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The development of mid-wavelength photoresponsivity in the mouse retina

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Abstract

Purpose—Photoreceptors in the mouse retina express much of the molecular machinery necessary for phototransduction and glutamatergic transmission prior to eye opening at postnatal day 13. Light responses have been observed collectively from rod and cone photoreceptors via electroretinogram recordings as early as postnatal day 13 (P13) in mouse, and the responses are known to become more robust with maturation, reaching a mature state by P30. Photocurrents from single rod outer segments have been recorded at P12, but no earlier, and similar studies on cone photoreceptors have been done, but only in the adult mouse retina. In this study, we wanted to document the earliest time point in which outer retinal photoreceptors in the mouse retina begin to respond to mid-wavelength light.

Methods—*Ex-vivo* electroretinogram recordings were made from isolated mouse retinæ at P7, P8, P9, P10 and P30 at seven different flash energies (561 nm). The a-wave was pharmacologically isolated and measured at each developmental time point across all flash energies.

Results—Outer-retinal photoreceptors generated a detectable response to mid-wavelength light as early as P8, but only at photopic flash energies. A-wave intensity response curves and kinetic response properties are similar to the mature retina as early as P10.

Conclusion—These data represent the earliest recorded outer retinal light responses in the rodent. Photoreceptors are electrically functional and photoresponsive prior to eye opening, and much earlier than previously thought. Prior to eye opening, critical developmental processes occur that have been thought to be independent of outer retinal photic modulation. However, these data suggest light acting through outer-retinal photoreceptors has the potential to shape these critical developmental processes.

Keywords

ex-vivo electroretinogram; photoreceptor; a-wave; postnatal; outer retinal dendrite; melanopsin

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Declaration of Interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Recent work in developmental neuroscience has demonstrated that light can facilitate circuit wiring of the early postnatal visual system. Specifically, studies in mice have shown that light can modulate retinal wave activity and assist in the segregation of retinogeniculate projections to discrete ocular domains even prior to eye opening at postnatal day 13 (P13) (1, 2). Other studies have shown that light can regulate vascular patterning in the retina (3) and can induce negative phototaxis in mice prior to eye opening (4). These light-mediated developmental processes and behaviors in every case have been shown to be due to melanopsin ganglion cells, a novel subclass of retinal ganglion cells that express the photopigment melanopsin within their cellular membrane (5, 6). Light is thought to penetrate through the semi-transparent, closed, pup eyelid and activate these melanopsin ganglion cells between the ages of P0 and P12 (4, 7).

Recent studies from our group have found anatomical evidence for a direct circuit from cones to a small population of M1-type melanopsin ganglion cells in the dorsal retina between P8 and P12 (8, 9). These studies suggest cones may be able bypass bipolar cells and signal ganglion cells directly, should these cones have the capacity to respond to light in this developmental window of time. However, one important missing link in this story is whether cones are functional prior to P10-P13.

Much work has been done to characterize the development of photoreceptors and document their function as the mouse begins to open its eyes and navigate its environment. Physiological studies in the outer retina of young adult mice using electroretinograms (ERGs) and suction electrode techniques have been performed over the past few decades (10-18). Findings have confirmed that photoresponses mature over the few weeks following eye-opening (P12-P14), becoming relatively stable in terms of kinetics and sensitivity around P30 (adults) (14, 19). Relatively few experiments have been done in the developing mouse retina prior to eye opening. *In-vivo* a-wave ERG responses from P9 and P10 retinas have been recorded in the anesthetized mouse, and bright white light flashes induce an a-wave with an amplitude of around 9 μ V at P10, with no detectable response found at P9 (20). These data suggest P10 is the earliest possible age at which the outer retina can detect white light, and downstream in the ganglion cell layer outer-retinal light-mediated signals have been detected in retinal ganglion cells as early as P10 in the mouse (21). With the development of more sensitive *ex-vivo* ERG recording configurations (14), the primary goal of this study was to determine the earliest developmental time point at which the outer retina can respond to mid-wavelength light in the mouse eye.

Methods

Animals

Experiments were conducted according to the NIH guidelines. Animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of Akron. 31 Male and female C57BL/6R mice of ages P7, P8, P9, P10, and P30 were used in this study. Animals were kept on a 12/12 dark/light cycle and prior to experiments, mice were

dark adapted for 6-12 hours. Mouse pups were sacrificed using a pentobarbital-sodium solution (0.2mL; Fatal-Plus, Vortech Pharmaceuticals) and P30 mice were sacrificed with CO₂ asphyxiation. All experiments were conducted between 12 pm and 3 pm EST.

Perfusion

Perfusion solution (pH=7.4) containing AMES medium (catalog A1372-25; US Biological, Salem, MA), glucose (10 mM; catalog G5416; Sigma Aldrich, St. Louis, MO), barium chloride to remove the slow PIII component of the wave (22) (100 μ M; catalog B0750; Sigma Aldrich, St. Louis, MO) and LAP4 to block the b-wave (40 μ M; catalog 0103; Tocris, Minneapolis, MN) was buffered with sodium bicarbonate (22.3 mM; catalog BP328-1; Fisher Scientific, Hampton, NH), bubbled with 95% O₂/5% CO₂ (14), and set with a flow rate of approximately 3mL/min. The solution was heated with an inline heater (SH-27B and TC-324C; Warner Instruments, Hamden, CT) to ensure that the solution in the chamber remained between 35-37 °C throughout the experiment.

Ex-vivo ERG configuration and transretinal recordings

All experimental procedures were completed under dim-red light (660 nm LED with a band pass range of 610-690nm; catalog LED660N-03; Roithner LaserTechnik, Vienna, Austria) to retain the dark-adapted state of the mice. Eyes were removed making sure that the optic nerve stayed connected to the back of the eye. Eyes were then placed in carbogenated AMES solution and further dissected for complete isolation of the retina from the eye cup. Retinas were then carefully placed photoreceptor-side up onto lens paper with a pre-made hole, and mounted onto a custom-made 2-part ERG chamber (23). A recording electrode (bare Ag/AgCl wire) sealed with wax inside a glass capillary was lowered onto the photoreceptor layer close to the center of the retina, making slight contact with the retina, as this orientation has been shown to significantly increase response amplitudes compared to recording from edges of the retina (24). This process was aided with the use of an OptiMOS IR camera (QImaging, Surrey, BC, Canada) fitted onto the microscope (BX51WI, Olympus, Center Valley, PA) and a micro manipulator (MP-220, Sutter Instruments; Novato, CA). Recordings were low-pass filtered at 300 Hz and data was acquired at 1000 Hz through the Multiclamp 700B amplifier, miniDigidata digitizer, computer, and Clampex 10 software (Molecular Devices, Sunnyvale, CA).

Stimulus Calibration and Configuration

Light from the LED system (X-Cite 120 LED, Lumen Dynamics; Mississauga, ON, Canada) was calibrated to desired flash energy using an S470 flexOptometer with photometric luminance sensor (265M; Gamma Scientific; San Diego, CA). The light source was bandpass filtered (561 nm center wavelength; 48 nm bandpass; catalog 49008; Chroma, Bellows Falls, VT). A series of neutral density filters (catalog 15358, 15164, 15201, Edmund Optics, Barrington, NJ) were placed in the path of light starting with -5 log (cd s m⁻²) and removed sequentially to achieve a range of flash energies ending with the maximal energy (0.85 cd s m⁻²). Flash energies ranged from -5.0 to -1.9 log (cd s m⁻²) for dim flashes, and -1.0 to 0.85 (cd s m⁻²) for saturating flashes. For each retina, 3 trials were conducted at the saturating flash energies and 5 trials were conducted at the dim flash energies and averages at each energy were used for data analysis.

Data Analysis

Responses were filtered, averaged, and analyzed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA). For all parameters, baseline was determined as the average voltage over the 1000 ms prior to stimulus onset. The light response was determined to begin at the time point at which the voltage differed by 5 standard deviations from the calculated baseline. A-wave responses were determined as the area under the curve between the points at which the voltage trace first crossed baseline in the negative direction and at the point where the trace first returned to baseline. Peak a-wave values were determined by comparing the maximum change in voltage to baseline. Time-to-peak values were calculated as the time between stimulus onset and the time point at which the peak amplitude occurred. Latency to onset was determined by subtracting the initial light response time (5x SD of baseline + baseline) from the start of the stimulus time.

Intensity response relationships were plotted and fitted with a model function as previously reported (24):

$$\frac{R}{R_{max}} = \alpha(1 - e^{-SI}) + (1 - \alpha)\left(\frac{I}{I + S^2I}\right)$$

Where R is the peak amplitude of the response, R_{max} is the maximum or saturating value of R, I the stimulus light intensity, S is fractional sensitivity, which is the inverse of the stimulus intensity that elicits a response with half of the saturating amplitude, and α is a temperature-dependent weighting coefficient ($0 < \alpha < 1$) (25). This model function is a weighted sum of Michaelis and exponential saturation functions curves and provided a better fit for our early post-natal photoresponse data in the low intensity range compared to either the Michaelis or exponential functions alone (24).

Statistics were calculated with a One-way ANOVA using the Tukey-Kramer Multiple Comparisons Test. Measurements for a-wave response and peak a-wave response were calculated at a saturating energy whereas time-to-peak and latency to onset measurements were made at dim (linear) energies.

Results

We found that a-wave photoresponses can be induced as early as P8 in the isolated dark-adapted mouse retina (Figure 1A). These responses were elicited in response to a 20 ms flash of mid-wavelength light (561 nm) in the presence of synaptic blockade of downstream retinal synapses. These photoresponses at P8 were small in amplitude, averaging 5.33 ± 0.95 μ V. However, the amplitude increased in size each subsequent day of postnatal development and reached an average response of 19.86 ± 2.28 μ V in P10 retinas (Figure 1A, Table 1). By comparison, the adult dark-adapted a-wave response evoked from P30 retinas were significantly larger than these early photoresponses (242.58 ± 39.74 μ V; Figure 1B, Table 1, $n = 7$). While there was a small, ($2.57 \pm .81$ μ V) but detectable response from the adult retina at the lowest energy of $-5 \log(\text{cd s m}^{-2})$, and maximal/saturating responses were evoked at $0.85 \log(\text{cd s m}^{-2})$; Figure 1C, Figure 1D). Overall, the a-wave amplitudes were dependent both on the flash energy as well as postnatal age (Figure 1 and 2).

The peak amplitudes in Figure 1C-D were used to construct intensity response relationships in Figure 2. An increase in response amplitude and an increase in sensitivity from P8 to P30 can be observed in Figures 2A-C. Based on the intensity-response model function of normalized data shown in Figure 2C, the flash energy that elicited a photoresponse with half of the saturating amplitude was 0.815, 0.082, 0.014 and 0.010 cd s m^{-2} at P8, P9, P10 and P30, respectively, indicating an increase in sensitivity from P8 to P10 (Figure 2C). Sensitivity increased by 1 log unit from P8 to P9 and by half a log unit from P9 to P10. The half saturating sensitivity is near the adult (P30) level of 0.01 cd s m^{-2} suggesting near adult – like photosensitivity at the 10th postnatal day, despite a much smaller total response amplitude in the P10 retina compared to adult (Figure 2C). However, caution should be taken when extracting, interpreting, and comparing exact values from sensitivity functions designed to interpret adult retina to the early postnatal retina when many developmental changes are occurring.

The kinetics of the dim flash response slowed over the course of development from P8 to P30 (Figure 3). In adult retina, the dim flash kinetics elicited in the linear range of the intensity response relation reflect phototransduction activity (26). The time-to-peak, shown in Figure 3C and Table 1, increased significantly over the course of development from 60.73 ± 13.35 ms at P8 to 83.98 ± 2.58 ms at P10, and 118.47 ± 7.49 ms at P30, suggesting a slowing of the phototransduction cascade over development. This is consistent with the increase in sensitivity from P8 to P10 (Figure 2C). In contrast, the latency to onset did not change significantly from P8 to P30 (Figure 3D and Table 1).

Discussion

We document outer retina photoresponses in the P8 mouse retina via *ex-vivo* electroretinogram recordings; this is the earliest outer-retinal light responses observed in a rodent (20, 27). Moreover, the development of photoresponsivity we observe is two days earlier than previously thought, and occurs at a time when the outer retina is presumably not yet connected to downstream retinal neurons. This work suggests that light has the ability to modulate critical developmental processes that occur prior to eye opening. Outer retinal photoreceptors in the mouse retina begin to respond to mid-wavelength light at P8. Over the next several postnatal days these response properties mature to the point at which, by P10, response kinetics and sensitivity begin to resemble those found in the mature retina (Figure 2C, Table 1 and (12, 14, 15), albeit at much lower total a-wave amplitudes. Responses at these young ages were considered to be photopic in nature as the flash energy required to reach threshold and evoke an a-wave at P8, P9 and P10 were greater than -1 log (cd s m^{-2}) (13, 14).

While mouse and human retina develop at vastly different rates (28), major developmental events of the ERG waveform can be compared between the two species. The a-wave (5-10 μV) and b-wave (30-80 μV) peak amplitudes have been recorded in premature infants of a 34 week gestational age (29). These data compare to amplitudes we have presented here with an average a-wave of around 5 μV in P8 mice. In newborn infants, scotopic and photopic a-wave responses reach 25-30 μV , while b-waves have increased to roughly 90 μV (30, 31). These values compare closely to waveform amplitudes seen in mice at around P10

(mouse age equivalence to human newborns), with our recorded a-wave averages $19.86 \pm 2.28 \mu\text{V}$ by P10. Additionally, these values compare closely to similar *in-vivo* studies in mice showing a-wave amplitudes around $50 \mu\text{V}$ in P13 mice, right at the time of eye opening (19). Taken together, these data suggest that light-mediated effects on the development of the mouse visual system could be a correlate of a light-mediated effect on the development of the human visual system.

Rhodopsin or m-opsin mediated response?

In the mature retina, both rods and m-opsin expressing cones respond to 561 nm light, raising the question as to which cell type might be generating the first outer-retinal photoresponses. Whole-retina quantitative RT-PCR experiments have found detectable amounts of rhodopsin mRNA at P7, though expression is less than 4% relative to expression in the mature retina (32). At P15 expression is at 50% and by P30 rhodopsin mRNA has reached adult expression levels (32). These mRNA expression levels compare favorably to immunohistochemical analysis of rhodopsin protein expression in retinal sections that suggest minimal amounts protein expression at P7 and significant amounts of protein expression at P14 (33). The G-protein activated by rhodopsin during phototransduction is rod transducin, which is coded by the *Gnat1* gene. There is no *Gnat1* mRNA expression at P7 as assayed in whole-retina quantitative RT-PCR experiments, and less than 20% expression relative to the mature retina by P15 (32). However, a more recent study examined *Gnat1* RNA expression with *in situ* hybridization in sections of retinal tissue and found low levels of *Gnat1* as early as P7 (33). This is surprising as rod outer segments have been reported as variable in length, not vertically aligned, and are less than $4 \mu\text{m}$ at P11, while they elongate to approximately $22 \mu\text{m}$ in the mature retina (34, 35). Cone outer segments begin forming by P4, their pedicles appear around P7, and overall morphology is adult-like by P10 (36). Taken together, these data suggest that the rod system has probably not matured to the point that it could generate a detectable light response in an *ex-vivo* ERG recording configuration between P8 and P10.

We speculate that the earliest detectable responses to mid-wavelength light are cone-mediated. M-opsin expression is driven by the gene *Opn1mw*. An *in situ* hybridization study found detectable levels of *Opn1mw* RNA in the dorsal retina as early as P0, and substantial amounts of expression in both the dorsal and ventral retina by P7 (33). Expression of m-opsin was consistent with *in situ* hybridization data. Appreciable amounts of m-opsin were immunohistochemically identifiable as early as P7, with significant staining at both P7 and P14 (33). A significant amount of cone arrestin3, a protein critical for the termination of a light response, was also immunohistochemically identifiable as early as P0 (33).

In addition to expression of the molecular machinery critical for m-opsin mediated phototransduction, the response properties we observed suggest m-opsin cone signals. The photoresponsivity we observed reflects the lower sensitivity of cones compared to rods, as rods would be expected to generate an a-wave at substantially lower flash energy (14, 15). Additionally, rods require the retinal pigment epithelium (RPE) for chromophore regeneration whereas cones are able to take advantage of the Mueller cell cycle (37). Early

in development when the RPE is not yet functional, rods would not be capable of regenerating functional rhodopsin (38). However, the threshold energy to elicit a rod or cone response during early postnatal development is not currently known, and could be less sensitive than in the mature retina. In other words, if rods can respond to light at P8, their threshold could be much higher than that for adult rods. Further, because the maturation process of a population of neurons is a gradual process, some rods may be more mature. Consequently, immature rods containing short outer segments may have contributed some portion of the detectable light response. While we cannot rule out some rod contribution, it is most likely that the first cells in the outer retina to respond to 561 nm light are m-opsin expressing cones. Future studies done in mice lacking functional rods or functional cones, or lacking the critical G-protein (*Gnat1*^{-/-}) would provide a more clear resolution to this discussion.

Response properties relative to other studies of the mature retina

The a-waves that we have recorded exhibited similar response characteristics to responses obtained in suction electrode recordings, which measure photocurrents from the outer segments of rod and cone photoreceptors (11, 39). The general waveform of the responses we obtained from both young mice and adult mice were similar to those seen in other *ex-vivo* ERG experiments (13, 14, 24), but were slightly slower and significantly smaller in younger P8-P10 mice. Smaller a-wave deflections in young mice (P8-P10) were expected, considering the low expression profiles of a number of critical players in the phototransduction cascade [(33) and for a review, see Swaroop, Kim (40)]. Two ERG studies in the anesthetized P13 mouse pup found an approximately 50µV a-wave response to a bright white light stimulus, which would have activated, both m-opsin cones and rods (17, 19).

In contrast to the early postnatal recordings, our a-wave recordings in the adult P30 retinas include a blend of rod and cone responses. Rods are fully functional by P30 and are responsible for (roughly 300 microvolts) the majority of the total a-wave deflection seen in the adult (14). In our experiment, we did not implement a double flash protocol or use a rod transducin knockout mouse (*Gnat1*^{-/-}) to isolate cone responses, so comparing our WT adult data with other studies is difficult. However, ERG experiments that have isolated cone responses either genetically or with a double flash, provide useful information about a mature cone response. Specifically, the a-waves we obtained at P10 look to be nearly mature as cones collectively produce an approximately 20 µV a-wave in the adult retina using a similar open chamber recording configuration (24). A more recent study used a novel enclosed *ex-vivo* recording configuration and found cone a-wave responses as large as 92 µV (14). These data, coupled with the nature of our recording configuration and the respective differences between major rod/cone-specific protein expression time points, suggests that this initial photoresponsiveness may be m-opsin mediated (11), as similar ERG responses have been characterized in isolated adult retinas and closely match our data (13-15).

Functional Implications

Cone photoreceptors begin to express markers of synaptic communication as early as P4 (41, 42) and synaptogenesis is mostly completed by eye-opening (43). The genes, presynaptic proteins, and ribbon synapses within cones reach functional levels of expression between P6-P10 (42-44). Connections between retinal ganglion cells and bipolar cells begin to form around P7 and mature in the third postnatal week (45-47). This culminates with the appearance of the first completed retinal circuits that have been observed in a mouse at P10, the earliest timepoint of light evoked ganglion cell spiking (21).

While a postnatal mouse does not open its eyes until P13, light penetrating through the eyelid is only attenuated by less than two orders of magnitude (4). Prior to eye opening, light modulates critical developmental processes through a small population of retinal ganglion cells in the inner retina that express the photopigment melanopsin. Photic activation of melanopsin expressing retinal ganglion cells between P4 and P7 modulates retinal wave activity, a critical mechanism for the segregation of retinogeniculate projections into discrete ocular domains (1, 2). These waves of activity switch from cholinergic propagation (P1-P7) to glutamatergic propagation between P8 and P12, however, any light-mediated effect on glutamatergic waves remains uninvestigated (48-51). Vasculature patterning and hyaloid vessel retraction between P1 and P8 is regulated by melanopsin phototransduction (3), and prior to eye opening mice as young as P6 have behaviorally adverse responses to light. This negative phototaxis behavior is mediated by melanopsin ganglion cells activity (4). Around this same developmental window of time, a small population of melanopsin ganglion cells extend dendrites through the inner retina into the OPL as early as P4 (8, 9). These Outer Retinal Dendrites (ORDs) are asymmetrically distributed to the dorsal retina, where cones predominately express m-opsin (9). ORDs are in close apposition to cone photoreceptor terminals and immuno-positive puncta labeling for post-synaptic density marker 95, an indicator of glutamatergic signaling, have been found (8). Taken together, early outer retinal photoreceptors currents have the potential to signal pre-synaptic partners as early as P8, five days earlier than previously thought. These currents, acting through ORDs or early bipolar cell circuits may influence critical developmental processes in the P8-P12 developmental time window.

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Figure 1.

Outer retina photoresponsivity to mid-wavelength light begins at P8 in the C57/BL/6 mouse retina. **A)** Developmental time course of the dark-adapted *ex-vivo* ERG a-wave at P7, P8, P9, and P10. A representative flash response from one retina is shown at each age. Each trace is an average of 3 recordings in response to a 20 ms saturating test flash (561 nm; $0.85 \log ((\text{cd s m}^{-2}))$). The earliest response can be seen at P8 and these responses increase in amplitude with each successive age. **B)** A representative flash response from a P30 retina under the same recording parameters as in A illustrate that a-wave photoresponses in the

mature retina are 10-15 times larger than those in the developing retina. **C)** Flash response families of ERG a-waves at different flash energies at P7, P8, P9, and P10 developmental stages. Representative traces are the average of 5 recordings each at dim energies (-5.0, -3.9, -3.0, and -1.9 log (cd s m⁻²)) and 3 recordings each at saturating energies (-1, 0, and 0.85 log (cd s m⁻²)). **D)** Similar flash response families in the adult retina under the same recording parameters as C show a-waves that increase in amplitude with increased flash energy.

Figure 2.

Intensity response relationships at various stages of early development illustrate outer retina photosensitivity across early postnatal development. **A)** Mean intensity response relationships were constructed from ERG a-wave flash response families in isolated mouse retina at P7 ($n = 6$; black squares), P8 ($n = 6$; red circles), P9 ($n = 6$; green triangles), P10 ($n = 6$; blue inverted triangles), and P30 ($n = 7$; grey diamonds) based on flash response families from Figure 1. **B)** Mean intensity response relationships similar to panel A, but

zoomed in and plotted without the adult (grey diamond). **C)** Data were normalized to peak amplitude and fitted with a model function as in (25) (see Methods). Error bars = SEM.

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Figure 3.

Outer retina photoresponse kinetics change across development. **A-B)** The mean total a-wave response (**A**, area under the curve) and mean peak a-wave amplitude (**B**) in response to a saturating 20 ms flash (561 nm, $0.85 \log (\text{cd s m}^{-2})$) were calculated across development. **C-D)** Mean time-to-peak (**C**) and mean latency to onset (**D**) were calculated from dim flash responses to 20 ms of 561 nm light elicited within the linear range of the intensity response relation in isolated retina. Dim flash energies were 0.85, -1.0, -1.9, and -3.0 $\log (\text{cd s m}^{-2})$ for P8, P9, P10, and P30, respectively. All calculations were from recordings in P8 ($n = 6$),

P9 (n = 6), P10 (n = 6), and P30 (n = 7) mouse retinas and can be found in Table 1. *** p 0.001, ** p 0.01, * p 0.05. Error bars = SEM.

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Table 1

a-wave photoresponse kinetics across development

Age	a-wave Response (mV * ms)	Peak Amplitude (μ V)	Time to Peak (ms)	Latency to Onset (ms)
P8	0.53 ± 0.07	5.33 ± 0.95	60.73 ± 7.15	27.9 ± 2.21
P9	0.97 ± 0.13	8.18 ± 1.02	71.60 ± 5.23	45.23 ± 4.27
P10	2.19 ± 0.22	19.86 ± 2.28	81.02 ± 2.27	39.42 ± 4.95
P30	23.04 ± 3.77	242.58 ± 39.74	118.47 ± 7.49	40.39 ± 3.74

Mean total a-wave response and mean peak a-wave amplitude in response to a saturating 20 ms flash (561 nm, $0.85 \log (\text{cd s m}^{-2})$). Mean time-to-peak and mean latency to onset were calculated from dim flash responses within the linear range of the intensity response relation in isolated retina. $\pm$ = SEM.