Melanopsin-Dependent Persistence and Photopotentiation of Murine Pupillary Light Responses

Yanli Zhu, 1,2 Daniel C. Tu, 1,2 Darcy Denner, 1 Thomas Shane, 1 Christine M. Fitzgerald, 1 and Russell N. Van Gelder 1,3

PURPOSE. To determine the relative contributions of inner and outer retinal photoreception to the pupillary light response.

METHODS. Wild-type, retinal degenerate (rd/rd), and melanopsin mutant (opn4+/−) mice were tested for pupillary light responsiveness by video pupillometry before, during, and after exposure to supersaturating light intensities. Similar lighting protocols were used to probe responses of intrinsically photosensitive retinal ganglion cells (ipRGCs) recorded with multielectrode arrays ex vivo.

RESULTS. Both outer retinal photoreceptors (rods and cones) and inner retinal photoreceptors (intrinsically photosensitive retinal ganglion cells [ipRGCs]) are sufficient to drive the pupillary light response in mice. After supersaturating light exposure, rather than bleaching or adapting, rd/rd mice showed paradoxical potentiation of responses to subsaturating light exposure. opn4+/− mice, in contrast, could not sustain pupillary constriction under continuous bright illumination, and showed desensitization after bright-light exposure. Both the intensity of light necessary to induce potentiation and the spectral sensitivity for sustained and potentiated responses differed from that necessary to trigger pupillary constriction, suggesting that photopotentiation is dependent on a pigment-state distinct from that triggering the pupillary light response itself. Multielectrode array recordings of ipRGCs from rd/rd retinas demonstrated persistent cell firing under continuous light exposure but did not show potentiation.

CONCLUSIONS. Unique photoreceptive properties of intrinsically photosensitive RGs confer resistance to bleaching and/or adaptation under continuous bright illumination to the pupillary light response and suggest the presence of a photopigment with multiple absorption states. (Invest Ophthalmol Vis Sci. 2007;48:1268–1275) DOI:10.1167/iovs.06-0925

From the Department of 1Ophthalmology and Visual Sciences and 2Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, Missouri.

1Contributed equally to the work and therefore should be considered equivalent authors.

Supported by National Eye Institute (NEI) Grant T32EY13360 and the Medical Scientist Training Program at Washington University Medical School (DCT), NEI Grant R01EY14988 (RVG), the Culppepper Clinician-Scientist Award of the Rockefeller Brothers Foundation (RVG), Research to Prevent Blindness Career Development Award (RVG), and a NARSAD (National Alliance for Research on Schizophrenia and Depression) Career Development Award (RVG).

Submitted for publication August 4, 2006; revised October 2, 2006; accepted January 19, 2007.

Disclosure: Y. Zhu, None; D.C. Tu, None; D. Denner, None; T. Shane, None; C.M. Fitzgerald, None; R.N. Van Gelder, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Russell N. Van Gelder, Department of Ophthalmology and Visual Sciences, Washington University Medical School, 660 S. Euclid Avenue, St. Louis, MO 63110; vangelder@vision.wustl.edu.
during light phase. Experiments were performed at the same time of day. All experiments were performed under institutional Animal Studies Committee approval, and all experiments met the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Pupilometry**

Mice were tested between Zeitgeber time (ZT)2 and ZT8 (where ZT0 is lights-on and ZT12 is lights-off). Although there is a significant circadian rhythm to pupillary light response, sensitivity is equivalent at these two time points (Owens et al., manuscript in preparation). Mice were dark adapted for >1 hour before the recordings. Pupillary light responses were recorded under infrared conditions with a charge coupled-device (CCD) video camera fitted with IR filter and macro lenses, as described. A halogen source was used for all light stimuli except for intense monochromatic lights which were provided by a 75-W xenon lamp (Nikon, Tokyo, Japan). Wavelength and intensity were manipulated via neutral density and narrow bandwidth (10 nm) interference filters (Oriel; Newport Corp., Irvine, CA). Irradiance measurements (in Watts per square meter) were made with a calibrated radiometer (Advanced Photonics International, White Plains, NY).

**Data Analysis**

**Time Course of Photopotentiation Investigation.** Normalized pupil area was calculated as (pupil area – minimum pupil area during saturating light exposure)/(average pupil area during seconds 30 to 60 of the first 470-nm probe light pulse – minimum pupil area during saturating light exposure).

**Time Course of Pupillary Relaxation Investigation.** The normalized pupil area was calculated as (pupil area – minimum pupil area during bright white light exposure)/(dark-adapted pupil area – minimum pupil area during bright white light exposure). The percentage potentiation was calculated as 100 × (1 – mean normalized pupil area during the second 470-nm “probe” light stimulus (i.e., seconds 150 – 180 of the 3-minute time course)). Normalized pupil area was calculated as described for the photopotentiation time course. The percentage of adaptation was taken as negative values of photopotentiation (i.e., the pupil is less constricted to the second subsaturating test pulse of light than the first).

**Percentage of pupil constriction** was calculated as 100 × (1 – minimum pupil area during light pulse/dark-adapted pupil area). **Relative response** was calculated by normalizing the maximum percentage of pupil constriction and percentage of photopotentiation to 1.0 and the minimum values to 0. Data were fit via a modified Naka-Rushton equation24 (SigmaPlot, Systat Software, Inc., San Jose, CA). Single irradiance relative spectral sensitivity for photopotentiation was generated by plotting the percentage of photopotentiation (calculated as described previously) as a function of the central wavelength of intense narrow-bandpass light used.

**Multielectrode Array Recordings.** For studies of wild-type mice, animals were killed on postnatal day 12 by CO2 narcosis followed by cervical dislocation. For studies of rd/rd mice, 5-week-old mice were killed in the same manner. The dissected retina was placed on an array of 60 electrodes (Multi-Channel Systems, Reutlingen, Germany) and perfused with a bicarbonate-buffered physiologic solution as described. Both recording environment and perfusion fluid were maintained at 30.8°C. For suppression of spontaneous activity during recording, the retina was kept under pharmacologic blockade by using both glutamatergic (50 mM d-APV and 10 mM CNQX) and cholinergic (4 nM epibatidine) inhibitors. Light was provided by a xenon source (Sutter Instruments, Novato, CA) and filtered with narrow-band-pass filters (Thor Laboratories, North Newton, NJ). Raw electrical signals in response to light stimuli were amplified, filtered, and digitized through an A/D card (National Instruments, Austin, TX) and analyzed off-line using custom spike-sorting software.26 Normalized response for each cell was calculated as (firing rate (Hz) – maximum firing rate during saturating light exposure)/(average firing rate during seconds 30 to 60 of the first 480-nm probe light pulse – maximum firing rate during saturating light exposure).

**RESULTS**

**Potentiation of the Pupillary Light Response of Retinal Degenerate Mice after Bright Light Exposure**

To determine the effect of bright light exposure on the subsequent sensitivity of outer and inner retinal photoreceptors, we used a protocol for comparing the PLR sensitivity before and after a saturating light stimulus. Unanesthetized, dark-adapted mice were immobilized by the scruff and exposed to 3 minutes of light in the following sequence: 1 minute of narrow bandpass 470-nm blue light at half-saturating stimulus strength for pupillary response, followed by 1 minute of bright white light (18 W/m², halogen source), followed by rechallenge with 1 minute of the initial 470-nm light (Fig. 1A). The irradiance of 470-nm light that yields 50% pupillary constriction (IR50) was ~1 × 10¹¹ photons/cm² per second for wild-type and 5 × 10¹² photons/cm² per second for rd/rd mice. Pupillary responses were measured by infrared video pupilometry.20,25 In retinal degenerate mice, the PLR during bright light exposure showed persistent constriction. During the 1-minute dim blue light exposure after bright light exposure, the PLR was ~50% augmented compared with the pre-bright-light PLR (Fig. 1A). We termed this phenomenon photopotentiation.

To ensure that this result was not caused by altered relaxation kinetics in the pupillary response of rd/rd mice, we monitored pupillary relaxation after a transition from bright white light stimulation to complete darkness. No difference in relaxation kinetics was observed for wild-type or rd/rd mice or mice lacking melanopsin (opn4−/−; Fig. 1B). Pupils returned to ~75% of dark-adapted dilation within 1 minute of termination of bright light. Photopotentiation was also observed when a dark period (allowing pupillary redilation) was interposed between the saturating white light pulse and second subsaturating 470-nm light pulse. To determine the time-course of persistence of the potentiated state, we systematically varied the duration of the interposed dark period. Photopotentiation decreased exponentially with increasing duration of the dark period, with a half-life of approximately 2 minutes (Fig. 1C).

Photopotentiation was initially observed only in rd/rd mice, whereas wild-type mice showed reduced pupillary responses (consistent with bleaching and/or adaptation) after bright light. This was unexpected, because presumably wild-type mice contain all photoreceptors present in an rd/rd animal. We reasoned that, if photopotentiation were a property of the inner retinal photoreceptor, it would only be manifest when that photoreceptor was stimulated. The light intensity causing 50% constriction in wild-type mice induces minimal pupillary constriction in rd/rd mice,20 thus, at this intensity rod and/or cone responses are likely to be primarily responsible for the observed PLR. We therefore tested wild-type mice for photopotentiation, given an initial 470-nm blue stimulus at the 50% threshold for rd/rd mice (5 × 10¹² photons/cm² per second). This intensity causes ~70% constriction of the wild-type pupil. It is thus more difficult to observe potentiation (as there is a physical limit of maximum pupillary constriction), but easier to see adaptation and/or bleaching. Under this paradigm, wild-type mice demonstrated photopotentiation (Fig. 1D). Thus, photopotentiation is operant in wild-type mice, but only under light conditions bright enough to stimulate inner retinal photoreception.
Photopotentiation Occurs on the Afferent Limb of the Pupillary Light Reflex

The pupillary light reflex originates in the neurosensory retina, and signals through the olivary pretectal nuclei where bilateral input is integrated, through the Edinger-Westphal nucleus of the third cranial nerve, and finally through the ciliary ganglion to the iris sphincter and dilator muscles.27 Photopotentiation could be occurring at any point along this pathway. Certain vertebrate irises, such as the isolated embryonic chicken iris, have intrinsic light responses28; thus, the iris itself could also mediate photopotentiation.

To localize the anatomic site of photopotentiation, we studied relative contralateral and ipsilateral pupillary light responses. Mice show symmetric ipsilateral and contralateral pupillary light responses.27 Bright light presented to the contralateral eye was unable to photopotentiate the ipsilateral response to dim blue light (Fig. 2A). Only bright light in the ipsilateral eye triggered potentiation, demonstrating that photopotentiation occurs either before integration of bilateral responses in the olivary pretectal nuclei or within the eye on the efferent limb. To test the latter hypothesis (whether the iris itself has photosensitivity that gives rise to photopotentiation) we monitored pupillary responses of both eyes during photopotentiation to one eye. If photopotentiation occurred at the level of the iris, we would see anisocoria or asymmetric constriction to the second blue light probe pulse, with increased constriction in the eye receiving the bright light stimulus (Fig. 2B). However, pupillary responses were essentially symmetric.
between the two eyes. Together, these results demonstrate that photopotentiation occurs before integration of photic information from the two eyes at the level of the olivary pretectal nucleus.

Role of Melanopsin in Photopotentiation and Maintenance of Pupillary Constriction

Inner retinal photoreponses are absolutely dependent on the opsin family member melanopsin. We tested mice lacking melanopsin (opn4<sup>−/−</sup>) for pupillary light responses under conditions that produced photopotentiation in wild-type and rd/rd mice. Mice lacking melanopsin showed no potentiation of pupillary light responses at any irradiance of white light or 470-nm blue light tested. Indeed, these mice showed escape of pupillary constriction after ~20 seconds of bright light (Fig. 3), probably reflecting bleaching and/or adaptation of outer retinal photoreceptors. Thus, both persistent pupillary light responses under bright light conditions and photopotentiation of the pupillary light response are dependent on melanopsin.

Intensity and Wavelength Dependence of Photopotentiation Versus the Pupillary Light Response Itself

If photopotentiation occurs due to stimulation of the inner retinal photopigment(s) underlying PLR, the intensity and wavelength of light that induce photopotentiation should correspond to that of the inner retinal-driven PLR itself. We first generated an intensity-response relationship white light to trigger the PLR, and compared this to the intensity of light required for photopotentiation in rd/rd mice. Approximately 100-fold higher light energy was necessary to elicit photopotentiation than was needed to generate the PLR itself (Fig. 4A). We next compared the action spectra for photopotentiation...
and PLR in rd/rd mice. The action spectrum for pupillary light responses to 30-second light exposure was fundamentally identical with that previously reported for rd/rd mice,4 with peak at 480 nm and shape consistent with an opsin-based pigment (data not shown). Because of the steepness of the irradiance–response relationship for photopotentiation, generation of a complete action spectrum for this response was prohibitive. We generated a single-irradiance relative spectrum for photopotentiation, using monochromatic light at 10^11 photons/cm² per second. This spectrum differed substantially from the action spectrum for pupillary light responsiveness itself, with photopotentiation occurring nearly equally at all blue-light wavelengths, but falling off at wavelengths longer than 500 nm (Fig. 4B).

Closer inspection of the time-course of pupillary constriction in rd/rd mice under bright monochromatic light revealed a related characteristic. Pupils remained constricted during a 60-second pulse of intense monochromatic light with wavelengths shorter than 500 nm (as well as for white light). However, with wavelengths longer than 500 nm, pupillary constriction escaped after ~20 seconds, rather than potentiat-
response to intense 500-nm light and shows subsequent bleaching and/or adaptation.

**Persistence and Photopotentiation of Isolated ipRGCs**

To determine whether photopotentiation occurs at the level of the ipRGC, we performed in vitro multielectrode array recordings of ipRGCs from rd/rd mice. Using a lighting scheme comparable to that used to measure potentiation in vivo in pupillary light response, ipRGCs showed persistent firing during the bright-light phase, with an initial peak firing of ~15 seconds after bright light exposure, with steady state firing occurring from 15 to 60 seconds. However, no potentiation of firing activity was seen during the second dim blue light exposure (Fig. 5). This result was consistent using bright light varying over 2-log intensity. To determine whether persistent firing was wavelength dependent, bright light of two wavelengths—one capable of eliciting photopotentiation in vivo (430 nm) and one incapable (530 nm)—were tested for their effects on sustained ipRGC firing. As shown in Figure 6, light of both wavelengths induced equivalent firing rates in the first 60 seconds of exposure; however, in the subsequent 5 minutes of bright-light exposure, firing rates declined significantly faster for the 530 nm light than for the 430 nm light, mirroring the effects of different wavelengths seen in the persistence of PLR in intact mice.

**DISCUSSION**

We have identified and characterized photopotentiation, a novel physiologic feature of the nonvisual inner retinal photoreception mechanism. Rather than attenuating responses after intense stimulation, the inner retinal photoreceptive mechanism instead potentiates responses to subsequent stimuli. Three lines of evidence suggest that this mechanism is innate to the intrinsically photosensitive RGCs: (1) Photopotentiation in wild-type mice only occurs in response to stimuli bright enough to stimulate the inner retinal photoreceptive system; (2) analysis of ipsilateral and contralateral pupillary responses demonstrates that photopotentiation occurs only in the eye receiving saturating stimulus (although light impinging on the potentiated eye drives enhanced responses to both pupils) and (3) photopotentiation is dependent on melanopsin, which is expressed predominantly in the intrinsically photoreceptive RGCs. However, potentiation does not appear to operate at the level of the photoreceptive event itself in the ipRGCs, as in vitro recordings of ipRGCs fail to demonstrate potentiated action-potential firing.

Pupillary light responses are seen in mice lacking outer retinal photoreceptor function, as well as in mice lacking melanopsin, however, no pupillary light responses can be elicited in mice lacking both outer retinal photoreceptors (or outer retinal function) and melanopsin. Thus, neither outer nor inner retinal photoreceptors are necessary, but each is sufficient to drive the pupillary light response. Lucas et al. first noted that melanopsin-mutant mice have diminished pupillary light responses at high irradiance levels, suggesting that the two photoreceptive systems are not completely redundant.

The current studies extend this finding. Under conditions of continuous broad-spectrum illumination, whereas outer retinal responses rapidly adapt (via bleaching and active adaptation mechanisms), inner retinal responses potentiate. Such a potentiating mechanism is likely to be advantageous for pupillary light responses. It is thought that the two functions of the PLR are to increase depth of field and image sharpness in bright light conditions and to protect the retina from phototoxicity under high ambient irradiation. The latter function cannot be performed by the outer retina alone (as the pupil dilates with adaptation, as seen in mice lacking melanopsin under continuous illumination). The inner retinal photoreceptive system generates sustained and enhanced responses under continuous high levels of illumination, thus conferring continual pupillary constriction under bright light conditions. As continued exposure to intense light is irreversibly toxic to the outer retina, it is possible that photopotentiation confers a selective advantage that has led to the preservation of two separate photoreceptive signaling pathways for the pupillary light response.

Perhaps the most intriguing aspect of photopotentiation is its unique action spectrum. If photopotentiation were an intrinsic property of the inner retinal photopigment that initiates...
pupillary light responses, one might expect the action spectra for photopotentiation and PLR initiation to be identical. The discordance between the two spectra is most obvious when comparing the time courses of PLR under bright, nearly monochromatic lights. Pulses of 450- and 500-nm light are equipotent at eliciting PLR; however, continuous illumination with 450-nm light results in persistent pupillary constriction and photopotentiation, whereas prolonged exposure to 500-nm light yields pupillary dilation, presumably secondary to bleaching and/or adaptation. This finding is not consistent with a single univariant pigment and suggests either the presence of multiple pigments in the ipRGC or multiple photoreceptive states of a single pigment. In a bistable pigment, the initial photon absorption both initiates signal transduction (by retinaldehyde isomerization) and also generates a pigment state that can absorb a second photon of different wavelength that drives reisomerization. Certain opsins (particularly insect opsins, but also lizard and lamprey opsins) can form such bistable pigments; indeed, photoisomerization is the mechanism used in insect opsins to reconstitute photopigment. There are several lines of evidence suggesting that melanopsin may be bistable, using light both to initiate the signaling cascade and to re-isomerize the retinal chromophore. Melanopsin has higher sequence homology to invertebrate than vertebrate opsins, and its expression pattern in the inner retina leaves it far from the chromophore-regenerating machinery of the retinal pigment epithelium. Recent studies have suggested that melanopsin does not require the enzymatic machinery of the outer retina for its chromophore regeneration. Studies examining photosensitivity in neuronal cell lines and Xenopus oocytes heterologously expressing melanopsin are potentially consistent with a such a bistable pigment/photoreisomerization model.

Partially purified, heterologously expressed, cephalochordate Amphioxus melanopsin has also been shown to be bistable in vitro; exposure to blue light results in a red shift in the absorption spectra of the pigment. Thus, photoisomerization may simultaneously explain the resistance to bleaching seen in this pigment while also providing a plausible mechanism for chromophore regeneration in the inner retina.

Of note, we did not see evidence of photopotentiation in multielectrode array recordings in vitro, although clear evidence for persistent firing under prolonged bright light stimulation was observed. This result suggests that the physiologic basis of photopotentiation is downstream of the photopigment and immediate phototransduction event itself. However, in our experiments examining the laterality of potentiation, it also appears that photopotentiation occurs on the afferent limb of the pupillary light response. Thus, the mechanistic basis of photopotentiation must occur between the phototransduction event and bilateral integration of light responses in the olivary pretectum (OPT). The logical place for this to occur is at the synapse between ipRGC and OPT, which leads us to the following model for photopotentiation (Fig. 7): light-dependent stimulation of melanopsin in ipRGCs leads to persistent cell firing, due to the equilibrium of active and inactive states of the bistable pigment. In turn, this persistent signaling generates short-term potentiation of the ipRGC-OPT synapse, which accounts for the observed photopotentiation of pupillary light responses. In this model, photopotentiation is thus a consequence of the persistence of cell firing conferred on the system by the unique photopigment properties of melanopsin.

Recent work has suggested that melanopsin-dependent inner retinal photoreception undergoes adaptation to background illumination. We see evidence of an adaptation process when we examine the in vitro ipRGC cell firing during prolonged light stimulation, in that cell-firing rates in response to the identical stimulus decline with continued illumination. (The adaptation process appears to be wavelength dependent, and shows more rapid decay with longer wavelength light; again, this may reflect differences in steady state equilibrium between two pigment states). Photopotentiation may partially counter this adaptation at the physiologic level, allowing continuous signal to reach the brain during prolonged light exposure despite adaptation of the pigment. Such a mechanism may explain the remarkable persistence of rat suprachiasmatic nucleus cell firing in vivo in response to light stimuli up to 30 minutes, despite apparent adaptation of the underlying pigment.

Acknowledgments

The authors thank Satchin Panda and John Hogenesch for supplying opn4−/− mice and Therese Gibler, Tianyang Yan, and Alisa P. Tu for assistance with the experiments.

References

2. Lucas RJ, Foster RG. Neither functional rod photoreceptors nor rod or cone outer segments are required for the photic inhibition of pineal melatonin. Endocrinology. 1999;140:1520–1524.