The Bioelectric Field of the Pattern Electroretinogram in the Mouse

Tsung-Han Chou and Vittorio Porciatti

PURPOSE. To compare the bioelectric field associated with the pattern electroretinogram (PERG) with that of the flash electroretinogram (FERG) in the mouse.

METHODS. PERGs and FERGs were recorded from each eye in 32 C57BL/6J mice using corneal silver loops referenced to a subcutaneous needle on the back of the head. PERG stimuli were horizontal gratings of 0.05 cycles per degree and 98% contrast reversing 2 times per second. Light-adapted FERG stimuli were bright strobe flashes. Stimuli were presented either monocularly or binocularly. In some experiments, TTX was injected in one eye and saline in the contralateral eye.

RESULTS. The PERG recorded from the contralateral, occluded eye had slightly larger amplitude ($1.14 \times$, $P < 0.01$) and longer latency ($+1.57$ ms, $P < 0.01$) compared with the ipsilateral eye. Under binocular stimulation, the PERG amplitude was much larger ($1.67 \times$, $P < 0.01$) than the monocular amplitude. TTX injected in the stimulated eye drastically reduced the PERG in both eyes. Monocular FERGs were recordable from the stimulated eye only and were moderately reduced by TTX. Binocular and monocular FERGs had similar amplitudes.

CONCLUSIONS. PERG and FERG generate different bioelectric fields in the mouse. The PERG bioelectric field is consistent with a dipole model whose axis is orthogonal to the eye axis, whereas the standard dipole model for the FERG is coaxial. Possible sources of the PERG bioelectric field are unmyelinated optic nerve axons adjacent to the sclera. Results provide new insights on the generators of the PERG signal and its alterations in mouse models of glaucoma and optic nerve diseases. (*Invest Ophthalmol Vis Sci.* 2012;53:8086–8092) DOI:10.1167/iov.12-10720

Neural processing in the retina in response to diffuse flashes of light generates an electric field throughout the eye and surrounding tissue that can be recorded noninvasively from the cornea as a flash-evoked electroretinogram (FERG). A well-established model for the FERG is that extracellular currents generated from radially oriented retinal elements, such as photoreceptors, bipolar cells, and Müller cells, will

sum up, thereby generating an electrical field whose axis is aligned with the eye axis, the positive pole being on the cornea and the negative pole on the back of the eye.^{1–3} The electroretinogram in response to patterned visual stimuli reversing in contrast at constant mean luminance (pattern electroretinogram, PERG) is fundamentally different from the FERG.^{4–6} Although retinal ganglion cells (RGCs) contribute little to the a- and b-wave of the standard FERG, they dominate the PERG. Indeed, the PERG signal is greatly reduced when RGCs are selectively degenerated after optic nerve lesion.^{4,7–9} The PERG, therefore, is extensively used to probe RGC function in clinical and experimental models of glaucoma and optic neuropathies.^{10–14}

Despite the widespread use of the PERG technique, the actual generators of the PERG signal are still not well understood. Concurrently, little is known on the nature of the bioelectrical field associated with the PERG signal. Intraretinal RGC axons are oriented orthogonally to photoreceptors, bipolar cells, and Müller cells, which are the main sources of extracellular currents underlying the FERG signal. Thus, it is possible the PERG bioelectric field differs from the FERG bioelectric field. We were drawn into this problem by the serendipitous finding that binocular pattern stimulation to record simultaneous PERGs from each eye in the mouse generated much larger signals compared with monocular stimulation. Binocular augmentation of the PERG signal did not occur with the FERG signal. Here we systematically investigate interocular interactions of PERG/FERG signals in the mouse model, in which the proximity of the two eyes may result in substantial overlap of the monocular bioelectric fields and emphasize the problem.

METHODS

Animals and Husbandry

All procedures were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals in ophthalmic and vision research. The experimental protocol was approved by the Animal Care and Use Committee of the University of Miami. A total of 32 mice (C57BL/6J; Jackson Laboratories, Bar Harbor, ME) were tested. Mice were maintained in a cyclic light environment (12 hours light at 50 lux–12 hours dark) and fed ad libitum.

Pattern Electroretinogram Recording

Detailed description of the PERG technique is reported elsewhere.¹⁵ In brief, mice were weighed and anesthetized with intraperitoneal injections (0.5–0.7 mL/kg) of a mixture of ketamine (42.8 mg/mL) and xylazine (8.6 mg/mL). Mice were then gently restrained in a custom-made holder that allowed unobstructed vision. The body of the animal was kept at a constant body temperature of 37.0°C using a feedback-controlled heating pad (TCAT-2LV; Physitemp Instruments, Inc., Clifton, NJ).

A PERG electrode (0.25-mm diameter silver wire [World Precision Instruments, Sarasota, FL] configured to a semicircular loop of 2-mm

From the Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida.

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Corresponding author: Vittorio Porciatti, Bascom Palmer Eye Institute, McKnight Vision Research Center, 1638 NW 10th Avenue, Room 201D, Miami, FL 33136; vporciatti@med.miami.edu.

radius) was leaned against the extrapupillary corneal surface by means of a micromanipulator. A small drop of balanced saline was topically applied every 30 minutes to prevent corneal dryness. The reference electrode was a subcutaneous stainless steel needle (Grass, West Warwick, RI) inserted in the scalp of the back of the head along the midline. The ground electrode was a subcutaneous stainless steel needle placed at the root of the tail. We considered the reference electrode as indifferent, as no consistent signal could be recorded in response to the pattern stimulus that elicited robust PERGs. PERG waveforms consisted of two main waves: a positive wave with a peak latency of 90 to 100 ms (P1) followed by a broad negative with peak latency in the range of 200 to 300 ms (N2).^{15,16} Peak latency of the N2 wave was sometimes difficult to identify with precision due to noise intrusion and we did not include it in the analysis. Miura and collaborators¹⁷ used a corneal electrode on the contralateral eye as reference to record the mouse PERG (interocular PERG). As it will be evident in the results section, the PERG recorded from the contralateral, nonstimulated eye is systematically larger in amplitude and longer in latency compared with the ipsilateral PERG as result of cross-talk. Thus, we did not use the Miura et al.¹⁷ interocular approach, as it produces a different waveform that is related to, but very different from, the true PERG signal.

Visual stimuli consisted of contrast-reversing (1 Hz, two reversals) horizontal bars generated by a programmable graphic card (VSG; Cambridge Research Systems Ltd., Rochester, UK) on a cathode-ray tube (CRT) display (Sony Multiscan 500; Sony Electronics Inc., San Diego, CA) with the center aligned with the projection of the pupil. The pupils were not dilated, and eyes were not refracted for the viewing distance since the mouse eye has a large depth of focus.^{18,19} At the viewing distance of 15 cm, the stimulus field covered an area of $69.4^\circ \times 63.4^\circ$. Patterns had fixed mean luminance of 50 cd/m² and 98% contrast, spatial frequency at 0.05 cycles per degree, and temporal frequency at 1 Hz. The luminance of the CRT display was γ -corrected using a photometer (OptiCal OP200-E; Cambridge Research Systems Ltd.). Contrast was defined as $C = (L_{\max} - L_{\min}) / (L_{\max} + L_{\min})$, where $L_{\max}$ = luminance of the bright stripes and $L_{\min}$ = luminance of the dark stripes. Three consecutive PERG responses to 600 contrast reversals each were recorded. The PERG responses were superimposed to check for consistency and then averaged (1800 sweeps). A light-adapted flash ERG (FERG) was also recorded with undilated pupils in response to strobe flashes of 20 cd/m²/s delivered at 0.5 Hz and superimposed on a steady background light of 12 cd/m² within a Ganzfeld bowl. Under these conditions, rod activity is largely suppressed, whereas cone activity is minimally suppressed. Three consecutive FERG responses to 60 light flashes each were recorded (180 sweeps). Averaged PERG and FERG waveforms were automatically analyzed with SigmaPlot version 11 software (Systat Software Inc., San Jose, CA) to identify the major positive and negative waves and calculate the sum of their absolute values (peak-to-trough amplitude). For both PERG and FERG, a "noise" response was also obtained with the stimulus occluded. The noise amplitude averaged over all mice ($n = 32$) was, PERG = 2.5 μ V; FERG = 4.7 μ V. In some experiments, tetrodotoxin (TTX, 1 μ L, 5 μ M) was intravitreally injected in the stimulated eye using a Hamilton syringe connected to a fine needle (33 gauge/0.375 inch/point style 4). An equal volume of saline was injected in the contralateral occluded eye. PERG and FERG were recorded 10 to 15 minutes after injections.

RESULTS

The PERG, but Not the FERG, Displays Interocular Cross-Talk and Binocular Summation

Our working hypotheses were the following: (1) under monocular stimulation, the PERG is recordable from both the ipsilateral and contralateral eye, whereas the FERG is recordable from the ipsilateral eye only, and (2) under

binocular stimulation the PERG signal is recordable from both eyes with larger amplitude compared with monocular stimulation, whereas the FERG signal has similar amplitude under monocular and binocular stimulation. Results obtained from testing these hypotheses are summarized in Figure 1.

As shown in the top panel of Figure 1A, pattern stimulation of the right eye elicited PERGs with similar waveform and amplitude from both ipsilateral and contralateral occluded eye. Binocular pattern stimulation elicited PERGs with similar waveform in either eye, whose amplitude was much larger than the monocular PERG amplitude (Fig. 1B). In contrast, flash stimulation of the right eye elicited a recordable FERG for the ipsilateral eye only (Fig. 1C). The PERGs in response to binocular flash stimulation had amplitudes similar to the monocular FERG (Fig. 1D). The bottom panel of Figure 1 shows the normalized averages of monocular and binocular PERGs/FERGs according to the sketch depicted in the top panel. The PERG recorded from the contralateral, occluded eye had an amplitude slightly larger ($1.14 \times$, 95% confidence interval [CI] 1.056–1.218, $P < 0.001$) and a longer latency ($+1.57$ ms, 95% CI 0.67–2.47, $P < 0.001$) than that recorded from the ipsilateral, stimulated eye. The PERG recorded under the binocular stimulation eye had an amplitude much larger ($1.67 \times$, 95% CI 1.232–2.113) than that of monocular PERG ($P < 0.001$). The FERG amplitude in the contralateral, occluded eye was much smaller ($0.16 \times$, 95% CI 0.096–0.23) than that of the ipsilateral eye ($P < 0.001$), and not different from the noise in response to an occluded stimulus. Under binocular stimulation, the FERG amplitude was not significantly different from that obtained under monocular stimulation. Altogether, results summarized in Figure 1 indicate that PERG, differently from FERG, displays clear interocular cross-talk and binocular summation.

Comparison between Ipsilateral and Contralateral PERG Waveforms

Ipsilateral and contralateral PERG waveforms were compared by calculating their difference. As shown in Figure 2, different waveforms were remarkably consistent among mice. This would mean that the contralateral PERG could be entirely predicted by the ipsilateral PERG and represents a slightly larger and delayed version of it. Small differences in the waveforms of the two eyes can be attributed to noise intrusion.

The PERG Cross-Talk Originates from Passive Spreading of Spiking Activity in the Inner Retina

As a control that the PERG cross-talk originated from PERG generators different from FERG generators, we blocked action potentials in the inner retina by intravitreally injecting TTX in the stimulated right eye, as sketched in Figure 3. An equal volume of saline was injected in the left eye. Previous studies have shown that TTX (a neurotoxin that abolishes action potentials in the inner retina) drastically reduces the PERG signal but to a lesser extent the FERG signal. Pattern stimulation of the TTX-injected eye did not generate a PERG significantly larger than the noise level, and the interocular cross-talk was also abolished (Fig. 3A). However, pattern stimulation of the saline-injected eye generated a normal PERG that was also recordable with similar amplitude and waveform from the TTX-injected eye (Fig. 3B). The FERG was moderately affected by TTX injection (Saline-FERG = 70.329 μ V [SEM 7.1]; TTX-FERG = 42.732 μ V [SEM 4.6], $P < 0.01$) and did not transfer to the contralateral, unstimulated eye (Figs. 3C, 3D). Altogether, this experiment suggested that the PERG signal originates from TTX-sensitive generators in the inner retina of

FIGURE 1. Cross-talk (A) and binocular summation (B) characterize the PERG but not FERG (C, D). *Top panel:* Sketch of the experimental protocol and representative examples of PERG and FERG responses obtained under either monocular or binocular stimulation. *Bottom panel:* Normalized mean ($\pm$ SEM) amplitudes of PERG and FERG signals obtained under either monocular or binocular stimulation ($n = 14$). For both PERG and FERG, the reference measure for normalization was the amplitude recorded from the right eye under monocular stimulation. For all conditions, active electrodes were silver loops leaned against the corneal surface, and the reference electrode was a subcutaneous stainless steel needle on the back of the head.

FIGURE 2. (A) Representative PERG waveforms recorded from the eye ipsilateral and contralateral to the pattern stimulus and their interocular (Ipsi minus contra) difference waveform. (B) Interocular difference waveforms for all mice tested.

FIGURE 3. Effect of TTX intravitreal injection on PERG and FERG. *Top panel:* Sketch of the experimental protocol and representative examples of PERG and FERG responses. *Bottom panel:* Mean ($\pm$ SEM) amplitudes of PERG and FERG signals obtained under monocular stimulation ($n = 4$). For all conditions, TTX was injected in the right eye and an equivalent volume of saline was injected in the left eye. Active electrodes were silver loops leaned against the corneal surface, and the reference electrode was a subcutaneous stainless steel needle on the back of the head. The *horizontal dashed lines* represent the average noise signal recorded in response to an occluded stimulus.

the stimulated eye and is passively transferred to the contralateral, unstimulated eye.

PERG Cross-Talk and Binocular Summation Are Consistent with a Dipole Model Orthogonal to That of the FERG

The electric field associated with the FERG consists of a distribution of currents around a charged object (dipole).^{1-3,20} In its simplest form, the charged object can be represented by a vector with a given orientation in space and with magnitude proportional to its charge. For the FERG, this can be depicted by an arrow whose orientation is coaxial with the eye, the positive pole being on the cornea. As the mouse eyes are oriented approximately 60 degrees lateral to the midline,²¹⁻²³ the geometry of FERG dipoles can be conceptually sketched (Fig. 4). With binocular flash stimulation, the electric fields associated with each eye dipole will generate an interaction field. In Figure 4, binocular interaction can be calculated as the vectorial sum of monocular dipoles. Monocular vectors are represented by two adjacent sides or a parallelogram whose diagonal will be equal to the resultant of these two vectors (binocular vector). The resulting binocular vector will be oriented along the midline, and its magnitude will be $Bin = (OD^2 + OS^2 + 2 \cdot OD \cdot OS \cdot \cos(\alpha))^{0.5}$, where α is the angle subtended by the two vectors. If the magnitude of monocular vectors is set = 1, then the magnitude of binocular vectors for the FERG will be $Bin = 1.0$. This prediction is indeed fulfilled

experimentally by calculating the ratio between binocular and monocular amplitudes (binocular FERG/monocular FERG = 1.0). For the PERG, however, the conventional dipole model of the flash-ERG is difficult to reconcile with experimental data. To account for interocular PERG cross-talk and binocular summation, we propose a PERG dipole whose orientation is orthogonal to the eye axis (Fig. 4). According to this proposed model, binocular pattern stimulation will generate a binocular vector of magnitude $Bin = 1.73$. This theoretical value is very close to the value determined experimentally by calculating the ratio between binocular and monocular amplitudes (binocular PERG/monocular PERG = 1.67). From Figure 4, it is also apparent that that monocular flash stimulation is expected to generate little or no FERG signal on the contralateral eye, whereas monocular pattern stimulation will generate a robust PERG signal on the contralateral eye, in agreement with experimental data.

The PERG Signal Is Widely Distributed over the Anterior Part of the Head

To better understand the distribution of the PERG signal over the head, we recorded from multiple locations, including temples and snout. Figure 5 shows that the PERG signal is widely distributed over the anterior part of the head, with a maximum at the level of the contralateral eye. These results are consistent with the PERG dipole model shown in Figure 4.

FIGURE 4. *Top panels:* Conceptual model for electrical dipoles associated with FERG and PERG. In mice, the eye axes are oriented approximately 60 degrees lateral to the midline. The FERG dipole is coaxial with the eye axis, whereas the PERG dipole is orthogonal to it. *Bottom panels:* Parallelograms showing the vectorial summation of monocular dipoles when both eyes are simultaneously stimulated. In the FERG, the magnitude of the sum vector (*thick vertical arrow*) is identical to that of the monocular vectors. In the PERG, the magnitude of the sum vector is 1.73 times larger than that of the monocular vectors.

DISCUSSION

The bioelectric field of the flash-evoked ERG is well described by a simple dipole model in which currents generated by active retinal elements are passively conducted throughout the eye and extraocular tissues. The dipole has the same orientation as the eye axis, with the positive pole at the center of the cornea and the negative pole at the back of the eye.^{2,3,20} The bioelectric field of the flash-evoked ERG has a limited lateral distribution, so that little signal is recordable from the contralateral unstimulated eye. Indeed, Flash-ERGs are commonly obtained in mice using simultaneous stimulation and

recording from both eyes,^{24,25} implying absence of measurable intraocular cross-talk and binocular summation. In keeping with this notion, the present results show that the FERG is not recordable from the contralateral, unstimulated eye. In addition, the FERG does not show summation under binocular stimulation.

In contrast, the PERG is recordable from the contralateral, nonstimulated eye with significantly larger amplitude and longer latency compared with the ipsilateral eye; moreover, the PERG displays strong summation under binocular stimulation. These results indicate that (1) the mouse PERG has an obvious interocular cross-talk, and (2) the monocular PERG bioelectric

FIGURE 5. Distribution of the PERG signal in response to monocular stimulation. Normalized means $\pm$ SEM of PERG amplitudes recorded from each eye, each temple, and snout. The reference measure for normalization was the amplitude recorded from the right eye. Active electrodes were silver loops (cornea) or subcutaneous stainless steel needles (other locations). The reference electrode was a subcutaneous stainless steel needle on the back of the head.

fields of the two eyes are oriented in such a way as to generate a binocular interaction field with larger currents compared with monocular fields. It should be taken into account that that, in mice, the eyes are oriented approximately 60 degrees lateral to the midline.^{21–23} Cross-talk and binocular summation can be explained if we assume that the bioelectric field of the monocular PERG is a simple dipole whose axis is orthogonal with eye axis, thus converging to the midline at an angle of approximately 30 degrees (Fig. 4). This would generate a signal on both eyes, with a bias on the contralateral eye. Binocular summation can be explained by vectorial summation of converging monocular bioelectric fields, the resulting dipole being oriented along the midline. The magnitude of binocular summation found experimentally ($1.67 \times$) virtually coincides with the theoretical calculation ($1.73 \times$).

Orthogonality of the PERG bioelectrical field compared with the eye axis requires an explanation different from that provided by the classical dipole model of for the Flash-ERG. The Flash-ERG is thought to be dominated by the summed activity of radially oriented neurons, such as photoreceptors, bipolar cells, and glial Müller cells. The PERG, in contrast, is dominated by the activity of RGCs and their axons. That electrical activity of RGC axons dominates the PERG is also demonstrated by the present finding that TTX drastically reduces the PERG signal but reduces the FERG to a smaller extent (in agreement with Miura et al.¹⁷). Miura and collaborators¹⁷ used a corneal electrode on the contralateral eye as reference to record the mouse PERG, similarly to the interocular approach frequently used in human studies. As shown by the present results, the contralateral eye is not indifferent. Thus, the interocular approach in mice results in a difference waveform that is related to, but very different from, the true PERG signal. It should be noted that the FERG of human and nonhuman primates includes a late negative wave (called photopic negative response or PhNR) that is selectively reduced in conditions that impair RGC activity, such as glaucoma and TTX treatment.^{26,27} There is little evidence that, in the mouse, the PhNR is selectively affected in the same conditions.^{15,17} The present study also shows that TTX does not selectively impair the mouse PhNR.

In the mouse retina, the optic disc is located centrally, coinciding with the eye axis; intraretinal RGC axons symmetrically converge to the optic disc from all retinal sectors. Therefore, lateral currents generated by intraretinal RGC axons are expected to cancel each other in the integrated PERG response. Once RGC axons enter the optic disc, however, their currents are expected to sum up and represent a substantial source of extracellular electrical current, particularly in the unmyelinated portion of the optic nerve immediately adjacent to the sclera (0.6–0.8 mm according to May and Lutjen-Drecoll²⁸). We propose that these extraretinal, unmyelinated RGC axons represent the main source of extracellular currents associated with the PERG. As intraorbital tissues, such as orbital fat, Harderian gland, and myelinated optic nerve all have high impedance, extracellular currents may find their path of least resistance by flowing tangentially to the back of the eye toward the snout and returning to the eye through extraocular tissues. This would explain orthogonality of the PERG electrical field compared with the eye axis and the widespread distribution of the PERG signal over the anterior part of the head. The presence of ERG signals originating from unmyelinated axons in the optic nerve head has been previously proposed by Sutter and Bearse,²⁹ based on their observations of signal delays in the human multifocal ERG (mfERG) related to distance of the optic nerve head (OHNC). Hood et al.³⁰ working on macaque mfERG OHNC, provided some supportive evidence that the signal travels out of the globe.

In conclusion, this study shows that PERG and FERG generate remarkably different bioelectric fields in the mouse, suggesting different electrogenesis. This is a novel finding that adds further interest to the PERG technique as an in vivo, noninvasive tool to probe the physiological activity of RGCs.^{13,14,31–35} Current models of flash-ERG electrogenesis do not appear adequate to explain how volume conduction of extracellular currents links the PERG source with its field. Here we propose that the main source of PERG signal originates from the unmyelinated portion of the optic nerve axons in the optic nerve head, as this hypothesis has a reasonable anatomical basis and accounts well for main experimental findings of cross-talk, binocular summation, and TTX effect. We are aware that the proposed PERG model is based on indirect evidence only. In addition, a more refined model should account for volume conductor inhomogeneities and provide a numerical solution for the bioelectrical field. This is a complex issue that requires knowledge of conductivities of different tissues included in the bioelectrical field as well as their relative geometry. We plan to address this issue in a future study. It is possible that the effects measured are emphasized in the mouse model due the proximity of the eyes, their laterality, and the unique morphology of the optic nerve head compared with other species.²⁸ In the human model, the PERG shows neither substantial cross-talk nor binocular summation.^{36,37}

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