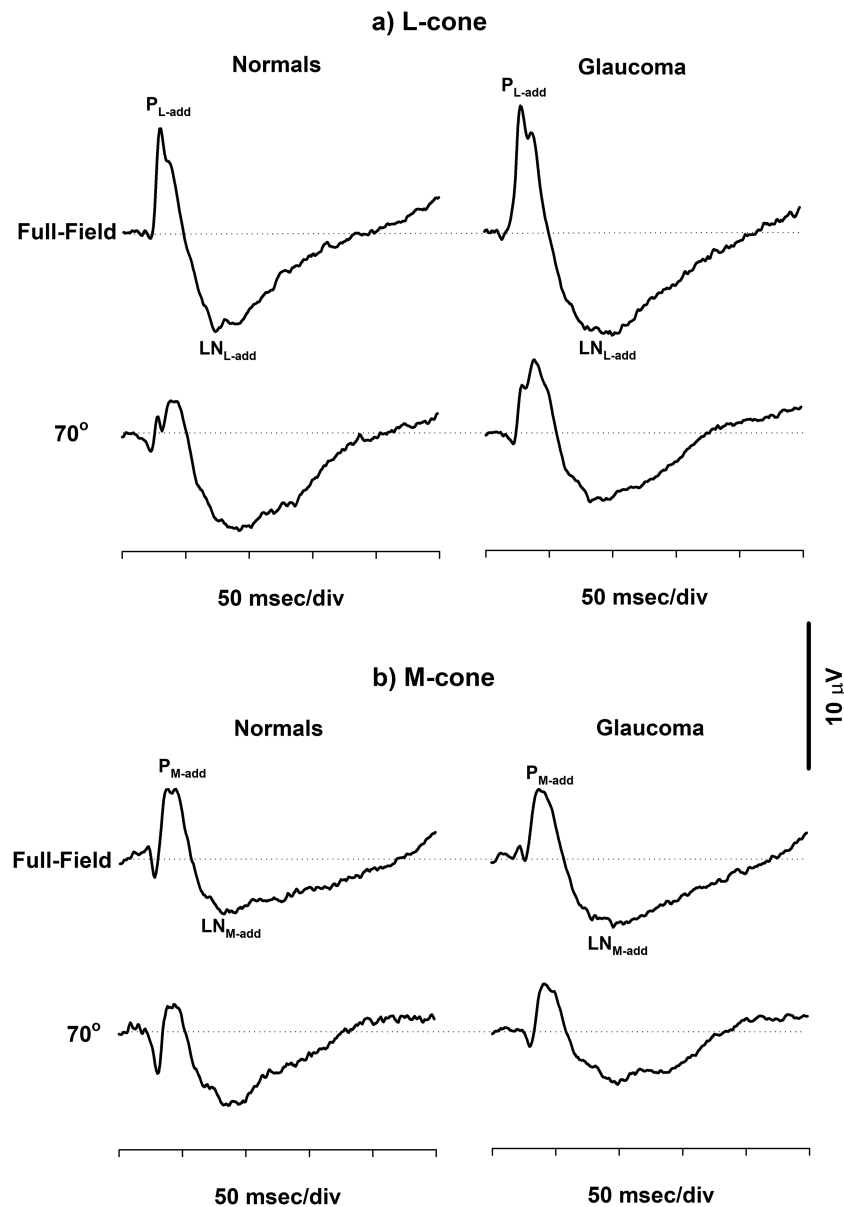
FIGURE 2. Group averaged L-add and M-add waveforms from the additions of ON- and OFF-ERGs elicited by L- and M-cone isolating sawtooth stimuli. Nomenclature of the ERG components and the baselines are also given.

diopters (range: −8.25 to +3.75 diopters). The refractive errors were not significantly different between the subject groups. The visual field mean defects (MDs) in the glaucoma patients [4.3 ± 3.9, (range: −1.8 to +20.6)] were significantly different ($P < 0.001$) from those of the normal subjects [0.92 ± 1.3, (range: −1.9 to +2.8)].

The retinal nerve fiber layer thickness (RNFLT) was measured in all participants using spectral-domain optical coherence tomography (SOCT) (Spectralis OCT, Heidelberg Engineering, Heidelberg, Germany). The RNFLTs in the glaucoma group [mean ± SD: 68.8 ± 14.2, (range: 40.32–96.21 μm)] were significantly different ($P < 0.001$) from those of the normal subjects [94.4 ± 11.8, (range: 70.3–112.7 μm)]

All normal participants and glaucoma patients had normal color vision at the time of the experiment according to the Farnsworth-Munsell D-15 color arrangement test and a Heidelberg Multi-Color anomaloscope (Oculus Optikgeräte GmbH, Wetzlar, Germany).

Figure 1 shows examples of ON- and OFF ERG responses elicited by 70° diameter L- and M-cone isolating 4 Hz ON- and OFF-sawtooth stimuli in a representative normal subject. The first column depicts the original 4 Hz ERG recording for L-ON, L-OFF, M-ON, and M-OFF stimuli, respectively. The original 4 Hz ERG recordings were further averaged and a single wave for L-ON, L-OFF, M-ON, and M-OFF of 250 ms length was constructed, as shown in the middle column. In agreement with previous results, the responses to L- and M-cone stimuli of opposite polarity (i.e., to L-ON and M-OFF and to L-OFF and M-ON) resemble each, whereas the responses to stimuli of the same polarities (i.e., to L-ON and M-ON and to L-OFF and M-OFF) have different morphologies. This was found for all observers (normal subjects and glaucoma patients).

The right column in Figure 1 shows the summed ON- and OFF-ERG response for L- and M-cone isolating stimuli (L-add and M-add, respectively). The summed responses for L- and M-cone are dominated by initial positive waves (P_{L-add}

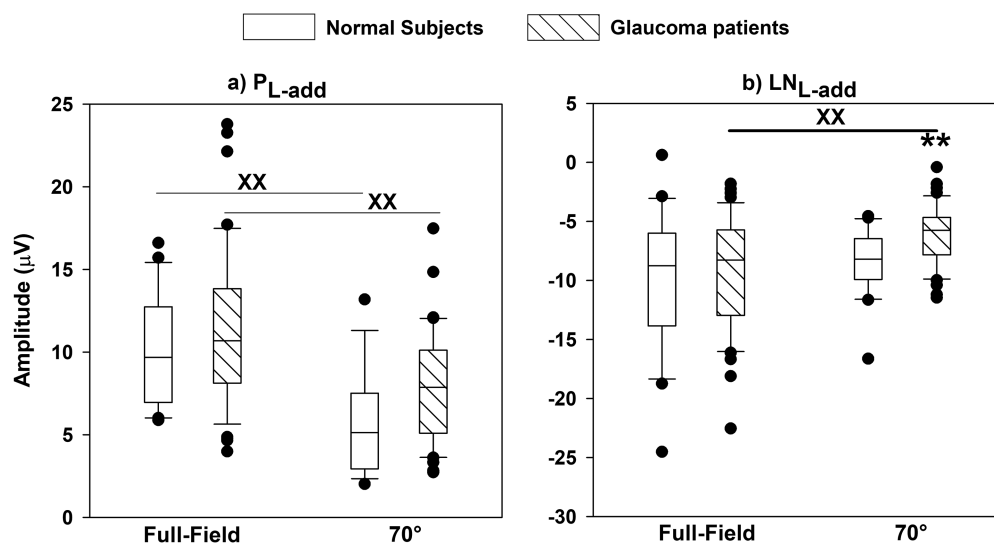**FIGURE 3.** Boxplots of **a)** P_{L-add} amplitudes and **b)** LN_{L-add} amplitudes in normal subjects (*open box*) and glaucoma patients (*hatched box*) recorded with the FF and 70° diameter stimuli. The boxes include 50% of measured values (5% and 95% percentiles) and show the position of the median (*horizontal line* in each box). Error bars indicate 1.5 times the interquartile distance from the upper and lower box edge. Outliers are denoted by *black-filled circles*. The *P* values from the Mann-Whitney test (**: $P < 0.01$) between the data from normal subjects and glaucoma patients are displayed on the top when the difference between the group was significant. "XX" denotes significant differences in Wilcoxon testing ($P < 0.01$) of the corresponding FF versus 70° amplitude comparison within the subject groups.
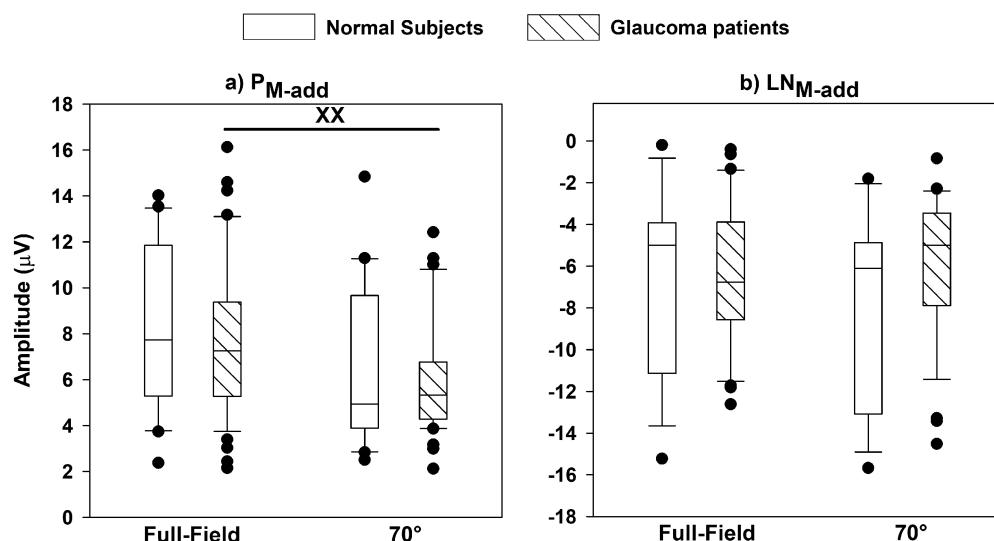

FIGURE 4. Boxplots of **a)** P_{M-add} amplitudes and **b)** LN_{M-add} amplitudes in normal and glaucoma patients recorded with the FF and 70° diameter stimuli.

and P_{M-add}) followed by late negative (LN_{L-add} and LN_{M-add}) components, which are possible correlates of the P_{50} and N_{95} components of the transient PERG, respectively.[11–13,29] We performed a statistical analysis on the components of summed responses to L- and M-cone isolating stimuli of normal subjects and glaucoma patients.

Group averaged L-add (a) and M-add (b) responses in normal subjects (left column) and glaucoma patients (right column) are given in Figure 2 for the FF and 70° diameter stimuli. Overall, the FF responses were larger than those to 70° diameter stimuli. The FF versu 70° amplitude difference was larger in the L-cone driven responses than those driven by the M-cones. Furthermore, the L-add and M-add

responses were similar in morphology. In agreement with previous data,[24] the latencies (data not shown) of P and LN components of the L- and M-cone responses were larger in the 70° diameter stimuli in normal subjects and glaucoma patients. However, the latencies were not significantly different between the subject groups.

Figure 3 shows the P_{L-add} (a) and LN_{L-add} (b) amplitudes in normal subjects and glaucoma patients. In FF condition, both P_{L-add} and LN_{L-add} amplitudes were not significantly different between the normal subjects ($P_{L-add} = 9.9 \pm 3.3$ µV, $LN_{L-add} = -9.9 \pm 5.9$ µV) and glaucoma patients ($P_{L-add} = 11.3 \pm 4.6$ µV, $LN_{L-add} = -9.2 \pm 4.7$ µV). For 70° diameter stimuli, the P_{L-add} was not significantly different. On the other

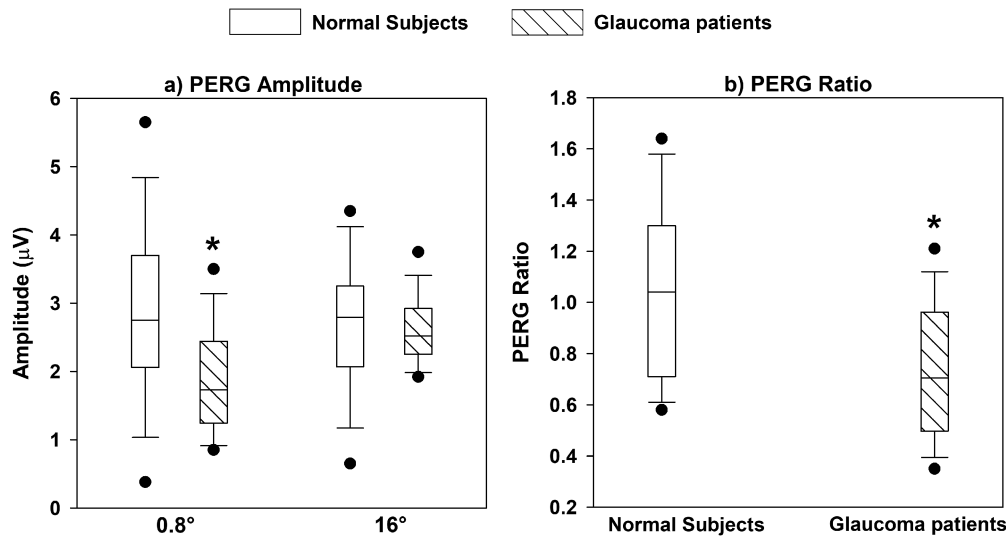**FIGURE 5.** **a)** PERG amplitudes obtained with 0.8° checks and 16° checks from 15 normal subjects and 18 glaucoma patients are given. **b)** The PERG amplitude ratio (0.8° amplitude/16° amplitude) is given for normal subjects and glaucoma patients. Outliers are indicated with *black-filled circles*. Significance between the groups is denoted by an asterisk (* if $P < 0.05$).
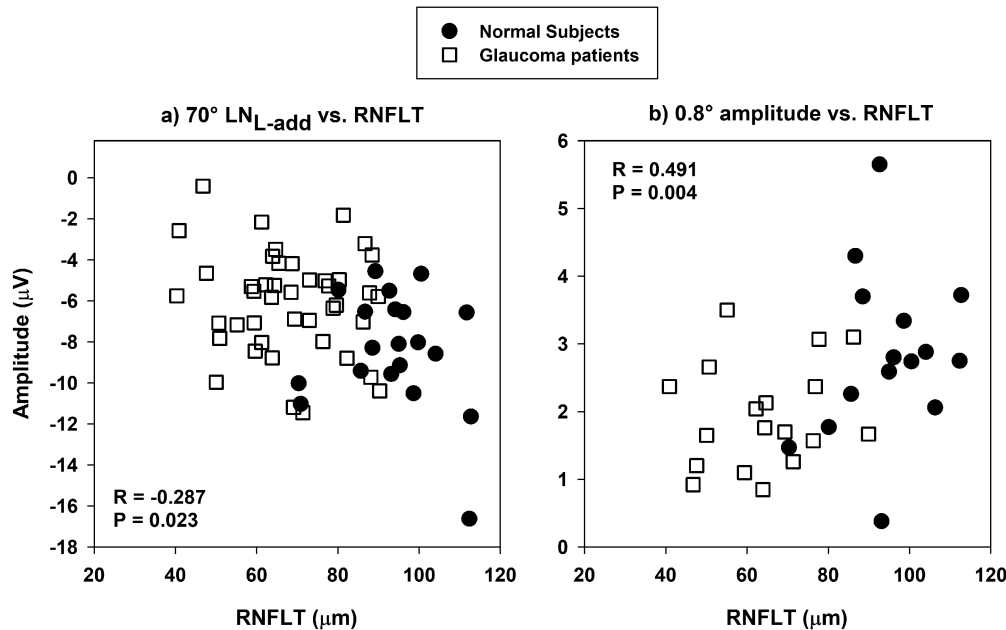

FIGURE 6. Individual LN_{L-add} amplitudes obtained with 70° diameter stimuli from normal subjects ($N = 21$) and glaucoma ($N = 44$) patients **(a)** and PERG amplitudes of responses to 0.8° checks obtained with a subpopulation of normal subjects ($N = 15$) and glaucoma ($N = 18$) patients **(b)** plotted as a function of retinal nerve fiber layer (RNFL) thickness. The Spearman-rho correlation coefficients (R) and the respective significance (P) levels are given.

hand, the LN_{L-add} amplitude in the glaucoma patients (-6.0 ± 2.4 μV) was significantly ($P < 0.01$) reduced relative to those in normal subjects (-8.36 ± 2.8), denoted as "**" (Note that the amplitudes are given as negative values, indicating that the LN troughs were below the baseline). Within the subject groups, there was a significant amplitude difference between the P_{L-add} amplitudes from FF and 70° diameter stimuli, denoted as "XX" ($P < 0.01$) in Figure 3. This finding is not surprising and in agreement with previously reported

data with normal subjects where the amplitude of an early positive peak of the summed responses for L-and M-cones showed a significant decrease with a decrease in stimulus size down to 10° diameter stimuli.[24] The LN_{L-add} amplitude in normal subjects was not significantly influenced by stimulus size, whereas it was significantly reduced ($P < 0.01$) in the glaucoma patients with the 70° stimuli.

Figure 4 shows the P_{M-add} **(a)** and LN_{M-add} **(b)** amplitudes obtained with FF and 70° diameter stimuli in the normal

subjects and the glaucoma patients. Interestingly, neither $P_{M\text{-add}}$ nor $LN_{M\text{-add}}$ amplitude were significantly different between the subject groups. In the glaucoma patients, the $P_{M\text{-add}}$ amplitude was significantly reduced ($P < 0.01$) with 70° diameter stimuli when compared to the FF response. The $LN_{M\text{-add}}$ amplitudes in normal subjects and glaucoma patients were nearly similar for both 70° diameter stimuli and FF.

The PERG data recorded from subpopulations of the normal subjects (15 subjects out of 21) and the glaucoma patients (18 patients out of 44) are shown in Figure 5. The PERG was recorded with two check sizes, 0.8° and 16° checkerboard. The amplitudes obtained after Fourier analysis for 0.8° checks and 16° checks are given in Figure 5a. In agreement with previous data ,[10,11] PERG responses to 0.8° checks were larger than those to 16° checks in normal subjects (open box) and resulted in 0.8°/16° PERG ratios larger than one. In glaucoma patients (hatched box), amplitudes of responses to 0.8° checks were reduced significantly (* = $P < 0.05$) as compared to normal subjects, whereas the amplitudes with 16° checks were similar to those measured in normal subjects. As a result, the 0.8°/16° PERG ratio was significantly (* = $P < 0.05$) reduced in the glaucoma patients.

The relations between the amplitudes of $LN_{L\text{-add}}$ component for 70° diameter stimuli and the 0.8° PERG amplitudes with the RNFLT are shown in Figures 6a and 6b, respectively. The 70° $LN_{L\text{-add}}$ amplitudes showed a significant ($R = -0.287$, $P = 0.023$) correlation with the RNFLT. Furthermore, the PERG amplitude with 0.8° checks were significantly ($R = 0.491$, $P = 0.004$) correlated with the RNFLT. As expected, the PERG ratio was also significantly ($R = 0.467$, $P = 0.006$) correlated with the RNFLT (data are not shown). There was no significant correlation between the amplitudes of the 70° $LN_{L\text{-add}}$ component neither with the 0.8° PERG amplitudes nor with the PERG ratios. Deming regression was also performed on the data presented in Figure 6. However, correlation coefficients obtained with Deming regression were identical to those obtained with Spearman's rho correlation.

The amplitudes of the $LN_{L\text{-add}}$ component for 70° diameter stimuli and the 0.8° PERG amplitudes were also correlated with mean defect values from the 30° SAP fields in normal subjects and glaucoma patients. However, there were no significant correlation between these parameters (data not shown).

DISCUSSION

Here, we studied if the reversal ERG responses differed between normal subjects and glaucoma patients. These were obtained by adding individual ON- and OFF- sawtooth responses, which were elicited by L- and M-cone isolating stimuli of two sizes (FF and 70° diameter). The symmetry between L-ON and M-OFF responses on the one hand and L-OFF and M-ON responses on the other hand indicated that responses are dominated by the parvocellular system. This notion is supported by the fact that differences between ERGs depending on field size were small. However, these parvocellular-mediated responses seem to correlate poorly with PERG and RNFLT. Only the $LN_{L\text{-add}}$ response was correlated with the retinal nerve fiber layer thickness and PERG data, probably because of an additional luminance component.

In agreement with previous data,[13,24] the cone isolating ON- and OFF ERGs of opposite polarity resembled each other for normal subjects (Fig. 1, middle column) and glaucoma patients (waveform not shown), indicating that the ERGs are to a large extent driven by a L-/M-cone-opponent mechanism. The cone opponency is particularly pronounced with 70° stimuli, suggesting that cone opponent signals are mainly present in the central retina. In contrast, luminance reflecting ERGs are particularly large with full field stimuli and decrease significantly with decreasing stimulus size, as previously demonstrated for cone isolating [35] and heterochromatic red-green sinusoidal stimuli.[36] Furthermore, luminance signals are L-cone dominated, explaining why the differences between the responses at FF and 70° are particularly significant for L-cone driven responses. Luminance signals are relatively small in M-cone driven responses.[19,35]

A significant difference between normal subjects and glaucoma patients was only found for the $LN_{L\text{-add}}$ component to 70° stimuli. This suggests that luminance driven responses are mainly affected by glaucoma because the luminance response is small in the M-cone driven responses. These findings are in agreement with previous suggestions that the magnocellular pathway (which is the luminance sensitive retinal pathway) is particularly affected by glaucoma.[37,38] Furthermore, previous data have shown that the PhNR-like LN components probably reflect retinal ganglion cell activity and is therefore probably particularly susceptible to glaucomatous changes.[12,29] A further finding was that the $LN_{L\text{-add}}$ component was smaller to the 70° stimuli than to FF in glaucoma patients but not in normal subjects. This indicates that the major glaucomatous changes are present in the central retina.

The reduction in amplitudes of the $LN_{L\text{-add}}$ component in glaucoma patients reported in the present study (Fig. 3) is consistent with previous data from Pangeni et al. [13] where the responses to ON- and OFF-sawtooth achromatic stimuli were employed to generate summed responses in normal human subjects and glaucoma patients. The data showed that the LN component of the summed response was significantly reduced in glaucoma patients and manifested a strong correlation with RNFLT.

The addition of individual ON- and OFF responses to L- and M-cone isolating (L-add and M-add) stimuli can be regarded as a reversal response (i.e., the response to a sudden change in the stimulus independent of the direction of the change) and therefore resembles the PERG response. This is in line with the current idea about the origins of the PERG, where ON- and OFF-responses to pattern reversals are considered to be asymmetric (and not mirror images of each other) and, therefore, do not cancel each other [10]. Attempts to simulate the transient PERG response by using ON- and OFF uniform fields [12,29] and 4 Hz sawtooth stimuli [13] have been reported before. Similar to the transient PERG with distinct positive (P_{50}) and negative (N_{95}) deflections, L-add and M-add responses showed positive ($P_{L\text{-add}}$ and $P_{M\text{-add}}$) and negative ($LN_{L\text{-add}}$ and $LN_{M\text{-add}}$) components as shown in Figure 2.

The steady-state PERG obtained from a subpopulation of normal subjects and glaucoma patients showed significantly reduced 0.8° PERG amplitudes and 0.8°/16° PERG ratios in glaucoma patients compared to normal subjects (Fig. 5). The PERG recording was performed as a parallel control experiment and the data agreed with previous results.[11] The black and white checkerboard stimulation to measure PERG responses has been used in clinical settings for many years to assess the retinal ganglion cell function in the case of

glaucoma.[11,39] The stimuli used in the PERG recording were achromatic and subtended an approximate angle of 32°. This supports our proposal that glaucomatous damage of the retina mainly affects the centrally located luminance mechanism.

Consistent with previous studies,[13,40] the RNFLT in glaucoma patients was significantly reduced and showed a significant correlation with reduced LN_{L-add} with 70° diameter stimuli, and 0.8° PERG amplitude in glaucoma patients as shown in Figure 6. The 0.8°/16° PERG ratio also showed a significant correlation with RNFLT (data not shown). The glaucoma patient group in the present study consisted of different stages of glaucoma (with or without visual field defect) but there was no correlation found with RNFLT within the glaucoma patient subgroup. The correlation of RNFLT with 70° LN_{L-add} further supports the notion that the LN component is sensitive to dysfunction of retinal ganglion cells which are centrally located. For the clinician, these findings implicate that luminance signals correlate much better with RNFLT as well as luminance-driven functional tests like standard automated perimetry (SAP) and PERG. While red-green color discrimination has been found to be impaired in patients with early, moderate, or severe glaucoma, the relevance of this remains unclear in light of our results and those of other groups. Therefore, luminance-driven ERGs might be useful for assessing glaucomatous damage in patients where RNFLT or SAP cannot be measured or interpreted, for example, in high myopia or in patients who cannot fixate well.

The present study has limitations. First, the ERGs elicited by silent substitution stimuli are generally smaller than those obtained with standard flashes because the stimuli are generally weaker (at 100% Michelson contrast the maximal output is only twice the background whereas flashes can be orders of magnitude brighter than the background). In addition, only a subpopulation of photoreceptors is stimulated. However, the signal-to-noise ratio is not very different in similar time intervals because interstimulus time intervals, as in flash ERGs, are not necessary. Furthermore, more data are necessary for a better comparison with, for instance, visual field data. Finally, RNFLT is known to correlate with axial length and refractive error. Although, the correlation was not significant in our data set, an effect should be considered.

CONCLUSIONS

The responses to cone isolating sawtooth stimuli elicited ERGs that strongly reflect (parvocellularly based) cone opponency. The cone opponent ERG responses do not correlate with the PERG nor with the RNFLT, and they are not significantly different between glaucoma patients and normal subjects, except for the LN_{L-add} responses at 70°. It is probable that these responses contain more luminance signals. FF responses contain contributions from the peripheral retina and therefore is less strongly affected by glaucoma.

References

1. Silverman SE, Trick GL, Hart WM, Jr. Motion perception is abnormal in primary open-angle glaucoma and ocular hypertension. *Invest Ophthalmol Vis Sci.* 1990;31(4):722–729.

2. Livingstone M, Hubel D. Segregation of form, color, movement, and depth: anatomy, physiology, and perception. *Science.* 1988;240(4853):740–749.

3. Livingstone MS, Hubel DH. Psychophysical evidence for separate channels for the perception of form, color, movement, and depth. *J Neurosci.* 1987;7(11):3416–3468.

4. Maunsell JH, Newsome WT. Visual processing in monkey extrastriate cortex. *Annu Rev Neurosci.* 1987;10:363–401.

5. Anderson RS, O'Brien C. Psychophysical evidence for a selective loss of M ganglion cells in glaucoma. *Vision Res.* 1997;37(8):1079–1083.

6. Kelly DH. Frequency doubling in visual responses. *J Opt Soc Am A.* 1966;56(11):1628–1633.

7. Maddess T, Goldberg I, Dobinson J, Wine S, Welsh AH, James AC. Testing for glaucoma with the spatial frequency doubling illusion. *Vision Res.* 1999;39(25):4258–4273.

8. White AJ, Sun H, Swanson WH, Lee BB. An examination of physiological mechanisms underlying the frequency-doubling illusion. *Invest Ophthalmol Vis Sci.* 2002;43(11):3590–3599.

9. Bach M, Poloschek CM. Electrophysiology and glaucoma: current status and future challenges. *Cell Tissue Res.* 2013;353(2):287–296.

10. Bach M, Hoffmann MB., Heckenlively J, Arden G (eds.), *Principles and Practice of Clinical Electrophysiology of Vision*. Cambridge, London: MIT Press; 2006:185–196.

11. Bach M, Hoffmann MB. Update on the pattern electroretinogram in glaucoma. *Optom Vis Sci.* 2008;85(6):386–395.

12. Lingley AJ, Kantungane AL, Coupland SG. Comparison of the uniform-field electroretinogram and the pattern electroretinogram to checkerboard and bar gratings. *Doc Ophthalmol.* 2019;140:13–21.

13. Pangeni G, Lammer R, Tornow RP, Horn FK, Kremers J. On- and off-response ERGs elicited by sawtooth stimuli in normal subjects and glaucoma patients. *Doc Ophthalmol.* 2012;124(3):237–248.

14. Kremers J. The assessment of L- and M-cone specific electroretinographical signals in the normal and abnormal human retina. *Prog Retinal Eye Res.* 2003;22(5):579–605.

15. Estevez O, Spekreijse H. The "silent substitution" method in visual research. *Vis Res.* 1982;22(6):681–691.

16. Estevez O, Spekreijse H. A spectral compensation method for determining the flicker characteristics of the human colour mechanisms. *Vis Res.* 1974;14(9):823–830.

17. Donner KO, Rushton WA. Retinal stimulation by light substitution. *J Physiol.* 1959;149:288–302.

18. Shapiro AG, Pokorny J, Smith VC. Cone-rod receptor spaces with illustrations that use CRT phosphor and light-emitting-diode spectra. *J Opt Soc Am A* 1996;13(12):2319–2328.

19. Aher AJ, McKeefry DJ, Parry NRA, et al. Rod- versus cone-driven ERGs at different stimulus sizes in normal subjects and retinitis pigmentosa patients. *Doc Ophthalmol.* 2018;136(1):27–43.

20. Scholl HP, Kremers J, Apfelstedt-Sylla E, Zrenner E. L- and M-cone driven ERGs are differently altered in Best's macular dystrophy. *Vision Res.* 2000;40(22):3159–3168.

21. Scholl HPN, Kremers J. Large phase differences between L-cone and M-cone driven electroretinograms in retinitis pigmentosa. *Invest Ophthalmol Vis Sci.* 2000;41:3225–3233.

22. Scholl HP, Kremers J. Alterations of L- and M-cone driven ERGs in cone and cone-rod dystrophies. *Vision Res.* 2003;43(22):2333–2344.

23. Scholl HP, Kremers J. L- and M-cone driven large-field and multifocal electroretinograms in sector retinitis pigmentosa. *Doc Ophthalmol.* 2003;106(2):171–181.

24. Tsai TI, Jacob MM, McKeefry D, Murray IJ, Parry NR, Kremers J. Spatial properties of L- and M-cone driven incremental (On-) and decremental (Off-) electroretinograms: evidence for the involvement of multiple post-receptoral mechanisms. *J Opt Soc Am A.* 2016;33(3):A1–11.

25. Bush RA, Sieving PA. Inner retinal contributions to the primate photopic fast flicker electroretinogram. *J Opt Soc Am A Opt Image Sci Vis.* 1996;13(3):557–565.

26. Sieving PA, Murayama K, Naarendorp F. Push-pull model of the primate photopic electroretinogram: a role for hyper-polarizing neurons in shaping the b-wave. *Vis Neurosci.* 1994;11(3):519–532.

27. Frishman LJ. Origins of the electroretinogram. In: Hecken-lively JR, Arden GB (eds.), *Principles and Practices of Clinical Electrophysiology of Vision.* Cambridge: MIT Press; 2006:139–183.

28. Luo X, Frishman LJ. Retinal pathway origins of the pattern electroretinogram (PERG). *Invest Ophthalmol Vis Sci.* 2011;52(12):8571–8584.

29. Viswanathan S, Frishman LJ, Robson JG. The uniform field and pattern ERG in macaques with experimental glaucoma: removal of spiking activity. *Invest Ophthalmol Vis Sci.* 2000;41(9):2797–2810.

30. Jonas JB, Budde WM, Panda-Jonas S. Ophthalmoscopic evaluation of the optic nerve head. *Surv Ophthalmol.* 1999;43(4):293–320.

31. Jonas JB, Gusek GC, Naumann GO. Optic disc morphometry in chronic primary open-angle glaucoma. I. Morphometric intrapapillary characteristics. *Graefes Arch Clin Exp Ophthalmol.* 1988;226(6):522–530.

32. Stockman A, MacLeod DIA, NE Johnson. Spectral sensitivities of the human cones. *J Opt Soc Am A.* 1993;10(12):2491–2521.

33. Huchzermeyer C, Martins CMG, Nagy B, et al. Photoreceptor-specific light adaptation of critical flicker frequency in trichromat and dichromat observers. *J Opt Soc Am A Opt Image Sci Vis.* 2018;35(4):B106–B113.

34. Meigen T, Bach M. On the statistical significance of electrophysiological steady-state responses. *Doc Ophthalmol.* 1999;98(3):207–232.

35. Jacob MM, Pangeni G, Gomes BD, et al. The spatial properties of L- and m-cone inputs to electroretinograms that reflect different types of post-receptoral processing. *PLoS One.* 2015;10(3):e0121218.

36. Martins CM, Tsai T, Barboni MT, et al. The influence of stimulus size on heterochromatic modulation electroretinograms. *J Vis.* 2016;16(8):13.

37. Quigley HA, Dunkelberger GR, Green WR. Chronic human glaucoma causing selectively greater loss of large optic nerve fibers. *Ophthalmology.* 1988;95(3):357–363.

38. Quigley HA, Sanchez RM, Dunkelberger GR, L'Hernault NL, Baginski TA. Chronic glaucoma selectively damages large optic nerve fibers. *Invest Ophthalmol Vis Sci.* 1987;28(6):913–920.

39. Pfeiffer N, Bach M. The pattern-electroretinogram in glaucoma and ocular hypertension. A cross-sectional and longitudinal study. *Ger J Ophthalmol.* 1992;1(1):35–40.

40. North RV, Jones AL, Drasdo N, Wild JM, Morgan JE. Electrophysiological evidence of early functional damage in glaucoma and ocular hypertension. *Invest Ophthalmol Vis Sci.* 2010;51(2):1216–1222.