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CRYPTOCHROME Is a Blue-Light Sensor That Regulates Neuronal Firing Rate

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Abstract

Light-responsive neural activity in central brain neurons is generally conveyed through opsin-based signaling from external photoreceptors. Large lateral ventral arousal neurons (ILNvs) in *Drosophila melanogaster* increase action potential firing within seconds in response to light in the absence of all opsin-based photoreceptors. Light-evoked changes in membrane resting potential occur in about 100 milliseconds. The light response is selective for blue wavelengths corresponding to the spectral sensitivity of CRYPTOCHROME (CRY). *cry*-null lines are light-unresponsive, but restored CRY expression in the ILNv rescues responsiveness. Furthermore, expression of CRY in neurons that are normally unresponsive to light confers responsiveness. The CRY-mediated light response requires a flavin redox-based mechanism and depends on potassium channel conductance, but is independent of the classical circadian CRY-TIMELESS interaction.

The *Drosophila melanogaster* circadian clock circuit is composed of 140 to 150 neurons in the central brain and includes PIGMENT-DISPERSING FACTOR (PDF)-expressing lateral ventral neurons. The large lateral ventral neurons (ILNvs) are arousal neurons (1–3) and increase spontaneous action potential firing in response to light (4), whereas the small lateral ventral neurons (sLNvs) are critical for circadian function (5). Light resets the circadian clock via two mechanisms (6): rhodopsin-based external photoreceptors [the compound eye, ocelli, and the Hofbauer-Buchner (HB) eyelet] and the blue-light photopigment CRYPTOCHROME (CRY). *Drosophila* CRY is best known for its light-activated targeting of TIMELESS (TIM) for degradation, resetting the clock (7–9). External photoreceptors and CRY entrain the *Drosophila* circadian circuit at vanishingly low light levels (10, 11). CRY also mediates magneto-sensitivity in flies and butterflies (11–13).

In addition to the circadian molecular clock, membrane excitability is a key component of normal maintenance of circadian rhythms (14). Electrophysiological characterization of the s- and ILNvs has shown that their membrane properties are circadian-regulated outputs as

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Materials and Methods

Figs. S1 to S3

Tables S1 and S2

References

well. Spontaneous firing frequencies are higher during the early day, gradually drop until dusk, and then rise again through the course of the night (1, 15). Additionally, the ILNv spontaneous firing frequency elevates 20 to 200% in response to moderately bright light (4). Given the plurality of light inputs to the ILNv, we investigated the ILNv electrophysiological light response (16) and found that the response is due to CRY acting by a cell-autonomous, redox-based mechanism, independent of CRY-TIM interactions, which requires the conductance of membrane potassium channels. Furthermore, ectopic expression of CRY optogenetically confers electrophysiological light responsiveness to neurons that ordinarily do not respond to light.

Results

Both tonic and burst firing ILNvs recorded in the whole-cell current clamp configuration in an acutely dissected whole-brain preparation from flies expressing the *pdfGAL4* driver and green fluorescent protein (GFP)-tagged nonconducting UAS-dORK, a *Drosophila* membrane-delimited potassium channel (4, 14), (*pdfGAL4-NC1-GFP*) under dark conditions (>0.02 mW/cm²) immediately increased their firing rate and their resting membrane potential in response to moderate-intensity white light (4 mW/cm²) (Fig. 1A, top) or high-intensity blue light (19 mW/cm²) from a mercury light source (450 to 490 nm) (Fig. 1A, bottom), then rapidly returned to baseline firing rate upon return to darkness. The strength of the firing frequency ILNv light response, expressed here as the firing frequency with the lights on divided by the firing frequency with the lights off (FF on/FF off), varied with light intensity, exhibiting significantly higher firing frequency during lights on compared with lights off at intensities of 2 to 3 mW/cm² or higher (Fig. 1B). FF on/off for 19 mW/cm² was 1.62 ± 0.14 ($n = 11$), for 4 to 5 mW/cm² was 1.51 ± 0.15 ($n = 18$), for 2 to 3 mW/cm² was 1.39 ± 0.06 ($n = 68$), for 1 to 2 mW/cm² was 1.18 ± 0.02 ($n = 27$), for 0.6 mW/cm² was 1.23 ± 0.06 ($n = 16$), and for 0.3 mW/cm² was 1.10 ± 0.04 ($n = 13$). Light responses to intensities of 19 mW/cm², 4 to 5 mW/cm², and 2 to 3 mW/cm² were significantly different from 1 to 2 mW/cm² [$P = < 0.0001$, 0.006, and 0.02, respectively, by analysis of variance (ANOVA)].

The ILNvs anatomically appear to receive input from the compound eyes and the HB eyelet. To determine whether the ILNv light response is due to synaptic inputs from external opsin-based photo-receptors, we recorded ILNv in *glass60j* (*gl60j*) mutant flies, which lack all external photoreceptors because of a null mutation in the *eyeless* gene (6). The ILNv response to moderate-intensity white light for *gl60j* flies was 1.37 ± 0.09 ($n = 14$; $P = 0.81$ versus control); under intense blue light, the response of *gl60j* flies was 1.59 ± 0.1 ($n = 5$, $P = 0.82$ versus control, as tested by ANOVA). Thus, the responses to white and intense blue light do not differ between control and *gl60j* mutant flies (Fig. 1C).

The intact ILNv light response in *gl60j* mutant flies suggests that the blue-light photo-pigment CRY expressed in the ILNv may underlie the response. *Drosophila* CRY is excited maximally at 450 nm and absorbs wavelengths no longer than 530 nm (17), so the ILNv response to discrete wavelength ranges was tested. The ILNvs significantly increased their firing rate in response to both moderate intensity blue-green light (<550 nm; 1.34 ± 0.04 , $n = 26$, $P < 0.001$ by paired *t* test of lights on versus off) and low-intensity blue-violet light (375 to 450 nm; 1.33 ± 0.02 , $n = 70$, $P < 0.001$), but not to red-orange light (>550 nm; 1.04

± 0.02 , $n = 23$, $P = 0.18$) (Fig. 1, D and E). The spectral profile of the ILNv light response does not differ when tested in *gl60j* mutant flies (Fig. 1E, FF on/off is 1.37 ± 0.09 , $n = 14$, $P = 0.001$ for white light; 0.94 ± 0.05 , $n = 9$, $P = 0.54$ for orange-red light; 1.21 ± 0.07 , $n = 12$, $P = 0.003$ for blue-green; 1.29 ± 0.05 , $n = 14$, $P < 0.001$ for blue-violet light.) For comparisons for a given wavelength range between control and *gl60j*, P values were 0.81, 0.42, 0.27, and 0.64 for white, orange, blue-green, and blue-violet light, respectively. The lack of ILNv responsiveness to wavelengths > 550 nm shows that infrared does not contribute to light-driven increases in firing frequency. The spectral profile of the ILNv light response matches that of CRY but requires about five orders of magnitude higher light intensity than that required for CRY's known role in resetting the circadian clock via TIM degradation and occur between four to five orders more rapidly than the first biochemical indications of CRY-mediated TIM degradation. Last, unlike CRY-mediated TIM degradation, the ILNv light response is reversible, suggesting that the two CRY-mediated phenomena occur via distinct mechanisms.

Hypomorphic *cry^b* mutant flies, which exhibit impaired circadian function because of a point mutation in the flavin chromophore binding site, display a weakened ILNv response to white light compared with that of their control counterparts (4). We quantified the light response of *cry^b* ILNvs and tested its spectral properties. Figure 1E (right) shows that, like in control and *gl60j* flies, white, blue-green, and blue-violet wavelengths evoke a significant increase in firing frequency (FF on/off = 1.14 ± 0.01 for white light; 1.13 ± 0.02 for blue-green light; 1.16 ± 0.03 for blue-violet light; $P < 0.01$ by paired t test in each case) in *cry^b* mutant flies' ILNvs. However, these *cry^b* light responses are significantly smaller than for their corresponding wavelengths in control flies ($P = 0.02$ for white, $P = 0.005$ for blue-green, and $P = 0.004$ for blue-violet by ANOVA).

To determine whether CRY is necessary for the ILNv light response, we recorded ILNvs from two CRY-null lines, *cry⁰¹* and *cry⁰²*. Immunocytochemistry confirms baseline native CRY expression in control flies (Fig. 2A), replicating previous results with this CRY antisera (18), and no staining of CRY-positive neurons in either CRY null line (Fig. 2B); however, CRY expression was selectively rescued in the LNvs in both CRY null lines when UAS-CRY was driven by *pdfGAL4* (Fig. 2C). The ILNvs of the *cry⁰¹* and *cry⁰²* null flies exhibited normal spontaneous firing but no light response (Fig. 2, D, top, and E) (*cry⁰¹* response = 1.03 ± 0.04 , $n = 26$, $P = 0.007$ versus control; *cry⁰²* response = 1.04 ± 0.03 , $n = 21$; $P = 0.01$ versus control, ANOVA), indicating that CRY is required for the ILNv light response. The ILNv light response was restored to levels indistinguishable from controls by LNv CRY expression in both *cry⁰¹* (FF on/off = 1.37 ± 0.15 , $n = 17$, $P = 0.99$ versus control) and *cry⁰²* (FF on/off = 1.48 ± 0.10 , $n = 14$, $P = 0.98$ versus control; Fig. 2, D, lower panels, and E) genetic background flies. Although the PDF driver does target the sLNvs in addition to the ILNvs, we have observed light sensitivity in the ILNvs of flies whose sLNvs have been genetically ablated (fig. S1) [also (1)]. Thus, we conclude that the CRY-driven ILNv light response is cell-autonomous and independent of CRY expression in external photoreceptors or other central brain neurons.

To determine whether CRY can optogenetically confer electrophysiological light responsiveness to inherently light-insensitive neurons, we targeted CRY expression to

olfactory projection neurons with the *GH146-GAL4* driver (fig. S2). This is a rigorous test of (i) CRY's ability to autonomously confer light responsiveness, (ii) a conserved mechanism for coupling CRY light activation to membrane changes in nonclock neurons, and (iii) independence of CRY/TIM interaction. CRY-expressing olfactory neurons increased firing rate recorded in voltage clamp mode in response to intense blue light (Fig. 3A), whereas control CRY-minus olfactory neurons were nonresponsive to white (FF on/off = 1.04 ± 0.02 , $n = 8$), blue-green (0.99 ± 0.04 , $n = 8$), blue-violet (1.01 ± 0.03 , $n = 8$), intense blue (1.04 ± 0.03 , $n = 8$), and orange-red (0.96 ± 0.06 , $n = 8$) light (Fig. 3B, left cluster of bars). The spectral profile of the light response of the CRY-expressing (*GH146-GAL4/UAS-CRY*) olfactory projection cells is almost identical to CRY-positive ILNvs (Fig. 3B, right) responding to white (FF on/off = 1.14 ± 0.02 , $n = 22$), blue-green (1.27 ± 0.04 , $n = 10$), blue-violet (1.18 ± 0.04 , $n = 18$), and intense blue light (19 mW/cm^2 to 35 mW/cm^2 , 1.47 ± 0.09 , $n = 10$), but not to orange-red light (1.03 ± 0.03 , $n = 10$). The light response of the CRY-expressing olfactory neurons was significantly higher for blue-green ($P < 0.0001$), blue-violet ($P < 0.005$), and intense blue light ($P < 0.0001$, one-way ANOVA) than counterparts measured in control (non-CRY-expressing) cells.

To probe the mechanism of the CRY-mediated light response, we assessed the ability of different CRY isoforms expressed in the LNV to rescue the light response in CRY-null flies. The *cry^m* mutant (a nine-amino acid C-terminal truncation of CRY) binds equally well to TIM in light and dark (19), whereas wild-type CRY interaction with TIM is light-dependent. This allowed us to ask whether a light-dependent CRY-TIM interaction is necessary for the acute light response of the ILNvs. The firing rate of CRY^M-expressing ILNV increased significantly in response to white (FF on/off = 1.19 ± 0.05 , $n = 14$) or blue-violet (1.28 ± 0.06 , $n = 12$, $P = 0.20$ versus white light) light, but not to red-orange light (1.00 ± 0.05 , $n = 9$, $P = 0.005$ versus white light) (Fig. 4, A and B). Significant large light responses were recorded in the ILNvs in *tim*-null flies for moderate-intensity white (FF on/off = 1.24 ± 0.03 , $n = 14$) and low-intensity blue-violet light (1.25 ± 0.04 , $n = 14$, $P = 0.89$ versus white light), but not for moderate-intensity orange-red light (1.00 ± 0.02 , $n = 14$, $p = 0.01$ versus white light) (Fig. 4C). Thus, TIM interaction is not necessary for the CRY-mediated ILNV light response.

In addition to having the *Drosophila*-like light-responsive CRY1, many insects express a second, vertebrate-like CRY2 that is a potent transcriptional regulator but lacks light sensitivity (20). Because *Drosophila* CRY may also act as a transcriptional regulator (21), we recorded ILNvs expressing either monarch butterfly (*Danaus plexippus*) dpCRY1 or dpCRY2 in the LNVs of *cry⁰¹* flies. The ILNvs expressing dpCRY1 were electrophysiologically responsive to moderate-intensity white (FF on/off = 1.19 ± 0.05 , $n = 15$) and low-intensity blue-violet (1.16 ± 0.04 , $n = 14$, $P = 0.27$ versus white light) light but not to moderate-intensity orange-red light (1.01 ± 0.05 , $n = 10$, $P = 0.007$ versus white light, ANOVA) (Fig. 4, B and C). In contrast, l-LNVs expressing dpCRY2 showed no significant response to white (FF on/off = 1.06 ± 0.04 , $n = 12$), blue-violet (1.02 ± 0.04 , $n = 14$, $P = 0.78$ versus white), or orange-red (1.02 ± 0.06 , $n = 8$, $P = 0.86$ versus white) light (Fig. 4, B and C). Paired *t* tests comparing firing frequency in light versus dark showed no significant response for any wavelength for dpCRY2 ($P = 0.62$ for white, 0.23 for blue-violet, and 0.74

for orange-red light). Thus, the light response requires CRY that is light-sensitive but not transcriptionally active.

Because a point mutation destabilizes the flavin chromophore binding site in *cry^b* mutants (9), we asked whether acute inhibition of the light-activated flavin redox reaction of CRY blocks the ILNv light response. The ILNv respond to blue-violet light at baseline (Fig. 4, D and E, FF on/off = 1.37 ± 0.08 , $n = 13$). Within 5 min of bath-applied flavin-specific redox inhibitor diphenyleiocationium chloride (DPI), the response was attenuated (FF on/off = 1.10 ± 0.06 , $n = 5$, $P = 0.16$ versus 5-min sham perfusion). Within 10 min of DPI exposure, blue-violet light no longer evoked a light response (FF on/off = 1.06 ± 0.06 , $n = 10$, $P = 0.0008$ for 10 min; 1.04 ± 0.08 , $n = 11$, $P = 0.01$ for 20 min; 1.06 ± 0.04 , $n = 13$, $P = 0.0008$ for 30 min; all comparisons by ANOVA versus same time in sham perfusion). In contrast, the ILNv light response in vehicle-treated controls was stable over 30 min (FF on/off = 1.39 ± 0.07 and $n = 10$ at zero; 1.27 ± 0.08 , $n = 6$ at 5 min; 1.42 ± 0.10 , $n = 10$ at 10 min; 1.30 ± 0.06 , $n = 10$ at 20 min; and 1.36 ± 0.06 , $n = 11$ at 30 min; $P > 0.31$ all time points compared by ANOVA) (Fig. 4D). Note that DPI does not alter spontaneous baseline firing frequency recorded from the ILNv for up to 30 min, indicating the absence of nonspecific metabolic effects on firing (Fig. 4E).

Light-activation of the flavin chromophore of CRY appears to couple to depolarization of neuronal membrane potential, resulting in increased firing rate. We measured the amplitude of intense blue light-evoked resting membrane potential changes (Δ RMP) in current clamp of opsin-free *gl60j* flies (Δ RMP = $2.85 \text{ mV} \pm 0.3$, $n = 45$) then tested the hypothesis that potassium channel modulation underlies coupling of light-activated CRY to membrane depolarization. To visualize light-evoked ILNv membrane potential changes more clearly, we blocked action potentials in these recordings well past saturation (fig. S3) with the voltage-gated sodium channel blocker tetrodotoxin (TTX), and we assessed the resting membrane potential (RMP) response to light. TTX did not significantly reduce these shifts at 100 nM (Δ RMP = 2.03 ± 0.3 , $n = 24$, $P = 0.4$ versus control), 500 nM (2.84 ± 0.5 , $n = 7$, $P = 1.0$ versus control), or 1 μ M (2.04 ± 0.4 , $n = 13$, $P = 0.7$) (Fig. 4F, right). Light-evoked membrane potential changes were, however, significantly decreased by a subsequent application of a voltage-gated and inward rectifier potassium channel blocker cocktail including 10 mM tetra-ethylammonium (TEA), 2 mM 4-aminopyridine (4-AP), and 2 mM cesium chloride in the presence of 100 nM TTX (Δ RMP = $0.41 \text{ mV} \pm 0.3$, $n = 34$, $P < 0.001$ versus control). We performed time-matched controls with 100 nM TTX only and found that RMP changes did not differ from control (Δ RMP = $2.77 \pm 0.3 \text{ mV}$, $n = 12$, $P = 0.9$). The K-blocker cocktail also significantly disrupted membrane potential shifts in the absence of TTX ($0.78 \text{ mV} \pm 0.2$, $P < 0.001$ versus control, $P = 0.9$ versus K blockers plus TTX). These results suggest that potassium channel modulation couples to the CRY-mediated ILNv light response.

To determine the precise timing of the CRY-mediated light response, we made recordings in ILNv of *gl60j* mutant flies illuminated with a software-triggered blue light source. After ~60 cycles of 4-s blue light pulses from four different ILNvs, records were analyzed in 100-ms bins for spike frequency, 1 s before and after light onset. Although a trend toward more action potentials can be observed during the light pulse, this measurement does not clearly

resolve the onset of increased firing in response to light at a millisecond time scale (Fig. 5A). However, application of episodic blue light pulse protocol followed by averaging 120 traces (30 each from four ILNvs) precisely registered by light-on and -off effectively filters the noise from individual records and yields a clear light-evoked RMP response (Fig. 5B). Kinetic analysis reveals that the averaged light-evoked response is best fit with two exponentials with a fast component ($\tau = 105$ ms) and a slower component ($\tau = 1.07$ s). Similarly, the averaged return to the baseline “dark” RMP is also best fit with two exponentials (fast, $\tau = 106$ ms; slow, $\tau = 1.27$ s). Notably, the fast components of the on and off response are nearly identical. The speed of the on response (≈ 100 ms) is within an order of magnitude of that of classical opsin-based phototransduction (22), suggesting that coupling light-activated CRY to depolarization may be diffusion-limited and require intermediate steps. Figure 5C shows individual records that contributed to the averages shown in Fig. 5, either A or B. Although most recordings show an appreciable rise in RMP (sweeps 2 to 5) within 50 ms after lights on (indicated by the arrow and blue shading), increases in firing frequency were difficult to resolve in time bins of this length. Taken together, the results in Figs. 4 and 5 are consistent with the idea that light-activated CRY couples to membrane depolarization via potassium channel modulation.

The white light intensity levels used to characterize the ILNv electrophysiological light response, although five times higher than intensities necessary to induce CRY-TIM interaction, correspond to natural light levels typically observed in the early to mid-morning on a clear day, consistent with recent findings that the ILNv are light-activated morning arousal neurons (1–4, 23). We asked what percentage of blue light permeates the cuticle and reaches central brain neurons. We found that the average transmittance through the head cuticle, when compared to phosphate-buffered saline mounting buffer alone, was $55.0\% \pm 4.3$ ($n = 7$). Importantly, the transmittance through the eye cuticle is similar at $57.2\% \pm 5.3$ ($n = 7$, $P = 0.74$ by t test).

CRY mediates a rapid electrophysiological light response that is distinct from classical opsin-based phototransduction. However, the present results do not rule out the possibility that light-activated synaptic inputs from external photo-receptors co-modulate ILNv firing rate in intact animals. Dye-filled individual ILNv cells show extensive arbors in the optic lobe and may reflect sites for external photoreceptor input (24). All lines of evidence herein indicate that the CRY-mediated electrophysiological light response is mechanistically distinct from the previously described CRY-TIM interaction. Qualitatively, the CRY-mediated electrophysiological light response bears some resemblance to melanopsin-based light activation of retinal ganglion cells (which underlies circadian entrainment in mammals), whereby light activation leads to increased action potential firing rather than graded potentials found typically in image-forming photoreceptors. The CRY-mediated electrophysiological response appears to exhibit a higher light threshold compared with opsin-based light sensing, which may bias its physiological functions to non-image-forming photosensitive cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Drosophila ILNvs rapidly increase spontaneous action potential firing rate in response to light independent of opsin-based classical photoreceptors. **(A)** (Top) Response of a representative tonic firing cell to 4 mW/cm² halogen white light. (Bottom) Light response of burst firing cell to 19 mW/cm² blue light. Alternating light/dark cycles denoted by white or blue versus black bars above traces. **(B)** Firing frequency in light/dark varies according to light intensity. **P* 0.05, ***P* 0.01, ****P* 0.005; error bars indicate SEM. **(C)** Genetic ablation of all external opsin-based photoreceptors has no effect on the ILNv light response. **(D)** Representative recordings of ILNv light response evoked by blue-violet and orange-red light. Alternating light/dark cycles denoted by violet, orange, and black bars. **(E)** Spectral profiles of light responses of ILNv from control versus *eyeless*-null *gl60j* mutant flies are indistinguishable, but responses to white and blue light of *cryb* hypomorphs are significantly reduced.

Fig. 2.

The ILNv light response is absent in *cry*-null flies but is functionally rescued by targeted expression of CRY in the LNvs. **(A)** Native CRY is detected by anti-CRY and colocalizes in the LNv to a *cryGAL4*-driven GFP signal. **(B)** Anti-CRY signal (red, middle) is absent in LNv (green *pdfGAL4/UAS-dORK-NC1-GFP*, left) in *cry*⁰¹ and *cry*⁰² null flies (overlay, right). **(C)** Verification of anti-CRY signal (red) expressed specifically in LNv (labeled in green, left, *pdfGAL4/UAS-dORK-NC1-GFP*) in *cry*⁰¹ and *cry*⁰² null flies that express CRY driven by *pdfGAL4* (overlay, right). **(D)** Representative recordings of *cry*-null flies (top) and genetic rescue of the ILNv light response in *cry*-null flies (bottom). **(E)** ILNv FF response evoked by 3 mW/cm² white light/dark does not differ between control and LNv specific expression of CRY in *cry*⁰¹ and *cry*⁰² null genetic background flies.

Fig. 3.

Ectopic expression of CRY in inherently light-insensitive neurons renders them light-responsive. **(A)** Representative voltage clamp recording of CRY-expressing olfactory projection neuron shows light response evoked by 30 mW/cm² blue light. **(B)** Spectral profile of light responses of olfactory projection neurons recorded from control (left) versus CRY-expressing (right) neurons. Control olfactory projection neurons are nonresponsive to all light wavelengths and intensities tested, whereas CRY-expressing olfactory neurons increase their firing rate in response to white, blue-green, and blue-violet light but not to orange light.

Fig. 4.

The CRY-mediated electrophysiological light response membrane depolarization by potassium channel modulation depends on flavin-specific redox reactions rather than TIM interaction. **(A)** Representative recordings of ILNv expressing CRY but *tim* null (*tim*⁰¹, top) and *cry*⁰¹ with *pdfGAL4*-driven CRY^M (CRY^M, bottom). **(B)** Recordings from ILNvs expressing UAS-driven *D. plexippus* dpCRY1 (top) or dpCRY2 exposed to blue-violet (purple bar) after darkness (black bar). **(C)** White and violet light evoke significant responses from each genotype except *dpCRY2*; *cry*⁰¹. **(D)** Treatment with the redox inhibitor DPI rapidly attenuates the light response. **(E)** Representative recordings of light-evoked responses in vehicle (top) versus 30 min of DPI treatment (bottom) for the same cell. **(F)** Light-evoked depolarization in ILNv is significantly decreased after treatment with potassium channel blockers TEA, 4-AP, and CsCl in the presence or absence of TTX.

Fig. 5.

RMP changes rapidly upon lights on and off. **(A)** Number of spikes in 100-ms bins shown for 1 s before and after the onset of an intense blue light pulse ($\sim 35 \text{ mW/cm}^2$), averaged for 60 sweeps from four ILNvs. **(B)** The RMP shows an evoked increase upon triggering of the intense blue light. Trace is an average of 120 sweeps recorded from four ILNvs. The evoked rise and fall were fitted with double exponential functions. **(C)** Individual records that contributed to the averages depicted in **(A)** and **(B)**. Four of five traces (bottom four traces) show an appreciable rise in RMP within 100 ms, but the increase in FF for the four cells that are firing is apparent only after several hundred milliseconds.