SUPPLEMENTAL INFORMATION

SUPPLEMENTAL DATA

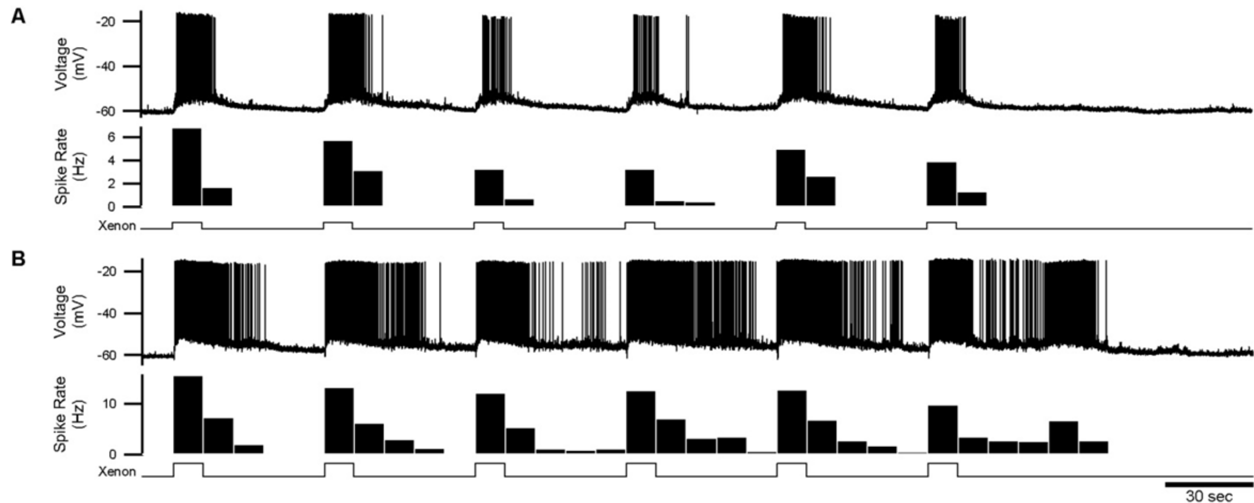

Figure S1, Related to Figure 1. Dependence of Temporal Integration on Light Intensity.
(A) Membrane voltage (top) and spike rate (bottom, in 10-sec bins) of an ipRGC illuminated with six pulses of xenon light (10-sec each, 2.0×10^{-6} μW μm^{-2}, equivalent to 2.6×10^{3} lux). (B) The same cell and protocol but with a higher intensity of xenon light (2.5×10^{-5} μW μm^{-2}, equivalent to 3.3×10^{4} lux). Recordings were made at 35 °C with synaptic transmission intact.

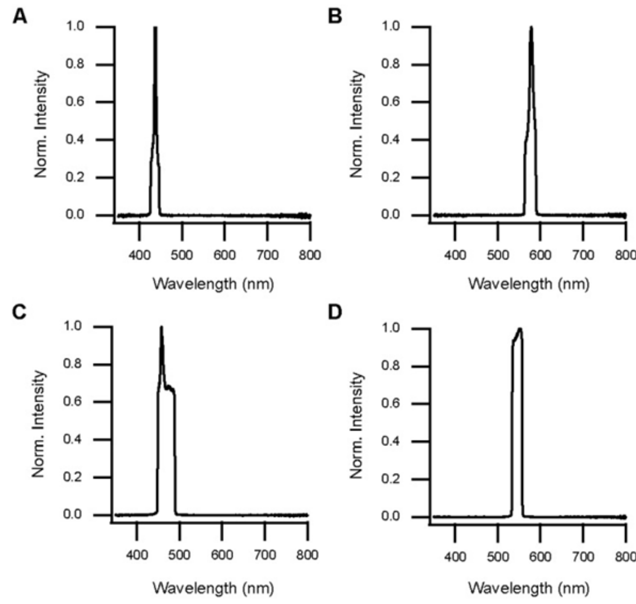

Figure S2, Related to Figures 2 and 3. Spectra and Intensities of Light Stimuli. (A) Short-wavelength stimulus used in Figures 2A, 2C, 2D, and 3A at an intensity of 4×10^{10} photons μm^{-2} sec^{-1}. This stimulus was also used in the current-clamp experiments that tested for persistent responses during pharmacological block of synaptic transmission. (B) Long-wavelength stimulus used in Figures 2A, 2C, 2D, and 3A at an intensity of 7×10^{10} photons μm^{-2} sec^{-1}. (C) Short-wavelength stimulus used in Figure 2B at an intensity of 3×10^{10} photons μm^{-2} sec^{-1}. (D) Excitation light used to identify tdTomato-positive ipRGCs at an intensity of 5×10^{10} photons μm^{-2} sec^{-1}. This is identical to the long-wavelength stimulus used in Figure 2B.

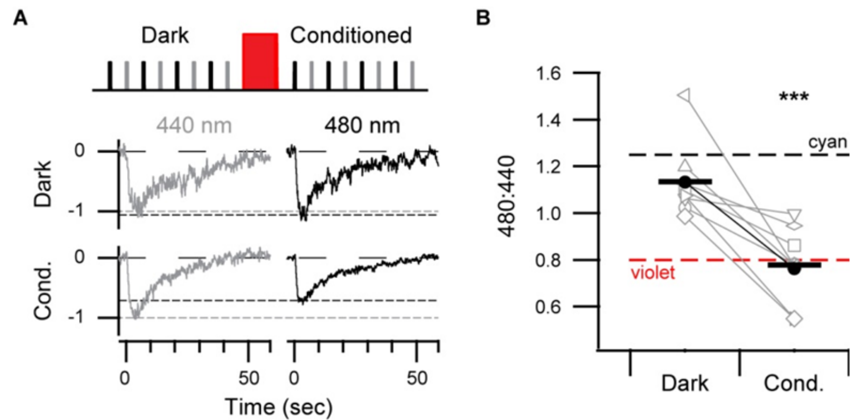

Figure S3, Related to Figure 4. Inducing the Violet State with a Period of Conditioning Light. (A) From the action spectra of the cyan and violet states, an ipRGC should be more sensitive to 480- than 440-nm light if all melanopsin is in the cyan state (ratio of sensitivities to 480- and 440-nm light 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.

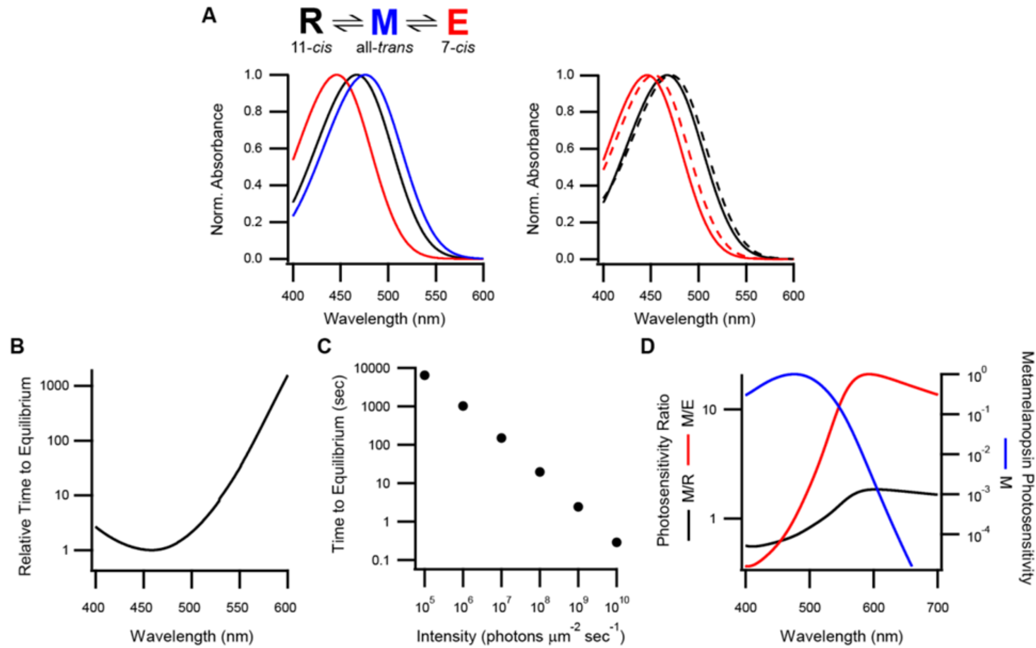

Figure S4, Related to Figure 7. Additional Details of the Melanopsin State Model. (A) State diagram (top) and normalized absorption spectra (bottom left) of the biochemically-defined melanopsin states (R, M, and E; Matsuyama et al., 2012). Bottom right: Normalized spectra of the R and E states (solid 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 deviation in λ_{max} 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.

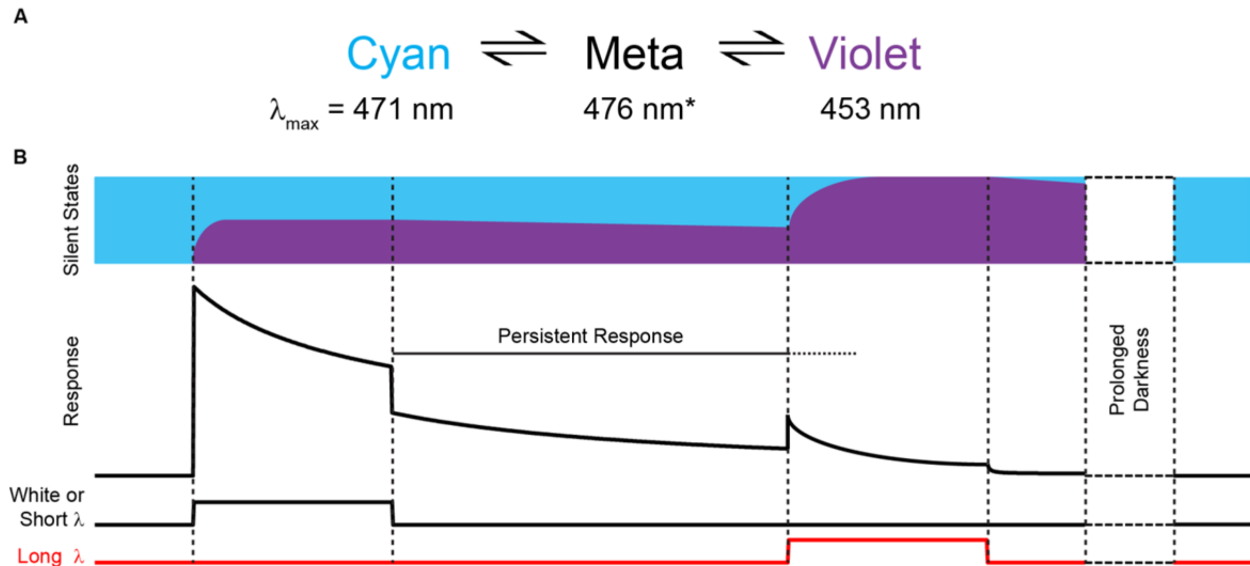

Figure S5. Summary Schematic. (A) State diagram of the three physiologically-defined melanopsin states and their light-driven transitions. The cyan and violet states are silent while the meta state is signaling. The wavelength of peak sensitivity (λ_{max}) of each silent state was measured from ipRGCs 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). 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 $MgCl_2$, 10 EGTA, 1 $CaCl_2$, 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 $NaHCO_3$, 3.1 KCl, 0.5 KH_2PO_4, 1.5 $CaCl_2$, 1.2 $MgSO_4$, 6 glucose, equilibrated with 95% O_2 / 5% CO_2 (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-sec intervals, to reduce the effect of biological noise.

Measurement of Gradations in Persistent Responses as a Function of Wavelength. Steps of light (60-sec duration) were delivered every 280 sec and the membrane current averaged from 150-160 sec 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 $1×10^9$ - $2×10^9$ photons μm^{-2} sec^{-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 sec at 35 °C and 70 sec 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-sec 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 μm^{-2}). For a dark-adapted ipRGC, dim flash responses are evoked by delivery of ~10^5 photons μm^{-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 λ_{max} as the only free parameter:

$$N(\lambda) = \frac{1}{\exp[69.7(0.88 - \lambda_{max}/\lambda)] + \exp[28(0.922 - \lambda_{max}/\lambda)] + \exp[-14.9(1.104 - \lambda_{max}/\lambda)] + 0.674}$$

$$+ 0.26 \times \exp[-\left(\frac{\frac{\lambda_{max}}{\lambda} - 189 - 0.315 \times \lambda_{max}}{-40.5 + 0.195 \times \lambda_{max}}\right)^2]$$

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 (λ_{max} = 471 nm) and the other describing the violet state (453 nm):

$$S(\lambda) = C \times \left(N(\lambda|\lambda_{max} = 471)\right) + V \times \left(N(\lambda|\lambda_{max} = 453)\right)$$

where $N(\lambda)$ are single-state nomograms with the λ_{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., 2004). 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 sec) 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 sec, 560 nm, 2×10^{9} photons μm^{-2} sec^{-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 sec 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).

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