Melanopsin Tristability for Sustained and Broadband Phototransduction

Highlights

- ipRGCs produce a persistent response that integrates light over minutes
- Long-wavelength light acutely decreases the persistent response of ipRGCs
- The intrinsic photosensitivity of ipRGCs exhibits chromatic integration
- Photoequilibration of melanopsin among three states accounts for these properties

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In Brief

Mammals sense light with melanopsin to regulate diverse aspects of physiology. Emanuel and Do report that melanopsin cells display exceptional integration over time and wavelength because melanopsin, unlike other opsins, possesses two silent states that photoequilibrate with a signaling state.
Melanopsin Tristability for Sustained and Broadband Phototransduction

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SUMMARY

Mammals rely upon three ocular photoreceptors to sense light: rods, cones, and intrinsically photosensitive retinal ganglion cells (ipRGCs). Rods and cones resolve details in the visual scene. Conversely, ipRGCs integrate over time and space, primarily to support “non-image” vision. The integrative mechanisms of ipRGCs are enigmatic, particularly since these cells use a phototransduction motif that allows invertebrates like Drosophila to parse light with exceptional temporal resolution. Here, we provide evidence for a single mechanism that allows ipRGCs to integrate over both time and wavelength. Light distributes the visual pigment, melanopsin, across three states, two silent and one signaling. Photoequilibrium among states maintains pigment availability for sustained signaling, stability of the signaling state permits minutes-long temporal summation, and modest spectral separation of the silent states promotes uniform activation across wavelengths. By broadening the tuning of ipRGCs in both temporal and chromatic domains, melanopsin tristability produces signal integration for physiology and behavior.

INTRODUCTION

The visual system resolves detail to support familiar tasks like recognizing objects and guiding action, but many processes have quite different requirements for sensing light. These “non-image” visual functions include the regulation of sleep, hormone levels, pupil contraction, and the circadian clock (reviewed by Do and Yau, 2010; Lucas et al., 2014). They tend to integrate rather than resolve, thereby smoothing fluctuations in light level across space and time to produce accurate representations of overall irradiance. The degree of integration can be remarkable. For instance, the circadian clock responds similarly to a given number of photons whether that number is delivered over milliseconds or minutes (Nelson and Takahashi, 1991). The clock uses irradiance to synchronize its endogenous rhythm with the solar day, thereby establishing normal patterns of gene expression in practically every tissue and allowing organisms to anticipate cycles of key parameters such as temperature and predator behavior (Mohawk et al., 2012). Dysregulation of the clock is linked to psychiatric illness, cardiovascular disease, metabolic disorders, and cancer (Takahashi et al., 2008).

Mammalian non-image vision begins in the retina and is supported by intrinsically photosensitive retinal ganglion cells (ipRGCs; Berson et al., 2002). IpRGCs are like conventional retinal ganglion cells (RGCs) in that they convey visual information to the brain that originates from the rod and cone photoreceptors. IpRGCs also sense light directly through their own mechanism of phototransduction. Selective elimination of ipRGC phototransduction has broad effects on the organism. Some visual functions are unable to reach their natural maxima. For instance, pupil constriction and circadian phase-shifting cannot be driven to completion; instead, they saturate at abnormally low light intensities (Lucas et al., 2003; Panda et al., 2002). Furthermore, some functions are abnormally fleeting. For example, pupil constriction and the acute modulation of locomotor activity are not sustained during steady illumination (Mrosovsky and Hattar, 2003; Zhu et al., 2007). Thus, ipRGCphototransduction appears to be particularly important at high light intensities (i.e., room light and above) and over extended timescales (i.e., seconds to hours).

IpRGCs sense light using a visual pigment called melanopsin (Provenzio et al., 1998, 2000). The wavelength sensitivity of melanopsin is precisely mirrored by that of ipRGCs, expression of melanopsin is required for all intrinsic light responses in ipRGCs, and heterologous expression of melanopsin in other cell types renders them photosensitive with the known characteristics of melanopsin (Berson et al., 2002; Dacey et al., 2005; Hattar et al., 2003; Lucas et al., 2003; Melyan et al., 2005; Qiu et al., 2005). Melanopsin is unusual in that it is expressed in vertebrates but is most homologous to the rhodopinic pigments that are typically found in invertebrates (Provenzio et al., 1998, 2000; Shichida and Matsuyama, 2009). Within ipRGCs, melanopsin drives a transduction cascade that is distinguished by its prolonged time course. For example, the unitary (i.e., single-photon) response of ipRGC phototransduction has an integration time of 8 s, which is approximately 300-fold longer than that of Drosophila photoreceptors, 100-fold longer than that of mammalian cones, and 20-fold longer than that of mammalian rods (Do et al., 2009; Henderson et al., 2000). Thus, melanopsin...
function appears tailored to the integrative nature of non-image vision.

We have investigated signal integration by ipRGCs and obtained evidence that it is greater than previously appreciated. Not only does the intrinsic light response integrate over minutes of time, it also integrates over wavelength. Furthermore, such integration appears to arise from molecular properties of melanopsin that have not been found in any other native visual pigment.

RESULTS

IpRGCs Generate a Persistent Response that Produces Temporal Integration

Using established techniques, we identified ipRGCs within the in vitro mouse retina and monitored the output of individual neurons using the perforated-patch mode of electrophysiological recording, which preserves the melanopsin-driven light responses of these cells (Do et al., 2009; Do and Yau, 2013; Xue et al., 2011). Our principal focus was the M1 subtype of ipRGC, which is strictly required for photoregulation of the circadian clock and influences other non-image visual functions (Güler et al., 2008; Hatori et al., 2008). M1 ipRGCs are strongly driven by a melanopsin-mediated response that is 10-fold greater in sensitivity and saturated amplitude than that of other subtypes (Ecker et al., 2010; Schmidt and Kofuji, 2009, 2011). Such responses allow for precise, quantitative analysis. We recognized M1 ipRGCs by standard criteria (Experimental Procedures; Do et al., 2009; Do and Yau, 2013; Xue et al., 2011) and will refer to them simply as “ipRGCs” unless otherwise noted.

We recorded from single ipRGCs near body temperature (35°C ± 1°C) with synaptic transmission intact. We stimulated ipRGCs with xenon light, which has a spectrum that resembles sunlight, at intensities within the physiological range. During illumination, ipRGCs generated a response in which synaptic and intrinsic components were often discernable, as expected from prior studies (Perez-Leon et al., 2006; Schmidt and Kofuji, 2009; Wong et al., 2007). Upon cessation of illumination, we observed that the response could persist for many minutes in darkness.

To evaluate the ability of these persistent responses to support temporal integration, we stimulated ipRGCs with pulses of light (10-s duration; intensity of 2.0 × 10^{-6} µW µm^{-2}, equivalent to 3.3 × 10^{4} lux) that were separated by extended intervals of darkness (40 s). An example of temporal integration is illustrated in Figures 1A and 1B: the persistent response increases across stimuli until reaching saturation, both at the level of spiking and the subthreshold membrane voltage (observed in 6 cells; Figure 1C). Some ipRGCs did not display temporal integration because their persistent responses were small or were already saturated with the first stimulus (n = 9 cells). Such variation in photosensitivity

Figure 1. Persistent Responses and Temporal Integration of IpRGCs

(A) Membrane voltage of an ipRGC in response to a series of 10-s pulses of white light (xenon at an intensity of 2.5 × 10^{-5} µW µm^{-2}, equivalent to 3.3 × 10^{4} lux). The corresponding spike rate is shown below in 10-s bins. Light monitor at bottom. Activation that continues beyond the period of illumination is referred to as the persistent response.

(B) Excerpts of the trace in (A) illustrating the response to the first and sixth light pulses. Dashed line represents the baseline (−60 mV).

(C) Subthreshold membrane voltage, averaged from 30–40 s after each light pulse, is displayed for individual cells (connected markers, n = 6 cells) and the population mean (bars) for this protocol. The difference from baseline, normalized to the maximum difference, is displayed for each cell. Closed markers represent the cell shown in (A).

(D) Subthreshold membrane voltage after the last pulse for the cell shown in (A) (in 5-s bins). Time point 0 corresponds to the end of illumination. Fit is a single exponential (τ = 107 s). All experiments were performed at 35°C with synaptic transmission intact. See also Figure S1.
is expected of ipRGCs, even within the M1 subtype (Do et al., 2009; Do and Yau, 2013; Xue et al., 2011). Indeed, altering the light intensity to change the magnitude of the persistent response could unmask temporal integration in such cases (n = 2 of 2 cells tested; Figure S1).

We estimated the lifetime of the persistent response by monitoring the subthreshold membrane voltage following illumination. The decay was well described by a single exponential with a time constant of 122 ± 34 s (mean ± SEM, n = 10 cells with saturated persistent responses; example in Figure 1D). Thus, the persistent response is expected to promote temporal integration in a window of ~5 min, which is unexpectedly long because the unitary response of ipRGCs decays with a time constant of 8.3 ± 1.0 s (inferred from the dim-flash response of 18 cells measured in voltage clamp; Experimental Procedures).

The Persistent Response Arises from Melanopsin Phototransduction

To investigate the mechanism of the persistent response, we stimulated ipRGCs with monochromatic light (440–480 nm; Figure S2) near the peak wavelength sensitivity (λ_{max}) of melanopsin while isolating the intrinsic response by blocking synaptic transmission. A persistent response was produced in every case, indicating that it is inherent to ipRGCs (n = 85 cells). In current clamp, the persistent response was evident as a depolarization of the analog membrane voltage. With regard to spikes, this depolarization drove tonic firing in the largest fraction of ipRGCs, was too small to do so in others, and was so large in the remainder that spikes were attenuated (n = 18, 5, and 13 cells, respectively, probed with the same saturating pulse of short-wavelength light; Figure S2). When measured at room temperature, the persistent depolarization only decayed slightly in darkness (observation period as long as 20 min; example in Figure 2A). Near body temperature, it lasted for minutes (n = 4 cells), as expected from experiments with synaptic transmission intact (Figure 1). Surprisingly, the persistent response was acutely decreased by light—provided that this light was of a longer wavelength than the preceding, activating stimulus (Figures 2A–2D; n = 4 and 81 cells at 35°C and 23°C, respectively). Indeed, the persistent response was repeatedly increased and decreased in magnitude with successive pulses of light in a wavelength-dependent manner (Figures 2B–2D).

Together with melanopsin phototransduction, voltage-gated ion channels produce the intrinsic excitability of ipRGCs. The
persistent response does not require these channels because it was neither increased nor decreased following pulses of depolarizing or hyperpolarizing current injection, respectively (all 36 cells tested). Furthermore, even when the membrane voltage was clamped, a robust persistent current was observed (n = 56 cells at -80 mV; example in Figure 3A). Comparison of persistent responses measured in current and voltage clamp (Figures 2B and 3A, respectively) indicates that phototransduction establishes a plateau of depolarization upon which voltage-gated channels primarily serve to drive action potentials.

We used voltage-clamp recording to evaluate the dependence of the persistent response on wavelength (Figure 3B; Supplemental Experimental Procedures). While all tested wavelengths activated ipRGCs during the period of illumination itself, the persistent response was largest following the shortest wavelength and smaller following longer wavelengths (Figure 3C). The noise accompanying the persistent response, which resembles dark regeneration between pulses, Light monitors are shown below trace. See Figure S2 for spectra and intensities of light.

Figure 3. Wavelength Dependence of Persistent Responses Measured in Voltage Clamp
(A) Example of a persistent response (i.e., the light-evoked current that continues to flow beyond the period of illumination) modulated by successive pulses of short- and long-wavelength light. This response does not show temporal integration because it is saturated with each short-wavelength pulse at the intensity used here, and there is negligible dark regeneration between pulses. Light monitors are shown below trace. See Figure S2 for spectra and intensities of light.

(B) Protocol for quantifying the magnitude of the persistent response as a function of wavelength. Test wavelengths were alternated with a "reset" wavelength of 560 nm to establish a baseline. The intensities of all stimuli (\(1 \times 10^8 - 2 \times 10^9\) photons \(\mu m^{-2} s^{-1}\)) were sufficient to produce a saturated persistent response at each wavelength tested.

(C) Population data from protocol in (B) for difference in current from baseline (left), difference in current normalized to minimum and maximum for each cell (middle), and current noise in the same period (standard deviation normalized to minimum and maximum; right). Connected markers are individual cells. All experiments were in voltage clamp (-80 mV) at 23°C with synaptic transmission blocked.

Melanopsin Activates from Two Silent States in IpRGCs

While the persistent response has no homolog in vertebrate rods and cones, it does resemble a feature displayed by many invertebrate photoreceptors, the "prolonged depolarizing afterpotential" (PDA; Hillman et al., 1983). The PDA is prolonged because the rhabdomeric type of visual pigment used by such photoreceptors has a stable signaling state. The pigment can be repeatedly switched between its signaling state and silent state by light, thereby activating and deactivating the PDA (Hillman et al., 1983). Such bistability has long been speculated to be a property of mammalian melanopsin (Melyan et al., 2005; Mure et al., 2007, 2009; Panda et al., 2005), though the topic is controversial (reviewed by Schmidt et al., 2011).

A defining feature of a bistable pigment is its activation from a single conformational state, and we tested whether this is true of melanopsin in ipRGCs. A pigment state can be defined by its spectral sensitivity, which is described by a mathematical nomogram whose only free parameter is the \(\lambda_{\text{max}}\) (Govardovskii et al., 2000). The spectral sensitivity of a pigment state is conferred upon the photoreceptor to give the cellular action spectrum (e.g., Govardovskii et al., 2000; Hillman et al., 1983; Makino et al., 1999). Electrophysiological measurement of the action spectrum is generally more sensitive, by orders of magnitude, than biochemical measurements of the absorption spectrum (Govardovskii et al., 2000). The sensitivity afforded by electrophysiology is particularly important for delineating the properties of native melanopsin because this pigment is expressed sparsely in ipRGCs (Do et al., 2009) and these cells are few in number (Berson et al., 2010; Ecker et al., 2010).

We measured the ipRGC action spectrum under two conditions: dark adaptation (no activation) and atop a background light of 600 nm (a wavelength that minimizes the persistent response). We found that the action spectrum of dark-adapted ipRGCs was described by a single-state nomogram with a \(\lambda_{\text{max}}\) of 471 ± 2 nm (n = 6 cells; Figure 4), comparable to most prior reports (Lucas et al., 2014). With the 600-nm background light, the action spectrum was again described by a single-state nomogram (Figure 4). Unexpectedly, this nomogram was blue.
shifted to have a λ_{\text{max}} of 453 ± 1 nm (n = 6 cells; same cells as above), which indicates the presence of a pigment state that is distinct from that observed in darkness. We observed this spectral shift in each cell tested (n = 4 cells at 35°C and 2 cells at 23°C with no detectable variation with temperature; λ_{\text{max}} differs between darkness and 600-nm background light with p < 0.001). A pulse of long-wavelength conditioning light was also effective in producing the blue-shifted pigment state (Figure S3). These experiments indicate that melanopsin activates from more than one state and is therefore not bistable.

When we delivered a background of 440-nm light (a wavelength that produces a large persistent response), ipRGCs exhibited an action spectrum that is broader than that of a single pigment state. Rather, it is described by the weighted sum of the 471-nm (69% ± 7%) and 453-nm (31% ± 7%) nomograms (n = 4 cells at 23°C; Figure S3). To test the effect of all visible wavelengths, we used a background of xenon light (1.5 \times 10^{-7} \text{ W m}^{-2} \text{ s}^{-1}, equivalent to 50 lux, an intensity similar to dim room light). Again, ipRGCs displayed an action spectrum that was described by the weighted sum of the 471-nm (59% ± 8%) and 453-nm (41% ± 8%) nomograms (n = 5 cells at 35°C and 4 cells at 23°C with no detectable variation with temperature; Figure 5B). Collectively, these data suggest that the ipRGC action spectrum reflects one pigment state, another, or both depending on illumination conditions (Figures 4 and 5). In other words, melanopsin activates from two states, which we refer to as cyan (λ_{\text{max}} = 471 nm) and violet (453 nm).

### Uniformity of Phototransduction Evoked from Two Silent States of Melanopsin

To determine whether ipRGCs respond differently to photon absorption by the cyan and violet states, we generated a dominant fraction of the violet state or a majority fraction of the cyan state using background lights (600 and 440 nm, respectively; Figures 4 and 5A) and probed transduction with dim flashes (480 nm; Figure 6A). We calibrated the intensities of the backgrounds to produce a comparable level of activation, and thus adaptation, in ipRGCs (Supplemental Experimental Procedures; Do and Yau, 2013; Wong et al., 2005). Without such calibration, differences in the responses could arise from a difference in adaptation rather than the identity of the pigment state. This consideration also precluded us from using darkness for comparison with the long-wavelength background—although darkness yields a pure cyan state, it produces no adaptation. Dim-flash responses evoked on these backgrounds had indistinguishable sensitivities and kinetics (Figures 6B and 6C). Comparing 440- and 600-nm backgrounds, sensitivities were 1.1 ± 0.3 \times 10^{-6} versus 1.0 ± 0.3 \times 10^{-6} pA photon^{-1} \text{ s}^{-1} (p = 0.56), while time constants were 1.4 ± 0.3 versus 1.6 ± 0.2 s (\tau_i of a fit using the convolution of two exponentials; p = 0.36) and 8.7 ± 2.8 versus 7.5 ± 1.3 s (\tau_t; p = 0.52, n = 5 cells). Thus, we did not detect a dependence of downstream signaling on silent states. Equivalent activation from the cyan and violet states, which are separated by ~20 nm, broadens the wavelength tuning of ipRGCs. Although modest, this spectral separation is comparable to that between the red (λ_{\text{max}} = 552 nm) and green (530 nm) cone pigments that serves human color vision (Merbs and Nathans, 1992). Here, this separation exists within a single pigment.

### Photoequilibrium of Melanopsin States Supports Temporal and Chromatic Integration

Our experiments suggest the following new view of melanopsin function in the mammalian retina. Dark-adapted ipRGCs contain melanopsin in the cyan state (Figure 4). Light produces an equilibrium of cyan, violet, and signaling “meta” states. The photoequilibrium fractions of these states are determined by their spectral sensitivities and the wavelength of illumination. Photon absorption by one state causes it to isomerize to another. Therefore, short wavelengths produce a photoequilibrium that favors the meta state (λ_{\text{max}} = 476 nm; Matsuyama et al., 2012) because it absorbs these wavelengths less effectively than the cyan (471 nm) and violet (453 nm) states. On the other hand, long wavelengths produce a photoequilibrium with a dominant violet state, because this state absorbs long wavelengths least effectively. The violet state is electrically silent, or largely silent, since it is formed by wavelengths that decrease ipRGC activity (Figures 2 and 3) and its isomerization activates ipRGCs (Figures 4 and 6). Thus, our biophysical measurements indicate that native melanopsin is bistable, possessing two silent states and one signaling state.

This view is inconsistent with biochemical experiments on melanopsin (Koyanagi et al., 2005; Newman et al., 2003; Shirzad-Wasei et al., 2013; Walker et al., 2008) with the exception of a study
by Shichida and colleagues (Matsuyama et al., 2012). This study demonstrated that melanopsin’s ground state (“melanopsin,” $\lambda_{\text{max}} = 467$ nm, containing 11-cis retinal) photoequilibrates with the signaling state (“metamelanopsin,” 476 nm, all-trans retinal). There was also evidence of photoequilibration between metamelanopsin and a third state, called “extramelanopsin” (446 nm) that contained 7-cis retinal. In other words, melanopsin $\not\rightarrow$ metamelanopsin $\not\rightarrow$ extramelanopsin, with direct conversion between melanopsin and extramelanopsin neither detected nor expected due to energetic constraints (Matsuyama et al., 2012). A physiological role for extramelanopsin was considered hypothetical because pigments containing 7-cis retinal have not been thought to exist in nature (Matsuyama et al., 2012; Sekharan and Morokuma, 2011) and these biochemical experiments were performed under highly reduced conditions (e.g., truncated pigment was expressed heterologously, solubilized in detergent, and tested at 0°C). Nevertheless, the spectrum of extramelanopsin closely resembles that of the violet state we observe in ipRGCs ($\lambda_{\text{max}} = 446$ nm and 453 nm, respectively; Figure S4).

To make this comparison between melanopsin biochemistry and ipRGC physiology quantitative, we developed a numerical model that uses parameters measured from purified melanopsin to predict the photoequilibrium of pigment states arising from any excitation spectrum (Experimental Procedures; Figures 7A and S4; Matsuyama et al., 2012). The model predicts that short wavelengths drive most pigment into the metamelanopsin (“M”) state, which parallels our experimental finding that these wavelengths generate the largest persistent responses (which reflect the M-like meta state; Figure 3). The remaining pigment is predicted to be divided between the melanopsin (“R”) and extramelanopsin (“E”) states (Figure 7B). For example, at 440 nm, the E state should compose 46% of the silent states. Our experiments on ipRGCs are in general agreement: illumination at this wavelength produces a photoequilibrium in which ~31% of the pigment occupies the E-like violet state (Figure 5A). For long wavelengths, the model predicts that there is a small fraction of the M and R states as well as a large fraction of the E state (Figure 7B), which accords with our observation that these wavelengths produce the smallest persistent responses in ipRGCs (Figures 2 and 3). At 600 nm, the E state is predicted to account for 92% of the silent states. When delivered to ipRGCs, light of this wavelength produces a photoequilibrium in which the only detectable silent state is the E-like violet state (Figure 4 and Experimental Procedures). Thus, mammalian melanopsin appears to be tristable in its native environment as well as when purified, and tristability can account for the integrative properties of ipRGCs that we observe.

Tristability Confers Unique Properties to Melanopsin
To investigate how tristability operates under diverse illumination conditions, we assessed model outputs for common light sources (Figure 8A; Johnsen et al., 2006). Remarkably, despite having diverse spectra, these sources are predicted to have practically identical effects on the photoequilibrium of melanopsin states, resembling monochromatic short-wavelength light in producing a majority fraction of the M state and roughly even fractions of the R and E states (Figure 8B).
We generated two additional models to compare the properties of tristable and bistable visual pigments. The first is a hypothetical, “bistable melanopsin” that lacks the E state (Figure 8C) and the second is *Drosophila* rhodopsin (Figure 8D). All relevant biochemical parameters are known for both models (Matsuyama et al., 2012; Ostroy, 1978; Stavenga, 2010). The principal difference between these bistable models is that the spectral sensitivities of the ground and signaling states are similar for melanopsin (λ_max = 467 and 476 nm, respectively) but different for rhodopsin (480 and 570 nm). We find that the photoequilibrium of bistable melanopsin, but not of rhodopsin, displays a high degree of invariance across diverse lighting spectra. By contrast, the photoequilibrium of rhodopsin, but not of bistable melanopsin, can have a nearly pure fraction of either the silent state or the signaling state. Tristable melanopsin is distinct from bistable pigments in that it displays both spectral invariance and state purity (Figure 8B). Furthermore, the approach of tristable melanopsin to photoequilibrium is slower than that of bistable melanopsin or *Drosophila* rhodopsin (by 1.6- and 6.1-fold, respectively), consistent with the integrative nature of non-image vision.

**DISCUSSION**

We have found that phototransduction in ipRGCs exhibits a high degree of signal integration that is consistent with the characteristics of non-image vision. Melanopsin activity generates persistent responses that support temporal integration over many minutes. We know of no other sensory cell that exhibits such a high degree of temporal integration; notably, ipRGCs accomplish this integration with signaling components that are employed in other systems to resolve signals on a millisecond timescale (e.g., Henderson et al., 2000). Furthermore, the persistent responses of ipRGCs are activated by a wider range of wavelengths than expected from a single visual pigment (Govardovskii et al., 2000).

We have provided evidence that this chromatric integration reflects activation of melanopsin from two spectrally distinct silent states, which we have called cyan and violet (Figure 5S).

The violet state of melanopsin resembles the E state that Shichida and colleagues defined biochemically for purified melanopsin (Matsuyama et al., 2012). The E state is unusual in using 7-cis retinal, a chromophore that has not yet been found in visual pigments under natural conditions. Molecular dynamics simulations have predicted that 7-cis retinal would produce an inactive pigment (Sekharan and Morokuma, 2011). Therefore, the E state is expected to be silent, like the violet state. Moreover, rhodopsins experimentally reconstituted with 11-cis or 7-cis retinal differ in the initial stages of photoactivation but converge before G protein engagement (Shichida et al., 1991). This is consistent with our observation of indistinguishable activation from the cyan and violet states in ipRGCs (Figure 6). Thus, extramelanopsin and the violet state are likely to be one and the same. Purification of 7-cis retinal from ipRGCs would provide a confirmation of this idea, though such experiments are challenging due to the miniscule amount of melanopsin in the retina (Berson et al., 2010; Do et al., 2009; Ecker et al., 2010). Such confirmation would motivate investigation of how melanopsin forms this isomer naturally, while other pigments require artificial conditions (Azuma and Azuma, 1985; Maeda et al., 1978, 1979); intriguingly, simulations already suggest that retinal has a distinct geometry when bound within melanopsin (Sekharan et al., 2012).

Some studies have suggested that mammalian melanopsin has only one silent state and is bistable (Meylan et al., 2005; Mure et al., 2007, 2009; Panda et al., 2005; Shirzad-Wasei et al., 2013). Other studies have not detected any photoequilibration of melanopsin among stable states (Do et al., 2009; Fu et al., 2005; Mawad and Van Gelder, 2008; Qiu et al., 2005; Sexton et al., 2012). Much of this controversy may stem from properties of tristability that we have described here. The largest persistent
responses we observe are approximately 30 pA and most are <10 pA, which can be difficult to detect. These responses are also associated with decreases in input resistance and photosensitivity (Figures 2C and 3A), which are typical criteria for terminating a recording session (Do et al., 2009; Do and Yau, 2013). Finally, even though persistent currents are small, they often can drive ipRGCs into depolarization block due to the high input resistance of these cells (Figure 2C; Do et al., 2009; Do and Yau, 2013; Schmidt and Kofuji, 2009); these activated but weakly or non-spiking cells would not be apparent in extracellular recordings (Mawad and Van Gelder, 2008; Sexton et al., 2012).

Our results suggest another reason that melanopsin’s nature has been elusive. The signaling state exhibits a monotonic decline in absolute sensitivity with increasing wavelength (past its λ_{max} of ~480 nm), which makes longer wavelengths less effective at depopulating it. On the other hand, the relative sensitivities of the three states are such that wavelengths near 600 nm are best for selectively depopulating the signaling state (Figure S4D). The optimal wavelength of cellular deactivation should reflect a balance of these absolute and relative sensitivities. Indeed, this optimal wavelength appears to be ~580 nm in our hands. A comparable optimum was deduced from behavioral experiments (Mure et al., 2009) and has been interpreted as the λ_{max} of a bistable melanopsin’s signaling state. Our data indicate that it is, instead, a consequence of melanopsin tristability. Notably, even light of the optimal wavelength must be intense or prolonged to produce an acute decrease of the persistent response, because all states of melanopsin absorb long wavelength poorly.

With regard to the spectral sensitivity of melanopsin activation, reported λ_{max} values range from ~420 nm to ~480 nm in studies conducted at biochemical, cellular, and behavioral levels (e.g., Berson et al., 2002; Brainard et al., 2001; Lucas et al., 2001; Matsuyama et al., 2012; Melyan et al., 2005; Newman et al., 2003; Panda et al., 2005; Qiu et al., 2005). The existence of the violet state helps explain this dispersion in values because its contribution to the observed spectral sensitivity can vary with illumination conditions; it is present during illumination and absent following prolonged darkness.

Melanopsin tristability appears to have advantages for non-image vision. First, photoequilibration of melanopsin among signaling and silent states supports the sustained activity of ipRGCs by maintaining the availability of pigment molecules for activation. By contrast, the monostable rod and cone pigments spontaneously dissociate into opsin and chromophore after a single activation, thereby losing photosensitivity. Because pigment regeneration requires a slow series of reactions that takes place in accessory cells, rods and cones have limited intrinsic capacities for sustained signaling (Wang and Kefalov, 2011). Our observations of the violet state and its activation provide evidence for light-driven regeneration of melanopsin in ipRGCs. Such regeneration may be particularly important to ipRGCs because they express relatively few melanopsin molecules (Do et al., 2009) yet must capture photons continuously over the long timescales of non-image visual responses (Mrosovský and Hattar, 2003; Wong, 2012).

With regard to sustaining photon capture, tristability is not expected to differ from bistability, which raises the question of whether tristability provides unique advantages. Our work indicates that bistable melanopsin displays an activation level that is similar across a variety of broadband spectra (“spectral invariance”) and can range from a small minimum to a large maximum (“state purity”). By contrast, a bistable pigment can display either spectral invariance or state purity—depending on the relative wavelength sensitivity of its two states—but not both. State purity facilitates the fine-tuning of pigment function to behavioral needs. For example, the balance between activation and deactivation can be flexibly altered through the expression of screening pigments that are upstream of the photoreceptors, a strategy that is used by many species for adaptation to diverse habitats (Cronin et al., 2001; Hardie and Postma, 2008). Tristable melanopsin has two additional features that distinguish it from bistable pigments. First, its spectral sensitivity is broadened by activation from two silent states, which confers a moderate degree of wavelength integration. Second, tristable melanopsin approaches photoequilibrium with an extended time course, which imposes a low-pass filter on visual signals. Thus, tristability endows melanopsin signaling
Figure 8. Melanopsin Tristability under Diverse Lighting Conditions

(A) Measured spectra of various, common light sources (in photons μm−2 s−1 nm−1 prior to normalization).

(B) Left: state diagram and relative photosensitivities as displayed in Figure 7A. Middle: predicted equilibrium fractions of melanopsin states for monochromatic illumination at two wavelengths (440 and 560 nm). Right: predicted equilibrium fractions of melanopsin states for broadband illumination by the sources shown in (A).

(C) Same as (B) but for a hypothetical bistable melanopsin with only the ground state (R) and metamelanopsin (M).

(D) Same as (B) but for Drosophila rhodopsin (R) and metarhodopsin (M). Midday and sunset spectra in (A) are courtesy of Sönke Johnson (Johnsen et al., 2006).
with a unique set of properties that is consistent with the integrative nature of non-image vision.

A noteworthy implication of our study is that light cannot produce a pure population of melanopsin’s ground state. Nevertheless, this is the only state detected in dark-adapted ipRGCs biochemically (Sexton et al., 2012; Walker et al., 2008) and in our own biophysical experiments (Figure 4). Therefore, melanopsin is likely to return to the ground state through a light-independent mechanism. We expect such dark regeneration to be slow because the persistent response (reflecting the meta state) can be observed for many minutes following its induction by light, and the action spectra of ipRGCs (reflecting the fractional occupancy of cyan and violet states) agree with predictions from our state model, which only includes light-driven transitions. Melanopsin can be regenerated by the administration of exogenous chromophore (Do et al., 2009; Fu et al., 2005), at least in some circumstances (Sexton et al., 2012), which raises the possibility that ipRGCs are likely to be of the M1 subtype based on their bright tdTomato labeling; large intrinsic light responses (typically >100 pA) wide, highly accommodating action potentials; and, often, dendrites that could be seen extended into the inner plexiform layer (Do et al., 2009; Do and Yau, 2013; Ecker et al., 2010; Schmidt and Kofuji, 2009; Xue et al., 2011). The few apparently non-M1 ipRGCs encountered were not overtly different with regard to melanopsin tristability, exhibiting persistent responses to short-wavelength light and shifted action spectra during or after long-wavelength light. Solutions are detailed in Supplemental Experimental Procedures.

EXPERIMENTAL PROCEDURES

Detailed procedures for individual experiments are included in Supplemental Experimental Procedures.

Tissue

All procedures were approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital. BAC-transgenic mice (either sex, postnatal days 20–140, housed in a 12-hr light/12-hr dark cycle) with ipRGCs labeled by expression of tdTomato from the melanopsin gene locus were used (Do et al., 2009). Mice were dark adapted for ≥1.5 hr and experiments were performed between zeitgeber times 3 and 10 (where 0 is lights on). Under dim red light, animals were anesthetized with Avertin, encuclated, and euthanized. The retina was mechanically freed from the retinal pigment epithelium and vitreous humor in carbonated Ames’ medium (i.e., equilibrated with 95% O2/5% CO2). The retina was flattened with peripheral cuts and held in the recording chamber, photoreceptors down, by a platinum–iridium frame strung with lycra fibers or a coverslip coated with poly-L-lysine.

Electrophysiology

The flat-mount retina was superfused with carbonated Ames’ medium at ~5 ml/min on the stage of an upright microscope. Cells were viewed with differential interference contrast optics using infrared transillumination (850-nm center wavelength and 30-nm width at half maximum). IpRGCs were visualized with ~1 s of light (25-nm bandpass centered on 545 nm, 1 × 1010 photons μm−2 s−1; Figure S2) and the overlying inner limiting membrane was mechanically removed. Cells were dark adapted for ≥15 min before data were collected. Pipettes (2–6 MΩ) were wrapped with paraffilm to reduce capacitance. Series resistance (generally ≤50 MΩ) was monitored. Integrity of the perforated-patch configuration was tested with periodic test flashes and, in some cases, brief visualization of Lucifer yellow (which does not permeate amphotericin B) at the end of the experiment. Recordings were performed near physiological temperature (35°C ± 1°C) or, for additional stability, at room temperature (~23°C). Temperature was monitored with a thermistor in the recording chamber. Recordings were low-pass filtered at 4–10 kHz (current clamp) or 2 kHz (voltage clamp) and sampling exceeded the Nyquist minimum. Analysis was performed using Clampfit and Igor Pro. Nearly all ipRGCs we recorded are likely to be of the M1 subtype based on their bright tdTomato labeling; large intrinsic light responses (typically >100 pA) wide, highly accommodating action potentials; and, often, dendrites that could be seen extended into the inner plexiform layer (Do et al., 2009; Do and Yau, 2013; Ecker et al., 2010; Schmidt and Kofuji, 2009; Xue et al., 2011). “Flashes” are impulse stimuli (i.e., duration and intensity can be interchanged to give the same response).

Numerical Model of Melanopsin Tristability

The distribution of biochemically defined melanopsin states (Matsuyama et al., 2012) as a function of wavelength was estimated with a numerical simulation in which occupancy of each state is calculated with each time step. The states are melanopsin (R), metamelanopsin (M), and extramelanopsin (E). A state depopulates if a molecule in that state absorbs a photon and isomerizes (thus converting to another state). A state populates if a molecule in an adjoining state absorbs a photon and isomerizes. R and E both interconvert directly with M but not each other, in accordance with biochemical data on purified melanopsin (Matsuyama et al., 2012) and the likelihood, given energetic constraints, that the transition from one cis isoform to another occurs via the all-trans conformation. For each state, photon absorption is governed by the extinction coefficient (ε in units of cm2 mol−1), which has a spectral dependence, A(λ). Following photon absorption, the probability of isomerization is given by the quantum efficiency (f). The equations are

\[ f_{M_{i-1}}^{M_i} = f_{M_{i-1}}^{M_{i-2}} + f_{M_{i-2}}^{M_{i-3}} \ln|10| \times \ln(\lambda) \times f_{\lambda} \times \epsilon_{\lambda} \times A(\lambda) \times \phi_\lambda \]

\[ f_{E}^{M_{i+1}} = f_{E}^{M_{i}} + f_{E}^{M_{i}} \ln|10| \times \ln(\lambda) \times f_{\lambda} \times \epsilon_{\lambda} \times A(\lambda) \times \phi_\lambda \]

where \( f_{M}^{M_{i+1}} \) and \( f_{E}^{M_{i+1}} \) are the fractional occupancies of each state and sum to 1. We typically begin our simulation with \( f_{E}^{M_{i+1}} = 1 \), reflecting dark adaptation but the equilibrium state is insensitive to the initial conditions. \( f_{M_{i+1}}^{M_{i+1}} \) is the fraction of M states (Sexton et al., 2012), which raises the possibility that ipRGCs are likely to be of the M1 subtype based on their bright tdTomato labeling; large intrinsic light responses (typically >100 pA) wide, highly accommodating action potentials; and, often, dendrites that could be seen extended into the inner plexiform layer (Do et al., 2009; Do and Yau, 2013; Ecker et al., 2010; Schmidt and Kofuji, 2009; Xue et al., 2011). The few apparently non-M1 ipRGCs encountered were not overtly different with regard to melanopsin tristability, exhibiting persistent responses to short-wavelength light and shifted action spectra during or after long-wavelength light. Solutions are detailed in Supplemental Experimental Procedures.

Optical Stimulation

Light from 75-W xenon arc lamps or a 100-W mercury halide lamp was filtered to deplete heat while selecting intensity and wavelength. Delivery through a 40× objective produced a uniform field (480-μm diameter) centered on the soma. Electromechanical shutters controlled stimulus timing. Light stimuli were measured at the site of the preparation using a calibrated radiometer and spectrometer. Light delivered through 10-nm bandpass filters were assumed to be of the center wavelength; for broader filters, photon flux was calculated from measured spectra (Figures 8 and S2). Photometric units were calculated using the CIE standard photopic luminosity function (Sharpe et al., 2005). “Flashes” are impulse stimuli (i.e., duration and intensity can be interchanged to give the same response).

Numerical Model of Melanopsin Tristability

The distribution of biochemically defined melanopsin states (Matsuyama et al., 2012) as a function of wavelength was estimated with a numerical simulation in which occupancy of each state is calculated with each time step. The states are melanopsin (R), metamelanopsin (M), and extramelanopsin (E). A state depopulates if a molecule in that state absorbs a photon and isomerizes (thus converting to another state). A state populates if a molecule in an adjoining state absorbs a photon and isomerizes. R and E both interconvert directly with M but not each other, in accordance with biochemical data on purified melanopsin (Matsuyama et al., 2012) and the likelihood, given energetic constraints, that the transition from one cis isoform to another occurs via the all-trans conformation. For each state, photon absorption is governed by the extinction coefficient (ε in units of cm2 mol−1), which has a spectral dependence, A(λ). Following photon absorption, the probability of isomerization is given by the quantum efficiency (f). The equations are

\[ f_{M_{i-1}}^{M_i} = f_{M_{i-1}}^{M_{i-2}} + f_{M_{i-2}}^{M_{i-3}} \ln|10| \times \ln(\lambda) \times f_{\lambda} \times \epsilon_{\lambda} \times A(\lambda) \times \phi_\lambda \]

\[ f_{E}^{M_{i+1}} = f_{E}^{M_{i}} + f_{E}^{M_{i}} \ln|10| \times \ln(\lambda) \times f_{\lambda} \times \epsilon_{\lambda} \times A(\lambda) \times \phi_\lambda \]

where \( f_{M}^{M_{i+1}} \) and \( f_{E}^{M_{i+1}} \) are the fractional occupancies of each state and sum to 1. We typically begin our simulation with \( f_{E}^{M_{i+1}} = 1 \), reflecting dark adaptation but the equilibrium state is insensitive to the initial conditions. \( f_{M_{i+1}}^{M_{i+1}} \) is the fraction of M states...
isomerizations that yields R and \( f^{M \rightarrow E} \) is the fraction that yields E; \( f^{M \rightarrow R} + f^{M \rightarrow E} = 1 \). \( f(l) \) is the light intensity in mol photons \( \text{cm}^{-2} \text{s}^{-1} \) (higher intensities simply give faster approaches to equilibrium; Figure S4). The \( \ln(10) \) term originates with the Beer-Lambert law governing absorbance of light. Equilibrium was defined at the point when the fraction of each state changes by \( <1.0 \times 10^{-20} \) between time steps (of at least 1 ms). For \( \phi(\lambda) \), we use the standard spectral template for A1-based pigments, including both \( \alpha \) and \( \beta \) absorption bands (Govardovskii et al., 2000), with the \( \lambda_{\text{max}} \) values reported for purified, mammalian melanopsin (\( R = 467 \text{ nm}, M = 476 \text{ nm}, \) and \( E = 446 \text{ nm}; \) Matsuyama et al., 2012). All model parameters except for \( \phi_E \) and \( f^{M \rightarrow R} \) (thus also \( f^{M \rightarrow E} \)) have been defined by Shichida and colleagues (Matsuyama et al., 2012). Most natural pigments have a quantum efficiency of activation near 0.2 (Cronin and Goldsmith, 1982; Matsuyama et al., 2012). There is little information available on the quantum efficiency of pigments containing 7-cis retinal (Shichida et al., 1991), like the E state. For our model, we selected a \( \phi_E \) of 0.4, which is intermediate between the values for melanopsin and metamelanopsin (Matsuyama et al., 2012), and an \( f^{M \rightarrow R} \) (and thus \( f^{M \rightarrow E} \)) of 0.5.

We do not consider light-independent transitions between states because the thermal decay of the M and E states appears to be negligible over the time scales of our experiments, especially at 23 °C. For instance, the persistent response (reflecting the signaling, or M state) is stable for minutes at 35 °C and 23 °C (Figures 1D and 2A) and the spectral sensitivity of the violet state (the physiologic hologram of the biochemical state) can be measured from iPrRGCs in a 10-min window following a conditioning light that produces it (at 23 °C, Figure S3). Furthermore, many of our experiments are performed with continuous illumination, which maintains the photoequilibrium distribution of melanopsin states.

**Numerical Models of Bistable Pigments**

The equations that compose the models of bistable pigments are

\[
\begin{align*}
\tau^R_{n+1} &= \frac{\tau^R_n + [\ln(10) \times f(l) \times f^M_{n+1} \times \epsilon_{AB} \times A_M(\lambda) \times \phi_R]}{[\ln(10) \times f(l) \times f^M_{n+1} \times \epsilon_{AB} \times A_M(\lambda) \times \phi_R]} \\
\tau^E_{n+1} &= \frac{\tau^E_n + [\ln(10) \times f(l) \times f^M_{n+1} \times \epsilon_{AB} \times A_E(\lambda) \times \phi_E]}{[\ln(10) \times f(l) \times f^M_{n+1} \times \epsilon_{AB} \times A_E(\lambda) \times \phi_E]}
\end{align*}
\]

Variables are identical to those given for the tristable melanopsin model. For the hypothetical “bistable” pigments, all the values are the same as for the tri-stable melanopsin model. For Drosophila rhodopsin, \( \epsilon_R = 35,000 \text{ cm}^2 \text{ mol}^{-1} \) and \( \epsilon_M = 56,000 \text{ cm}^2 \text{ mol}^{-1} \) (Ostroy, 1978), \( \phi_R \) has been measured to be 0.71 of \( \phi_R \) (Stavenga, 2010). \( \phi_R \) itself has not been directly measured but its proportionality with \( \phi_R \) is sufficient for accurate prediction of photoequilibrium pigment fractions. \( A_M(\lambda) \) and \( A_E(\lambda) \) are Govardovskii nomograms (Govardovskii et al., 2000) with \( \lambda_{\text{max}} \) values of 480 and 570 nm, respectively, which fit Drosophila pigment states (Stavenga, 2010).

**Statistical Methods**

We used non-parametric statistics, employing the Mann-Whitney U test for unpaired data and the Wilcoxon signed-rank test for paired data. To compare more than two groups with repeated measures, we used the Friedman test and a post hoc Wilcoxon signed-rank test with Bonferroni correction.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.02.011.

**AUTHOR CONTRIBUTIONS**

A.J.E. and M.T.H.D. designed experiments, which were conducted by A.J.E., and wrote the paper.

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Supplemental Information

Melanopsin Tristability for Sustained and Broadband Phototransduction

Alan Joseph Emanuel and Michael Tri Hoang Do
Figure S1, Related to Figure 1. Dependence of Temporal Integration on Light Intensity.

(A) Membrane voltage (top) and spike rate (bottom, in 10-s bins) of an ipRGC illuminated with six pulses of xenon light (10 s each, 2.0×10^{-6} \mu W \mu m^{-2}, equivalent to 2.6×10^{3} lux). (B) The same cell and protocol but with a higher intensity of xenon light (2.5×10^{-5} \mu W \mu m^{-2}, equivalent to 3.3×10^{4} lux). Recordings were made at 35 °C with synaptic transmission intact.
Figure S2, Related to Figures 2 and 3. Spectra and Intensities of Light Stimuli. (A) Short-wavelength stimulus used in Figures 2A, 2C, 2D, and 3A at an intensity of $4 \times 10^{10}$ photons $\mu$m$^{-2}$ s$^{-1}$. This stimulus was also used in the current-clamp experiments that tested for persistent responses during pharmacological block of synaptic transmission. (B) Long-wavelength stimulus used in Figures 2A, 2C, 2D, and 3A at an intensity of $7 \times 10^{10}$ photons $\mu$m$^{-2}$ s$^{-1}$. (C) Short-wavelength stimulus used in Figure 2B at an intensity of $3 \times 10^{10}$ photons $\mu$m$^{-2}$ s$^{-1}$. (D) Excitation light used to identify tdTomato-positive ipRGCs at an intensity of $5 \times 10^{10}$ photons $\mu$m$^{-2}$ s$^{-1}$. This is identical to the long-wavelength stimulus used in Figure 2B.
**Figure S3, Related to Figure 4. Inducing the Violet State with a Period of Conditioning**

**Light.** (A) From the action spectra of the cyan and violet states, an ipRGC should be more sensitive to 480- than 440-nm light if all melanopsin is in the cyan state (ratio of sensitivities to 480- and 440-nm light of 1.25) but the opposite should be true for the violet state (ratio of 0.80). Illustrated is the protocol for measuring dim-flash sensitivities to 480-nm and 440-nm light before and after a 560-nm conditioning step (top). Representative dim-flash responses (bottom). All responses are normalized to the peak of the 440-nm responses. The peaks of the 440- and 480-nm responses are marked by gray and black dashed lines, respectively, for ease of comparison. (B) The 480:440 sensitivity ratio for all cells in each condition. Closed symbols denote the cell in A and bars are population means. Dashed black and red lines represent the ratio expected if all pigment is in the cyan and violet state, respectively. Asterisks signify statistical significance. Sensitivity ratios were 1.15 ± 0.05 during dark adaptation and 0.78 ± 0.05 following the conditioning light (n = 9 cells, p < 0.001). The slight deviation from the theoretical ratios is likely due to a small amount of the violet state remaining from the fluorescence-identification of ipRGCs; that is, incomplete dark adaptation. These experiments suggest that the violet state has a high degree of stability in ipRGCs (at 23 °C where these experiments were performed) because measuring it required a period of >10 min after the conditioning light ceased (Supplemental Experimental Procedures). Synaptic transmission was blocked in these experiments.
Figure S4, Related to Figure 7. Additional Details of the Melanopsin State Model. (A) State diagram (top) and normalized absorption spectra (bottom left) of the biochemically-defined melanopsin states (R, M, and E; Matsuyama et al., 2012). Bottom right: Normalized spectra of the R and E states (solid black and red lines, respectively) plotted with those of the cyan and violet states that we measured electrophysiologically from ipRGCs (dashed black and red lines, respectively; Figure 4). The slight deviations in $\lambda_{\text{max}}$ values may be due differences in the environments of the purified and native pigments. (B) Time required for the melanopsin state model to reach photoequilibrium as a function of wavelength, normalized to the minimum (which occurs at 456 nm). (C) Time to photoequilibrium as a function of light intensity for 480-nm light. Model parameters are as described for Figure 7. (D) Photosensitivity of the M state (normalized to the peak; right axis), compared with the photosensitivity of the M state relative to each of the silent states (i.e., M/R and M/E; left axis), all plotted as functions of wavelength.
**Figure S5. Summary Schematic.** (A) State diagram of the three physiologically-defined melanopsin states and their light-driven transitions. The cyan and violet states are silent while the meta state is signaling. The wavelength of peak sensitivity ($\lambda_{\text{max}}$) of each silent state was measured from ipRGCs in the present study; that of the meta state was measured from purified melanopsin by Matsuyama and colleagues (2012). (B) Diagram illustrating fractional occupancy of the two silent states (top) and the cellular response (middle) produced by visual stimulation with different wavelengths (bottom), as functions of time. The depicted cellular response has features that are not overtly related to state transitions of the pigment, such as transient responses that accompany periods of illumination. Common sources of broadband ("white") light produce occupancy of both cyan and violet states, leading to a modest broadening of ipRGC spectral sensitivity. Monochromatic, short-wavelength illumination has a similar effect. Long-wavelength illumination produces a dominant fraction of the violet state, acutely decreasing the persistent response. Pigment states change gradually between periods of illumination due to dark regeneration. Prolonged darkness is required to fully deactivate the persistent response and restore all melanopsin to the cyan state.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Solutions. The intracellular solution for perforated-patch recordings was (in mM): 110 K-Methanesulfonate, 13 NaCl, 2 MgCl₂, 10 EGTA, 1 CaCl₂, 10 HEPES, 0.1 Lucifer Yellow (dipotassium salt), and 0.125-0.25 amphotericin B. The pH was adjusted to 7.2 with KOH for a final [K⁺] of 139 mM. Amphotericin B was stored in the dark at -20 °C for several weeks as a 100X stock in DMSO. Amphotericin-containing internal solution was sonicated before each recording. A liquid-junction potential of +7 mV has been corrected (Neher, 1992). The extracellular solution was bicarbonate-buffered Ames’ medium, or ionic Ames’ medium (in mM): 120 NaCl, 22.6 NaHCO₃, 3.1 KCl, 0.5 KH₂PO₄, 1.5 CaCl₂, 1.2 MgSO₄, 6 glucose, equilibrated with 95% O₂ / 5% CO₂ (Do et al., 2009). Fast synaptic transmission was blocked in most experiments by adding to the external solution (in mM): 3 kynurenate, 0.1 D,L-AP4, 0.1 picrotoxin, and 0.01 strychnine (Do et al., 2009).

Measurement of Subthreshold Membrane Voltage. Subthreshold membrane voltage was isolated by detecting spikes, excising a 20-ms interval surrounding the peak of each spike, and averaging the remaining voltage within the time window of interest. The decay of the persistent response was measured from subthreshold membrane voltage that was binned in 5-s intervals, to reduce the effect of biological noise.

Measurement of Gradations in Persistent Responses as a Function of Wavelength. Steps of light (60-s duration) were delivered every 280 s and the membrane current averaged from 150-160 s after each pulse. Test steps of various wavelengths alternated with a "reset" step of 560-nm light. The current following 560-nm light is used as a baseline rather than the dark-adapted holding current to correct for any drift over the extended time course of the experiment (>30 min). The persistent response that remains after 560-nm light is estimated to be slightly
larger than 3 pA on average. Light intensities for all wavelengths were $1 \times 10^9 - 2 \times 10^9$ photons $\mu$m$^{-2}$ s$^{-1}$, which was sufficient to produce a saturated persistent response at each wavelength.

**Analysis in the Linear Range.** "Dim-flash" responses are impulse responses. They are obtained in the linear range of the ipRGC intensity-response relation, where the response magnitude scales arithmetically with flash intensity while the response waveform remains invariant (Baylor and Hodgkin, 1973; Do et al., 2009; Do and Yau, 2013). Dim-flash responses are identical in waveform to single-photon responses (Do et al., 2009). Sufficient time was given between dim flashes (35 s at 35 °C and 70 s at 23 °C) for full, observable response decay and recovery from adaptation (Do et al., 2009; Do and Yau, 2013; Wong et al., 2005). For analysis, dim-flash responses were digitally refiltered to 2-10 Hz and resampled at 100 Hz. Baselines, measured in a 1-s window prior to flash onset, were subtracted. Measurements were made from the average of 3-6 responses, to reduce unavoidable Poisson variations in magnitude (Do et al., 2009), and amplitude was calculated as the mean current in a 400-ms window centered on the response peak. Dim-flash sensitivity is the amplitude (in pA) divided by flash intensity (in photons $\mu$m$^{-2}$). For a dark-adapted ipRGC, dim flash responses are evoked by delivery of $\sim 10^5$ photons $\mu$m$^{-2}$ of 480-nm light (Do et al., 2009; Do and Yau, 2013; Xue et al., 2011); light-adapted ipRGCs require higher intensities (Do and Yau, 2013).

**Measurement of Action Spectra.** Action spectra were constructed by calculating dim-flash sensitivity for various wavelengths (Baylor and Hodgkin, 1973). Due to the extended time scale of the measurement, sensitivity was normalized to that of a periodic reference wavelength (480 nm) to correct for drift, then normalized to the maximum sensitivity. Action spectra were fit with standard, single-state nomograms (Govardovskii et al., 2000) using least-squares regression with $\lambda_{max}$ as the only free parameter:
The action spectra obtained during ongoing xenon or 440-nm illumination were each fit with a weighted sum of two single-state nomograms, one describing the cyan state ($\lambda_{\text{max}} = 471$ nm) and the other describing the violet state (453 nm):

$$N(\lambda) = \frac{1}{\exp[69.7(0.88 - \lambda_{\text{max}}/\lambda)] + \exp[28(0.922 - \lambda_{\text{max}}/\lambda)] + \exp[-14.9(1.104 - \lambda_{\text{max}}/\lambda)] + 0.674} + 0.26 \times \exp[-\left(\frac{\lambda_{\text{max}} - 189 - 0.315 \times \lambda_{\text{max}}}{-40.5 + 0.195 \times \lambda_{\text{max}}} \right)^2]$$

where $N(\lambda)$ are single-state nomograms with the $\lambda_{\text{max}}$ parameter as designated and $C$ and $V$ are coefficients describing the weight of each single-state nomogram (constrained so that neither coefficient is less than 0). Applying this weighted-sum nomogram to the average action spectra measured in darkness (where we expect a dominant cyan state) and atop 600-nm (where we expect a dominant violet state) light yielded cyan/violet fractions of 0.90/0.10 and 0.00/1.00, respectively.

**Interpretation of Action Spectra.** Because the probability of photon absorption during a dim flash is low, any melanopsin molecule should isomerize only once (Do et al., 2009). In principle, the isomerization could occur from a silent or signaling state and result in activation or deactivation, respectively. Because dim flashes caused no detectable deactivation, isomerization of the signaling state is unlikely to contribute to our spectral measurements. Indeed, the action spectra we obtained for the cyan and violet states were each fit well by a single-state nomogram (Govardovskii et al., 2000; Makino et al., 1999).

The action spectra we measured from ipRGCs do not reflect the tdTomato that is expressed in these cells because these action spectra are well-described by the nomograms of pigments that employ retinaldehyde chromophores (Govardovskii et al., 2000). These nomograms are distinct from the excitation and emission spectra of tdTomato (Shaner et al., 2003).
Indeed, electrophysiological measurements of the cellular action spectrum have consistently isolated the melanopsin absorption spectrum regardless of whether the cells expressed melanopsin alone or together with fluorophores that are as spectrally distinct as fluorescein and rhodamine (Berson et al., 2002; Dacey et al., 2005; Qiu et al., 2005; Tu et al., 2005).

**Ratio of Sensitivities to 480- and 440-nm Light.** The relative sensitivity of ipRGCs to 480- and 440-nm photons was measured from dim-flash responses. Wavelengths were interleaved, and the responses to five 50-ms flashes (separated by 70 s) were averaged for each; either 440- or 480-nm was given first (5 and 4 cells, respectively). This probe series was delivered after prolonged dark adaptation or a step of conditioning light (30 s, 560 nm, $2 \times 10^9$ photons $\mu m^{-2} s^{-1}$). 560-nm light is predicted to produce a similar photoequilibrium to 600-nm light (Figure 7) but to do so ten-fold faster (Figure S4). Therefore, 560-nm light is preferable for use as a discrete conditioning step to generate a dominant fraction of the violet state. The first two dim flashes after the conditioning step were excluded from analysis because these were diminished by transient adaptation (Do and Yau, 2013; Wong et al., 2005). Measuring dim-flash responses required >10 min after illumination ceased because dim-flash responses are prolonged (i.e., having an integration time of ~20 s at 23 ºC) and need to be averaged to obtain a reliable measurement (due to Poisson variations in their amplitude; Do et al., 2009). Two cells were excluded from analysis because the 480:440 ratio in darkness was <0.95, indicating insufficient dark adaptation.

**Comparison of Activation from the Cyan and Violet States.** We evoked dim-flash responses atop backgrounds of 440- or 600-nm illumination. To compare these responses, we matched the background intensities to produce a similar level of steady, cellular activation. Because we found the dark-adapted, dim-flash sensitivity of ipRGCs to be 130-fold greater with 440- than
600-nm light, we delivered a background that was 111-fold lower in intensity at 440 than 600 nm; a closer match was not permitted by our optical instruments. The accuracy of matching would be further limited if there were a difference in photosensitivity between the cyan and violet states. Presently this information is not known. Nevertheless, we found that the steady-state photocurrents produced by the 440- and 600-nm backgrounds were similar, regardless of the order in which these backgrounds were given (Figure 6, n = 5 cells). The kinetics of dim-flash responses were measured by fitting the average response from each cell (calculated from 7-13 responses) to the convolution of two exponentials, $A(e^{-t/\tau_1} - e^{-t/\tau_2})$, as previously described (Do et al., 2009; Xue et al., 2011).
SUPPLEMENTAL REFERENCES

