View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Neuron, Vol. 43, 293–300, January 20, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2004.12.038

Circadian Pacemaker Neurons Transmit and Modulate Visual Information to Control a Rapid Behavioral Response

Esteban O. Mazzoni, Claude Desplan, and Justin Blau*
Department of Biology
New York University
100 Washington Square East
New York, New York 10003

Summary

Circadian pacemaker neurons contain a molecular clock that oscillates with a period of ~24 hr, controlling circadian rhythms of behavior. Pacemaker neurons respond to visual system inputs for clock resetting, but, unlike other neurons, have not been reported to transmit rapid signals to their targets. Here we show that pacemaker neurons are required to mediate a rapid behavior. The *Drosophila* larval visual system, Bolwig's organ (BO), projects to larval pacemaker neurons to entrain their clock. BO also mediates larval photophobic behavior. We found that ablation or electrical silencing of larval pacemaker neurons abolished light avoidance. Thus, circadian pacemaker neurons receive input from BO not only to reset the clock but also to transmit rapid photophobic signals. Furthermore, as clock gene mutations also affect photophobicity, the pacemaker neurons modulate the sensitivity of larvae to light, generating a circadian rhythm in visual sensitivity.

Introduction

Daily rhythms of behavior are controlled by circadian pacemaker neurons found in the brain of most animals (Reppert and Weaver, 2002). Evidence from *Drosophila* and mammals indicates that diffusible factors are rhythmically released from pacemaker neurons and are at least partially responsible for regulating the 24 hr rhythms in the behavior of the whole animal (Handler and Konopka, 1979; Silver et al., 1996).

Each pacemaker neuron contains a molecular clock that oscillates with a period close to 24 hr, even in constant darkness (DD) (Reppert and Weaver, 2002). The molecular clock in *Drosophila* consists of two transcription/translation negative feedback loops tied together by the transcription factors CLOCK (CLK) and CYCLE (CYC) (reviewed by Allada [2003] and Stanewsky [2003]). In one *Drosophila* clock loop, CLK and CYC directly activate transcription of two other essential clock genes, *period* (*per*) and *timeless* (*tim*). PER and TIM proteins heterodimerize in the cytoplasm and enter the nucleus where PER inhibits CLK/CYC activity, which represses further *per* and *tim* expression. Repression is maintained until PER and TIM are degraded, allowing CLK and CYC to resume transcription of *per* and *tim*. Multiple post-translational events help to separate the phases of transcription and repression of *per* and *tim* and other genes directly activated by CLK/CYC, which ensures accurate and sustained molecular rhythms.

Although molecular clocks free-run with a period of approximately 24 hr in DD, they can be entrained by environmental light:dark (LD) cycles. Resetting the *Drosophila* clock occurs via the rapid degradation of TIM protein in response to light (reviewed by Stanewsky, 2003). Progression of the molecular clock is delayed if TIM is degraded during its cytoplasmic accumulation phase prior to midnight, since this delays PER/TIM nuclear entry. Conversely, TIM degradation in the second half of the night advances the clock if TIM has already entered the nucleus, since CLK/CYC activity is derepressed earlier.

In *Drosophila*, two pathways convey light information to the pacemaker lateral neurons (LNs) for TIM degradation. In one pathway, light activates CRYPTOCHROME (CRY), a cell-autonomous circadian photoreceptor in the LNs, which then interacts with TIM and initiates TIM degradation via the proteasome (Busza et al., 2004; Naidoo et al., 1999). In a second pathway, light information from retinal and extraretinal photoreceptor cells is transmitted to the LNs and also synchronizes the clock, presumably via TIM degradation (Stanewsky et al., 1998). These two pathways are partially redundant, since the rhythmic locomotor activity of adult flies lacking either functional CRY or a functional visual system can still be entrained by light, whereas the locomotor activity of flies lacking both CRY and photoreceptors cannot be synchronized by light (Helfrich-Forster et al., 2001).

Adult *Drosophila* have photoreceptor cells in the retina and in an extraretinal structure, the Hofbauer-Buchner eyelet. Since the eyelet photoreceptors project directly to the LNs, the eyelet's main function is presumably to entrain the clock rather than to play a role in vision (Helfrich-Forster et al., 2001, 2002). However, *Drosophila* larvae have only one visual structure—Bolwig's Organ (BO). Like the eyelet, BO directly contacts the LNs and is involved in light entrainment of the molecular clock in the larval LNs (Kaneko et al., 1997, 2000; Malpel et al., 2002, 2004). However, BO is also necessary for foraging larvae to perform certain visual tasks such as rapidly avoiding light (Sawin-McCormack et al., 1995).

It is well established that pacemaker neurons receive signals from the visual system to entrain the molecular clock (Stanewsky, 2003). However, in contrast to most other neurons, there is not yet evidence that they can rapidly transmit and modulate sensory signals. We used the simple visual and circadian system in *Drosophila* larvae to test whether the LNs can rapidly transmit visual information. Here we show that pacemaker neurons not only receive light information for entrainment of their clock, but also relay this information to produce a rapid photophobic response. We found that larvae in which the pacemaker neurons were either ablated or electrically silenced were as defective in light avoidance as larvae lacking all photoreceptor cells. This indicates that pacemaker neurons in *Drosophila* larvae are an essential part of a neuronal circuit that leads to a rapid behavioral

*Correspondence: justin.blau@nyu.edu

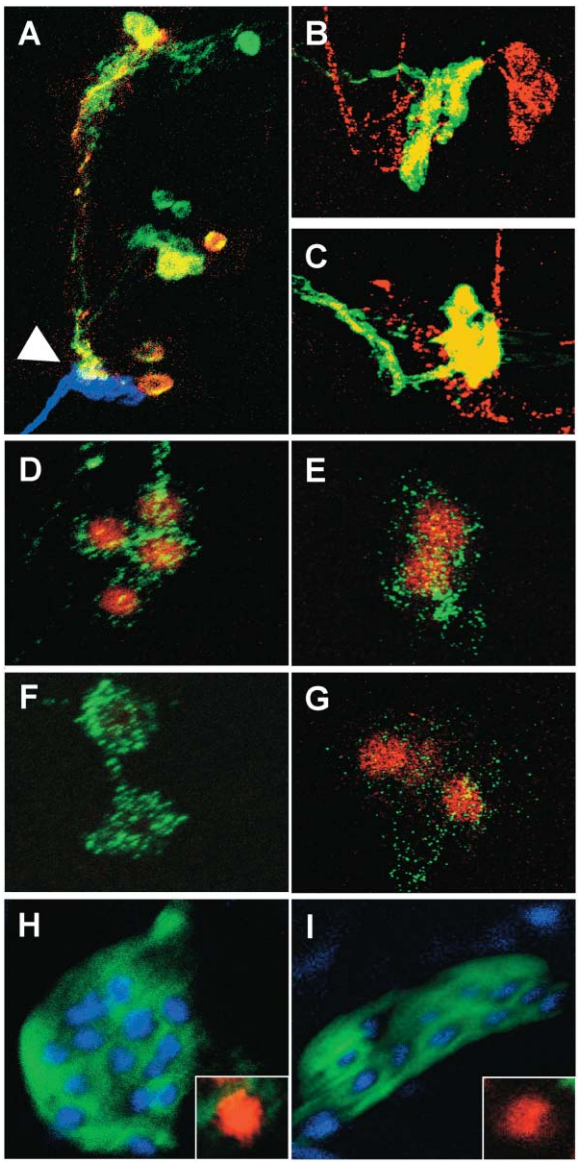

Figure 1. Characterization of Bolwig's Organ, the Larval Visual System

(A) Larval brain expressing membrane-targeted CD8-GFP under control of the clock gene driver *tim(UAS)-Gal4* (green) and stained with antibodies to the neuropeptide PDF to mark LNs (red) and with Rh6 to mark BO (blue). GFP expression is absent from larval visual system axons projecting into the brain. BO axons project to the LNs (arrow). *tim(UAS)-Gal4* and PDF staining only overlap in the LNs (yellow).

(B and C) Higher magnification images of the termini of BO axons (arrow in [A]). *rh5-Gal4* (B) and *rh6-Gal4* (C) driving *UAS-n-Synaptobrevin-GFP* (Syb-GFP, green) to mark BO synapses. Brains were also stained with antibodies to PDF (red) to label LNs. Both BO populations project to the dendritic arborizations of LNs, since Syb-GFP colocalized with PDF in the BO-LN synapse (yellow).

(D–G) Larval LNs stained with antibodies to TIM (red) and PDF (green). In the absence of a light pulse, control *yellow white (y w*, [D]) and *GMR-hid* (E) LNs show nuclear TIM at ZT24, as expected. After a 1 hr light pulse starting at ZT23, TIM was undetectable in wild-type LNs (F), but present in *GMR-hid* larval LNs (G) in which BO is missing.

(H and I) BO photoreceptors expressing GFP (green) under the control of GMR stained for the essential clock proteins (red) PER (H) and TIM (I) and the neuronal marker ELAV (blue) at ZT21. PER and TIM were not detected in BO photoreceptor cells. Insets are LNs from the same larvae, showing that PER and TIM are expressed in pacemaker neurons as expected. The blue ELAV signal was removed from the insets for simplicity.

response. In addition, the pacemaker neurons regulate the degree of this light-avoidance response in a circadian manner, acting as a circadian filter for visual information.

Results

The Larval Visual System Entrains the Molecular Clock in the Pacemaker Neurons

Previous studies in *Drosophila* larvae suggested that BO transmits light to the LNs to entrain the molecular clock (Malpel et al., 2004). The cell bodies of the 12 photoreceptors that comprise each BO are located on both sides of the head. BO neurons express either *rhodopsin-5* (*rh5*) or *rhodopsin-6* (*rh6*) (Malpel et al., 2002; F. Pichaud, E.O.M., and C.D., unpublished data). BO axons project into the central brain where they terminate at the dendrites of the four circadian pacemaker lateral neurons (LNs) in each brain lobe (Kaneko et al., 1997; Malpel et al., 2002; Figure 1A). We used *rh5*- or *rh6-Gal4* drivers to express a synaptic marker, n-Synaptobrevin-GFP, in each of the two BO subtypes to visualize their synapses. This revealed synapses of both BO populations in the immediate vicinity of the LN dendrites (Figures 1B and 1C). Most BO axons stopped at the LNs, although there was one axon from the *rh5*-expressing cells that defasciculated after reaching the LNs and projected deeper into the brain, where we could not identify its target cells. Direct innervation of the LNs by BO suggested that BO is required for the light-induced degradation of the essential clock protein TIM, which entrains the clock (Stanewsky, 2003).

To test this idea, we analyzed the response of TIM to light at the end of the night, when TIM is located in the nucleus of the LNs (Figures 1D and 1E). A 1 hr light pulse applied to wild-type larvae starting 1 hr before the end of the night led to TIM degradation (Figure 1F). In contrast, the same treatment applied to larvae lacking BO (via a *GMR-hid* transgene that drives expression of the proapoptotic gene *head involution defective* [*hid*] in all photoreceptors) did not cause detectable TIM degradation (Figure 1G). Although dendritic branching of the LNs is altered when BO is eliminated via *GMR-hid*, no such change was observed when BO synaptic transmission was blocked with *UAS-Tetanus Toxin* (*TNT*) under the control of *GMR-Gal4* (*GMR>TNT*) (Malpel et al., 2002). We observed that TIM degradation at the end of the night was also defective in *GMR>TNT* larvae (Supplemental Figure S1 [http://www.neuron.org/cgi/content/full/45/2/293/DC1/]). Therefore we conclude that BO is required for rapid light-induced degradation of TIM in the LNs and that delayed TIM degradation in *GMR-hid* larvae is not due to a developmental defect in the LNs. Our data confirm previous studies that suggested that the visual system, in addition to CRY, is required to entrain the LN clock (Kaneko et al., 2000; Malpel et al., 2002, 2004). The existence of a functional connection between BO

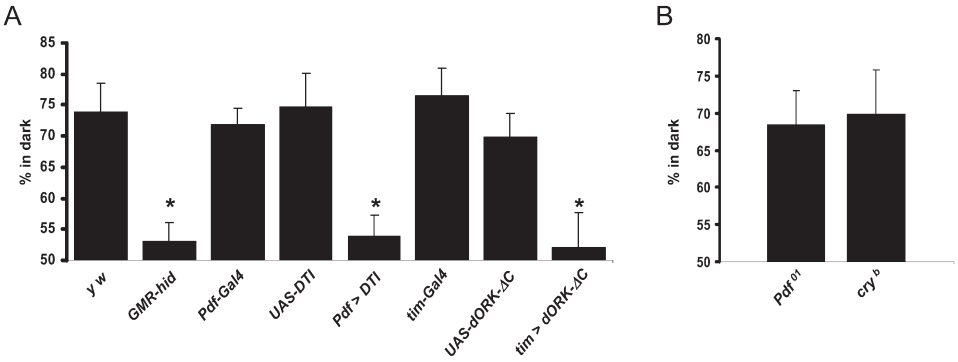

Figure 2. The Larval Visual System and Pacemaker Neurons Are Both Essential for Photophobic Behavior

(A) Foraging third instar larvae were tested in a light versus dark preference test at 750 lux (see Experimental Procedures). The percentage of animals on the dark side of the dish after 15 min is shown as a measure of photophobicity. Control *y w* larvae showed a clear preference for darkness, while larvae lacking a visual system (*GMR-hid*) displayed no photophobic behavior. Ablation of pacemaker cells with *Pdf-Gal4* and *UAS-DTI* (*Pdf>DTI*) or electrically silencing clock cells with *tim(UAS)-Gal4* and *UAS-dORK-ΔC* (*tim>dORK-ΔC*) phenocopied ablation of BO. Parental lines were as photophobic as *y w* controls. This experiment was performed at ZT6. Bars indicate averages of six replica plates of 20 larvae each, with error bars showing standard deviation (SD). (*$p < 0.001$ versus *y w*, χ^2 test).

(B) Larvae lacking the LN neuropeptide PDF (Pdf^{01}) or the circadian photoreceptor CRY (cry^b) display normal photophobic behavior. This experiment was performed as in (A).

and LNs is also supported by the presence of choline acetyltransferase, the enzyme that synthesizes acetylcholine (ACh), in some BO photoreceptor neurons (Gorczyca and Hall, 1987; Yasuyama et al., 1995; F. Pichaud, E.O.M., and C.D., unpublished data), and by the presence of a functional nicotinic ACh receptor in the LNs (Wegener et al., 2004).

Larval Light Avoidance Is Dependent on Both the Visual System and Pacemaker Neurons

In addition to entraining the LN clock, BO is also required for foraging larvae to avoid light (Sawin-McCormack et al., 1995). This photophobic behavior probably keeps larvae in food and away from predators. However, the neural circuit for this behavior has not been described. Light avoidance can be quantified experimentally by placing larvae in the middle of an agar Petri dish and then counting larvae on the illuminated and dark halves of the dish 15 min later. Typically, most wild-type larvae are found on the dark side, while larvae lacking BO are "blind" and distribute evenly between the illuminated and dark sides (Dettman et al., 2001; Figure 2A).

Since the great majority of BO projections terminate at the LNs, we tested whether the LNs were part of a neural circuit downstream of the photoreceptors, leading to light avoidance. First, the LNs were ablated by expressing an intracellular form of Diptheria toxin (Han et al., 2000) with a *Pigment dispersing factor* (*Pdf*)-*Gal4* driver whose expression is restricted to the four LNs in each lobe and to six nonclock neurons in the ventral ganglion (Renn et al., 1999). We found that while individual *Pdf-Gal4* and *UAS-DTI* strains behaved indistinguishably from wild-type, larvae carrying both constructs lacked LNs and were as blind as larvae lacking BO (Figure 2A). Similar results were obtained when the LNs were present but electrically silenced by expression of an open rectifier K^+ channel that hyperpolarizes neurons (*UAS-dORK-ΔC*) (Nitabach et al., 2002) under the control of a *tim(UAS)-Gal4* driver (Blau and Young, 1999). This driver is expressed in all larval clock neurons (four LNs plus approximately eight other neurons in each lobe, Figure 1A). Importantly, the *Pdf-Gal4* and *tim-(UAS)-Gal4* drivers are not expressed in BO, and their expression only overlaps in the LNs (Figure 1A). It should also be noted that ablation of the LNs does not affect BO development (Malpel et al., 2002). Thus, two independent methods of inactivating LN function lead to the same conclusion: the circadian pacemaker neurons are an essential part of the neural circuit leading to the rapid behavioral response of larvae to light.

Light Avoidance Is Independent of CRY and PDF

Although cry^b mutant larvae are partially defective in light entrainment of the molecular clock (Ivanchenko et al., 2001; Malpel et al., 2004; E.O.M., C.D., and J.B., unpublished data), they exhibited wild-type photophobicity (Figure 2B). Therefore, larvae do not require *cry* function for photophobicity, and the LNs presumably receive visual information for light avoidance solely from BO, consistent with the blind phenotype of larvae lacking BO.

Next, we tested whether photophobic outputs of the LNs use the same neurotransmitter that mediates rhythms of adult locomotor activity. The neuropeptide PDF is the only output molecule of the LNs so far described and is required for circadian rhythms of locomotor activity in adult flies (Renn et al., 1999). However, Pdf^{01} mutant larvae showed no significant differences from wild-type larvae in the larval light avoidance assay (Figure 2B). Pdf^{01} mutants, like wild-type larvae, displayed circadian modulation of photophobic behavior (see below and Supplemental Figure S2 [http://www.neuron.org/cgi/content/full/45/2/293/DC1/]). Therefore we conclude that PDF is not required for photophobic behavior. Thus, the LNs use PDF to modulate the 24 hr rhythms of sleep/wake cycles in the adult (Renn et al., 1999) and, consistent with the two roles of LNs in behavior, must use a second unidentified neurotransmitter to convey the rapid light avoidance signal.

The Larval Visual System Has No Molecular Clock

Why would a rapid behavior be transmitted via the master circadian pacemaker neurons? One explanation could be that the visual output, like that of all sensory systems, needs to be modulated in a circadian manner. For instance, adult *Drosophila* photoreceptors and antennae, as well as vertebrate retinal cells, have autonomous molecular clocks that regulate their sensitivity and physiology (Claridge-Chang et al., 2001; Krishnan et al., 1999; Tosini and Fukuhara, 2002). However, BO is a sensory system without a molecular clock, as demonstrated by the absence of staining with antibodies to PER, TIM, and PDP1 clock proteins and by the lack of expression of Gal4 driver constructs for *per*, *tim*, and *cry* (Figures 1A, 1H, and 1I and data not shown). Therefore we hypothesized that the LNs provide circadian modulation of the BO visual signal via their own molecular clock and do not simply function as relay neurons.

Mutations in Clock Genes Alter the Photophobic Response

As described above, the molecular clock is a transcriptional/translational feedback loop (Stanewsky, 2003). Mutations in the genes encoding the positive (*Clk*/*cyc*) and negative (*per*/*tim*) arms of the clock clamp the clock at two distinct molecular states: CLK/CYC activity is derepressed in *per* and *tim* null mutants, leading to constitutively high levels of *per* and *tim* transcription; in contrast, the RNAs of *per*, *tim*, and other similarly regulated genes are at constant low levels in *Clk* and *cyc* mutants.

To test the hypothesis that the LNs modulate the output of BO, we compared the performance of wild-type larvae with that of per^0, tim^{01}, Clk^{Jrk}, and cyc^0 mutant larvae in the light avoidance assay, using three different light intensities (Figures 3A–3C). For uniformity, all larvae were entrained to LD cycles and assayed in the middle of the day (ZT6, see below). *per* and *tim* null mutants were insensitive to light under these experimental conditions, even at the highest light intensity used (750 lux, Figure 3A). The ability of wild-type larvae to distinguish between light and dark diminished as the light intensity was decreased, such that by 150 lux, wild-type larvae could no longer differentiate between the light and dark sides of the plate (Figures 3A–3C). However Clk^{Jrk} and cyc^0 mutant larvae remained highly sensitive to light, even at 150 lux (Figure 3C). Thus, mutations in the positive clock components (*Clk* and *cyc*) increase light sensitivity of larvae, while mutations in the negative components (*per* and *tim*) render larvae insensitive to light. These results also support the conclusion that PDF is not required for light avoidance, since PDF is absent from larval LNs in the highly light-sensitive Clk^{Jrk} mutants (Blau and Young, 1999; Park et al., 2000).

per^0 mutant larvae were insensitive to light in two different genetic backgrounds with the same results (Figures 3A–3C). Furthermore, the blindness of per^0 mutants at 750 lux was rescued by a *per* transgene, whose expression is limited to the LNs (7.2:2, Frisch et al., 1994), and the blindness of tim^{01} mutants was also rescued by a genomic *tim* rescue construct, *tim7B* (Ousley et al., 1998) (Figure 3D). Therefore, the defects in photophobicity observed in Figures 3A–3C are due to the clock mutations themselves and do not depend on genetic background.

Increasing the light to 1100 lux also partially rescued the photophobic defects of *per* and *tim* null mutant larvae (Figure 3E), indicating that these mutants are not completely blind and that the molecular clock regulates visual sensitivity. In contrast, increasing the light to 1100 lux did not rescue photophobicity in larvae lacking LNs (via *Pdf-Gal4* and *UAS-DTI* transgenes). Since BO does not possess a clock (Figures 1A, 1H, and 1I), we conclude that the state of the molecular clock in the LNs modulates the rapid behavioral responses to signals from BO.

Circadian Modulation of Photophobic Behavior

The ability of all four of the clock mutants tested to affect visual sensitivity led to the prediction that the sensitivity of wild-type larvae to light follows a circadian rhythm. To test this prediction, larvae were entrained in LD cycles for 2–3 days, shifted into DD, and tested for their ability to avoid light at 3 hr intervals on the second day of DD. In this experiment, 150 lux were used to maximize differences between genotypes. Wild-type larvae were minimally sensitive to light toward the end of the subjective day and maximally sensitive toward subjective dawn (Figure 4A). In contrast, there was no detectable rhythm in per^0 or cyc^0 mutant larvae, with per^0 larvae constitutively insensitive and cyc^0 larvae constitutively sensitive to light at all time points measured (Figure 4A). When entrained *y w* larvae were tested at CT0 and CT12 across a range of light intensities, these larvae were more photophobic at CT0 than at CT12 at both 150 and 350 lux (Figure 4B). However, the differences in photophobicity were minimal when larvae were tested with either 70 or 600 lux (Figure 4B). These results confirm the conclusion that the molecular clock in the LNs determines the extent to which larvae avoid light.

Control of Light Sensitivity in Biological Conditions

Larvae in DD exhibit circadian modulation of their visual sensitivity, with a peak at the end of the subjective night and a trough at the end of the subjective day. This difference is increased in LD cycles, with larvae even less sensitive during the day (compare Figures 3C and 4D). One explanation for this difference is desensitization of BO after exposure to a strong light stimulus, in addition to the circadian modulation of visual sensitivity by the LNs.

To test for an effect of desensitization on photophobic behavior, we compared the photophobic behavior of tim^{01} null mutants, which lack a functional clock, in LD and DD. The results (Supplemental Figure S3 [http://www.neuron.org/cgi/content/full/45/2/293/DC1/]) revealed that although tim^{01} larvae were essentially blind during the light part of an LD cycle, they recovered some photophobic behavior during the dark portion of LD when tested with 750 lux. The same level of photophobic behavior was maintained during the first day in DD. Low sensitivity in light and rapid recovery in the dark, without any modulation after adaptation, is characteristic of visual desensitization.

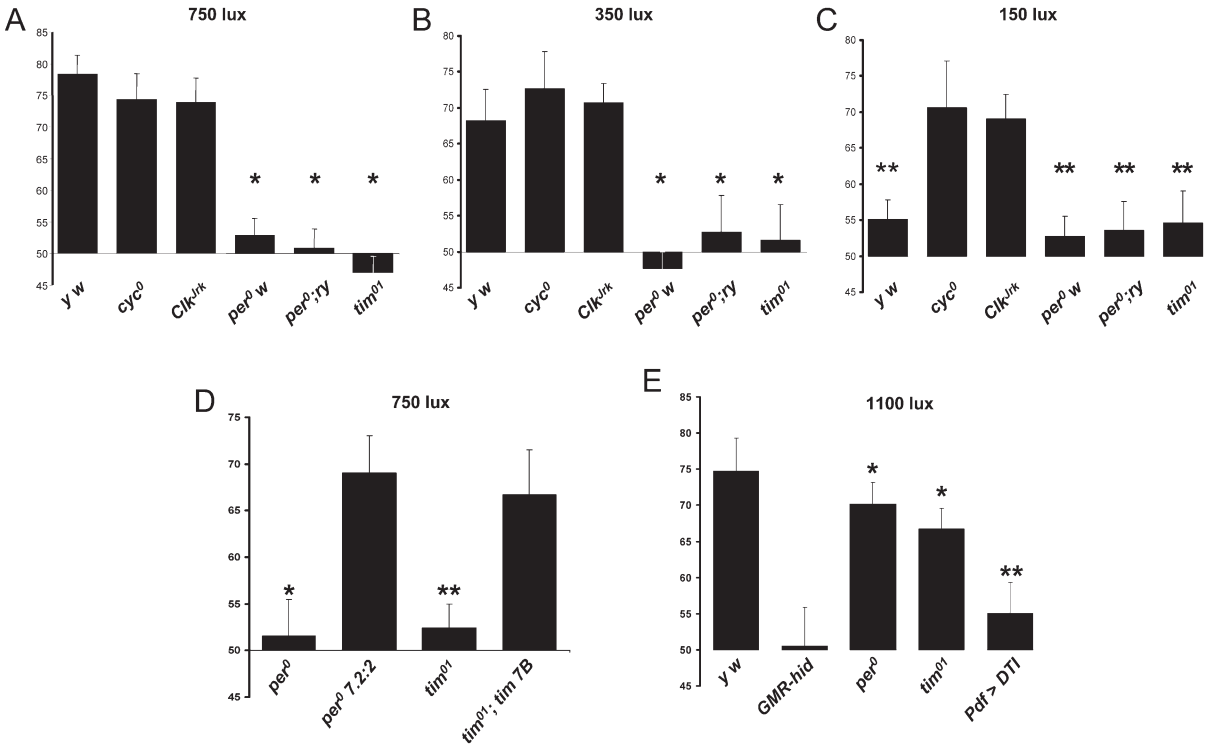

Figure 3. The Larval Visual Response Is Regulated by the Molecular Clock

Photophobicity was measured as in Figure 2. (A–C) Analysis of clock gene mutants in the photophobic assay. Photophobicity was measured at three light intensities: 750 lux (A), 350 lux (B), and 150 lux (C). *y w* larvae were able to distinguish the illuminated from the dark side of the plate at high and medium light intensities (A and B), but not at a lower intensity (C). In contrast, Clk^{Jrk} and cyc^0 mutant larvae were photophobic at all intensities used, with a majority found in the dark at the end of the assay (A–C). per^0 and tim^{01} larvae are essentially blind, even at the highest intensity used, and distributed themselves without preference for light or dark (A–C). Bars are averages of three replica plates, with error bars showing SD (*$p < 0.01$ versus *y w*, **$p < 0.01$ versus Clk^{Jrk} and cyc^0; χ^2 test). This experiment was performed at ZT6. (D) The blind phenotype of per^0 and tim^{01} mutant larvae was rescued to photophobicity with appropriate *per* or *tim* transgenes at 750 lux (*$p < 0.01$ versus per^0, **$p < 0.01$ versus tim^{01}; χ^2 test). This experiment was performed at ZT6. (E) The blind phenotype of per^0 and tim^{01} mutant larvae was rescued to photophobicity by increasing the light intensity to 1100 lux (*$p < 0.01$ versus *GMR-hid*, χ^2 test). However, even at this higher light intensity, larvae lacking pacemaker cells, *Pdf>DTI*, are not different from *GMR-hid* (**$p > 0.3$ versus *GMR-hid*; χ^2 test).

The difference between tim^{01} larval photophobic behavior in light and dark means that photophobicity scores at equivalent times in LD and DD cannot be directly compared. It also means that only experiments performed in DD analyze the circadian regulation of light avoidance in the absence of the confounding issue of desensitization. The results in Figure 4 reveal that larval visual sensitivity exhibits a bona fide circadian rhythm in *Drosophila* larvae.

The intensity and the composition of the light that larvae experience under natural conditions change continuously from dawn to dusk so that larvae are never exposed to only one light intensity or color for any significant length of time. The combination of regulated photophobic behavior (circadian plus desensitization) and the characteristics of the light at that time of day (intensity and color) will generate a "wild-type" photophobic response.

Discussion

We have shown that circadian pacemaker neurons in *Drosophila* larvae directly mediate a rapid behavioral response on the order of minutes and that they modulate this response via their internal molecular clock. This is in addition to the well-characterized role of LNs in regulating behavior over 24 hr, and the LNs appear to use different neurotransmitters for the two behaviors they control. The LNs act as a circadian gate for BO sensory neurons by directly transmitting and controlling the strength of the sensory signal rather than by regulating the general physiology of the organism.

How Could Circadian Pacemaker Neurons Control a Rapid Behavior?

We favor a cell-autonomous mechanism involving circadian changes in the membrane excitability of the LNs. Such rhythmic changes in membrane potential would allow the LNs to respond differentially to light. We propose that the membrane is hyperpolarized during the day and in *per* and *tim* null mutants. Thus, the BO input at dusk needs to be much stronger to trigger an action potential in the LNs than at the end of the night (and in *Clk* and *cyc* mutants), when the neurons are more depolarized. Consequently, a single intensity of light stimulus applied to larvae at different times of day or to larvae carrying mutations in different clock genes leads to varying abilities to avoid light, depending on the state

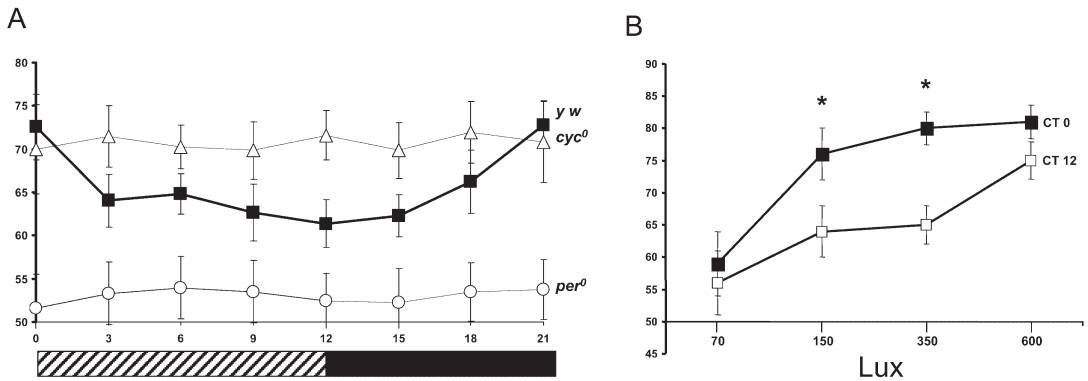

Figure 4. Circadian Modulation of Photophobic Behavior

(A) Time course of photophobicity of wild-type *y w* (black squares), *cyc*^{0} (open triangles), and *per*^{0} (open circles) larvae. Entrained larvae (from parents kept in LD) were shifted to DD and tested for photophobicity using 150 lux on the second day in the dark. *y w* larvae showed a circadian modulation of photophobicity, with a peak response toward the end of the night and lowest responses toward the end of the day. *y w* larvae were always more photophobic than *per*^{0} larvae, but less photophobic than *cyc*^{0} larvae, except at ZT0 and ZT21, when they were as sensitive as *cyc*^{0}. Each point is the average of six replica plates, with error bars showing SD. The effect of genotype was significant ($p < 10^{-8}$; two-way ANOVA). The effect of time of day was significant only for *y w* ($p < 10^{-7}$; one-way ANOVA).

(B) Analysis of photophobic behavior at the peak (CT0) and trough (CT12) with different light intensities. At very low intensity (70 lux), larvae at CT0 and CT12 perform equally poorly. However, at higher light intensities (150 and 350 lux), larvae at CT0 perform better than larvae at CT12 (*$p < 0.05$, CT0 versus CT12; χ^2 test). By 600 lux, there was no significant difference between the photophobicity scores of larvae from CT0 and CT12.

of the molecular clock in the LNs (see Figure 5). An oscillation in the membrane potential of neurons containing a molecular clock may be a general mechanism by which clock output signals are modulated, since mammalian pacemaker neurons show a circadian rhythm in their resting membrane potential (Pennartz et al., 2002). In fact, the resting membrane potential in adult and larval *Drosophila* LNs is probably regulated by, and itself regulates, the molecular clock (Nitabach et al., 2002, 2005).

Circadian Control of Sensory Information

Circadian gating of sensory information seems to be a widespread phenomenon (Barlow et al., 1977; Krishnan et al., 1999; Tosini and Fukuhara, 2002). Presumably, this helps a sensory neuron find the correct dynamic range to measure changes in the environment, since the strength of a stimulus may change dramatically over 24 hr. For most *Drosophila* and mammalian sensory neurons, this modulation is autonomous to the sensory receptors since they possess their own molecular clock. For example, the expression of some genes involved in phototransduction, such as *trpL*, is under circadian regulation in *Drosophila* (Claridge-Chang et al., 2001).

A second mechanism for circadian control of visual sensitivity has been described in *Limulus*. Here the photoreceptor cells are innervated by efferent neurons that control photoreceptor light sensitivity in a circadian manner (Battelle, 2002). However, it has not yet been established whether these efferent neurons are pacemaker neurons or even if they contain a molecular clock (Battelle, 2002). In any case, in *Limulus*, the clock directly or indirectly feeds back to the photoreceptors rather than filtering information.

The data presented here identify a third mechanism for circadian control of visual sensitivity. Indeed, the BO-LN system may represent a more primitive and extremely simplified mechanism for allowing a sensory organ to track time using the relationship between the visual system and pacemaker neurons that entrains the molecular clock. The tight relationship found between the visual and circadian systems across evolution can

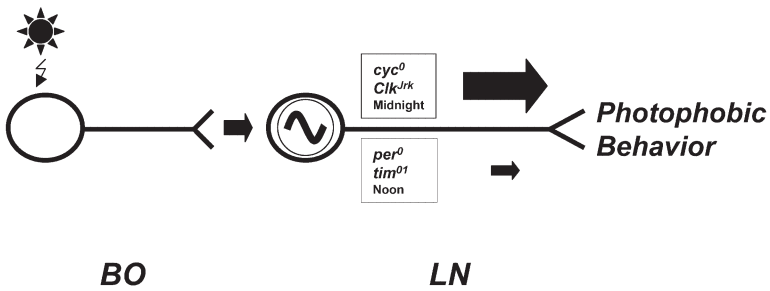

Figure 5. Model for How BO and LNs Regulate Larval Light Avoidance

BO transfers light information directly to the LNs. The LNs then transfer this information to downstream neurons that regulate rapid light avoidance. The same signal strength from BO leads to differing amounts of photophobic behavior depending on the state of the molecular clock in the LNs: at the end of the night (dawn), or in *cyc*^{0} and *Clk*^{Jrk} mutant LNs, a strong signal is passed on by the LNs, leading to strong light avoidance; at the end of the day, or in *per*^{0} and *tim*^{01} mutant LNs, a weaker signal is passed on, leading to lower levels of light avoidance. It is unknown how the molecular clock controls the strength of the signal that it communicates to downstream neurons, but one idea is that the molecular clock controls the resting membrane potential of the LNs (see text for details).

now be viewed as advantageous for both systems: the visual system helps to entrain the clock, and the clock provides circadian gating for the visual system. It is only in this configuration that the LNs are as essential for light avoidance as the photoreceptors themselves, since the LNs form part of the neuronal circuit and directly transmit (and modulate) visual signals. For *Drosophila* larvae, the function of the single deeper-projecting, *rh5*-expressing cell remains to be elucidated. This neuron may affect another light-induced behavior, and it may also explain the subtly different photophobicity scores in bright light between larvae lacking BO and those lacking LNs.

Our observation that the state of the molecular clock sets the ability of a neuron to respond to stimuli leads us to speculate that a molecular clock could perform the same function within any neuron. One example may be the mammalian forebrain neurons involved in cued and contextual long-term memory (LeDoux, 1995), since circadian regulation of contextual memory has been reported (Chaudhury and Colwell, 2002). Forebrain neurons contain molecular clocks that are stopped in mice with a knockout of *NPAS2*, the forebrain equivalent of *Clock* (Reick et al., 2001). Interestingly, these *NPAS2*$^{-/-}$ mice also display defects in their ability to form cued and contextual memory (Garcia et al., 2000). Thus, forebrain neurons, in an analagous manner to the *Drosophila* larval LNs, may have differing abilities to modulate learning and memory formation at different times of day via their own internal molecular clocks.

Experimental Procedures

Fly Strains

Flies and larvae were housed in 12 hr:12 hr LD cycles at 25°C, except for the DD experiments in which entrained larvae (from parents kept in LD) were shifted to DD for 2 days at 25°C. *y w* larvae were used as controls because all transgenes were in a *y w* background. *rh5-Gal4, rh6-Gal4*, *tim(UAS)-Gal4*, *Pdf-Gal4*, *per-Gal4*, and *cry-Gal4* driver lines were described previously (Blau and Young, 1999; Cook et al., 2003; Emery et al., 2000; Plautz et al., 1997; Renn et al., 1999). *UAS-DTI* and *UAS-dORK-ΔC* were also described previously (Han et al., 2000; Nitabach et al., 2002). *UAS-n-Synaptobrevin-GFP*, *per*^{0}*; per7.2:2*, *tim*^{01}*; tim7B*, and *yw;Clk*^{Jrk} flies were generously provided by M. Ramaswami, M. Rosbash, A. Sehgal, and R. Allada, respectively. *UAS-CD8-GFP* and *UAS-nGFP* were obtained from the Bloomington Stock Center.

Immunohistochemistry

Immunohistochemistry of larval heads and brains was as described in Blau and Young (1999). Antibodies were described in Blau and Young (1999) and in Cook et al. (2003), except anti-PDF, which was a generous gift of F. Rouyer. Images were obtained using a Leica TCS S2 confocal system.

Clock Entrainment by Light

Entrained third instar larvae were exposed to a 1 hr light pulse of 1000 lux from ZT23 to ZT24 (ZT [zeitgeber time]: time in LD). Entrained larvae at ZT24 without any light treatment were used as controls.

Photophobic Behavior

We tested larval photophobic behavior as described (Dettman et al., 2001) with minor modifications. A 10 cm Petri dish with 15 ml of 1.5% BactoAgar was used, and half of the lid was covered with black electrical tape. Early third instar larvae were removed from food and washed with PBS for ~30 s. Twenty larvae were distributed along the junction between light and dark, and plates were illuminated from above using either a fluorescent light (TFC Daylight) or a Phillips cool light with the same results. After 15 min at room temperature, the number of larvae in each half was counted. Plates with larvae burrowing into agar were discarded. Larvae crawling on the walls of Petri dishes were not included. Different light intensities produced different scores, with the strongest photophobicity at the highest intensities. The highest light intensity that we could use without heating up the plates was 1100 lux (Figure 3E). A neutral 70% transmittance filter (Roscolux #397: Pale Grey) was used to reduce light intensity. No gross morphological or general locomotor defects were observed in larvae of any genotype used in this study. Larvae of all genotypes were able to find food on a Petri dish and remain in that area. Furthermore, blind larvae moved and distributed themselves in a similarly random manner on Petri dishes as did control larvae that were fully exposed to light (data not shown). We did not detect any morphological defect in BO or LNs when LNs were electrically silenced.

Acknowledgments

We are very grateful to R. Allada, J. Hall, M. Ramaswami, M. Rosbash, F. Rouyer, A. Sehgal, L. Vosshall, M. Young, the Bloomington Stock Center, and the Developmental Studies Hybridoma Bank for antibodies and fly stocks and to M. Drapeau for advice on statistical analysis. We also thank R. Allada, B. Battelle, M. Nitabach, F. Pichaud, A. Rothenfluh, S. Small, S. Yamaguchi, M. Young, and members of the Blau and Desplan labs for their invaluable contributions and comments on the manuscript. Work in the authors' laboratories is supported by NIH grants EY013010 (C.D.) and GM063911 (J.B.).

Received: July 19, 2004
Revised: October 28, 2004
Accepted: November 23, 2004
Published: January 19, 2005

References

Allada, R. (2003). Circadian clocks: a tale of two feedback loops. Cell *112*, 284–286.

Barlow, R.B., Jr., Bolanowski, S.J., Jr., and Brachman, M.L. (1977). Efferent optic nerve fibers mediate circadian rhythms in the *Limulus* eye. Science *197*, 86–89.

Battelle, B.A. (2002). Circadian efferent input to *Limulus* eyes: anatomy, circuitry, and impact. Microsc. Res. Tech. *15*, 345–355.

Blau, J., and Young, M.W. (1999). Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell *99*, 661–671.

Busza, A., Emery-Le, M., Rosbash, M., and Emery, P. (2004). Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception. Science *304*, 1503–1506.

Chaudhury, D., and Colwell, C.S. (2002). Circadian modulation of learning and memory in fear-conditioned mice. Behav. Brain Res. *133*, 95–108.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., and Young, M.W. (2001). Circadian regulation of gene expression systems in the *Drosophila* head. Neuron *32*, 657–671.

Cook, T., Pichaud, F., Sonneville, R., Papatsenko, D., and Desplan, C. (2003). Distinction between color photoreceptor cell fates is controlled by Prospero in *Drosophila*. Dev. Cell *4*, 853–864.

Dettman, R.W., Turner, F.R., Hoyle, H.D., and Raff, E.C. (2001). Embryonic expression of the divergent *Drosophila* beta3-tubulin isoform is required for larval behavior. Genetics *158*, 253–263.

Emery, P., Stanewsky, R., Helfrich-Forster, C., Emery-Le, M., Hall, J.C., and Rosbash, M. (2000). *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron *26*, 493–504.

Frisch, B., Hardin, P.E., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1994). A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system. Neuron *12*, 555–570.

Garcia, J.A., Zhang, D., Estill, S.J., Michnoff, C., Rutter, J., Reick, M., Scott, K., Diaz-Arrastia, R., and McKnight, S.L. (2000). Impaired

cued and contextual memory in NPAS2-deficient mice. Science *288*, 2226–2230.

Gorczyca, M.G., and Hall, J.C. (1987). Immunohistochemical localization of choline acetyltransferase during development and in *Cha*^{ts} mutants of *Drosophila melanogaster*. J. Neurosci. *7*, 1361–1369.

Han, D.D., Stein, D., and Stevens, L.M. (2000). Investigating the function of follicular subpopulations during *Drosophila* oogenesis through hormone-dependent enhancer-targeted cell ablation. Development *127*, 573–583.

Handler, A.M., and Konopka, R.J. (1979). Transplantation of a circadian pacemaker in *Drosophila*. Nature *279*, 236–238.

Hassan, J., Iyengar, B., Scantlebury, N., Rodriguez Moncalvo, V., and Campos, A.R. (2005). Photic input pathways that mediate the Drosophila larval response to light and circadian rhythmicity are developmentally related but functionally distinct. J. Comp. Neurol. *481*, 266-275.

Helfrich-Forster, C., Winter, C., Hofbauer, A., Hall, J.C., and Stanewsky, R. (2001). The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron *30*, 249–261.

Helfrich-Forster, C., Edwards, T., Yasuyama, K., Wisotzki, B., Schneuwly, S., Stanewsky, R., Meinertzhagen, I.A., and Hofbauer, A. (2002). The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. J. Neurosci. *22*, 9255–9266.

Ivanchenko, M., Stanewsky, R., and Giebultowicz, J.M. (2001). Circadian photoreception in *Drosophila*: functions of Cryptochrome in peripheral and central clocks. J. Biol. Rhythms. *16*, 205–215.

Kaneko, M., Helfrich-Forster, C., and Hall, J.C. (1997). Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. J. Neurosci. *17*, 6745–6760.

Kaneko, M., Hamblen, M.J., and Hall, J.C. (2000). Involvement of the *period* gene in developmental time-memory: effect of the *per*^{Short} mutation on phase shifts induced by light pulses delivered to *Drosophila* larvae. J. Biol. Rhythms *15*, 13–30.

Krishnan, B., Dryer, S.E., and Hardin, P.E. (1999). Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature *400*, 375–378.

LeDoux, J.E. (1995). Emotion: clues from the brain. Annu. Rev. Psychol. *46*, 209–235.

Malpel, S., Klarsfeld, A., and Rouyer, F. (2002). Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. Development *129*, 1443–1453.

Malpel, S., Klarsfeld, A., and Rouyer, F. (2004). Circadian synchronization and rhythmicity in larval photoperception-defective mutants of *Drosophila*. J. Biol. Rhythms *19*, 10–21.

Naidoo, N., Song, W., Hunter-Ensor, M., and Sehgal, A. (1999). A role for the proteasome in the light response of the Timeless clock protein. Science *285*, 1737–1741.

Nitabach, M.N., Blau, J., and Holmes, T.C. (2002). Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. Cell *109*, 485–495.

Nitabach, M.N., Sheeba, V., Vera, D.A., Blau, J., and Holmes, T.C. (2005). Membrane electrical excitability is necessary for the free-running larval *Drosophila* circadian clock. J. Neurobiol. *62*, 1–13.

Ousley, A., Zafarullah, K., Chen, Y., Emerson, M., Hickman, L., and Sehgal, A. (1998). Conserved regions of the *timeless* (*tim*) clock gene in *Drosophila* analyzed through phylogenetic and functional studies. Genetics *148*, 815–825.

Park, J.H., Helfrich-Forster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J.C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. Proc. Natl. Acad. Sci. USA *97*, 3608–3613.

Pennartz, C.M., de Jeu, M.T., Bos, N.P., Schaap, J., and Geurtsen, A.M. (2002). Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. Nature *416*, 286–290.

Plautz, J.D., Kaneko, M., Hall, J.C., and Kay, S.A. (1997). Independent photoreceptive circadian clocks throughout *Drosophila*. Science *278*, 1632–1635.

Reick, M., Garcia, J.A., Dudley, C., and McKnight, S.L. (2001). NPAS2: an analog of Clock operative in the mammalian forebrain. Science *293*, 506–509.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell *99*, 791–802.

Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature *418*, 935–941.

Sawin-McCormack, E.P., Sokolowski, M.B., and Campos, A.R. (1995). Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. J. Neurogenet. *10*, 119–135.

Silver, R., LeSauter, J., Tresco, P.A., and Lehman, M.N. (1996). A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature *382*, 810–813.

Stanewsky, R. (2003). Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J. Neurobiol. *54*, 111–147.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M., and Hall, J.C. (1998). The *cry*^{b} mutation identifies Cryptochrome as an essential circadian photoreceptor in *Drosophila*. Cell *95*, 681–692.

Tosini, G., and Fukuhara, C. (2002). The mammalian retina as a clock. Cell Tissue Res. *309*, 119–126.

Wegener, C., Hamasaka, Y., and Nassel, D.R. (2004). Acetylcholine increases intracellular Ca^{2+} via nicotinic receptors in cultured PDF-containing clock neurons of *Drosophila*. J. Neurophysiol. *91*, 912–923.

Yasuyama, K., Kitamoto, T., and Salvaterra, P.M. (1995). Localization of choline acetyltransferase-expressing neurons in the larval visual system of *Drosophila melanogaster*. Cell Tissue Res. *282*, 193–202.

Note Added in Proof

Hassan et al. (2005) have recently shown that only the *rh5*-expressing photoreceptor cells of Bolwig's Organ are required for the light-induced ON-OFF response of *Drosophila* larvae. Since larvae do not require the *rh6*-expressing photoreceptor cells or the LNs for this behavior, it is likely that the ON-OFF response is mediated by the single *rh5* axon that projects deeper than the LNs.