Flicker-Evoked Changes in Human Optic Nerve Blood Flow: Relationship with Retinal Neural Activity

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PURPOSE. Visual flicker induces a response in human optic nerve blood flow (F_{onh}) and inner retinal activity, as assessed by laser Doppler flowmetry and electroretinogram (ERG), respectively. In this study the relationship was examined between the flicker-evoked changes in F_{onh} (RF_{onh}) and ERG when various parameters of the stimulus were varied.

METHODS. In five normal observers (mean age, 41; range, 25–62 years) F_{onh} and ERG were recorded simultaneously in response to pure red (R) or pure green (G) flicker, as well as heterochromatic R-G flicker (30° field at the posterior pole). RF_{onh} and the changes in the first (1F) and second (2F) harmonic amplitudes of the ERG were documented as a function of the frequency of pure luminance and equiluminant R-G flicker, the mean illuminance of 10-Hz luminance flicker, and the color ratio $r = R/(R + G)$ of a 15-Hz heterochromatic R-G flicker.

RESULTS. Frequency-dependent changes in RF_{onh} were similar to those in both 1F and 2F amplitudes for equiluminant R-G flicker. RF_{onh} and 2F amplitude increased and then saturated with increasing mean illuminance of luminance flicker. They both decreased similarly as the R-G flicker approached the **r** value corresponding to equiluminance. RF_{onh} was positively correlated with both 1F and 2F amplitudes ($r = 0.55$ and 0.31, respectively, $P < 0.05$) when these quantities were recorded as a function of frequency of R-G equiluminant flicker. RF_{onh} was positively correlated with 2F amplitudes when both quantities were recorded as a function of mean illuminance of luminance flicker and **r** of heterochromatic R-G flicker ($r = 0.52$ and 0.48, respectively, $P \leq 0.01$).

CONCLUSIONS. Under specific experimental conditions, changes in human RF_{onh} are similar to and correlated with those of the flicker ERG 1F and 2F amplitudes. These findings support a relationship between vaso- and neural activity changes in the neural tissue of the human eye. (*Invest Ophthalmol Vis Sci.* 2002;43:2309–2316)

Recent experimental data obtained by laser Doppler flowmetry (LDF) in both animals and humans demonstrate that optic nerve head blood flow (F_{onh}) responds to modulation of retinal neural activity.[1–5] When this activity is modulated by luminance or chromatic flicker stimulation,[5] the variation of the F_{onh} response (RF_{onh}), plotted as a function of various parameters of the stimulation (such as temporal frequency, modulation depth and relative chromaticity), is comparable to that of the retinal ganglion cell activity evoked by the same stimulus.[6] For example, for luminance flicker, RF_{onh} is maximal at intermediate temporal frequencies (**Tf** = 10–20 Hz) and decreases at both low and high **Tf**, whereas, for equiluminant chromatic flicker RF_{onh} decreases at **Tf** > 8 Hz.[5,7]

Investigating the relationship between RF_{onh} and retinal neural activity is relevant to the understanding of retinal neurovascular coupling, the existence of which has been suggested by the studies just mentioned.[1–5,7] In humans, presumably the most direct noninvasive approach would be to compare the changes in RF_{onh} and those in the pattern-evoked electroretinogram (ERG), because the latter is correlated with ganglion cell activity.[8,9] However, RF_{onh} responses to pattern stimuli have yet to be reported.

Although the ERG to flickering of uniform fields originates from a less localized retinal region than the pattern ERG, its use in the investigation of the neurovascular coupling is nevertheless legitimate, because it contains, in both monkeys[10–13] and humans,[14,15] a component that strongly reflects neural activity in the inner retina. More specifically, whereas the first harmonic component of the response (i.e., the component at the stimulus frequency, 1F) is dominated by on- and off-bipolar cell activity (with a contribution, at low frequencies, from photoreceptors),[13] the second harmonic (i.e., the component at twice the stimulation frequency, 2F) is generated mainly from the inner retina, perhaps with a contribution of ganglion cells themselves.[12,15] The presence of a correlation of RF_{onh} with ERG components' amplitude, when the flicker stimulus characteristics are varied, would provide evidence in support to the hypothesis that, in humans, visually evoked F_{onh} changes are coupled with retinal neural activity. A nonselective correlation of RF_{onh} with both 1F and 2F amplitudes would suggest a neuro-vascular coupling involving both outer (1F) and inner (2F) retinal activity, whereas a selective correlation with the 2F component would support a specific role of inner retinal activity in the neurovascular coupling. In this study, F_{onh} and the ERG were therefore simultaneously recorded in response to various flicker stimuli with the purpose of determining whether a coupling exists between RF_{onh} and the 1F and/or 2F amplitude components, how specific this coupling is with either ERG component, and whether the strength of this coupling depends on the type of stimulus.

Physiological flicker responses can be elicited by both luminance and chromatic modulation at various frequencies.[6] A detailed analysis of these responses may therefore be accomplished by varying flicker luminance, frequency, and relative chromaticity. With the use of this approach, the study design included different experimental protocols in which stimuli of pure luminance modulation, presented at different mean illuminances and frequencies, pure chromatic modulation (equiluminant flicker) presented at different frequencies, and mixed luminance–chromatic modulation, presented at different relative chromaticities, were used. These types of stimuli provided a broad range of experimental conditions under which the association between vascular and neural responses was evaluated.

MATERIALS AND METHODS

Subjects

Five normal volunteers (four men, one woman; mean age, 41; range, 25–62 years), participated in the study. They had excellent target fixation and had taken part in several previous LDF studies. In all subjects, general and ophthalmic examinations produced normal findings, including color vision, as evaluated by Ishihara plates and Farnsworth 100 Hue test. The corrected visual acuity was 20/20, and refractive errors were below 1 spherical diopter in all subjects. Only one eye for each subject was tested with simultaneous F_{onh} and ERG recordings. Signed informed consent was obtained from all subjects. The research adhered to the tenets of the Declaration of Helsinki.

Stimulation

Stimuli were generated by two independently controlled ultrabright LEDs (dominant wavelengths at 524 and 630 nm). The light was delivered in Maxwellian view to generate at the fundus a diffuse, circular uniform field of 30° diameter. The currents of the LEDs were square-wave modulated, 180° out of phase, between zero and a maximum illuminance of 2.6 lux for red (R) and 13.5 lux for green (G). **Tf** of the modulation ranged from 2.3 to 64 Hz. The color ratio defined as **r** = R/(R + G) illuminances was varied from 0 to 1. The color ratio **r** = 0 corresponded to a G-black and **r** = 1 to an R-black illuminance modulation. Intermediate values of **r** defined an R-G chromatic modulation. International Commission on Illumination (CIE) coordinates were ($x = 0.7; y = 0.3$) for the R and ($x = 0.17; y = 0.7$) for the G LED. Under the present experimental conditions, the medium (M)- and long (L)-wavelength–sensitive cones were mainly stimulated by the LEDs. The excitation of each cone type was estimated by multiplying each LED emission spectrum with the psychophysically based cone fundamentals[16] and integrating over the wavelength range. These M- and L-cones did not modulate at **r** = 0.34 and **r** = 0.72, respectively, and their responses were equal and opposite at **r** = 0.46.

Blood Flow Recordings

F_{onh} was measured using a near-infrared laser Doppler system.[2,17] Briefly, a probing laser beam (wavelength, 810 nm; power at the cornea, 90 μW; diameter at the fundus, approximately 150 μm) was directed at a temporal site on the neuroretinal rim of the optic disc. The laser light scattered by the tissue and red blood cells was collected by an optical fiber at the image plane of the fundus camera and guided to a photodetector. The aperture of the light-collecting optical fiber (diameter at the fundus approximately 180 μm) was centered on the site illuminated by the probing laser beam. An infrared video camera placed in the retinal image plane of the fundus camera allowed the operator to monitor the location of the beam and of the light-collecting aperture and, when necessary, reposition both at the desired site. The camera was positioned in front of the eye so that an edge of the pupil of the tested eye could also be observed on the video monitor. In this manner, the observer could monitor the position of the camera relative to the pupil of the subject's eye and perform the necessary corrections to keep it steady. Guided by color photographs of the disc and video monitoring of the fundus image, we took care to aim the laser beam at the same site of the disc in all experiments in each subject, avoiding the visible vessels; to obtain a similar DC value as the first one of the series; and to maintain the same image of the pupil on the video monitor. The DC component of this signal, which is proportional to the intensity of the light reaching the detector was monitored continuously in real time throughout the experiment. The output signal of the detector was analyzed using a software implemented on a computer (NeXT Computer, Inc., Redwood City, CA).[18]

ERG Recordings

The flicker ERG was recorded as previously published.[19] Briefly, signals were monocularly derived, with an interocular reference, by means of Ag-AgCl superficial cup electrodes taped over the skin of the lower eyelids, amplified (100,000-fold), band-pass filtered between 1 and 250 Hz (6 dB/octave) and averaged (12-bit resolution, 2-kHz sampling rate, 600 repetitions). The averaging time (i.e., the sweep duration) was varied according to the stimulus period. Single sweeps exceeding a threshold voltage (25 μV) were rejected, to minimize noise coming from blinks or eye movements. A discrete Fourier analysis was performed off-line to isolate the ERG 1F and 2F harmonic components, whose peak-to-peak amplitudes (in microvolts) and phases (in degrees) were estimated. Averaging and Fourier analysis were also performed on signals sampled asynchronously at 1.1 times the temporal frequency of the stimulus, to give an estimate of the background noise.[20] Noise amplitudes ranged from 0.05 to 0.08 μV. In all experimental conditions, both 1F and 2F components were above the noise level (signal-to-noise ratio, >2.8) and sufficiently reliable (i.e., the SE of amplitude was typically <30% of the average amplitude, and the phase SE was within ±20°).

Experimental Protocols

A baseline F_{onh} recording was obtained for approximately 40 seconds before exposing the eye to a defined flicker stimulus. During this recording period, the eye was exposed to an unmodulated uniform field whose luminance was matched to the mean luminance of the flickering stimulus. While the recording of F_{onh} was pursued, the flicker stimulus was presented and the ERG signal was acquired for 60 seconds. The recording of F_{onh} was continued for 40 to 60 seconds after turning off the stimulation. A rest period of approximately 1 minute during which the subject could move his or her head off the head rest was inserted between each F_{onh} recording.

Four flicker stimulation protocols were followed: R flicker with luminance modulation at different **Tf** (2.3–64 Hz); G flicker with 10-Hz luminance modulation at different mean illuminances (1–13.5 lux); R-G flicker at 15 Hz with mixed luminance-chromatic modulation at different **r** (0 – 1); and equiluminant R-G flicker at different **Tf** (2.3–64 Hz). Equiluminance was determined psychophysically from the minimum flicker perception at 15 Hz. For every subject, experiments were performed over several days. In a typical recording session with one subject, one or two experimental protocols were completed (for a total duration of 90–120 minutes). In each protocol, the order of presentation for the different stimulus conditions (e.g., mean illuminances of 10-Hz G-flicker) was randomized.

Potential fatigue effects on the magnitude of RF_{onh} during sequential recordings were assessed by analyzing series of successive 2-minute recordings, as well as three continuous, extended recordings of F_{onh} from three temporal sites of the optic disc in the same subject in response to a G luminance stimulus of 13.5 lux.

Data Analysis

For each experimental condition, RF_{onh} was defined as the percentage change in F_{onh} averaged over the last 20 seconds of exposure to a specific flicker stimulus condition relative to the F_{onh} averaged over the last 20 seconds of exposing the eye to an unmodulated uniform field of the same mean luminance as the flicker stimulus. The results of previous experiments[7] and control recordings, evaluating the time course of flicker-evoked RF_{onh} (Riva CE, manuscript in preparation), indicated that in the different subjects, most of the increase in F_{onh} occurs during the first 30 seconds Therefore, the time segment corresponding to the last 20 seconds of the 60 seconds flicker exposure was confidently selected as that containing information relative to the full vascular response to flicker.

Before determining the average F_{onh} during baseline and during flicker, spikes due to blinks and microsaccadic eye motion were removed from the recordings, as described elsewhere.[21] In most experiments in all subjects, the changes in the DC component during an experiment (baseline and flicker) were less than 10%.

Reproducibility of baseline average F_{onh} was evaluated for every subject and recording session by using two different approaches: by
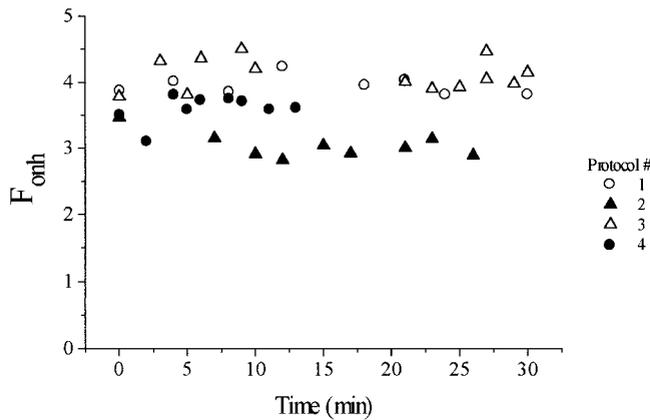
FIGURE 1. Baseline F_{onh} obtained in one control subject, during the different recording sessions, plotted as a function of experimental time.

determining with linear regression analysis whether baseline F_{onh} changed over time and by calculating the variation coefficient of different baseline F_{onh} levels.

One simultaneous RF_{onh} and ERG recording was obtained in each subject under a given condition of flicker stimulus. However, reproducibility of RF_{onh} under the different conditions was estimated by determining the coefficient of variation of the response, based on at least three measurements, for the reference conditions of 15-Hz R luminance flicker at 13.5 and 5.6 lux mean illuminance, as well as of 3.2-Hz equiluminant chromatic flicker.

ERG 1F and 2F amplitudes were evaluated for each condition. Although phase data were also collected to check intrasession response consistency, they were not used in the correlation analysis with RF_{onh} results, given the uncertainty of the phase measurements, which are bound to $\pm180°$ changes when interpreting responses to a series of stimulus conditions (e.g., different flicker frequencies). Reproducibility of the ERG measurements was estimated similarly to that for the RF_{onh} estimates. The RF_{onh} and ERG data were evaluated by two observers, each masked to the results of the other, and plotted as a function of the various stimulus conditions. To compare the response functions of RF_{onh} directly with those of ERG component amplitudes, obtained with the four stimulation protocols, the individual responses were normalized by dividing them by the responses obtained under an arbitrarily chosen condition in a given protocol (e.g., the responses obtained at 10 Hz in protocol 1). This procedure also reduced the confounding influence of intersubject variability on absolute measurements of both RF_{onh} and ERG amplitudes. RF_{onh} and ERG data are presented in all figures as the mean $\pm$ SE, unless stated otherwise, based on the measurements from five subjects. Correlation analyses between RF_{onh} and ERG amplitudes were assessed by Pearson's correlation on the normalized values, averaged across subjects. In the analyses, two-tailed $P < 0.05$ was considered significant.

RESULTS

Continuous F_{onh} recordings in normal humans in response to luminance and chromatic flicker stimuli have been shown.[5,7] Figure 1 shows the baseline F_{onh} levels obtained in one control subject, during the different recording sessions, plotted as a function of experimental time. No significant trend of F_{onh} change throughout the experiments was apparent. The coefficient of variation of baseline F_{onh} data ranged from 3.7% to 18.5%, with an average of 9.95%, for the five observers included in the study. Test–retest coefficient of variation of RF_{onh} data, determined for the responses to the reference conditions, ranged from 10% to 48% (average, 26%).

Regarding fatigue effects that may affect RF_{onh} during a recording session, five successive recordings of F_{onh} in response to the 15-Hz, 13.5-lux G luminance flicker obtained in one of the subjects within 14 minutes provided RF_{onh} between 46.1% and 66.6% (mean $\pm$ SD, 56% $\pm$ 9%). A linear regression demonstrated no significant trend during this period ($r = 0.27$, NS). The same behavior was observed in another series of nine successive recordings in response to the same stimulus (mean, 59% $\pm$ 29%; $r = 0.59$, NS).

Figure 2 shows a smoothed recording of F_{onh} (approximately 11 minutes) in response to a 10-Hz, 13.5-lux G luminance flicker. Each F_{onh} represented an average over 2 seconds recording. A 10-point moving average procedure was used to further smooth the data. The average increase of F_{onh} between 125 and 525 seconds was 51% of the baseline value. A linear regression of the F_{onh} data versus time calculated between these time limits provided a correlation coefficient of 0.35 ($n = 189$, $P < 0.01$), demonstrating a slight, but significant increase in RF_{onh} amounting, in the average, to approximately 3% RF_{onh} increase per minute. Two other F_{onh} recordings of 8 minutes flicker duration from a different temporal site of the disc showed no significant trend when the linear regression was determined in the interval of time between 30 seconds after the start and the end of the flicker

Figure 3 shows representative examples of flicker ERG recordings, obtained from one subject in response to luminance flicker and equiluminant chromatic flicker modulation at 12 Hz. In Figure 3A, the four partial blocks obtained during a given stimulus have been superimposed. The sweep duration corresponds to one stimulus cycle (i.e., 83.3 ms). Figure 3B shows the 1F and 2F harmonics of the grand average of the partial blocks shown. Mean 1F amplitude was 0.33 $\pm$ 0.09 (SE) μV for the luminance and 1.48 $\pm$ 0.06 μV for the chromatic ERG. Mean 2F amplitude was 1.76 $\pm$ 0.03 and 0.52 $\pm$ 0.03 μV for the luminance and chromatic ERG, respectively. Test–retest coefficient of variation of the 1F and 2F amplitudes ranged from 10% to 36%, with an average of 20%.

In Figure 4, mean RF_{onh} and ERG 1F and 2F amplitudes obtained in response to luminance flicker are plotted as a function of stimulus **Tf**. In this and the following figures (Figs. 5, 6, and 7), the data points without error bars indicate those responses to which all the data obtained in a specific protocol have been normalized (see the Materials and Methods section,
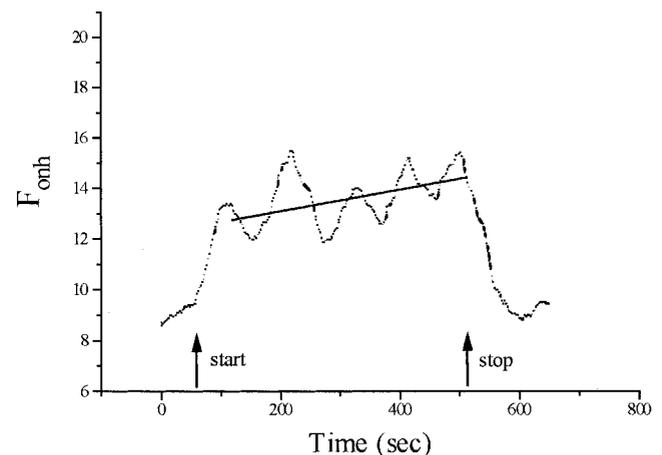

FIGURE 2. Smoothed recording of F_{onh} (approximately 11 minutes) obtained from one subject in response to a 10-Hz, 13.5-lux G luminance flicker. Each data point represents an average during 2 seconds recording. A 10-point moving average procedure was used to smooth the data. Linear regression is fitted to the data points recorded between 125 and 525 seconds (start and stop, respectively), corresponding to the period of flicker stimulation.
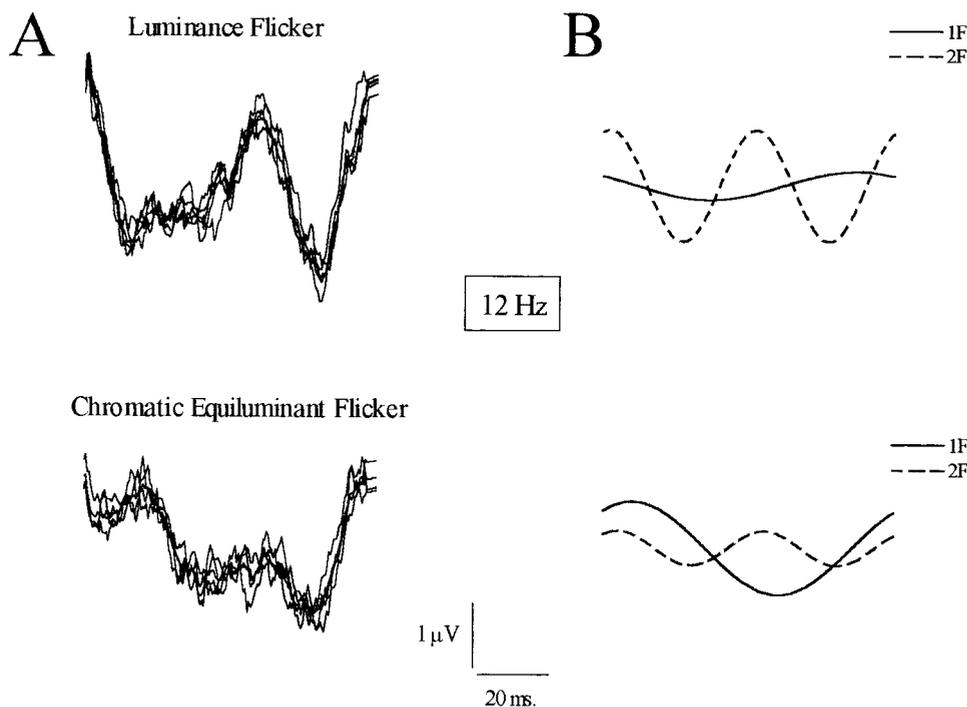
A   Luminance Flicker

B ——— 1F
--- 2F

12 Hz

Chromatic Equiluminant Flicker

——— 1F
--- 2F

1 μV

20 ms.

FIGURE 3. (**A**) Representative examples of flicker ERG recordings, obtained from one subject in response to luminance flicker and equiluminant chromatic flicker modulation at 12 Hz. Several blocks of responses are superimposed to demonstrate reproducibility. The sweep duration corresponds to one stimulus cycle (i.e., 83.3 ms). (**B**) 1F and 2F components of the grand average of the responses shown in (**A**).

Data Analysis). The RF_{onh} function (A) shows a band-pass behavior with a broad maximum at approximately 12 to 20 Hz and attenuation at higher and lower frequencies. The ERG 2F amplitude function (B, bottom panel) shows a maximum at approximately 9 Hz and is more low-pass in shape, compared with the RF_{onh} function. Attenuation occurred above 9 Hz. The 1F amplitude function (B, top panel) differed substantially from either the 2F or RF_{onh} functions, with peaks in the low- and high-frequency ranges.

In Figure 5, mean RF_{onh} and ERG 1F and 2F amplitudes obtained in response to 10-Hz luminance flicker are plotted as a function of the stimulus's mean luminance. It can be noted that RF_{onh} and the 2F amplitude first increase and then saturate

with increasing stimulus's mean illuminance, whereas the 1F amplitude showed no systematic trend. Characterizing RF_{onh} and 2F versus mean illuminance by a general Hill model of the form $Y = Y_{max} \cdot X^n/(X^n + K_{50}{}^n)$, where Y_{max} is the maximum amplitude, K_{50} the Hill parameter for 50% of the maximum, and n the Hill coefficient (least-squares method), RF_{onh} and 2F reached 50% of maximum at 4.95 ± 1.47 (SD) and 4.5 ± 1.8 (SD) lux, respectively.

Figure 6 shows the changes in mean RF_{onh} and ERG 1F and 2F amplitudes as a function of color ratio (**r**) of the 15-Hz R-G chromatic stimulus. RF_{onh} decreased substantially when **r** approached the psychophysical equiluminant point of 0.46 (Fig. 6A). Similar to RF_{onh}, both 1F and 2F amplitudes (Fig. 6B)
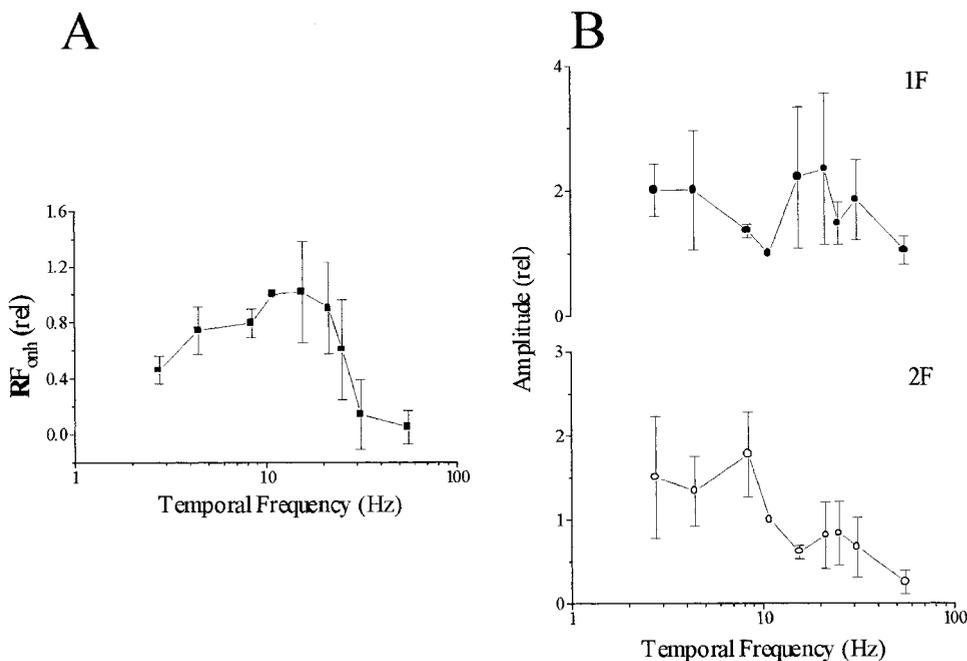
A

1.6

1.2

0.8

0.4

0.0

RF_{onh} (rel)

1   10   100

Temporal Frequency (Hz)

B

4

2

0

1F

3

2

1

0

2F

Amplitude (rel)

1   10   100

Temporal Frequency (Hz)

FIGURE 4. Protocol 1. (**A**) Mean ($\pm$SE) RF_{onh} obtained in response to luminance flicker, plotted as a function of stimulus frequency. (**B**) Mean ($\pm$SE) ERG 1F ($\bigcirc$) and 2F ($\bullet$) amplitudes for the same stimulus conditions.
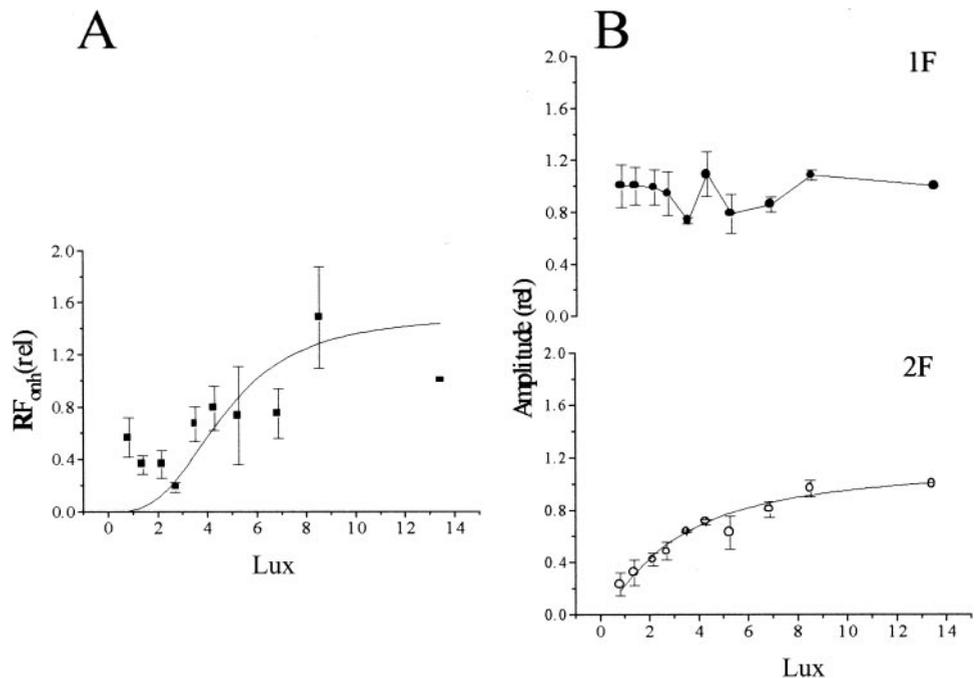
FIGURE 5. Protocol 2. **(A)** Mean (±SE) RF_{onh} obtained in response to 10-Hz luminance flicker plotted as a function of the stimulus's mean luminance. **(B)** Mean (±SE) ERG 1F (○) and 2F (●) amplitudes for the same stimulus conditions. For both RF_{onh} and ERG amplitudes, the response-versus-illuminance functions are fitted by hyperbolic saturation functions.

decreased when **r** approached equiluminance. Both the 2F and RF_{onh} functions were more symmetrical around the equiluminance minimum, compared with the 1F function.

In Figure 7, mean RF_{onh} and ERG 1F and 2F amplitudes in response to equiluminant chromatic flicker are plotted as a function of **Tf**. The RF_{onh} function (Fig. 7A) displayed, unlike the luminance flicker functions (Fig. 4), an attenuation at **Tf** > 10 Hz. The 1F and 2F amplitude functions (Fig. 7B) were similar to the corresponding RF_{onh} functions, with an attenuation beyond 10 Hz.

The relationship between RF_{onh} and flicker ERG 1F and 2F amplitudes obtained under the various experimental condi-

tions are reported in Figures 8A and 8B. All data collected from the five subjects are shown in the scatterplots. Correlation results are reported only for conditions in which 1F and 2F amplitudes and RF_{onh} changes showed similar functional shapes. It can be seen that Pearson's correlations between mean RF_{onh} and 1F and 2F amplitudes are significant for R-G equiluminant flicker responses recorded as a function of **Tf** (Fig. 8B). A significant correlation between RF_{onh} and 2F amplitude is also present for the condition of 10-Hz G luminance flicker (changing mean illuminance, Fig. 8A, left) and for the condition of 15-Hz R-G flicker (changing the **r,** Fig. 8A, right). Removing the potential outlier data point in Figure 8A (left)
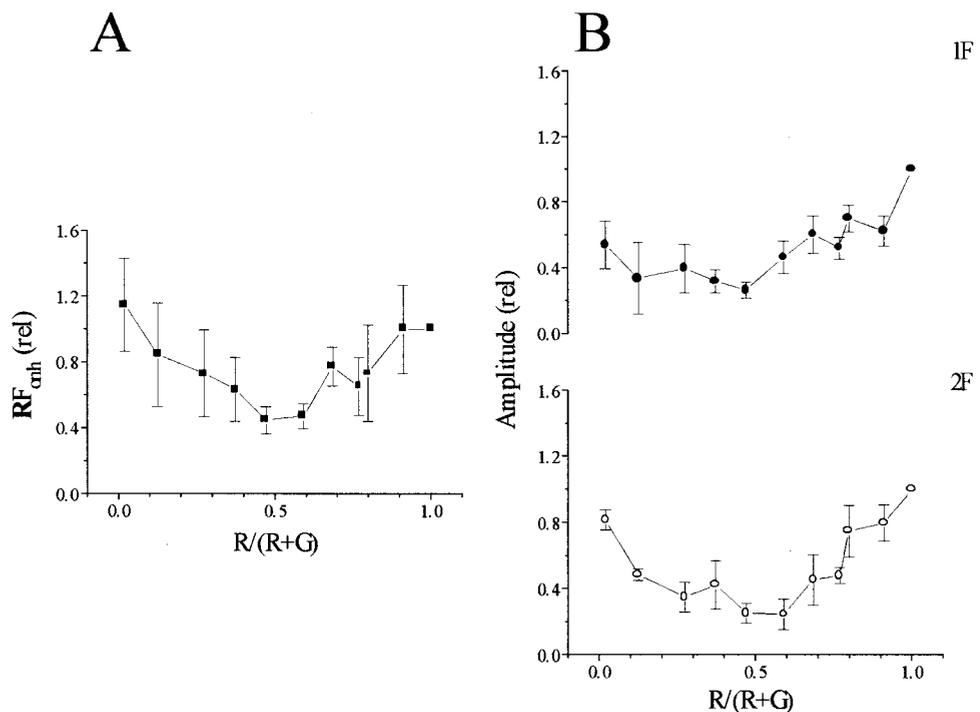

FIGURE 6. Protocol 3. **(A)** Mean (±SE) RF_{onh} plotted as a function of color ratio (**r**) of the 15-Hz R-G mixed chromatic–luminance flicker. **(B)** Mean (±SE) ERG 1F (○) and 2F (●) amplitudes for the same stimulus conditions.
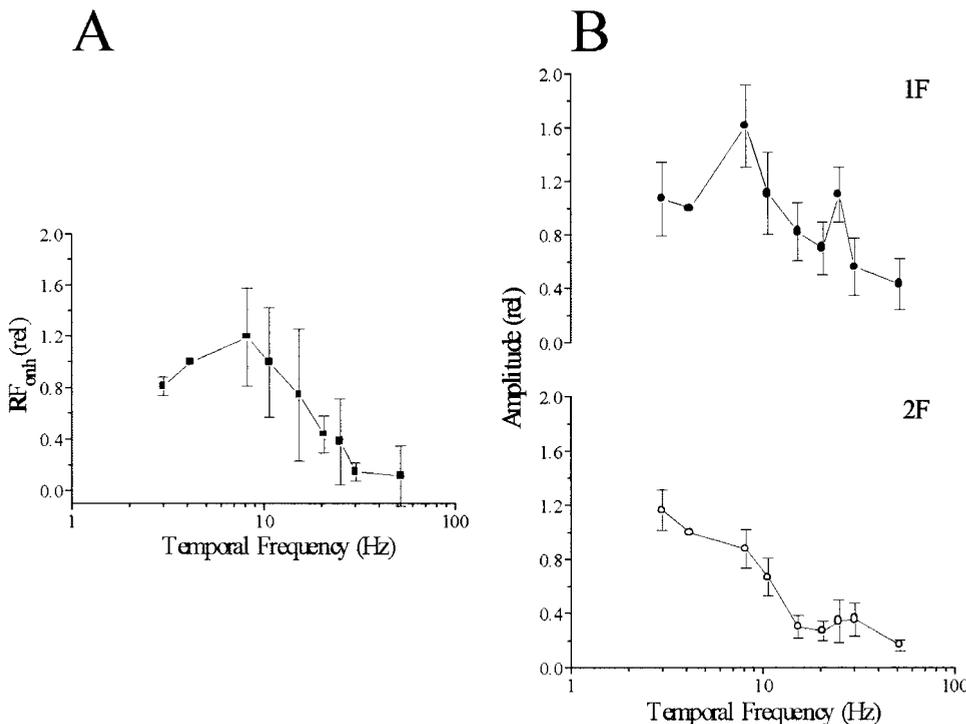
A

B

FIGURE 7. Protocol 4. (A) Mean (±SE) RF_{onh} in response to R-G equiluminant chromatic flicker, plotted as a function of stimulus **Tf**. (B) Mean (±SE) ERG 1F (○) and 2F (●) amplitudes for the same stimulus conditions.

reduces the corresponding correlation coefficient from 0.52 to 0.40, a value that is still statistically significant ($P = 0.02$). Correlation analyses performed on the RF_{onh} and ERG data averaged across the five subjects for each condition (i.e., with a reduced number of data points for each correlation) yielded statistically significant results similar to those shown in the figure.

DISCUSSION

Previous studies in animals have demonstrated a strong correlation between local RF_{onh} and neural activity response, the latter obtained from the flicker-induced changes in the electrical signal[22] and K^+ ion-concentration,[3] both measured at the surface of the optic disc. In humans, luminance and heterochromatic flicker stimulation generate an RF_{onh} that displays characteristics comparable to those of ganglion cell spiking activity evoked by the same stimuli, when RF_{onh} is plotted as a function of frequency, modulation depth, and relative chromaticity.[7] Although this finding suggests the existence of a coupling between neural and vasoactivity, the relationship between both physiological activities remains to be established. With this goal in mind, we chose the 1F and 2F components of the flicker ERG to assess changes in neural activity for the following reasons. First, noninvasive methods to measure activity at the optic nerve, the site of recording of F_{onh}, are not available. Second, although the pattern ERG appears to be the most appropriate stimulus because it is generated mainly by ganglion cell activity, no data are available yet on RF_{onh} evoked by contrast-reversal patterns. In contrast, a relatively large amount of data on flicker-induced RF_{onh} have been collected from experiments in animals and humans. Third, the hypothesis of an association between RF_{onh} and the 1F and 2F harmonics of the flicker ERG is justified, based on the origin of these components of retinal neural activity (see the introduction).

The results of this study indicate that, under specific experimental conditions, the stimulus-evoked changes in RF_{onh} are similar and significantly correlated to the changes in the flicker

ERG harmonic components. In particular, the associations were the most significant (1) when the **Tf** of the stimulus was changed in the condition of R-G equiluminant flicker (protocol 4, Fig. 7), (2) when the mean illuminance was varied for a G illuminance flicker (protocol 2, Fig. 5), and (3) when the **r** was varied for the heterochromatic R-G flicker (protocol 3, Fig. 6). In case (1), RF_{onh} correlated with both 1F and 2F amplitudes. In the cases (2) and (3), RF_{onh} correlated with only the 2F amplitude.

The experiments designed to assess the influence of potential fatigue effects demonstrated no effect on the magnitude of RF_{onh} that would significantly alter the conclusions drawn from the collected data. In all subjects, no decrease was apparent in the quality of the recordings obtained within a given session.

The significant correlation found between RF_{onh} and each of 1F and 2F amplitudes for the case of R-G equiluminant flicker responses recorded as a function of **Tf** (protocol 4, Fig. 7), as well as between RF_{onh} and 2F amplitudes for R-G flicker responses as a function of **r** (protocol 3, Fig. 6), suggests a coupling arising from the pooled activity of the M- and L-cones. Many lines of experimental evidence (reviewed in the introduction) indicate that the flicker ERG 1F and 2F reflect the activity of different postreceptoral retinal layers. In addition, clinical results in humans[15,23–25] have shown that the 2F component may be selectively altered (i.e., with normal 1F component) in diseases affecting primarily the inner retina, such as glaucoma, optic neuritis, or optic nerve compression,[15] and appears to be highly sensitive to retinal vascular disorders, such as diabetic retinopathy[26] and occlusion of a branch of the central retinal artery or vein.[24,27] Given the retinal origin of the flicker ERG 2F, the present findings provide support to the hypothesis that blood flow changes recorded at the ONH in response to flicker stimulation parallel the corresponding changes in the neural function of the inner retina. Under some experimental conditions (chromatic flicker modulated at different temporal frequencies), however, RF_{onh} changes may also be similar to changes in the 1F amplitude, implying com-
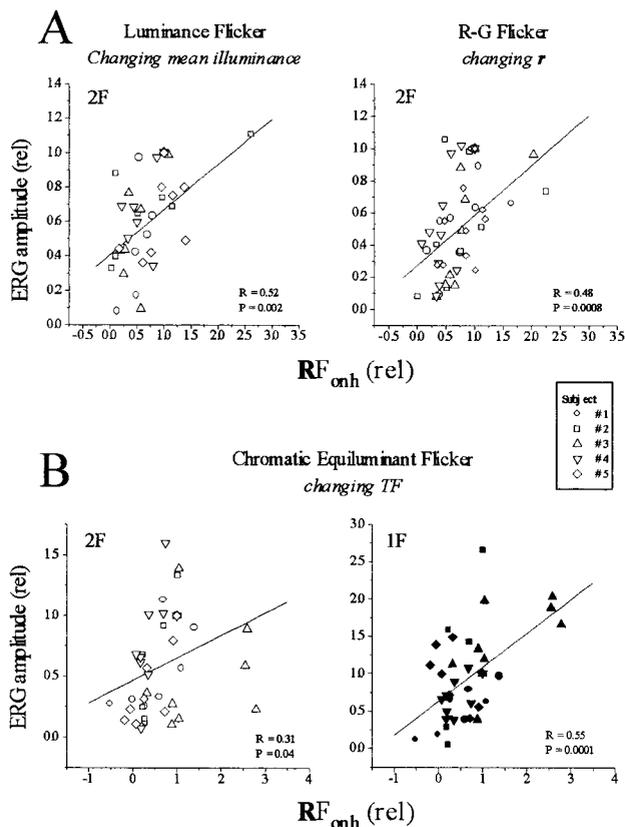
FIGURE 8. Mean RF_{onh} changes plotted as a function of corresponding ERG component amplitudes obtained in response to (**A**, *left*) G luminance flicker presented at different mean illuminance levels (protocol 2); (*right*) R-G hetero-chromatic flicker presented at different **r** (protocol 3); (**B**) R-G chromatic equiluminant flicker modulated at different **Tf** (protocol 4). All data recorded from the five subjects are shown in the scatterplots (each subject represented by a separate symbol). Correlation analyses performed only when RF_{onh} changes and ERG amplitudes showed similar functional shape. Coefficients (*r*) of the linear regressions to the data are reported in each scatterplot. Slopes of the regression lines are as follows: (**A**) Luminance flicker (protocol 2): 0.26; (**A**) R-G flicker (protocol 3): 0.31; (**B**) chromatic equiluminant flicker (protocol 4), RF_{onh} versus 2F: 0.18; (**B**) chromatic equiluminant flicker (protocol 4), RF_{onh} versus 1F: 0.45.

mon properties of optic nerve vascular and neural activity changes in the middle retina (i.e., ON and OFF bipolar cells).

With the G luminance flicker, correlation was found between RF_{onh} and 2F, but not between RF_{onh} and 1F amplitude. Furthermore, both RF_{onh} and 2F amplitude varied similarly as a function of mean illuminance. In the tested subjects, the variation of the 1F amplitude with mean illuminance relative to its variability may have been too small for a potential correlation between RF_{onh} and 1F to be revealed. It is possible that the amplitude of the 1F at low luminance, which is similar in magnitude to that at the high luminance, represents the contribution of a rod-mediated signal. Indeed, it is known[14] that, although the 2F is mainly cone-mediated, the 1F component has a contribution from both rod and cone systems, depending on the stimulus luminance and adaptation conditions.

It may be of interest to compare the results of both pure and equiluminance flicker, when the **Tf** is varied (Figs. 4, 7). In the case of pure luminance, although the changes in the 1F and 2F amplitudes extended over a relatively large range, no significant association was found between RF_{onh} and both components' amplitudes. A possible physiological basis for this finding is that the generators underlying RF_{onh} and ERG response

to luminance flicker may differ, depending on flicker frequency. Multiple generators for both responses, whose relative contribution changes according to stimulus frequency, may obscure the correlation when measurements are obtained over a range of frequencies. By contrast, it is possible that the blood flow and neural responses to a specific stimulus frequency (i.e., the luminance flicker at 10 Hz, share common generators in the inner retina) thus revealing the association between RF_{onh} and ERG 2F amplitude. In contrast, the R-G heterochromatic flicker (protocol 4, Fig. 7) reveals a significant coupling between RF_{onh} and both 1F and 2F amplitudes, suggesting a contribution from both retinal layers to the coupling between the neural and vascular responses.

Although several previous studies have suggested a neurovascular coupling in the human retina,[5,28,29] direct evidence of it has not been provided in the past. The observed correlations may not directly prove an effect of retinal neural activity on blood flow measured at the optic disc. It is however reasonable to suggest that the pooled response of neural generators underlying the 1F and 2F components may induce a vasoactive mechanism resulting in a corresponding blood flow change. Putative mediators underlying this neurovascular response have been discussed in detail in previous reports,[3,22,30,31] the most prominent being nitric oxide (NO). The present findings support the hypothesis that the strength of neurovascular coupling may be dependent on the type of flicker stimulus. Indeed, from the data shown in Figure 8, it can be concluded that both luminance and chromatic flicker may be appropriate to study the coupling between RF_{onh} and 2F amplitude, whereas the chromatic equiluminant stimulus appears to be more suitable for investigating the association between RF_{onh} and 1F.

In conclusion, the results of this study provide direct evidence that, in humans, the retinal vascular and neural changes elicited by flicker stimulation are quantitatively associated. RF_{onh} changes can be correlated with both 1F and 2F amplitude changes, depending on the stimulus condition, suggesting that both outer and inner retinal generators may contribute to the observed associations. These findings support the presence of a neurovascular coupling in the human retina, and warrant further investigation to provide more detailed insights into the characteristics of this coupling, and its potential value in studying normal and diseased retinas.

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