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Evidence that the TIM Light Response Is Relevant to Light-Induced Phase Shifts in *Drosophila melanogaster*

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Summary

Light is a major environmental signal for the entrainment of circadian rhythms. In *Drosophila melanogaster*, recent experiments suggest that photic information is transduced to the clock through the *timeless* gene product, TIM. We provide genetic and spectral evidence supporting the relevance of TIM light responses to clock resetting. A missense mutant TIM, TIM-SL, exhibits greater sensitivity to light in both TIM protein disappearance and locomotor activity phase shifting assays. We show that the wavelength dependence of light-induced decreases in TIM levels and that of light-mediated phase shifting are virtually identical. Analysis of dose response of TIM disappearance in a variety of mutant genotypes suggests cell-autonomous light responses that are largely independent of the canonical visual transduction pathway.

Introduction

Fundamental processes in biological systems include daily fluctuations in many aspects of biochemistry, physiology, and behavior. These changes are governed by internal clocks present in virtually all eukaryotes and even some prokaryotes (Edmunds, 1988). Circadian clocks are self-sustaining timers in constant conditions and can be entrained or reset by certain environmental stimuli such as light and temperature changes (Saunders, 1982).

In *Drosophila melanogaster*, the *period* gene (*per*) is the best characterized circadian clock component (Rosbash et al., 1996). *per* RNA and protein (PER) fluctuate in abundance with ~24 hr periodicity (Hardin et al., 1990). PER undergoes phosphorylation changes with time (Edery et al., 1994b), and its nuclear entry is also temporally regulated (Curtin et al., 1995). Expression of *per* in per^{01}, per^{S}, and per^{L} mutants is affected in a manner that is consistent with a feedback regulatory loop model (Rosbash, 1995). It has also been shown that PER's phosphorylation state is a very early biochemical change following a light pulse (Lee et al., 1996).

The recently identified *timeless* gene (*tim*) shares many features with *per*. Both *tim* RNA and the protein it encodes (TIM) fluctuate in abundance with 24 hr periods similar to *per* RNA and PER protein (Sehgal et al., 1995; Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996), and TIM phosphorylation also changes with time (Zeng et al., 1996). The tim^{0} null mutation, which results in arrhythmic behavior, manifests neither *per* nor *tim* mRNA cycling (Sehgal et al., 1994, 1995), and PER is present at constant low levels (Price et al., 1995). The similar features of PER and TIM cycling are likely related to the fact that both proteins are present in a heterodimeric complex (Zeng et al., 1996).

Stimuli that phase shift the clock might cause rapid changes in one or more clock components, such as PER and TIM (Aronson et al., 1994; Edery et al., 1994a). Light is one such stimulus and affects several features of circadian oscillations: phase, period, and amplitude. TIM appears more closely connected to light than PER, because a decrease in TIM levels appears to be the first detectable response of a molecular clock component to acute light exposure (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). TIM levels respond to illumination even in arrhythmic per^{01} flies, i.e., in the absence of PER, and it has been proposed that a signal transduction pathway from a light receptor to TIM is an important part of the light-mediated entrainment pathway in *Drosophila* (Myers et al., 1996; Zeng et al., 1996).

In many organisms, the circadian light receptor is unknown. Dose-response curves and action spectra for light effects on circadian rhythms have been determined in plants (Hastings and Sweeney, 1960; Sargent et al., 1966) and animals (Frank and Zimmerman, 1969; Takahashi et al., 1984). During certain times of day, there is little or no response to light, whereas at other times there are pronounced delays or advances (dead zone, delay zone, and advance zone, respectively). Frank and Zimmerman (1969) reported 30 years ago the action spectrum for light-induced phase shift of the circadian rhythm of adult emergence (eclosion) in *Drosophila pseudoobscura*. A more detailed study of the action spectrum for the eclosion delay zone phase shift of this same species was subsequently published by Klemm and Ninnemann (1976). The main conclusion from those experiments is that the action spectra for advance and delay phase shifts are very similar. The most effective wavelengths are between 420 and 480 nm, with a sharp decline above 550 nm (Frank and Zimmerman, 1969); the eclosion clock is virtually insensitive to wavelength above 570 nm. This similarity suggests that both phase advances and phase delays are mediated by the same type of photoreceptor. The spectrum is significantly different from that of the major route of visual photoreception in *D. melanogaster* (Stark et al., 1976), which involves a rhodopsin species in all of the outer photoreceptors (Montell, 1989), suggesting that a different type of photopigment may be involved in the circadian gating of eclosion (Zimmerman and Goldsmith, 1971). During the past 25 years, *Drosophila melanogaster* has generally replaced *Drosophila pseudoobscura* as the most common experimental fly species for circadian rhythm research. Locomotor activity rhythms have also replaced

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adult eclosion as the most common *Drosophila* rhythm assay. This is due in part to the fact that the periods and responses to environmental stimuli of individual flies can be measured in the locomotor activity assays. But no action spectra have been reported for locomotor activity rhythms in *D. melanogaster*. Moreover, no action spectra data exist for the recently described light-mediated decrease in TIM levels. Since TIM is proposed to be the key clock molecule mediating light reception, the TIM disappearance spectrum should parallel the behavioral action spectrum. Our results reveal that this is indeed the case and further support the notion that the visual phototransduction pathway is dispensable for TIM degradation. We also provide genetic evidence that light-mediated decrease in TIM levels is relevant to the behavioral effects of light.

Results

Locomotor Activity Action Spectra

To measure the effect of light on the phase of adult locomotor activity, we used a modified phase response curve (PRC) protocol, called the anchored PRC (APRC; Aschoff, 1965; Levine et al., 1994). Figure 1A shows the APRC for wild-type flies.

To measure the spectral profile of the APRC, flies were irradiated with 400–700 nm monochromatic light, at times corresponding to maximal phase delays (ZT15) and maximal phase advances (ZT21) (Saunders et al., 1994). The action spectra are very similar for both cases: maximal effects are observed with wavelengths of 400–500 nm, and the flies were almost unresponsive to wavelengths of 600 nm or greater (Figure 1B). The action spectrum correlates very well with that reported for the *Drosophila pseudoobscura* eclosion rhythm (Frank and Zimmerman, 1969).

The TIM Light Response

The clock component TIM has been proposed to be an important link between light stimuli and the circadian pacemaker (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). To provide additional evidence for such a hypothesis, we examined the action spectrum for light-mediated TIM disappearance using the same protocol used to generate the behavioral PRCs: flies were pulsed with light at ZT15 (Figure 2A) or at ZT21 (Figure 2B) and then put in the dark for 1 hr to allow sufficient time for TIM levels to change in response to illumination prior to freezing and extract preparation (Myers et al., 1996; Zeng et al., 1996).

The spectra were quite similar to each other and to the action spectra of the behavioral experiments (compare Figure 2D with Figure 1B). At both times, the highest amount of TIM disappearance occurred at wavelengths of 450 and 500 nm with little effect at or above 600 nm (Figure 2D; the bump at 650 nm in the ZT15 action spectra was not reproducible). The amount of TIM decrease at ZT15 is somewhat less than what is observed at ZT21 ($<$40% for ZT15 and 50%–60% for ZT21; see below), in agreement with previous reports (Myers et al., 1996; Zeng et al., 1996).

Previous results indicated that illumination causes a decrease in TIM levels in a per^{01} strain, i.e., in arrhythmic flies devoid of PER protein (Myers et al., 1996; Zeng et al., 1996). We therefore examined the TIM disappearance action spectrum in this genetic background (Figure 2C). The spectrum is very similar to what is observed in wild-type flies and very similar to the behavioral action spectrum (Figures 2D and 1B, respectively). Moreover, the extent of TIM decrease, 50%–60%, was very similar to what was seen in a per^{+} background. The results suggest that neither PER nor a functional circadian clock is necessary for the normal circadian photoreception and signal transduction effects on TIM levels.

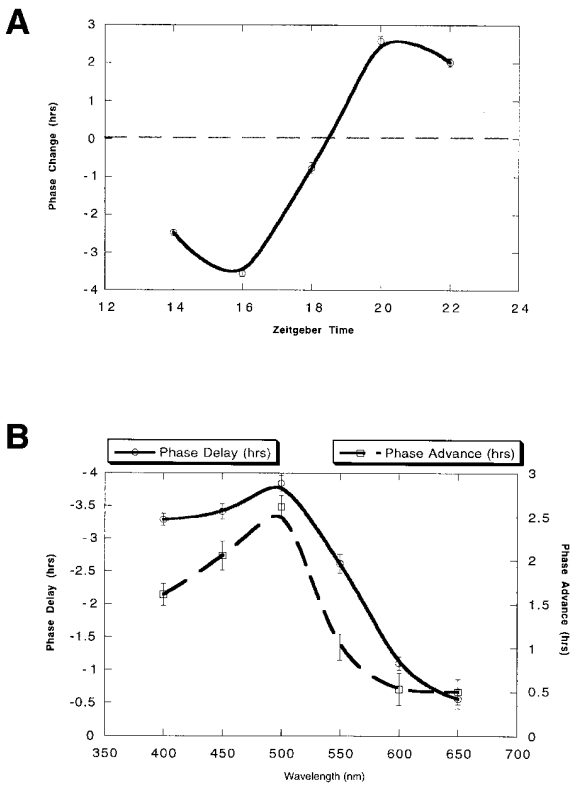

Figure 1. Action Spectra of Locomotor Activity Phase Shift

(A) Anchored phase response curve of wild-type flies. Flies were entrained for 4 light–dark cycles, pulsed with 5 mW/cm^2 white light for 10 min at the indicated zeitgeber times, and transferred to constant darkness. Phase change was calculated by comparing the phase of light pulsed flies a day after the pulse to a control group of flies. Data was pooled from the following number of flies: 30 (control), 27 (ZT14), 28 (ZT16), 24 (ZT18), 22 (ZT20), and 25 (ZT22). The error bars denote SEMs, which were calculated by taking the square root of the sum of squares of standard deviation weighted by the number of animals for the control and experimental groups. The graph was plotted using the smooth curve option in the KALEIDAGRAPH software version 3.0.2 (Abelbeck Software, PA).

(B) Action spectrum of locomotor activity phase delays and advances. Flies were entrained as in (A). Phase delay and phase advance action spectra were generated by pulsing flies for 10 min with monochromatic light of different wavelengths at ZT15 and ZT21, respectively. In both cases, the intensity of light used was 1 mW/cm^2. Data were an analyzed as in (A). Data were pooled from the following number of flies: phase delay action spectrum: 27 (control), 25 (400 nm), 18 (450 nm), 22 (500 nm), 14 (550 nm), 23 (600 nm), and 17 (650 nm); phase advance action spectrum: 23 (control), 26 (400 nm), 23 (450 nm), 27 (500 nm), 21 (550 nm), 26 (600 nm), and 19 (650 nm). Error bars represent SEMs calculated as in (A). Curves were drawn as in (A).

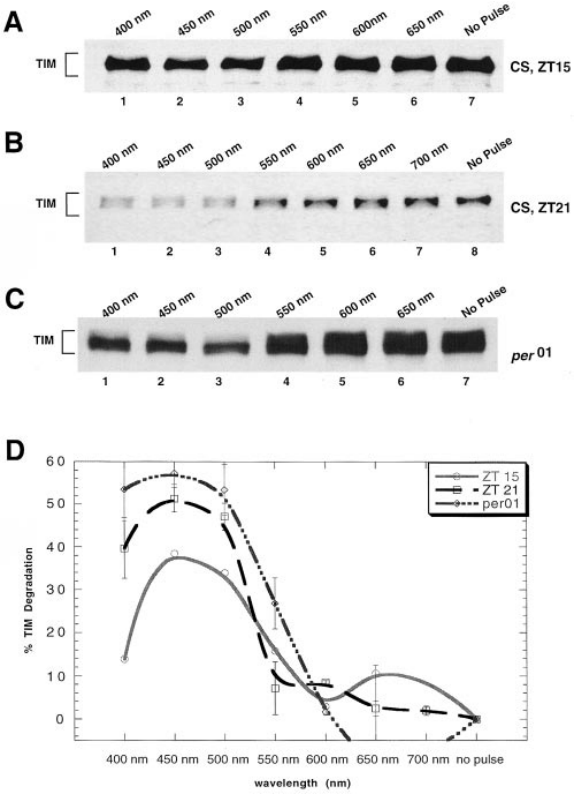

Figure 2. TIM Degradation Spectra in the Delay Zone and Advance Zone and in *per*^{01} Flies

(A) Western blot of TIM disappearance spectrum at ZT15. Flies were pulsed for 10 min at ZT15 with monochromatic lights of different wavelengths followed by 1 hr of recovery in the dark before Western blotting analysis. "No pulse" control (lane 7) indicates that flies did not receive the 10 min light pulse; therefore, it is equivalent to the ZT16 sample.

(B) Western blot of TIM disappearance spectrum at ZT21. The experiment was done exactly as described above for the ZT15 spectrum.

(C) Western blot of TIM disappearance spectrum in *per*^{01} mutant. Flies were given monochromatic light pulses of 15 min duration followed by 1 hr of recovery in the dark before Western blotting analysis. "No pulse" control (lane 7) indicates that flies did not receive the light pulse.

(D) TIM disappearance action spectrum in delay zone (ZT15) and advance zone (ZT21) and in *per*^{01} background. To generate the curves, the amount of TIM in the nonpulsed lane was set as 100%; therefore, this sample lane was taken as 0% disappearance. The rest of the data points were normalized against this value. The curve was fitted using the smooth curve option in KALEIDAGRAPH software version 3.0.2 (Abelbeck Software, PA). For the ZT15 action spectrum, three independent experiments gave visually identical results on Western blots. However, due to poor blot quality, only one of the blots could be unambiguously quantitated. For the ZT21 and *per*^{01} action spectra, the error bars indicated SEMs from three independent experiments.

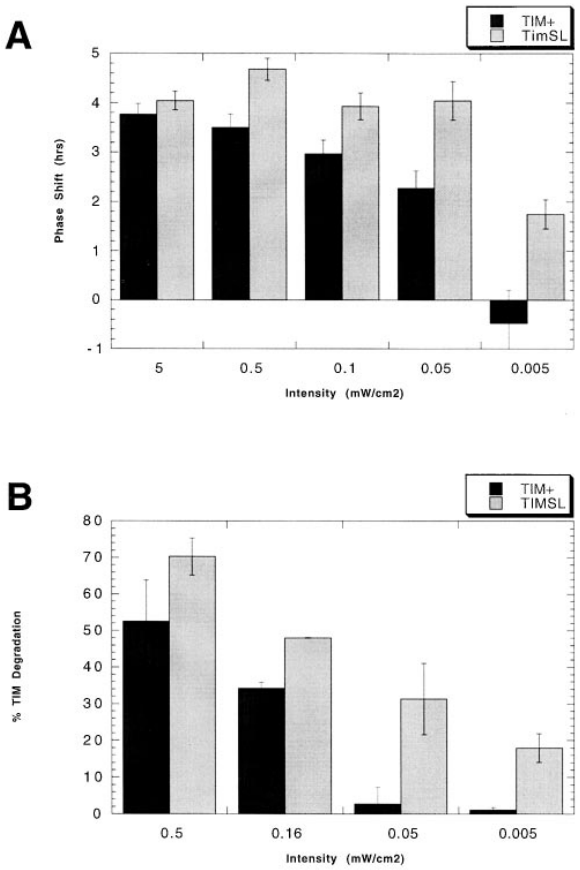

Figure 3. *tim*^{SL} Mutation Causes Increased Light Sensitivity of Both Phase Shifting and TIM Disappearance Responses

(A) Comparison of intensity response of locomotor activity phase delay between *tim* and *tim*^{SL} flies. Flies were entrained and data analyzed as in Figure 1A. Flies were pulsed with monochromatic light of 450 nm at different intensities at ZT15. Data were pooled from the following number of flies: *tim*: 27 (control), 28 (5 mW/cm^2), 27 (0.5 mW/cm^2), 29 (0.1 mW/cm^2), 29 (0.05 mW/cm^2), and 24 (0.005 mW/cm^2); *tim*^{SL}: 27 (control), 27 (5 mW/cm^2), 27 (0.5 mW/cm^2), 29 (0.1 mW/cm^2), 22 (0.05 mW/cm^2), and 23 (0.005 mW/cm^2). A two-tailed Student's t test performed using TTEST function in EXCEL version 5.0 (MICROSOFT, WA) gave $p \leq 0.0187$, indicating statistically significant differences between the two genotypes.

(B) Comparison of TIM disappearance in *tim* versus *tim*^{SL} flies in *per*^{01} background. Flies were pulsed for 10 min with 450 nm monochromatic light of the indicated intensity and allowed to recover in the dark for 1 hr. Extracts were run on Western blot, and TIM levels were quantitated using densitometry (see Experimental Procedures). To generate the curve, the amount of TIM in the nonpulsed lane was set as 100%; therefore, this sample lane was taken as 0% disappearance. The rest of the data points are normalized against value. A two-tailed Student's t test done using TTEST function in EXCEL gave $p \leq 0.009$, indicating statistically significant differences between the two genotypes.

Mutant TIM-SL Protein Alters the Light Sensitivity of TIM Disappearance and the Behavioral Response

We compared the light sensitivities for TIM disappearance and behavioral phase shifts (Figure 3). To optimize sensitivity in such experiments, 450 nm light was used. Effects on the protein and on behavior were maximal at 0.5 mW/cm^2, with little or no light response at 0.005 mW/cm^2. The behavioral response appears to be more sensitive, which may reflect the fact that only a modest effect on TIM levels is sufficient to cause a phase shift (see Discussion). The sensitivity difference in the two assays may also be due to technical difficulties in accurately assessing minor differences in TIM levels on Western blots.

To make a tighter connection between light-induced TIM disappearance and phase shifting, we assayed the one period-altering allele of *tim*, which encodes the TIM-SL protein (Rutila et al., 1996). Experiments with *tim*^{SL}

mutant adults indicate that TIM-SL is more sensitive to light than wild-type TIM; there was somewhat more TIM disappearance (compared to that observed in *tim*$^+$), and this was especially apparent at low intensities: at 0.05 mW/cm^2, there was little or no effect on wild-type TIM but a robust response by TIM-SL. This hypersensitivity correlates with the effect on the behavioral response: TIM-SL causes a larger phase shift at ZT15, which is most apparent at low intensities, i.e., at 0.05 mW/cm^2 and at 0.005 mW/cm^2. These results provide the first genetic connection between *tim* gene function and the light response and further support the relevance of the light-induced TIM decrease to the behavioral phase shift.

TIM Decrease in the Eye Is Independent of Brain Pacemaker Neurons

PER's expression pattern in the fly head has been extensively studied. It is present in photoreceptor cells, putative glia in various ganglia, and neurons in lateral as well as dorsal regions of the central brain (Saez and Young, 1988; Liu et al., 1988; Siwicki et al., 1988; Zerr et al., 1990). Expression in lateral neurons is probably necessary and may be sufficient for locomotor activity rhythms (Ewer et al., 1992; Frisch et al., 1994; Vosshall and Young, 1995) PER undergoes robust cycling in all of these tissues as assayed by histochemistry (Zerr et al., 1990). Although less well studied, the expression pattern of TIM is similar (Hunter-Ensor et al., 1996), as expected from the biochemical relationship between the two proteins. Multiple expression sites raise a problem for comparing the behavioral and biochemical action spectra: the former probably reflects changes in TIM levels in lateral neurons, whereas the latter largely reflects changes in TIM levels in photoreceptor cells. Indeed, a comparison of the Western blot TIM signal from eyeless heads with that from wild-type flies indicates that about two-thirds of TIM in head extracts comes from the eye (i.e., the photoreceptor cells; data not shown). The similar action spectra for behavior and TIM level changes might therefore reflect a shared photoreceptor tissue, which detects light and relays the appropriate circadian information to the relevant tissues. Alternatively, they might reflect the fact that the eye and pacemaker neurons in the CNS have independent circadian photoreceptors but share the same photopigment. The extreme version of this second possibility is that the TIM response to light is entirely cell autonomous and reflects a ubiquitous photoreceptor (e.g., Plautz et al., 1997).

To begin to address this question, we evaluated the TIM degradation pattern in *disco* flies. In this mutant strain, the lateral neurons are largely eliminated (Zerr et al., 1990; Helfrich-Forster, 1998), ganglia normally located between the brain and the eyes are disrupted or eliminated (Steller et al., 1987), and free-running locomotor activity behavior is largely arrhythmic (Dushay et al., 1989; Helfrich-Forster, 1998). This implies that signals that normally emanate from the pacemaker neurons are largely eliminated and that the same or perhaps a slightly greater fraction of TIM in *disco* extracts is still derived from eyes. To optimize sensitivity, TIM levels were examined as a function of intensity with 450 nm light (Figure 4).

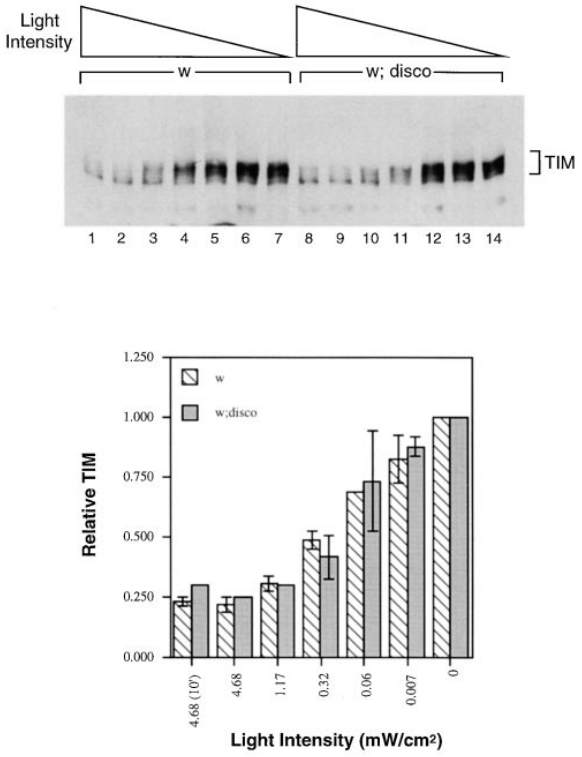

Figure 4. Comparison of TIM Disappearance in *white* and *w;disco* Flies

(A) Western blot of TIM disappearance in *white* (*w*) and white-eyed *disco* (*w;disco*) flies. Flies were given a series of monochromatic light pulses (450 nm) at ZT21 for 2 min before returning back to dark for 1 hr. The series of different light intensities are: 4.68 mW/cm^2 (10 min) (lanes 1 and 8), 4.68 mW/cm^2 (lanes 2 and 9), 1.17 mW/cm^2 (lanes 3 and 10), 0.32 mW/cm^2 (lanes 4 and 11), 0.06 mW/cm^2 (lanes 5 and 12), 0.007 mW/cm^2 (lanes 6 and 13), and 0 mW/cm^2 (lanes 7 and 14). Wedge shapes on top of the gel indicate decreasing light intensities.

(B) Quantitation of Western blot shown in (A). Hatched bars represent data series for *white* and solid bars represent data series for *w;disco*. The plot was generated as described in the legend to Figure 3B.

First, in both *disco* and wild-type genotypes, the TIM level decrease is a function of light intensity. Second, there was little or no effect of *disco*, indicating that the pacemaker neurons have little or no effect on light-mediated decrease in TIM levels in the eye (Figure 4). This is consistent with the second possibility just discussed, that the TIM light response in photoreceptors may be cell autonomous; i.e., that the eye may contain all of the components necessary for a light-mediated decrease in TIM levels.

TIM Is More Sensitive to Light in *white* Flies than in Wild-Type Flies

To test the notion that photoreceptor cell TIM responds directly to light, we compared TIM levels in wild-type and *white* flies in response to illumination at different light intensities. The rationale is that the mutant eyes are missing screening pigment and therefore these photoreceptor cells could be more light sensitive than those from wild-type eyes. Indeed, TIM disappearance was

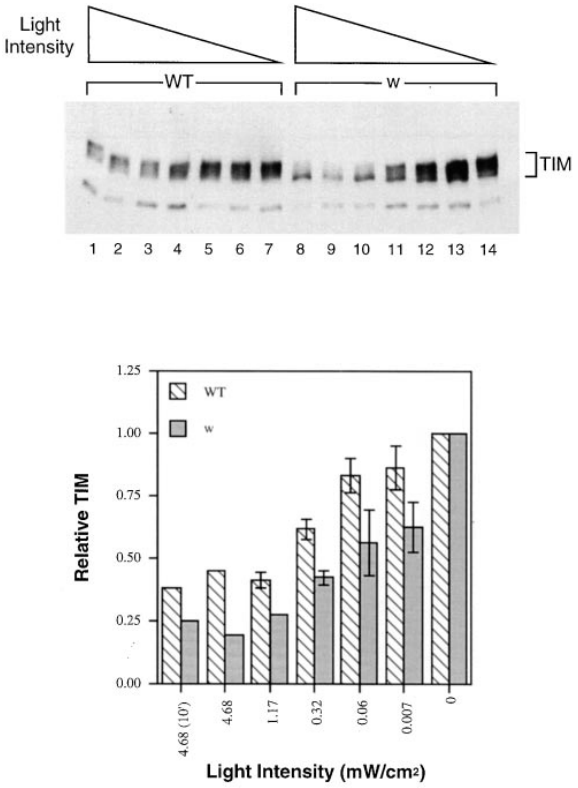

Figure 5. Comparison of TIM Disappearance in Wild-Type and White-Eyed Flies

(A) Western blot of TIM disappearance in wild-type (WT) and *white* (*w*) flies. Flies were pulsed as described in the legend to Figure 4A. (B) Quantitation of Western blot shown in (A). Hatched bars represent data series for wild-type and solid bars represent data series for *white*. The plot was generated as described in the legend to Figure 3B. A two-tailed Student's t test performed using TTEST function in EXCEL gave $p \leq 0.013$, indicating statistically significant differences between the two genotypes.

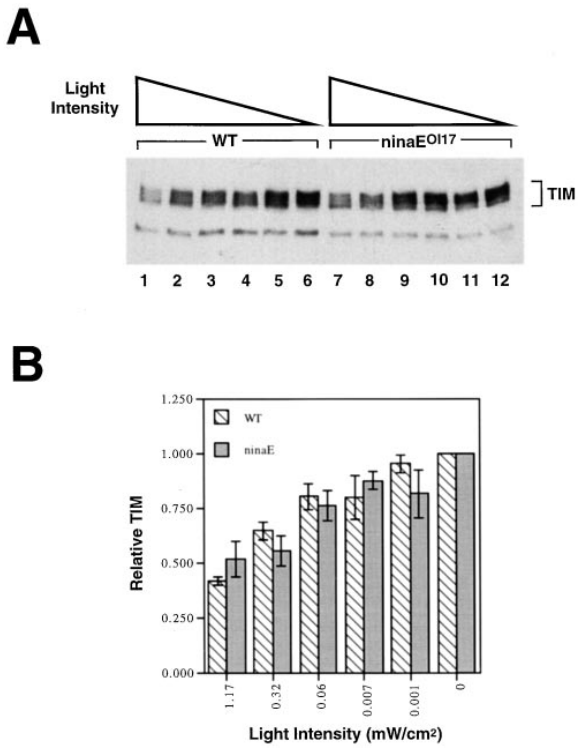

Figure 6. TIM Disappearance in *ninaE*^{OI17} Mutant

(A) Western blot analysis of TIM disappearance in wild-type and *ninaE*^{OI17}. Flies were pulsed as described in the legend to Figure 4A. (B) Quantitation of Western blot shown in (A). Hatched bars represent data series for wild-type and solid bars represent data series for *ninaE*^{OI17}. The plot was generated as described in the legend to Figure 3B.

more extensive in *white* flies than in wild-type flies at all intensities tested (Figure 5). We also noticed that at high intensities there appeared to be an effect on TIM phosphorylation state, as if there was a greater decrease in the level of hyperphosphorylated eye TIM in *white* as compared to wild-type flies (Figure 5; compare lanes 1 and 8, 2 and 9, and 3 and 10, respectively). Given that the Western blots predominantly examine TIM in photoreceptor cells, we conclude that the relevant circadian photoreceptor is within or near these cells.

TIM Light Response in the Eye Does Not Require the Canonical Phototransduction Pathway

Previous histochemical studies on PER protein cycling examined visual system mutants and indicated that the standard phototransduction pathway of the compound eye is not necessary for circadian variations in eye PER levels (Zerr et al., 1990). Because of the less quantitative nature of the histochemical approach, because our experiments suggest the possibility of eye-autonomous circadian phototransduction, and because effects of the classical phototransduction mutants on TIM have not been examined, we asked whether the response of eye TIM levels to light requires the known phototransduction cascade. We looked at TIM responses in two different well-characterized visual mutants, namely, *ninaE*^{OI17} (O'Tousa et al., 1985) and *norpA*^{p24} (Bloomquist et al., 1988).

Figure 6 compares light-mediated TIM decrease in visually normal and *ninaE*^{OI17} flies. The results indicate that there is little or no effect of the mutation, suggesting that the photoreception mechanisms involved in TIM responses do not use the major rhodopsin encoded by the *ninaE* gene. Also, in the *norpA* mutant, TIM levels decreased to the same extent as in wild-type flies after 1, 2, and 4 hr of white light pulses (data not shown). This indicates that the standard photoreceptor transduction cascade is not involved in the circadian light response in the eye.

A Direct Comparison of Visual and Circadian Action Spectra

To confirm this surprising conclusion, we directly compared action spectra for vision and for TIM disappearance in photoreceptor cells. To this end, we took advantage of a recently identified and cloned novel *Drosophila* protein, PIP82, which shows light-dependent dephosphorylation. This response is dependent on a functional visual transduction pathway (see Experimental Procedures).

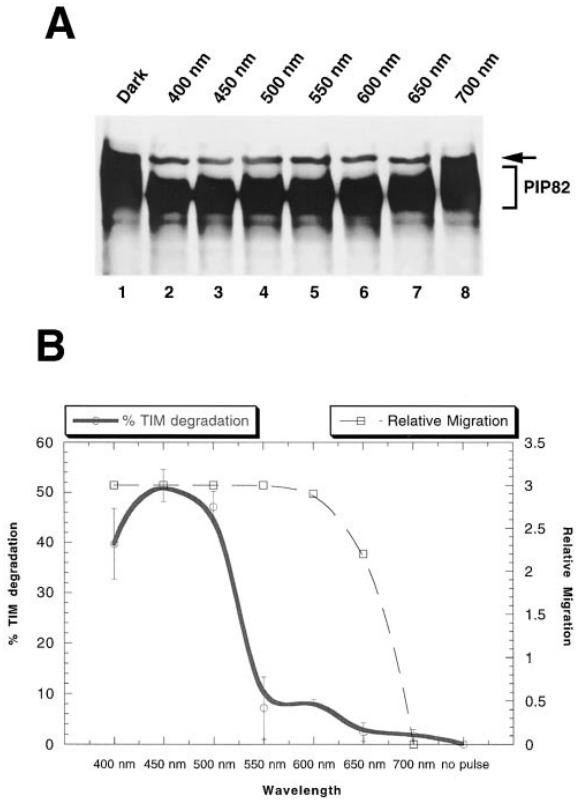

Figure 7. Comparison of Visual and Circadian Action Spectra

(A) Western blot analysis of PIP82 phosphorylation. Flies were given monochromatic light pulses of different wavelengths for 5 min and subjected to Western analysis immediately at the end of the pulses as described in the Experimental Procedures. "Dark" (lane 1) indicates that flies were not pulsed. In this lane, PIP82 was fully phosphorylated.

(B) Comparison of visual and circadian action spectra. Visual action spectra were assayed by measuring the relative migration curve of PIP82. The distance between the cross-reacting band (arrow) and the slowest migrating form of PIP82 was used to calculate the relative migration. Basically, the distances for 400–550 nm (lanes 2–5) are the same and therefore arbitrarily set to 3. TIM degradation action spectrum at ZT21 was used to generate the curve for circadian action spectra.

To measure the visual action spectrum in the context of light effects on PIP82, we measured the mobility change for this protein as a function of illumination wavelength. This involved a direct comparison of the PIP82 and TIM action spectra, using the same flies, methods, and even the same blots. As expected, administration of monochromatic light pulses converted PIP82 to more hypophosphorylated forms as a function of wavelength (Figure 7A; see Experimental Procedures): at 400–600 nm PIP82 had the same mobility (lanes 2–6), at 650 nm it had an intermediate mobility (lane 7), and at 700 nm it was virtually unaffected by light (compare lanes 8 and 1). The action spectrum is virtually identical to the known electrical response curve for photoreceptor cells (Stark et al., 1976). Importantly, the spectrum is substantially different from the spectrum for TIM disappearance or that for locomotor activity phase shifting (Figure 7B), consistent with the notion that the circadian responses use a specialized photopigment as well as a different signal transduction pathway.

Discussion

To compare circadian light effects between *Drosophila* behavioral and molecular assays, we first examined the wavelength dependence of locomotor activity phase shifts. Since all of our biochemical experiments were done on flies entrained to a 24 hr light–dark cycle, we used a modified protocol for generating phase response curves, which generally administers light pulses during free-running cycles (Saunders et al., 1994). The APRC assays the phase shift of entrained rhythms, as the flies are pulsed during the dark phase of the last light–dark cycle. The light–dark cycle maintains greater synchronization of the individual clocks and individual flies. It also optimizes the possibility of assessing effects on any light-dependent clock components, which may dampen or otherwise change during long periods in constant darkness.

The PRCs thus generated closely resemble previously published PRCs (Saunders et al., 1994), showing phase delays early at night (ZT14 and ZT16) and phase advances late at night (ZT20 and ZT22), with no response at around ZT18 (the so-called crossover point; Figure 1A). We assayed the wavelength dependence of phase delay and phase advance responses by analyzing the PRC at ZT15 and ZT21. Both responses have very similar if not identical action spectra, suggesting that similar and perhaps common photoreception mechanisms mediate both phase advance and phase delay responses. Minor differences may have gone undetected owing to the broad bandwidth of the filters we applied (50 nm).

To probe the spectral profile of molecular components involved in phase shifting, we assayed TIM levels in flies illuminated with specific wavelengths, in the phase delay zone (ZT15) and the phase advance zone (ZT21) and in arrhythmic (per^{01}) flies. The profiles were quite similar to each other as well as to the behavioral action spectra, suggesting that all of these responses are downstream of a common photoreceptor.

In addition, we generated dose-response curves for behavioral phase shifting at ZT15 as well as TIM disappearance in the arrhythmic per^{01} flies. The two curves were similar, consistent with the notion that both are downstream from a common photoreceptor and signal transduction pathway and consistent with the notion that the behavioral response is related to the TIM response. Dose-response curves were generated for the TIM-SL protein, which was more light sensitive and also gave rise to a more sensitive behavioral response. This provides the first genetic evidence that the TIM light response in the delay zone is relevant to the behavioral response. The biochemical assays were done in the per^{01} flies. In this genetic background, there were no confounding effects of a phase shift to the clock; i.e., in a wild-type background, the light-induced phase shift at ZT15 caused subtle differences in TIM levels. These were variable with illumination intensity, which were superimposed on the subtle TIM-SL effects (data not shown).

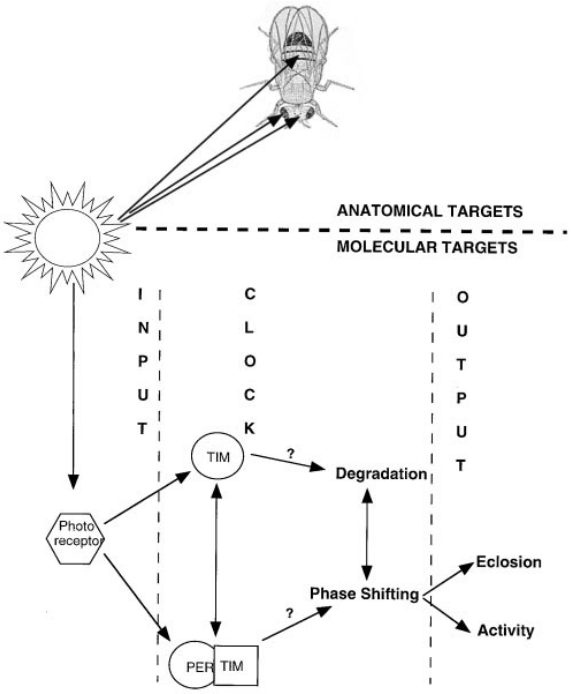

Figure 8. A Model Showing Putative Anatomical Targets and Molecular Mechanisms Involved in Circadian Photoreception

Different clock tissues are potentially independently light responsive (Giebultowicz and Hege, 1997; Plautz et al., 1997). The photoreceptor targets TIM or the PER-TIM dimer, resulting in TIM disappearance and phase shifting. It is unclear whether TIM disappearance response and phase shifting are serial or parallel responses (see Discussion).

We do not know why TIM-SL is more light sensitive. First, it could be a more efficient degradation substrate than wild-type TIM, if the light-mediated decrease in TIM levels is largely due to degradation. Second, it could reflect some TIM-SL–mediated alterations of *tim* RNA levels or TIM translation. Third, the mutant could also give rise to a more efficient light capture mechanism, due perhaps to an indirect effect on other clock components (the photoreceptor itself?). Distinguishing between these possibilities will require learning more about the mechanism that leads to these light-induced changes in TIM levels as well as the nature of the photoreceptor.

Consistent with a previous report, we found a modest difference in the extent of TIM disappearance between ZT15 and ZT21 (Myers et al., 1996; Zeng et al., 1996): at ZT21, the TIM signal was ~60% weaker than the no-pulse control, whereas at ZT15 it was only ~40% weaker. As our light-pulse protocol includes a subsequent 1 hr incubation in the dark, differences in TIM synthesis rates (due, for example, to higher RNA levels at one time than another) could confound differences in apparent decrease in protein levels. It should be emphasized that it is not certain that the differences in TIM levels are due to light-induced degradation; there is no compelling argument against light-mediated inhibition of translation. We also cannot rule out the possibility that these temporal differences are due to clock-regulated modifications of the photoreception mechanism, of the degradation machinery, or of PER or TIM themselves. The time-dependent phosphorylation of both PER and TIM as well as their heterodimeric association make the latter two possibilities quite reasonable (Zeng et al., 1996). It may be relevant in this context that the TIM degradation response in per^{01} background is very similar to that observed at ZT21, where much of PER is in monomeric form (Zeng et al., 1996).

The traditional *Drosophila melanogaster* PRC manifests bigger phase delays at ZT15 than phase advances at ZT21 (Saunders et al., 1994), which is the opposite of what one might predict from the relative magnitude of the TIM response. However, the behavioral responses may reflect a more complex combination of molecular changes with as yet uncharacterized responses to illumination. More importantly, perhaps, our assays indicate that most of the TIM signal in head extracts comes from the eyes rather than the behaviorally more relevant lateral neurons. The head Western blot signals may therefore misrepresent the magnitude of the TIM response in pacemaker neurons, which is presumably most relevant to the extent of the behavioral phase shift. Finally, the TIM decrease may be a parallel rather than a serial response to illumination. In the former case especially, it may have no impact on behavior and occur subsequent to a light effect on TIM activity and behavior. This is particularly relevant to the accelerated disappearance of TIM observed at ZT21, where recent evidence suggests that TIM disappearance may be insufficient to generate a phase shift (Sidote et al., 1998).

Previous reports indicate that PER's phosphorylation status also changes in response to light pulses (Lee et al., 1996). But the PER response times vary as a function of circadian time and are longer than those of TIM. Some change in PER is observed 1 hr after a light pulse at ZT21, but even this is slower than the TIM response to light (easily detectable 15 min after a light pulse; Hunter-Ensor et al., 1996; Lee et al., 1996). Importantly, there is no detectable PER phosphorylation difference until ~3–4 hr after a light pulse at ZT15, and there is no detectable light effect on PER in the absence of TIM (data not shown). Although these results may reflect a number of technical issues (e.g., the inability of the mobility shift assay to detect small but important light-induced phosphorylation differences), the slower light response of PER may indicate that it is downstream of the TIM response. But the PER light response is probably also relevant to the behavioral phase shift: the light-induced changes in PER phosphorylation are bidirectional (Lee et al., 1996), and the per^{S} and per^{L} mutants affect the magnitude of the phase response curve (Konopka et al., 1989; Saunders et al., 1994).

It has recently been suggested that the PAS domain of PER constitutes an evolutionary link between clock molecules and photoreceptors (Crosthwaite et al., 1997). An implication of this primary sequence conservation is that PER is at or near the circadian photoreceptor. Our observations and those of others (Myers et al., 1996; Zeng et al., 1996) are not easily compatible with this view, at least in its simplest form. This is because they link TIM more closely to circadian photoreception than PER, and this link is independent of PER. However, we cannot exclude the possibility that other PAS proteins

may be involved in circadian light perception, and TIM might be associated with some of these proteins.

There are no reports of light-induced circadian gene expression in *Drosophila*, and the rapidity of the TIM response (detectable decrease in levels after 10–30 min of light exposure) suggests that it is probably upstream of, or at least independent of, any light effects on immediate-early gene expression in this organism.

Because 60%–70% of TIM is in the eye, we wanted to identify the machinery involved in the TIM light response in this tissue. Although the obvious candidates were rhodopsin and other components of the phototransduction pathway in the external eyes, we observed no effect of the relevant visual system mutations on TIM degradation; these biochemical results are consistent with a previous report that this pathway is dispensable for PER protein cycling in the eye (Zerr et al., 1990). The simplest interpretation is that the canonical eye phototransduction pathway, including the *ninaE*-encoded rhodopsin itself, is dispensable for TIM light response. Similar conclusions have also been reported in an independent study by another group (Yang et al., 1998 [this issue of *Neuron*]). The novel phototransduction pathway might reside within the eye, or some of it might reside in another tissue and make neural or humoral connections to the eye. Our in vivo assays do not distinguish between these possibilities in a satisfactory manner, but we did eliminate the pacemaker neurons as a possible source of photic information. This is because *disco* mutant flies had a very similar TIM light response to illumination. This result is consistent with both the robust PER cycling in eyes in light–dark conditions (Zerr et al., 1990) and *per* RNA cycling in both light–dark and dark–dark conditions (Hardin et al., 1992). It is also consistent with the suggestion that *disco* flies have an active, light-responsive pacemaker but are defective in output machinery (Hardin et al., 1992).

The simplest interpretation of our results is that all cycling tissues contain cell-autonomous oscillators with the same, as yet unidentified, photoreceptor (Figure 8). The data also suggest that the same oscillator tissues contain signal transduction components that impact rapidly on TIM. This interpretation is supported by a report of robust circadian cycling and light-mediated phase shifting in the malphigian tubules of decapitated flies, where no cross-talk with the brain or eye is possible (Giebultowicz and Hege, 1997). It is also consistent with a more recent report demonstrating in vitro light sensitivity of a number of oscillating adult *Drosophila* tissues (Plautz et al., 1997). The data indicate that *Drosophila* has a specialized circadian photoreceptor, and the action spectra suggest that flavin- and/or pterin-based molecules such as cryptochromes may be involved (Ahmad and Cashmere, 1996; Guo et al., 1998). Every oscillator tissue, perhaps every cycling cell, may contain the same machinery for light entrainment as well as for free-running rhythms. The machinery may include novel components for photoreception, signal transduction, protein processing, and gene regulation. This raises the exciting possibility that other components of the light-input pathway may be encoded by novel clock genes. The light effects on TIM levels should aid in the identification of the photoreceptor as well as the unknown signal transduction pathways that link it to the PER–TIM clock cycles.

Experimental Procedures

Fly Strains

The following fly strains were used in this study: Standard wild-type Canton-S (CS); white-eyed w^{1118}; $w;disco^{2}f$, a neuroanatomical mutant that is arrhythmic and missing most of the fly circadian pacemaker neurons (Steller et al., 1987; Dushay et al., 1989); $ninaE^{OI17}$, a null mutant for the major opsin species (O'Tousa et al., 1985); $norpA^{p24}$, a phospholipase C mutant (Bloomquist et al., 1988); per^{01}, a null mutant for per (Konopka and Benzer, 1971); and tim^{SL}, a *tim* allele isolated as a suppressor of per^{L} (Rutila et al., 1996).

Phase-Shift Action Spectra Analyses

Wild-type flies were entrained to a 12 hr light:12 hr dark cycle for 4 days as previously described (Hamblen et al., 1986). During the fifth dark phase of the cycle, flies were given either a white light pulse (intensity, 8 mW/cm^2) at ZT14, ZT16, ZT18, ZT20, and ZT22 or monochromatic light pulses (1 mW/cm^2) at either ZT15 or ZT21 for 10 min (where ZT12 is lights off, and ZT0 is lights on). A separate control group of flies was not pulsed. Flies were then put into constant darkness for the next 5 days.

The phase of locomotor activity peaks after the light pulse was determined as described previously (Edery et al., 1994a), using activity offset at 50% of the peak as the phase reference point. To calculate the phase shift of these activity peaks, the average phase value after the light pulse for a group of flies was compared to the average phase values of the nonpulsed controls on the second day after the light pulse. By this time, essentially all of the flies had completely phase shifted.

Fly Entrainment and Light Pulse Protocol for Biochemical Analyses

Flies were entrained in plastic vials (Applied Scientific, CA) containing agar at 25°C in light–dark (12 hr:12 hr) cycle for 3 days. On the fourth dark phase, flies were given a light pulse for either 2 or 10 min at ZT15 or ZT21 and allowed to recover in the dark for 1 or 2 hr before being collected on dry ice. Monochromatic light was produced using a Q convective lamp housing 60,000 (Oriel Instruments, CT) and a series of wavelength cutoff filters (bandwidth, 50 nm). Light intensity was measured using a radiometer IL1350 (International Light, MA). Experimentally varying light intensities were adjusted using neutral density filters (Oriel Instruments, CT) in combination with adjusting the distance between target and light source.

Western Blotting

Protein extractions and Western blotting were carried out essentially as previously described (Edery et al., 1994b). The samples were electrophoresed on 6% or 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, using a semi-dry electroblotting apparatus (Integrated Separation Systems, MA). After transfer, membranes were stained with ponceau S to ensure equal loading of lanes.

For quantitation of chemiluminescent Western blots, each blot was exposed to a chemiluminescence screen (Bio-Rad Labs, CA) and quantified with a GS-363 phosphorimager (Bio-Rad Labs, CA). In some cases, the quantitation was done by densitometry using an ARCUS II scanner (Agfa, NV) and Molecular Analyst software (Bio-Rad Labs, CA). Antisera containing TIM antibody were obtained from rat immunized with a bacterial recombinant C-terminal TIM fragment (amino acids 1083–1376). To generate PIP82 antibody, a peptide fragment KVNKLISRFEGGRPRLCP (corresponding to amino acids 968–987) was injected into rabbits, and antibody was purified from antisera by affinity columns conjugated with the above peptide.

Analysis of PIP82, a Novel Light-Responsive *Drosophila* Protein

PIP82 is a novel 1171 amino acid *Drosophila* protein that is predominantly expressed in photoreceptor cells of the compound eye (Z. Q.

and M. R., unpublished data). Mobility shift on Western blots indicated rapid dephosphorylation of PIP82 when the animals were exposed to light. The mobility change was found to be a function of illumination intensity. Analysis of PIP82 in various visual mutants indicated that PIP82 dephosphorylation response is downstream of the canonical phototransduction pathway, as no light-dependent dephosphorylation could be seen in either *ninaE* or *norpA* mutants. Additionally, spectral characterization of dephosphorylation response indicated that PIP82 dephosphosphorylation spectra are virtually indistinguishable from the spectra of visual sensitivity (Figure 8). PIP82 sequence is available from GenBank (accession number AF067153).

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