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Photoreceptor Adaptation in Intrinsically Photosensitive Retinal Ganglion Cells

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Summary

A rare type of mammalian retinal ganglion cell (RGC) expresses the photopigment melanopsin and is a photoreceptor. These intrinsically photosensitive RGCs (ipRGCs) drive circadian-clock resetting, pupillary constriction, and other non-image-forming photic responses. Both the light responses of ipRGCs and the behaviors they drive are remarkably sustained, raising the possibility that, unlike rods and cones, ipRGCs do not adjust their sensitivity according to lighting conditions ("adaptation"). We found, to the contrary, that ipRGC sensitivity is plastic, strongly influenced by lighting history. When exposed to a constant, bright background, the background-evoked response decayed, and responses to superimposed flashes grew in amplitude, indicating light adaptation. After extinction of a light-adapting background, sensitivity recovered progressively in darkness, indicating dark adaptation. Because these adjustments in sensitivity persisted when synapses were blocked, they constitute "photoreceptor adaptation" rather than "network adaptation." Implications for the mechanisms generating various non-image-forming visual responses are discussed.

Introduction

The mammalian image-forming visual system can operate over a wide spectrum of ambient light intensities covering more than ten orders of magnitude, from dim starlight to bright daylight. This capacity is based in part on the complementary operating ranges of two types of photoreceptors: the rods, which function in dim lighting conditions and can detect single-photon events, and the cones, which operate under daylight conditions. Both rods and cones have limited dynamic ranges under dark-adapted conditions. A light stimulus that is about 3 $\log_{10}$ units above threshold evokes a maximal response and brighter stimuli cannot elicit a larger one. However, when the same stimulus is presented as a continuous background, rods and cones gradually reduce their sensitivity so that the background light is no longer saturating, and the cell can respond to further increments in light intensity. This process, termed light adaptation, thus extends the dynamic range of these receptors by normalizing their sensitivity to a temporally integrated measure of background light level. Conversely, when rods and cones return to darkness after prolonged exposure to bright light, they gradually recover from light adaptation by regaining sensitivity in a process called dark adaptation (Dowling, 1987; Rodieck, 1998).

It is unknown whether similar adaptational processes occur in the third class of mammalian retinal photoreceptors, the intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson et al., 2002). These cells differ from rods and cones in many respects. For example, they respond to light much more sluggishly, are less sensitive, depolarize in response to light by opening cation channels, use an invertebrate-like photopigment (melanopsin), generate action potentials, and connect directly to thalamic and brainstem visual centers. Their primary functional roles relate to non-image-forming visual reflexes, such as circadian entrainment, the pupillary light reflex, and photic regulation of pineal melatonin release (Berson et al., 2002; Hattar et al., 2002; Berson, 2003; Gooley et al., 2003; Lucas et al., 2003; Warren et al., 2003; Hannibal and Fahrenkrug, 2004; Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005).

The requirements of non-image-forming behavioral responses to light are fundamentally different from those of pattern vision and may not include a capacity for light adaptation. The pattern vision system must rapidly generate a robust representation of local image contrast under the entire range of naturally encountered ambient light intensities, and the ability to light adapt is thus a critical requirement. By contrast, the behavioral responses driven by the non-image-forming visual system are remarkably tonic and can be maintained by hours of continuous light exposure (e.g., the constriction of the pupil and the suppression of nocturnal melatonin release) or exhibit temporal integration over very long time scales (e.g., circadian phase resetting). Because non-image-forming photic reflexes appear to call for a stable representation of absolute light intensity, it is unclear that light adaptation is an obligatory or desirable feature of the photoreceptors that mediate them.

Here, we provide the first direct evidence that ipRGCs display both light and dark adaptation like that observed in conventional photoreceptors. These findings have implications for the properties of the various non-image-forming visual responses and of the neuronal pathways involved and for the functional roles of ipRGCs.

Results

We tested the effects of lighting history and current background illumination on light-evoked responses of ipRGCs in intact retinas. These studies were all conducted under synaptic blockade (see Experimental Procedures), and so the adaptational mechanisms they revealed are presumably intrinsic to the ipRGCs.

Light Adaptation

Background-Induced Desensitization

A standard feature of photoreceptor adaptation to background illumination is "desensitization," a reduction in sensitivity relative to that observable in the dark-adapted state (Perlman and Normann, 1998). Light exposure

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clearly desensitizes ipRGCs. This is shown in Figures 1A–1C, which compare the currents evoked in an ipRGC by brief light flashes either after being held in darkness for about an hour (Figure 1A) or in the presence of steady illumination with a background of initially nearly saturating intensity (Figure 1B). Plots of response amplitude as a function of stimulus irradiance (Figure 1C) clearly reveal a rightward shift of the curve obtained under light-adapting conditions, corresponding to a reduction in sensitivity of 1.6 ± 0.1 log units (S.E.; n = 3) based on a criterion response amplitude of −20 pA (arrow in Figure 1C). Similarly, after partial (10–15 min) dark adaptation, when two short light flashes of similar intensities were presented about 1 min apart, the response to the second flash was 58% ± 3% smaller than that to the first flash (n = 3) (Figure 1D), suggesting that exposure to the first light flash caused a desensitization that persisted well after the light had been extinguished.

Response Decay and Progressive Resensitization during Steady Illumination

Background-induced desensitization in photoreceptors can arise from "light adaptation" or "response compression." In light adaptation, the gain of the phototransduction cascade is reduced, allowing the cell to respond to further light increments. In response compression, by contrast, the strong effect of the bright background on light-gated channels (or other transduction elements) limits the cell's ability to encode further increases in illumination (Perlman and Normann, 1998).

The desensitization phenomena illustrated in Figure 1 cannot be fully explained by response compression because the steady inward current evoked by the adapting light (~10 pA in Figure 1B) is far too small to account for the reduced responses to light increments (~170 pA smaller for a −1 log light step in Figure 1B than in Figure 1A). Instead, the data imply a bona fide reduction in the gain of the signaling pathways linking photopigment to channel ("light adaptation"), and we performed additional experiments to test this. In rods and cones, light adaptation can be distinguished from mere response compression in two ways. First, the response to a prolonged, constant stimulus decays over time, a phenomenon not explicable on the basis of response compression alone. Furthermore, while the response to that constant stimulus is decaying, the cell gradually becomes more sensitive to light flashes superimposed on that stimulus, a process sometimes termed "resensitization" (Perlman and Normann, 1998).

Figures 2 and 3 show that both of these phenomena were readily observable in ipRGCs. The current-clamp recording in Figure 2A shows the depolarization evoked in an ipRGC by a light step of near-saturating intensity. The response peaked within about 3 s and began to decay almost immediately, rapidly within the first few seconds and more gradually thereafter (n = 13). We considered the possibility that this decay results from voltage-dependent channel gating rather than from a change in phototransduction gain (i.e., light adaptation), but reject this interpretation on two grounds. First, when we used direct current injection to simulate a slowly developing but otherwise constant photocurrent, the voltage response was flat (Figure 2B) (n = 7), rather than slowly decaying as during actual light stimulation (Figure 2A). Second, in voltage-clamp recordings (Figure 2C)

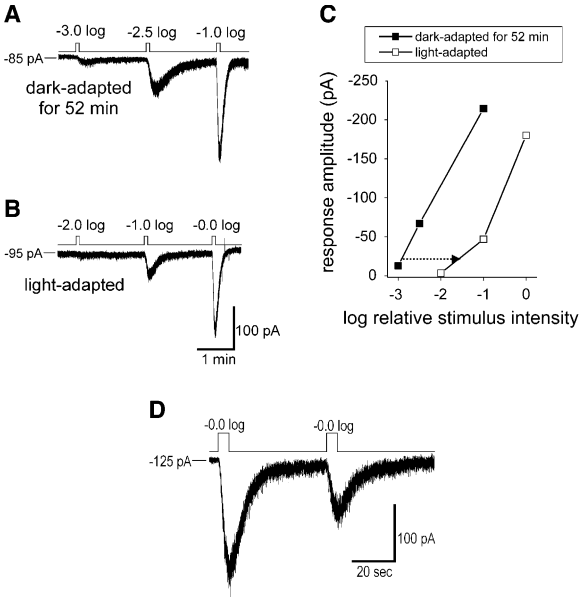

Figure 1. Light-Induced Desensitization of ipRGCs

(A and B) Current responses of an ipRGC to 5 s light pulses (indicated by the steps in the top trace) at three different intensities presented immediately after 52 min of dark adaptation (A) and then while exposing the same cell to a bright background light (B). Unattenuated intensity of test pulses ("−0.0 log") was 2.2×10^{14} photons cm^{-2} s^{-1} sampled at 480 nm, and intensity of green background light was 1.0×10^{15} photons cm^{-2} s^{-1}. Trace in (B) begins 5 min after the background light was turned on. The slightly larger basal inward current in (B) (−95 pA) relative to that in (A) (−85 pA) is what remains at steady-state of the inward current induced by the background.

(C) Plots of peak evoked current as a function of stimulus intensity for the data shown in (A) and (B). Desensitization induced by the background light shifted the curve to the right. The amount of the rightward shift was measured with −20 pA as the criterion response, as indicated by the arrow, to calculate the average change in sensitivity for all cells tested (see text).

(D) Paired-flash experiment in another cell, showing that exposure to one flash desensitized the response to a second flash of identical intensity (1.7×10^{13} photons cm^{-2} s^{-1} sampled at 480 nm) presented 50 s later.

(n = 16), the light-evoked current decayed with a time course paralleling that of the voltage response in Figure 2A. We conclude that the decay in the photoresponse reflects a gradual attenuation in the gain of the phototransduction cascade rather than a secondary effect of the light-evoked depolarization.

This reduction in gain gradually resensitizes ipRGCs to stimulus increments in the presence of an initially near-saturating background light. This is documented in Figure 3A, which shows the voltage responses of a dark-adapted ipRGC to a series of probe light pulses of fixed amplitude superimposed on a near saturating background. As the response to the background stimulus decayed, responses to the probe flashes grew in amplitude. Group data for a total of five cells studied in this way, summarized in Figure 3B, show that this was a consistent phenomenon and that it reached steady state within about 5 min after background onset with a time constant of 64 ± 33 s.

Voltage-clamp recordings yielded similar gradual (time constant = 71 ± 38 s) increases in the increment responses as the background-induced current decayed

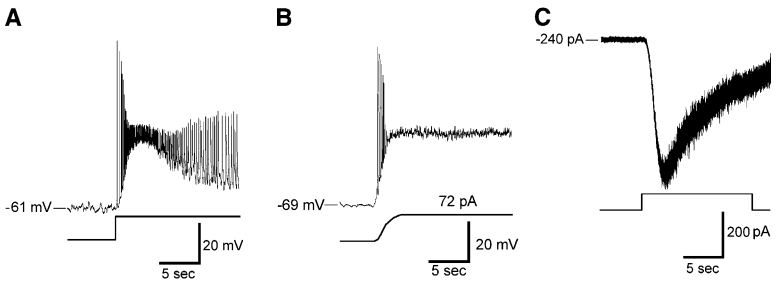

Figure 2. Evidence that Decay of the Response to Steady Light Cannot Be Explained by Voltage-Dependent Channel Gating

(A) Current-clamp response of an ipRGC to a prolonged light stimulus (2.2×10^{14} photons cm^{-2} s^{-1} sampled at 480 nm). The membrane reaches its maximal depolarization ~3 s after stimulus onset and then gradually repolarizes during steady illumination. Spikes fail during the response peak, presumably through depolarization block, but reappear during the subsequent repolarization.

(B) Voltage response of another cell to a direct current injection (indicated by the step in the bottom trace) simulating a photocurrent that develops relatively slowly but is constant thereafter. The flat voltage profile at steady state indicates that the decay in (A) reflects a reduction in photocurrent, not a voltage effect on ion channels.

(C) The light response of a third cell recorded under voltage clamp, showing that the relaxation of the response observed in current clamp mode (A) is also evident when the membrane potential is held constant (intensity = 1.7×10^{13} photons cm^{-2} s^{-1} sampled at 480 nm).

(n = 8) (Figures 3C and 3D). This argues against passive or active electrical properties of the membrane as the basis for these phenomena. For example, it makes it unlikely that the growth of the incremental responses is attributable to the activation of an increasing number of voltage-gated channels or that the gradual increase in increment responses merely reflects the increased driving force for the light-evoked current as the cell repolarizes.

Acceleration of Light Responses

Another hallmark of light adaptation in both vertebrate rods and cones and in invertebrate photoreceptors is the acceleration of responses to dim flashes, enhancing temporal resolution. The time to response peak is shortened and poststimulus recovery is more rapid (Fuortes and Hodgkin, 1964; Baylor and Hodgkin, 1974; Fain et al., 2001).

We found a similar phenomenon in ipRGCs (n = 5). Under voltage clamp and after partial dark adaptation (15–40 min), we presented a 5 s light flash evoking a relatively small response (40–70 pA). A dim background light was then turned on and, after adaptation to that background for at least 5 min, the same 5 s flash was presented again. Light adaptation significantly reduced the time to peak, from 9.0 ± 0.7 to 6.9 ± 0.7 s (p value = 0.0058). Light adaptation had a more pronounced effect on the decay of the flash response back to baseline, with the decay time constant reduced from 41 ± 4 to 18 ± 3 s (p value = 0.0052). An example is shown in Figures 4A and 4B. Light adaptation speeds response decay more than time to peak in other photoreceptors as well (Baylor and Hodgkin, 1974; Matthews et al., 1990; Gomez and Nasi, 1997).

The acceleration of response decay by background light became more prominent when cells were more fully dark adapted. This can be clearly seen in Figure 4C, which plots the ratio of the light-adapted and dark-adapted time constants as a function of duration of dark adaptation. In other words, dark adaptation progressively retards the recovery of ipRGCs from dim flashes.

Dark Adaptation

Recovery from Light Adaptation

Having demonstrated that light reduces the sensitivity of ipRGCs (Figures 1 and 2) and that this desensitization persists after the light is extinguished (Figure 1D), we now sought to track the increase in sensitivity as ipRGCs move from light into darkness and undergo dark adaptation. For the experiment shown in Figures 5A and 5B, we first light adapted a voltage clamped ipRGC by illuminating it with a bright background light. The background was then extinguished, and, 15–22 min later, sensitivity was assessed from current responses to a series of moderate-intensity probe flashes varying in irradiance (Figure 5A, "1st round," left). The cell was then left in darkness for ~20 min and retested with the same series of probe flashes (Figure 5A, "1st round," right). Dark adaptation increased the response to both the −2.5 log and the −2.0 log flashes, as shown most clearly in Figure 5B ("1st round"), which plots the peak response amplitude as a function of stimulus irradiance for this cell. Similar results were obtained from all cells tested, with the intensity-response curve shifting leftward by an average of 0.27 ± 0.01 log units over the 15–20 min of dark adaptation separating the first ("brief") and the second ("prolonged") intensity series (based on a criterion response of −10 pA; arrow in Figure 5B; n = 3). Similar results were obtained with this protocol in current-clamp recordings, with the intensity-response function shifting to the left by 0.35 ± 0.08 log units during the 20–30 min of dark adaptation based on a criterion response of 10 mV (n = 5; not shown).

This modulation of sensitivity by lighting history could be demonstrated repeatedly in individual cells. When the cell shown in Figures 5A and 5B was subjected to two more rounds of the same stimulus protocol, sensitivity was reduced shortly after each exposure to the background light, and prolonged dark adaptation induced a leftward shift of the intensity-response curve (Figure 5A, "2nd round"; Figure 5B, "2nd round" and "3rd round"). This is important because it excludes the possibility that the increase in sensitivity detected in the first round is entirely due to some generalized sensitization of the cell over time, as might occur, for example, as a consequence of intracellular dialysis by the recording pipette. Figures 5C and 5D document the voltage responses of another ipRGC to a probe flash of fixed intensity at various times after a periodically presented bright adapting light. The representative voltage traces in Figure 5C show that, after each of two rounds of adaptation, responses were invariably larger after a prolonged period in darkness (right) than shortly after the adapting light was extinguished (left). More complete data from the same cell are illustrated in Figure 5D, which

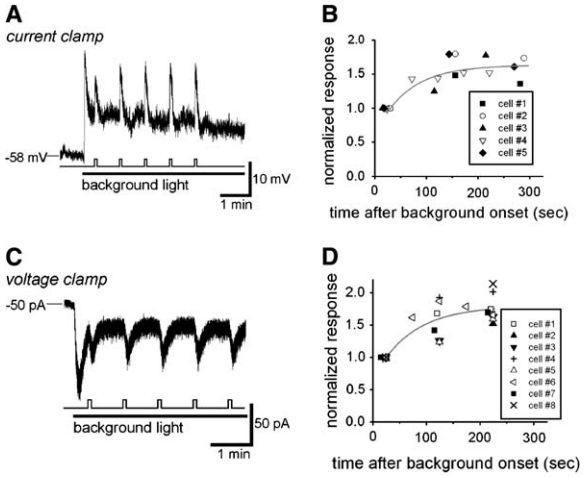

Figure 3. Evidence for Light Adaptation in ipRGCs, Revealed by the Gradual Resensitization of the Response to Periodic Probe Flashes Superimposed on a Bright Background Light

(A and B) Current-clamp recordings. (A) After this cell had been in darkness for 15 min, a background light (indicated by the horizontal bar at the bottom of the trace) was presented. At five different time points after the onset of the background, a probe flash was superimposed on the background (green background intensity: 1.0×10^{15} photons cm^{-2} s^{-1}; probe flash intensity: 4.3×10^{14} photons cm^{-2} s^{-1} sampled at 480 nm). (B) A summary of the results from five cells, with cell number four corresponding to the cell shown in (A). Durations of dark adaptation prior to background onset ranged from 10–25 min. The responses for each cell were normalized to the amplitude of the response to the first test flash. The gray curve is a first-order exponential decay fit for the data points from all five cells and has a time constant of 64 ± 33 s. Resensitization is evident for all cells.

(C and D) Voltage-clamp recordings. (C) The protocol was similar to that for (A) except that the probe flash had an irradiance of 1.7×10^{13} photons cm^{-2} s^{-1} sampled at 480 nm, and the cell had been kept in darkness for 10 min. (D) A summary of the results from eight cells, all showing resensitization. Cell number six represents the cell shown in (C). The durations of dark adaptation prior to background onset ranged from 10–15 min. Resensitization is again evident and the first-order exponential decay fit (gray curve) has a time constant of 71 ± 38 s.

plots the progress of sensitization at a finer time scale over a total of four rounds of adaptation. Similar data demonstrating multiple cycles of light/dark adaptation were obtained in a total of nine cells. The sensitivity of ipRGCs continued to increase at an unabated rate in the dark for at least 40 min under the conditions of these experiments (Figure 5D), suggesting that dark adaptation in ipRGCs may proceed for much longer than 40 min. The time course of ipRGC dark adaptation is examined further in the following section.

The dark adaptation data in Figures 5A and 5C provide further evidence for the ability of ipRGCs to light adapt. If the gradual increase in responsiveness were merely due to recovery from background-induced response compression, then there should be a parallel relaxation of the resting current or potential back to dark levels. Instead, after termination of the background light, the resting current and potential returned to dark levels within several minutes, and the responses measured after brief and prolonged dark adaptation had similar holding currents (Figure 5A) and resting membrane potentials (Figure 5C). In other words, all or nearly all of the cation channels previously opened by the background light had already closed after brief dark adaptation, and the time-dependent increase in the amplitude of flash responses (Figures 5A and 5C) most likely reflects a progressive increase in the gain of the phototransduction pathway. Thus, one effect of the background light is to reduce phototransduction gain, as expected for a light-adapting photoreceptor.

Time Course of Dark Adaptation

The data shown in Figures 4C and 5D suggest that after light adaptation, ipRGCs progressively regain responsiveness for at least 40 min. To assess more fully the time course and extent of recovery of sensitivity, we turned to recordings in the cell-attached configuration, an extracellular recording method that spares the cell from intracellular dialysis, which can affect sensitivity. Six cells were given an initial 1–5 min exposure to bright light, and then flashes of a fixed intensity were presented periodically to monitor the gradual increase in response amplitude. For all cells, the response to test flashes continued to increase as long as we were able to maintain recordings, which lasted between 40 and 160 min. Figure 6 shows data from the two recordings of longest duration. For the first cell (Figures 6A and 6B), a single −1 log flash was presented after 10 min of dark adaptation, evoking a response of 3.0 Hz. Thereafter, the test flash intensity was reduced to −2 log, and a flash was presented once every 10 min. The response to this weaker flash was barely measurable initially but became much more vigorous with time and by 70 min, slightly exceeded the response to the initial −1 log test flash. This indicates that this cell's sensitivity increased by about 1 log unit during the first hour of dark adaptation. Sensitivity appeared to be increasing even after 90 min, when the recording was lost. For the second cell (Figures 6C–6E), we sought to minimize any light adaptation that might be triggered by the test flashes by reducing their intensity whenever the response exceeded ~1 Hz. The cell was clearly still dark adapting after being in darkness for 160 min. With a criterion response of 0.8 Hz, threshold dropped by approximately 1 log unit in the first hour of dark adaptation (as did the first cell) (Figures 6A and 6B) and by another log unit between 70 and 160 min, with a time constant of 198 min (Figure 6E). In conclusion, after exposure to just a few minutes of bright background light, ipRGCs regain their sensitivity very slowly, with full recovery taking at least several hours.

Discussion

This study provides the first direct evidence that mammalian ipRGCs exhibit light and dark adaptation. Such adaptation is a virtually ubiquitous feature of both vertebrate and invertebrate photoreceptors, and analogous adaptation occurs in nearly all sensory receptors. In this sense, adaptation in ipRGCs was perhaps to have been expected. On the other hand, behavioral observations had suggested that neuronal pathways mediating non-image-forming visual responses may light adapt little, if at all, and so the data resolve a substantive functional question.

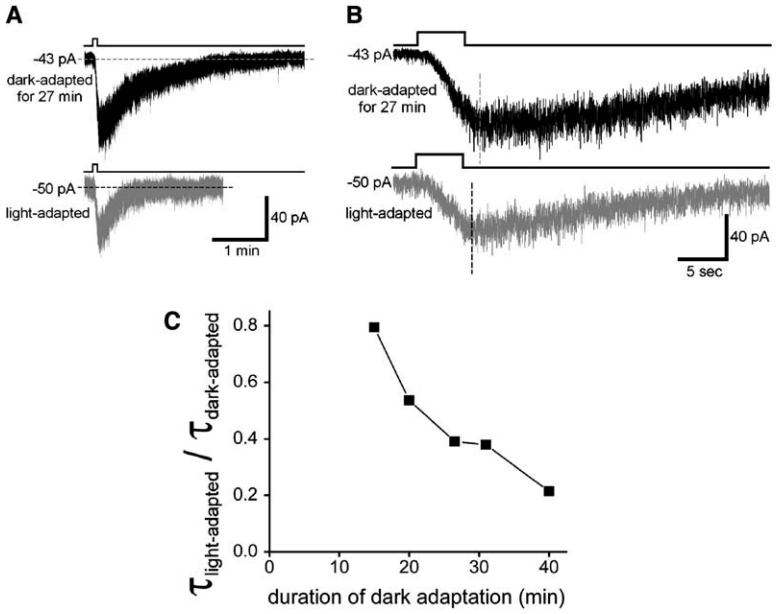

Figure 4. Light Adaptation Accelerates Light Responses

(A) Voltage-clamp responses of an ipRGC to a 5 s light flash (8.5×10^{11} photons cm^{-2} s^{-1} sampled at 480 nm) after 27 min of dark adaptation (top response trace) and then again 5 min after adapting to a dim white background (7.4×10^{10} photons cm^{-2} s^{-1} sampled at 480 nm; bottom trace). The response obtained in the light-adapted state decayed from the peak to the baseline (indicated by the horizontal dashed line) more rapidly than that obtained in the dark-adapted state. The time constants of the decay are 22 and 55 s for the light- and the dark-adapted responses, respectively. Notice also that the light-adapted response is smaller in amplitude than the dark-adapted one, indicating desensitization by the background.

(B) The same responses as those shown in (A) but on an expanded time scale to show that light adaptation shortens the time to peak (indicated by the vertical dashed lines), from 7.4 to 6.2 s.

(C) A summary of the data from all five cells tested, showing that the longer a cell was allowed to dark-adapt (x axis), the more dramatic was the subsequent background-induced shortening of the response decay time constant, resulting in a lower ratio of light-adapted to dark-adapted time constants (y axis).

Properties and Mechanism of ipRGC Adaptation

The light and dark adaptation reported here were observed under blockade of glutamatergic synaptic transmission, effectively interrupting signaling between rod and cone photoreceptors and the inner retina. Thus, the changes in sensitivity we report reflect processes intrinsic to the ipRGCs. Stated in the nomenclature of classic retinal adaptation literature, they represent a form of "photoreceptor adaptation" rather than "network adaptation" (Dowling, 1987). Both photoreceptor and network adaptation are evident in the rod and cone systems, and our data do not exclude a network contribution to ipRGC adaptation. Indeed, conventional photoreceptors appear to exert both excitatory and inhibitory influences on ipRGCs (Belenky et al., 2003; Dacey et al., 2005) (K.Y.W., F.A.D., and D.M.B., unpublished data), and rod/cone adaptation would seem very likely to modulate these influences.

The adaptation observed appears to reflect a change in the signaling pathway coupling melanopsin to the light-activated ion channel in the plasma membrane. Thus, it represents a form of "light adaptation" or "biochemical adaptation." It is not simply a consequence of response compression, which can be expected as the pool of light-gated channels available for responding to further increments of light dwindles with progressive increases in background lighting. We know this because the depolarization evoked by an initially saturating background stimulus decays while responses to superimposed light increments concurrently reemerge (Figure 3). Further, the gradual repolarization in the face of steady light reflects a dynamic alteration in the phototransduction cascade itself rather than an unrelated electrical effect on the membrane, such as gating of voltage-sensitive channels because it is observable under voltage clamp (Figure 2C) and cannot be mimicked by injection of steady current (Figure 2B). In addition, background light accelerates the responses to superimposed flashes (Figure 4). Thus, all the hallmarks of light adaptation in rods and cones are observable in rat ipRGCs. ipRGCs in the primate retina are likely also capable of light adapting because their responses to light steps display the kind of relaxation observed here (Dacey et al., 2005).

The kinetics of rat ipRGC light adaptation (with a time constant of ~1 min and completion within ~5 min) is somewhat slower than that for rods and cones of various vertebrate species, which have been reported to fully light adapt within anywhere from 1 s to 3 min (Normann and Perlman, 1979; Cervetto et al., 1985; Silva et al., 2001; Calvert and Makino, 2002). Because of technical difficulties inherent in cell-attached recordings, we were unable to determine the full time course of ipRGC dark adaptation, although we found that after exposure to several minutes of bright backgrounds, the rate of dark adaptation remained constant for at least 40 min for all cells tested, and the recording of longest duration revealed a dark adaptation time constant of ~3 hr (Figures 6C–6E). By contrast, the cones in albino rats have been reported to complete dark adaptation within about 30 min, whereas the rods in these animals dark adapt with a time constant of ~40 min and reach the final steady state in about 3 hr (Perlman, 1978; Behn et al., 2003). Thus, the kinetics of dark adaptation is much slower for ipRGCs than for cones and probably also considerably slower than for rods. Such slow kinetics are unlikely to be an artifact of our recording conditions. We measured from the same preparation the

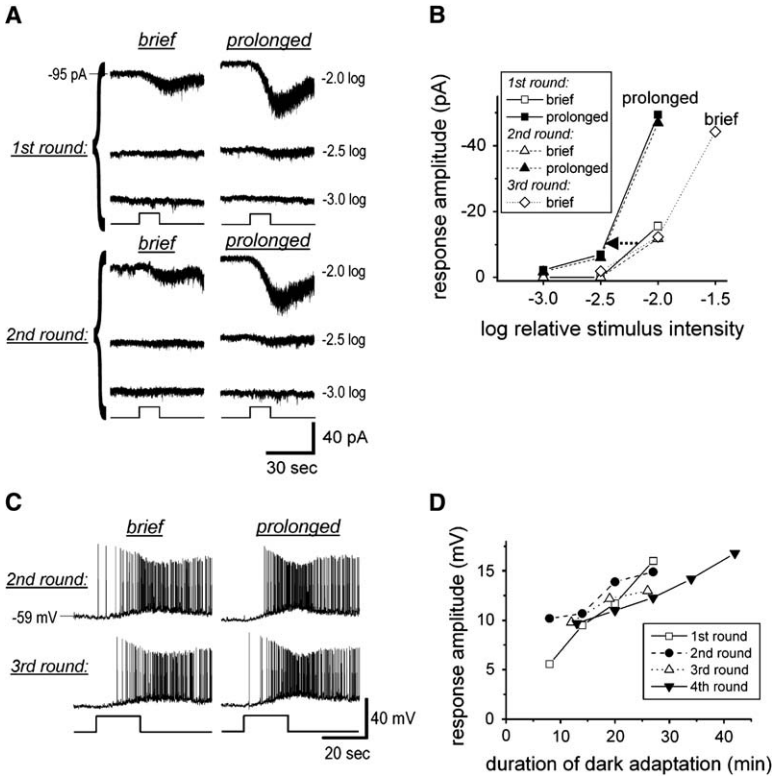

Figure 5. Dark Adaptation in Whole-Cell-Recorded ipRGCs

(A and B) Increases in sensitivity with dark adaptation are apparent when light-evoked currents are recorded under voltage clamp. Three rounds of adaptation were carried out for this cell. In each round, a green adapting light (1.0×10^{15} photons cm^{-2} s^{-1}) was first presented for 10 min, after which a series of dim light flashes (unattenuated irradiance: 1.7×10^{13} photons cm^{-2} s^{-1} sampled at 480 nm) was presented after brief dark adaptation and again after more dark adaptation. (A) shows representative data from the first two rounds of adaptation. Brief dark adaptation (left) corresponds to 15–22 min of darkness after the adapting light and prolonged adaptation to 35–45 min (round 1) or 39–47 min (round 2) of darkness. (B) Intensity-response plot of all the data from this cell, showing that both epochs in darkness shifted the curve to the left, indicating increased sensitivity. The arrow indicates the criterion response (−10 pA) used for calculating the average sensitivity increase for all cells tested (see text).

(C and D) Data from another cell showing that dark adaptation can also be observed repeatedly in the same cell under current clamp. For each iteration of adaptation, this cell was exposed to a white light-adapting background light (2.2×10^{14} photons cm^{-2} s^{-1} sampled at 480 nm; 1 min). This was then extinguished and fixed-intensity test flashes (1.7×10^{11} photons cm^{-2} s^{-1} sampled at 480 nm) presented periodically. The process was repeated three times. (C) Representative voltage records from the second and third rounds of adaptation. Responses were smaller after brief dark adaptation (left, 8 min in round 2, 12 min in round 3) than after prolonged dark adaptation (right column, 20 and 26 min, respectively). (D) A summary of all responses obtained from the same cell.

pharmacologically isolated PIII component of the electroretinogram, which reflects rod and cone activity (Dowling, 1987), and found full recovery from bleaching background lights required about 5 hr (K.Y.W. and D.M.B., unpublished data), comparable to that found in vivo (Perlman, 1978; Behn et al., 2003). A potential functional relevance for such a slow ipRGC dark adaptation time course is discussed below.

Although the adaptational phenomena observed thus appear traceable to the phototransduction cascade within the ipRGCs themselves, the precise loci and mechanisms for the modulation of sensitivity remain to be determined. Progress in this area is currently hampered by our ignorance of the biochemical properties of the photopigment melanopsin, of the signaling pathways linking it to the light-activated channel and of the identity of that channel. Calcium plays a key role in the adaptation process in other photoreceptors (Fain et al., 2001; Lisman et al., 2002), and because light appears to elevate intracellular calcium in ipRGCs (Sekaran et al., 2003, 2005), it is conceivable that calcium is also involved in ipRGC adaptation.

Signs of Adaptation in Non-Image-Forming Visual Behaviors

Very sustained responses and long-term temporal integration are hallmarks of both ipRGCs and the non-image-forming behaviors to which they contribute. At the cellular level, these qualities are evident in the ability of constant light to drive continuous depolarization and sustained expression of the activity-dependent gene *c-fos* in ipRGCs (Hannibal et al., 2001; Berson et al., 2002). A striking behavioral correlate is the remarkably long interval over which increases in stimulus duration can compensate for reductions in intensity to produce a constant circadian phase shift (Takahashi et al., 1984). Another behavioral correlate is the unique capacity of the melanopsin system to suppress nocturnal locomotor activity for many hours when stimulated by constant light ("negative masking") (Mrosovsky and Hattar, 2003). Similarly, light-evoked changes in pupil diameter are remarkably tonic and track ambient light intensity (Lucas et al., 2001, 2003; Hattar et al., 2003).

Although these data emphasize the capacity of this system for signaling the presence of light over the very long term, they do not preclude some degree of adaptation. Indeed, evidence for adaptation has been reported for the three best characterized non-image-forming visual behaviors, namely circadian entrainment, pupillary light reflex (PLR), and acute photic suppression of pineal melatonin release. Nelson and Takahashi (1991) found that light stimuli lasting ~300 s were the most effective in inducing phase shifts of circadian wheel-running activity in hamster, whereas longer-duration stimuli were less effective and proposed that light adaptation might explain such a finding. Furthermore, it has been reported that the magnitude of photically induced circadian phase shifts was correlated with the time spent in

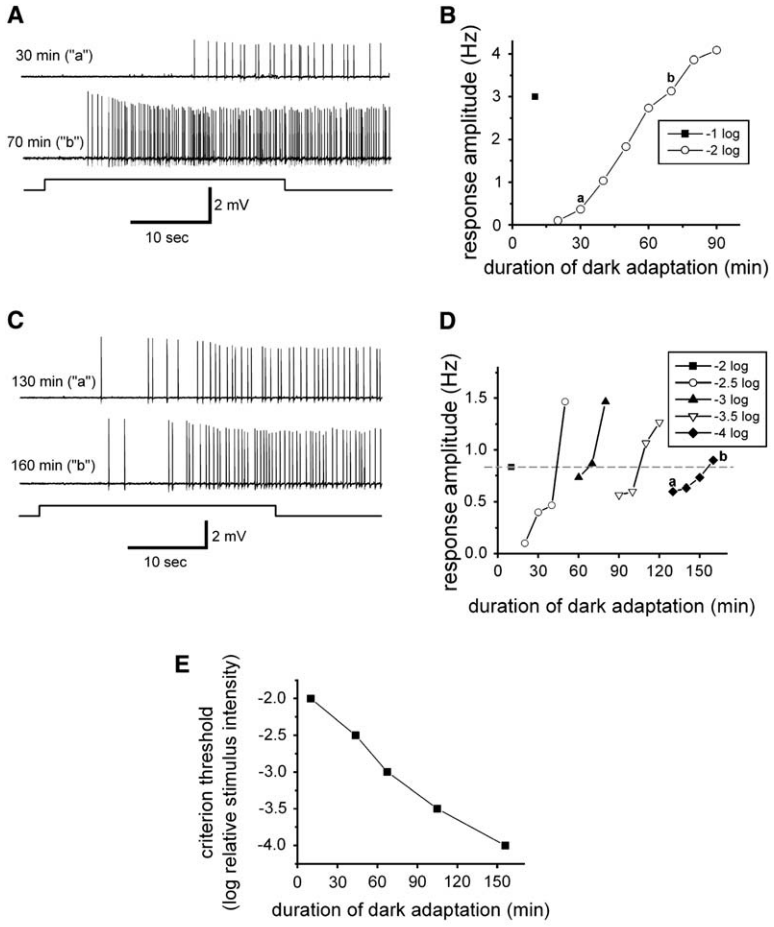

Figure 6. Cell-Attached Recordings from Two Cells Reveal Very Slow Kinetics of ipRGC Dark Adaptation

(A and B) Data from the first cell, whose sensitivity increase during dark adaptation after exposure to a green background (5.1×10^{17} photons cm^{-2} s^{-1}; 5 min) was probed mainly with fixed-intensity white flashes. Between 20 and 90 min of dark adaptation, the intensity of the test flashes was 1.7×10^{11} photons cm^{-2} s^{-1} sampled at 480 nm, although the flash presented at 10 min was 1 log unit brighter. (A) shows the raw data for the data points labeled "a" and "b" in (B).

(C–E) Data from the second cell, for which the intensity of the probe flashes (unattenuated intensity: 1.7×10^{13} photons cm^{-2} s^{-1} sampled at 480 nm) was gradually reduced during dark adaptation to keep the responses relatively small. The same green background was used to light-adapt this cell. (D) is a summary of the result, and the raw traces for data points "a" and "b" are shown in (C). The horizontal dashed line in (D) is used to determine the time points when each of the stimulus intensities triggered the same response amplitude as that evoked by the −2 log flash at 10 min, enabling generation of the criterion threshold versus dark adaptation duration plot shown in (E).

darkness prior to the light pulse (Shimomura and Menaker, 1994; Refinetti, 2003). Likewise, the PLR gradually regains sensitivity as the duration of dark adaptation after prior exposure to adapting light increases (Ohba and Alpern, 1972; Trejo and Cicerone, 1982). Finally, there is some evidence that the ability of light to acutely suppress pineal melatonin release is modulated by lighting conditions in the hours preceding the experimental light pulse, suggestive of light adaptation (Hebert et al., 2002; Smith et al., 2004). Consistent with this observation, light responses recorded from the pineal gland of rats often adapt rapidly (Thiele and Meissl, 1987; Martin and Meissl, 1990). In seeking to relate such evidence to the photoreceptor adaptation we report in ipRGCs, it is important to remember that rods and cones as well as melanopsin contribute to all three visual behaviors (Lucas et al., 1999, 2001, 2003; Panda et al., 2003; Hattar et al., 2003). It will be of interest to reexamine these phenomena in rodless-coneless and in melanopsin knockout mice to determine whether the classical outer retinal photoreceptors, the melanopsin system, or both contribute to these adaptational phenomena.

Conversely, several other findings seem to suggest a lack of adaptation in photic entrainment and the PLR. Nelson and Takahashi (1999), analyzing hamster locomotor rhythms, found that although background light reduced the amount of circadian phase resetting induced by a light pulse, this appeared to result from response saturation rather than true light adaptation. They further showed that when an initial light stimulus triggered such saturation, the circadian system was virtually unresponsive to subsequent light stimulation for at least an hour, suggesting a very slow recovery of sensitivity after extinction of that stimulus. By contrast, in ipRGCs, though full dark adaptation takes hours, resting potentials return to their dark levels within 10 min even after intense illumination (e.g., Berson et al., 2002), and responsiveness returns within minutes (Figures 5 and 6). Turning to the rodent PLR, prolonged light steps trigger a pupillary constriction that rises rapidly to a peak, with no significant relaxation over at least 1 min. This contrasts sharply with the decaying depolarization that ipRGCs exhibit in response to such light steps, a decay we attribute at least partly to light adaptation. The lack of decay in the pupillary responses has been observed both in mice lacking functional rods and cones, in which the response is driven by melanopsin, and in melanopsin knockout mice in which only rods and cones can drive the PLR (Hattar et al., 2003; Lucas et al., 2001, 2003).

It is difficult to determine why these two behaviors, which are driven by a combination of inner and outer retinal photoreceptor signals, fail to reflect some of the adaptational phenomena that we detect in the ipRGCs and that are well known for rods and cones. Certainly, different species were used, and there were disparities in

adaptation conditions and stimulus protocols. The most important difference, however, may be that the non-image-forming visual behaviors do not always faithfully retain all the information generated by the photoreceptors driving these behaviors. For photic effects on circadian phase, the loss of such information most likely occurs beyond the first synapse in the SCN, because the light responses of SCN neurons share many similarities with those of ipRGCs, rods and cones. For example, SCN cells recover from previous light stimulation much more rapidly than does circadian phase-resetting by light and can thus respond to multiple light flashes presented in relatively quick succession (e.g., with 2 min intervals). Further, increased firing rates evoked by light in SCN cells often decay during steady illumination in a manner suggestive of light adaptation (Groos and Mason, 1980; Meijer et al., 1992, 1998; Aggelopoulos and Meissl, 2000). In addition, lighting history has been shown to affect the photic responses of SCN cells, assessed either electrophysiologically or from patterns of c-Fos expression (Meijer et al., 1986; Aggelopoulos and Meissl, 2000; Coogan and Piggins, 2005). The slow recovery of photic effects on circadian phase measured behaviorally (Nelson and Takahashi, 1999) thus probably reflects rate-limiting, long-lasting biochemical events downstream from the SCN light responses. Similarly, the discrepancy between the temporal profile of light responses of cells in the olivary pretectal nucleus (OPN) (which typically show a marked decay from an initial peak; Trejo and Cicerone, 1984) and of the pupillary response (which largely lacks such decay) suggests temporal filtering by mechanisms downstream of the OPN light responses.

Potential Functions of ipRGC Adaptation

Various functional roles of ipRGC adaptation can be contemplated. First, the adaptation-based decay of the response to prolonged light may be required to maintain spiking. With constant inward current injection, ipRGCs stop spiking rapidly (Figure 2B; see also Warren et al., 2003), presumably because of depolarization block. Similarly, spiking is often suppressed at the peak of the light response and resumes only after partial repolarization (Figure 2A). Second, it is possible that the ability of ipRGCs to light adapt helps photic entrainment operate under a variety of ambient light levels, such as those caused by seasonal and weather changes. For example, daylight is less intense in winter than in summer, and thus ipRGCs would generate weaker light responses in winter unless they can adjust their sensitivity. The long nights of winter may provide a basis for such a sensitivity adjustment, however, because they may permit more complete dark adaptation of ipRGCs than is possible in short summer nights. We have demonstrated that dark adaptation of ipRGCs is very slow and continues for at least 2 hr 40 min (Figure 6). If further increases in sensitivity occur over many hours, sensitivity at dawn should be higher in winter than in summer, and this could compensate for the weaker winter light, stabilizing light response amplitudes across the seasons. Third, light adaptation in conventional photoreceptors has been shown to make their intensity-response curves shallower than if light adaptation were absent, thus broadening their response dynamic ranges (Baylor and Hodgkin, 1974; Matthews et al., 1988). It is plausible that light adaptation in ipRGCs likewise serves to enable them to discriminate a wider range of light intensities, e.g., from dawn light to noon light. Fourth, the outputs of ipRGCs to the brain are diverse (Gooley et al., 2003; Morin et al., 2003; Hannibal and Fahrenkrug, 2004), and the functional roles served by many of these projections are obscure. The functional relevance of the adaptation identified here may vary significantly among these circuits. In those subject to extensive temporal integration, such as circadian entrainment, the adaptational changes may be difficult to detect. In others that operate on a faster time scale, the contribution of adaptation may be more evident, as in possible interactions with the geniculostriate circuit in primates (Dacey et al., 2005). Finally, there has been some evidence suggesting a role for ipRGCs in intraretinal processing (Van Gelder, 2001; Hankins and Lucas, 2002), and the adaptational properties of ipRGCs might therefore contribute to the adaptational states of other retinal cells. In summary, ipRGC-driven visual pathways and behaviors may be more complex than previously thought, and further studies will be needed to address the above possibilities.

Experimental Procedures

All procedures conformed to NIH guidelines for work with laboratory animals and were approved by the Institutional Animal Care and Use Committee at Brown University.

Retrograde Labeling and In Vitro Preparation

Rats (male, Sprague-Dawley, 56–70 days of age, 265–355 grams) were anesthetized with ketamine (60 mg/kg i.p.; Wyeth, Madison, NJ) and medetomidine (0.4 mg/kg i.p.; Orion Corporation, Finland). Fluorescent latex microspheres (rhodamine labeled, alone or mixed with fluoroscein-labeled microspheres; Lumafluor; 0.1–0.3 μl; Naples, FL) were deposited stereotaxically into the hypothalamus unilaterally through glass pipettes tilted 10° from vertical. 5–60 days after tracer injection, animals were anesthetized (Beuthanasia, 360 mg/kg i.p.; Schering-Plough Animal Health, Union, NJ), and eyes removed and hemisected. After removal of the vitreous humor, eyecups were maintained at room temperature (20°C–25°C) in Ames medium gassed with 95% O_2 5% CO_2.

Electrophysiology

Flattened eyecups were put on a coverslip, anchored by a weighted nylon mesh with the vitreal surface up, and mounted in a chamber (Warner RC-26GLP; Hamden, CT). The chamber was mounted on a fixed-stage upright microscope (Nikon E600FN; Melville, NY). Retrolabeled RGCs were located by epifluorescence (530–550 nm, 5.1×10^{17} photons cm^{-2} s^{-1}; Chroma filter 31002A; Rockingham, VT) and then visualized by transillumination and IR-DIC optics during mechanical exposure of the soma with an empty patch pipette. Because of this relatively intense epifluorescence light, all cells were partially light adapted when recording started. Whole-cell patch recordings were established under visual control with pipettes pulled from thick-walled borosilicate tubing (tip resistances 3–7 MΩ) on a Flaming/Brown P-97 puller (Sutter Instruments, Novato, CA). Recordings were made in voltage or current clamp mode with a Multiclamp 700A amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Series resistance was partially compensated and typically reduced to ~20 MΩ. Recordings were discarded if series resistance became excessive. PClamp 9 (Axon Instruments/Molecular Devices) was used for data acquisition. Signals were low-pass filtered at between 200 Hz and 4 kHz, and the sampling frequency was at least four times higher than the low-pass filter cutoff. Liquid junction potentials (14 mV for the K^+-based and 10 mV for the Cs^+-based internal solutions; see below) were corrected for whole-cell recordings.

For cell-attached recordings, pipettes were filled with the K^+-based internal solution, and seals were relatively loose (typically

around 100–500 MΩ) to help prevent spontaneous rupture of the cell membrane. Data were high-pass filtered at 3–100 Hz and low-pass filtered at 4 kHz, and the sampling frequency was at least 16 kHz. The light response was determined by subtracting the average spike frequency during the 10 s just before stimulus onset from the average spike frequency during the entire light stimulus. Microcal Origin software (OriginLab Corporation, Northampton, MA) was used for statistical calculations and for generating data plots.

Electrode and Bathing Solutions

Internal solutions contained the following (in mM): K-gluconate or Cs-methanesulfonate, 120; NaCl, 5; KCl or CsCl, 4; HEPES, 10; EGTA, 2; ATP-Mg, 4; GTP-Tris, 0.3; phosphocreatine-Tris, 7. pH was adjusted to 7.3 with KOH or CsOH. The potassium-based solution was used in current-clamp and cell-attached experiments, and the cesium-based solution in voltage-clamp experiments to improve space clamp. In the voltage-clamp experiments, all cells were held at −70 mV.

The extracellular solution during recording was Ames medium gassed with 95% O_2 5% CO_2. In addition, to isolate the intrinsic photosensitivity of ipRGCs, we suppressed activity in rod and cone-driven networks by including 100 μM L(+)-2-Amino-4-phosphonobutyric acid (L-AP4) to saturate and thus block signal transfer at group III metabotropic glutamate receptors essential for photoreceptor-to-ON-bipolar cell transmission, 40 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 30 μM D-2-Amino-5-phosphonovaleric acid (D-AP5) or 50 μM DL-2-Amino-5-phosphonovaleric acid (AP5) to block ionotropic glutamate receptors essential for communication between photoreceptors and OFF bipolar cells and between all bipolar cells and both amacrine and ganglion cells. Tetrodotoxin (TTX; 500 nM) was added to the extracellular solution to block voltage-gated sodium channels in all voltage-clamp experiments to further improve space clamp; however, because of the relatively long dendrites of ipRGCs (Berson et al., 2002), space clamp at distal dendrites may still be inadequate, and thus interpretations of the voltage-clamp data should be regarded with caution. TTX was also used in some current-clamp experiments (e.g., Figure 3A) to block spikes, facilitating the analysis of graded voltage responses.

This extracellular solution was gravity fed into the recording chamber at 2–6 ml min^{-1}. All experiments were performed at room temperature. Chemicals were dissolved in distilled water to generate stock solutions. L-AP4, CNQX, DNQX, D-AP5, and AP5 were purchased from Tocris (Ellisville, MO). All other chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Light Stimuli

In the light adaptation experiments, two independent light sources were used, one to set the background irradiance and a second to deliver briefer light stimuli ("test pulses") superimposed on this background. Two kinds of background light were used. For the experiments shown in Figures 1 and 3, the background light was the same full-field green epifluorescence light used for identifying retrolabeled ganglion cells except that it was attenuated 512-fold with neutral density filters, giving an intensity of 1.0×10^{15} photons cm^{-2} s^{-1}. For the experiments shown in Figure 4, the background was full-field white light generated by a tungsten lamp (FO-6000; World Precision Instruments, Sarasota, FL). This was presented through the camera port and objective lens and provided an irradiance at the tissue of 7.4×10^{10}–7.4×10^{11} photons cm^{-2} s^{-1} when sampled at 480 nm (see below). In all experiments, test pulses were full-field, broadband white light introduced from below, with the microscope's 100 W tungsten-halogen lamp and transillumination optics, and were presented at intervals long enough (typically 1–10 min) for the membrane potential or holding current to recover from the previous flash. An electromechanical shutter regulated the timing of test pulses. Neutral density filters (Newport/Oriel, Stratford, CT) were used to control stimulus energy. In the dark adaptation experiments, both the green epifluorescence light and the transilluminating broadband white light were used as light-adapting backgrounds.

Stimulus energy was measured with a calibrated radiometer (UDT Instruments, Baltimore, MD). The irradiance of the green epifluorescence background light was measured by placing the photodiode directly under the objective lens and by assuming that all photons were at 540 nm. For white light, the irradiance was sampled at 480 nm, the λ_{max} for melanopsin (Berson et al., 2002; Hattar et al., 2003; Qiu et al., 2005; Dacey et al., 2005), by placing a 480-nm narrowband filter (Newport/Oriel, Stratford, CT; 10 nm width at half height) between the light source and the photodiode. For test pulses delivered from the scleral side, stated intensities have been corrected for the attenuation (~1 log unit) by the sclera, choroid, pigment epithelium, and retina, assessed by placing a flattened eyecup between the light source and the photodiode.

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