Noninvasive Assessment of Retinal Function in Rats Using Multifocal Electroretinography

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PURPOSE. To assess the applicability of multifocal electroretinograms (mfERGs) for evaluation of function in this small-eyed animal with a rod-dominant retina that is often used to model retinal diseases.

METHODS. Noninvasive monocular mfERGs were recorded in anesthetized albino (Sprague–Dawley) and pigmented (Long Evans) rats. Achromatic stimuli subtending a 49° by 53° field consisted of 61 hexagons that were generated and presented (at varying rates and luminances) using a Visual Evoked Response Imaging System (VERIS; EDI, San Mateo, CA). The VERIS also was used to calculate individual responses and for analysis.

RESULTS. mfERGs were recorded from pigmented and albino rats by slowing the rate of stimulus presentation to allow for the slow recovery time of the rod system. In each rat strain, responses varied systematically with changes in stimulus parameters. Peak response amplitude increased as the rate of stimulation was slowed and as stimulus luminance was increased. Response latency decreased as stimulus intensity was increased. The local nature of the response was assessed by several independent measures.

CONCLUSIONS. The present work demonstrated the feasibility and limitations of using mfERG to assess topographical changes in the rat retina. It showed that despite the problems of the unavoidable self-adapting nature of the stimulus, the small eye of the animal, and the high potential for light scatter within the retina, multifocal responses with a good signal-to-noise ratio can be obtained from the rat. (Invest Ophthalmol Vis Sci. 2000;41:610–617)

Retinal degenerative diseases such as retinitis pigmentosa and cone–rod dystrophy are primary causes of blindness. To determine the progression of such diseases, it is important to evaluate retinal function in terms of its rate of deterioration and/or the success of drug therapy or other interventions. Because these diseases often show topographical patterns of degeneration, localized retinal response patterns are important in the accurate diagnosis and treatment of these diseases.

Conventional electroretinograms (ERGs) have been useful for assessing changes in retinal function. The ERG is noninvasive, allows repeated assessment, and can be performed in a relatively short period. Clinical ERGs recorded in response to a full-field flash provide a quantitative assessment of global retinal function but do not provide local response information.

Focal ERGs permit assessment of localized retinal activity but require detailed procedures to minimize the effects of stray light and require considerable time and effort to obtain responses from multiple retinal regions.1,2 Recently, a technique using nonlinear systems analysis to extract local responses from a continuous ERG was developed.3 This approach, termed multifocal electroretinography (mfERG), permits simultaneous assessment of local responses from a large number of retinal areas. Because it can provide topographical analysis of retinal function in a relatively short period, this technique has already been used in a variety of basic and clinical studies of human vision.4–13

In brief, the mfERG technique involves the presentation of multiple local flashes in a predefined order (m-sequence) and subsequent extraction of the corresponding ERGs from the field potential by computation of the cross correlation between the stimulus m-sequence and the response cycle (for detailed description, see References 3 and 4). Typically, a single recording session lasts only a few minutes and can provide a topographical map of local retinal responses, making it a practical way to evaluate retinal function in the clinic.

Direct comparisons of mfERGs with full-field ERGs have provided evidence that the two are comparable.5 When stimulus and recording conditions are closely matched for the two methods, changes in stimulus parameters result in comparable changes in “b-wave” amplitude and latency, suggesting that the waveforms represent similar cellular responses.5 In a study examining the rod mfERG in humans, waveforms were found to contain an early component not seen in full-field ERGs.6 Although slightly shorter, this early component more closely matched the full-field ERG in latency compared with the later
peak response. When the stray-light response was reduced by adding a surround, the larger peak response decreased, whereas the early component increased in amplitude. Thus, the small early component was attributed to a local rod response. In patients with diseased retinas, multifocal, full-field, and focal ERGs yield compatible results in area and type of dysfunction observed.  

Measurement of mfERGs has been useful in providing a topographical and quantitative functional assessment of normal and diseased retinas. In normal humans, the amplitude of the individual mfERG responses varied with the density of cone receptors. Clinically, the mfERG has been used to map the topographical pattern of deterioration in patients with retinitis pigmentosa, myopia, diabetic retinopathy, and white dot syndrome. Application of mfERGs to evaluate topographical changes in animal models of various retinal diseases would be extremely useful for describing the progression of these diseases and for testing the efficacy of various treatments. However, mfERG was developed as a photopic test to assess local cone-related function, whereas most animal models have been developed in rod-dominant rodents. Until recently, the application of mfERGs to test rod function had been equivocal because of the self-adapting nature of the stimulus (repeated, high-luminance flashes). However, by adjusting stimulus parameters to accommodate for the slow recovery of rod photoreceptors, Hood et al. have successfully applied the technique to evaluate human rod function. Because mfERGs have not been demonstrated in any small-eyed and rod-dominant animals, the primary objective of the present study was to determine whether the mfERG technique can be further developed to assess retinal function in the rat. First, optimal stimulus and recording parameters were determined, the local nature of the responses was assessed, and the potential influences of dark adaptation and anesthesia type were evaluated. The results showed that with the use of appropriate temporal rates and stimulus intensities, a consistent and easily measurable mfERG response can be obtained from the rod-dominant retinas of both pigmented and albino rats.

**METHODS**

**Subjects**

Ten adult female albino rats (Sprague–Dawley) and 10 pigmented rats (Long Evans; ca. 250 g) were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital or with 40 mg/kg ketamine and 5.4 mg/kg xylazine. Animals anesthetized with ketamine-xylazine were given supplemental doses approximately every 30 minutes. This ensured the deep level of anesthesia necessary to prevent eye movements. Animals anesthetized with sodium pentobarbital did not require supplemental treatments. corneas were further treated with proparacaine hydrochloride (Ophthaine; Apothecon, Princeton, NJ), and the pupil of the tested eye was dilated with tropicamide ophthalmic solution (Mydriacyl 1%; Alcon, Humacao, Puerto Rico). Animals anesthetized with ketamine-xylazine were given supplemental doses approximately every 30 minutes. This ensured the deep level of anesthesia necessary to prevent eye movements. Animals anesthetized with sodium pentobarbital did not require supplements.

**Stimulus**

Figure 1A shows an example of the 61-hexagon stimulus array. The stimulus was generated by the Visual Evoked Response Imaging System (VERIS; EDI, San Mateo, CA) and presented on a 21-In. monochrome display monitor (P104 phosphor; Nortech Imaging, Plymouth, MN) using a customized Macintosh (Apple Computer, Cupertino, CA) video card. Stimulus luminance was calibrated at a 15° angle using a photometer (model 350; United Detector Technologies, Hawthorne, CA) with a photometric filter. Note that because of the small size of the rat eye and its physiological optics, the effective retinal illuminance of the rat eye would be approximately 10 times methylcellulose (artificial tears; Butler, Columbus, OH). No optical correction was used, because pilot experiments showed no difference in the mfERG response when different refractive lenses were used, presumably because of the large depth of field of the rat eye. After recording was complete, animals were placed in an incubator to recover before they were returned to their cages. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
higher than that of a human. More specifically, after the approach of Remtulla and Hallett,\textsuperscript{15} retinal illuminance varies as the square of the posterior to nodal difference in the rat eye (3.35 mm)\textsuperscript{16} and human eye (16.70 mm)\textsuperscript{17} (e.g., 16.70\textsuperscript{2}/3.35\textsuperscript{2} = 25.15 times greater in the rat). However, based on dilated pupil areas for human (50.27 mm\textsuperscript{2})\textsuperscript{18} and rat (19.63 mm\textsuperscript{2}),\textsuperscript{17} the human eye should capture approximately nine times more photons (50.27/19.63 = 2.56). Thus, a fully dilated rat retina should receive 9.82 times the irradiance of a similarly stimulated human retina (i.e., 25.15/2.56 = 9.82).

On a given stimulus frame, each hexagon in the array had a 0.5 probability of being white or dark. Each stimulus frame was followed by 6 to 12 blank frames (F indicates blank frames) after which each hexagon again had a 0.5 probability of being white or dark according to a defined order, or m-sequence. The identical m-sequence was pseudorandomly repeated \(2^{12} - 1\) times for each local hexagon in the array, but the starting point within the m-sequence was lagged at each hexagon location to allow the reconstruction of the response to each stimulus location using a cross-correlation technique.\textsuperscript{3}

The number of blank frames following a stimulus frame determined the maximum rate at which an individual hexagon flashed white. The addition of each \(F\) added a minimum of 13.3 msec and an average of 26.6 msec between white stimulus frames. For example, for a stimulus with \(6F\) the average time between white frames (e.g., interstimulus interval, ISI) was 13.3 msec, whereas \(6F\) added a minimum of \(6 \times 13.3\) msec and an average of \(6 \times 26.6\) msec. When blank frames are added to each element in an m-sequence, a smaller number of stimuli are presented per unit time, and a smaller number of responses are recorded per unit time. Thus, as \(F\) was increased, the stimulation rate was slowed, and a longer recording period was required to collect an equal number of responses. Values greater than \(12F\) were not used, because this would have required a recording time of almost 30 minutes.

To determine the temporal parameters necessary to obtain an optimal response, recordings were made over a range of temporal rates. The following rates were used: \(12F\), mean ISI = 332.5 msec; \(10F\), mean ISI = 279.3 msec; \(8F\), mean ISI = 226.1 msec; and \(6F\), mean ISI = 172.9 msec. The signal-to-noise ratio was unsatisfactory for recordings made at less than \(6F\). In a given recording session, rats were tested at each stimulus rate beginning with the slowest rate, followed by progressively increasing rates. The photopic luminance of each white hexagon was kept at 81 candelas [cd]/m\textsuperscript{2} for all temporal rates, whereas the background and blank frames were set to 0.4 cd/m\textsuperscript{2}. A rate of \(10F\) was found to elicit large responses, yet permitted a relatively short recording time (10 minutes).

Once the optimal temporal rate for rod stimulation was determined, responses at four different luminances were measured using the \(10F\) rate. The intensity of the white hexagons was varied, whereas the background and blank hexagons remained black (0.04 cd/m\textsuperscript{2}) resulting in a mean screen luminance during presentation of the stimulus elements equal to approximately one eleventh that of the white hexagons. All luminance levels reported here refer to the luminance of the white hexagons and not the mean luminance. To obtain an intensity–response series, the photopic luminance of the stimulus frames was increased in increments of 0.5 log steps (i.e., by a factor of 3) beginning with 3 cd/m\textsuperscript{2} and increasing successively to 9 cd/m\textsuperscript{2}, 27 cd/m\textsuperscript{2}, and 81 cd/m\textsuperscript{2}.

**Recording**

Retinal responses were recorded using DTL electrodes\textsuperscript{19} placed on the vertical midline of the cornea of each eye. Recordings from the stimulated eye were referred to the contralateral eye. The animal was grounded using a needle electrode. Responses were amplified (\(\times 10,000\)) and filtered (1 Hz/1 kHz; amplifier model 12, Neurodata Acquisition System; Grass, West Warwick, RI), and sampled 15 times within each frame interval (15 times every 13.3 msec) with an analog-to-digital board. The ERG signal was continuously monitored during recording to ensure signal quality. Signals with high noise levels were rejected and rerecorded. Recording duration varied from 2 to 11 minutes depending on temporal parameters. All recording sessions were divided into two overlapping segments.

**Partial Retinal Bleaching**

To evaluate whether the responses represented localized retinal activity, a small retinal area was bleached by presentation of a full-spectrum light (1000-W xenon source; \(240\) J/cm\textsuperscript{2}) for 35 seconds. The light beam was focused on a localized portion (approximately 2 mm in diameter) of the retina, by using a Maxwellian view system. An mfERG was recorded before bleaching and several times after bleaching.

**Local Lesions**

After prior VERIS testing, a pigmented rat was anesthetized with sodium pentobarbital (50 mg/kg) and positioned so that its right eye would be aligned in the optical axis of an argon laser beam. A pulse (0.15 W) was then delivered to the right eye for 1 second. Postlesion recordings were made approximately 2 hours after delivery of the lesion. The m-sequence of the stimulus was composed of one white hexagon (81 cd/m\textsuperscript{2}) followed by 10 blank frames.

**Data Analysis**

A first-order response was calculated that reflected activity at each retinal area. Response amplitudes were measured from baseline set to 0.0 mV at the beginning of the 150-msec averaged response to individual stimulus flashes. Latency was measured from stimulus onset to time of peak response amplitude. One iteration of an artifact rejection manipulation\textsuperscript{3} was performed on the obtained signals by using the VERIS software. This manipulation identified segments that contained significant deviations not temporally related to the stimulus and replaced such segments with a response estimate computed from the entire record.\textsuperscript{3} Each response was averaged with 17% of all surrounding responses with the VERIS software. To generate a composite ERG, the 61 responses comprising a trace array were summed. All response arrays shown are from a single recording session, not averages of multiple sessions. Although averages of multiple sessions may have provided a cleaner response array, we chose to use each day’s recording session to obtain a complete series of recordings (e.g., an intensity series or blank-frame series) rather than repeat individual conditions. Because minor deviations in eye position and electrode location were likely to occur from day to day (unlike humans, our subjects did not fixate the target) averaging across days would not have produced valid topography. (Note: Individual responses are local only if the subject is fixated on the stimulus. Any eye movements during a recording...
session would result in a blurring of the responses. For recordings in humans, a fixation point is often added to the center of the stimulus matrix. Because the rats could not be instructed to fixate and were not paralyzed, a stable eye position was dependent on anesthetic. To test for eye movements, a 1-mm² thin mirror was secured to the cornea of the anesthetized rat. A laser beam directed to the mirror was used to track any positional changes in the eye over a period of 15 minutes. Eye movements were found to be negligible. Another indicator that eye movements were not a cause of concern came from online monitoring of the continuous ERG. If the depth of anesthesia began to decrease during a recording session, eye movements quickly returned, resulting in obvious artifacts and noise in the recording.

RESULTS

mfERGs were recorded from albino and pigmented rats by using relatively low luminances and slowing the rate of stimulus presentation to allow for the slow recovery time of the rod system. Figures 1B and 1C show typical response arrays obtained from albino and pigmented rats, respectively. The most consistent feature of the responses was a positive wave (similar to the b-wave of the full-field ERG) that occurred at approximately 60 msec from the time of stimulus onset. Peak amplitude and latency varied systematically with changes in stimulus rate and intensity. Responses varied in a similar fashion for pigmented and albino rats. No clear localization of the optic nerve head was apparent; however, inactivation of retinal regions by photic bleaching and by laser lesion produced localizable areas of dysfunction. Although responses in the two bottom rows of the response array often showed reduced amplitude, the source appeared to be an occlusion of the lower part of the stimulus by the animal’s cheek.

Temporal Parameters

In Figure 2, summed responses are shown for stimuli incorporating a range of blank frames: 6F, 8F, 10F, and 12F. The fastest stimulation rate shown (6F) yielded the lowest response amplitudes. Rates less than 6F resulted in an unacceptable signal-to-noise ratio (data not shown). Figure 3 plots the relationship between number of blank frames and response amplitude for five individual albino rats (Fig. 3A) and for five pigmented rats (Fig. 3C). As more blank frames were used, producing a slower stimulation rate, the amplitude of the peak showed little consistent change, but often slightly increased. Figures 3B and 3D show that, in general, response latency decreased as the number of blank frames increased.

Stimulus Intensity

Examples of the summed responses obtained at each of the four luminance levels tested (3, 9, 27, and 81 cd/m²) are shown in Figure 4. As is apparent in Figure 4 and plotted in Figures 5A and 5C, the peak amplitude increased with increasing luminance up to 27 cd/m² for albino rats (Fig. 5A) and up to 81 cd/m² for three of five pigmented rats. In both albino and pigmented rats, luminances greater than 81 cd/m² resulted in a decreased peak amplitude (data not shown). As plotted in Figures 5B and 5D, peak latency decreased with increasing stimulus intensity in pigmented and albino rats.

Assessment of the Local Nature of the Response

Critical to the interpretation of mfERGs is an assessment of how well individual traces truly represent local activity. The cross-correlation analysis merely identifies the portion of the gross response that is correlated with the changes in illumination at each stimulus hexagon. If the light emitted from a single hexagon is subject to significant scatter within the eye, then neurons from distant areas could be contributing to the response of unassociated hexagons. To test this, a portion of the retina was inactivated either temporarily by photic bleaching or permanently by laser lesion, and mfERGs were recorded before and after the inactivation. Figure 6A shows a pretest trace array recorded from a pigmented rat before localized photic bleaching. After bleaching, the affected area was identified on the response array shown in Figure 6B (post-bleach...
test 1) as the group of relatively flat traces (in the upper left of the array) adjacent to remaining traces with normal-looking responses. Thus, after bleaching no response was obtained by stimulating this particular retinal location. If light emitting from the hexagons corresponding to the bleached area was scattering to other unbleached retinal regions, then a signal (generated by peripheral regions, but attributed to the particular hexagon) should have resulted in the corresponding areas. This was not the case, confirming that extracted responses are indicative of activity in a localized area of the retina due to stimulation from the corresponding hexagons. Figure 6C shows a trace array (post-bleach test 2) recorded from the same animal 20 minutes after the recording shown in 6B. The recovery of the response in the upper left region indicated that the flat responses in Figure 6B were not due to external factors (e.g., movement of the eye between the time before and after test 1). The results of a localized retinal laser lesion are shown for a pigmented rat in Figures 7A (prelesion) and 7B (postlesion). A seven-hexagon region (on the right, slightly above center) identifies an area of diminished signal amplitude compared with surrounding traces above, below and to the left. (That this local decrease was not caused by poorer recording conditions is evident from the quite large responses compared with the prelesion recordings at the left of the array.) Notable differences in elemental responses between Figure 6A and Figures 6B and 6C are most likely because of differences in the alignment of the retina with the stimulus monitor. To induce the lesion, the animal was taken out of the mfERG recording setup and moved to another apparatus for photic bleaching. However, for Figures 6B and 6C the animal was not moved.

Differences between the nonbleached regions in Figures 6B and 6C may be because of the scatter of bleaching light.

A consistent result obtained across stimulus conditions was that traces of considerably reduced amplitude corresponding to the lower two rows of the stimulus array were consistently recorded. It is most likely that this reduction was due to a blocking of the light from these rows of hexagons by the animal’s cheek. To show that the reduction in response was not an artifact related to the order in which the screen was painted (i.e., from top to bottom), some recordings were made with the monitor turned upside down. The pre- and post-bleaching recordings shown in Figure 6 were obtained using this configuration. Note that these results show diminished responses in the top two rows of the trace array. Because the VERIS software assumes the monitor is positioned upright, elemental responses shown at the top of each trace array correspond with stimulation in the lower portion of the rat’s visual field. Thus, regardless of the orientation of the monitor (and the order in which the screen was painted) the stimulus hexagons located at the bottom of the rat’s lower visual field consistently produced reduced-amplitude responses.

**Effect of Anesthesia Type**

Initially, all animals were anesthetized with sodium pentobarbital. In later sessions, ketamine-xylazine was used because it allows a faster recovery time. As shown in Figure 8, recordings obtained in animals under sodium pentobarbital anesthesia were similar to those obtained in those under ketamine-xylazine anesthesia, for both albino and pigmented rats.

**Effects of Dark Adaptation**

Traditionally, rod ERGs are obtained in the dark-adapted state. However, because the recording of mfERGs requires repeated presentation of a stimulus, it is by nature self-adapting. To determine the importance of dark adaptation, two albino rats were housed in the dark (during their normal light cycle) for 3 hours before testing and not exposed to light before the...
mfERG recording began. Figure 9 shows that the latency and amplitude of the peak were comparable in responses measured: after 3 hours of dark adaptation, after a subsequent 20 minutes of light adaptation, and after 40 minutes of light adaptation.

DISCUSSION

By using the procedures described in this report, functional topography of the rod-dominant rat retina can be assessed with mfERG. Despite some limitations associated with the small size of the rat eye and the inability to define precise retinal landmarks, this approach will be useful in assessing function in rat models of human retinal diseases (e.g., Reference 20).

The self-adapting nature of the mfERG stimulus provided a major challenge to the application of this technique to the rat. Because light adaptation has been shown to greatly reduce the amplitude of the rat full-field ERG,21 rod responses have been typically recorded from dark-adapted rats using single full-field flashes.22 This has produced optimal responses because the rod system is very slow to recover from light adaptation caused by prior stimulation, although adaptation, even to intense lights, does not saturate the rod responses.23 The standard recording conditions for the mfERG stimulus (successive flashes presented at high frequency) were designed to evaluate faster, photopic cone responses, and thus were inappropriate for use with rods. To maximize the rod response in the multifocal paradigm, the stimulus rate was slowed to allow for recovery of the rod system by the addition of dark blank frames to each element of the m-sequence (as described in Reference 6). This procedure resulted in easily measurable, positive b-wave-like responses that varied systematically with the intensity and temporal frequency of the stimulus. Prior dark adaptation had no effect on responses. The stimulus–response relationships and resultant waveforms were quite similar to rod mfERGs obtained in humans by Hood et al.6 The exception was that in rats, response latency decreased as the number of blank frames was increased. This was not reported in human rod mfERGs. In comparison with full-field ERGs, rat and human mfERG data were similar, in that the negative-going “a-wave” was either missing (rats) or negligible (humans), and in that the amplitude of the maximal response was reduced. The “a-wave” was most likely absent because the stimulus luminance was below a-wave threshold. The overall reduction in amplitude likely reflects the continued adaptation of some portion of the rod system, as well as reflecting the smaller retinal area that is activated by the multifocal stimulus compared with full-field stimulation.

The waveform obtained in rats is most certainly reflective of rod-mediated activity, which is not surprising, given the small proportion of cones in the rat retina.24 The absence of response to stimulus rates faster than 6F (cone mfERGs can be recorded at 0F) and the slow latency and small amplitude of the peak support the conclusion that this response originates from rods and not cones. The response latencies obtained (50–80 msec) were comparable to human rod mfERGs (60–100 msec) and were clearly longer than those obtained in human cone mfERGs5 (26–33 msec) or in mfERG recordings from the cone-dominated retina of the tree shrew.25 (ca. 20–30 msec). The particular cell type(s) that generate the positive-going responses cannot be identified from this study. It has
been suggested that the positive component of the full-field ERG is likely to be generated by rod bipolar cells.26

Light scatter presented a second major concern regarding the application of the mfERG to the rat. This problem is prevalent even in large eyes. For example, in humans, an mfERG response can be obtained “from” the optic disc, presumably representing activity in adjacent areas due to light scatter from nerve fibers at the disc. Also, because rods, unlike cones, are not sensitive to stimulus direction,27 they are especially sensitive to intraocular stray light. Consequently, both focal and multifocal human rod ERGs contain a large, slower response elicited by stray light.6,28 Although a slower stray-light component was not observed in rats, the small size of the rat eye increases the prevalence of light scatter, and an absence of pigmentation in the albino eye exacerbates the problem. It is likely that this is why no obvious reduction in amplitude was seen in the hexagon corresponding to the location of the optic nerve head. Because of these limitations it is not possible to define the exact size of a prescribed retinal area responding to an individual stimulus hexagon. However, the partial retinal bleaching experiment and the laser lesion experiment reported here in the pigmented rat showed that these manipulations produced clear localized changes in the response array. In fact, the appearance of the trace arrays resulting from these manipulations resembled published mfERG recordings from patients with retinitis pigmentosa (see Fig. 3 in Reference 9).

In sum, the present work demonstrated the feasibility and limitations of using mfERG to assess topographical changes in the rat retina. It showed that despite the problems of the unavoidable self-adapting nature of the stimulus, the small eye of the animal, and the high potential for light scatter within the retina, multifocal responses with a good signal-to-noise ratio can be obtained. The addition of blank frames increased recording time, but even with the addition of 10 blank frames and an m-sequence of $2^{12} - 1$ elements, a complete recording was obtainable in only 10 minutes.

Authors’ Note

Since the acceptance of our paper, a report of mfERG recordings in another rod-dominant rodent retina (the mouse) was published in this journal. (Nusinowitz S, Ridder WH III, Heckenlively JR. Rod multifocal electroretinograms in mice. Invest Ophthalmol Vis Sci. 1999;40:2848–2858.)

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References


