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Two Distinct Processes are Evident in Rat Cone Flicker ERG Responses at Low and High Temporal Frequencies

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Abstract

The electroretinogram (ERG) provides a noninvasive, objective measure of retinal function, and is one of the most widely used diagnostic tools in the study of visual disorders. Although rodents are often used in the study of retinal disease, the properties of the flicker ERG of the rodent retina have not been fully characterized. Here, we show that the fundamental response of the rat ERG to sine-wave flicker exhibited a low-pass pattern in the frequency range from 2 to 30 Hz, whereas the second harmonic (F2) showed a more complex frequency response relation. The F2 component represented only a small fraction of the ERG response at low temporal frequencies (below 12 Hz), but it made a substantial contribution to responses at high frequencies. The contrast-response relation was linear when tested with a low-frequency (6 Hz) stimulus, but saturated in response to a high-frequency (20 Hz) stimulus. After intravitreal injection of L-AP4, a specific blocker of the retinal ON pathway, the flicker responses elicited by either 6- or 20-Hz stimuli were greatly reduced in amplitude, whereas only a very slight enhancement was seen after the application of PDA, a drug that blocks retinal OFF-pathway activity. Based on the observed differences in the degree of non-linearity, and contrast-response properties of the rat flicker ERG at low and high frequencies, as well as the pharmacological results, we postulate that sustained and transient ON bipolar cells generate the flicker ERG responses elicited at low and high temporal frequencies, respectively.

Keywords

Electroretinogram; flicker ERG; rodent; bipolar cell; pharmacology; sinusoidal stimuli; temporal frequency

The electroretinogram (ERG) affords a quantitative, objective, and noninvasive method by which to examine light-evoked neuronal activity, and is commonly used to study the functional integrity of normal and diseased retinas. Decades of studies from a number of laboratories have provided a clearer understanding of the origins of the various potentials that summate to give rise to the ERG waveform elicited by a brief pulse of light (Penn and Hagins, 1969; Lamb and Pugh, 1992; Breton et al., 1994; Hood and Birch, 1997) Robson and Frishman, 1995; Hood and Birch, 1996). ERG responses elicited by flickering light stimuli, either to a series of pulses or to sinusoidally modulated light, have also been studied, and the results have provided

informative insights into the temporal properties of retinal signal processing mechanisms. However, most flicker ERG studies have been carried out on primates, where the presence of ON and OFF retinal pathways has complicated analysis of the underlying events (Kondo and Sieving, 2001; Viswanathan et al., 2002). Although mice and rats provide useful models for the study of retinal disorders (Peachey and Ball, 2003), far fewer investigations have dealt with the ERG responses to sine-wave modulated light in the rodent retina, where the ERG responses are dominated by ON retinal activity (Krishna et al., 2002). In the present study, we characterized the flicker ERG responses elicited by sinusoidally modulated light from pigmented rat eyes under photopic conditions. Pharmacological tools were used to assess the contributions of ON and OFF retinal pathways to the responses elicited at low and high temporal frequencies, and special emphasis was placed on the analysis of nonlinearities (second harmonic response) and contrast-response relations.

Adult pigmented Long Evans rats (both sexes, weight 250–500g) were used for this study. All experimental procedures conformed to the statement on animal care of the Association for Research in Vision and Ophthalmology, and adhered to the guidelines for the Care and Use of Laboratory Animals formulated by the Animal Care Committee of the University of Illinois at Chicago. The procedures for recording the rat ERG were similar to those described previously (Ramsey et al., 2006). Briefly, animals were anesthetized with intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine (5 mg/kg), and their pupils were maximally dilated with topical phenylephrine HCl (2.5%) and tropicamide (1%). ERG responses, picked up by a chlorided silver electrode placed in the center of the cornea, were fed to the input stage of a Grass AC amplifier (Model P511) with a bandwidth of 0.3 to 300 Hz (without a 60 Hz notch filter), and were digitized at 2 kHz. Body temperature was maintained at ~37°C with a heating pad. Throughout the preparatory procedures, the animals were light adapted by exposure to room light.

Photoc stimuli were delivered by multiple light-emitting diodes (LEDs) with a peak wavelength of 505 nm (Nichia NSPE590S, Tokushima, Japan) mounted in a small integrating sphere (Oriol 70500, Newport Corp., Stratford, CT) to provide a full-field stimulus. The current driving the LEDs was pulse-width modulated under computer control, and the luminance was calibrated with a Minolta LS-100 photometer. The output of the diode array was modulated sinusoidally at temporal frequencies ranging from 2 to 30 Hz and a mean luminance of 350 cd/m². At this mean luminance, rat rods are saturated (Xu et al., 2003), and ERG responses reflect the activity of cone-mediated neural pathways in the retina. The stimulus contrast was defined as:

$$\frac{L_{\max} - L_{\min}}{L_{\max} + L_{\min}} \quad \text{Eq. 1}$$

where $L_{\max}$ and $L_{\min}$ are the maximum and minimum stimulus luminances, respectively. In most experiments, the contrast was 90%. For experiments examining the contrast-response relation, flicker ERGs were also measured at stimulus contrasts ranging from 5% to 90% at temporal frequencies of 6 and 20 Hz. Each stimulus was presented for approximately 10 s, and the ERG recording was synchronized with the stimulus onset and offset. For data analysis, segments of approximately 500 ms were deleted from the beginning and end of the recorded waveforms. The fundamental and second-harmonic amplitudes and phases were derived from discrete Fourier transforms of the approximately 9-s waveform segments using the Matlab Signal Processing Toolbox (The Mathworks, Boston, MA).

After applying a topical anesthetic (proparacaine, 0.5%), pharmacological agents were delivered to the eye by intravitreal injection through a 30-gauge needle introduced into the vitreous cavity by piercing the sclera 3 mm posterior to the temporal limbus at approximately a 45-degree angle to the optical axis. The injection site was monitored under a dissecting microscope and a 3-μl aliquot solution was injected into each eye. The final vitreal

concentrations listed in the text and figure legends were derived by assuming complete mixing in the rat vitreous with an estimated volume of 38 μl (Xu et al., 2003). Control experiments were conducted with injections of an equivalent volume of physiological saline.

Fig. 1A illustrates flicker ERG responses elicited from a rat eye by sine-wave modulated light stimuli with temporal frequencies ranging from 2 to 30 Hz at 90% contrast. The amplitude of the ERG response was maximal at low temporal frequencies and gradually declined with increasing stimulus frequency. In the range of temporal frequencies analyzed (2 to 30 Hz), the signal-to-noise ratio was excellent (> 10 fold). Above 30 Hz, ERG responses were smaller than 5 μV and were not included for further analysis in this study. This frequency-response relation is commonly observed with nocturnal animals (Krishna et al., 2002; Rosolen et al., 2005). The flicker ERG was then analyzed in the frequency domain, and Fig. 1B summarizes the mean fundamental response amplitudes ($n = 20$) of the rat sine-wave flicker ERG. Similar to the findings reported for the mouse photopic flicker ERG (Krishna et al., 2002), the mean fundamental amplitude exhibited a monotonic low-pass pattern in the frequency range from 2 to 30 Hz. The mean phases of the fundamental responses (Fig. 1C) were well fit by a linear function with a slope of $-15.6^\circ/\text{Hz}$ (depicted by a curved line on these log-linear coordinates), which corresponds to a simple response delay of about 43 ms. This slope is similar to that observed for the mouse flicker ERG (Krishna et al., 2002).

The mean frequency-response properties of the second harmonic of the rat flicker ERG ($n = 20$) are presented in Fig. 2A–C. Unlike the fundamental response function shown in Fig. 1, the response function for the second harmonic did not show a monotonic decline in amplitude (Fig. 2A). At low stimulus frequencies (< 12 Hz), the amplitude of the second harmonic decreased with increasing temporal frequency to reach a minimum at about 12 Hz. The amplitude then increased to a peak at approximately 20 Hz before falling off again at still higher stimulus frequencies. The relative proportion of the second-harmonic response in the full-field flicker ERG is apparent when the second-harmonic amplitude is normalized to the amplitude of the fundamental (Fig. 2B). Within the frequency range of 2 to 12 Hz, the second harmonic contributed only a small fraction (< 0.25) to the flicker ERG response. On the other hand, the second-harmonic response made a substantial contribution to the flicker ERG at higher temporal frequencies, reaching a maximum proportion of about 0.5 at 25 Hz. These results are a clear indication that the ERG responses elicited at high temporal frequencies display a much greater nonlinearity than those elicited with low-frequency stimuli.

Differences between the responses elicited at low and high temporal frequencies are also reflected in the phase plots for the second harmonic. As shown in Fig. 2C, there was a discontinuity in the phase data for the second-harmonic response at a stimulus frequency of about 12 Hz. Although plotted here on log-linear coordinates, the phase values for both the low and high temporal frequency regions could be described by linear functions, but with different slopes. The slope at stimulus temporal frequencies of 12 Hz and below was $-21.7^\circ/\text{Hz}$, whereas the slope at stimulus frequencies above 12 Hz was $-19.2^\circ/\text{Hz}$. Thus, analysis of the second harmonic suggests that there are two distinct processes contributing to the rat flicker ERG: a quasi-linear process that predominates at low temporal frequencies, and a non-linear process within the higher-frequency range.

To investigate further the differences seen in these temporal domains, we determined the contrast-response relation for stimuli presented at a low (6 Hz) and a high (20 Hz) temporal frequency. The averaged data ($n = 20$) shown in panels D and E of Fig. 2 indicate that the responses at 6 Hz (D) exhibited a linear contrast-response relation over the entire range of contrast, whereas the responses to 20-Hz stimuli (E) showed a saturating contrast-response relationship. These results provide additional support for the presence of two distinct processes in the rat ERG response to sinusoidally modulated stimuli: a process with a linear contrast-

response function at a low temporal frequency, and a process with a saturating contrast-response function at high temporal frequencies.

We used pharmacological tools to examine the contributions of the ON and OFF retinal pathways to the flicker ERG responses elicited by low- and high-frequency stimuli. The agents we introduced intravitreally to selectively block these pathways were 2-amino-4-phosphonobutyric acid (L-AP4, 1 mM), a selective blocker of the ON pathway, and *cis*-2,3-piperidinedicarboxylic acid (PDA, 10 mM), which blocks OFF-pathway activity. The effect of L-AP4 was examined in 6 rats and the effect of PDA was tested in 5 rats. For each animal, the test drug was injected into one randomly selected eye, and an equal amount of saline was injected into the other eye, which served as control. Photopic ERG recordings in response to single (200 msec) light pulses were recorded initially in each animal to ensure that the drugs produced the desired effects. Light stimuli were presented on a rod-saturating, steady background luminance of 5 cd/m². As shown in Fig. 3A, intraocular injection of L-AP4 eliminated the cornea-positive component (*b*-wave) of the photopic ERG, leaving only a small negative residual response that is considered to be the summed activity of photoreceptors and OFF-type retinal neurons (Xu et al., 2003). On the other hand, intravitreal injection of the OFF-pathway blocker (PDA) produced little change in the response amplitude to the pulsed stimulus, although the example illustrated in Fig. 3B shows a slight enhancement of the *b*-wave response.

The effects of L-AP4 and PDA injections on the rat flicker ERG were then examined at temporal frequencies of 6 and 20 Hz. For each temporal frequency, the responses elicited after intravitreal injection were normalized to those recorded from the control eye of the same animal injected with saline. As shown in Fig. 3, intravitreal injection of L-AP4 had a profound effect on the flicker ERG responses. The mean fundamental response amplitudes were reduced by 75% and 60% compared to the saline control at 6 Hz and 20 Hz, respectively (Fig. 3C) and mean phase shifts of -121° and -78° relative to the saline control were observed at these two stimulus frequencies (Fig. 3D). By comparison, blocking the OFF pathway by the application of PDA had no appreciable effect on either the mean amplitude (Fig. 3C) or the mean phase (Fig. 3D) of the fundamental responses to 6-Hz and 20-Hz stimuli. These findings indicate that the retinal ON pathway is a major source of the responses at these stimulus frequencies.

To summarize our results, flicker ERG responses elicited from rat retina at relatively high temporal frequencies (> 12 Hz) contained a larger proportion of a second-harmonic component than those elicited at lower stimulus frequencies, an indication of a greater degree of nonlinearity in the high-frequency response. There was also a transition at approximately 12 Hz in the phase plot of the second harmonic, such that the slope for the low-frequency stimulus was greater than that for the high-frequency stimulus. A further indication of a functional difference in the responses elicited at low and high temporal frequencies was evident in the contrast-response relationship. Our data showed a linear contrast-response function at the relatively low temporal frequency of 6 Hz, whereas the contrast-response function at the high temporal frequency of 20 Hz approached saturation with increasing contrast. Using pharmacological agents to probe the contributions of ON and OFF retinal pathways in the flicker ERG responses, we found that the response amplitudes and phases elicited at both low and high temporal frequencies were highly sensitive to the metabotropic glutamate receptor agonist L-AP4, but were resistant to the ionotropic glutamate receptor antagonist PDA. Thus, the ERG responses elicited at both low and high temporal frequencies are most likely driven by retinal ON bipolar cells.

The differences in the degree of nonlinearity and the shapes of the contrast-response functions that we observed in the rat flicker ERG have similarities to those reported for sustained and transient ganglion cells, which are thought to receive their inputs from sustained and transient bipolar cells, respectively (Awatramani and Slaughter, 2000). Sustained or X-type ganglion

cells have a low temporal resolution, a small non-linear component, and weak contrast gain control, whereas transient or Y-type ganglion cells exhibit greater temporal resolution, a large non-linear component, and high-contrast gain control that shows saturation (Enroth-Cugell and Robson, 1966; Cleland et al., 1971; Shapley and Enroth-Cugell, 1984; Stone and Pinto, 1993; Rodieck, 1998). Furthermore, ganglion cell responses are shaped to a large extent by the response properties of bipolar cells (Awatramani and Slaughter, 2000; Demb, 2002; Zaghloul et al., 2005; Manookin and Demb, 2006). Therefore, we postulate that the sustained ON bipolar cells may be the source of the quasi-linear flicker ERG responses elicited at low temporal frequencies, whereas the transient ON bipolar cells may generate the nonlinear flicker ERG responses to high-temporal-frequency stimuli.

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Figure 1.

A. Examples of typical ERG waveforms elicited from a rat eye by sinusoidally modulated light stimuli at 90% contrast, presented at temporal frequencies ranging from 2 to 30 Hz. Each trace represents the average of eighteen 500-ms segments from a 10-s recording. **B.** Mean amplitudes (n = 20) of the ERG fundamental responses derived from discrete Fourier transforms and plotted on log-log coordinates. **C.** Mean phases of the ERG fundamental responses of the same group of animals, plotted on log-linear coordinates. On linear coordinates, the curve represents a straight line with a slope of $-15.6^\circ/\text{Hz}$. In this and subsequent figures, error bars represent standard errors of the means (SEMs) and were omitted when smaller than the symbols.

Figure 2.

A. Mean second-harmonic amplitudes ($n = 20$) plotted on log-log coordinates. **B.** Mean ratios of second-harmonic amplitude to fundamental amplitude obtained from the same group of rats as in A. **C.** Second-harmonic phases derived from the ERG recordings from the same group of rats as in A and B. Curves represent least-squares best fits to the mean data when plotted on linear coordinates, with the data points above and below 13 Hz fit separately. **D.** Mean fundamental response amplitude vs. percent stimulus contrast ($n = 20$) at a temporal frequency of 6 Hz. **E.** Mean fundamental response amplitude vs. percent stimulus contrast ($n = 20$) at a temporal frequency of 20 Hz. Lines are least-square best fits of either a linear function (D) or an exponential function (E).

Figure 3.

Examples of rat photopic ERG waveforms in response to a 200-ms pulse from the control eye (saline injected, upper traces), and from the fellow eye injected with either **A.** 1 mM L-AP4 (lower trace) or **B.** 10 mM PDA (lower trace). Recordings in A and B are from two different animals. The stimulus, which was presented on a steady background of 5 cd/m², is indicated below the traces. **C.** Mean ERG fundamental response amplitudes obtained at 6 Hz (open bars) or 20 Hz (hatched bars) following intravitreal injection of either L-AP4 (left pair of bars) or PDA (right pair of bars), with the amplitude from the injected eye normalized to that of the saline-injected control eye of the same animal. **D.** Mean phase differences between the ERG responses obtained from the drug-injected eye and those of the saline-injected fellow eye induced by intravitreal injection of either L-AP4 (left pair of bars) or PDA (right pair of bars) at either 6 Hz (open bars) or 20 Hz (hatched bars).