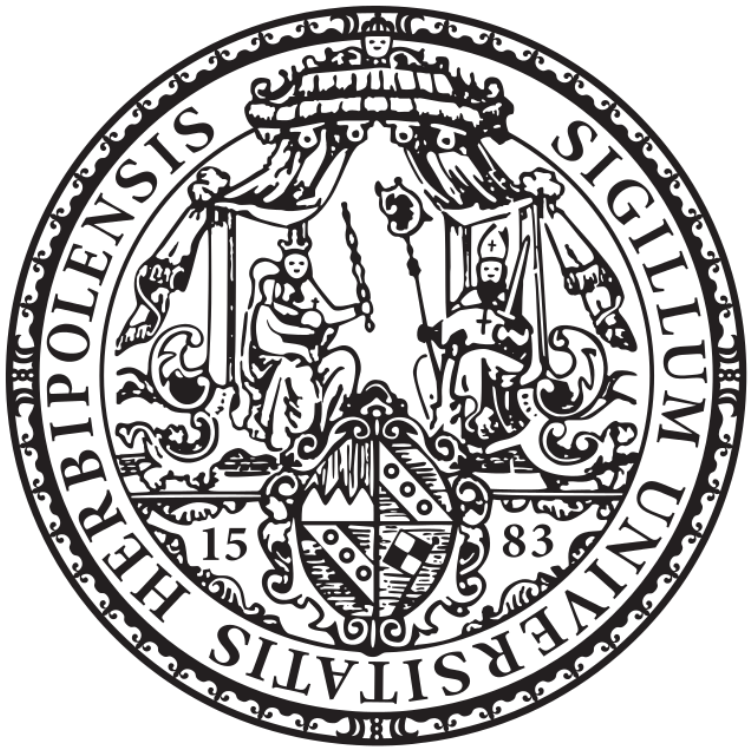
Light entrainment of the circadian clock: the importance of the visual system for adjusting *Drosophila melanogaster´*s activity pattern.

Lichtentrainment der inneren Uhr: Die Bedeutung des visuellen Systems für die Anpassung des Aktivitätsmusters von *Drosophila melanogaster*.



Doctoral thesis for a doctoral degree

at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

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Würzburg, April 2015

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Review: Schlichting M, Helfrich-Förster C (2015) Photic Entrainment in *Drosophila* Assessed by Locomotor Activity Recordings. In Circadian Rhythms and Biological Clocks, B Sehgal, ed, pp 105-123, Elsevier.

Paper 1: Mazzotta G, Rossi A, Leonardi E, Mason M, Bertolucci C, Caccin L, Spolaore B, Martin AJ, Schlichting M, Grebler R, Helfrich-Förster C, Mammi S, Costa R, and Tosatto SC (2013) Fly cryptochrome and the visual system. Proceedings of the National Academy of Sciences of the United States of America 110:6163-6168.

Paper 2: Schlichting M, Grebler R, Peschel N, Yoshii T, & Helfrich-Förster C (2014) Moonlight detection by *Drosophila*'s endogenous clock depends on multiple photopigments in the compound eyes. Journal of biological rhythms 29(2):75-86.

Paper 3: Schlichting M, Grebler R, Menegazzi P, and Helfrich-Förster C (in press) Twilight dominates over moonlight in adjusting *Drosophila´*s activity pattern. Journal of biological rhythms.

Paper 4: Schlichting M, Menegazzi P, and Helfrich-Förster C (submitted) Normal vision can compensate for the loss of the circadian clock. BMC biology.

Paper 5: Schlichting M, Grebler R, Mason M, Fekete A, Menegazzi P, Mazzotta G, Costa R, and Helfrich-Förster C (submitted) Cryptochrome plays different roles in the fruit fly´s compound eyes and clock neurons. Proceedings of the National Academy of Sciences of the United States of America.

Paper 6: Schlichting M, Lelito KR, Denike J, Helfrich-Förster C, and Shafer OT (close to submission) Rhodopsin five and six expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms of *D. melanogaster*.

Zusammenfassung

Der Wechsel von Tag und Nacht stellt für viele Organismen eine große Herausforderung dar, da sie ihre Physiologie und auch das Verhalten den äußeren Gegebenheiten anpassen müssen. Um dieser Aufgabe gerecht zu werden, haben viele Organismen innere Uhren entwickelt, welche es ihnen erlauben, den Wechsel von Tag und Nacht vorherzusehen. Diesen inneren Uhren liegt ein molekularer Mechanismus zugrunde, welcher einen Rhythmus von etwa 24 Stunden generiert. Eine wichtige Eigenschaft dieser Uhren ist es, dass sie durch äußere Faktoren, so genannte Zeitgeber, an den Tag-Nacht-Wechsel angepasst werden können. Viele Studien an Mensch, Tier und Pflanze weisen darauf hin, dass Licht der wichtigste Zeitgeber ist, wobei auch Temperatur, Luftfeuchtigkeit oder soziale Interaktionen die innere Uhr an den Tag-Nacht-Wechsel anpassen können. Ziel dieser Arbeit ist es, die Auswirkung von Licht auf das Lauf-verhalten und die innere Uhr genauer zu beleuchten, wozu der Modellorganismus *Drosophila melanogaster* herangezogen wird.

Zahlreiche Forschergruppen haben sich bereits mit der Synchronisation der inneren Uhr durch Licht beschäftigt, wobei klar hervorgeht, dass die Taufliege verschiedene Möglichkeiten hat, Lichtinformationen für die Synchronisation der Uhr zu verwenden. Der wohl am besten untersuchte Prozess ist die Synchronisation durch das Pigment Cryptochrom. Dieses Molekül ist in etwa der Hälfte der Uhrneuronen exprimiert und greift direkt in den molekularen Uhrmechanismus ein, wodurch dieser an den Tag-Nacht-Wechsel angepasst werden kann. Schaltet man jedoch das Gen für dieses Molekül aus so zeigt sich, dass die Tiere dennoch dazu in der Lage sind sich an den Licht-Dunkel-Wechsel anzupassen. Dies bedeutet, dass die visuellen Organe Informationen an die innere Uhr weiterleiten können, wobei der Mechanismus dafür noch nicht vollständig entschlüsselt werden konnte. Selbiges trifft auf sogenannte Maskierungseffekte zu: Maskierung beschreibt eine Veränderung des Verhaltensmusters, welches nicht durch die innere Uhr gesteuert, sondern direkt durch äußere Reize hervorgerufen wird. Diese direkten Effekte komplettieren das Verhalten der Tiere, da sie dadurch selbst zu endogen ungünstigen Zeiten adäquat auf äußere Reize reagieren können.

In dieser Arbeit wird sich beider Phänomene angenommen: Zum einen soll die Bedeutung des visuellen Systems für die Synchronisation der inneren Uhr genauer untersucht, und zum anderen soll uhrgesteuertes Verhalten von Maskierung getrennt werden. Zu diesem Zweck wurden Lichtbedingungen simuliert, die den natürlichen ähnelten und die Untersuchung beider lichtabhängiger Effekte ermöglichten. Die Untersuchung von Dämmerung und Mondlicht zeigte deutlich, dass diese starke Veränderungen im Lauf-Verhalten hervorrufen. Die Simulation von Mondlicht bewirkte

einen Anstieg der Nachtaktivität und ein Verschieben der Aktivitätsmaxima der Fliege in die Nacht. Das Gegenteil war bei Dämmerungssimulation zu beobachten, da die Tiere mehr Aktivität in den Tag legten. Bei gleichzeitiger Simulation von Mondlicht und Dämmerungsphasen zeigte sich, dass die Dämmerung ein stärkerer Zeitgeber ist als Mondlicht ist. Dieses Ergebnis geht einher mit der Annahme, dass die Dämmerung ein wichtiges Signal für die Synchronisation der inneren Uhr ist, da der Anstieg der Lichtintensität am frühen Morgen unabhängig von der Jahreszeit sehr ähnlich ist. Die Untersuchung von verschiedensten Mutanten konnte zudem zeigen, dass die Komplexaugen der Fliege von größter Bedeutung für die beobachteten Veränderungen im Verhaltensmuster und die Anpassung der inneren Uhr an "natürliche" Lichtbedingungen sind. Dabei stellte sich heraus, dass vor allem die inneren Rezeptorzellen wichtig für die Synchronisation der inneren Uhr und somit uhrgesteuerter Verhaltensänderungen sind. Für Maskierungseffekte scheint eine komplexe Interaktion von mehreren Rezeptorzellen für die Anpassung an Dämmerungs- und Mondlichtbedingungen vorzuliegen, da diese nur bei Mehrfachmutationen verschiedener Rhodopsine, den lichtabsorbierenden Molekülen der Fliege, verschwanden. Jedoch scheinen nicht nur die Komplexaugen das rhythmische Verhalten in Mondlichtnächten zu beeinflussen. Wird das Gen für Cryptochrom, dem Photorezeptor der inneren Uhr, ausgeschaltet, verschieben die Tiere ihre Abendaktivität noch stärker in die Nacht als es bereits beim Wildtyp der Fall ist. Durch verschiedene genetische Manipulationen konnten wir den Grund dieses Verhaltens auf die Expression von Cryptochrom in nur vier Uhrneuronen pro Hemisphäre zurückverfolgen. Zugleich zeigten unsere Ergebnisse, dass die Komplexaugen und Cryptochrom entgegengesetzte Wirkung auf das Timing der Abendaktivität haben. Während die Komplexaugen die Abendaktivität in die Nacht hinein schieben, bewirkt Cryptochrom, dass die Aktivität noch während des Tages stattfindet. Dies bedeutet, dass das wildtypische Verhalten eine Mischung aus beiden Lichteingängen ist und sich die Tiere somit ideal an die äußeren Gegebenheiten anpassen können.

Cryptochrom wird jedoch nicht nur in den Uhrneuronen, sondern unter anderem auch in den Komplexaugen der Tiere exprimiert. Um die Funktion in den Augen genauer zu untersuchen, konnten wir in Kollaboration mit Prof. Rodolfo Costa (University of Padova) zunächst zeigen, dass CRY mit der Phototransduktionskaskade über das Protein INAD interagiert und dadurch visuelles Verhalten, wie zum Beispiel Phototaxis oder die optomotorische Antwort, beeinflussen kann. In weiteren Experimenten konnten wir zudem zeigen, dass CRY in den Augen die lokomotorische Aktivität der Fliegen beeinflusst. Dabei trägt es zur Wahrnehmung von Licht bei, ohne jedoch per se ein Photopigment zu sein. Vielmehr scheint CRY die Phototransduktion dahingehend zu verändern, dass es

den Phototransduktionskomplex an das Cytoskelett innerhalb der Rhabdomere bindet und somit die Umwandlung von Lichtenergie in elektrische Signale erleichtert.

Zusammen mit Prof. Orie Shafer (University of Michigan) ist es uns zudem gelungen, die Rolle des extraretinalen Hofbauer-Buchner-Äugleins für die Synchronisation der Uhr genauer zu beleuchten. Die Anregung des Äugleins führte dabei zu einem Anstieg der Ca^{2+} und cAMP Mengen in bestimmten Uhrneuronen und dies bewirkte eine Phasenverschiebung des Verhaltens der Taufliege.

Somit konnten in dieser Arbeit neue Erkenntnisse über die Funktionen von Cryptochrom und verschiedener Augenstrukturen für das Verhalten der Fliege gewonnen werden. Dabei konnten die Bedeutungen der inneren Uhr sowie von Maskierungseffekten für das Verhalten der Tiere in der Natur herausgearbeitet werden.

Summary

The change of day and night is one of the challenges all organisms are exposed to, as they have to adjust their physiology and behavior in an appropriate way. Therefore so called circadian clocks have evolved, which allow the organism to predict these cyclic changes of day and night. The underlying molecular mechanism is oscillating with its endogenous period of approximately 24 hours in constant conditions, but as soon as external stimuli, so called Zeitgebers, are present, the clocks adjust their period to exactly 24h, which is called entrainment. Studies in several species, including humans, animals and plants, showed that light is the most important Zeitgeber synchronizing physiology and behavior to the changes of day and night. Nevertheless also other stimuli, like changes in temperature, humidity or social interactions, are powerful Zeitgebers for entraining the clock. This thesis will focus on the question, how light influences the locomotor behavior of the fly in general, including a particular interest on the entrainment of the circadian clock. As a model organism *Drosophila melanogaster* was used.

During the last years several research groups investigated the effect of light on the circadian clock and their results showed that several light input pathways to the clock contribute to wild-type behavior. Most of the studies focused on the photopigment Cryptochrome (CRY) which is expressed in about half of the 150 clock neurons in the fly. CRY is activated by light, degrades the clock protein Timeless (TIM) and hence entrains the clock to the light-dark (LD)-cycle resulting from changes of day and night. However, also flies lacking CRY are still able to entrain their clock mechanism as well as their activity-rest-rhythm to LD-cycles, clearly showing that the visual system of the fly also contributes to clock synchronization. The mechanism how light information from the visual system is transferred to the clock is so far still unknown. This is also true for so-called masking-effects which are changes in the behavior of the animal that are directly initiated by external stimuli and therefore independent of the circadian clock. These effects complement the behavior of the animals as they enable the fly to react quickly to changes in the environment even during the clock-controlled rest state.

Both of these behavioral features were analyzed in more detail in this study. On the one hand, we investigated the influence of the compound eyes on the entrainment of the clock neurons and on the other hand, we tried to separate clock-controlled behavior from masking. To do so "nature-like" light conditions were simulated allowing the investigation of masking and entrainment within one experiment. The simulation of moonlight and twilight conditions caused significant changes in the locomotor behavior. Moonlit nights increased nocturnal activity levels and shifted the morning (M) and evening (E) activity bouts into the night. The opposite was true for the investigation of twilight, as

the activity bouts were shifted into the day. The simulation of twilight and moonlight within the same experiment further showed that twilight appears to dominate over moonlight, which is in accordance to the assumption that twilight in nature is one of the key signals to synchronize the clock as the light intensity during early dawn rises similarly in every season. By investigating different mutants with impaired visual system we showed that the compound eyes are essential for the observed behavioral adaptations. The inner receptor cells (R7 and R8) are important for synchronizing the endogenous clock mechanism to the changes of day and night. In terms of masking, a complex interaction of all receptor cells seems to adjust the behavioral pattern, as only flies lacking photopigments in inner and outer receptor cells lacked all masking effects. However, not only the compound eyes seem to contribute to rhythmic activity in moonlit nights. CRY-mutant flies shift their E activity bout even more into the night than wild-type flies do. By applying *Drosophila* genetics we were able to narrow down this effect to only four CRY expressing clock neurons per hemisphere. This implies that the compound eyes and CRY in the clock neurons have antagonistic effects on the timing of the E activity bout. CRY advances activity into the day, whereas the compound eyes delay it. Therefore, wild-type behavior combines both effects and the two light inputs might enable the fly to time its activity to the appropriate time of day.

But CRY expression is not restricted to the clock neurons as a previous study showed a rather broad distribution within the compound eyes. In order to investigate its function in the eyes we collaborated with Prof. Rodolfo Costa (University of Padova). In our first study we were able to show that CRY interacts with the phototransduction cascade and thereby influences visual behavior like phototaxis and optomotor response. Our second study showed that CRY in the eyes affects locomotor activity rhythms. It appears to contribute to light sensation without being a photopigment per se. Our results rather indicate that CRY keeps the components of the phototransduction cascade close to the cytoskeleton, as we identified a CRY-Actin interaction *in vitro*. It might therefore facilitate the transformation of light energy into electric signals.

In a further collaboration with Prof. Orie Shafer (University of Michigan) we were able to shed light on the significance of the extraretinal Hofbauer-Buchner eyelet for clock synchronization. Excitation of the eyelet leads to Ca^{2+} and cAMP increases in specific clock neurons, consequently resulting in a shift of the flies´ rhythmic activity.

Taken together, the experiments conducted in this thesis revealed new functions of different eye structures and CRY for fly behavior. We were furthermore able to show that masking complements the rhythmic behavior of the fly, which might help to adapt to natural conditions.

1. Introduction

1.1. A functional model of the circadian clock

All animals and plants are exposed to environmental changes which affect their daily life. Some of these events occur suddenly, e.g. thunderstorms. Others appear in a predictable way like the gradual changes of the seasons or the alterations of day and night. To predict the latter most organisms have evolved circadian clocks, either on the single cell level or several clocks in specific tissues like brain, liver or kidneys. In mammals the suprachiasmatic nucleus (SCN) in the brain was shown to be the master clock synchronizing peripheral clocks in other tissues (Saunders, 1985). Depending on the species the SCN consists of several thousand neurons. Such concentrated clocks in the central nervous system are not restricted to mammals as in all so far investigated higher animals, like insects or fish, clock neurons were found in the brain.

Even though the molecular components of the circadian clock differ between species its basic functioning is well conserved: In constant conditions the clock generates a period of approximately 24 hours and is therefore free-running in its own speed. However, as soon as external stimuli, so-called Zeitgebers, are presented in a rhythmic fashion, the clock can adjust its period to exactly 24h, which is referred to as entrainment (Figure 1).

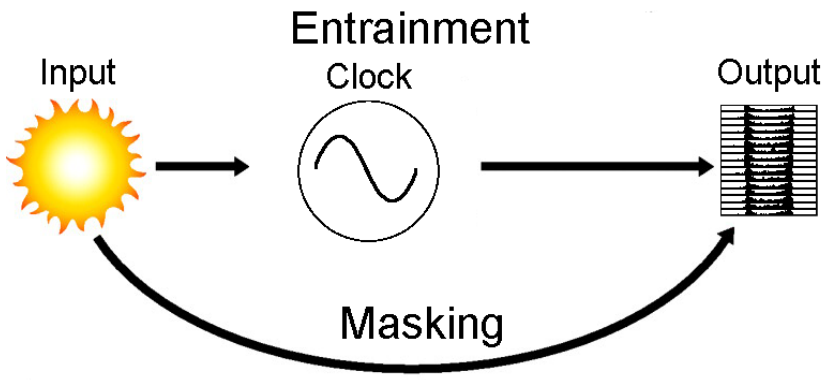

Figure 1: A functional model of the circadian clock. In constant conditions the circadian clock runs in its own period close to 24h. However, input from the environment (Input), such as cycles in light, temperature or humidity, are able to adjust the period to exactly 24h, which is called entrainment. As a consequence the clock generates rhythms in physiology or behavior of the animal. The latter can easily be measured as locomotor activity rhythms. However, not all components of the behavior are clock controlled, as changes in the environment can directly affect the activity of the animals, which is referred to as masking (modified from Golombek and Rosenstein, 2010).

Several studies demonstrated that light is the most important Zeitgeber synchronizing the clock (Golombek and Rosenstein, 2010). However, also other environmental factors like temperature, vibration or social interactions can synchronize the clock to the changes of day and night. Once synchronized to the environment the clock generates outputs as rhythmic changes in physiology, metabolism or behavior.

In this study I used *Drosophila melanogaster* as a model organism to investigate the importance of light for rhythmic locomotor behavior and entrainment of the circadian clock.

1.2. The molecular clock of *Drosophila melanogaster*

The first clock gene, *period* (*per)*, was already described in 1971 by Ron Konopka and Seymour Benzer. They identified mutants with altered free-running periods of adult emergence under constant conditions (Konopka and Benzer, 1971). It took another 13 years until the *per* gene was finally isolated and the first mechanism of PER protein acting as a transcriptional repressor of *per* m-RNA was proposed (Bargiello et al., 1984; Bargiello and Young, 1984; Reddy et al., 1984; Zehring et al., 1984; Hardin et al., 1990; Hardin et al., 1992; Zeng et al., 1994). In the following years genetic screens uncovered many additional clock genes including *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*) and *Cryptochrome* (*Cry*) and others (Sehgal et al., 1994; Allada et al., 1998; Rutila et al., 1998; Stanewsky et al., 1998). The whole circadian clock mechanism consists of several interlocked feedback loops (Hardin, 2011). However, for this study the "core" feedback loop is most relevant and therefore the different interlocked feedback loops will not be discussed.

Between Zeitgeber Time (ZT) 4 and ZT18 *per* and *tim* transcription is activated by the binding of CLK and CYC to the E-boxes of the *per*- and *tim*-genes (Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). 6-8 hours after transcription PER and TIM proteins start to accumulate in the cytoplasm of the cell. This delay is thought to be caused by the balance between phosphorylation-mediated degradation of PER and the stabilization of PER by an interaction with TIM. The degradation of PER is mediated by *doubletime* (DBT) which phosphorylates PER and hence leads to its degradation via the proteasome. In the presence of TIM the DBT-PER-TIM complex is built in the cytoplasm and thereby protects PER from degradation (Curtin et al., 1995; Gekakis et al., 1995; Price et al., 1995; Kloss et al., 1998; Price et al., 1998; Kloss et al., 2001). More recent studies also demonstrated that translational regulation of PER mediates the delay of protein accumulation in addition to phosphorylation dependent degradation (Chiu et al., 2008; Lim et al., 2011). Further phosphorylation of PER and TIM promotes the nuclear entry of DBT-PER and TIM into the

nucleus, where the PER-DBT and/or DBT-PER-TIM complex binds CLK (Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003). This binding induces a phosphorylation of CLK and the CLK-CYC complex is released from the E-boxes of the *per*- and *tim*-genes and thereby their transcription is inhibited between ZT18 and ZT4 (Lee et al., 1998; Lee et al., 1999; Bae et al., 2000; Yu and Hardin, 2006; Menet et al., 2010). Once PER and TIM protein levels drop, CLK and CYC bind to the E-boxes of the *per*- and *tim*-genes again and a new cycle starts.

1.3. Influence of light on the clock mechanism

Light was shown to be the most important Zeitgeber synchronizing the circadian clock of several animal species (Foster and Helfrich-Förster, 2001). Unlike in mammals, in which the master clock (SCN) synchronizes the peripheral clocks via humoral signals (Dibner et al., 2010; Golombek and Rosenstein, 2010), the *Drosophila* circadian clocks seem to work cell autonomously, as single tissues can be entrained to light-dark (LD) cycles even if they are separated of the rest of the body (Plautz et al., 1997). Several studies showed that TIM is rapidly degraded after illumination, as TIM levels out of head extracts are drastically reduced already after a light pulse of 30 min duration (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). This light-dependent degradation of TIM is mediated cell-autonomously via the blue-light absorbing pigment Cryptochrome (CRY), which is expressed in several clock neuron clusters and the retina of adult flies (Emery et al., 1998; Stanewsky et al., 1998; Benito et al., 2008; Yoshii et al., 2008). CRY directly binds TIM in a light-dependent manner and leads to its degradation in the proteasome (Ceriani et al., 1999; Naidoo et al., 1999; Busza et al., 2004; Dissel et al., 2004).

This CRY mediated degradation of TIM is also thought to reset the clock of *Drosophila* at the beginning of each day. At ZT0 PER and TIM levels are high in the nucleus and DBT-PER and/or DBT-PER-TIM is bound to CLK and leads to the release of the CLK-CYC complex from the E boxes of the *per*- and *tim*-genes. Upon illumination, TIM is degraded and PER is "deprotected", thus progressively phosphorylated by DBT, and afterwards targeted for degradation via the proteasome at around ZT4 (Naidoo et al., 1999; Kloss et al., 2001; Grima et al., 2002; Ko et al., 2002). Upon degradation of PER, hypo-phosphorylated CLK accumulates in the clock neurons, the CLK-CYC dimer forms again and starts a new cycle by binding to the E-boxes of the *per*- and *tim*- genes (Hardin, 2011).

Light does not only synchronize the clock to the changes of day and night. Short light pulses (LPs) are further able to shift the clock, when applied at certain times within the 24h cycle (Pittendrigh, 1967; Egan et al., 1999). However, depending on the time of

day the clock "interprets" a given LP differently (Suri et al., 1998). A LP during the subjective day (between circadian time (CT) 0-12) does not shift the endogenous clock, as TIM levels are low and thereby TIM cannot be degraded. A LP in the early evening (around CT15) phase-delays the clock. At that time of the subjective day TIM is accumulated in the cytoplasm and starts to enter the nucleus. The LP induced TIM-degradation delays its nuclear entry but TIM levels can be replenished within a few hours as *tim*-mRNA levels are high. This changes, when the LP is applied in the late night (around CT22). Due to low *tim*-mRNA levels degraded TIM protein cannot be re-synthesized and the LP therefore mimics "early dawn" and resets the core mechanism and phase advances the clock (Golombek and Rosenstein, 2010; Hardin, 2011). When the LP-induced phase shifts are plotted against the time of day a phase-response-curve is generated with a dead zone, an advance zone and a delay zone (Figure 2).

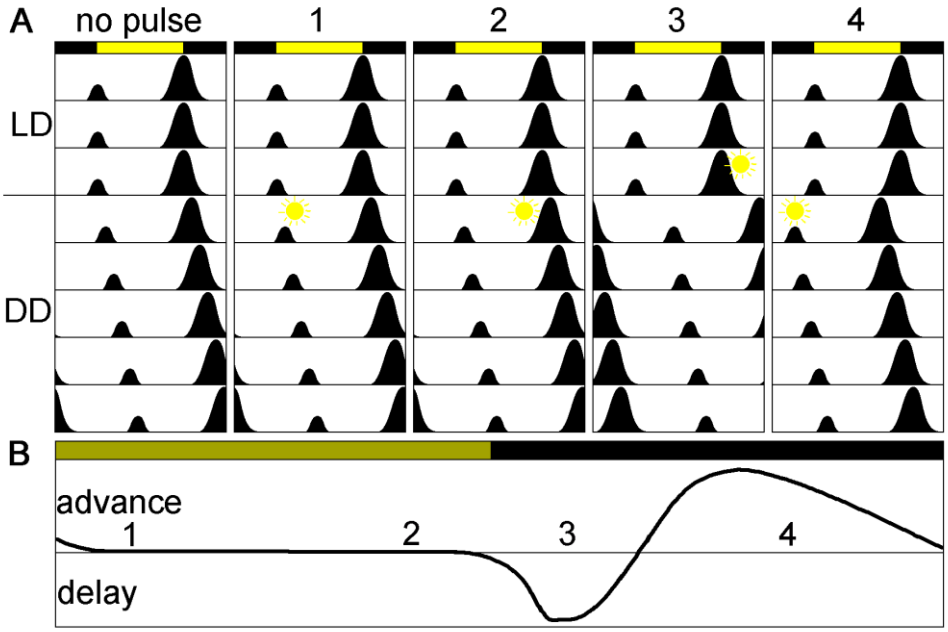

Figure 2 Photic phase response curve. Depending on the time of day a light pulse (LP) can phase shift the clock in different directions: A LP in the early night phase-delays the clock, a LP in the late night phase-advances the clock, whereas a LP during the subjective day does not affect the clock.

Even though the light-resetting function of CRY is widely accepted, it is not the only pathway synchronizing the clock neurons in the brain of *Drosophila*. Flies without CRY (cry^{01}) or with a mutated form of CRY, that renders its photoreceptive ability out of function (cry^{b}), are still able to synchronize to light/dark-cycles or respond to light pulses applied in the subjective night (Stanewsky et al., 1998; Dolezelova et al., 2007; Kistenpfennig et al., 2012). However, flies lacking photoreceptive CRY show rather weak phase shifts upon a LP and need more time to entrain to LD regimes (Emery et al., 2000;

Kistenpfennig et al., 2012). This shows that input from the opsin-based visual system is able to synchronize the clock neurons upon illumination, which is supported by the fact that only flies lacking all photoreceptive organs as well as CRY are not able to entrain to LD regimes (Helfrich-Förster et al., 2001). The mechanism of how light from the opsin-based photoreceptors is integrated by the clock remains so far unknown (Dubruille and Emery, 2008).

1.4. The visual system of *Drosophila melanogaster*

Even though the pathway mediating light information from the visual organs to the clock is still unknown in *Drosophila,* the above mentioned findings in *cry*-mutants demonstrated the importance of the visual system for clock synchronization. In total the fruit fly possesses seven eye structures: Three ocelli, two Hofbauer-Buchner (H-B) eyelets and two compound eyes (Hofbauer and Buchner, 1989).

The ocelli of the adult fly are arranged in a triangle between the compound eyes and the vertex of the head. The receptor cells of the ocelli contain microvilli structures, so-called rhabdomers, with Rhodopsin 2 (Rh2) being the photopigment to absorb light (Pollock and Benzer, 1988). They provide the fly with information about its horizontal position and help to maintain the orientation in space, to stabilize flight and to keep the gaze level (Krapp, 2009). The ocelli signal into the lateral protocerebrum of the fly where the signals are transmitted to descending neurons which connect to motor systems.

The H-B eyelets consist of only four photoreceptor cells per hemisphere at the posterior margin of the compound eye (Hofbauer and Buchner, 1989). The eyelets derive from the larval Bolwig organ and appear during adult development (Helfrich-Förster et al., 2002). The receptor cells constitute various microvilli, which are arranged into coherent rhabdomeres that contain Rhodopsin 6 (Rh6) as a pigment, suggesting that the eyelets are functional photoreceptors. There is evidence that histamine and acetylcholine are synthesized as transmitter in these cells, which can be released into the accessory medulla at the axonal terminals (Yasuyama and Meinertzhagen, 1999; Yasuyama and Salvaterra, 1999). In the accessory medulla the axons overlap with fibers of clock neurons, implying a role in clock synchronization (Helfrich-Förster et al., 2002; Veleri et al., 2007).

The most prominent visual organs of the fly are the compound eyes which allow the fly to see 85% of its surrounding environment with only a small blind spot in the back (Heisenberg and Wolf, 1984). They are comprised of approximately 750-800 hexagonal ommatidia, each of which contains 20 cells, including eight photoreceptor cells. Six receptor cells (R1-6) are arranged in the periphery of each ommatidium and span the whole depth. In the center of the ommatidium receptor cell seven (R7) is situated in the

distal and receptor cell eight (R8) in the proximal half of the retina (Wolken et al., 1957). The ommatidia are shielded from each other, with the secondary pigment cells being the main shielding cells. Tertiary pigment cells as well as mechanosensory bristle cells are located at the vertices of the ommatidia in an alternating fashion. On top of each ommatidium a cornea and primary pigment cells regulate the exposure to light (Borst, 2009; Montell, 2012) (see Figure 3). In *Drosophila* the phototransduction cascade is localized in the rhabdomeres, a specialized area of each receptor cell which is formed by approximately 30.000 tightly packed microvilli. In this part of the receptor cells the light absorbing molecules, the rhodopsins, as well as the necessary molecules to transfer light information into an electrical signal are located (Hardie and Raghu, 2001). Each rhodopsin consists of a protein, the opsin, which is fixed in the membrane of the receptor cell via seven trans-membrane domains, and the chromophore 11-cis-retinal. Upon illumination 11-cis-retinal changes its conformation and the rhodopsin turns into the activated state, the metarhodopsin. This transition activates the phototransduction cascade, which, over several steps, leads to the opening of Trp- and Trpl-channels and hence to the depolarization of the cell and to the release of histamine at the synapse (Vogt and Desplan, 2014). To return to the rhodopsin state the metarhodopsin has to absorb another photon of different wavelength or an enzymatic "visual cycle" recycles the activated retinal and thereby restores normal rhodopsin function (Wang et al., 2010; Wang et al., 2012).

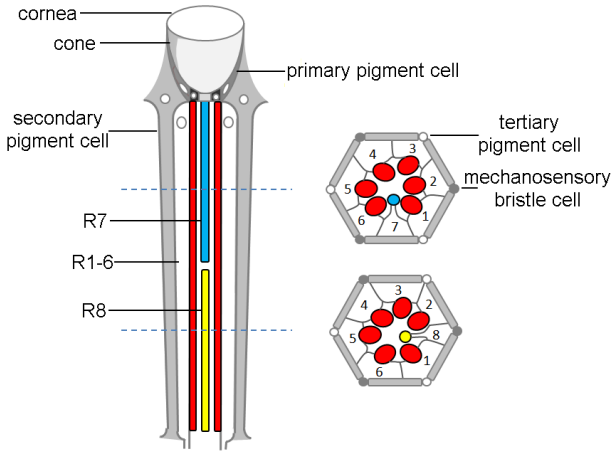

Figure 3: The ultrastructure of an ommatidium. Each ommatidium is comprised of 20 cells including eight photoreceptor cells. R1-6 are arranged in the periphery of the ommatidium and span its whole depth. R7 and R8 are located in the center with R7 being located above R8. Pigment cells shield the ommatidia from each other with the secondary pigment cell being the main shielding cell between the different ommatidia. Tertiary pigment cells as well as mechanosensory bristle cells are located in the vertices of the ommatidia in an alternating fashion.(modified from Wang and Montell (2007)).

Besides the structural difference between outer and inner receptor cells they also differ in rhodopsin content and function (Figure 4). The outer receptors R1-6 are homogenous in morphology and function. They express Rhodopsin 1 (Rh1) as a pigment which shows two sensitivity peaks at 360 nm (UV light) and 486 nm (blue light) (O'Tousa et al., 1985; Zuker et al., 1985; Feiler et al., 1988). The UV sensitivity of the molecule is achieved by an additional sensitizing pigment or a second chromophore, presumably a vitamin A derivate (Kirschfeld and Franceschini, 1977). These cells appear to be important for motion and dim light detection (Yamaguchi et al., 2008).

The rhodopsin constitution in the inner receptors is more complex, including four different rhodopsins, and they are therefore ideal candidates for color processing (Yamaguchi et al., 2008). The retina of the fly is mainly composed of two types of ommatidia which are distributed in a stochastic fashion (Bell et al., 2007) (see Figure 4). The pale subtype occurs in about 30% of the ommatidia. In this subtype R7 express the UV-sensitive Rh3 with its absorption maximum at 331 nm and R8 express the blue-sensitive Rh5 with its absorption maximum at 442 nm (Feiler et al., 1992; Chou et al., 1996; Chou et al., 1999; Salcedo et al., 1999; Bell et al., 2007). In approximately 70% of the ommatidia the yellow subtype can be found, with R7 expressing the UV-sensitive Rh4 with an absorption maximum at 355 nm, and the green sensitive Rh6 with its maximum at 515 nm in R8 (Montell et al., 1987; Feiler et al., 1992). Rh6 is to some degree special: whereas the metarhodopsins of all other *Drosophila* rhodopsins absorb light of higher wavelength, the metarhodopsin of Rh6 absorbs in a shorter range (Salcedo et al., 1999). The above described rhodopsin constitution is present in most of the regions in the compound eyes with 2 exceptions: In the dorsal third of the retina yellow R7 co express Rh3 and Rh4 (dorsal yellow) and in the dorsal rim area, a specialized part for detecting polarized light, R7 and R8 express Rh3 (Fortini and Rubin, 1991; Wernet et al., 2003; Mazzoni et al., 2008; Wernet et al., 2012).

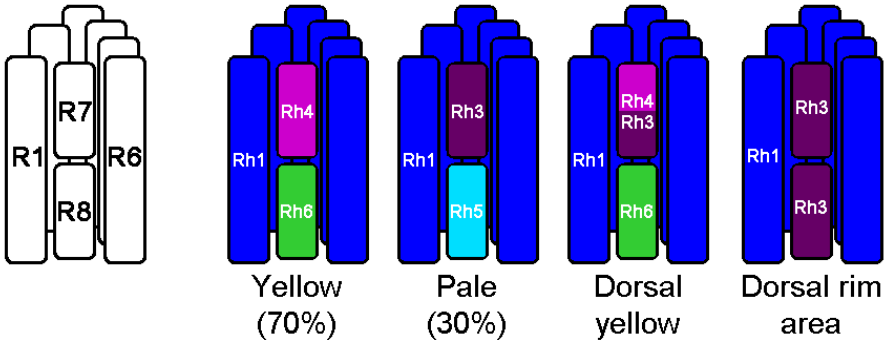

Figure 4: Rhodopsin expression in the *Drosophila* compound eyes. All outer receptors express Rh1 whereas the expression pattern of the inner receptors is more complex, which is attributed to their role in color vision. 70% of the ommatidia show Rh4 expression in R7 and Rh6

expression in R8, whereas about 30% of the ommatidia show Rh3-expression in R7 and Rh5-expression in R8. In the dorsal part of the retina specialized regions show ommatidia of altered rhodopsin expression (dorsal yellow and the dorsal rim area). For details see text. (Modified from Mazzoni et al. (2008)).

1.5. The neuronal network of the clock and its light input pathways

The molecular mechanism described in section 1.2 is expressed in certain neurons in the brain of the fly, which are called clock neurons. This clock consists of approximately 150 neurons, which can be subdivided into several lateral and dorsal neuron clusters (see Figure 5) (Helfrich-Förster, 1995).

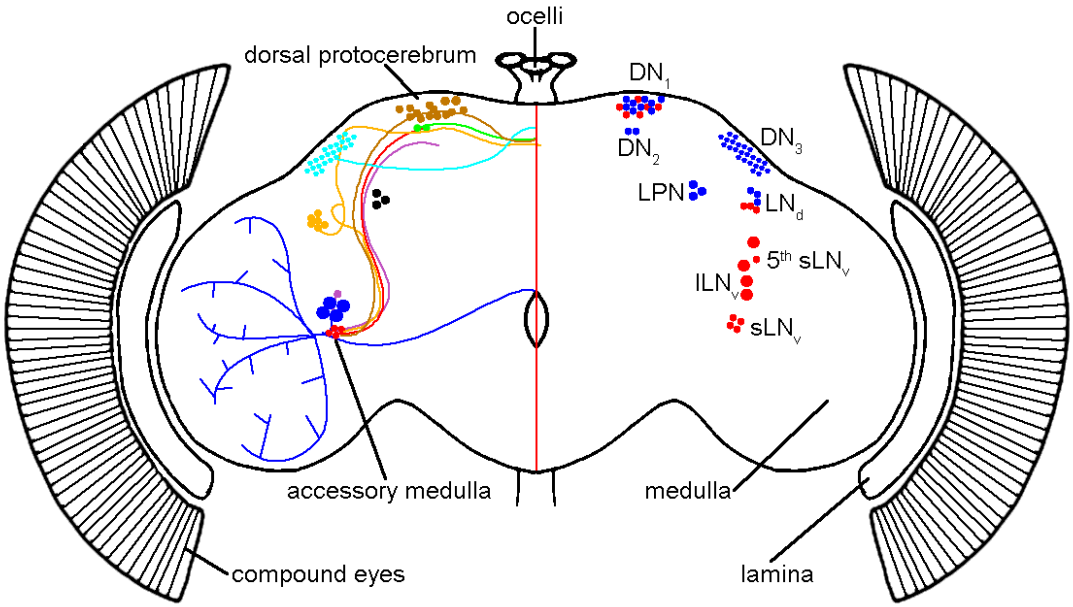

Figure 5: Overview of the neuronal clock network in *Drosophila*. The *Drosophila* clock consists of about 150 clock neurons, which can be subdivided into five different lateral neuron clusters (LPN, sLN_v, 5^{th} sLN_v, lLN_v and LN_d) and three dorsal neuron clusters (DN_1, DN_2 and DN_3). Left panel: Schematic overview of the arborizations pattern of the different clock neuron clusters. Arborizations of each neuron cluster are depicted in different colors. The accessory medulla, as well as the dorsal protocerebrum are centers of high fiber density and therefore putative communication sites. Right panel: Expression pattern of CRY within the clock neuron network. CRY-positive neurons are depicted in red, CRY-negative neurons in blue. The s-LN_v, lLN_v, 5^{th} sLN_v, half of the LN_d as well as half of the DN_1 are CRY-positive and can sense light cell-autonomously. For details see text.(modified from Helfrich-Förster et al. (2007a) and Yoshii et al. (2008)).

In the lateral brain five different neuron clusters can be found: three lateral posterior neurons (LPN) per hemisphere with yet unknown projection pattern. A further group is comprised of the ventro-lateral neurons (LN_v) which can be further subdivided due to their size or the expression of the pigment dispersing factor (PDF). Four of the small ventro-lateral neurons (sLN_v) express PDF and send their projections into the dorsal

protocerebrum and the accessory medulla, which is an important pacemaker center of many insects (Reischig and Stengl, 2003). The fifth small ventro-lateral neuron (5^{th} sLN_v) is PDF-negative, but shows similar arborizations in the accessory medulla and the dorsal protocerebrum. The large ventro-lateral neurons (lLN_v) express PDF but show a different projection pattern: They innervate the ipsilateral accessory medulla and its ventral elongation as well as outer medulla layers in both sides of the brain, thereby allowing the communication between the two hemispheres. Another group comprising six neurons, the dorsal-lateral neurons (LN_d), is located more dorsally. Even though the size of these cells is the same this group appears to be very heterogeneous: Only three of the six cells express CRY, three cells express the neuropeptide F (NPF), one expresses the ion transport peptide (ITP) and 2 express the short neuropeptide F (sNPF) (Lee et al., 2006; Johard et al., 2009). They send their projections into the dorsal protocerebrum and some fibers also innervate the ipsilateral accessory medulla (Helfrich-Förster et al., 2007b).

In the dorsal brain of *Drosophila* three different groups of dorsal neurons (DN) can be identified (DN_1, DN_2 and DN_3). As with the LN_d also the DN_1 are a heterogeneous group, as only about half of the neurons express CRY and are therefore light-sensitive (Benito et al., 2008; Yoshii et al., 2008). All dorsal neurons also innervate the dorsal protocerebrum and some fibers project to the ipsilateral accessory medulla. Taken together the arborizations of the different neuron clusters are closely packed in the area of the accessory medulla and the dorsal protocerebrum allowing communication among the different clusters (Helfrich-Förster et al., 2007b).

As described in section 1.3, CRY is one of the key molecules synchronizing the circadian clock to LD-cycles. Focusing on the expression pattern of CRY in the clock neurons, it is expressed in all ventro-lateral neurons, in 3 of the six LN_d and in some DN_1 (Yoshii et al., 2008) (see Figure 5). However, only flies lacking CRY as well as all photoreceptor organs are not able to entrain to LD-cycles, clearly showing that the visual system is contributing to entrainment (Helfrich-Förster et al., 2001). The H-B-eyelet sends its axons towards the accessory medulla, where it most probably shares synapses with the lateral clock neurons. Several studies already showed a significance of the H-B-eyelet for clock synchronization (Helfrich-Förster et al., 2002; Mealey-Ferrara et al., 2003; Veleri et al., 2007). The ocelli are so far not known to contribute to light entrainment even though the anatomical vicinity to the dorsal neuron clusters would suggest a connection of the two systems. In case of the compound eyes it is widely accepted that they contribute to the entrainment of the clock, even though the exact mechanism is so far unknown. The candidate receptor cells are R7 and R8, as they send their projections directly into the medulla, close to the arborizations of the lLN_v and thus could communicate with these cells either directly via histamine or via inter-neurons (Helfrich-Förster, 2014).

1.6. *Drosophila* behavior under light-dark conditions

Input to the clock via Zeitgebers is able to synchronize the circadian clock to the environment. As light is the most important Zeitgeber most studies focus on the behavior of flies in light-dark conditions, in which flies show a bimodal activity pattern with a morning (M) and an evening (E) activity bout and only low levels of activity during midday or night. Based on a hamster study Pittendrigh and Daan (1976) proposed the so called dual oscillator model, which explains the behavior of animals in entrained conditions. According to this model the first oscillator tracks dawn and is therefore called M oscillator whereas the second one tracks dusk and is therefore called E oscillator. This model perfectly fits to the behavior of *Drosophila* as the M oscillator can control the morning activity bout and the E oscillator the E activity bout. It further allows interpretation of the behavioral changes under long or short photoperiods: Under short photoperiods the M and the E peaks get closer together whereas they move apart from each other under long photoperiods (Rieger et al., 2003; Rieger et al., 2012). This can be explained by the different properties of the M and E oscillators which control the timing of the two activity bouts. Upon illumination the period of the M cells is shortened thereby allowing to phase-advance the M peak under long photoperiods. On the contrary the period of the E cells is lengthened by light thereby allowing to phase-delay the E peak in long day conditions (Yoshii et al., 2012).The neuronal basis of this hypothesis was established by the studies of Grima et al. (2004) and Stoleru et al. (2004) in *Drosophila*, in which they manipulated or ablated specific clock neurons. By this they identified the four PDF positive sLN_v being necessary and sufficient to drive M-activity and rhythmic behavior in DD, whereas the three CRY positive LN_d drive E-activity. More recent studies showed, however, that this view of distinct M and E cells appears to be too simplified: It seems as if complex interactions between the different neuronal clusters are necessary in order to drive rhythmic locomotor activity (Sheeba et al., 2010; Yoshii et al., 2012; Dissel et al., 2014; Yao and Shafer, 2014).

However, not all aspects of fly behavior are clock controlled as flies also respond directly to changes in the environment. These direct responses are referred to as masking, as they often hide the clock-controlled output of the clock. Nevertheless, masking often complements the behavior of the animals and enables them to respond to sudden changes in the environment (Mrosovsky, 1999). In *Drosophila* the endogenous timing of M and E peaks is often masked by lights-on or lights-off, when rectangular LD-cycles are applied, as the flies respond with a strong increase of activity to the sudden change of light intensity. In addition flies show only low levels of activity in completely dark nights, which is caused by an inhibition of activity by darkness.

1.7. Aim of the study

The aim of this study was to investigate the significance of the visual system for the locomotor activity rhythm of *Drosophila melanogaster*. Using different light regimes and mutants I aimed to segregate clock controlled behavior from direct responses to light (masking). To do so, I focused on the investigation of more "natural" light conditions, including the simulation of twilight and moonlight.

Previous studies already showed that the simulation of moonlight leads to an increase of nocturnal activity, a phase advance of the M and a phase delay of the E peak (Bachleitner et al., 2007; Kempinger et al., 2009). These effects were shown to depend on photoreceptors of the compound eyes as they did not occur in eyeless flies. Therefore one aim was to unravel which rhodopsins are necessary for moonlight detection.

In addition, I focused on the investigation of twilight conditions. This part of the study appears to be very interesting as dawn is the first light stimulus the organisms are confronted with in the beginning of the day. While in the course of a year the average temperatures and photoperiods change, the properties of dawn and dusk remain rather stable throughout the year (Bünning, 1969). Consequently it was predicted that dawn might be the most important light cue synchronizing the clock in nature. A previous study already showed that twilight is able to alter the behavior of *Drosophila* in comparison to rectangular LD-cycles: the activity maxima occur during dawn or dusk, meaning the flies delay the M and advance the E peak, respectively (Rieger et al., 2007). Therefore, we investigated which input to the clock is necessary to evoke those behavioral changes and whether these effects are caused by changes of clock synchronization or are directly triggered by changes of light during dawn or dusk (masking).

As described previously, moonlight or twilight have different effects on the timing of the M or E peak: Moonlight generates an advanced M and a delayed E peak compared to completely dark nights. On the contrary, twilight simulation induces a phase delay of the M and a phase advance of the E peak. In nature these light stimuli never happen in isolation and also completely dark nights never occur. Thus we asked in a third study, if twilight or moonlight is the dominant factor triggering the activity of *Drosophila* and investigated the simultaneous simulation of twilight and moonlight. We again focused on the light input via rhodopsins from the compound eyes as these were shown to be important to detect light of moonlight intensity (Bachleitner et al., 2007).

So far I have only focused on the input of rhodopsins of the compound eyes on the circadian clock. However, Yoshii et al. (2008) had shown that Cryptochrome is not only expressed in about half of the clock neurons, but also in the compound eyes. In collaboration with Prof. Rodolfo Costa, we aimed to unravel the biological significance of

CRY in the eyes. Our first aim was to identify, if CRY can affect photoreception in the fly. This question arose as CRY is activated by blue light in the clock neurons and appears to be very light sensitive as already a blue light intensity of 0.03 nW/cm^2 is able to synchronize the molecular clock (Hirsh et al., 2010). Therefore its ability to absorb photons might also have an effect on photoreception in the fly retina. Then we aimed to separate the effects of CRY in the compound eyes and CRY within the clock neurons on the locomotor activity of the fly.

In the last study we focused on the input from another photoreceptor, the H-B eyelet, on the clock. This organ is the residue of the larval photoreceptor, Bolwig organ, which is originally comprised of 12 photoreceptor cells, but only 4 cells survive through metamorphosis. The axons of the eyelet terminate in the area of the accessory medulla and therefore the eyelet is thought to be important for clock synchronization by light. In collaboration with Prof. Orie Shafer we tried to identify the mechanism, how this eyelet is able to transfer light information to the clock of *Drosophila*.

2. Material and Methods

In this section I will introduce different ways of monitoring the flies´ locomotor activity and the analysis of the obtained data. The exact light conditions and the investigated genotypes of each experiment will be displayed in the Materials and Methods sections prior to the respective projects. The same applies to the immunocytochemical experiments: In this part of the thesis I will introduce the general protocols used for staining of adult retinas and adult brains, whereas the used antibodies and genotypes will be listed in the particular projects.

2.1. Fly rearing

All flies were raised on standard *Drosophila* medium (0.8% agar, 2.2% sugar beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, and 0.3% hydroxybenzoicacid). Prior to each experiment and for crossings flies were raised at 25°C, for long-time storage flies were kept at 18°C.

2.2. Binary expression systems

Besides the fact that the number of clock neurons in *Drosophila* is much smaller than in mammals, a second reason for studying the fruit fly is its rather simple genetic accessibility in targeting specific neurons in the brain or also cells of interest in the whole body. To do so Brand and Perrimon (1993) introduced the GAL4-UAS system which allows the expression of gene constructs in cells of interest *in vivo*. This system consists of 2 transgenic fly lines: The GAL4 (driver) line and the UAS (responder) line. In order to target gene expression to specific cells the promotor region of the gene of interest is cloned upstream of the GAL4 sequence, which encodes a transcriptional activator of yeast. Thus the transcription of GAL4 is under the control of the promotor of interest and is thereby restricted to cells in which the gene of interest is expressed. On the contrary the responder line contains an upstream-activating-sequence (UAS) which is cloned upstream of any kind of effector gene (for example RNA interference (RNAi) constructs, reporters like the green fluorescent protein (GFP), cell death genes and many more). GAL4 is able to bind to the UAS sequence and thereby activates the transcription of the effector gene.

As the expression pattern of GAL4 lines can be rather broad, Lee and Luo (1999) added a third element to this binary expression system which is called GAL80. This transgenic fly strain again contains a promotor of interest which is cloned upstream of the sequence of GAL80, which acts as a transcriptional regulator in yeast. GAL80 is able to

bind to the active domain of the GAL4 protein and prevents its binding to the UAS sequence of the responder line. As soon as all three components are combined in one fly, the effector gene is only expressed in those cells that express GAL4 but not GAL80, thereby allowing to narrow down GAL4 expression to much smaller cell populations.

In order to express different effector genes in different cell types a second binary expression system was introduced by Lai and Lee (2006), the so called *lexA-lexAop* system. This system uses the *lexA* DNA-binding domain from a bacterial transcription factor which can be linked to a strong activation domain from the herpes simplex virus. The working principle is the same as in the GAL4-UAS-system: *lexA* is produced under the control of a promotor of interest and binds to and activates the *lexA* operator (*lexAop*) which is fused to an effector of choice (del Valle Rodriguez et al., 2012).

2.3. Locomotor activity recording

2.3.1. Home-made system

This system was described first in Helfrich-Förster (1998). To monitor locomotor activity 2-6 days old male flies were singularly transferred into specially prepared photometer-half-cuvettes (Figure 6).

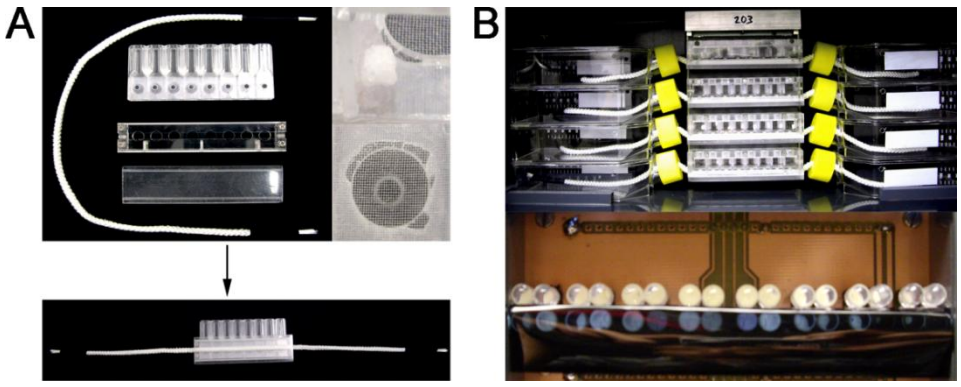

Figure 6 Home made system for locomotor activity recording. A Structure of the half-cuvettes with water and food supply as well as opening for ventilation on the broad end of the cuvette. Eight of these specially prepared cuvettes are glued next to each other building a rack for monitoring eight flies. **B** Four of the racks are placed in one light box in which an infra red (IR) light beam crosses at the narrow side of the cuvette and a computer samples the number of beam crosses in one minute intervals. Water tanks on both sides supply the flies with water. Light is provided using white LEDs and additional neutral density filters. In front of each cuvette four LEDs simulate the changes of day and night for one single fly. The upper row simulates daylight intensity whereas the lower row was used to simulate moonlit nights .

At the tight end an infra red (IR) light beam crossed the cuvette which was interrupted, whenever a fly passed this section of the cuvette. A computer counted the number of beam interruptions caused by the fly in one minute intervals. At the other end of the cuvette a small and a big hole were introduced. The big one was closed by a cotton net in order to provide air ventilation. In front of the second one a glass-fiber rope was arranged in order to close the cuvette. Both ends of this rope were placed into a water reservoir and supplied the flies with water. At the same side of the cuvette a big piece of sugar was fixed as food. Eight of these specially prepared cuvettes were glued in a row as a rack. Finally four of these racks were placed into the recording system at the same time, allowing the recording of 32 flies in one experiment. As a light source white light emitting diodes (LEDs, Lumitronix LED-Technik GmbH, Jungingen, Germany) were placed in front of each single cuvette. The additional use of neutral density filters (Lee Filters Worldwide, Andover, UK) broadened the range of light intensities without disturbing the spectral composition of the LEDs. Using this setup the range of light intensity (LI) was adjustable between 0.001 lux and 10000 lux. All experiments were conducted in a climate controlled chamber at 20°C. In order to avoid heating up of the light boxes, each box was additionally connected to a ventilation system.

2.3.2. *Drosophila* Activity Monitor (DAM) system

The DAM system (Trikinetics, Waltham, MA) is the most commonly used method to record locomotor activity of *Drosophila* or also bigger insects (see Figure 7). It consists of activity monitors that can simultaneously record the activity of 32 individual flies. To do so, 2-6 days old male flies were singularly transferred into glass tubes with food on one end (consisting of 4% sucrose and 2% agar) and a plug to close the tube on the other end. An IR light beam crossed the tube in the center and a computer measured the number of beam crosses in one minute intervals. Light was provided using white LEDs (Lumitronix LED-Technik GmbH, Jungingen, Germany) that were fixed above the monitors. Neutral density filters (Lee Filters Worldwide, Andover, UK) were used for fine tuning light intensity. In this system the range of possible light intensities was, however, smaller compared to the home-made system, since the neutral density filters had to be installed in front of all LEDs of the whole box, containing 6 monitors, whereas this was variable in the above described system. Additionally, the LEDs in the home-made system were placed directly in front of the cuvette whereas the LEDs in the DAM system illuminated the flies from the top of the box resulting in a bigger distance between LEDs and flies. All experiments were conducted in a climate controlled chamber at a constant temperature of

20°C. Using this setup it was possible to record the activity pattern of individual flies for up to 30 days without disturbance.

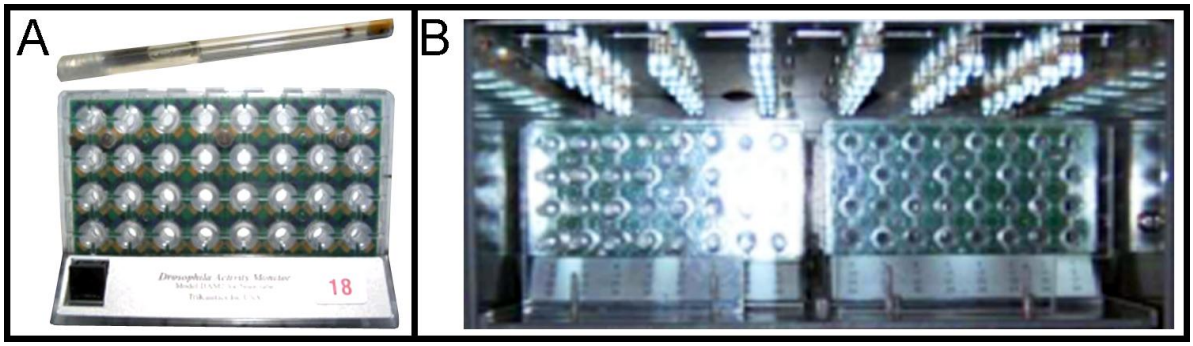

Figure 7 Recording locomotor activity using the DAM-system. A Individual flies are transferred into glass tubes which are filled to 1/3 with an agar/sucrose mixture at one end and closed with a plug at the other end. 32 of these tubes are placed in one monitor allowing the simultaneous monitoring of these flies. **B** Six monitors are placed into one light box. Above the monitors white LEDs are installed, which were used to simulate the changes of day and night. Additional neutral density filters (removed in this picture) are introduced for fine-tuning the light intensity without changing the spectrum of the LEDs.

2.3.3. Camera based system

In the above described systems the activity was monitored by measuring light-beam interruptions caused by the fly in one minute intervals, allowing already a quantitative way of measuring activity. However, camera based systems are the only way to really determine the distance covered by the fly in a certain time interval. They further provide the possibility to define special regions of interest in the investigated area, leading to a more detailed analysis of behavior. In this study the commercially available Noldus-tracking system (Noldus Information Technology, www.noldus.com) was used.

To record activity, 2-6 days old male flies were transferred into photometer cuvettes. At the closed end a sponge soaked with 4% sucrose solution was provided and the open end was closed with a plug. Eight of these cuvettes were placed on a glass table and the focus of the camera was adjusted. Pictures were sampled every 500 ms and the distance covered by the fly during each time interval was determined. To enable activity recording also during darkness IR LEDs illuminated a white light-reflecting platform below the table. The reflected light passed the photometer cuvettes and was recorded by the IR sensitive camera. Illumination was provided with white LEDs (Lumitronix LED-Technik GmbH, Jungingen, Germany) from above simulating the changes of day and night. The range of light intensity was even smaller in this system, as the distance between the LEDs

and the flies was even bigger compared to the DAM system and the number of white LEDs was reduced.

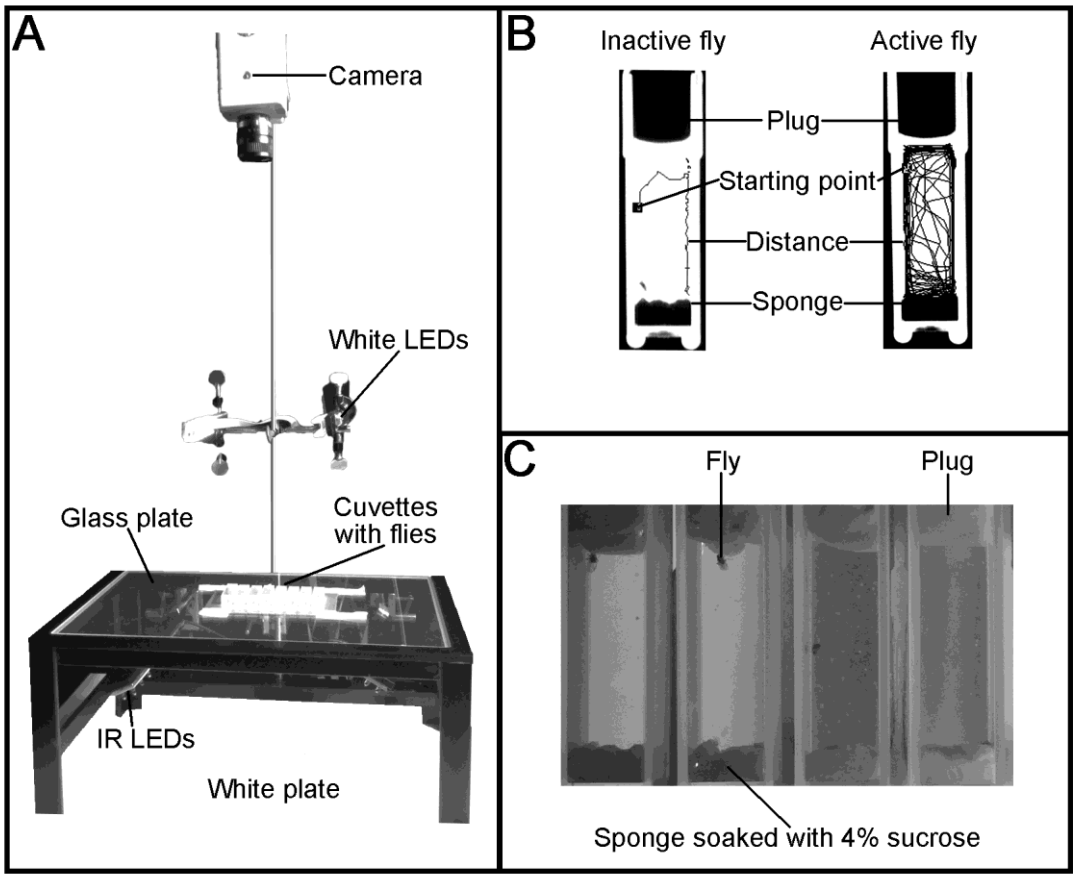

Figure 8 Recording locomotor activity using the Noldus-system. A Flies are transferred into photometer cuvettes and placed on a glass plate. IR-LEDs expose the cuvettes with reflected light from below that is detected by the IR sensitive camera and white LEDs above are used to synchronize the flies to specific light conditions. The camera takes a picture every 500 ms and the software calculates the distance covered by the fly. **B** Two examples of flies recorded in this system. The left fly is rather inactive, whereas the right fly shows high levels of activity. The distance covered by each fly is depicted by the black line. **C** Cuvettes used for recording locomotor activity. Singular flies are transferred into photometer cuvettes containing a sponge soaked with 4% sucrose solution at one end as food supply. The open end of the cuvette is closed using a foam plug (Schlichting and Helfrich-Förster, 2015).

2.3.4. Data analysis

In this section I will shortly explain the most commonly used ways of analyzing locomotor activity data throughout this study. Whenever more detailed or a special analysis was applied in any of the projects, this will be explained in the material and methods section prior to each project summary in section 3.

Average activity profiles

In each experiment the respective light regime was at least applied for six consecutive days so that the animals were able to entrain their clock as well as their rhythm in locomotor activity to the given light condition. To calculate the average activity profile the first day of the raw data was cut off and an actogram for each single fly was displayed using the FIJI (free distribution of ImageJ, available at www.fiji.sc) plugin ActogramJ (available at actogramj.neurofly.de, Schmid et al. (2011)). The actograms show the activity of the flies day by day and were used to check 1) if the flies survived over the whole experiment, as only flies surviving throughout the whole experiment were used for the analysis and 2) how long the flies needed to entrain to the given light regime, as average activity profiles should only be calculated if the flies´ behavior is stable. Depending on the genetic background of the flies entrainment took up to three days, so that in some experiments only the data of the last three days of each light condition were used for the analysis. To calculate the average activity profiles of single flies an excel makro-sheet (constructed by Prof. Taishi Yoshii, University of Okayama) was used, which averaged the activity of each minute of the day in single flies. To calculate the average activity profile of one experiment the single fly average days were averaged and smoothened using a moving average of 11. As the timing of the activity maxima was the main focus of this study, the average activity profiles were normalized to 1 and plotted using the program QtiPlot (version 9.8.8, Ion Vasilief, Craiove, Romania). Thereby differences in absolute activity level were not visible any longer, but these were not subject of our experiments.

Further analysis based on single fly activity profiles

To investigate the effect of light on the behavior of the flies in more detail further analysis was necessary. One important feature of the flies´ activity profile is the timing of the M or E peak. To determine this, the single average activity profiles were smoothened by a moving average of 30, thereby reducing the number of spontaneous spikes in the recording. Then the single smoothened average activity profiles were plotted and the

timing of M and E peak was determined fly by fly. This is necessary as the "real" peak is often masked by activity caused by lights-on or lights-off but can be distinguished independently when single fly activity profiles are analyzed (reviewed in Mrosovsky (1999)). The single peak timing values were averaged and a standard error of the mean (SEM) was calculated for each peak.

We further evaluated how light influences the distribution of diurnal or nocturnal activity of the flies. To do so, we determined 1) the mean activity level of the flies during the day by calculating the mean activity between ZT0-ZT12, 2) the mean activity level of the flies during the night by calculating the mean activity between ZT12-ZT24 and 3) the relative nocturnal activity calculated by the sum of activity between ZT12-ZT24 divided by the sum of activity of the whole day. For each value the average activity, either in beam-crosses/minute (case 1 and 2) or in percent of whole activity (case 3), was calculated including the SEM.

Statistics

Statistical analysis was performed using Systat11. We either applied a one-way ANOVA, two-way ANOVA, Kruskal-Wallis-test or a Wilcoxon-test after testing for normal distribution using a Kolmogorov-Smirnov test. For details see material and methods sections of the different projects.

2.4. Immunocytochemistry (ICC)

2.4.1. ICC on adult *Drosophila* brains

Depending on the aim of each staining, the flies were entrained for 5-7 days to the light condition of interest. When flies were expressing GFP, the whole flies were subsequently fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 3h at room temperature (RT). In any other case fixation was performed for 2.5h in PBS including 0.5% TritonX (PBST). After fixation, the flies were rinsed 4x 15 min in PBS and dissected in PBS. Afterwards the brains were blocked with 5% normal goat serum (NGS) in PBST for 2 hours at RT, followed by the first antibody solution, which was applied overnight at RT. This included the selected antibodies (see different projects) in a solution consisting of 5% NGS and 0.02% NaN_3 in PBST. The next day the brains were rinsed 5x 10 min with PBST and the secondary antibody solution (secondary antibodies of choice 1:200 in 5% NGS in PBST) was applied for 3h at RT excluding illumination. This step was followed by washing the brains 5x 10 min in PBST and a last washing step with PBST with a reduced level of TritonX (0.1%). In the last step the brains were mounted on glass slides with the

anterior side up using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and the slide was sealed using Fixogum. The slides were stored at 4°C until microscopy.

2.4.2. ICC on *Drosophila* retinas

To stain the retina of *Drosophila* the whole flies were fixed for 2.5 h in 4% PFA in PBS. Then the flies were rinsed 4x 15 min with PBS and afterwards dissected in PBS. The dissected retinas were blocked 30 min in 5% NGS in PBST and afterwards transferred into the first antibody solution consisting of the antibody of interest (see different projects), 5% NGS and 0.02% NaN_3 in PBST. After incubation at RT for 2 nights the retinas were rinsed 5x 20 min with PBST and the secondary antibody solution (antibody of interest 1:200 including 5% NGS in PBST) was applied overnight at RT. On the next day the retinas were washed 6x in PBST for 10 min. Flies in w^- background (including flies carrying a *miniwhite* construct) were embedded at this stage of the staining as the pigmentation was washed out completely. Flies in w^+ background were washed for several days in PBST until the red eye pigmentation disappeared. Afterwards the retinas were mounted on glass slides in a way that the cornea laid on the slide using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Slides were closed using Fixogum and stored at 4°C until microscopy.

2.4.3. Confocal laser scanning microscopy and image analysis

For image acquisition the Leica TCS SPE (Leica, Wetzlar, Germany) was used. Using three different laser diodes (488nm, 532nm and 635nm) either GFP or the fluorophores of the secondary antibodies were excited and confocal stacks of 2 µm thickness were obtained. Experimental strains and controls were scanned using identical laser settings. The same applied for experiments in which a time-series was investigated and the staining intensity had to be determined. Image analysis was performed using FIJI. Brightness and contrast were adjusted using Photoshop CS5.1 (Adobe Corporation, San José, CA, USA). For detailed description of specific analyses see section 3.

3. Results

In this section I will summarize the results we obtained in the different projects. To do so I will first give a short introduction to each topic to mention previously published results and to give an overview, why the experiments were conducted. As I did not give detailed material and methods in section 2, each paragraph will include a material and methods part, which describes fly strains, antibodies and further details on the conducted experiments. Then short summaries will emphasize the main results of each project. For details please refer to the full-text paper or manuscript attached in section 6.

3.1. Photic Entrainment in *Drosophila* Assessed by Locomotor Activity Recordings

Published in Methods in Enzymology (2015)

3.1.1. Introduction

Light is the most important Zeitgeber for synchronizing the circadian clock of *Drosophila* and other animals to the changes of day and night (Foster and Helfrich-Förster, 2001). In *Drosophila* the approximately 150 clock neurons in the brain get direct and indirect input from the H-B eyelet and the compound eyes. In addition, half of the clock neurons express the blue light sensitive photopigment CRY, which resets the molecular clock mechanism upon illumination. Therefore the circadian clock of *Drosophila* is very light sensitive and most studies often apply a rectangular light dark cycle of 12h light and 12h darkness (LD 12:12) to entrain the circadian clock (Hardin, 2011). Using this LD regime, flies show a bimodal activity peak with an M peak shortly after lights-on and an E peak shortly before lights-off. However, the endogenous peak timing is often masked by the sudden transitions in light condition as flies react to these events with a strong increase of activity. The first aim of this study was to show different possibilities of using light as a Zeitgeber for synchronizing the activity pattern of the flies, which might be more useful in specific cases. In the second part we aimed to show different possibilities of recording locomotor activity and provide a possible way of analyzing locomotor activity data in entrained conditions.

3.1.2. Material and Methods

Fly strains

Table 1 Fly strains used in Schlichting and Helfrich-Förster 2015

Genotype	Source	Reference
$WT_{CantonS}$	stock collection	
per^{s}	stock collection	(Konopka and Benzer, 1971)
per^{l}	stock collection	(Konopka and Benzer, 1971)

Locomotor activity recording

In order to show the advantages of using different photoperiods for separating the endogenous peak of activity from responses to lights-on or lights-off, all genotypes were monitored in LD12:12 followed by LD 16:8 and LD 20:4 using the DAM system. From these data average activity profiles were calculated as described in section 2.3.4.

In order to show different ways of monitoring activity, we analyzed the behavior of $WT_{CantonS}$ in LD12:12 using all monitoring devices described in section 2.3. In every experiment white LEDs were used to simulate the changes of day and night and the light intensity was set to 100 lux. Analysis was performed as described previously. In addition, we calculated the Morning-anticipation Index (MI) by dividing the sum of activity 3h before lights-on (ZT21-24) by the sum of activity 6h before lights-on (ZT18-24). Whenever this value is significantly higher than 0.5, the flies anticipate lights-on. We further analyzed the duration of the siesta as the time between offset of M and onset of E activity.

3.1.3. Summary

In this study we showed different ways of monitoring locomotor activity rhythms in flies. Two of the systems count the number of beam crosses in a given time interval, whereas the Noldus-system directly tracks the movement of the flies and records the activity in a much smaller time window (500ms). Our results show that the activity pattern appears different in the three systems even though the same light conditions and the same wildtype were used. The overall activity measured with the home-made system was drastically reduced compared to the DAM system, which can be explained by more available space for moving inside the cuvettes. An interesting result was that the timing of the M peak was exactly the same in all systems, whereas the flies showed a later E peak in the DAM system compared to the other ones. This is mainly due to the much stronger

response to lights-off in this system whereby the real E peak is strongly masked compared to the other systems. These results clearly show that data obtained with different recording systems strongly influences the activity pattern of the fly and thus a comparison of data obtained with different systems should be considered with caution.

3.2. Moonlight Detection by *Drosophila´*s Endogenous Clock Depends on Multiple Photopigments in the Compound Eyes

Published in the Journal of Biological Rhythms (2014)

3.2.1. Introduction

Researchers usually use rectangular LD cycles to simulate the changes of day and night. However, absolutely dark nights never occur in nature, as they are illuminated by the moon or even by the stars. Several studies demonstrated an alteration of the daily pattern of activity, foraging or predation in moonlit nights in a variety of species (Kronfeld-Schor et al., 2013). Also *Drosophila* alters its behavior in light-moonlight (LM) conditions. The most prominent effect is an increase of nocturnal activity, which was shown to be directly mediated by the light input from the compound eyes and to be independent of a functional clock (Kempinger et al., 2009). On the other hand the M peak was significantly advanced, whereas the E peak was significantly delayed in LM compared to LD, which resulted in changes in PER immunoreactivity in M and E cells controlling the timing of the activity peaks (Bachleitner et al., 2007). These studies clearly show that the activity pattern of *Drosophila* depends strongly on the input from the compound eyes in LM conditions. Therefore we asked, whether we are able to narrow down the effects on behavior to specific receptor cells in the compound eyes.

3.2.2. Material and Methods

Fly strains

Table 2 Genotypes used in Schlichting et al., 2014

Genotype	Source	Reference
$WT_{CantonS}$	stock collection	
WT_{ALA}	R. Costa	(Sandrelli et al., 2007)
$WT_{Lindelbach}$	R. Wolf	
$rh3^{1}rh4^{1}$	C. Desplan	(Vasiliauskas et al., 2011)
sev^{LY3}	stock collection	(Benzer, 1967)
$rh5^{2}$	stock collection	(Yamaguchi et al., 2008)
$rh6^{1}$	C. Desplan	(Cook et al., 2003)

rh5^{2};rh6^{1}	C. Desplan	(Yamaguchi et al., 2008)
rh5^{2};rh3^{1}rh4^{1}rh6^{1}	C. Desplan	
ninaE^{17}	C. Schnaitmann	(Kumar and Ready, 1995)
ninaE^{17}rh6^{1}	F. Rouyer	
cli^{eya}	stock collection	(Bonini et al., 1993)
w;rh5^{2};cry^{b}	R. Stanewsky	(Szular et al., 2012)
+;CyO/Sco;MKRS/TM6B	stock collection	
+;Cyo/Sco	stock collection	
+;ls-tim;MKRS/TM6B	stock collection	

ICC

In order to avoid differences in the yellow/pale distribution in the retina of the investigated flies, we determined the Rh5/Rh6 ratio in $WT_{CantonS}$, *sev^{LY3}*, *rh3^{1}rh4^{1}*, *rh6^{1}* and *ninaE^{17}rh6^{1}*. As there is an age dependent de-repression of Rh5 in *rh6^{1}* mutants we determined the distribution at the age of 4 and 11 days (Vasiliauskas et al., 2011). To do so the ICC protocol described in section 2.4.2 was used. We applied three different antibodies: anti-pigment-cell enriched dehydrogenase (PDH) was used to stain the pigment cells, whereas anti-rhodopsin 5 (Rh5) and anti-rhodopsin 6 (Rh6) stained the two photopigments in R8 (see Table 3).

Table 3 Antibodies used in Schlichting et al., 2014

Antibody	**Dilution**	**Reference/Source**
rat anti-PDH	1:100	(Wang et al., 2012) C. Montell
mouse anti-Rh5	1:50	(Salcedo et al., 1999) S. Britt
rabbit anti-Rh6	1:1000	(Tahayato et al., 2003) C. Desplan
Alexa Fluor 488 (goat anti rat)	1:200	Invitrogen
Alexa Fluor 555 (goat anti-mouse)	1:200	Invitrogen
Alexa Fluor 635 (goat anti-rabbit)	1:200	Invitrogen

To determine the yellow/pale ommatidia ratio, the number of Rh5 and Rh6 expressing cells of at least 7 retinas was counted manually. In case of flies carrying the *rh6*^{1} mutation the PDH staining was used in order to determine the whole number of counted ommatidia.

DNA extraction and Polymerase Chain Reaction (PCR)

Recent studies showed a natural polymorphism of *tim* splicing. Flies either carry the *s-tim* allele resulting only in the short m-RNA isoform, whereas flies carrying the *ls-tim* allele produce small and long mRNA isoforms (Sandrelli et al., 2007; Tauber et al., 2007). These studies further showed that the clock of *s-tim* flies appears to be more light sensitive compared to *ls-tim* flies. In order to avoid differences in behavior as a result of *s/ls-tim* polymorphism, we checked all investigated mutants for the *tim*-allele. To do so, the DNA of 5 individual flies per genotype was extracted using 50 µl Squishing buffer (consisting of 10 mM Tris-HCl (pH=8.2), 1 mM EDTA and 25 mM NaCl) and 5 µl Proteinase K (final concentration of 20 mg/ml). The suspension was heated to 56°C for 30 min using a Thermo Shaker TS-100 followed by an inactivation step of 92°C for 2 min. The suspensions were then put on ice until the PCR reaction (composition see Table 4) was performed. The following primers were used for the PCR:

tim 5´: TAGGTATCGCCCTCCAAG
tim 3´: TAGGCAGCTCCACAATCA

Table 4 PCR mix for 1 reaction

Reagent	Amount
RedTaq Readymix	10 µl
H_2O	5 µl
tim 5´ (10mM)	2 µl
tim 3´ (10mM)	2 µl
DNA template	1 µl

The PCR was performed using the PeqLab PeqStar Universal 96 thermocycler using the temperature protocol described in Table 5. Afterwards the PCR-product was separated using a 1% agarose gel including Midori Green (Nippon Genetics Europe) as a dye. As expected the PCR resulted in only one band which represented an amplified part of the *tim*-gene. Then the PCR reaction was purified using the standard protocol of the MSB Spin PCRapace (Invitec GmbH & Co KG, Düsseldorf, Germany) kit and the samples were sent to LGC Genomics for sequence analysis.

Table 5 Standard PCR steps to amplify small DNA fragments (up to 1000bp)

Step	Temperature [°C]	Duration (mm:ss)	
Initialization	94	5:00	
Denaturation	94	0:30	35x
Annealing	60	0:30	
Elongation	72	0:30	
Final elongation	74	5:00	

Genetic fly crosses for the project

As the *rh5*^{2} mutant of our stock collection carried the *s-tim* allele, we decided to replace it by a strain carrying the *ls-tim* allele. To do so I back-crossed the *w;rh5*^{2}*;cry*^{b} strain (*ls-tim*) twice to the *Cyo/Sco;TM6B/MKRS* (+DB) strain resulting in a stable stock with the following genetics: *rh5*^{2};*TM6B/MKRS* (*ls-tim*). To remove the balancers/markers on the III. chromosome this strain was crossed once to *CyO/Sco* and *rh5*^{2}*/CyO;TM6B/*+ flies were finally crossed with each other until all balancers were removed.

Similar to *rh5*^{2} also the *ninaE*^{17} mutant was back-crossed twice to the +DB resulting in the stable *CyO/Sco;ninaE*^{17} strain. To remove the balancer/marker of the II. chromosome and to introduce the *ls-tim* allele the flies were crossed once to *ls-tim;MKRS/TM6B*. *ls-tim/CyO;ninaE*^{17}*/TM6B* flies were collected and crossed to each other until final removal of the balancers.

Locomotor Activity Recording

To analyze the locomotor activity the home-made system described in 2.3.1 was used. The flies were either recorded in a light-dark cycle of 12 h light and 12 h darkness (LD12:12) or in a light-moonlight cycle (LM12:12, moonlight intensity of 0.01 lux). We further analyzed 4 different daylight intensities (10, 100, 1000 and 10000 lux). Each light condition was given for one week.

3.2.3. Results

The *tim* polymorphism was shown to be important for the fly´s behavior with flies carrying the *s-tim* allele being more light sensitive (Sandrelli et al., 2007; Tauber et al., 2007). In *s-tim* flies the deletion of a G nucleotide at position 294 of the *tim* cDNA results in the

generation of a stop codon immediately 5´ of the translational start of *s-tim* (Myers et al., 1995; Peschel et al., 2006). The amplification and sequencing of the region of interest in the *tim* allele showed that all investigated mutants, except $rh5^{2}$ and $ninaE^{17}$, carried the *ls-tim* allele. Therefore these mutants were crossed into the *ls-tim* background as described above. For further results of this study please see supplement.

3.2.4. Summary

In contrast to the studies of Bachleitner et al. (2007) and Kempinger et al. (2009) we were able to analyze the behavior of the flies in a quantitative way. In the previous studies an older version of the home-made system was used allowing only yes (1) or no (0) activity counts in a 4 minute interval. Nevertheless, we were able to reproduce the data of both studies: The flies delayed their E peak and increased nocturnal activity levels in moonlit nights, although this increase was less prominent in the updated system. Both effects depended on the daylight intensity applied: the higher the light intensity, the later the E peak and the stronger the increase of nocturnal activity upon moonlight simulation. We were further able to confirm that all of these effects require light input from the compound eyes, as eyeless flies neither delayed their E peak, nor increased the nocturnal activity level in moonlit nights. By analyzing several photoreceptor mutants, we could show that a complex interaction of inner (R7 and R8) and outer receptor cells (R1-6) is necessary for wild-type behavior. As soon as either of them was not functional (due to the manipulation of rhodopsins), the flies were not able to delay their E peak in moonlit nights. On the contrary, with functional inner or outer receptor cells the flies were still able to increase the nocturnal activity level when moonlight was simulated, but to a much lower extent compared to wild-type flies. Only when Rh1 and Rh6 were absent, the flies did not respond to moonlight at all, indicating an important role of these rhodopsins. This was rather unexpected, as only R1-6 were reported to be important for dim light detection. Our data clearly show the influence of especially Rh6, which is expressed in R8. The importance of Rh6 got even more obvious in sev^{LY3} mutants: Due to the lack of R7 these flies only express Rh6 in R8. When we compared this mutant to wild-type flies, we observed a significant increase of nocturnal activity. On the contrary, as soon as only Rh6 was absent, we found strong decreases in nocturnal activity in LM conditions.

3.3. Normal vision can compensate for the loss of the circadian clock

Submitted to BMC Biology

3.3.1. Introduction

Circadian clocks were thought to be essential for timing the animals´ daily activity and hence increase their fitness (DeCoursey et al., 2000). Recent studies challenged this point of view as the behavior of clock-less flies or mice was almost indistinguishable from WT behavior when they were recorded in "natural" conditions (Daan et al., 2011; Vanin et al., 2012). However, in nature many factors like humidity, temperature, light or the emergence of predators may synchronize the clock and/or provoke direct responses of the animals, which bypass the clock. In this study we went back to the lab and investigated WT flies as well as photoreceptor- and clock-mutants by singularly changing the light regime, whereas all other external cues were kept constant. As twilight was predicted to be the most important light stimulus for synchronizing the circadian clock in nature, we investigated the flies in rectangular LD cycles, in conditions including the simulation of twilight and in conditions with gradual in- and decreases of the light intensity within a longer time period to simulate the course of the sun within one day. Previous studies already showed that twilight simulation significantly altered the behavior of the flies. In twilight conditions the M peak was significantly delayed, whereas the E peak was significantly advanced. Both peaks took place at a light intensity of approximately 5 lux, at which the flies prefered to be active (Rieger et al., 2007). Our aims of the present study were 1) to identify behavioral changes caused by prolonged twilight simulation (4.5h for dawn and dusk each) 2) to clarify if the effects we observed were clock controlled or directly mediated by light (masking) and 3) whether the circadian photoreceptor CRY or the compound eyes mediate the behavioral adaptations.

3.3.2. Material and Methods

Fly strains

Table 6 Fly strains used in "Normal vision can compensate for the loss of the circadian clock"

Genotype	Source	Reference
$WT_{CantonS}$	stock collection	
per^{01}	stock collection	(Konopka and Benzer, 1971)

tim^{01}	stock collection	(Sehgal et al., 1994)
cry^{01} *(CS)*		(Dolezelova et al., 2007)
eya^{2}	stock collection	(Bonini et al., 1993)
per^{01}*;eya*^{2}	stock collection	
per^{01}*;;cry*^{01}		
+;CyO/Sco;MKRS/TM6B	stock collection	

ICC

In order to examine the effect of more natural light conditions on the clock, we investigated $WT_{CantonS}$ and *cry*^{01} flies in rectangular light dark cycles (LD 12:12) with a daylight intensity of 100 lux and compared the expression pattern of the clock protein TIM with a light condition simulating the course of the sun via increasing light intensity within 4.5 h in the morning and decreasing it within 4.5 h in the evening (LDR2). To do so, the flies were entrained either in LD or LDR2 and collected every 2h between ZT10 and ZT24, and every 1h after lights-on (between ZT0 and ZT2). As TIM protein levels are low during the day we only focused on time-points of high protein levels (ZT10-ZT2). Flies were fixed and stained as described in section 2.4.1. For analyzing TIM cycling in the lateral neuron clusters we used anti-TIM and anti-PDF in order to be able to distinguish the 5th sLN_v from the other lateral neurons (see Table 7 for used antibodies). Laser settings were adjusted using ZT22 and were kept constant for all samples to avoid differences in staining intensity due to different scanning settings. The staining intensity was analyzed using FIJI. To do so we defined a square shaped area of 9 pixels and analyzed the staining intensity of single neurons by measuring the mean grey value of the 9 pixel area. We concentrated on the analysis of the lateral neurons as they are known to be important for the timing of M and E activity peaks. For each time point at least 5 different brains were analyzed.

Table 7 Antibodies used in "Normal vision can compensate for the loss of the circadian clock"

Antibody	Dilution	Reference/Source
mouse anti-PDF	1:1000	C7, Developmental Studies Hybridoma Bank, Iowa, USA
rabbit anti-PER	1:500	R. Stanewsky
rat anti-TIM	1:2000	J. Giebultowicz

Alexa Fluor 488 (goat anti rabbit)	1:200	Invitrogen
Alexa Fluor 555 (goat anti-rat)	1:200	Invitrogen
Alexa Fluor 635 (goat anti-mouse)	1:200	Invitrogen

Genetic fly crosses for the project

To generate $per^{01};;cry^{01}$ flies, chromosomes II and III of the per^{01} mutant and chromosome II of the cry^{01} mutant were balanced using the +DB. The two balanced lines were crossed together and residual balancers and markers were removed using standard *Drosophila* genetics.

To "Cantonize" the cry^{01} mutant the +DB was backcrossed to $WT_{CantonS}$ seven times to exchange chromosomes and allow recombination events to take place. Afterwards two balancer lines (*CyO/Sco* and *MKRS/TM6B*) were generated with all chromosomes, except the balanced one, being in $WT_{CantonS}$ background. The cry^{01} mutant was backcrossed to $WT_{CantonS}$ for 5 generations allowing recombination events and thereby exchanging the genetic background. Afterwards individual male flies were checked for the cry^{01} mutation using PCR (see below). To clearly identify the single flies, they were kept isolated in Eppendorf tubes for the duration of DNA extraction and PCR. Males still carrying the mutation were crossed to the *MKRS/TM6B* (CS) balancer. In the next generation only males and virgins carrying *TM6B* as a balancer were analyzed for the presence of the mutation using PCR and crossed together until removal of all balancers.

DNA extraction and PCR

As the flies carrying the cry^{01} mutation had to be used for ongoing crossings it was not possible to use the whole fly to extract DNA. To minimize violation of the flies they were anaesthetized with CO_2 and a single haltere was ripped off with a forceps. These were transferred into a DNA extraction solution consisting of 20µl squishing buffer including Proteinase K. The samples were treated as previously described in section 3.2.2. For the PCR reaction we used two pairs of primers at the same time: As the flies were either heterozygous cry^{01} or homozygous WT we used one set of primers amplifying a part of the *cry* gene as positive control whether the PCR worked (*cry* primers). We further generated primers that selectively amplify a part of the cry^{01} mutation (cry^{01} primers). In cry^{01} the whole *cry* gene was replaced by a *miniwhite* gene using homologous recombination (Dolezelova et al., 2007). Therefore we placed one primer into the *miniwhite* construct and the other one downstream of the *cry* locus into one of the recombination sites. The sequence of the primers were:

cry 5´: CGGAGTTGATGAATGTGCC

cry 3´: GCATGTTTCGCTTTACGG

cry^{01} 5´: AACGAAACCAAATAAAACGGTACCC

cry^{01} 3´: CGTAAACCGCTTGGAGCTTCGTCAC

Due to the lower amount of DNA the composition for the PCR reaction was slightly changed (see Table 8).

Table 8 PCR composition to amplify *cry* and *cry*^{01} fragments out of haltere DNA extracts

Reagent	Amount
RedTaq Ready Mix	10 µl
cry 5´ (10mM)	1.5 µl
cry 3´ (10mM)	1.5 µl
cry^{01} 5´ (10mM)	1.5 µl
cry^{01} 3´ (10mM)	1.5 µl
DNA template	4 µl

To amplify these fragments the PCR settings described in Table 5 were used. The samples were then separated using a 1% agarose gel including MidoriGreen as a dye.

Locomotor activity recording

To analyze the locomotor activity the home made system was used, which was described in section 2.3.1. In the first set of experiments we applied the standard rectangular light dark cycle for one week. In the second week we simulated twilight by a gradual increase of the light intensity within 1.5h in the morning and a gradual decrease within 1.5h in the evening (LDR1). In the third week the course of the sun was mimicked by gradually increasing/decreasing light intensity within 4.5h each (LDR2). For each condition the light and dark phases were set to 12h and the maximal light intensity was set to 100 lux.

In a second set of experiments we tested, to which degree the observed changes in behavior are clock regulated or direct responses to light. To do so we monitored the flies in LD12:12 (100 lux) for 5 days. On day 6 we applied a "night pulse" after lights-off: Light intensity was gradually increased between ZT15.5-ZT17, kept constant at 100 lux for 2h and finally gradually decreased between ZT19-ZT20.5. This light pulse was applied at

a time, in which the clock normally inhibits activity and we could therefore investigate the contribution of the clock to the behavior in twilight conditions.

3.3.3. Results

After back-crossing for 5 generations to $WT_{CantonS}$ we checked in total 26 male flies (Figure 9), whether or not they still carry the *cry*^{01} mutation. As a control we used $WT_{CantonS}$ and *cry*^{01} flies. As expected we only got one band with a length of 380bp in $WT_{CantonS}$ flies, as the *cry*^{01} primer pair, which was designed to detect the *miniwhite* construct, was not able to bind. On the contrary, we only got one band of 194bp in the *cry*^{01} mutant, since in this case the *cry* primer pair was not able to bind the *cry* locus as this had been exchanged by *miniwhite*. In the back-crossed flies we always observed the band deriving from the *cry* primer pair, since at least one of the 2 chromatides was still WT. In 4 of the 26 flies we additionally found a band at about 200bp deriving from the *cry*^{01} specific primer pair, which confirmed that these 4 flies were heterozygous for *cry*^{01}.

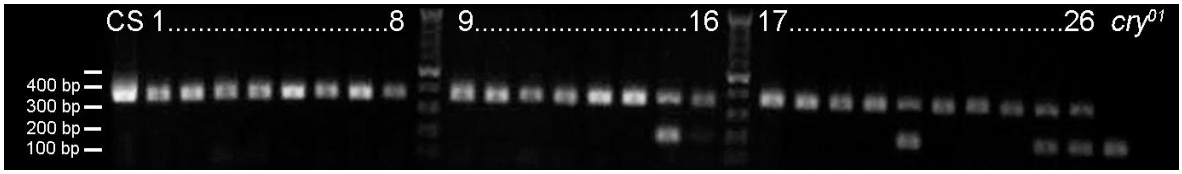

Figure 9 PCR results after back-crossing 5x to $WT_{CantonS}$. As a control we also performed the PCR using DNA-extracts from $WT_{CantonS}$ (first lane on the left) and *cry*^{01} (first lane on the right). Only 4 out of 26 investigated flies still carried the *cry*^{01} mutation. For details see text.

These four flies were then crossed to *MKRS/TM6B* (CS) and we determined the genotype of single male and female virgin flies carrying *TM6B* as a balancer using the same set of primers. As 50% of those flies were expected to carry the mutation, the number of analyzed flies was reduced: We determined the genotype of 8 male flies (1-8) and 18 (9-26) virgin females (Figure 10).

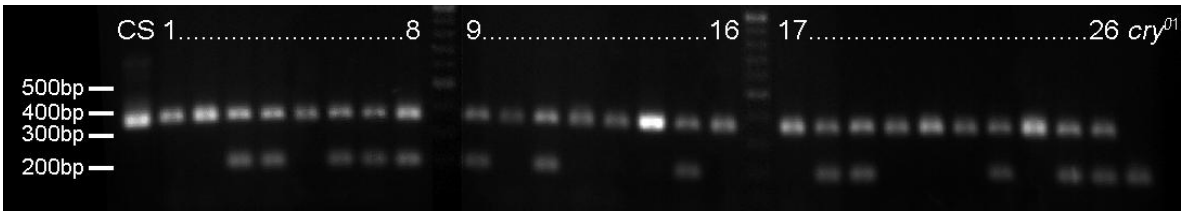

Figure 10 PCR results after crossing *cry*^{01}/+ flies to *TM6B/MKRS* (CS) balancer. $WT_{CantonS}$ (first lane on the left) and *cry*^{01} (first lane on the right) served as controls for this PCR. Lanes 1-8 represent 8 individual male flies with flies 3,4,6,7 and 8 still carrying the *cry*^{01} mutation. Lanes 9-26 represent 18 virgins with flies 9,11,15,18,19,23,25 and 26 carrying the *cry*^{01} mutation.

Again we used $WT_{CantonS}$ and *cry*^{01} as controls which resulted in single bands of 380bp (CS) and 194bp (*cry*^{01}), respectively. As expected the number of flies carrying the *cry*^{01} mutation was much higher in this screen. 5 out of 8 males and 8 out of 18 virgin females carried the *cry*^{01} mutation. These flies were then mated to generate the *cry*^{01} (CS) strain. For further results of this study please see supplement.

3.3.4. Summary

In this study we investigated the behavior of $WT_{CantonS}$ in three different light conditions: In LD12:12 the flies showed a bimodal activity pattern with the M peak occurring shortly after lights-on and the E peak shortly before lights-off. Upon the simulation of twilight (LDR1) flies delayed the M and advanced the E peak, which is in accordance to the study of Rieger et al. (2007). The peaks occurred at a light intensity of 2-7 lux, at which the flies prefer to be active. In the third light condition, LDR2, the shifts of M and E activity bouts were even stronger as the preferred light intensity was reached later in the morning and earlier in the evening. To test whether the sharp peaks during twilight are mediated by the clock, we investigated null-mutants for the clock genes *per* and *tim*. In LD these flies showed an increase of activity during the day, but lacked activity peaks and siesta. In LDR1/LDR2 the flies developed a bimodal activity pattern with the M peak occurring during dawn and the E peak during dusk. Especially in LDR2 the activity profiles were almost identical to WT, indicating that the sharp peaks during dawn and dusk may be direct responses to light. Therefore we wondered which light input pathway might cause these effects, and tested, whether CRY or the compound eyes cause this bimodal activity pattern. To investigate the contribution of the clock we further analyzed clock-less *cry*^{01} and *eyes absent* mutants. Our results showed that flies lacking CRY, independently of a functional clock, exhibit sharp M and E peaks in LDR1/LDR2 conditions, whereas *eyes absent* mutants do not respond to the simulation of twilight. This strongly indicates that the compound eyes are necessary and sufficient for detecting twilight and causing a WT-like activity pattern. To further test, whether the sharp peaks were only caused by the gradual changes of light intensity or if the molecular clock was also shifted, we investigated 1) the cycling of TIM in the lateral neuron clusters in LD and LDR2 and 2) the effect of a midnight pulse simulating dawn/dusk in the middle of the night. The results of the TIM staining showed that twilight is able to significantly broaden the TIM protein bout over the day, whereas the time point of maximal TIM staining was not affected. The broadening of TIM appeared to be mediated by CRY as we did not observe this in *cry*^{01} flies. On the other hand, the night pulse experiment showed that all clock mutants responded to dawn and

dusk with sharp activity peaks even during the middle of the night (strong masking), whereas *cry*^{01} and WT flies only showed increased activity levels and *eyes absent* flies did not respond at all to the midnight pulse. Taken together our results show that CRY mediates clock related adaptations, whereas the compound eyes mediate the timing and occurrence of the sharp M and E peak in twilight conditions that can be regarded as masking. The fine-tuning of the activity profile by masking results in normal rhythmicity of clock-less flies, which might explain the observed quasi-normal activity rhythms of flies and mice in the wild (Daan et al., 2011; Vanin et al., 2012).

3.4. Twilight dominates over moonlight in adjusting *Drosophila´*s activity pattern

In press in the Journal of Biological Rhythms

3.4.1. Introduction

The previous studies (section 3.2 and section 3.3) investigated the effects of moonlight or twilight simulation on fly rhythmic behavior. This separate examination allowed us to judge the behavioral changes evoked by either moonlight or twilight alone. The observed effects were quite opposite: moonlight shifted M and E peaks into the night and strongly increased nocturnal activity levels whereas twilight shifted M and E peaks into dawn and dusk of the day and thereby also reduced the level of nocturnal activity (Bachleitner et al., 2007; Rieger et al., 2007; Kempinger et al., 2009). Both effects were shown to be mediated by the compound eyes, as *eyes absent* mutants neither shifted activity into moonlit nights nor showed activity peaks during dawn or dusk. Even though our studies focusing separately on either of the two light conditions had resulted in new insights into masking and entrainment pathways influencing the fruit fly´s activity pattern, in nature both light stimuli are coupled. Consequently, we asked which of the two light cues dominates the fly´s activity pattern and investigated the behavior in standard LD cycles followed by the simulation of twilight (LDR), as well as in LM cycles followed by the combination of moonlight and twilight (LMR). Our results showed that twilight dominates over moonlight, as both activity peaks take place during dawn or dusk. We were further able to support the above described studies, showing that the compound eyes are required for dim light detection, with a special role of Rh6.

3.4.2. Material and Methods

Fly strains

Table 9 Fly strains used in "Twilight dominates over moonlight in adjusting *Drosophila´*s activity pattern."

Genotype	Source	Reference
$WT_{CantonS}$	stock collection	
WT_{ALA}	R. Costa	(Sandrelli et al., 2007)
$WT_{Lindelbach}$	R. Wolf	
$rh3^{1}rh4^{1}$	C. Desplan	(Vasiliauskas et al., 2011)

sev^{LY3}	stock collection	(Benzer, 1967)
rh5^{2}	stock collection	(Yamaguchi et al., 2008)
rh6^{1}	C. Desplan	(Cook et al., 2003)
rh5^{2};*rh6*^{1}	C. Desplan	(Yamaguchi et al., 2008)
rh5^{2};*rh3*^{1} *rh4*^{1}*rh6*^{1}	C. Desplan	
ninaE^{17}	C. Schnaitmann	(Kumar and Ready, 1995)
cli^{eya}	stock collection	(Bonini et al., 1993)

$WT_{CantonS}$ served as a control for all investigated photoreceptor mutants as all of them carried the *ls-tim* allele. To further address the contribution of the *s-/ls-tim* polymorphism on more natural light regimes we investigated $WT_{Lindelbach}$ (*s-tim*) as well as WT_{ALA} (mixture of *s-/ls-tim*) under the same conditions. WT_{ALA} is a mixture of 37 iso-female lines, which were collected in Alto Adige, Italy. Due to the mixture they contain both *tim*-isoforms.

ICC

To investigate the yellow/pale distribution in adult retinas we entrained $WT_{CantonS}$, $WT_{Lindelbach}$, *rh3*^{1}*rh4*^{1}, *sev*^{LY3} and *rh6*^{1} mutants for 18 days in LD 12:12. Afterwards the whole flies were fixed and retinas were stained as described in section 2.4.2 using anti-Rh5 and anti-Rh6 (Table 10).

Table 10 Antibodies used in "Twilight dominates over moonlight in adjusting *Drosophila´*s activity pattern."

Antibody	**Dilution**	**Reference/Source**
mouse anti-Rh5	1:50	(Salcedo et al., 1999) S. Britt
rabbit anti-Rh6	1:1000	(Tahayato et al., 2003) C. Desplan
Alexa Fluor 555 (goat anti-mouse)	1:200	Invitrogen
Alexa Fluor 635 (goat anti-rabbit)	1:200	Invitrogen

To determine the ratio of yellow/pale ommatidia we manually counted the number of Rh5 or Rh6 expressing cells of at least 7 retinas.

Locomotor activity recording

To record the locomotor activity of the flies the home-made system described in section 2.3.1 was used. 2-6 days old male flies were transferred into photometer cuvettes and entrained by white LEDs.

In the first set of experiments we applied a light-dark cycle of 12h light and 12h darkness for one week, followed by a week of LD cycles with increasing light intensity during 1.5h in the morning and decreasing light intensity during 1.5h in the evening, simulating twilight (LDR). In the second set of experiments flies were recorded for one week in light-moonlight cycles (LM12:12, moonlight intensity: 0.01 lux) followed by one week with the same twilight simulation as in LDR but additional simulation of moonlight (LMR). WT flies were investigated at 4 different daylight intensities (10, 100, 1000 and 10000 lux) to unravel an appropriate light intensity for investigating the behavior of the different photoreceptor mutants. Thereafter, the latter were only recorded at a daylight intensity of 100 lux.

For each experiment we calculated the average activity profiles, the levels of nocturnal activity and the peak timing as described in section 2.3.4. For details on statistical analysis please see section 6.4.

3.4.3. Summary

In this study we aimed to investigate the effects on behavior when moonlight and twilight are simulated within the same experiment. In LD WT flies showed a bimodal activity pattern with the M peak occurring shortly after lights-on and the E peak shortly before lights-off. Dawn and dusk simulation shifted the peaks into daytime with the M peak taking place significantly later and the E peak significantly earlier compared to LD, as it had already been reported previously (Rieger et al., 2007). Moonlight simulation, however, caused the advance of the M and the delay of the E peak as well as an increase of nocturnal activity as it had been described previously (Bachleitner et al., 2007). The simultaneous simulation of twilight and moonlight (LMR) resulted in a bimodal activity pattern with the M peak taking place during dawn and the E peak during dusk, meaning that they come closer together compared to LM conditions, as it is the case when LD and

LDR are compared. Also the level of nocturnal activity was decreased in LMR compared to LM, indicating that the effect of twilight dominates over moonlight.
However, the light intensity during the day seems to play an essential role in the flies´ behavior. Already in LD flies delayed their E peak and shifted activity into the night with increasing light intensity. Also the *s-tim/ls-tim* polymorphism plays a role in this kind of adaptation. Flies in which the clock is more light-sensitive (*s-tim*) increasingly shifted their activity into the night corresponding to the increasing day light intensity, whereas *ls-tim* flies increased nocturnal activity in a less linear way. The peak-delaying effect caused by the increasing day light intensity was not restricted to LD conditions. We observed a delay of the E peak with increasing light intensity in all four investigated light regimes. As the phase differences between LD/LDR and LM/LMR were biggest at 100 lux, we investigated the different photoreceptor mutants at this light intensity.

Our aim was to further unravel the significance of specific photoreceptors for detecting twilight. In the "moonlight" study (section 3.2) we were already able to show that Rh1 and Rh6 are very important for dim light detection, whereas we so far only showed that the compound eyes are necessary for twilight detection in our "twilight" study (section 3.3). However, dim light detection and the detection of gradual changes in light intensity may be achieved by different photoreceptors. Thus, we first re-investigated the *eyes absent* mutant under all light conditions and did not find any changes in peak timing or levels of nocturnal activity confirming our previous results. The investigation of several photoreceptor mutants unraveled the importance of the inner receptor cells (R7 and R8) for WT-like peak timing, as only eyeless flies and $rh5^{2};rh3^{1}rh4^{1}rh6^{1}$ mutants did not advance their E peak upon twilight simulation (comparison between LD/LDR or LM/LMR, respectively). The reduction of nocturnal activity by twilight seems to depend on a complex interaction of all photoreceptor cells as it was only absent in eyeless flies. A special role in this process seems to apply to Rh6, as sev^{LY3} flies, which express Rh6 in all R8, were the only flies that shifted significantly more activity into moonlit nights compared to WT flies. In all other mutants the level of nocturnal activity was either decreased or at WT level.

3.5. Fly Cryptochrome and the visual system

Published in the Proceedings of the National Academy of Sciences (2013)

3.5.1. Introduction

The previous studies investigated the significance of the compound eyes and its rhodopsins on the adaptation to more natural light regimes. However, the study of Yoshii et al. (2008) clearly showed that also the blue light photopigment Cryptochrome is strongly expressed in the retina. In *Drosophila* most of the studies involving CRY focused on its mediation of the light-dependent degradation of TIM within the molecular mechanism of the circadian clock (see section 1.3). CRY is not only present in the fly genome, it can widely be found in the animal and plant kingdom. Cryptochromes consist of an N-terminal domain homologous to photolyases and a very divergent C-terminal tail (Hemsley et al., 2007). Even though the molecular mechanism of how the activation of CRY by light works is widely unknown, several studies showed that the C-terminus region seems to be mediating this process (Rosato et al., 2001; Dissel et al., 2004; Ozturk et al., 2011). Structural analysis of the C-terminal region showed that it contains several molecular interaction motifs among which two class III PDZ-binding motifs were found (Hemsley et al., 2007). These motifs contain modular domains, which are essential for the formation of large protein complexes and the structure of these motifs are rather conserved: They consist of five to six β-strands and two or three α-helices forming a β-stranded sandwich. In this study we focused on potential interaction partners of the CRY C-terminus which are involved in phototransduction and the resulting question, whether CRY is involved in visual processes in *Drosophila*. For this study we collaborated with Prof. Rodolfo Costa.

3.5.2. Material and Methods

Fly strains

Table 11 Fly strains used in the study Mazzotta et al., 2013

Genotype	Source	Reference
$WT_{CantonS}$	stock collection	
w^{1118}	D. Dolezel	(Dolezelova et al., 2007)
cry^{01}	D. Dolezel	(Dolezelova et al., 2007)
cry^{M}	R. Costa	(Busza et al., 2004)

Determination of Phototaxis

The phototaxis experiments were conducted as described in Benzer (1967). The setup consists of a stationary row of six Plexiglas vials. On top a flexible part, that can be pushed left and right, is installed consisting of five Plexiglas vials. To investigate the phototaxis, flies were starved for 3 h prior to the experiment in order to improve the activity level. Flies were then transferred into the first tube of the lower vial row, which was closed by the mobile upper part so that the flies were unable to escape. Then the flies were shook down to the bottom of the tube and the mobile vial row was shifted allowing the flies to walk into the first glass of the upper part. To do so the whole apparatus was placed horizontally on a table with the upper part being directed towards a fluorescent lamp (light intensity approximately 3000 lux) allowing the flies to run towards the light source for 15 sec. Afterwards the mobile part was shifted back to its original position, the flies were shook to the bottom of the vials again and the process was repeated four times. Like this the flies were distributed over six vials at the end of the experiment with flies remaining in the first vial showing no phototaxis and flies reaching the sixth vial showing five times positive phototaxis. The flies in each vial were then counted resulting in the number of flies in each vial (n(1) to n(6)) and the number of all flies investigated (n(total)). Out of these values a performance index (PI) was calculated using the following equation:

$$PI = \frac{n(1)x0+n(2)x1+n(3)x2+n(4)x3+n(5)x4+n(6)x5}{n(total)x5}$$

A PI of 1 indicates that all flies showed five times positive phototaxis (all flies in the last vial) whereas a PI of 0 indicates that none of the flies showed positive phototaxis (all flies in vial one). For each genotype the experiment was repeated 10 times using approximately 40 male flies resulting in a number of about 400 investigated flies per genotype.

Determination of optomotor response (OR)

To measure the OR flies were starved for 3 h in order to increase the general activity level and dark adapted for 10 min prior to the experiment. Without anesthesia flies were transferred into a Plexiglas walking chamber (circular arena: ø 3 cm, height: 0.15 cm) which was placed in the center of an upright cylinder (ø 8 cm, height: 4.5 cm). The walls of the outer cylinder were vertically striped with six equally spaced black stripes on white background (width: 30°). The outer cylinder was rotated with a velocity of ω =60°/s, which means that 10 revolutions per minute were generated. Illumination was provided using white LEDs, which were arranged in a ring surrounding the striped cylinder (15 LEDs, light

intensity in the center: 23 µW/cm^2). To record the OR of single flies the outer cylinder was first rotated 5 minutes clock-wise (cw), followed by 5 s darkness and 5 min counter clock-wise (ccw) rotation. The number of the fly´s cw revolutions (rev_{cw}) was counted during the first period of 5 min and in the second period the number of ccw revolutions (rev_{ccw}) was counted. Then a performance index was calculated using the following equation:

$PI = (rev_{cw} + rew_{ccw}) / (n_{cw}+n_{ccw}) \times 100\%$

n_{cw} indicates the number of cw revolutions by the outer cylinder in the first 5 min (5x 10 revolutions/min are given as a stimulus) and n_{ccw} the number of revolutions within the second 5 min period. A PI of 1 indicates perfect OR with the fly following the striped pattern all the time and a PI of 0 indicates no OR with the fly showing no revolution in the right direction.

3.5.3. Summary

In this study we were focusing on the C-terminal domain of *Drosophila* CRY. Using *in silico* analysis we were able to show that the class III PDZ-binding sites are selectively maintained in the animal kingdom. In a coimmunoprecipitation assay we investigated possible interaction partners of CRY using *tim*-GAL4 which is expressed in clock neurons, glia cells and photoreceptor cells. Using this technique we were able to identify CRY in two complexes containing the visual signaling molecules Retinal Degeneration A (RDGA) and Neither Inactivation Nor Afterpotential C (NINAC). Using bioinformatical and molecular tools we further demonstrated that CRY is able to interact with NINAC through the scaffolding protein Inactivation No Afterpotential D (INAD), which is an important protein keeping phototransduction running. This interaction appears to be light dependent, as the interaction was significantly stronger after illuminating the flies for two hours compared to a time point in the dark. In order to show which part of CRY is necessary for the interaction with INAD, we expressed different parts of CRY and INAD in yeast cells. Using the yeast-2-hybrid system we were able to show that the C-terminal region of CRY is required for the interaction with the phototransduction protein INAD. This already suggests that CRY might act in *Drosophila* vision but in order to show a biological relevance for this interaction, we tested CRY mutants in visual behavior. To do so we investigated cry^{01} mutants, in which the whole gene is replaced by *miniwhite* (Dolezelova et al., 2007), and a cry^{M} mutant, in which a part of the regulatory C-terminal region is missing (Busza et al., 2004). Our phototaxis analysis revealed that both CRY mutants showed a significantly reduced phototactic response. Using two different assays for analyzing OR we were further able to show that the OR is significantly reduced in both

mutants compared to controls. This phenotype was rescued by expressing CRY only in R1-6, which had been shown to be essential for motion vision in the fly (Yamaguchi et al., 2008). By monitoring the electro-retinogram-response (ERG) and OR at several times of the day, we showed that the daily cycling in strength of the two responses is absent in cry^{01} mutants. Taken together our results show that CRY is able to interact with INAD and is thereby able to alter visual behavior and rhythms in ERG and OR in adult flies.

3.6. Cryptochrome plays different roles in the fruit fly´s compound eyes and clock neurons

Submitted to the Proceedings of the National Academy of Sciences (PNAS)

3.6.1. Introduction

The study of Mazzotta et al. (2013) nicely showed that CRY is able to interact with components of the phototransduction cascade and that this interaction is able to alter visual behavior. Together with the fact, that CRY in the clock neurons mediates entrainment of the circadian clock to light, these findings suggest that CRY in the compound eyes may contribute to this. With the help of different rhodopsin mutants (see sections 3.2 and 3.4), we could already show that the compound eyes have different roles in the entrainment of activity rhythms: On the one hand the compound eyes fine tune the activity pattern by causing direct light-responses in behavior. On the other hand the compound eyes signal most probably to the lateral clock neurons and thereby alter entrainment of the clock. In this study we asked whether we are able to separate effects on rhythmic activity caused by CRY in the compound eyes from those caused by CRY expressed in the clock neurons. Using CRY-rescue and -knockdown experiments we were able to show that CRY in the compound eyes contributes to the fine-tuning of *Drosophila´*s activity pattern, whereas CRY in the clock neurons sets the phase of the clock with a special importance for timing the E peak.

3.6.2. Material and Methods

Fly strains

Table 12 Fly strains used "Cryptochrome plays different roles in the fruit fly´s compound eyes and clock neurons".

Genotype	Source	Reference
$WT_{CantonS}$	stock collection	
w^{1118}	D. Dolezel	(Dolezelova et al., 2007)
$w;cry^{01}$	D. Dolezel	(Dolezelova et al., 2007)
cry^{01} *(CS)*		
w;ninaE-GAL4		BL: 30540
w;UAS-cry	P. Emery	(Emery et al., 1998)

w UAS-dcr2		VDRC: 60012
w;;UAS-cryRNAi	P. Emery	BL: 25859
w;R78G02-GAL4		BL: 40010
w;UAS-stinger2		(Barolo et al., 2000)

ICC

To investigate the expression pattern of *R78G02-GAL4* we expressed nuclear GFP using *UAS-stinger2*. In order to be able to determine co-localization of GFP in specific clock neurons we co-labeled with anti-PDF and anti-CRY. This staining showed that *R78G02*-GAL4 is expressed in a subset of the clock neurons, which were the CRY-positive LN_d and the 5^{th} sLN_v (E cells). Therefore we analyzed in a second set of experiments the efficiency of CRY knockdown in these cells. To do so we expressed *UAS-cryRNAi* using *R78G02-GAL4* and determined the CRY staining intensity in the LN_d and the 5^{th} sLN_v. To mark the clock cells we additionally stained against PDF and Vrille (VRI). Prior to staining flies were entrained in LD12:12 (light intensity: 100lux) and fixed at ZT21. Brain staining was performed as described in section 2.4.1. For dilution of the antibodies see Table 13.

We further investigated the subcellular location of CRY in the receptor cells of the compound eyes. To do so cry^{01} (CS) and $WT_{CantonS}$ were kept in complete darkness to allow accumulation of CRY. As Mazzotta et al. (2013) had shown that the CRY-INAD interaction was light dependent, we analyzed CRY staining after complete darkness or after a 2h light pulse of 1000 lux. To mark the rhabdomeres of R1-6 we used the Rh1 antibody. The CRY staining intensity in the rhabdomeres was analyzed from at least 7 retinas by measuring the staining of at least 10 ommatidia per retina using a 9-pixel area. We used the staining protocol previously described in section 2.4.2. For dilution of the used antibodies see Table 13.

Table 13 Antibodies used in "Cryptochrome plays different roles in the fruit fly´s compound eyes and clock neurons".

Antibody	Dilution	Reference/Source
mouse anti-PDF	1:1000	C7, Developmental Studies Hybridoma Bank, Iowa, USA
guinea pig anti-VRI	1:2000	(Glossop et al., 2003) P. Hardin
chicken anti-GFP	1:2000	abcam®

mouse anti-Rh1	1:30	4C5, Developmental Studies Hybridoma Bank, Iowa, USA
rabbit anti-CRY	1:1000	(Yoshii et al., 2008) T.Todo
Alexa Fluor 488 (goat anti-chicken)	1:200	Invitrogen
Alexa Fluor 555 (goat anti-guinea pig)	1:200	Invitrogen
Alexa Fluor 635 (goat anti-mouse)	1:200	Invitrogen
Alexa Fluor 488 (goat anti-rabbit)	1:200	Invitrogen

Locomotor activity recording

To analyze the behavior of the flies the home-made system described above in section 2.3.1 was used. We investigated two different light conditions: In the first week a rectangular light-dark cycle of 12h light and 12h darkness (LD12:12) was applied and in the second week we additionally simulated moonlight at night (LM12:12, moonlight intensity: 0.01 lux). The behavior was investigated at 4 different light intensities (10, 100, 1000 and 10000 lux) using white LEDs as a light source. For all experiments only flies surviving the whole experiment were analyzed. We determined the average activity profiles, relative nocturnal activity levels and peak timing as described in section 2.3.4.

3.6.3. Summary

In collaboration with Prof. Rodolfo Costa we continued investigating the significance of CRY with a special focus on the impact of CRY in the compound eyes on the entrainment in LD and LM conditions. Using ICC we were able to show that CRY is expressed in every receptor cell of the ommatidium and that it is stably expressed even after a 2h light pulse. Therefore the interaction with INAD, which had been shown to be light dependent in the study of Mazzotta et al. (2013), opens the possibility that CRY might enhance light-sensitivity of the compound eyes even after longer illumination. Further molecular studies revealed that CRY is able to interact with 3 different forms of Actin in a light independent fashion. As mentioned in section 1.4 the rhabdomeres of the photoreceptor cells are composed of several thousand microvilli, which are built of Actin filaments. As the different Actin proteins, which are encoded in the genome of *Drosophila*, show high similarity, a CRY-Actin-interaction in the compound eyes is very likely. Together with the CRY-INAD-interaction we had described previously this suggests that CRY keeps the signalplex in the rhabdomers attached to the cytoskeleton. This hypothesis is further supported by our ERG recordings: Whereas we do not observe any difference in the ERG after raising the

flies in complete darkness, we find a slight reduction of the receptor potential in cry^{01} flies after a 2h light pulse followed by 15 min of darkness. These data suggest that CRY does not work as a photoreceptor per se, but enhances light-sensitivity of the compound eyes by keeping the signalplex together.

To test this hypothesis we investigated the locomotor activity of the flies in LD and LM with increasing daylight intensity. In our previous study (section 3.2) we already demonstrated that the compound eyes are essential for dim light and daylight detection and that the flies tend to be active at rather low light intensities. Also in the present study, the flies responded sensitively to light and increased their relative level of nocturnal activity with increasing daylight intensity. This behavioral response is mediated by receptor cells R1-6 and was significantly lower in cry^{01} flies. Expressing *cry* only in R1-6 fully rescued relative nocturnal activity. This indicates that CRY contributes to the light sensitivity of the compound eyes and thus to setting diurnal/nocturnal activity levels in LD conditions.

In a next step we analyzed the behavior of WT flies and cry^{01} mutants in LM conditions. We expected cry^{01} flies to shift less activity into moonlit nights, as our previous findings in LD conditions suggested a reduction of light sensitivity of the compound eyes, which are responsible for the delay of the E peak and the increase in nocturnal activity. Surprisingly, the cry^{01} mutant shifted more activity into the night compared to its respective control (w^{1118}). This appeared to be caused by a significant delay of the E peak, which was overlooked in the previous study (Bachleitner et al., 2007). This delay of the E peak was even more prominent in cantonized cry^{01} mutants, which was puzzling, as our previous results clearly showed that the compound eyes mediate this effect on E peak timing. In collaboration with Dr. Agnes Fekete we found, however, that cry^{01} flies in w^{1118} background have an impaired compound eye signaling as the levels of histamine out of head extracts were significantly reduced compared to WT flies (Borycz et al., 2008). Thus the difference in E peak timing between cry^{01} mutants in $WT_{CantonS}$ and w^{1118} background can be explained by reduced histamine signaling derived from the compound eyes and therefore a reduced phase delay in w^{1118} flies. The question remained, however, why cry^{01} mutants display a significantly delayed E peak timing in LM compared to WT flies. As the knockdown of CRY in the compound eyes did not result in a change of peak timing this suggests a role of CRY within the clock neurons for phasing the E peak in LM conditions. Indeed, we found that the knockdown of CRY in the E neurons was sufficient to reproduce the delayed E peak phenotype of cry^{01} mutants in LM. This suggests that CRY in the clock neurons partly counterbalances the phase-delaying effect of the compound eyes on timing the E peak in LM. In summary we were able to show that CRY has different roles in the compound eyes and in the clock neurons. On the one hand, CRY in the compound eyes

contributes to the light sensitivity as it keeps the signalplex together. On the other hand, CRY within the clock neurons partly counter-balances the phase-delaying effect of the compound eyes in LM conditions. Consequently, $WT_{CantonS}$ flies have only a slightly delayed E peak in LM, whereas *cry*^{01} mutants show a strong delay and *eyes absent* mutants a strong advance of the E peak. For a model of these interactions please see the attached manuscript in section 6.6.

3.7. Rhodopsin 5 and 6 expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms of *Drosophila melanogaster*.

3.7.1. Introduction

So far I have only focused on the importance of the compound eyes and CRY on the light-entrainment of the rhythmic activity in *Drosophila*. However, also the Hofbauer-Buchner (H-B) eyelet was shown to be an important light input pathway for synchronizing the lateral clock neurons in *Drosophila* (Helfrich-Förster et al., 2001; Veleri et al., 2007). These studies mainly investigated the effect of the H-B eyelet on the behavior of the fly, whereas a physiological connection between the eyelets and the clock was not yet shown. The H-B eyelet consists of 4 receptor cells expressing Rh6. These four cells derive from the larval visual system, the Bolwig organ, and are strongly modified during metamorphosis. Whereas the Bolwig organ consists of 12 receptor cells, with 8 cells expressing Rh6 an 4 cells expressing Rh5, the adult eyelet consists of only 4 Rh6 expressing neurons (Sprecher et al., 2007). Interestingly the adult receptor cells derive from the Rh5 positive larval cells, which switch their rhodopsin expression (Sprecher and Desplan, 2008). Besides the change in rhodopsin content the adult eyelet also differs in its transmitter constitution: Besides histamine, which is the common neurotransmitter in the *Drosophila* visual system, a choline acetyltransferase is expressed in the eyelet, suggesting that it uses a second transmitter, acetylcholine, to communicate with postsynaptic cells (Yasuyama and Meinertzhagen, 1999). These postsynaptic cells, among others, were shown to be the clock neurons in both, larval and adult flies, indicating an important function in clock synchronization (Malpel et al., 2002). This function has already been demonstrated in several studies in both developmental stages (Helfrich-Förster et al., 2001; Wegener et al., 2004; Hassan et al., 2005; Veleri et al., 2007; Keene et al., 2011).

However, the previous studies mainly investigated different photoreceptor mutants and suggested the connection between the eyelet and the clock neurons to be functional. In this study we aimed to unravel, how the eyelet contacts the clock neurons and which effect this connection has on the behavior of the fly. This study was done in collaboration with Prof. Orie Shafer.

3.7.2. Material and Methods

Fly strains

Table 14 Fly strains used in "Rhodopsin 5 and 6 expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms in *Drosophila melanogaster*".

Genotype	Source	Reference
w;;rh6-GAL4		BL: 7464
w;UAS-GFPS65T	stock collection	BL: 1522
w^{1118}	stock collection	
w;hdc^{JK910}	stock collection	(Burg et al., 1993)
w;hdc^{JK910}*;rh6-GAL4*		
w; hdc^{JK910}*;UAS-TrpA1*		
w; LexAop-CD4::spGFP11; UAS-CD4::spGFP1-10		(Gordon and Scott, 2009)
w;pdf-lexA		(Shang et al., 2008)
w;;UAS-TrpA1	O. Shafer	(Hamada et al., 2008)

ICC and GRASP

In this study we aimed to use the GRASP (GFP Reconstitution Across Synaptic Partners) technique in order to show direct interaction of the Rh6 expressing photoreceptors and the PDF-neurons. To apply this technique the GFP is split into two parts, which can be separately expressed in two different clusters of cells. To do so we used the UAS-GAL4-system to express UAS-CD4::spGFP1-10 in the *rh6*-expressing neurons. To express the second fragment of GFP we employed the *lexA-lexAop*-system and expressed the fragment in the PDF-positive lateral neurons. However, to avoid false positive signals the lexA and GAL4 lines may not express in the same cells as otherwise both GFP constructs are expressed within the same cell and would allow the split-GFP molecules to reconstitute. Therefore we investigated the expression pattern of *UAS-GFPS65T/+;rh6-GAL4/+* and the proper controls and compared it to PDF staining using the protocol described in section 2.4.1. As primary antibodies we used chicken anti-GFP and mouse anti-PDF. For dilution of antibodies see Table 15.

As GFP and PDF were not expressed in the same subset of cells we applied the GRASP technique using *rh6*-GAL4 and *pdf*-lexA. Brains were dissected and stained as described in section 2.4.1. As the GFP antibody also recognizes the un-reconstituted parts of the GFP protein we only applied anti-PDF in order to avoid false positive signals.

Table 15 Antibodies used in " Rhodopsin 5 and 6 expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms in *Drosophila melanogaster*".

Antibody	Dilution	Reference/Source
mouse anti-PDF	1:1000	C7, Developmental Studies Hybridoma Bank, Iowa, USA
chicken anti-GFP	1:2000	abcam®
Alexa Fluor 488 (goat anti-chicken)	1:200	Invitrogen
Alexa Fluor 635 (goat anti-mouse)	1:200	Invitrogen

Locomotor activity recording

To monitor locomotor activity the DAM system, described in section 2.3.2, was used. In order to investigate the cholinergic input of the H-B eyelet on the clock of *Drosophila* we aimed to eliminate signals deriving from the compound eyes and monitored the behavior in hdc^{JK910} mutant background (Burg et al., 1993). This mutation renders histidine-decarboxylase, an essential enzyme for histamine synthesis, out of function, which hence leads to the absence of histamine in the fly. As described previously, histamine is the neurotransmitter of all eye structures, thus the signaling from these organs is abolished in hdc^{JK910} mutants with exception of the cholinergic signaling from the H-B eyelet. To stimulate the H-B eyelet we expressed the temperature sensitive UAS-*TrpA1* channels using *rh6*-GAL4. At low temperatures this channel is inactive, but as soon as the temperature rises (above 28°C) the ion channels open and depolarize the targeted cells (Hamada et al., 2008). To apply this technique we recorded our experimental (*w;*hdc^{JK910}*;rh6-GAL4/UAS-TrpA1*) and control flies (*w;*hdc^{JK910}*;rh6-GAL4/*+ and *w;*hdc^{JK910}*;UAS-TrpA1/*+) in an incubator (SANYO Incubator MIR-154) for 7d in LD 12:12 at 20°C. In the first night after this LD period we applied a heat pulse (HP, temperature: 30°C) for 2h and afterwards recorded the behavior in DD for 10 more days at 20°C. The

HP was applied at 5 different time points: Two in the subjective day (CT 2-4 and CT 8-10) and three in the subjective night (ZT 14-16, ZT 18-20 and ZT 21-23).

To determine phase differences in behavior caused by the HP we used Chronoshop (freely available, developed by J. Spoelstra). As the offset of activity could not be determined reliably in all flies, we used the center of gravity (COG) to determine the phase of the activity rhythm. First we determined an average COG for entrained conditions (COG_E) by averaging the COGs of the last 3 days in LD. We further analyzed the COG of one day after the HP (COG_1) and two days after the HP (COG_2). For each of the two COGs we subtracted the phase difference caused by the free-running period in order to avoid calculating phase shifts due to a shorter or longer period. We then calculated the phase shift (PS) using the following equation:

$$PS = [(COG_E-COG_1)+(COG_E-COG_2)]/2$$

This analysis was done on single flies and only flies, in which we could reliably determine all COGs plus the free-running period, were used for analyzing the phase shift caused by the activation of the H-B eyelet.

3.7.3. Summary

The aim of this study was to show the physiological connection of the H-B eyelet to the clock neuron network and to unravel its physiological significance for the animal. In order to show possible interaction sites we investigated the *rh6*-GAL4 expression pattern. The synaptic terminals of the Rh6-positive R8 of the compound eyes terminate in the medulla, whereas the axons of the H-B eyelet directly innervate the accessory medulla, in which also the PDF neurons arborize (Helfrich-Förster et al., 2007a). In order to show direct interaction of the PDF-positive neurons, which are located in the lateral brain, and the eyelet we used the GRASP technique and observed reconstituted GFP in the accessory medulla and its ventral elongation, suggesting a direct interaction of the PDF-neurons with the H-B eyelet. In order to investigate, whether the interaction is functional in the adult brain we used an *ex vivo* live-imaging approach on adult *Drosophila* brains with attached lamina and H-B eyelets. We expressed an ATP-receptor, which functions as a ligand-gated ion channel, in the Rh6-positive receptor cells and were thereby able to activate these neurons upon application of ATP (Yao et al., 2012). Employing both the GAL4/UAS-system and the *lexA/lexAop*-system, we could simultaneously image from the lateral clock neurons. Using different second messenger sensors we demonstrate a significant increase of cAMP and Ca^{2+} after the activation of the Rh6-positive neurons, which was restricted to the sLN_v. This response could principally derive from histamine signaling via

the compound eyes and/or the H-B eyelet or from cholinergic signaling stemming from the H-B eyelet. Imaging experiments, in which histamine was bath-applied, did not show any increase of either cAMP or Ca^{2+} in any of the lateral neurons, whereas a previous study by Lelito and Shafer (2012) demonstrated the increase of cAMP and Ca^{2+} upon stimulation with cholinergic agonists, indicating that the responses observed after the activation of the Rh6-positive neurons in our experiments derives from the cholinergic input from the eyelet.

To test whether this cholinergic input of the eyelet is relevant for phase-shifting the circadian clock of *Drosophila*, we investigated locomotor activity rhythms of adult flies after temporal activation of the eyelet. As described in section 1.3*,* a light pulse at a certain time of the subjective night phase shifts behavioral rhythms in the fly. We aimed not to excite Cryptochrome, which causes the main phase shifts by light, and therefore took advantage of UAS-*TrpA1*, an ion channel, which is closed at low, but opens at high temperatures. By applying a heat-pulse to w^{1118};hdc^{JK910};*rh6*-GAL4/UAS-*TrpA1* flies in the subjective night, we temporally activated the Rh6-positive neurons, but simultaneously impaired histamine signaling due to the hdc^{JK910} mutation that blocks histamine synthesis. In this way we could investigate the consequences of the cholinergic input from the H-B eyelet on the phase of the clock. Our experiments show that flies significantly phase-advance their behavioral rhythms, upon activation of the H-B eyelet at two different time-points in the subjective night. This indicates that the cholinergic connection of the H-B eyelet is relevant for phase advancing the clock.

4. Discussion

4.1. The compound eyes mediate behavioral adaptations to more natural light regimes

Two main factors influence spontaneous locomotor activity of animals: 1) environmental stimuli to which animals respond immediately and that allow rapid, adequate changes of behavior (e.g. increasing or decreasing activity) 2) clock controlled processes, which affect the activity cyclically on a longer time scale. Both factors have the adaptive value of confining the animals to their temporal niche (Redlin, 2001). However, in terms of locomotor activity it is often hard to separate these two factors, as spontaneous behavioral responses frequently hide the output of the endogenous clock. Therefore, direct responses of the animals to the environment are also referred to as "masking"-effects (Mrosovsky, 1999). In this study I only focused on the effects of light on locomotor activity of flies, but already simple changes in light conditions evoke both, masking and entraining effects in behavior. As mentioned above, separating these effects is most of the time hard, but studies in various animals already attributed different photoreceptors to masking and entrainment responses: In silk moths and fruit flies an increase in the eclosion rate directly after lights-on was observed. This is regarded as a masking effect, as it disappears as soon as the animals are transferred into constant darkness. Furthermore, this effect was attributed to the compound eyes of the animals, as the effect was absent in *eyes absent* mutants (fruit fly) or when the eyes were removed surgically (moths) (Engelmann and Honegger, 1966; Truman, 1972). Masking effects cannot only be found in the eclosion rhythm; also the adult locomotor activity is composed of masking and entrainment effects, especially under rectangular LD regimes. First of all, absolute darkness during the night strongly suppresses activity, but the most prominent “masking”-effect is a sudden increase in activity when lights are switched on, which is referred to as "startle response" (Hamblen-Coyle et al., 1992; Wheeler et al., 1993). This effect was also shown to depend on functional compound eyes, but not on the blue-light pigment Cryptochrome (Rieger et al., 2003). These results indicate that the compound eyes play an essential role in direct responses of the animals´ behavior and physiology. But their role is certainly not restricted to masking, as only flies lacking all known photoreceptors were not able to entrain to LD-regimes, whereas flies lacking either the compound eyes or Cryptochrome were still able to, indicating that the compound eyes entrain the clock neurons via an up to now unknown mechanism. It was suggested by several groups, that the large ventro-lateral neurons receive light information from the compound eyes, but this has not been proven so far (Helfrich-Förster, 2014).

As the endogenous output of the clock is often masked by direct responses to light, there are different light regimes applicable that encounter this problem and that can be used in behavioral experiments instead of rectangular LD-cycles. One possibility is to change the photoperiod, as the endogenous M and E activity bouts are often uncoupled from lights-on or lights-off under short or long days, respectively. Another possibility is to mimic more natural light conditions, a strategy that we pursued in this study. On the one hand, this provides the possibility to understand the behavioral data of fly locomotor activity recorded in nature-like conditions (Vanin et al., 2012; De et al., 2013) and on the other hand it might enable us to separate masking from real entrainment. Our first attempt was to investigate the effect of moonlight, as our lab already obtained promising results in the past: moonlight simulation at night significantly reduced the startle response at lights-on and the inhibition of activity during the night vanished completely. On the contrary, moonlight even stimulated nocturnal activity (Kempinger et al., 2009). In addition, it advanced the M and delayed the E activity peak, respectively. (Bachleitner et al., 2007). Both effects depend on the presence of the compound eyes, as eyeless flies neither shift their activity maxima nor increase nocturnal activity in LM conditions. Even though both effects are caused by the compound eyes, they belong to different pathways: The shifts in peak timing are caused by an entraining signal, as it coincides with phase changes of PER/TIM cycling in clock neurons controlling M and E activity (Bachleitner et al., 2007). On the other hand the increase of nocturnal activity is a direct response to dim light at night, as it is also present in clock mutants (Kempinger et al., 2009). Therefore LM-cycles appear to be an ideal model for studying the contribution of different receptor cells in masking and/or entrainment. In the present study WT flies showed in general the same behavioral changes as described previously. However, the differences in nocturnal activity and E peak timing were not as severe as described in Bachleitner et al. (2007), probably because of the more precise way of monitoring locomotor activity in the present study (Schlichting et al., 2014). Whereas in the former study only a "yes-or-no"-response within a four-minute interval was counted as activity, we were able to register the exact number of beam crosses in one-minute intervals, leading to a quantitative measurement of activity levels. Nevertheless, even though there are quantitative differences between the two studies, we could again demonstrate that the compound eyes are essential for the increase of nocturnal activity as well as the shift of M and E peaks. By investigating different rhodopsin mutants, we aimed to separate masking from entrainment and to confine a certain function to each of the receptor cells, which, unfortunately, turned out to be impossible even under LM conditions: only flies, in which we manipulated outer and inner receptors (*ninaE*^{17}*rh6*^{1}) were neither able to increase nocturnal activity nor delay their E peak, whereas mutants lacking inner or outer receptors (*ninaE*^{17} and

rh5^{2};*rh3*^{1}*rh4*^{1}*rh6*^{1}) alone were not able to delay their E peak but were still able to slightly, but significantly, increase nocturnal activity levels upon moonlight simulation (Schlichting et al., 2014). These findings suggest that inner and outer receptors work together in fine-tuning behavioral adaptations to moonlight simulation. A "cooperation" between different photoreceptor cells as the here reported one between inner and outer receptors had already been proposed by several studies (Jacob et al., 1977; Yamaguchi et al., 2008; Yamaguchi et al., 2010). The sophisticated study of Wardill et al. (2012), which combined genetic manipulation of rhodopsin expression with electrophysiological and behavioral experiments, finally confirmed a connection between R8 and R1-6. Hence, we were not able to assign masking/entrainment pathways to specific receptor cells of the compound eyes, but were able to show a complex interaction of all receptor cells in adjusting *Drosophila´*s activity pattern.

Along our aim to unravel the significance of more natural light conditions for the locomotor activity pattern of *Drosophila*, we continued with the investigation of twilight conditions. We did so, as a previous study had shown that the M peak is significantly delayed, whereas the E peak is significantly advanced when twilight is simulated and that both peaks occur during dawn and dusk, respectively, at a light intensity of approximately 5 lux (Rieger et al., 2007). However, this previous study left some open questions: 1) Are the shifts of M and E peaks directly mediated by the increase/decrease of the light intensity (masking) or are they caused by a shifted PER/TIM cycling in the clock neurons as observed in moonlight conditions? 2) Which photoreceptors contribute to these behavioral changes? To answer the first question, we recorded the clock mutants *per*^{01} and *tim*^{01} under rectangular light-dark-cycles (LD) and light regimes simulating gradual increases/decreases of light intensity in the morning or evening, respectively (LDR). Under LD conditions the mutants showed the startle response to lights-on, but lacked WT-like siesta or the anticipation of E activity. This, however, changed upon simulation of twilight: Both mutants exhibited an activity pattern with an M activity maximum around dawn and an E activity maximum around dusk. Especially when light intensity increased/decreased within 4.5 h each, the activity patterns of WT and clock mutants became almost indistinguishable from each other (with the exception of reduced activity during midday and night in WT) (Schlichting et al., submitted-b). This is in accordance to studies of flies and mice in the wild, as these experiments revealed that the behavior of clock mutants is rather similar to WT animals under natural conditions, a finding that had not been observed in standard rectangular LD regimes in the lab (Daan et al., 2011; Vanin et al., 2012). Interestingly, we observed the same phenomenon in the lab by changing only the light regime to a more natural condition. This indicates that especially the gradual changes of light intensity during dawn and dusk are important signals guiding

the behavior of animals. In nature, the activity pattern of clock mutants might appear even more similar to WT, as other Zeitgebers like temperature and humidity also cycle in the course of a day and therefore also influence the animals behavior either directly (masking) or as an entraining signal. Taken together these results suggest that the sharp peaks during dawn and dusk observed in Rieger et al. (2007) are masking effects with the increase/decrease of light intensity directly influencing the fly´s activity. However, not all of the fly´s behavioral changes are masking effects, as we also observed differences in daily TIM cycling in M and E cells. Even though the timing of the staining intensity maximum (= protein maximum) did not change, TIM accumulated earlier in the cytoplasm and was detectable for longer time after lights-on in the nucleus (Schlichting et al., submitted-b).

But, are these changes in TIM-cycling mediated by the compound eyes or by CRY which is present in half of the clock neurons? We were able to show that CRY is responsible for the change in TIM cycling, whereas the sharp peaks in activity around dawn/dusk are singularly caused by the compound eyes. This again suggests that the compound eyes are important organs causing direct responses such as the startle response, the increase of nocturnal activity in moonlit nights as well as the shift of the activity into dawn/dusk when twilight is simulated (Wheeler et al., 1993; Kempinger et al., 2009).

Summing up, the results obtained so far show one thing very clearly: Twilight and moonlight have antagonistic effects on the behavior of *Drosophila* with twilight shifting the activity peaks into the day and moonlight shifting them into the night. We further know that a high portion of the behavioral changes are directly mediated by light input deriving from the compound eyes. However, the question remains, which light stimulus is the dominant one and which photoreceptors of the compound eyes mediate twilight responses. To unravel this, we investigated flies in a condition simulating dawn and dusk during the day and applying moonlight at night (LMR). Our results indicate that twilight dominates over moonlight in adjusting the activity pattern of *Drosophila*. Even though the nocturnal activity was still increased in moonlit nights compared to dark nights, the activity spent during the night was significantly reduced in LMR compared to rectangular LM cycles. On the other hand all activity peaks occurred during dawn or dusk, respectively, and were not shifted into the night. The investigation of different photoreceptor mutants confirmed our previous results with the compound eyes being essential for twilight and moonlight detection. However, the phase-advancing effect of the E peak seems to depend on the inner receptor cells R7 and R8, whereas the reduction of nocturnal activity again seems to be due to an interaction of inner and outer receptor cells, with a special importance of Rh6 (Schlichting et al., in press).

The open question remains, whether the behavior we observed in LMR conditions is comparable to the behavior of flies in nature. A previous study showed that in spring and autumn flies display a bimodal activity pattern in nature with an M and an E peak occurring during dawn/dusk respectively, which we were able to reproduce in our experiments in the lab (Vanin et al., 2012; Schlichting et al., submitted-b). In summer a third activity peak appeared in nature, the so called afternoon (A) peak, which seemed to be clock controlled, as it was differently phased in short and long *period* mutants. In our experiments, in which exclusively overall light intensity and the dynamics of light intensity were altered, we were not able to observe this A peak in any of our experiments. This appears to be due to the low temperature of 20°C that we applied in our studies. By mimicking hot summer days in the lab Menegazzi et al. (2012) demonstrated that the A peak only occurs when temperature rises above 30°C, which appears to be disadvantageous for the flies and cause an escape response. The clock appears to modulate (suppress) this response, as clock mutants showed a significantly higher A peaks compared to WT flies. A second astonishing result of Vanin et al. (2012) was that the clock mutants per^{01} and tim^{01} appeared to behave almost identically to WT flies in nature. This is not only restricted to *Drosophila*, but identical results were also observed in mice being monitored outdoors (Daan et al., 2011). In our twilight experiments we were able to observe similar things. Especially when the increase/decrease of light intensity was performed over 4.5 hours each, WT flies and clock mutants behaved almost identically. This astonishing behavior appeared to be mediated by light input from the compound eyes, which directly stimulates the activity of the fly, independent of a functional clock (Schlichting et al., submitted-b). Similar mechanisms might have caused the WT behavior of clock mutants in the outdoor experiments. However, the synchronization of the animals´ behavior might have been masked even stronger in the experiments of Vanin et al. (2012) as not only light was altered in this case, but also temperature, humidity and noise are cycling during the change of day and night. These factors might also directly affect the behavior of flies and mice in the wild. However, the almost WT-like behavior of per^{01} and tim^{01} might not only be mediated by masking, as several studies discuss a residual clock in these mutants (Helfrich and Engelmann, 1987; Yoshii et al., 2002; Collins et al., 2005). As the molecular clock mechanism is composed of several interlocked feedback loops, these mutations only interfere with the core-loop, allowing the second interlocked loop to retain some residual clock function. The reason for this discussion is that single tim^{01} or per^{01} mutants sometimes still exhibit a bimodal activity patterns and are able to maintain a rhythm for one or two days in DD before they get arrhythmic (Bywalez et al., 2012). Also in the present study especially some of the tim^{01} flies showed M and E activity peaks, even though the reduction of the siesta was not

as prominent as in WT flies (Schlichting et al., submitted-b). If these mutants indeed possess a residual clock, multiple Zeitgebers in nature might additionally improve the synchronization of the fly´s behavior. Therefore it might be worth to investigate mutants, which are expected to display a stronger phenotype, as their gene products represent key features in several feedback loops, such as clk^{JRK}, clk^{AR} or cyc^{01}. We also recorded clk^{JRK} flies in twilight conditions (data not shown) and just like tim^{01} and per^{01} the flies showed sharp peaks around dawn and dusk. However, this particular mutant also carries some disadvantages: On the one hand it is marked by a *scarlet* (st^{1}) mutation affecting the pigmentation of the eye, which is disadvantageous when investigating an effect mediated by the compound eyes. On the other hand the flies show a significantly higher dopamine signaling, which is thought to cause high levels of activity during the night (Kumar et al., 2012). Nevertheless the mutant shows an M activity peak during dawn and an E activity peak during dusk, again stressing the hypothesis that these sharp peaks in twilight conditions are caused by the gradual changes of light intensity.

Even though a lot of our results fit to the results of Vanin et al. (2012), flies recorded outdoors never became nocturnal like WT flies did under LM conditions in the lab. Furthermore, Vanin et al. (2012) did not observe a difference in nocturnal activity in full-moon or new-moon nights, which argues against our theory of nocturnal light stimulating the activity of the flies. One reason for this difference might be the low temperatures during the night outside, which would cause a waste of energy for the flies when being active, in contrast to the temperature in our lab experiments, which was set to 20°C during day and night. A second reason, why the authors were not able to identify any difference between full-moon and new-moon nights, might be that in nature complete darkness is never achieved: Even though moonlight intensity varies, starlight or "light pollution" from the surrounding cities/villages is always present. More studies in the lab systematically varying "moonlight" intensity would be necessary to investigate this further. In this respect, another interesting question arises: how big does the difference in light intensity have to be between day and night in order to allow the fly to interpret the light regime as "day and night" or constant light. On the one hand the *Drosophila* clock is extremely light sensitive due to the photon-integrating function of CRY (Hirsh et al., 2010; Vinayak et al., 2013). On the other hand similarly high light intensities during day and night would degrade TIM in similar ways, thereby strongly interfering with the molecular clock mechanism. A third reason why these high levels of nocturnal activity are not present in outdoor experiments might be due to the dominating effect of twilight over moonlight. In our lab experiments we were able to show that the E peak of WT flies always occurs during dusk as soon as twilight is simulated (Schlichting et al., in press). Another effect of simulated twilight is the

reduction of activity during the night, which might be even more obvious in nature as low temperatures during the night inhibit fly activity.

4.2. The compound eyes and CRY in the clock neurons have antagonistic effects on timing *Drosophila* behavior

Even though the compound eyes mediate many direct responses to light, their function is not restricted to masking. Only flies lacking signaling from Cryptochrome and the visual system are "circadianly blind", meaning that they fail to entrain to the changes of light and dark (Helfrich-Förster et al., 2001). Flies lacking CRY (cry^{01}, cry^{b}) are still able to entrain to LD cycles, although the re-entrainment to shifted LD-cycles takes longer than in WT flies (Stanewsky et al., 1998; Emery et al., 2000; Dolezelova et al., 2007). Furthermore, flies lacking CRY still show shifts in their free-running behavior when a light pulse is applied during the subjective night, even though the amplitude of this shift is severely reduced compared to WT flies (Kistenpfennig et al., 2012). All of these results indicate that CRY within the clock neurons is an essential component of the *Drosophila* light entrainment pathway, but that the compound eyes suffice to entrain the circadian clock to the changes of day and night via a so far unknown mechanism. It is proposed that the eyes contact the large ventro-lateral neurons, as these send their projections into the medulla, the region in which the axons of receptor cell 7 and 8 terminate (Helfrich-Förster, 2014). However, it is unknown, whether this contact is direct or mediated by inter-neurons exacerbating the investigation of visual input to the clock.

The opposite is true for CRY, for which the interaction with the core clock components is well understood: CRY is activated by light and directly binds TIM. This interaction leads to the degradation of TIM in the proteasome and hence resets the clock to the changes of day and night (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996; Ceriani et al., 1999). The investigation of *trp*- or *trpl*-mutants, which prevent the photoreceptor cells from depolarizing, revealed, that the flies are still able to synchronize to the changes of day and night but show a perturbed entrainment (Yang et al., 1998). The same was true for flies lacking the compound eyes (cli^{eya}) or compound eyes plus ocelli (so^{1}). Even though CRY is still present in the different clock neuron clusters, these flies showed perturbed entrainment under LD12:12 (Helfrich-Förster et al., 2001). Especially the investigation of eyeless mutants under long and short photoperiods revealed that CRY alone is not able to entrain the behavioral rhythms of *Drosophila* in an appropriate way, as 70% of the cli^{eya} and so^{1} flies were not able to entrain to LD 20:4, but showed free-running behavior with a longer period instead (Rieger et al., 2003). Taken together, these studies strongly suggest that both, the visual system and CRY contribute

to the entrainment of the fly. However, under certain conditions, none of these pathways is essential for entraining the flies´ behavior, as either of them suffices to entrain the locomotor activity rhythm of the fly. How the two different mechanisms interact or lead to wild-type behavior remains unknown.

Here, I investigated the behavior of WT, eyeless and CRY-less flies under LD and LM conditions. Already under LD 12:12 the flies appeared to behave differently: whereas the E peak of $WT_{CantonS}$ was masked by lights-off, the E peak of eyeless flies was significantly advanced compared to the control, indicating that the light input from the eyes to the clock phase-delays the E peak (Schlichting et al., 2014). On the contrary, the E peak of cry^{01} flies was not significantly different from WT. However, the onset of E activity appeared later in the mutant and the E peak appeared not to have reached its full height, when lights were switched off. This indicates that cry^{01} mutants have a late E activity but that the inhibition of activity by complete darkness might hinder the E peak from appearing during the night (Schlichting et al., submitted-a). This inhibition of activity in darkness disappears, however, in LM conditions. As described previously, moonlit nights lead to an increase of nocturnal activity, an advance of the M and a delay of the E peak compared to completely dark nights (Bachleitner et al., 2007; Kempinger et al., 2009). Investigating the above mentioned flies under LM conditions showed a more severe phenotype: WT flies significantly delayed their E peak by approximately 30 min, meaning the E peak occurred shortly after lights-off at the beginning of the night. On the contrary, *eyes absent* mutants did not change their E peak timing upon moonlight simulation, clearly showing that the compound eyes, but not Cryptochrome are needed for phase delaying the E peak in moonlit nights (Schlichting et al., 2014). As the E peak was already significantly advanced in *eyes absent* flies compared to $WT_{CantonS}$ in LD, this difference appeared to be even bigger under LM conditions. Eyeless flies still showed an advanced E peak, whereas WT flies significantly delayed it. However, from this we are not able to conclude that this phase-delaying effect is singularly mediated by the compound eyes. Flies lacking CRY showed a significantly delayed E peak in LM conditions compared to WT flies, which is consistent with the finding that the E peak appeared to be not at its real maximum in LD. These findings strongly suggest that the compound eyes and CRY have antagonistic effects on timing the E peak: Whereas CRY phase advances the E peak, meaning it keeps the peak during the day, the compound eyes appear to have a delaying effect (Schlichting et al., submitted-a). Assuming this hypothesis is right we are able to explain our results in an appropriate way: *Eyes absent* flies lack the phase-delaying effect from the compound eyes and therefore the phase-advancing effect remains, leading to an advanced E peak compared to WT flies. In contrast, cry^{01} flies only retain the phase-delaying effect of the compound eyes but lack the phase advancing-effect of CRY and

therefore show an E peak significantly later compared to WT flies. In WT flies both effects are present, which leads to an intermediate response, which is the case as the activity peak of $WT_{CantonS}$ was in between *cry*^{01} and *eya*^{2}. Along this argumentation the investigation of long photoperiods should give similar results with an E peak occurring significantly earlier in eyeless flies and significantly later in *cry*^{01} flies. Indeed, eyeless flies have been shown to have an early peak under long photoperiods (Rieger et al., 2003). Furthermore, very recently, Yoshii et al. (2015) were able to show that the E peak of *cry*^{01} flies appeared significantly later than in controls under long photoperiods. Moreover this appeared to be independent of the compound eyes, but singularly caused by CRY in the E cells (3 LN_d and 5th sLN_v), which was shown by rescuing CRY using different GAL4 driver lines that express in different neuronal clusters. These results strengthen our hypothesis.

Interestingly, Yoshii et al. (2015) found that it is CRY in the evening neurons that controls the timing of the E peak. In their experiments they used the GAL4-UAS system to rescue CRY in these neurons. In our moonlight experiments we were also able to show that CRY in the E neurons is essential for phase-advancing the E peak by using a different approach. In our experiments we used RNA interference to knock down CRY specifically in the CRY positive LN_d and the 5th sLN_v that belong to the E neurons. We were able to show by ICC that this knockdown drastically reduces CRY levels in these cells, showing that the UAS-*cryRNA*i line is sufficiently working. By knocking down CRY in the E neurons we were able to reproduce the phenotype of *cry*^{01} in LM, strongly supporting the idea of Yoshii et al. (2015) that CRY in the E cells is important for phase-advancing the E peak (Schlichting et al., submitted-a). The question remains, however, how the compound eyes contribute to clock synchronization. For a long time it has been hypothesized that the compound eyes signal to the lLN_v which then integrate these signals (Helfrich-Förster, 2014). Electrophysiological recordings from lLN_v showed that the neurons respond to the application of histamine, the neurotransmitter of the compound eyes, with a reduction in firing rate (personal communication of Dr. Edgar Buhl), strongly supporting this hypothesis. Furthermore, *hdc*^{JK910} mutants showed a significantly advanced E peak already in LD 12:12, indicating that the histamine signaling from the compound eyes via the lLN_v is able to phase delay the E peak (Schlichting et al., close to submission). However, the lLN_v do not belong to the neurons controlling the phase of the E peak and therefore a connection between the lLN_v and the E neurons must exist. This connection is most probably mediated via PDF, as *pdf*^{01} mutants show a significantly advanced E peak in LD, just like flies lacking input from the compound eyes do (Renn et al., 1999; Yao and Shafer, 2014). The inhibition of the PDF-neurons by histamine might affect PDF release and therefore alter the communication to PDF-receptor (PDFR)

positive neurons. Further experiments rescuing the PDFR in specific groups of neurons will be useful to answer this question. An indication for this is already included in the study of Bachleitner et al. (2007): In this study the PER staining intensity maximum appeared significantly later under moonlight conditions compared to rectangular LD cycles in the 5th sLN_v (one of the E neurons). Latest studies showed, however, that PDF leads to an increase of cAMP in PDFR-positive neurons and hence stabilizes PER (Li et al., 2014). Therefore, the delay of PER cycling might be caused by the stabilization of PER via PDF signaling from the lLN_v. Considering the present results and those of Yoshii et al. (2015), Bachleitner et al. (2007) and Rieger et al. (2003) together, it becomes evident that CRY in the E neurons and the compound eyes work antagonistically on timing the E activity bout.

4.3. CRY in the compound eyes contributes to visual input

As described in section 1.3, CRY is an essential component of the light-resetting mechanism of the circadian clock. Upon illumination CRY undergoes a conformational change, gets activated, binds TIM and leads to its degradation via the proteasome. However, the mechanism how this conformational change is achieved remains so far unknown (Ozturk et al., 2014). Studies ablating the C-terminal 20 amino acids (aa) tail render CRY constitutively active, which led to the suggestion that light at least affects these 20 aa in wild-type CRY (Dissel et al., 2004). However, several studies focused on the conformational change of Cryptochrome. Purified dCRY contains the two-electron oxidized FAD_{ox} form. Upon illumination with blue light, which is the main absorption range for CRY, this oxidized form is transferred into a semiquininone (FAD^{-}) and this transition has been thought to cause the conformational change (Berndt et al., 2007). However, a recent study challenged this point of view, suggesting that the oxidized form of dCRY is caused by the purification protocol of the protein, assuming that some other mechanism must be responsible for the conformational change (Kavakli and Sancar, 2004; Ozturk et al., 2014). Even though the detailed mechanism of how CRY is activated by light is still not understood, its function in clock synchronization or magneto-sensation has been described by several studies (Yoshii et al., 2009; Fedele et al., 2014). In collaboration with Prof. Rodolfo Costa we were now able to unravel an additional role of CRY in the visual system. Yoshii et al. (2008) already demonstrated that CRY is highly expressed in the compound eyes, although the sub-cellular location was not determined. In our studies we were able to show that CRY is present in all photoreceptor cells and that it is not only located in the cell bodies, but also within the rhabdomeres, the site where the phototransduction cascade takes place (Schlichting et al., submitted-a). Whereas light leads to rapid CRY degradation in the clock neurons (Hunter-Ensor et al., 1996; Myers et

al., 1996; Zeng et al., 1996), CRY appeared to be stable within the rhabdomeres of the compound eyes. This suggests that either the ubiquitin-ligases necessary for degradation via the proteasome are not expressed in the compound eyes or that CRY is actively bound to the rhabdomere and hence is protected from degradation. The stability of CRY even after illumination suggests that the molecule could indeed be involved in phototransduction (Mazzotta et al., 2013). This hypothesis is strengthened by *in silico* analysis as well as yeast-2-hybrid and CoIP experiments, confirming that CRY is able to interact with members of the phototransduction cascade via the scaffolding protein inaD as well as with F-Actin. Together with the subcellular localization of CRY within the rhabdomeres this strongly suggests that CRY might affect vision (Mazzotta et al., 2013; Schlichting et al., submitted-a). And indeed cry^{01} mutants showed significantly reduced levels of phototaxis as well as optomotor response (Mazzotta et al., 2013). However, these behaviors are far downstream of the processing cascade of visual input to the brain and many other aspects might contribute to changes in behavior of cry^{01} mutants. One of these factors might also be the processing of light information in the different optical neuropils. The axons of R1-6 terminate in the lamina, the first optic neuropil. Recent studies showed that these are important for dim light and motion detection, indicating that the processing within the lamina might already influence the performance index in optomotor response experiments (Yamaguchi et al., 2008). Several studies showed that the lamina is a site of pronounced circadian plasticity and that rhythmical morphological changes in interneuron- and glia-size take place (Pyza and Meinertzhagen, 1995). Further analysis revealed that CRY mediates the cycling of a Na+/K+-ATPase α subunit in the lamina, which could affect visual behavior (Damulewicz et al., 2013). The contribution of this neuronal plasticity on the phototaxis and/or the optomotor response should be, however, rather negligible, as we were able to fully rescue the behavioral phenotype of cry^{01} flies by expressing CRY only in R1-6. The same is true for locomotor activity rhythms: WT flies significantly increased nocturnal activity with increasing daylight intensity, which was less pronounced in cry^{01} flies. This phenotype was also rescued by expressing CRY within R1-6 (Schlichting et al., submitted-a). With the help of our newly cantonized cry^{01} mutant we were further able to investigate the ERG response of this mutant. Interestingly CRY seems not to work as a photoreceptor per se, but appears to modulate light adaptation. One possible mechanism would be that CRY keeps the signalplex close to the F-Actin filaments in the rhabdomere. This would be possible as yeast-2-hybrid and CoIP assays showed that CRY is able to interact with three different forms of F-Actin, which are encoded by different genes in *Drosophila*. Further studies proving the role of CRY in phototransduction will be necessary, but our data strongly suggest that CRY is able to alter visual inputs from the compound eyes to the central

nervous system. This input appears to be strong enough to alter visual behavior and influence the ratio of diurnal/nocturnal activity in locomotor activity rhythms.

4.4. The Hofbauer-Buchner eyelet acts as a functional photoreceptor to entrain the clock

Besides the compound eyes and CRY, *Drosophila* possesses two additional types of photoreceptive organs: three ocelli on the vertex of the head in between the two compound eyes and two Hofbauer-Buchner eyelets which are located on the surface of the lamina in both hemispheres. Even though the ocelli are located rather close to the dorsal neuron clusters of the circadian clock, so far no connection between the two systems is known. On the contrary, the axons of the H-B eyelets directly innervate the accessory medulla, to which most clock neuron clusters send arborizations. Such an overlap with fibers from the clock neurons is also observed for the Bolwig organ (BO), the precursor of the H-B eyelet in larvae (Helfrich-Förster et al., 2002). In this case, even a functional connection between BO and the clock neurons (sLN_v) has been demonstrated (Wegener et al., 2004) that might persist into adulthood. However, during metamorphosis BO undergoes several changes: 1) Its location moves from anterior to the margin of the lamina, 2) only four of the 12 larval cells building up Bolwig organ persist into adulthood, 3) these four neurons switch their photopigment from Rh5 to Rh6, and 4) a switch of the neurotransmitter from acetylcholine to histamine occurs (Helfrich-Förster et al., 2002; Sprecher et al., 2007; Sprecher and Desplan, 2008). Nevertheless, the switch in neurotransmitters may be incomplete, since the adult H-B eyelet still expresses the enzyme choline acetyltransferase that is necessary for acetylcholine synthesis (Yasuyama and Meinertzhagen, 1999). Thus, the H-B eyelet may use acetylcholine in addition to histamine. Indeed, Lelito and Shafer (2012) found that the sLN_v of adult flies still respond to acetylcholine as they do in larvae; but, they did not respond to histamine. To test, whether this acetylcholine stems from the H-B eyelet, we electrically activated the eyelet and simultaneously measured cAMP and Ca^{2+} in the sLN_v and lLN_v. This study was done in collaboration with Prof. Orie Shafer and clearly showed that the eyelet activation provokes a rise of cAMP and Ca^{2+} in the sLN_v but not in the lLN_v (Schlichting et al., close to submission). This strongly suggests that the release of acetylcholine from the H-B eyelet is responsible for this neuronal response. This would make sense, as our investigation of the *rh6*-GAL4 expression pattern clearly showed that the axons of the eyelet terminate in the accessory medulla close to the sLN_v. GRASP experiments further suggested that the eyelet and the PDF-neurons share synapses (Schlichting et al., close

to submission). Even though GRASP is not a direct proof of synaptic interaction, the physiological response in the sLN_v to the eyelet activation strongly suggests that this interaction is direct. Furthermore, in the blowfly *Protophormia terraenovae* electron microscopy studies revealed synapses between the Pt-eyelet (the extra-retinal eyelet of the blowfly) and the PDF-neurons in the fly, suggesting that this connection might also be direct in *D. melanogaster* (Yasuyama et al., 2006). To further confirm the role of this acetylcholine mediated response of the clock, we also recorded locomotor activity rhythms of the fly. By activating the *rh6*-GAL4 neurons in the hdc^{JK910} mutant background we were able to narrow down the signaling to the eyelet via acetylcholine. Our results show that the activation of the eyelet slightly phase shifts the activity rhythm of the fly. We found significant advances of the rhythm when the eyelet was activated around ZT14 or ZT22 (Schlichting et al., close to submission). This suggests that the H-B eyelet is most probably only able to phase-advance the clock, which is in contrast to previous studies showing that the eyelet mainly mediates phase-delays (Helfrich-Förster et al., 2002). The main difference between the two studies lies in the investigated mutants: In order to draw conclusions on the eyelet Helfrich-Förster et al. (2002) compared so^1 (lacking compound eyes and ocelli) and so^1 gl^{60j} (lacking compound eyes, ocelli and H-B eyelet) flies in their ability to phase delay their activity rhythm and showed, that the eyelet is important for phase-delaying the clock. However, a more recent study showed that *glass*-mutants also lack a part of the dorsal clock neurons, which could be one reason for the differences in phase shifting between the two investigated genotypes (Helfrich-Förster et al., 2007a). The significance of the H-B eyelet for clock synchronization, especially in phase delaying the clock, was further analyzed by Veleri et al. (2007). They did not use anatomical mutants in order to exclude signaling from the compound eyes, but used the $norpA^{P41}$ mutant, which is a mutant for phospholipase C and hence blocks phototransduction without degeneration of the compound eyes. In addition they introduced a cry^b mutation so that only input via the H-B eyelet is left. Their study showed that flies lacking signaling from the compound eyes and CRY are still partially able to entrain to a light-dark cycle. Surprisingly these flies showed a lights-on response, which we did neither see in our *eyes absent* flies nor in flies being deficient in histamine signaling. When the authors further blocked signaling of the *rh5*-expressing neurons using TTX, none of the flies was able to re-entrain to a phase delay of 6 hours. The authors attributed this to the signaling of the H-B eyelet on the clock, but by expressing TTX using *rh5*-GAL4 also 30% of R8 in the compound eyes are affected. In our study we were nicely able to show that R8 does contribute to the entrainment of the clock, as flies lacking the photopigments in R8 ($rh5^2;rh6^1$ mutants) showed a significantly advanced E peak in LD 12:12, just like flies lacking signaling from the eyes (Schlichting et al., 2014; Schlichting et al., in press).

Therefore the influence of the compound eyes cannot be neglected, especially since a recent study showed that signaling from Rh5 and Rh6 expressing neurons might be independent of phospholipase C (Szular et al., 2012). If true, this would mean that *norpA*^{P41} flies still retain reduced signaling from the compound eyes, which would fit to the behavioral lights-on response observed in the mutants. Therefore, the lack in phase delaying might also derive from the input of the compound eyes, as we were nicely able to show that input from the compound eyes is important for phase delaying the activity of the flies (Schlichting et al., submitted-a).

Whereas all up to now published data rather suggest a phase-delaying effect of the eyelet, our data strongly suggest a phase advancing effect. In the previous studies the conclusions were drawn either by completely disrupting the visual system or by blocking the signaling from the H-B eyelet using TTX, whereas we activated the eyelet in the *hdc*^{JK910} mutant background. This means that the previous studies compared flies with an intact eyelet and flies lacking the eyelet, whereas we only blocked one of the possible signaling mechanisms via histamine, but retained acetylcholine signaling. Thus, a possible explanation for the discrepancy between our data and previous studies is that the eyelet is able to phase-delay the clock via histamine and phase advance the clock via acetylcholine. This may be possible by contacting different neurons, as our GRASP staining did not only show labeling in the accessory medulla but also in its ventral elongation. Only the lLN_v arborize in the ventral elongation suggesting that the eyelet is also able to communicate to the lLN_v via histamine thereby causing phase delays. As stated above Dr. Edgar Buhl observed a reduced firing rate in the lLN_vs when applying histamine (personal communication), which can also explain the different responses of the clock to the two neurotransmitters: whereas ACh activates the neurons and leads to increases of cAMP and Ca^{2+} in the sLN_v, histamine reduces the firing and therefore silences the lLN_v. Further studies will be necessary to unravel the function of the H-B eyelet in clock synchronization. However, for such future studies an eyelet-specific driver line would be necessary as the differentiation between signaling from the compound eyes and/or the eyelet will be hard to achieve otherwise.

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6. Papers and manuscripts

6.1. Photic Entrainment in *Drosophila* Assessed by Locomotor Activity Recordings

CHAPTER FIVE

Photic Entrainment in *Drosophila* Assessed by Locomotor Activity Recordings

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Abstract

Light is the most important Zeitgeber to entrain the circadian clock of the fruit fly *Drosophila melanogaster* to the 24-h cycle on earth. The fruit fly's circadian clock is very light sensitive, mainly because about half of the 150 clock neurons in the fly's brain express the blue-light photopigment, Cryptochrome, which provokes an immediate degradation of the clock protein Timeless upon activation by light. Consequently, *Drosophila's* molecular clock can reset very fast to measure the changes in environmental-lighting conditions. However, usually the responses of the molecular clock to light are not directly measured, but conclusions about entrainment of the circadian clock are drawn from recording the flies' locomotor activity rhythms. Here, we review how the flies' locomotor activity can be recorded under different light regimes and how entrainment can be analyzed and properly judged. We also summarize the influence of different recording and lighting methods on the flies' activity pattern, highlight their advantages and disadvantages, and stress general pitfalls.

Methods in Enzymology, Volume 552
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2014.10.017

1. INTRODUCTION

The fruit fly *Drosophila melanogaster* is a powerful model organism to understand entrainment of the circadian clock on the molecular, cellular, and behavioral levels. Like a mammal, the fly possesses molecular circadian oscillators throughout its body (most organs and virtually all sensory cells) and its head (eyes, olfactory system, and ~150 central brain clock neurons). The clock neurons in the brain get direct and indirect light input from the Hofbauer–Buchner Eyelets and the compound eyes, and about half of them express the blue-light pigment Cryptochrome (dCRY; reviewed in Hall, 2005; Helfrich-Förster, 2002). Altogether, this makes the fly's clock neurons rather light sensitive. Upon light, dCRY gets activated and leads to immediate degradation of Timeless (TIM; reviewed by Dubruille & Emery, 2008). Since period (PER), the dimer partner of TIM, is rather unstable without TIM; the entire molecular cycle is reset every morning when lights turn on (under light–dark cycles of 12 h:12 h). TIM and PER stay low during the day, start to accumulate at the beginning of the night in the cytoplasm of the clock neurons, enter the nucleus in the middle of the night and reach their maximum at the end of the night, before they get degraded again in the morning (reviewed by Hardin, 2011). The clock neurons control the behavioral rhythms of the fly, of which the locomotor activity rhythm is easy to record and, therefore, best investigated.

Usually the locomotor activity rhythm is taken as clock output suited to judge the state of the circadian clock in the brain—including its entrainment to light–dark cycles. In most cases, this simplification is justified, but one has always to bear in mind that the locomotor activity of the fly is not only controlled by the clock. The fly has to respond with activity or inactivity to immediate changes in its environment, including changes in irradiation. Furthermore, activity does strongly depend on the internal state of the fly: A hungry or thirsty fly will search for food and thus be very active, perhaps not caring at all about the signals of the clock. In summary, the clock's control on the locomotor activity can be completely masked by responses of the fly to external and internal cues. Here, we will focus on putative "masking effects" of light (reviewed by Mrosovsky, 1999) and try to distinguish these from real clock entrainment by light.

This is a practical manual on how to use locomotor activity for judging entrainment of the fly's circadian clock. We will not provide a review of the literature of how photoentrainment works in the fly, because this is covered

by others (e.g., Dubruille & Emery, 2008; Hall, 2005; Johnsson, Helfrich-Förster, & Engelmann, in press). For monitoring an action spectrum to reveal the relevant photopigments of circadian entrainment, please see Peirson, Thompson, Hankins, and Foster (2005).

In order to investigate entrainment by light it is essential to keep all other putative Zeitgebers constant during the whole experiment and to completely shield the flies from external light. To avoid fluctuations in temperature, the experiments should be performed in a temperature-controlled chamber or an incubator. Temperature cycles are known to entrain the clock quite well (Glaser & Stanewsky, 2007; Lee & Montell, 2013; Sehadova et al., 2009). Regular vibrations should be avoided as well (e.g., regularly switching on/off the ventilation system), since very recent studies showed that vibrations can work as a Zeitgeber (Simoni et al., 2014). Last but not least, humidity should be kept constantly at ~60%, which can be achieved by placing a small water tank into the incubator. Please also note that not all commercially available incubators are completely light tight. Light may enter through the exits of the electrical cables as well as through the seal around the incubator door. Due to the high light sensitivity of the flies, such tiny openings are enough to influence the flies' activity and should be closed by black tape or covered by other light-tight material.

2. DIFFERENT LIGHT REGIMES USED TO ENTRAIN LOCOMOTOR ACTIVITY OF FRUIT FLIES

In the following, we will give an overview over the different light regimes that have been used to entrain the flies' activity. As a general rule, each light regime should be applied for at least 1 week to get a stable entrainment. To distinguish whether a fly is entrained or just free-running with a period of 24 h, the light regime can be shifted by several hours after 1 week of entrainment. In case of real entrainment, the activity should follow the shift, which may take several days depending on the flies' genetic background. Another possibility to test for real entrainment is to release the flies into constant darkness (DD) after entrainment. Under DD, the free-running activity should continue from the entrained activity (for examples, see Helfrich-Förster, Winter, Hofbauer, Hall, & Stanewsky, 2001).

2.1. Rectangular light–dark cycles

During rectangular light–dark cycles the lights are simply switched-on and -off. Since this is easy to do, most studies use such light regimes for

synchronizing the flies. However, the lights-on and -off switches cause prominent "masking effects" of light: a sudden pronounced activity increase after lights-on (also called startle effect) and a sudden shorter activity increase after lights-off (Fig. 1). Furthermore, activity is often suppressed by complete darkness (Rieger, Stanewsky, & Helfrich-Förster, 2003).

2.1.1 12 h light and 12 h darkness

This light regime is the most commonly used one and appears adequate for *D. melanogaster* with tropical origin. The molecular clock is best studied

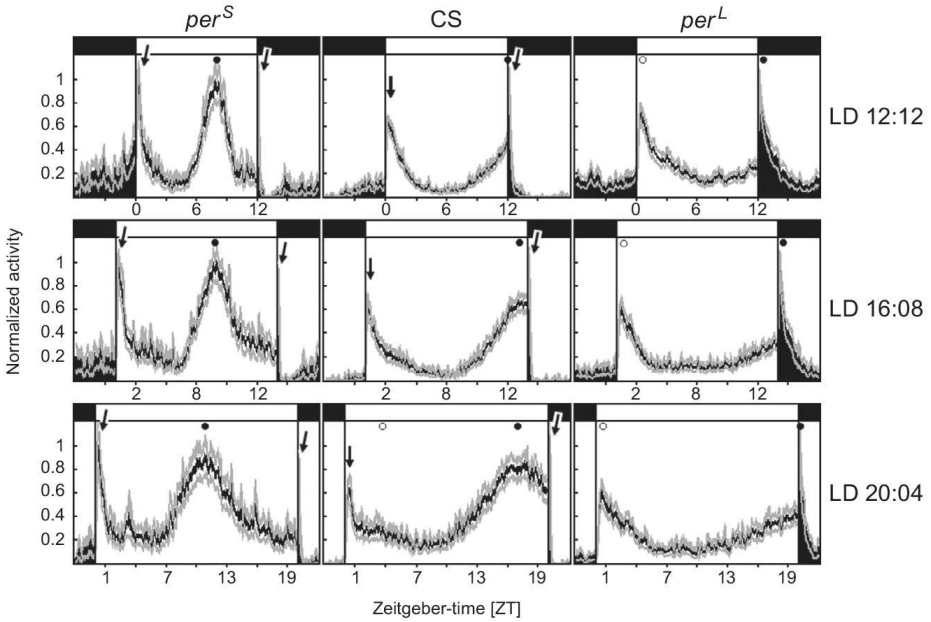

Figure 1 Average activity profiles of wild-type (WT) flies, *per*^{s} and *per*^{l} mutants (Konopka & Benzer, 1971) under different photoperiods (LD 12:12, LD 16:08, and LD 20:04). The activity of the flies was recorded by the Trikinetics system (Waltham, MA). Morning (M, open circles) and evening (E, closed circles) peaks of WT flies occur at lights-on and lights-off under LD 12:12 and are difficult to distinguish from the lights-on and -off masking effects (arrows). Under long days, M and E peaks can follow the lengthening of the light phase to some degree, but from a certain light/dark threshold M and E peaks uncouple from lights-on and lights-off and occur during the day. This L:D threshold depends on the speed of the clock: *per*^{s} flies showing a free-running period of about 18 h are not able to follow lights-off with their E peak already at LD 12:12. On the contrary, *per*^{l} flies with an endogenous period of approximately 26 h show a delayed E peak in LD 12:12 but the latter can easily follow lights-off under long photoperiods. This means that the simulation of different photoperiods is a powerful tool in order to unravel clock-controlled behavior: the endogenous M and E peaks can often be calculated independently of the lights-on and lights-off responses.

under this light regime and even mutants with different speed of the clock are usually able to entrain (Fig. 1). Under 12 h light and 12 h darkness (LD 12:12), wild-type (WT) flies show a bimodal activity pattern with a morning (M) peak and an evening (E) peak and a siesta in between (Wheeler, Hamblen-Coyle, Dushay, & Hall, 1993). The M peak appears shortly after lights-on and the E peak appears shortly before lights-off making it sometimes difficult to distinguish them unequivocally from the lights-on and lights-off masking effects. Since one of the key features of a circadian clock is to prepare the organism to the changes of day and night, the flies should be able to anticipate the beginning of the light and dark phases. Therefore, many labs judge whether the flies' activity anticipates lights-on and lights-off and use this as a criterion for real entrainment in contrast to masking where the flies just respond to the change in light (see Section 4.3.2).

2.1.2 Long and short photoperiods

Long photoperiods are suited to partly uncouple the lights-on and lights-off "masking" responses from the endogenous M and E activity peaks in WT flies (Fig. 1). M and E peaks try to follow lights-on and lights-off, respectively, but they are only able to do so until a certain day length (Shafer, Levine, Truman, & Hall, 2004). Under LD 20:04, M and E peaks of WT flies occur after lights-on and before lights-off, respectively, and are clearly distinct from the lights-on and lights-off responses (Fig. 1). In short-period mutants, the E peak is already distinct from the lights-off response under LD 12:12 and does not delay further under long days, whereas in long-period mutants, the E peak occurs after lights-off under LD 12:12 and at lights-off under LD 20:04. In this mutant, the E peak would probably be distinct from the lights-off effect under short days. In any case, applying different photoperiods proves the endogenous clock-controlled nature of M and E peaks. When the flies are transferred to DD, the free-running rhythm was shown to start from the previous M and E peaks and not from the lights-on and -off peaks, respectively (Rieger et al., 2003).

2.1.3 Varying light intensity or wavelength

In humans, too little or low light (especially in short winter days) may result in partial free-run and cause seasonal affective disorders. Exposing people to extra light (light therapy) can significantly reduce the symptoms, and this light exposure seems to work best when the light is short-wavelength enriched (Wirz-Justice, Benedetti, & Terman, 2013). For

entraining the activity of *Drosophila* also blue-green light is most effective (Helfrich-Förster et al., 2002), but low light is not a problem, because the circadian clock of *Drosophila* is very light sensitive since dCRY can integrate photons over a period of 12 h and longer (Vinayak et al., 2013): WT flies are still able to entrain to 12:12 blue light:dark cycles at a blue-light intensity of 0.03 nW/cm^2 (Hirsh et al., 2010). WT flies can even entrain to red light/dark cycles, because the spectral sensitivity of rhodopsin 6 and 1 (especially metarhodopsin 1) extends into the long-wavelength ranges (Hanai, Hamasaka, & Ishida, 2008). The activity pattern under red and blue light differs slightly with E activity increasing earlier under red light (Helfrich-Förster et al., 2002). Furthermore, the light intensity affects the overall activity level of the flies: low light intensities appear to promote activity, whereas high light intensities inhibit activity (Rieger et al., 2007). Therefore, it is of significant importance that all flies are treated with the same light intensity and light quality within one experiment. It is also important to always indicate the light source in the papers, since white light coming from LEDs, halogen lamps, or fluorescence tubes has different emission spectra.

2.1.4 Light–moonlight cycles

As complete darkness at night will never happen in nature, labs started to use dim light of moonlight intensity to simulate moonlight. In light–moonlight (LM) cycles, flies still show a bimodal activity pattern with an M peak and an E peak, whereas lights-on and lights-off effects appear considerably reduced (Bachleitner, Kempinger, Wülbeck, Rieger, & Helfrich-Förster, 2007; Kempinger, Dittmann, Rieger, & Helfrich-Förster, 2009). Compared to LD, the M peak is advanced and the E peak is delayed, which is reflected in the changes of PER cycling in the clock neurons controlling M and E activity bouts. Nevertheless, the most prominent effect is a rise of activity during the night, which is caused by the compound eyes, in particular rhodopsin 1 and rhodopsin 6, and which is not dependent on a functional clock and can thus be regarded as "masking" effect (Kempinger et al., 2009; Schlichting, Grebler, Peschel, Yoshii, & Helfrich-Förster, 2014). Already nocturnal light of 0.01 lux provokes rather huge changes in nocturnal activity and E peak timing and allows the comparison of clock-modulated entrainment with masking effects. It seems, however, that the increase of nocturnal activity during moonlight nights occurs only in the lab, as flies do not show a difference between new-moon and full-moon nights under seminatural conditions (Vanin et al., 2012).

2.2. Simulating gradual changes in light intensity

2.2.1 Simulating dawn and dusk

Another option to approach more natural-like light regimes is the simulation of dawn and dusk by a gradual increase of light intensity in the morning and a gradual decrease of light intensity in the evening. Investigations in several species could show that this simulation improves the synchronization of the animals significantly and enabled even entrainment to unusual photoperiods (reviewed in Rieger, Peschel, Dusik, Glotz, & Helfrich-Förster, 2012). Flies recorded under simulated twilight of 1.5 h duration shifted their activity almost completely into dawn and dusk (Rieger et al., 2007). In comparison to LD, the flies were more diurnal under simulated twilight and they had significantly delayed M and significantly advanced E peaks, meaning that the maxima came closer together. M and E peaks occurred always at a light intensity of ~7 lux, and this seems to coincide with the irradiance the flies prefer when they can choose between different light intensities (Rieger et al., 2007).

2.2.2 Simulating more natural conditions

During the last years, several groups focused on the entrainment of the clock and the activity rhythm under semi- or quasinatural conditions meaning that the locomotor activity of flies was recorded outdoors in the usual activity monitors (see later), well shielded from direct sunlight and rain (De, Varma, Saha, Sheeba, & Sharma, 2013; Menegazzi, Yoshii, & Helfrich-Förster, 2012; Vanin et al., 2012). Under such conditions, depending on the seasons, light intensity gradually increases during the first 4–7 h of the day, stays maximal only for 2–3 h, and then decreases again during the last 4–7 h. In addition, temperature oscillates showing maximal values about 2 h after midday and minimal values around dawn. The recordings showed that the timing and expression of the M peak strongly depend on the environmental temperature and are completely suppressed under cold days, whereas the E peak appears more clock controlled and starts when light and temperature are decreasing (Vanin et al., 2012). In addition to the well-known M and E activity peaks, a pronounced afternoon activity peak appeared under warmer days. This afternoon peak puzzled the fly community, because it suggests that the bimodal activity pattern of flies entrained under laboratory conditions may be completely artificial. In the meantime, researchers returned to more controlled lab conditions and varied light and temperature systematically. These studies showed that the afternoon

peak occurs also in other *Drosophila* species and that it is most likely an escape response of the flies from high temperatures and high light intensities during midday (e.g., Prabhakaran & Sheeba, 2014). Most likely, it is facilitated by the method used to record the flies' activity (Trikinetics system, which is very sensitive to even small movements, see Section 3.2).

3. METHODS TO MEASURE LOCOMOTOR ACTIVITY

In the following, we will compare several systems that record the locomotor activity of fruit flies and can lead to small qualitative and quantitative differences in the entrained activity pattern.

3.1. Home-made recording systems

When experiments to record locomotor activity were first initiated, a commercial system was not available, which is the reason why several labs have developed their own solutions to monitor activity. One of the first methods was used by Konopka and Benzer (1971): they placed individual flies in chambers sized 3 mm × 4 mm × 45 mm and monitored locomotor activity using two silicon solar cells. In those days, activity could only be recorded in a qualitative way (just an all-or-nothing response was monitored in a certain time interval by an Esterline Angus event recorder). The same was true for the system described by Helfrich-Förster (1998), in which individual flies were confined to half photometer cuvettes (10 mm × 10 (5) mm × 45 mm) in which on the wide end (10 mm × 10 mm) sugar and water were supplied, whereas on the narrow end (10 mm × 5 mm) an Infra Red (IR) light beam was installed that allowed monitoring of activity (yes or no) in consecutive 4-min intervals. Illumination via a halogen lamp came from the side of the cuvette that provided water and sugar. Though the system was computerized, the software reduced the amount of data stored by registering only "yes" or "no" beam crossings over a specific interval. Later the same system was modernized (Rieger et al., 2007): Light was provided by light-emitting diodes (LEDs) placed in front of each cuvette, and more importantly the software measured how often the fly crossed the IR beam in defined intervals enabling now quantitative measurements. The differences between the qualitative and quantitative measurements are already elaborated in Rieger et al. (2007) and will not be reviewed here. Important to mention is, however, that the flies have plenty of space to move freely in the cuvettes and that only real "running" activity of the flies is recorded as the fly has to cross the whole cuvette in order to cause a light-beam interruption. Movements

between food and water are not registered. An advantage of the system is that water and sugar are supplied independently from each other so that the fly can decide whether it wants to drink or eat. The possibility to choose between water and sugar prolongs the lifespan of the flies leading to recordings up to 40 days. Another advantage of the system is that the time interval during which the IR beam crosses were counted could be reduced to 1 min leading to a high-temporal resolution that facilitates the distinction between lights-on/lights-off effects and M and E activity peaks of the flies.

3.2. Commercially available Trikinetics system

Since several years ago, the Trikinetics system (Waltham, MA) is commercially available. This system consists of activity monitors that can simultaneously record the activity of 32 individual flies, an interface device and the software for computerized data collections (nicely depicted in Tataroglu & Emery, 2014). This assay has become by far the most common behavioral assay in flies. Individual flies are placed in glass tubes (4 mm × 65 mm) that contain food on one end and a porous plug on the other end (larger glass tubes for larger insects are also available). An IR light beam crosses the tube and is detected by a photodetector on the other side. The software automatically generates text files in which the number of beam crosses is saved in a chosen time span for each individual fly. Most labs choose 30 min bins, but it is also possible to save the data at higher resolution, e.g., in 1-min intervals. In comparison with the above-described home-made system, the flies have less space and cross the IR beam even when moving a little bit. On the other hand, locomotor activity cannot be recorded for such a long time as with the home-made system, because the food tends to dry out. Nevertheless, providing 25 mm of food and a careful sealing of the glass tubes enables recordings for 30 days.

3.3. Camera-based recording system

With the methods just described, it is only possible to record activity if the fly crosses the IR beam, whereas movements outside of it are not monitored. This is different with a camera-based system (e.g., Rieger et al., 2007). Here, we will describe the commercial Noldus-tracking system originally designed for larger animals (Noldus Information Technology; www.noldus.com), but which is also perfectly suitable to monitor activity in *D. melanogaster*. In our lab, flies are transferred into full photometer cuvettes (10 mm × 10 mm × 45 mm), provided with sugar water, illuminated by IR

light from below and recorded by an IR-sensitive camera from above (Fig. 2). White LEDs provide the desired light regime. During the experiment, the camera takes a picture every 500 ms and the software stores the position of the fly. Later, the movements of the fly can be tracked and the distance the fly has covered within a certain time interval can be

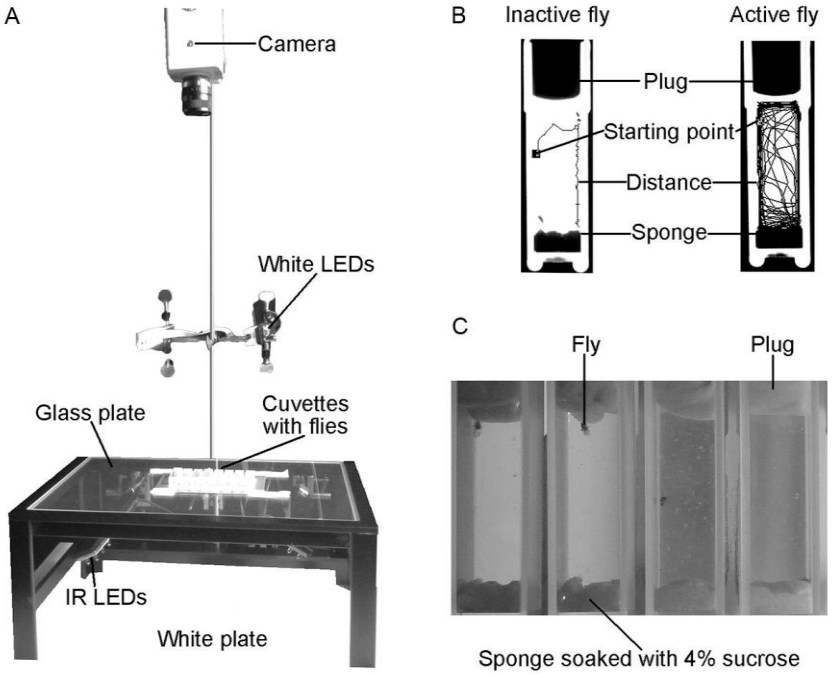

Figure 2 Monitoring the flies' activity with the tracking system of Noldus. (A) The flies are confined to photometer cuvettes; eight cuvettes can be recorded at the same time and are placed on a small glass table. Above the table an IR-sensitive camera is adjusted in a way that all cuvettes are in the picture and in focus. Illumination comes from IR LEDs placed underneath the table shining onto a white plate that reflects the IR light and this way illuminates all cuvettes evenly from below. The LD cycles are provided by white LEDs placed above the glass plate. The IR-sensitive camera is equipped with a filter that lets only IR pass so that the LD cycle is not visible for the camera and does not disturb recording. During the experiment, the camera takes a picture every 500 ms and the software analyzes the distance covered within this time interval and tracks the path of the fly. (B) Two cuvettes showing the tracks of an inactive and active fly. Each cuvette contains a piece of sponge with sucrose solution. The sponge was cut before to the right size and placed at the closed side of the cuvette. Around 200 µl of 4% sucrose solution were added on the sponge (*note*: any water drop on the side of the cuvette would interfere with the camera recording and give wrong results). On the other side, the cuvette is closed by a plug to prevent the flies from escaping (make sure that the plug is placed with the same depth inside the cuvette on all sides as otherwise the fly can hide behind the plug and cannot be recorded by the camera). (C) Pictures of four cuvettes show the fly, the sponge, and the plug.

calculated. Due to the short-time interval and the exact tracking of the flies, this system is excellently suited for analyzing the flies' locomotor activity (or also sleep) in more detail. A further advantage of this system is that several areas of interest can be defined and separately analyzed; e.g., a small area around the food source can be defined in order to analyze when and how long the flies prefer to stay there. The disadvantage, however, is that the length of the experiments is much shorter compared to the two systems using the light-beam method: As the food consists of only 200 μl of sugar water the flies can only survive for 2–3 days, making it impossible to test different lighting conditions in the same fly or to record the free-running behavior under constant conditions.

4. DATA ANALYSIS AND SYSTEM COMPARISON

In this section, we will describe appropriate methods to analyze the behavior of flies under entrained conditions including the design of actograms, average activity profiles, as well as further analyses based on the averaging single days. In order to analyze all these features of locomotor activity, the raw data of the experiments are needed. All of the described systems provide text files (.txt) which contain the activity of each single fly; while the first two recording methods give the number of IR beam crosses per time interval, the tracking system gives distances covered by the fly per time interval.

4.1. Designing actograms

Several programs are available that can build actograms from the raw data (e.g., Clocklab from actimetrics (Evanston, IL, http://www.coulbourn.com/v/vspfiles/assets/manuals/ACT-500%20ClockLab%20Analysis%20Manual.pdf), El Temps (http://www.el-temps.com/), and ChronoShop (https://www.nioo.knaw.nl/sites/default/files/ChronoShop%20manual.pdf)). We will concentrate on one program, ActogramJ, that builds on the free distribution of ImageJ (Fiji, available at: http://fiji.sc/Downloads) and can be downloaded as a plugin (available at: http://132.187.25.13/actogramj/versions.html) and be copied into the "plugin" folder of Fiji (Schmid, Helfrich-Förster, & Yoshii, 2011). One advantage of this software is that the format of the actograms can be freely chosen and these can be later exported as PDF files without any loss of resolution. For judging entrainment, it is important that the actograms are not too narrow, so that a deviation from the period of 24-h can be easily seen by eye. Furthermore, a wide actogram

may allow the distinction of lights-on and lights-off effects from M and E activity peaks, at least when the data are collected in 1-min bins.

The following steps will lead you to an actogram in ActogramJ (with Trikinetics data/sampling interval 1 min):

1. For better visualization, the raw data should be modified to cut the values of the first day so that noon appears in the middle of the average day. This means if lights-on occur at 8 a.m. and lights-off at 8 p.m., the data until 2 a.m. of the first day should be cut. Save this modified file as a new .txt file and open ActogramJ.
2. File → open data → open the modified .txt file.
3. Choose start column "11" and end column "42".
4. Set start row to "1" and end row to $X \times 1440$ (with X being the duration of the experiments in days).
5. Leave "calibration" sections unchanged and press ok.
6. ActogramJ automatically generates double plotted actograms, which are useful in DD conditions to follow the free-running period of the fly. However, in entrained conditions a single plot is sufficient to show the flies' behavior. To change the double plot into a single plot press edit → properties and change no. of plots to 1.
7. In order to show general tendencies of entrained behavior, use an average actogram of all investigated flies. To do so select all flies which survived the whole experiment, press analyze → analysis and chose "average". ActogramJ will automatically generate an average actogram.
8. Either single or average actograms can be exported as PDF files.

4.2. Creating average activity profiles

Actograms are very important to judge whether the activity of each single fly is entrained to the light regime of interest. Only if the fly is stably entrained, the calculation of an average activity profile (=average day) over the number of entrained days makes sense. In many cases, the flies are not fully entrained during the initial days of the experiments. As visible in Fig. 3, even WT flies take 2 days to be fully synchronized to a new LD 12:12 cycle, as they show either unnaturally high levels of activity during the siesta or differently shaped M or E peaks. Therefore, one has to judge the duration of entrainment for each single experiment and genotype, as the average day should only represent the stable part of the activity. The necessary steps to create daily activity profiles for individual and groups of flies are illustrated in Fig. 3.

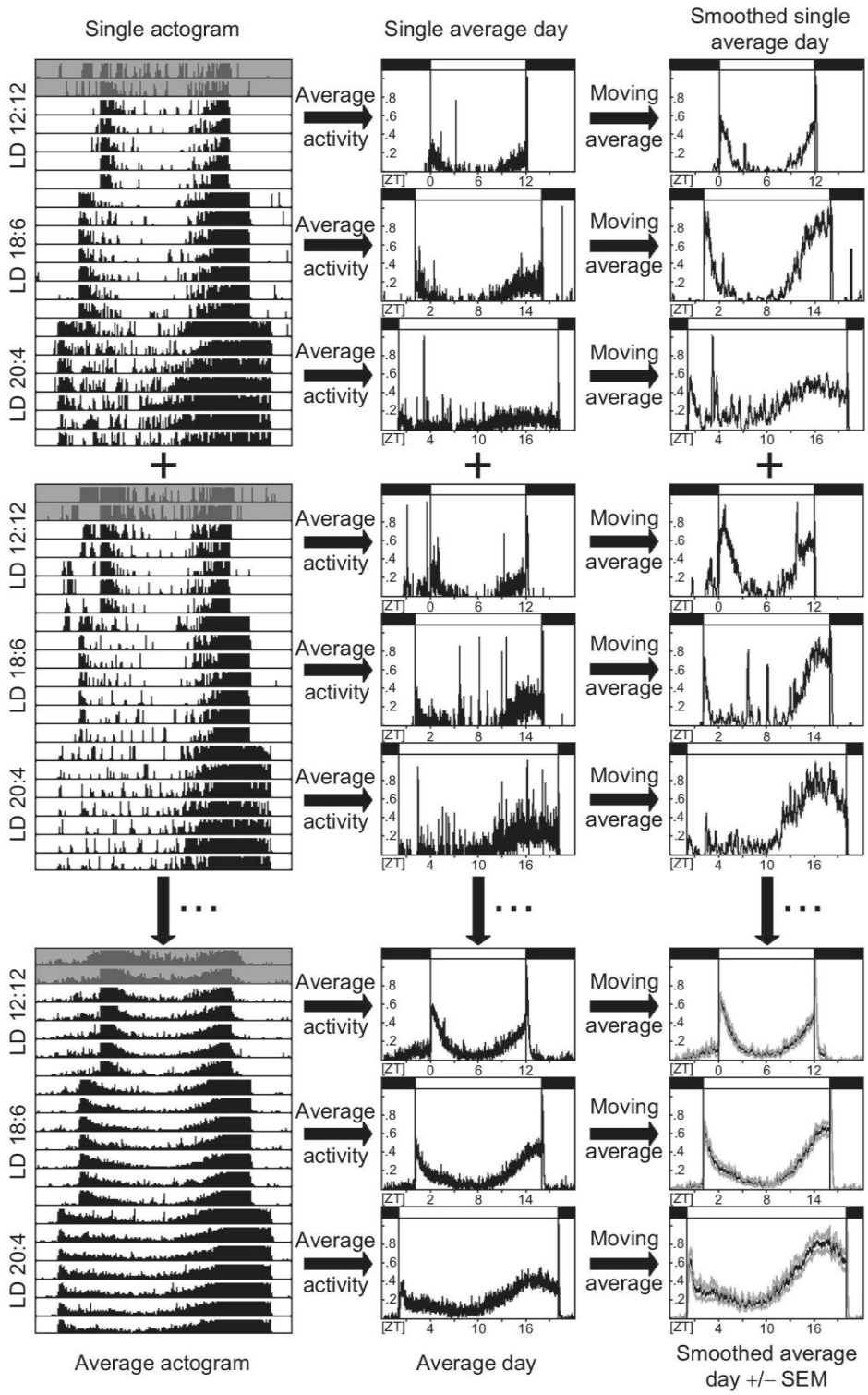

Figure 3 (See legend on next page.)

The average activity profiles show the general activity pattern of the flies' behavior under the given light conditions. When comparing those of WT flies and *period* mutants under LD 12:12, which were generated from data obtained with the Trikinetics system (Fig. 1) and data obtained with the home-made system (Fig. 4), clear differences are evident: flies in the home-made system show barely any M anticipation and only a small lights-off response, whereas the M anticipation and lights-off effect are very strong in the Trikinetics system.

4.3. Further analyses based on average days of individual flies

In order to compare the activity level at different times of the day statistically, one has to go back to the single fly data. In the following section, we will show how to analyze several important features of the flies' behavior under entrained conditions.

4.3.1 Calculating activity levels

The general activity level can be quite different in individual flies, but it does also depend on the recording system. Calculating the mean out of the 1440 min of the individual average day will give the average beam crosses/minute (for IR based systems) or the average distance covered in cm/min (for the camera based system). Comparing the two IR-beam based methods reveals that the Trikinetics system is much more sensitive than the home-made system leading to a significantly higher general activity level (Fig. 5). The activity level obtained with the Noldus system cannot be quantitatively compared with the activity obtained by the other systems, but reveals interesting details. It shows, for example, that the flies travel much larger distances during the day than during the night: though they seem not as frequently active during the siesta (as revealed by the IR-based systems), they seem to move larger distances if they move. On the contrary,

Figure 3 Calculating average activity profiles from individual flies recorded under three different photoperiods in the Trikinetics system as well as calculation of average activity profiles from all flies of one genotype. First, the duration of entrainment for each single fly has to be judged (here, the first 2 days marked by gray are omitted from the calculation). Then, the activity of minute 1 of all 5 days in LD 12:12 is averaged, and this is continued for minutes 2, 3, 4, etc. The same is repeated for the other light conditions (LD 18:6 and LD 20:4). The obtained 1440 activity values are plotted as single average day for each light condition and each fly (middle column). By smoothing with a moving average filter (over 11 values), one gets the smoothed curves shown to the right. To evaluate the behavior of a whole fly group, average actograms as well as average activity profiles out of all flies can be calculated (bottom).

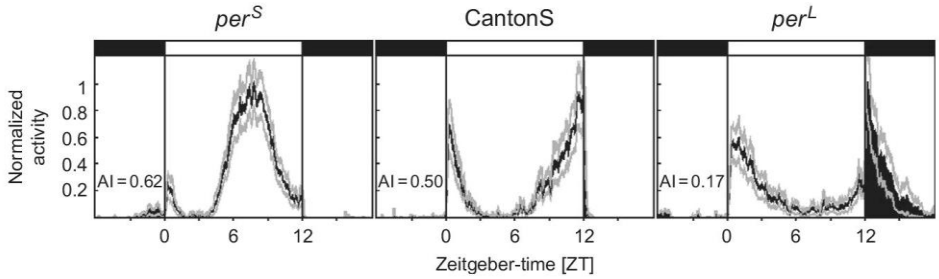

Figure 4 Average activity profiles of *per*^{s} mutants, wild-type flies, and *per*^{l} mutants under LD 12:12 recorded with the home-made system. Note that there is virtually no M anticipation and no lights-off effect.

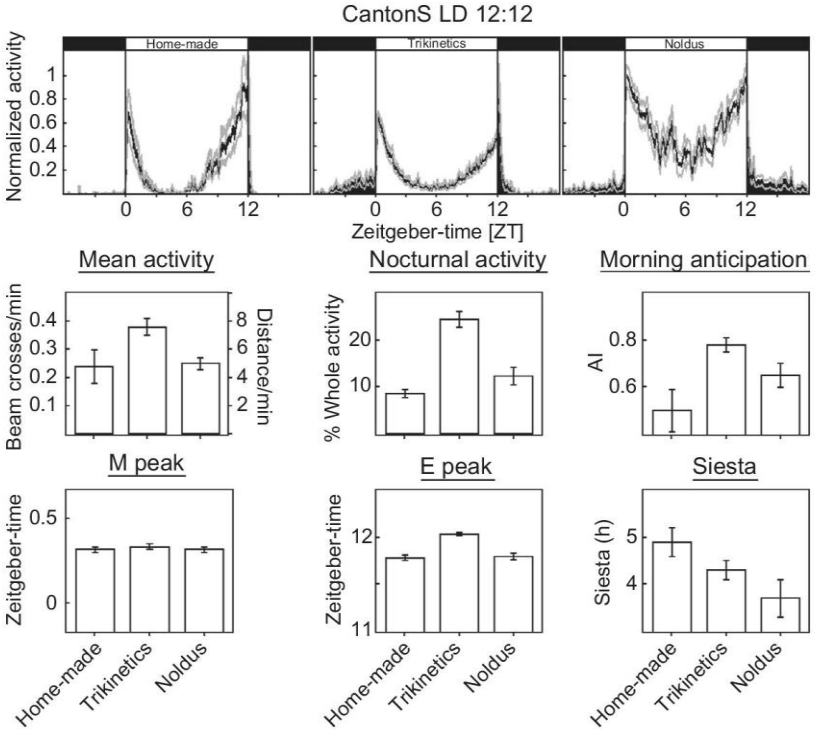

Figure 5 Comparison of the activity pattern, activity level, M and E peak timing as well as the length of the siesta of wild-type flies recorded with the home-made, the Trikinetics, and the Noldus system. Distinct differences are visible that are explained in detail in the text.

they do not seem to walk large distances during the night and especially not during their morning anticipation (see later).

Determining diurnal and nocturnal activity levels is useful to calculate the degree of diurnality and nocturnality, and as we discussed above this can be quite different under different light regimes. Nocturnal activity

can be expressed in percent of whole daily activity, and then it becomes comparable between the three systems. Figure 5 shows that even the values of this relative nocturnal activity depend on the recording system. The home-made system shows rather low levels of relative nocturnal activity, which is due to the fact that the fly has to cross the whole cuvette in order to interrupt the light beam. The Noldus system shows already higher percentage of nocturnal activity and the Trikinetics system shows the highest level. The very high levels of nocturnal activity in the Trikinetics system can be explained by the small volume inside the tubes leading to a high number of beam crosses even during the night.

4.3.2 Analysis of morning anticipation

As mentioned earlier, the morning anticipation describing the increase of activity already before lights-on is often taken as criterion for clock-controlled activity. A morning anticipation index (AI) is usually calculated by the sum of activity within the last 3 h of night phase divided by the sum of activity within the last 6 h of night phase (Sheeba, Fogle, & Holmes, 2010). If this value is significantly higher than 0.5, the flies show morning anticipation and the higher the value the stronger the anticipation. This index is a powerful way in order to describe the increase of activity before lights-on. However, the results gained with this index should be interpreted carefully, as the AI depends on the endogenous period of the flies investigated (Figs. 1 and 4): flies having a short period (per^s) show their M peak before lights-on in LD 12:12 and, therefore, the AI is high, whereas flies with a long period (per^l) do not show activity before lights-on leading to a low index. Although there is a huge difference in morning anticipation, both flies possess an intact morning oscillator and, therefore, concluding from the morning AI on the presence or absence of the morning oscillator is not straight forward. The AI also depends on the recording system used. In the home-made system, the flies barely show any M anticipation, which is due to the fact that the flies generally show almost no activity at night. The flies would have to cover the whole distance of the cuvette, which almost never happens during the night based on analyzing the running profile of flies using the Noldus system. In the latter, the flies show a significant AI and this is even stronger in the Trikinetics system, which might again be explained by the small tube volume that allows IR beam crossings with little activity.

4.3.3 Determining M and E peaks

According to the dual oscillator model, the timing of M and E activity peaks is controlled by M or E clock neurons in the fly's brain (reviewed by

Helfrich-Förster, 2014). As a consequence, the peak timing allows drawing conclusions on the clock status. To determine the timing of the peaks, smooth the data for the single day average by a moving average of 30. Through this process, randomly occurring spikes are reduced and the real maximum of the fly's activity can be determined. During peak determination, one has to be careful not simply to take the highest values, because these are often the lights-on or the lights-off responses of the fly.

Regarding the M peak, there is no significant difference between all three recording systems (Fig. 5): in any case, the M peak appears about 20 min after lights-on independent of the morning anticipation. The E peak, however, appears shortly before lights-off in the home-made and the camera-based system, whereas it appears directly at lights-off in the Trikinetics system. The reason for that is that the E peak in the Trikinetics system is strongly masked by lights-off, which is already visible in the average activity profile (Fig. 5). Therefore, a better method is to determine the onset of E activity (Vanin et al., 2012; see later).

4.3.4 Siesta determination

Another way of analyzing the entrained state is to determine the offset of M activity and the onset of E activity, as well as the length of the siesta. To do so, the average day data have to be plotted as bar diagrams with each bar representing the sum of activity within 20 min. This is necessary as the raw data are often noisy and thereby it would be impossible to reliably determine the offset of M activity (the first time a bar reaches the level of activity which is stable during noon) and the onset of E activity (the first bar when activity starts to rise consecutively). To determine the siesta simply calculate E activity onset − M activity offset.

In the siesta also some differences are obvious between the three systems (Fig. 5): due to the generally reduced level of activity in the home-made system, the activity during noon is almost 0, whereas it does not go down to 0 in both other systems. In the calculation, the siesta is therefore also significantly longer in the home-made system.

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6.2. Moonlight Detection by *Drosophila*´s Endogenous Clock Depends on Multiple Photopigments in the Compound Eyes

Moonlight Detection by *Drosophila*'s Endogenous Clock Depends on Multiple Photopigments in the Compound Eyes

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Abstract Many organisms change their activity on moonlit nights. Even the fruit fly *Drosophila melanogaster* responds to moonlight with a shift of activity into the night, at least under laboratory conditions. The compound eyes have been shown to be essential for the perception of moonlight, but it is unknown which of the 5 rhodopsins in the eyes are responsible for the observed moonlight effects. Here, we show that the outer (R1-R6) and inner (R7 and R8) photoreceptor cells in a fly's ommatidium interact in a complex manner to provoke the moonlight effects on locomotor activity. The shift of the evening activity peak into the night depends on several rhodopsins in the inner and outer photoreceptor cells. The increase in relative nocturnal activity in response to moonlight is mainly mediated by the rhodopsin 6–expressing inner photoreceptor cell R8 together with the rhodopsin 1–expressing outer receptor cells (R1-R6), whereas just rhodopsin 1 of R1 to R6 seems necessary for increasing nocturnal activity in response to increasing daylight intensity.

Keywords circadian clock, entrainment, masking, rhodopsins

Light is the most important environmental time cue to synchronize endogenous clocks to the cyclic environment. Consequently, the clocks of most species are very sensitive to light, especially to nocturnal light. The 29.5-day moonlight cycle is used by some animals as a zeitgeber for semilunar and lunar reproductive cycles; others modify their daily pattern of activity, foraging, predation, and communication on moonlit nights (Kronfeld-Schor et al., 2013). Moonlight can increase the activity of nocturnal mammals, as shown for the lemur *Eulemur fulvus albifrons* and the owl monkey *Aotus azarae boliviensis* (Erkert and Cramer, 2006), and it can affect the sleep of diurnal mammals as revealed for humans (Cajochen et al., 2013).

Moonlight does also significantly influence the activity pattern of diurnal fruit flies in the laboratory (Bachleitner et al., 2007; Kempinger et al., 2009). Moonlight (0.03 lux) exposure at night causes significant phase shifts of the flies' morning (M) and evening (E) activity into the night (Bachleitner et al., 2007). In addition, moonlight stimulates nocturnal activity dramatically (Kempinger et al., 2009). The phase shifts of M and E activity under light-moonlight (LM) cycles correlate with phase shifts of the molecular clock (oscillations of the clock proteins

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol 29 No. 2, April 2014 75–86
DOI: 10.1177/0748730413520428

PERIOD and TIMELESS) in respective M and E clock neurons, indicating that the observed moonlight effects on activity are at least partially mediated by the circadian clock (Bachleitner et al., 2007). In contrast, the circadian clock turned out to be dispensable for the nocturnal activity increase during moonlit nights because clock mutants respond in the same way (Kempinger et al., 2009). Such direct light effects on activity that bypass the clock are known as masking effects (Mrosovksy, 1999). Masking complements the circadian clock in fine-tuning activity patterns in response to environmental stimuli and therefore represents an important pathway for proper synchronization of the activity rhythms to the day-night cycles (Hut et al., 1999). Although it is doubtful that fruit flies are nocturnal under full moon conditions in nature (Bachleitner et al., 2007; Vanin et al., 2012), *Drosophila*'s activity patterns in the laboratory can be used as a powerful tool to understand the effects of dim light on activity rhythms.

The moonlight effects on *Drosophila*'s activity have previously been shown to depend on functional compound eyes and not on the blue-light photopigment cryptochrome (Bachleitner et al., 2007). Yet, it is still unknown which photoreceptor cells and rhodopsins in the eyes are responsible for moonlight detection. The flies' compound eyes consist of approximately 800 hexagonal ommatidia, containing pigment cells as well as 8 receptor cells (Rister et al., 2013). Receptor cells 1 to 6 (R1-R6) are arranged in the periphery of each ommatidium, span its entire length, and express rhodopsin 1 (Rh1), which has a broad sensitivity to blue-green light (Suppl. Fig. S1). Receptor cells 7 (R7) and 8 (R8) are located in the center of the ommatidium, with R7 being arranged above R8. R7 and R8 define 2 subtypes: In the "pale" cluster R7 expresses the ultraviolet (UV)–sensitive rhodopsin 3 (Rh3) and R8 the blue-sensitive rhodopsin 5 (Rh5). In the "yellow" cluster R7 contains rhodopsin 4 (Rh4) that is sensitive to longer UV wavelengths and R8 the green-sensitive rhodopsin 6 (Rh6). The pale and yellow subtypes are statistically distributed in a 30:70 ratio throughout most of the retina, whereby rhodopsin expression is regulated by sophisticated molecular mechanisms (Rister et al., 2013).

To answer the question of which photoreceptor cells and photopigments mediate the flies' responses to moonlight, we performed a systematic study monitoring the activity of wild-type (WT) flies and selected photoreceptor mutants under 12:12 LD and LM cycles at 4 different daylight intensities. Our results demonstrate an interaction between inner and outer photoreceptor cells in irradiance detection and WT-like timing of activity.

MATERIALS AND METHODS

Strains and Rearing

As a control, we used the laboratory strain $WT_{CantonS}$ and 2 different strains caught in the wild. The first strain, named WT_{ALA}, was isolated in Val Venosta (Alto-Adige, North Italy, 45°N) and is a mixed culture of 37 isofemale lines (Sandrelli et al., 2007; Rieger et al., 2012). The second one was caught in a small village near Würzburg (Lindelbach, Germany, 50°N) and is referred to as $WT_{Lindelbach}$.

The following photoreceptor mutants were used: cli^{eya} mutants that lack the entire compound eyes (Bonini et al., 1993) served as negative controls. $rh3^1$ $rh4^1$ double mutants lack photopigments in photoreceptor cell 7 (R7) (Vasiliauskas et al., 2011), whereas *sevenless*, sev^{LY3} mutants lack the entire R7 cell (Benzer, 1967). sev^{LY3} mutants are additionally devoid of Rh5 but express more Rh6 than WT flies because pale R7 cells are needed to instruct the underlying R8 to become pale (Rh5-expressing) R8 cells (Chou et al., 1999); consequently, all R8 of sev^{LY3} mutants are of the yellow type (expressing Rh6). $rh5^2$ mutants lack Rh5, rendering 30% of R8 out of function (Yamaguchi et al., 2008), whereby $rh6^1$ mutants lack Rh6, leading to a loss of function in 70% of the R8 cells (Cook et al., 2003). The latest studies showed, however, an age-dependent de-repression of Rh5 in the $rh6^1$ mutant background, leading to a higher level of R8 containing Rh5 as a pigment (Vasiliauskas et al., 2011). Therefore, less than 70% of R8 should be out of function in $rh6^1$. To investigate the effects of a total loss of R8 function, we used $rh5^2;rh6^1$ double mutants (Yamaguchi et al., 2008). Due to the additional knockout of *rh5*, de-repression is not possible in this mutant. To render both inner receptor cells out of function, a quadruple mutant $rh5^2;rh3^1$ $rh4^1$ $rh6^1$ was generated. Our starting point was the already recombined $rh3^1$ $rh4^1$ $rh6^1$ triple mutant that we generously received from Nina Vogt (New York University, New York, NY). To knock out the function of photoreceptor cells R1 to R6, we used the *rh1*-null mutant $ninaE^{17}$ (neither inactivation nor afterpotential E) (Kumar and Ready, 1995), and to eliminate in addition the function of 70% of the R8 cells, we used $ninaE^{17};rh6^1$ double mutants (Yamaguchi et al., 2010). Because many $ninaE^{17}$ mutants carry in addition a mutation in the gene for the histamine-gated chloride channel *ora transientless ort* (CG7411), we made sure by PCR that our used $ninaE^{17}$ and $ninaE^{17};rh6^1$ double mutant were fine with respect to this (see below).

To minimize differences in light sensitivity caused by the genetic background, all mutants had red eyes, and all were crossed into the *ls-timeless* background (the L-TIM protein expressed only in *ls-tim* flies is less

light sensitive compared to S-TIM, which is expressed in both *ls-tim* and *s-tim* flies) (Sandrelli et al., 2007). Of the WT strains, $WT_{CantonS}$ flies were *ls-tim*, WT_{ALA} flies contained a mixture of both *tim* forms but were predominantly *ls-tim*, and $WT_{Lindelbach}$ flies were *s-tim* (as determined by PCR) (Rieger et al., 2012). All flies were raised on a *Drosophila* medium (0.8% agar, 2.2% sugar beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, and 0.3% hydroxybenzoic acid) at 25 °C in LD 12:12.

PCR

The *timeless* and *ort* genes of the different strains were partly sequenced using genomic DNA. To distinguish between *s-tim* and *ls-tim* animals, we used the following oligonucleotide primers to amplify (and later to sequence) the genomic DNA: sense TAGGTATCGCCCTCCAAG and antisense TAGG CAGCTCCACAATCA. ort^1 is a null mutant carrying a deletion of 569 nucleotides overlapping intron 2 (110 bp) and 459 nucleotides of exons 2 and 3 (Iovchev et al., 2002). The deletions lead to loss of the sequence encoding a substantial portion of the N-terminal extracellular domain and the first 2 membrane-spanning segments and also introduce a frameshift. To exclude the ort^1 mutation in $ninaE^{17}$ mutants, we used the following oligonucleotide primers to amplify the genomic DNA: sense AATATGACACAGGAA TACCGCCTGCTC and antisense CGTGTCGCTCAG GACGATGTTTATTAG.

Recording

The locomotor activity of the flies was recorded in a homemade system described first by Helfrich-Förster (1998) and refined by Rieger et al. (2007). Briefly, 2- to 4-day-old single male flies were confined to photometer cuvettes, which were placed with one end in an infrared light beam that recorded the number of walk-throughs in 1-min intervals; on the other end of the cuvette, water and sugar were supplied. Locomotor activity was recorded at 20 °C in a climate-controlled chamber. Illumination was provided by "white" light-emitting diodes (LEDs) (Lumitronix LED-Technik GmbH, Jungingen, Germany) and additional neutral density filters (Lee Filters Worldwide, Andover, UK) for fine adjustment of light intensity. The emission spectrum of the LEDs allowed the excitation of Rh1, Rh5, and Rh6 but not of Rh3 and Rh4 (Suppl. Fig. S1). Thus, photoreceptor cell R7 was not activated by our light conditions.

We applied 2 different light conditions, all consisting of 12 hours of day and 12 hours of night. Locomotor activity was recorded for 7 days either in a rectangular LD cycle or in an LM cycle (moonlight intensity = 0.01 lux) at 4 different daylight intensities (10, 100, 1000, and 10,000 lux).

For every experiment, 32 flies of each genotype were recorded. We only used flies surviving the whole experiment for the calculations, so that the number of analyzed flies varies between 17 and 32.

Data Analysis

The raw data were displayed as actograms using ActogramJ (University of Wuerzburg, Germany; Schmid et al., 2011). In the next step, we analyzed the mean activity profiles of single flies using at least 5 days of recording. To create the average day for 1 experiment, the mean values of at least 17 single activity profiles were calculated and smoothened by a moving mean of 11 values (Helfrich-Förster, 2000; Rieger et al., 2003; Rieger et al., 2012). All average days were normalized and plotted the same size using the program QtiPlot (version 9.8.8, Ion Vasilief, Craiove, Romania) for better visualization. For determination of the E peak phase, we smoothed the activity profiles of single flies over a moving mean of 30. We then determined the times of the E peak manually for each single fly as described by Rieger et al. (2003). Mean diurnal and nocturnal activity levels were calculated out of the unsmoothed average days as described by Rieger et al. (2003).

For statistical analysis, we used a 1- or 2-way ANOVA after testing for normal distribution by the Kolmogorov-Smirnov test. If the data were not normally distributed, p values were adjusted by multiplication with 5 according to Glaser (1978).

Immunohistochemistry

To estimate the de-repression of *rh5* in the $rh6^1$ mutant background, we performed retina stainings in $rh6^1$, $ninaE^{17}$ $rh6^1$, and $WT_{CantonS}$ flies. We also checked the distribution of Rh5 and Rh6 in $rh3^1$ $rh4^1$ and sev^{LY3} mutants and Rh1 expression in the outer photoreceptor cells in most mutants. The flies were entrained for 4 (oldest age of the flies at the beginning of the behavior experiments) or 11 days (end of experiments, respectively) in LD 12:12 at a light intensity of 100 lux. On days 4 and 11, the whole flies were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (pH = 7.4). After washing 5 times for 10 min in PBS, we dissected 15 retinas for each genotype and blocked for 20 min in 5% normal goat serum (NGS) in PBS. In the next step, the retinas were incubated over 2 nights in the primary antibody solution containing rat anti–pigment cell–enriched dehydrogenase (PDH) (1:100; gift of C. Montell, University of California, Santa Barbara,

CA, USA) (Wang et al., 2012), mouse anti-Rh5 (1:50; gift of S. Britt, University of Colorado, Aurora, CO, USA) (Salcedo et al., 1999), and rabbit anti-Rh6 (1:1000; gift of C. Desplan , New York University, New York, NY, USA) (Tahayato et al., 2003) or anti-Rh1 (1:30; 4C5; obtained from Developmental Studies Hybridoma Bank, Iowa City, IA) including 5% NGS in PBS with 0.1% Triton X-100 (PBST) (pH = 7.4). After washing the retinas 5 times with PBST for 20 min each, the secondary antibody was applied consisting of Alexa Fluor 488 (goat anti-rat), Alexa Fluor 555 (goat anti-mouse), and Alexa Fluor 635 (goat anti-rabbit) each in a dilution of 1:200 in PBST containing 5% NGS. After incubating overnight, the retinas were washed for up to 4 days in PBST until the red eye pigment disappeared. At the end, the retinas were embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) in a way that the cornea of the eye laid on the glass slide.

In a second set of experiments, we investigated the brains of cli^{eya} flies to confirm the presence of Hofbauer-Buchner (H-B) eyelets (Hofbauer and Buchner, 1989). For this staining, the protocol described by Hermann et al. (2013) was used. We applied a primary antibody solution consisting of rabbit anti-Rh6 (1:1000) and mouse anti–pigment-dispersing factor (PDF) (1:1000; obtained from Developmental Studies Hybridoma Bank) containing 5% NGS in PBST. Alexa Fluor 488 (goat anti-rabbit) and Alexa Fluor 635 (goat anti-mouse) were used as secondary antibodies in a dilution of 1:200 and 5% NGS in PBST.

Microscopy and Image Analysis

The brains and retinas were analyzed using laser scanning confocal microscopy (Leica TCS SPE, Leica, Wetzlar, Germany). To excite the fluorophores of the secondary antibodies, we used 3 different laser diodes (488, 532, and 635 nm) and obtained confocal stacks of 2 µm thickness. All images were analyzed using the program Fiji (an ImageJ distribution software). In the case of the retinas, the percentage of ommatidia showing Rh5 or Rh6 staining was analyzed by manually counting the number of Rh5- or Rh6-positive R8 of at least 9 retinas. In cli^{eya} brains, we analyzed the presence of the H-B eyelets as well as the course of their tracts towards the accessory medulla (aMe) of 30 flies.

RESULTS

WT Behavior

So far, the response to moonlight was only shown for the laboratory WT strain CantonS ($WT_{CantonS}$) (Bachleitner et al., 2007; Kempinger et al., 2009). To ensure that the behavioral changes in response to moonlight are also observed in other WT strains, 2 additional strains were tested, WT_{ALA} and $WT_{Lindelbach}$, which originally stem from 2 different locations in Europe. Furthermore, for the first time, the absolute activity levels of the flies under LD and LM were measured. The recording system used by Bachleitner et al. (2007) and Kempinger et al. (2009) only allowed us to determine whether a fly was active (value of 1) or inactive (value of 0) during 4-min intervals. Thus, only a qualitative judgment of the nocturnal activity increase in response to moonlight was possible. The quantitative analysis of the present study revealed that moonlight significantly increased the nocturnal activity of all 3 WT strains but that the effects appeared less dramatic as compared to the previous study (Fig. 1). Especially, M activity appeared much lower than in the previous studies. This was because the flies crossed the light beam only once or twice within 4 min in the morning but up to 20 times per minute in the evening. The old system could not distinguish between the different activity levels in the morning and evening: Because the flies crossed the light beam at least once per 4 min at both times, an activity level of "1" was recorded (Bachleitner et al., 2007; Kempinger et al., 2009). Due to the low M activity, the phase of the M peak was hard to determine in the present study. Therefore, we decided to analyze only the timing of the E peak (Fig. 1B).

Furthermore, we found that the absolute diurnal and nocturnal activity levels were quite different among the 3 WT strains (Fig. 1C and 1D and Table 1): $WT_{Lindelbach}$ was about twice as active as $WT_{CantonS}$, and WT_{ALA} ranged in between the two (Table 1). Similar large differences in the activity level were also revealed for the different photoreceptor mutants (see below). In spite of the differences in overall activity, nocturnal activity increased and diurnal activity decreased in response to moonlight in all 3 WT strains (Fig. 1C and 1D). Therefore, we normalized the activity profiles (maximal activity was set to 1) (Fig. 1A) and calculated the relative nocturnal activity in the percentage of whole daily activity (Fig. 1E) to compensate for differences in absolute activity.

We found the following for all 3 WT strains:

1. Moonlight simulation (LM) delayed the E peak in comparison to LD. Upon moonlight, the $WT_{CantonS}$ phase delayed the E peak on average by 18.5 ± 1.4 min, WT_{ALA} by 22.7 ± 2.7 min, and $WT_{Lindelbach}$ by 14.9 ± 3.1 min (Fig. 1B), giving no significant difference between the 3 investigated WT strains of what indicates a minor role of *timeless* polymorphism on the delay of the E peak on moonlit nights.
2. Moonlit nights increased nocturnal activity and decreased diurnal activity as compared to dark nights (Fig. 1C and 1D).

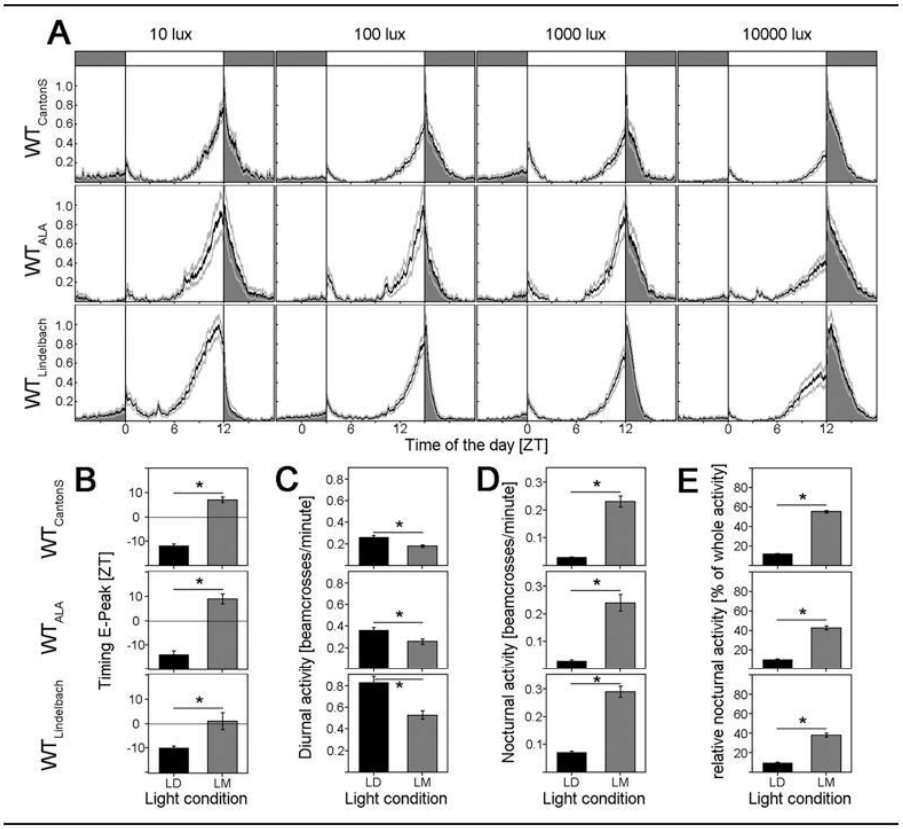

Figure 1. Activity patterns of $WT_{Lindelbach}$, WT_{ALA}, and $WT_{CantonS}$ under 12:12 LM cycles of different daylight intensities. (A) Mean activity profiles (±SEM): Activity during the night is indicated in dark gray. From left to right, the light intensity was varied between 10, 100, 1000, and 10,000 lux during the day. All profiles were normalized to 1. (B) Timing of the E peak in LD and LM in minutes after or before lights-off. The value 0 represents the time of lights-off, negative values display the minutes before lights-off, and positive values display the minutes after lights-off, respectively. All WT strains show a significantly delayed E peak in LM compared to LD. The differences range between 15 and 23 min. (C) Mean diurnal activity (±SEM) in LD (black bars) and LM (dark gray bars) calculated as the mean number of beam crosses between ZT0 and ZT12. All genotypes show a significant reduction in diurnal activity upon moonlight simulation. (D) Mean nocturnal activity (±SEM) in LD (black bars) and LM (dark gray bars) calculated as the mean number of beam crosses between ZT12 and ZT0. All genotypes show a significantly higher activity in LM compared to LD. (E) Relative nocturnal activity calculated as the sum of nocturnal activity (exhibited between ZT12 and ZT0) divided by the whole activity of the flies. All 3 WT strains show a significant increase in relative nocturnal activity, which is caused by a decrease in absolute diurnal activity and an increase in absolute nocturnal activity (B, C).

3. The relative nocturnal activity increased with increasing daylight intensity. This was most prominent in $WT_{Lindelbach}$ (Fig. 1A) and the least evident but still significant in $WT_{CantonS}$ (Fig. 1A and 4C).

Rhodopsin Expression in the Photoreceptor Mutants

To evaluate rhodopsin expression in the mutants, we performed immunostainings for Rh1, Rh5, and Rh6 in flies that were the same age as our experimental animals (4 days and 11 days). As expected, Rh1 was present in the outer photoreceptor cells of all fly strains except for $nina^{E17}$ and $ninaE^{17}$ $rh6^{1}$ mutants (not shown). The relation between Rh5 and Rh6 expression needed to be determined because de-repression of Rh5 was reported for $rh6^{1}$ mutants (Vasiliauskas et al., 2011), and the absence of pale R7 cells was shown to increase Rh6 expression in R8 (Chou et al., 1999). We found the expected 70%:30% Rh6:Rh5 distribution in $WT_{CantonS}$ flies (Fig. 2A) as well as in $rh3^{1}$ $rh4^{1}$ mutants (Suppl. Fig. S2), whereas sev^{LY3} expressed Rh6 in approximately 99% of R8 cells (Suppl. Fig. S2). $rh6^{1}$ mutants and $ninaE^{17}$ $rh6^{1}$ double mutants revealed the expected de-repression of Rh5 that increased slightly with age (Fig. 2A). Nevertheless, the latter appeared less extreme in our flies than was previously reported. We found Rh5 expression in a maximal 50% of R8 cells in 11-day-old flies (Fig. 2A), whereas Vasiliauskas et al. (2011) revealed Rh5 expression in 80% of R8 cells in 14-day-old flies.

Next, we tested whether cli^{eya} mutants still have normal extraretinal H-B eyelets. The H-B eyelets are extraretinal visual organs composed of 4 Rh6-expressing photoreceptor cells that directly project to the fly's circadian pacemaker center: the aMe (Yasuyama and Meinertzhagen, 1999; Helfrich-Förster et al., 2002) (Fig. 2B). The H-B eyelets have been shown to be present in eyeless flies and to project into the aMe (Hofbauer and Buchner, 1989; Helfrich-Förster et al., 2002), but so far, it has not been

Table 1. Absolute and relative daily activity levels of the WT strains and the photoreceptor mutants under the LM condition.

	n	*Mean Daily Activity*[a] ± SEM	*Activity Normalized to* $WT_{CantonS}$[b] ± SEM, %
$WT_{CantonS}$	192	0.2088 ± 0.0152	100 ± 7
WT_{ALA}	106	0.2465 ± 0.0251	118 ± 12
$WT^{Lindelbach}$	121	0.4094 ± 0.0278	196 ± 13
$rh3^1\ rh4^1$	100	0.1962 ± 0.0181	94 ± 9
sev^{LY3}	123	0.1921 ± 0.0175	92 ± 8
$rh5^2$	99	0.0487 ± 0.0044	23 ± 2
$rh6^1$	102	0.0485 ± 0.0060	23 ± 3
$rh5^2;\ rh6^1$	95	0.0540 ± 0.0044	26 ± 2
$rh5^2;\ rh3^1\ rh4^1\ rh6^1$	113	0.0495 ± 0.0047	24 ± 2
$ninaE^{17}$	100	0.0761 ± 0.0075	36 ± 4
$ninaE^{17}\ rh6^1$	106	0.4073 ± 0.0339	195 ± 16
cli^{eya}	80	0.0756 ± 0.0084	36 ± 4

n = number of analyzed flies.
a. Absolute mean activity levels (infrared light beam crosses per minute), pooled over all daylight intensities.
b. Relative activity levels, normalized to $WT_{CantonS}$ (the activity level of $WT_{CantonS}$ is set to 100%).

investigated whether these always find their targets in the aMe. Therefore, we immunostained 30 cli^{eya} brains with anti-Rh6 and anti-PDF. PDF is expressed in *Drosophila*'s most important circadian clock neurons, and it was shown that the terminals of the H-B eyelets overlap with the PDF neurons in the aMe of WT flies and eyeless so^1 mutants (Helfrich-Förster et al., 2002, 2007). We found that cli^{eya} mutants possessed 4 H-B eyelet cells per brain hemisphere (Fig. 2C) that had quite variable locations but projected into the aMe in 59 of the 60 investigated hemispheres (Fig. 2B). Thus, there was just 1 brain that was only unilaterally innervated, indicating that photoreception via the H-B eyelets is still functional in cli^{eya} mutants.

Contribution of Different Photoreceptors to the Flies' Responses to Moonlight

To find out which photoreceptor cells (and rhodopsins) in the compound eyes mediate WT-like responses to moonlight under increasing daylight intensities, we recorded the locomotor activity of the different photoreceptor mutants (Fig. 3). $WT_{CantonS}$ flies served as internal controls because these flies were *ls-tim* as all the mutants. cli^{eya} mutants without compound eyes but normal H-B eyelets served as negative controls.

As found for the WT strains, the different mutants showed quite different activity levels (Table 1). The severely impaired photoreceptor mutants $ninaE^{17}\ rh6^1$ were most active among the photoreceptor mutants, whereas $rh5^2$, $rh6^1$, and $rh5^2;rh6^1$ mutants showed very low activity levels, although these lacked just single rhodopsins. We conclude that the overall activity level is probably neither caused by deficits in photoreception nor by the *s-/ls-tim* polymorphism but by other genetic factors not of interest in the present study and did not consider these further.

Similar to WT flies, all photoreceptor mutants showed a WT-like bimodal activity pattern with a sometimes very weak M activity followed by a midday siesta and a pronounced E activity (Fig. 3). We noticed that the E activity was differently phased in the different photoreceptor mutants: It occurred earlier in the mutants with severely impaired photoreception as $rh5^2;rh3^1\ rh4^1\ rh6^1$, $ninaE^{17}$, $ninaE^{17}\ rh6^1$, and cli^{eya} under LD and LM (Fig. 3A). This general phase advance of the E peak seemed to depend on photoreceptor input to the clock and will be addressed in detail in another paper (Schlichting et al., in preparation). In the present study, we were mainly interested in the effects of moonlight on the phase of the E peak and the nocturnal activity level. Therefore, we determined for each genotype whether moonlight significantly delayed the phase of the E peak and increased the nocturnal activity level.

Delay of the E peak upon moonlight. ANOVA revealed a significant delay of the E peak in all photoreceptor mutants except the eyeless flies and the ones that lacked photoreception in all inner or all outer photoreceptor cells ($rh5^2;rh3^1\ rh4^1\ rh6^1$, $ninaE^{17}$, and $ninaE^{17}\ rh6^1$) (Fig. 4A). We conclude that all photoreceptor cells of the compound eyes contribute to delaying the E peak upon moonlight. Interestingly, photoreceptor cell R7 appeared to influence the delaying effect of moonlight, although this cell was not activated by our light (Suppl. Fig. S1). When comparing the phase of the E peak under moonlight in all mutants that responded with a delay, ANOVA followed by a post hoc test revealed that the time of the E peak was the same in $rh3^1\ rh4^1$ and sev^{LY3} mutants ($p = 1.0$) and that it occurred significantly later than that of all the other strains ($p < 0.003$).

Increase in nocturnal activity upon moonlight. Figures 3 and 4B show that moonlight seemed to stimulate nocturnal activity in a WT manner in the mutants that lacked photoreception in R7 ($rh3^1\ rh4^1$ and sev^{LY3}).

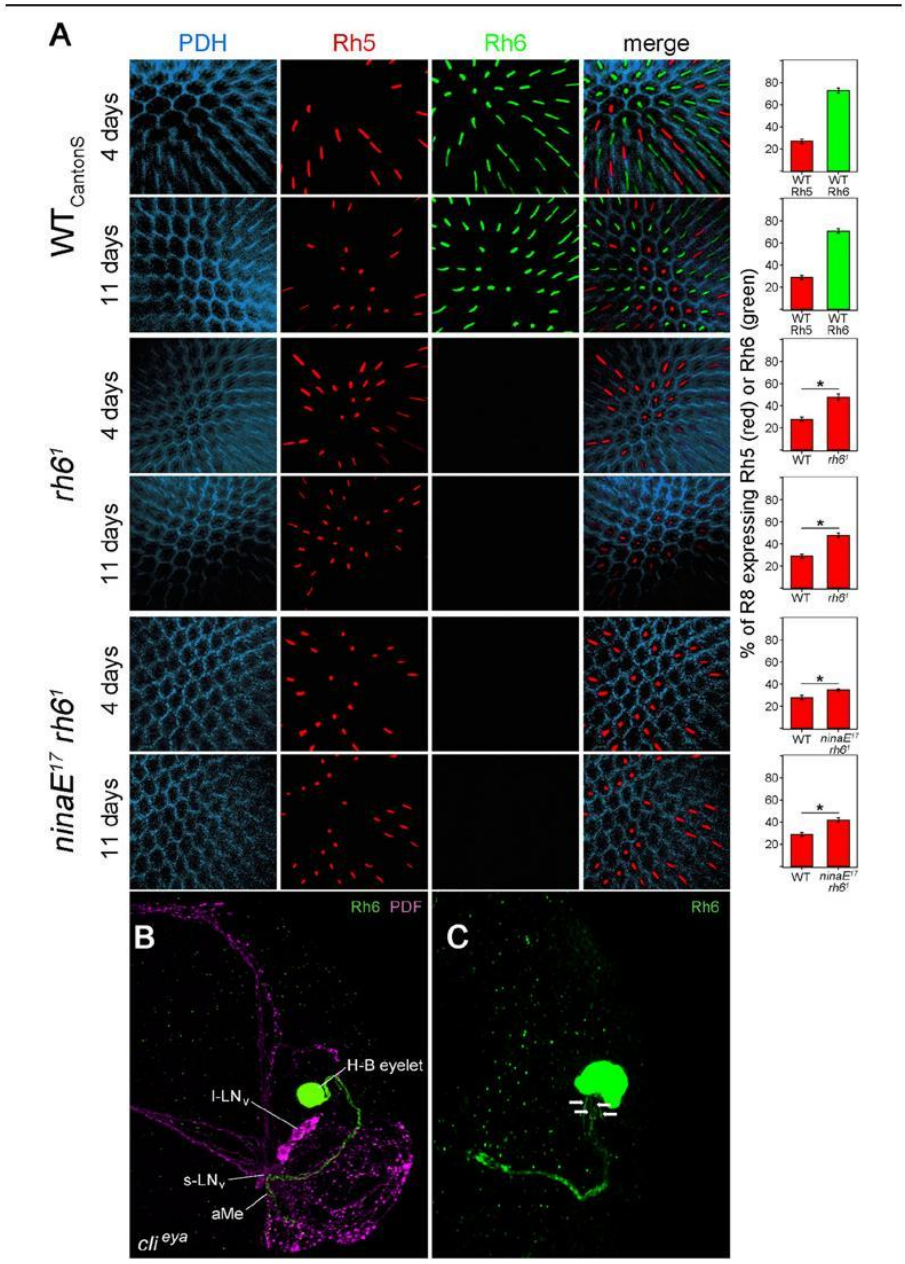

Figure 2. (A) Retinas immunostained with anti-PDH (blue), anti-Rh5 (green), and anti-Rh6 (red) of the genotypes $WT_{CantonS}$, *rh6*^{1}, and *ninaE*^{17} *rh6*^{1} at the age of 4 and 11 days. Anti-PDH labels the pigment cells surrounding the photoreceptor cells, whereas anti-Rh5 and anti-Rh6 label the rhabdomeres of photoreceptor cell R8 that are located in the centers of the ommatidia. $WT_{CantonS}$ shows Rh5 expression in 30% ± 3% (red bar) and Rh6 expression in about 70% ± 3% (green bar) of R8 as expected from the distribution of pale and yellow ommatidia throughout the retina (Rister et al., 2013), and there was no difference with the age of the flies. In *rh6*^{1} and *ninaE*^{17} *rh6*^{1} mutants, we could not detect any Rh6 staining, but the number of Rh5-positive R8 cells was significantly increased as compared to WT flies, and this was more pronounced in 11-day-old flies than in 4-day-old flies. (B) The H-B eyelet and PDF-positive lateral neurons (s-LN_v and l-LN_v) in the right brain hemisphere of a *cli*^{eya} fly. The neurites of the H-B eyelet (green, marked by anti-Rh6) project into the aMe, where they overlap with fibers from the PDF-positive LN_v (magenta). (C) As true for WT flies, 1 H-B eyelet of *cli*^{eya} mutants consists of 4 photoreceptor cells. This can be judged from the 4 neurites (arrows) originating from it.

The 2 mutants without functional photoreception in R7 had even the tendency for a higher relative nocturnal activity as compared to $WT_{CantonS}$, but this was not significant. In *rh5*^{2} mutants, moonlight provoked an increase in nocturnal activity that was about half as high as in $WT_{CantonS}$ (Figs. 3 and 4B). In all other mutants, the stimulation of nocturnal activity by moonlight was strongly reduced, or it was completely absent (Fig. 3). The latter was true for *ninaE*^{17} *rh6*^{1} and *cli*^{eya} mutants (Fig. 4B). *ninaE*^{17} *rh6*^{1} double mutants are furthermore exceptional with respect to their extremely low relative nocturnal activity (Figs. 3 and 4), although their general activity level was very high (Table 1). Already, the presence of Rh6 in the H-B eyelets was enough to bring the relative nocturnal activity back to the level of *ninaE*^{17}, suggesting that the H-B eyelet does also contribute to normal nocturnal activity levels. Overall, we conclude again that all photopigments of the compound eyes contribute to the increase in nocturnal activity upon moonlight, whereby Rh1 and Rh6 seem to be most important.

Dependence of nocturnal activity on daylight intensity. cli^{eya} mutants, *ninaE*^{17} mutants, and

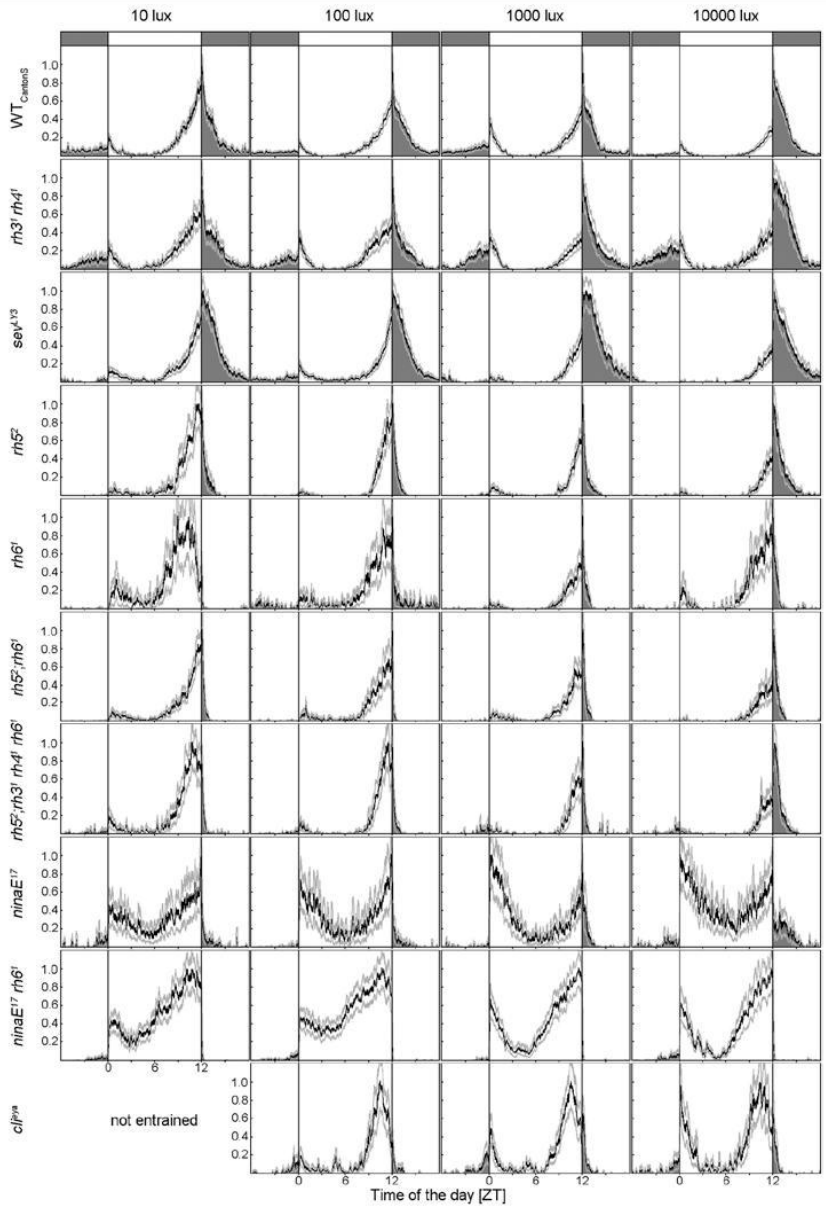

Figure 3. Mean normalized activity profiles (±SEM) of $WT_{CantonS}$ and different photoreceptor mutants under 12:12 LM cycles of different daylight intensities. cli^{eya} mutants (bottom) are completely eyeless; $rh3^1$ $rh4^1$ mutants lack the photopigments in the inner photoreceptor cell R7, and sev^{LY3} mutants lack the inner photoreceptor cell R7 as a whole; $rh5^2$, $rh6^1$, and $rh5^2;rh6^1$ mutants lack photopigments in R8; in $rh5^2;rh3^1$ $rh4^1$ $rh6^1$ mutants, photoreception is impaired in both inner photoreceptor cells (R7 and R8); $ninaE^{17}$ mutants lack Rh1 in the outer photoreceptor cells (R1-R6); and $ninaE^{17}$ $rh6^1$ mutants lack Rh1 and Rh6. Except for cli^{eya} that could not entrain to LM with 10-lux daylight intensity, all genotypes were able to synchronize to all simulated light conditions but differ mainly in their nocturnal activity. For detailed information, see text. Labeling as in Figure 1A.

$ninaE^{17};rh6^1$ double mutants did not change the ratio of diurnal/nocturnal activity with increasing daylight intensity (Fig. 4C), indicating that especially Rh1 is needed for measuring daylight intensity.

DISCUSSION

The aim of this study was to investigate the importance of rhodopsins for a WT response to moonlight and increasing daylight intensities. First, we showed that the activity rhythms of 2 independent WT strains responded qualitatively in the same way to high daylight intensities as well as to moonlight simulation as did the laboratory strain $WT_{CantonS}$: All 3 strains shifted activity into the night when artificial moonlight was present, and this was more prominent the higher the daylight intensity. This is especially interesting because the 3 WT strains expressed different proportions of the short (*s*) and long (*ls*) forms of the *tim* gene that play a major role in the circadian clock's response to light (Kyriacou et al., 2008). The clock of *s-tim* flies was shown to be more light sensitive than that of *ls-tim* flies (Sandrelli et al., 2007; Tauber et al., 2007). Consequently, we may expect that the *s-tim* $WT_{Lindelbach}$ flies shift activity stronger into moonlit nights than the *ls-tim* $WT_{CantonS}$ flies. Yet, this was not the case, suggesting that the input from the photoreceptor cells is more important for the present investigated light effects on the clock than is *s-tim* or *ls-tim*. We conclude that the evident abnormal responses to moonlight that we observed in the photoreceptor mutants are most likely due to the absence

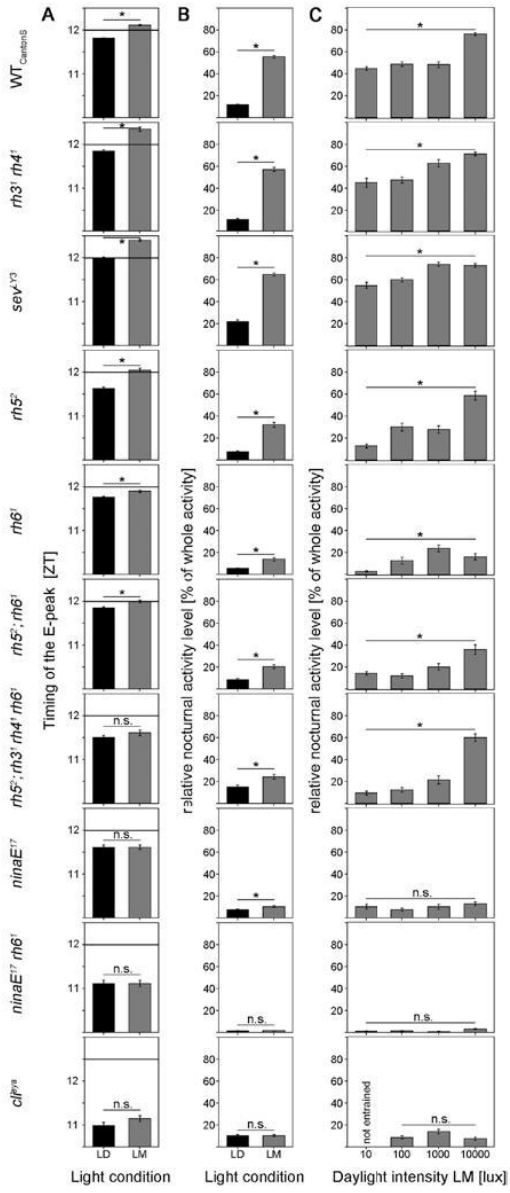

Figure 4. Timing of the E peak and relative nocturnal activity under LD and LM conditions in $WT_{CantonS}$ and 9 different photoreceptor mutants (alignment as in Fig. 2). (A) $WT_{CantonS}$ flies significantly delay the E peak upon moonlight simulation, whereas cli^{eya} mutants and mutants with severely impaired photoreception in the inner or outer photoreceptor cells of the compound eyes show no significant difference between LD and LM. (B) The relative nocturnal activity of $WT_{CantonS}$ and most photoreceptor mutants was significantly higher under LM than under LD. Only mutants without compound eyes (cli^{eya}) and mutants lacking rhodopsin 1 and 6 ($ninaE^{17}$ $rh6^{1}$) show no rise in nocturnal activity on moonlit nights, indicating an important role of these 2 rhodopsins within the compound eyes for dim light detection. (C) Relative nocturnal activity in dependence of daylight intensity of 10, 100, 1000, and 10,000 lux. The higher the light intensity, the higher the nocturnal activity in $WT_{CantonS}$ (ANOVA: $F_{3,188} = 64.168$, $p < 0.001$). The same is true for mutants affecting the inner receptors. As soon as the outer receptors are out of function, no increase in the relative nocturnal activity is visible, indicating an important role of R1 to R6 and Rh1 for measuring daylight intensity. See text for further details.

of particular photoreceptor cells (or photopigments) and not caused by different WT genetic backgrounds.

Second, we showed that the Rh6-expressing inner photoreceptor cell R8 together with the outer photoreceptor cells R1 to R6 are responsible for moonlight detection necessary for phase delaying of the E peak and stimulating general nocturnal activity. These photoreceptor cells are also the ones that finally determine the ratio of diurnal and nocturnal activity. The outer photoreceptor cells R1 to R6 are additionally needed for measuring daylight intensity.

It is already known that photoreceptor cells R1 to R6 are important for dim light detection in motion vision and phototaxis (Heisenberg and Buchner, 1977; O'Tousa et al. 1985; Yamaguchi et al., 2008; Zuker et al., 1985), whereas the inner photoreceptor cells were assumed to be less light sensitive and mainly responsible for color vision (Yamaguchi et al., 2010). This situation reminds us of the scotopic and photopic visual systems in mammals represented by the light-sensitive rods and the color-sensitive cones. Both systems signal via rod and cone bipolar cells on melanopsin-positive retinal ganglion cells that are light sensitive on their own and directly innervate the central clock in the suprachiasmatic nuclei (Hattar et al., 2003). Most interestingly, Altimus et al. (2010) found that, at higher irradiances when the classic rod pathway is not working anymore, rods signal through cones to the cone bipolar cells. This implies a direct interaction between the scotopic and photopic pathways in irradiance detection serving synchronization of the endogenous clock.

In the fruit fly, an interaction between the inner and outer photoreceptor cells was predicted for a long time for the phototactic response (Jacob et al., 1977) and was more recently confirmed (Yamaguchi et al., 2008, 2010). Whereas Jacob et al. (1977) proposed

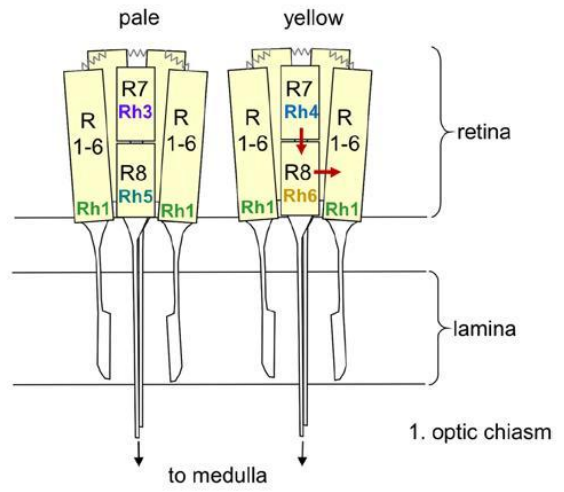

Figure 5. Rhodopsin expression in pale and yellow ommatidia of the fly retina. The 8 photoreceptor cells are shown in a schematic manner with the outer receptor cells R1 to R6 spanning the entire depth of the retina and the inner receptor cells R7 and R8 with R7 arranged above R8. R1 to R6 terminate in the fly's first optic neuropil, the lamina, whereas R7 and R8 run toward the medulla. R1 to R6 are connected via gap junctions (zigzag lines). The arrows indicate the putative interaction between the Rh6-expressing R8 cell and R1 to R6 as well as between R7 and R8. See text for further details.

that R8 is inhibiting R1 to R6, Yamaguchi et al. (2010) could not find a general inhibition of the R1 to R6 receptor subsystem by R7/R8. This applies also for our results. If R8 would inhibit R1 to R6, $rh5^2;rh6^1$ mutants should be more light sensitive than WT flies and shift more prominently into moonlight. However, the opposite happened. Furthermore, we found that $ninaE^{17}$ mutants without functional outer photoreceptor cells R1 to R6 still shift activity into moonlit nights, whereas $ninaE^{17},rh6^1$ double mutants are not able to do so anymore. This clearly indicates that Rh6 in R8 adds to moonlight detection. A direct functional connection between R8 and the outer photoreceptor cells was recently discovered by Wardill et al. (2012). These authors showed by sophisticated genetic manipulations of rhodopsin expression paired with electrophysiological and behavioral recordings that R8 signals either to the neighboring R6 and/or to R1 and by this way add to the optomotor responses elicited by R1 to R6. This supports our hypothesis that R8 and R1 to R6 work in the same direction in terms of dim light detection for synchronizing the flies' activity rhythms (Fig. 5).

Wardill et al. (2012) found also that input from R7 cells converged on the outer photoreceptor cells. Our data do not permit a definitive conclusion about R7 because we did not stimulate the UV-sensitive rhodopsins Rh3 and Rh4 with our "white light" LEDs. Nevertheless, our results suggest that R7 may also be involved in the interaction between R8 (and/or R1-R6) but rather in the opposite direction. After ablation of R7 by the *sevenless* mutation and after elimination of Rh3 and Rh4, we did not see any reduction in moonlight sensitivity. On the contrary, sev^{LY3} and $rh3^1\ rh4^1$ mutants showed a stronger shift of the E peak into moonlit nights and had the tendency to increase relative nocturnal activity more than WT flies. This result is especially remarkable for sev^{LY3} mutants because the R7 rhabdomere should serve as a light guide to the R8 rhabdomere: The rhabdomere tip of R7 is positioned in the focal plane of the facet lens, where the light flux is most intense, and subsequently, the light flux is trapped in the rhabdomere in an optical waveguide mode down to the rhabdomere of R8 (Snyder and Menzel, 1975). Without R7, R8 is expected to receive much less light, and as a consequence, photoreception of R8 should be reduced. Because we found the contrary, we have to conclude that at least regarding synchronization of the clock, intact (not excited) R7 cells inhibit R8 and/or R1 to R6 via unknown mechanisms. This inhibition seems to need the presence of the photopigments because it was similarly lost when R7 was present, but just Rh3 and Rh4 were absent. Future studies with light sources containing UV light are necessary to further unravel this interesting interaction.

Another interesting detail of our results is the fact that Rh5-expressing R8 cells seem to contribute only marginally to dim light detection. Although the number of Rh5-expressing R8 cells was increased in the absence of Rh6 (in $rh6^1$ and $ninaE^{17}\ rh6^1$ mutants), these mutants did not increase their response to moonlight. We could also exclude a prominent role of the H-B eyelet in moonlight detection. Although the H-B eyelets may contribute to general phasing of the E peak as well as to the relative nocturnal activity level of the flies, they were clearly neither sufficient for the shift of the E peak into moonlit nights nor for the nocturnal activity increase in response to moonlight.

In summary, we show here that the inner and outer photoreceptor cells of *Drosophila*'s eyes interact in adapting the flies' activity patterns to variable diurnal and nocturnal light conditions. In mice, similar mechanisms are present in the retina, and among other factors, these determine whether the animal is nocturnal or diurnal (Doyle et al., 2008; McNeill et al., 2008). The latter may also be true in *Drosophila*, confirming the fruit fly as a suited model organism to unravel the mechanisms of synchronizing the circadian clock to light.

ACKNOWLEDGMENTS

The authors thank Nina Vogt, Claude Desplan, Francois Rouyer, Ralf Stanewsky, Erich Buchner, Rodolfo Costa, and Christopher Schnaitmann for providing fly lines, Christiane Hermann and Pingkalai Senthilan for critical comments on the paper, and the German Research Foundation (DFG; Fo207/10-3 and SFB1047, INST 93/784-1) as well as the European Community (6th Framework Project EUCLOCK no. 018741) for funding. They also thank Craig Montell, Steve Britt, and Claude Desplan for providing antibodies as well as the Graduate School for Life Sciences Würzburg and the Hanns-Seidel-Foundation for supporting Rudi Grebler and Matthias Schlichting, respectively.

CONFLICT OF INTEREST STATEMENT

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

NOTE

Supplementary material is available on the journal's website at http://jbr.sagepub.com/supplemental.

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Supplementary Online Material

Moonlight detection by *Drosophila's* endogenous clock depends on multiple photoreceptors in the compound eyes

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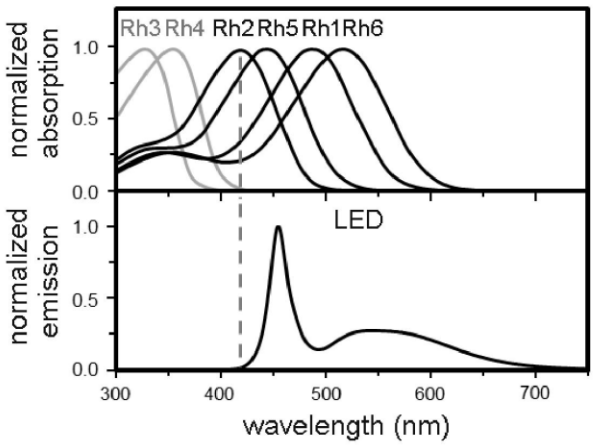

Figure S1

Normalized absorption spectra of *Drosophila's* rhodopsins and emission spectrum of the white LEDs used for illumination of the flies. The LEDs can excite all rhodopsins except for the UV-sensitive rhodopsins Rh3 and Rh4 that are expressed in photoreceptor cell R7. The rhodopsin absorption spectra are derived from Stavenga and Arikawa (2008). Rh2 is expressed in the ocelli and not in the compound eyes.

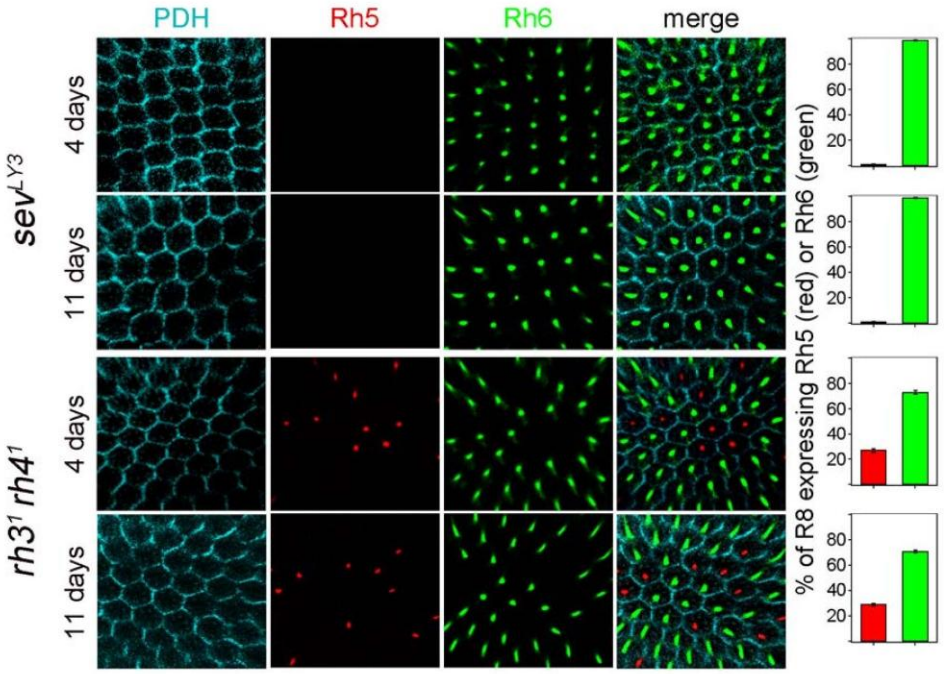

Figure S2

Retinas of *sev*^{LY3} and *rh3*^{1} *rh4*^{1} mutants immunostained with anti-Pigment-cell enriched Dehydrogenase (PDH, blue), anti-Rh5 (green) and anti-Rh6 (red) at the age of 4 and 11 days. Anti-PDH labels the pigment cells surrounding the photoreceptor cells, whereas anti-Rh5 and anti-RH6 label the rhabdomeres of photoreceptor cells R8 that are located in the centers of the ommatidia. *rh3*^{1} *rh4*^{1} mutants show a wild-type Rh5 and Rh6 expression with a relation between Rh5 and Rh6 of 30% to 70%. *sev*^{LY3} mutants expressed Rh6 in all R8 cells. Rh5 was almost completely absent. In both mutants there was no difference in Rh5 and Rh6 expression between young and older flies.

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6.3. Normal Vision Can Compensate for the Loss of the Circadian Clock

Normal Vision Can Compensate for the Loss of the Circadian Clock

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Classification: BIOLOGICAL SCIENCES, Neuroscience

Author contributions: CHF and MS conceived the study and wrote the paper. MS performed and analyzed the experiments. PM helped with the immunocytochemical studies and provided substantial intellectual input. The authors declare no competing financial interests. Correspondence should be addressed to CHF.

Significance Statement

The well-established view that the circadian clock is necessary for normal rhythmic behavior was recently challenged for mice and fruit flies, since clock-less mutants showed astonishingly normal activity rhythms in nature. Therefore, compensatory mechanisms enable clock mutants to live a normal life under natural conditions. Here we show that, in fruit flies, normal vision can largely compensate for the loss of the clock and almost enable normal daily locomotor activity patterns, although the circadian clock would help to reduce futile activity at unfavorable times. Our results are generally encouraging for clock mutants: regular exposure to natural light-dark cycles may guarantee a rather normal adaptation to cyclic changes in the environment.

Circadian clocks are thought to be essential for timing the daily activity of animals and consequently increase fitness. This view was recently challenged for clock-less mice and fruit flies that exhibited astonishingly normal activity rhythms under outdoor conditions. Compensatory mechanisms appear to enable even clock mutants to live a normal life in nature. Here, we aimed to unravel these mechanisms. We show that gradual daily increases/decreases of light in the lab, with all other environmental conditions kept constant, suffice to provoke normally timed sharp morning (M) and evening (E) activity peaks in clock-less flies. We also show that the compound eyes but not cryptochrome (CRY), mediate the precise timing of M and E peaks, since this timing is absent in eyeless but unchanged in CRY-less mutants. Eyeless *period*^{01} mutants completely lack sharp M and E peaks, whereas CRY-less *period*^{01} mutants behave essentially like *period*^{01} single mutants. We conclude that the precisely timed M and E peaks under natural-like conditions are not controlled by the circadian clock but by signals coming from the compound eyes. Nevertheless, the circadian clock appears critical for keeping activity low during midday and midnight, since clock-less flies exhibit a less pronounced siesta than wild-types and respond with high activity to twilight exposure in the middle of the night. Thus, possessing a clock may help to save energy by reducing futile activity. Furthermore, the circadian clock responds to natural-like light-cycles by significantly broadening Timeless (TIM) abundance in the lateral clock neurons, and this effect is mediated by CRY.

The daily pattern of animal behavior is thought to be of critical importance for fitness. It is generally assumed that the circadian clock times activity to the optimal time of day and that possessing a circadian clock is important for survival and reproductive fitness (e.g.(1-3)). This view was recently challenged in mice and fruit flies, because clock-less mutants showed almost wild-type activity patterns when exposed to natural-like conditions (4-7). In particular, wild-type mice and mutants for the *period2* gene (*Per2*^{Brdm1}) were kept in a semi-natural outdoor environment over two years and the differences in activity patterns between the two genotypes turned out to be negligible compared to the very similar seasonal changes observable in both genotypes (4). Furthermore, the clock mutation had no persistent negative effects on fitness. In fruit flies, locomotor activity of wild-type and different clock-less mutants was recorded in the traditional glass tubes placed outdoors in an area sheltered from rain and direct sunlight (5-7). All genotypes showed typical morning (M) and evening (E) activity and virtually no differences in activity patterns were observed between clock-less mutants and wild-type flies.

To determine the timing cues that enable clock-less flies to time locomotor activity in a wild-type manner in nature, we recorded locomotor activity of wild-type strain

CantonS ($WT_{CANTONS}$) and of previously studied clock mutants – per^{01} and tim^{01} – in the lab, where we could precisely define the cyclic environmental conditions. We kept temperature and day length constant and only varied the daily light-profile: all flies were first exposed to usual lab light-dark cycles (LD) with the light being switched-on/ -off suddenly, then to light-dark cycles with simulated twilight (LDR1, R=ramp; (8, 9)) and finally to light-dark cycles closely mimicking the light profile occurring in nature (LDR2, (10, 11)). We found that the natural-like light profile was sufficient to provoke almost wild-type like activity patterns in the mutants.

Next we aimed to unravel the light-input pathways that are responsible for the wild-type like activity pattern of the mutants. *D. melanogaster* has several photoreceptors, the compound eyes, the ocelli, the extraretinal eyelets and CRY (reviewed in (12)). Among these, CRY and the compound eyes have the greatest impact on locomotor activity rhythms. CRY is expressed in the majority of *Drosophila's* lateral clock neurons (13, 14). Upon light-activation, CRY interacts directly with the molecular feedback loop that generates circadian oscillations by provoking degradation of the clock protein TIM (15, 16) and consequently, the molecular clock is set to a new phase (17). The compound eyes only have moderate effects on rhythm phase (18), but they are necessary to adapt fly activity to long days (19), to nocturnal dim light (20, 21), as well as to twilight (22).

To investigate whether CRY or the compound eyes are necessary for a wild-type (WT)-like behavior under natural-like light cycles, we recorded mutants without CRY (cry^{01} mutants; (23)) or without eyes (eya^{2} mutants; (24)), as well as mutants that lack the clock and additionally either CRY or the eyes under the above mentioned light schedules. Furthermore, we measured TIM oscillations in the lateral clock neurons of WT flies and cry^{01} mutants to reveal the effect of natural-like light cycles on the molecular clock. We found that CRY is responsible for slight effects of natural-like light cycles on the molecular clock, but that the compound eyes are responsible for fly wild-type like activity patterns.

Results

M and E peak timing under simulated twilight is independent of a functional clock

WT flies showed the typical bimodal activity pattern with M and E activity bouts and a pronounced siesta between them under all tested conditions (LD, LDR1 and LDR2; Fig. 1). In LD, M activity peaked shortly after lights on, E activity shortly before lights off and the flies were strongly diurnal as maximally 11.7% of the activity took place at night. Dawn and dusk simulation even augmented fly diurnality (Fig. 1C), since the M peak significantly delayed and the E peak significantly advanced in LDR1 and even further in LDR2 (Fig. 1D, E; M peak: $F_{(2,51)}=62.573$ $p<0.001$; E peak: $F_{(2,52)}=84.966$; $p<0.001$).

As reported previously (25-27), *per*^{01} and *tim*^{01} clock mutants behaved very differently from WT flies under conventional LD cycles: they showed no clear M and E activity bouts, lacked the siesta and were slightly more active during the night (Fig. 1). However, when light-intensity was gradually increased/decreased (LDR1 and LDR2) the locomotor activity of *per*^{01} and *tim*^{01} mutants became more similar to the one of WT flies: WT-like M and E peaks appeared (Fig. 1B) and nocturnal activity decreased (Fig. 1C). Mutant flies delayed the M peak and advanced the E peak in LDR2 compared to LDR1 as wild-type flies did (Fig. 1D, E). Under LDR2 the activity pattern of the mutants was virtually indistinguishable from that of WT flies, only the activity during the siesta remained higher in the mutants (Fig. 1B).

Taken together, our findings show that the timing of M and E peaks, as well as the shift of activity out of the night into daytime upon simulation of natural-like light conditions, is independent of a functional clock. The two clock mutants are able to precisely track changes in light intensity with their activity. Consequently, the activity patterns of *per*^{01} and *tim*^{01} mutants look surprisingly similar to those of WT flies. When calculating the light intensity at which M and E peaks occurred, we found that they always took place between 1 and 10 lux, independently of the presence or absence of the clock.

The compound eyes are necessary to time M and E peaks in a wild-type manner

We subsequently wanted to elucidate the mechanisms by which flies precisely time their activity peaks under LDR even in absence of a functional clock. We therefore tested *eya*^{2} mutants and *cry*^{01} mutants under LD, LDR1 and LDR2 conditions (Fig. 2 A-H). We found that both mutants still entrained to all applied light conditions, but that their activity pattern differed substantially from each other. *cry*^{01} mutants behaved essentially indistinguishably from WT flies, whereas *eya*^{2} mutants exhibited rounded M and E activity bouts, but no sharp M and E peaks. Furthermore, *eya*^{2} mutants neither reduced nocturnal activity when exposed to LDR1 and LDR2 conditions (Fig. 2C; $F_{(2,48)}=0.670$; $p=0.517$) nor significantly altered the timing of E activity bouts upon these conditions (Fig. 2D; $F_{(2,48)}=0.976$;

p=0.384): The percentage of nocturnal activity stayed constant in *eya*^{2} mutants under all light conditions and the same was true for the timing of maximal E activity. We could not reliably calculate the phase of the M activity bout in *eya*^{2} mutants, because M activity was drastically reduced under LDR1 and even more so under LDR2 (Fig. 2A). This shows that *eya*^{2} mutants can still detect the different light conditions but that the compound eyes are absolutely essential for the presence of sharp M and E peaks and their precise timing to gradually increasing/decreasing light. CRY, on the other hand, appears unnecessary for this timing.

Functional eyes are also necessary for WT-like activity patterns in clock mutants

To test whether losing the compound eyes but not CRY also leads to a loss of M and E activity peaks in clock-less flies, we recorded *per*^{01}*;eya*^{2} and *per*^{01}*;;cry*^{01} double mutants under LD, LDR1 and LDR2 conditions (Fig. 2I-O). Indeed, the activity pattern of *per*^{01}*;;cry*^{01} double mutants was very similar to that of *per*^{01} single mutants (compare Fig. 2L-O with Fig. 1). In contrast, *per*^{01}*;eya*^{2} double-mutants lacked M and E peaks (Fig. I-K), showing that the occurrence of these peaks depends on functional compound eyes but not on CRY. We conclude that the normal timing of M and E peaks in *per*^{01} mutants is solely caused by the ability of the compound eyes to measure and integrate the regularly changing light intensity and to provoke almost normal activity patterns under LDR2 even in the absence of a circadian clock.

The clock is needed for a normal siesta and for suppressing activity during the night

In spite of the identical timing of M and E peaks in WT flies and clock mutants under LDR conditions, some differences in the activity pattern of the two strains were evident. The typical WT-like siesta seemed less pronounced in the mutants (Fig. 1B) and, at least under LD, clock mutants were slightly more active during the night. This suggests that a functional clock may suppress activity during midday and midnight. If true, it should be more likely to induce nocturnal activity in mutant than in WT flies. To test this hypothesis, we simulated 1.5h dawn followed, two hours later, by 1.5h dusk in the middle of the night while recording fly activity.

We found that *per*^{01} and *tim*^{01} mutants responded to “midnight twilight” exactly as they did to morning/evening twilight (Fig. 3). The same was true for clock-less flies that lacked, in addition, CRY (*per*^{01};;*cry*^{01} mutants). Flies with functional clock as WT and *cry*^{01} also increased activity in response to light, but lacked the sharp activity peaks during midnight twilight whereas eyeless flies did not respond at all to the nocturnal light. Together this shows that light driven behavior at midnight is mediated by the compound

eyes but suppressed by a functional clock. Consequently, eyeless flies with functional clock are not at all stimulated by nocturnal light. WT flies and *cry*^{01} mutants that have functional eyes and clocks show an intermediate response and clock-less flies with functional eyes show the strongest response.

LDR2 alters TIM-cycling in clock neurons in a CRY-dependent way

As the clock seems to contribute to the activity pattern of the flies, we investigated next how LDR2 affects the molecular clock in the lateral clock neurons known to control M and E activity bouts (s-LN$_v$, 5th s-LN$_v$, and LN$_d$; Fig. 4A). We assessed TIM cycling by immunocytochemical means in wild-type flies and *cry*^{01} mutants under LD and LDR2.

As reported previously for wild-types flies under LD (16, 28-34), we found TIM to rise after lights off reaching its maximum between ZT 18 and ZT 21 and then to decrease again (Fig. 4B). After lights on, TIM disappeared and started to rise again after lights off (ZT 12). More natural-like light conditions (LDR2) significantly affected TIM cycling in WT flies. ANOVA revealed that the increase and decrease of TIM depended on the light-condition (LD/LDR2) in two of the three groups of clock neurons. Furthermore, there was a significant interaction between time of day and light-condition on TIM, meaning that the slopes of TIM increase and decrease were different between LD and LDR2: TIM accumulated earlier and stayed stable for a longer time under LDR2 than under LD (Fig. 4B). Only small differences in TIM cycling were visible between M and E neurons. In the s-LN$_v$, that belong to the M neurons, TIM reached its peak levels slightly earlier than in the 5th s-LN$_v$, that belongs to the E neurons.

cry^{01} mutants did not show any difference in TIM cycling between LD and LDR (Fig. 4B) indicating that CRY is necessary for mediating the observed effects of twilight on the clock. In addition, the absence of CRY influenced the general TIM cycling profile. TIM persisted clearly longer after lights-on in *cry*^{01} mutants than it did in WT flies. Furthermore, TIM rose later and reached its peak significantly later in the 5th s-LN$_v$ cell (E neuron) than in the s-LN$_v$ cells (M neurons). Consistent with earlier studies, the shape of TIM cycling was flattened in the LN$_d$ cells, which seem to consist of a mixture of M and E neurons (20, 35-37).

Discussion

In nature, many environmental factors oscillate during the 24-hour day, among which irradiance, temperature and humidity are most important. Animals sense these regular fluctuations and respond immediately in an adequate way. For example, a diurnal animal whose visual system cannot tune to darkness will stop moving after night onset. In addition, the circadian clock will prepare the animal for the coming night allowing it to anticipate inactivity already before darkness onset. Consequently, an animal's daily behavior is a mixture of immediate responses to environmental changes and clock-controlled processes. It is not always easy to distinguish between the two contributions, especially not under natural conditions where multiple environmental factors are changing in parallel. Immediate responses to the environment are usually important for fine-tuning clock-controlled responses, but they can be strong enough to conceal the clock-controlled processes, a phenomenon known as "masking" of the endogenous clock output (38).

Transferring animals to the lab, and especially to constant conditions, helps to see which part of activity is clock controlled. This procedure has been carried out for many animals in the last century. Under constant conditions, fruit flies exhibit bimodal activity with a smaller activity bout in the subjective morning (M, best visible at temperatures above 25°C) and a larger activity bout (E) spanning the subjective afternoon and evening [28,29]. Cycling environmental conditions in the lab (especially LD-cycles) modify the shape of M and E activity bouts: they become higher and narrower, couple to lights on and off, respectively, and are clearly separated by a siesta (e.g., (39, 40)). The phase of these sharp M and E activity peaks in LD can easily be determined. Under LDR1, M and E activity bouts become even sharper and the peaks occur at specific irradiances: ~7.5 lux in a previous study (8)and between 1 to 10 lux in the present study. Yet, are these sharp peaks the output of the entrained circadian clock or induced by light?

The sharp M and E peaks are not controlled by the circadian clock

The present study clearly shows that sharp M and E peaks provoked by twilight simulation are not outputs of the circadian clock. Activity of the clock mutants peaked at the same irradiance as activity of WT flies (1-10 lux). Moreover, when irradiance was gradually increased/decreased to/from the same maximal intensity but within 4.5 hours instead of 1.5 hours, the activity of all flies still peaked at 1-10 lux. Since 1-10 lux was now reached significantly later/earlier, the peaks were shifted, respectively. This behavior can be fully explained by an immediate response of the flies to the increasing/decreasing irradiance sensed by the compound eyes. Indeed, the sharp peaks completely disappeared in eyeless flies. Obviously, fly activity is stimulated by the increasing/decreasing irradiance and flies prefer to be active at rather low irradiances. This observation fits to previous

results obtained in the lab that have shown that flies show a preference for rather dim light (8) and clock mutants exhibit the same light preference (41).

The perhaps most surprising result of this study is the fact that the activity pattern of per^{01} and tim^{01} clock mutants looks virtually identical to that of WT flies when irradiance is slowly increased/decreased, closely mimicking the natural time course of irradiance during the 24-hour day. Thus, we could largely reproduce the results of Vanin*, et al.* (7), who recorded flies outside the lab under natural-like light and temperature cycles and found similarly timed onsets of M and E activity in WT flies and per^{01} and tim^{01} mutants. Our results also suggest that, in the outdoor experiments of Vanin*, et al.* (7), clock mutants may have mainly responded to the cyclic environment. Furthermore, our study indicates that natural-like light-dark cycles are sufficient to provoke these quasi-normal activity patterns in clock mutants, additional temperature cycles seem not to be necessary.

Nevertheless, outdoor temperature cycles have most likely contributed to the WT-like activity pattern of clock mutants. High temperatures have furthermore caused the so-called ‘afternoon peak’ that appeared in the outdoor experiments during warm days and that was interpreted as an escape response of the flies (5, 7). Menegazzi, Yoshii and Helfrich-Förster (6) found the afternoon peak to be more pronounced in clock mutants than in WT flies supporting the idea that it is a direct response to high temperature that may be partly repressed by the circadian clock of WT flies that usually rest at this time. Here, we found that clock mutants were more active during siesta than WT flies, which fits to this idea. In addition, we show that clock mutants responded stronger than wild-type flies to twilight simulations in the middle of the night, a time at which flies normally sleep (42, 43). These findings support our conclusion that a functional clock suppresses activity at unfavorable times. Activity during the hottest part of the day would require a cooling system, whereas activity during the coldest part of the day would need an internal heating system consuming considerable amounts of energy. Consequently, activity suppression during these times prevents flies from wasting energy. Amazingly, this suppression also works at the here applied constant temperature of 20°C clearly speaking for an endogenous control.

The circadian clock responds to twilight

Although the circadian clock is not necessary for the exact timing of M and E peaks under twilight conditions, the molecular clock is receptive to twilight as we show here for TIM-cycling in the lateral clock neurons. TIM accumulated earlier and was present longer under LDR2 than under LD in two lateral groups of the clock neurons. TIM plays a relevant role in clock sensitivity to light because it gets degraded via the proteasomal

pathway after interaction with CRY and JETLAG, as soon as the fly perceives light in the morning (16, 17, 26, 44-48). Similarly, TIM cannot re-accumulate before dusk. In our study, the gradual increase/decrease in irradiance under LDR2 obviously allowed TIM to remain longer in the morning and to increase earlier in the evening. Interestingly, this difference disappeared in *cry*^{01} mutants supporting the idea that CRY is critical for timing the molecular clock in response to twilight. However, the absence of CRY did not only impair sensitivity of the molecular clock to twilight, but also delayed the timing of TIM accumulation and degradation in the 5th s-LN$_v$ that belongs to the E neurons (under LD and LDR2). Interestingly, the delay of this E neuron had only minor consequences on the timing of the E activity bout. Only under LD, E activity of *cry*^{01} mutants rose slower than in WT flies and the E peak appeared not quite at its maximal level at lights-off (Fig. 2E). However, under LDR1 and LDR2 the differences between *cry*^{01} mutants and WT flies disappeared suggesting that M and E peak timing by the compound eyes dominated over the effects of CRY on the molecular clock.

The eyes as important light-sensing organs

Here we show that light is the key signal for adapting the activity pattern of *Drosophila* to natural-like conditions and the compound eyes play a major role in sensing gradual changes in light-intensity. This makes sense, as the compound eyes mediate other immediate responses of light reported previously, such as the startle response after lights-on in LD-cycles and the nocturnal increase in activity upon moonlight (41). To measure gradually changing light intensity, an irradiance-detecting system employing several photopigments is required (49). Indeed, we showed recently that fruit flies use multiple photopigments within their compound eyes to detect dim light (21) and twilight (22).

In summary, our study is encouraging for organisms carrying clock gene mutations. Obviously, nature provides mechanisms that allow for almost normal activity rhythms. The natural light-dark cycle and functional eyes seem to be sufficient for normal timing. In nature, temperature cycles and other cycling Zeitgebers will contribute to fine tune activity, making it difficult to distinguish clock mutants from animals with normally ticking clocks.

Material and Methods

Fly strains and rearing

To investigate the impact of a functional clock on locomotor activity we studied per^{01} and tim^{01} flies that are null mutants for the core clock genes *per* and *tim* (50-52). $WT_{CantonS}$ served as control. eya^{2}-mutants, that lack compound eyes but have normal ocelli as well as the extraretinal Hofbauer-Buchner-Eyelets (24), were used to investigate the role of compound eyes in timing activity to the appropriate time of day. cry^{01} mutants (23) were used to test the role of CRY in this process. Double mutants, $per^{01};eya^{2}$ and $per^{01};;cry^{01}$, were used to investigate the importance of both intact vision and a functional clock on timing of behavior. All flies were raised on *Drosophila* medium (0.8% agar, 2.2% sugarbeet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, and 0.3% hydroxybenzoic acid) at 25 °C in LD 12:12.

Locomotor activity recordings and data analysis

Fly locomotion was measured in a home-made system described first in Helfrich-Förster (53) and refined in Rieger*, et al.* (8). 2 to 5 days old male flies were singly transferred into photometer cuvettes with water and food supply on one end and an infrared light-beam recording the number of infrared light-beam interruptions caused by the fly in 1 minute intervals on the other end. All experiments were performed in a climate controlled chamber at 20°C. Illumination was provided by tunable “white” LEDs (Lumitronix LED-Technik GmbH, Jungingen, Germany). In addition, neutral density filters (Lee Filters Worldwide, Hampshire, United Kingdom) were used for fine-adjustment of light intensity. We simulated 3 different light conditions, each consisting of 12 hours light and 12 hours darkness and a maximal light intensity of 100 lux. In LD we simulated a rectangular light-dark-cycle, in LDR1 the light intensity increased within 1.5 hours in the morning and decreased within 1.5 hours in the evening to simulate dawn and dusk and in LDR2 the light intensity increased or decreased within 4.5 hours each to simulate the course of the sun within one day. Each light condition was given for 7 days with LD being present from day 1 to 7, LDR1 from day 8 to 14 and LDR2 from day 15 to 21. In each experiment 32 flies per genotype were recorded, but only flies surviving until day 21 were analyzed. To further test the contribution of masking in the response to simulated twilight we entrained flies in LD12:12 for 6 days. On day six we additionally applied a light pulse in the night with light intensity rising to 100 lux between ZT15.5 and ZT17 and decaying between ZT19 and ZT20.5.

Raw data were plotted as actograms using ActogramJ (54). Behavioral analysis was performed as described in Schlichting and Helfrich-Förster (55). Besides the average activity profile, we analyzed relative nocturnal activity and peak timing of individual flies.

Statistical analysis was performed using Systat11. After testing for normal distribution by a Kolmogorov-Smirnov-test, data were compared using a 1- or 2-way-ANOVA. In case of not normally distributed data, p-values were adjusted by multiplication with 5 (56).

Fluorescent immunohistochemistry

To analyze the molecular cycling of TIM in the brain, 1-4 days old male $WT_{CantonS}$ and cry^{01} flies were entrained for 5 days either in LD or LDR2 with a maximal light intensity of 100 lux and sampled every 2 h. Whole flies were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) containing 0.1% Triton X-100 (PBT, pH=7.4) for 2.5 hours at room temperature. After washing the flies 4 times for 15 min in PB, the brains were dissected in PB and afterwards transferred into blocking solution (5% normal goat serum (NGS) in PBT) over night at 4°C. On the following day, brains were transferred into the first antibody solution containing rat anti-TIM (dilution 1:1000, provided by Isaac Edery, (48)) and mouse anti-PDF (dilution 1:2000, Developmental Studies Hybridoma Bank, Iowa), 5% NGS and 0.02% NaN_3 in PBT. After incubating overnight at 4°C, brains were washed 5 times in PBT for 10 min. In the next step, the secondary antibody solution was applied for 3 hours at room temperature consisting of Alexa Fluor 555 (goat anti-rat) and Alexa Fluor 635 (goat anti-mouse), each in a dilution of 1:200 in PBT containing 5% NGS. After washing 5 times for 10 minutes each in PBT, brains were embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) with the anterior surfaces upside on the slide.

Microscopy and image analysis

Brains were analyzed using laser scanning confocal microscopy (Leica TCS SPE; Leica, Wetzlar, Germany). To excite the fluorophores of the secondary antibodies we used 2 different laser diodes (532 nm and 635 nm) and obtained confocal stacks of 2 µm thickness. To quantify and compare the intensity of TIM staining, laser settings were kept constant for all samples. Staining intensity was analyzed in 3 different clock neuron clusters (s-LN_v, 5^{th} s-LN_v, and LN_d) using the ImageJ distribution Fiji (http://fiji.sc/Fiji). For quantification, we determined the brightness of single clock neurons using a 9 pixel-area and subtracted three different background intensities to compensate for unspecific staining as described in Menegazzi*, et al.* (57). For each time-point, one hemisphere of at least 5 different brains was analyzed. TIM cycling was normalized to 1 and plotted using Qtiplot. To show general tendencies in TIM staining intensity cycling, a polynomial fit of the 4^{th} order considering the value's standard error of the mean was applied (internal function of Qtiplot). A two-way ANOVA was used to calculate the dependency of TIM increase and decrease on time (ZT) and light-regime (LD/LDR2).

Acknowledgements

We thank Alois Hofbauer and Erich Buchner for providing fly lines, Isaac Edery and Ralf Stanewsky for providing antibodies, Marta Beauchamp, Christiane Hermann-Luibl, Nicolai Peschel, William Schwartz and Christian Wegener for critical comments on the manuscript as well as Verena Dusik and Christiane Hermann-Luibl for the help with brain dissection. We thank the German Research Foundation (DFG; Fo207/10-3 and SFB1047, INST 93/784-1) as well as the European Community (6th Framework Project EUCLOCK no. 018741) and the Graduate School of Life Sciences for funding and support. Matthias Schlichting was further sponsored by a Hanns-Seidel-Foundation excellence grant funded by the BMBF (German Ministry for Education and Research).

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Figure Legends:

Figure 1

Rhythmic activity of wild-type flies ($WT_{CantonS}$) and the clock mutants *per^{01}* and t*im^{01}* in LD (light-dark cycle with lights-on and -off), LDR1 (LD with 1.5 hour twilight simulation) and LDR2 (LD with 4.5 hours increasing/decreasing light). For each strain average actograms (A), average activity profiles (B), nocturnal activity (C), timing of morning (M) and evening (E) activity peaks (D and E) were calculated. In the average actograms, the mean activity of 25 flies is indicated in black without error bars. LD was simulated from day 1-7, LDR1 from day 8-14 and LDR2 from day 15-21. The gray vertical lines indicate Zeitgeber Time (ZT) 0 (= beginning of day) and ZT 12 (= beginning of night). The average activity profiles indicate the average activity of all flies (black curve) ±SEM (light gray) under the relevant light condition (LD, LDR1, LDR2), which is given on top of each diagram (black: complete darkness, dark gray: time of increasing or decreasing light intensity, white: time of maximal light intensity (100 lux)). The WT strain shows bimodal activity patterns with M and E activity bouts under all three light conditions. Upon LDR1 and more so upon LDR2, nocturnal activity decreases (C), M and E peaks delay/advance, respectively, (D, E). Nocturnal activity is given in percentage of whole daily activity (± SEM), timing of M and E peak in ZT. The clock mutants lack bimodal activity patterns under LD, but develop them under LDR1 and LDR2 (A,B). Nocturnal activity was higher than in WT flies under LD, but was reduced under LDR1 and LDR2 in a WT-like manner (C). Timing of M and E peaks under LDR1 and LDR2 was also WT-like in the mutants (D, E). Consequently, the activity pattern of *per^{01}* and *tim^{01}* mutants is virtually indistinguishable from that of wild-type flies in LDR2 (B). Only activity during the siesta was higher in the mutants. For direct comparison, the activity profiles of LD (black) and LDR2 (gray) are plotted together in the lowest panel of B (without SEM).

Figure 2

Rhythmic activity of mutants with impaired photoreception (*eya^2* and *cry^{01}* mutants) and with impaired photoreception plus circadian clock (*per^{01};eya^2* and *per^{01};;cry^{01}* double mutants) in LD, LDR1 and LDR2. Average actograms, average activity profiles as well as nocturnal activity and the timing of the E peaks are shown (labeling as in Figure 1).Only for *per^{01};eya^2* double-mutants E peak times could not be calculated, because E peaks were simply absent (I, J). *eya^2* mutants lack the sharp M and E peaks, but still show M and E activity bouts although the M bout was small under LDR1 and LDR2 (A, B). *eya^2*

mutants neither reduce nocturnal activity (C) nor advance their E activity maxima (D) in response to LDR1 and LDR2. *cry*^{01} mutants behaved in principal WT-like (E-H). Only their E activity rose slower under LD (arrow). *per*^{01}*;eya*^{2} double-mutants still responded to the light-regimes and even modified their activity pattern in response to LDR1 and LDR2, but they did not show M and E activity bouts nor sharp M and E peaks under any condition (I, J). Nocturnal activity levels also did not change in response to LDR1 and LDR2 (K). *per*^{01}*;;cry*^{01} double mutants behaved in principle like *per*^{01} single mutants, with the exception that their activity after lights-on was lower (arrow) under LD, and the M peak rather small under LDR1 (L, M).

Figure 3

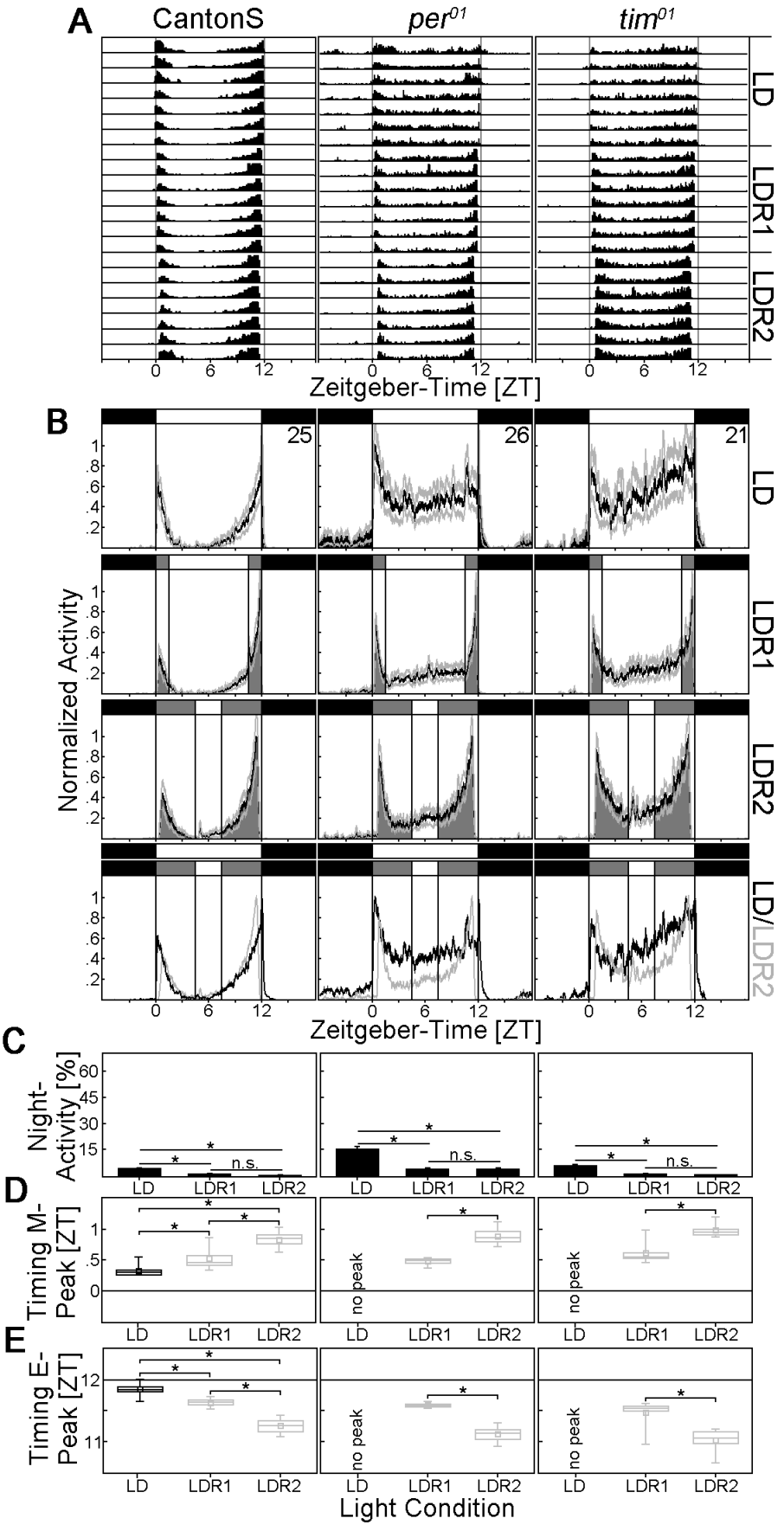
Clock-less mutants can be stimulated to high activity by simulating twilight in the middle of the night. Flies were entrained in LD12:12 for 5 days. On day 6 we applied a light pulse in the middle of the night with dawn from ZT15.5-17 and dusk from ZT19-20.5. The activity profiles depicted represent the mean average activity profiles of days 6 and 7 (black line) ± SEM (gray lines). Times of total darkness are depicted in black, dawn/dusk simulation in dark gray and times of highest light intensity (100 lux) in white. Most genotypes respond to the "midnight pulse" by increasing activity, only eyeless flies do not respond at all. Clock mutants (*per*^{01}, *tim*^{01} and *per*^{01}*;;cry*^{01}) show a bimodal pattern during dawn and dusk simulation in the middle of the night, whereas WT and *cry*^{01} flies only show an increase of activity without sharp peaks. This indicates that the sharp M and E peaks in LDR1/LDR2 are direct effects of light caused by the compound eyes. For details see text.

Figure 4

TIM cycling in the lateral clock neurons under LD and LDR2 in WT flies and *cry*^{01} mutants. The position of the lateral clock neurons (s-LN$_v$, 5th s-LN$_v$ and LN$_d$) in the brain relative to the dorsal clock neurons (DN$_1$, DN$_2$ and DN$_3$) of the fly is indicated in A, TIM cycling in these neurons in B. Black circles (± SEM) connected by thin broken black lines represent the measured staining intensity in LD, whereas gray circles (± SEM) connected by thin gray broken lines represent the staining intensity in LDR2. Polynomial fits of the cycling in LD and LDR2 are added in thick black and gray lines, respectively. The polynomial fits were characterized by $R^2 \geq 0.98$ indicating that they nicely match the original cycling (only exception: LN$_d$ in *cry*^{01} $R^2=0.89$). In WT flies, TIM accumulates earlier and stays stable for longer time in LDR2 as compared to LD in the sLN$_v$ and the LN$_d$ lateral neurons ($F>9.105$; $p \leq 0.003$). In *cry*^{01} mutants the LDR2 effects were absent. Besides the broadening of TIM distribution we do not observe a significant shift of TIM protein maximum in LDR2 compared to LD ($F \leq 0.735$; $p \geq 0.393$) neither in WT nor in *cry*^{01}.

However, we found differences between WT and *cry*^{01} flies: the TIM protein peak was significantly later in the sLN_v ($F_{(1,77)}$=11.636; p=0.001) and in the 5th sLN_v ($F_{(1,17)}$=5.44; p=0.032) whereas it did not change in the LN_d ($F_{(1,117)}$=2.941; p=0.089). The cycling amplitude of the LN_ds is reduced in *cry*^{01} as these neurons are composed of different neuronal subsets that cycle out of phase.

Figure 1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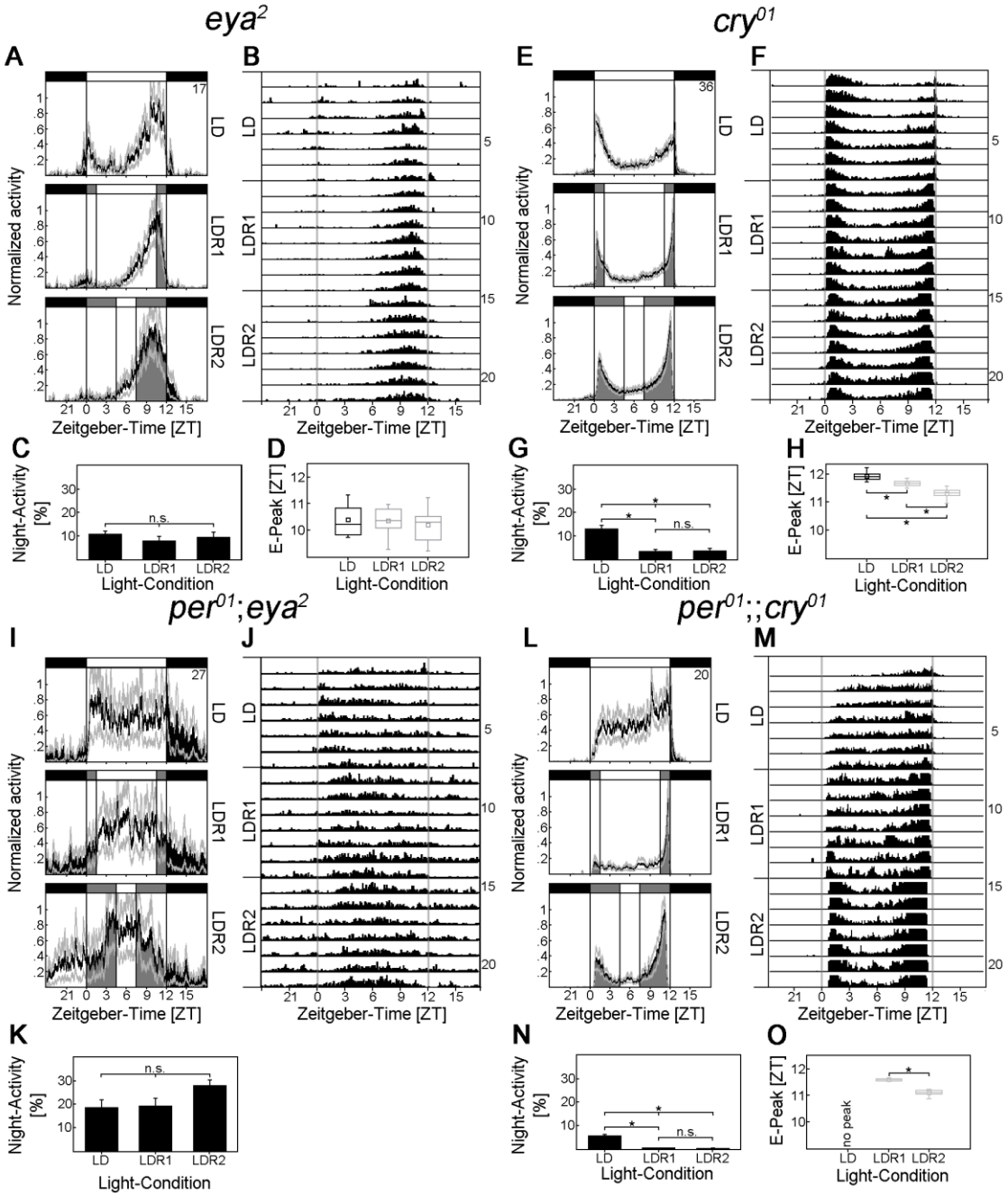
CantonS
per01
tim01
LD
LDR1
LDR2
0
6
12
Zeitgeber-Time [ZT]
B
25
26
21
Normalized Activity
LD/LDR2
C
Night-Activity [%]
n.s.
D
Timing M-Peak [ZT]
no peak
E
Timing E-Peak [ZT]
Light Condition

Figure 2


eya2
cry01
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per01;;cry01
A
B
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K
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N
O
Normalized activity
Zeitgeber-Time [ZT]
LD
LDR1
LDR2
Night-Activity [%]
E-Peak [ZT]
Light-Condition
n.s.
no peak

Figure 3

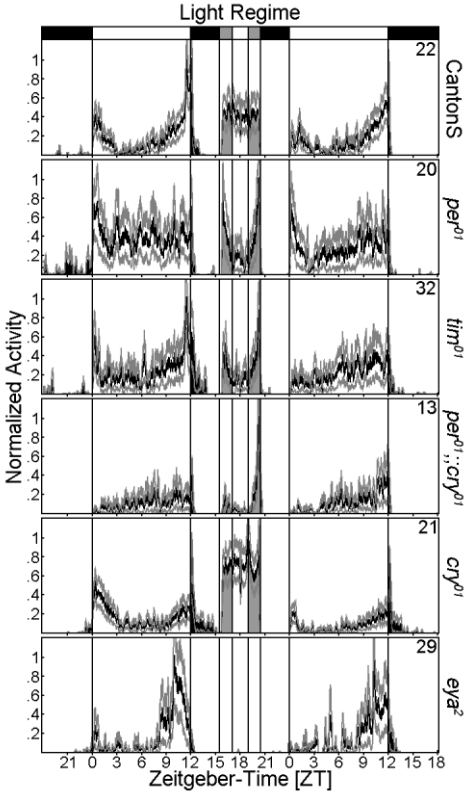
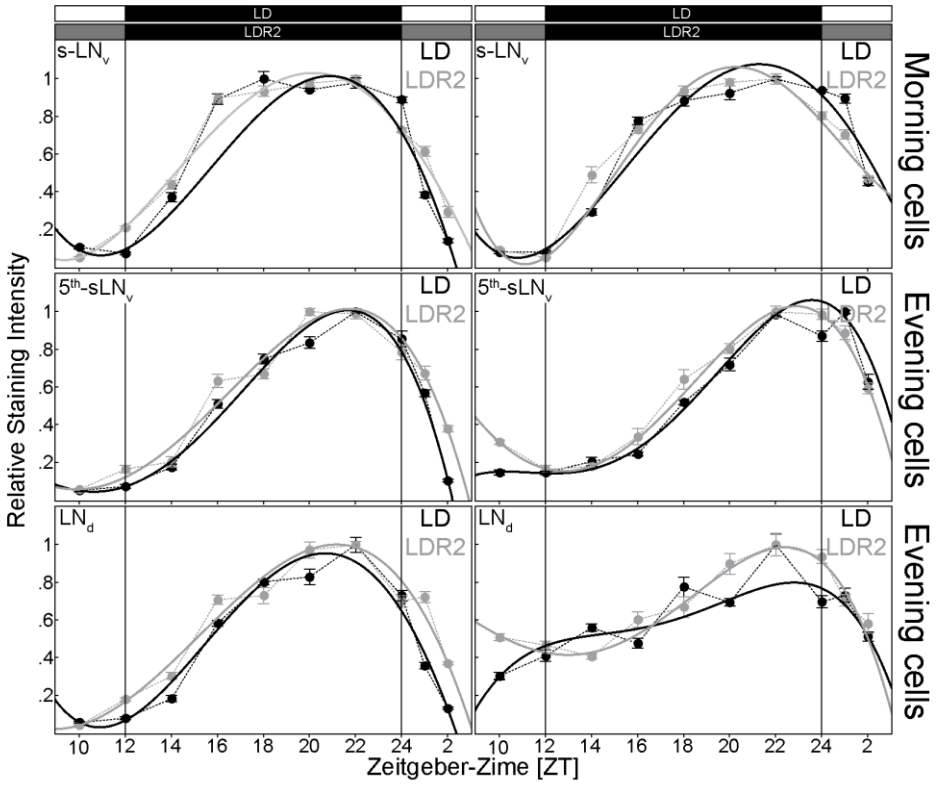


Figure 4



6.4. Twilight dominates over moonlight in adjusting *Drosophila´*s activity pattern

Twilight dominates over moonlight in adjusting *Drosophila*'s activity pattern

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Running title: Entrainment in combined moonlight and twilight

Number of Pages: 20
Number of Illustrations: 4
Number of Tables: 0

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ABSTRACT

Light is the most important Zeitgeber for the synchronization of the D. melanogaster circadian clock. In nature, there is twilight and the nights are rarely completely dark a fact that is usually disregarded in lab experiments. Recent studies showed contrary effects of simulated twilight and moonlight on fly locomotor activity with twilight shifting morning and evening activity into the day and moonlight shifting it into the night. A currently unanswered question is what may happen to locomotor activity when flies are exposed to more natural conditions in which both moonlight and twilight are simulated? Our data demonstrate that flies are able to integrate twilight and moonlight. However, twilight seems to dominate over moonlight as both morning and evening activity peaks take place at dawn or at dusk, respectively and not during the night. Furthermore, nocturnal activity decreases in the presence of twilight. The compound eyes are essential for this behavior and by investigating different photoreceptor mutants we unraveled the importance of photoreceptor cells 7 and 8 for wild-type phases of the activity peaks. In order to adjust nocturnal activity levels to a wild-type manner, all photoreceptor cells work together in a complex way with rhodopsin 6 having a prominent role.

Key words: circadian clock, entrainment, twilight, moonlight, rhodopsins

INTRODUCTION

In nature, many Zeitgebers cycle within the span of a 24h day, with light being the most important environmental cue for synchronizing the circadian clocks of animals and plants. Lab studies mostly apply rectangular light-dark cycles of 12h light and 12h darkness (LD 12:12) to simulate day-night changes, which do not reflect the actual changes of light in nature as dawn, dusk and moonlight are consequently disregarded. All of these light features cause significant changes in animal behavior and physiology: moonlit nights lead to an accelerated recovery from jetlag, can increase the activity of nocturnal animals and, in humans, can affect sleep (Erkert and Cramer, 2006; Evans et al., 2009; Cajochen et al., 2013). Moreover, the simulation of twilight by nature-like increases or decreases of light intensity improves entrainment compared to abrupt lights-on and lights-off in many species and is able to advance *Per1*, *Per2* and PER1, PER2 oscillation profiles under long photoperiods in mice (Boulos et al., 1996a; Boulos et al., 1996b; Boulos et al., 1996c; Boulos et al., 1996d; Fleissner and Fleissner, 1998; Danilenko et al., 2000; Fleissner and Fleissner, 2002; Boulos and Macchi, 2005; Comas and Hut, 2009; Sosniyenko et al., 2009).

In *Drosophila melanogaster*, simulation of moonlit nights leads to an advance of the morning (M) and a delay of the evening (E) activity peak which correlates with changes in PER cycling in specific clock neurons (Bachleitner et al., 2007). Additionally, the level of nocturnal activity increases significantly in moonlit nights. The latter is independent of a functional clock and is especially mediated by rhodopsin 1 (Rh1) and rhodopsin 6 (Rh6) of the compound eyes (Kempinger et al., 2009; Schlichting et al., 2014). The effect of twilight simulation goes instead in the opposite direction: dawn and dusk provoke a delay of the M and an advance of the E peak, and the flies reduce their nocturnal activity to a minimum (Rieger et al., 2007). In addition, twilight is able to improve the ability to phase-delay the E peak under long photoperiods (Rieger et al., 2012).

To fulfill the difficult sensory task of using light as a Zeitgeber, a complex visual system is necessary (Foster and Helfrich-Förster, 2001). To perceive light, *Drosophila* uses cryptochrome as well as six rhodopsins (Stanewsky et al., 1998; Ceriani et al., 1999; Emery et al., 2000; Helfrich-Förster et al., 2001; Rieger et al., 2003; Busza et al., 2004). Cryptochrome is a circadian blue-light photoreceptor and it is expressed in the compound eyes as well as in several clock neurons (Benito et al., 2008; Yoshii et al., 2008). Rhodopsins are expressed in seven eye structures: two compound eyes, two Hofbauer-Buchner (H-B) eyelets and three ocelli (Hofbauer and Buchner, 1989; Salcedo et al., 1999; Rister et al., 2013). This study focusses on the compound eyes, which are the most

prominent visual organs of the fly; they consist of about 800 ommatidia, each containing pigment cells as well as eight photoreceptor cells (R1 to R8). The photoreceptor cells express specific rhodopsins. In particular, the outer photoreceptor cells, R1-R6, express rhodopsin 1, whereas 70% of the inner photoreceptor cells (R7 and R8) express rhodopsin 4 in R7 and rhodopsin 6 in R8 (yellow subtype) and 30% express rhodopsin 3 in R7 and rhodopsin 5 in R8 (pale subtype) (Rister et al., 2013). Only few specialized ommatidia in the dorsal rim area express only rhodopsin 3 in both inner photoreceptor cells (Wernet and Desplan, 2014).

The first aim of this study was to investigate fly behavior when moonlight and twilight are simulated at the same time. We show that twilight dominates over moonlight in terms of peak timing and nocturnal activity, even though the moonlight-induced nocturnal activity increase was still present. The second aim was to unravel the contribution of the different photoreceptor cells and rhodopsins in perceiving gradual changes in irradiance during twilight. We demonstrate that flies lacking compound eyes fail to adjust their behavior in a normal way and that inner and outer photoreceptor cells cooperate in adapting fly behavior to combined twilight and moonlight with a prominent role of rhodopsin 6 in dim light detection.

Materials and Methods

Fly strains and rearing

Light sensitivity of the circadian clock is known to be influenced by a naturally occurring polymorphism in the *timeless (tim)* gene (Sandrelli et al., 2007; Tauber et al., 2007). To exclude possible effects of this *tim* polymorphism we crossed all photoreceptor mutants into the *ls-tim* background (Schlichting et al., 2014) and used the wild-type strain $WT_{CantonS}$ with the same *ls-tim* background as control. In addition, we recorded two additional wild-type strains with completely different backgrounds to see whether they responded similarly to the combination of twilight and moonlight. These are WT_{ALA} and $WT_{Lindelbach}$ that have been described in Rieger et al. (2012) and Schlichting et al. (2014), respectively. $WT_{Lindelbach}$ is *s-tim* and WT_{ALA} carries a mixture of both *tim* forms.

cli^{eya} mutants, which lack the compound eyes but retain the H-B-eyelets and the ocelli, served as negative controls (Bonini et al., 1993; Schlichting et al., 2014). To eliminate R1-R6 we used $ninaE^{17}$ (*neither inactivation nor afterpotential E*) mutants (O'Tousa et al., 1985; Kumar and Ready, 1995). To impair R7 function we used 2 different strains: (1) sev^{LY3} mutants, in which R7 do not develop and therefore all R8 express rhodopsin 6 and (2) $rh3^{1}rh4^{1}$ double mutants, which leave the rhodopsin distribution in R8 unchanged (Benzer, 1967; Chou et al., 1999; Vasiliauskas et al., 2011). To manipulate R8 we used rhodopsin 5 and rhodopsin 6 single mutants ($rh5^{2}$ and $rh6^{1}$) as well as the relevant double mutant ($rh5^{2};rh6^{1}$) (Cook et al., 2003; Yamaguchi et al., 2008). To render both inner photoreceptor cells out of function $rh5^{2};rh3^{1}rh4^{1}rh6^{1}$ quadruple mutants were investigated.

All flies were raised on standard *Drosophila* medium (0.8% agar, 2.2% sugar beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, and 0.3% hydroxybenzoicacid) at 25 °C in LD 12:12.

Behavior recording and light programs

For recording locomotor activity, a home made system was used (Helfrich-Förster, 1998; Schlichting and Helfrich-Förster, 2014). 2-4 days old single male flies were transferred into photometer cuvettes with food and water supply on one end. On the other end an infra red beam was installed and a computer measured the number of beam crosses in 1 minute intervals. Behavior was recorded in a climate controlled chamber at 20°C. As light source "white" LEDs (Lumitronix LED-Technik GmbH, Jungingen,

Germany) were used with additional neutral density filters (Lee Filters Worldwide, Andover, UK) for finetuning of light intensity.

To distinguish the effect of simulated dawn and dusk on behavior we recorded the flies at 4 different light conditions: In the first week we applied a rectangular light-dark cycle of 12h light and 12h darkness (LD 12:12) and in the second week twilight was simulated using a gradual increase of light intensity in the morning and a gradual decrease in the evening within 1.5 h each (LDR) (Rieger et al., 2007). In the same way we investigated conditions including moonlight: Flies were recorded for one week in light-moonlight conditions (LM, moonlight intensity of 0.01 lux) and in the second week twilight simulation was added (LMR condition). For wild-type flies we investigated 4 different daylight intensities (10 lux, 100 lux, 1000 lux and 10000 lux) in order to evaluate effects that depended on daylight intensity and to find the optimal intensity for testing the photoreceptor mutants. Experiments were repeated at least twice. Finally we had 17 to 32 flies for each genotype that survived the entire experiment and could be used to analyze the behavior.

Data analysis and statistics

The raw data were plotted as actograms using the ActogramJ plugin for Fiji (available at http://fiji.sc/Downloads, Schmid et al. (2011)). In the next step, average activity profiles were analyzed using the last 4 days of each light condition. All activity profiles were normalized to 1 and plotted using the program Qtiplot (version 0.9.8.9, Ion Vasilief, Craiova, Romania). To analyze peak timing the activity profiles of single flies were smoothened by a moving mean of 30 and the timing of the peak was determined manually as desribed in Schlichting and Helfrich-Förster (2014). In addition we calculated the relative nocturnal activity for each experiment by dividing the sum of activity between ZT12 and ZT0 by the whole daily activity. The analysis was done for each single fly and the values ontained were then averaged.

Statistical analysis was performed using Systat11. Normal distribution was tested using a one-way Kolmogorov-Smirnov test. In case of normal distributed data a 1- or 2-way ANOVA was applied. For non-normally distributed data a Mann-Whitney-U test (2 groups) or a Kruskal-Wallis test (more than 2 groups) followed by a Wilcoxon comparison including Bonferroni adjustment was used.

Immunocytochemistry and image analysis

To assess the distribution of rhodopsin 5 and 6 in the retina, 18-day-old male flies were entrained in LD 12:12 at 20°C and fixed for 2.5 hours in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). After rinsing 4x15 min each with PBS containing 0.5% Triton-X (PBST) the retinas were dissected in PBST and the lamina was removed. Retinas were blocked for 30 min in 5% normal-goat-serum (NGS) in PBST and subsequently incubated in the primary antibody solution consisting of rabbit anti-rhodopsin 6 (1:1000, gift of C. Desplan, New York University, New York, NY, USA, Tahayato et al. (2003)) and mouse anti-rhodopsin 5 (1:50, gift of S. Britt, University of Colorado, Aurora, CO, USA, Salcedo et al. (1999)) in PBST containing 5% NGS and 0.02% NaN_3. Samples were incubated in the primary antibody for 2 nights at room temperature (RT). After rinsing 5x20 min each with PBST, the secondary antibody was applied overnight (Alexa Fluor 555 goat anti-mouse and Alexa Fluor 635 goat anti-rabbit, each in a dilution of 1:200 in PBST and 5% NGS). For the following 3-5 days, the retinas were rinsed several times per day until the red pigmentation of the eyes was fully washed out to eliminate autofluorescence. Retinas were embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) in a way that the cornea of the eye laid on the glass slide.

The retinas were analyzed using laser scanning confocal microscopy (Leica TCS SPE, Leica, Wetzlar, Germany). To excite the secondary antibody fluorophores two different lasers were sequentially used (532 and 635 nm) and of 2 µm thick stacks were obtained. All images were analyzed using Fiji. We counted manually the number of ommatidia expressing Rh5 or Rh6 in the entire retina and did so for 7 different retinas.

RESULTS

Wild-type behavior under LD, LDR, LM and LMR with different daylight intensities

Our previous studies have shown that fly activity pattern depends not only on the presence of twilight or moonlight but also on daylight intensity (Rieger et al., 2007; Schlichting et al., 2014). Therefore, we recorded activity of the wild-type strains under LD, LDR, LM and LMR at four different daylight intensities (10, 100, 1000 and 10000 lux), respectively. Consistent with previous studies, all three wild-type strains showed bimodal activity patterns with M and E peaks, with the phase of the M peak occuring earlier and that of the E peak later with increasing daylight intensity (shown for $WT_{CantonS}$ in Fig. 1A and $WT_{Lindelbach}$ and WT_{ALA} in Fig. S1A, F). Here, we determined only the phase of the E peak and found that it significantly delayed with increasing light intensity in all wild-types and at all four light regimes (LD, LDR, LM, LMR) ($p<0.001$; Fig. 1B, C; Fig. S1). This delay was most pronounced under moonlight conditions in $WT_{Lindelbach}$ (Fig. S1C) and the least pronounced in $WT_{Cantons}$ (Fig. 1C). In addition, E peak timing depended significantly on the light regime, and this was again similar in all wild-type strains (Fig. 1B, C; Fig. S1): simulated twilight advanced the E peak, whereas moonlight delayed it. Consequently, the earliest E peaks were found under LDR (Fig. 1B) and the latest under LM conditions (Fig. 1C) (see also Fig. S1). Most importantly, twilight appeared to prevent the E peak from occurring during the night (Fig. 1B, C; Fig. S1B, C, G, H). Under LMR the E peak occurred always during dusk (between 10 and 1000 lux) or directly at lights-off (at 10000 lux), whereas under moonlight alone (LM) the E peak was after lights-off when daylight intensity exceeded 100 lux. This indicates that advancing the E peak by twilight dominates over delaying the E peak by moonlight.

Daylight intensity and light regime influenced also nocturnal activity. The flies tended to reduce diurnal and increase nocturnal activity with increasing daylight intensity under all light regimes. As already found for the daylight-intensity-dependent delay of the E peak, this tendency was slightly different in the three wild-type strains and the least pronounced, though still significant, in $WT_{CantonS}$ (Fig. 1D, E). $WT_{Lindelbach}$ flies that carry the *s-tim* allele showed a linear and steep increase of nocturnal actvity with increasing daylight intensity, whereas WT_{ALA} flies that carry a mixture of *s-tim* and *ls-tim* behaved in-between $WT_{CantonS}$ and $WT_{Lindelbach}$ flies (Fig. S1).

In all strains the highest nocturnal activity was present under LM conditions, followed by LMR, LD and LDR (Fig. 1D, E; Fig. S1D, E, I, F) and these differences were significant ($p\leq0.004$). Thus, moonlight could even shift activity into the night in the presence of twilight, though nocturnal activity in LMR was lower compared to LM alone (Fig 1E; Fig. S1I, F).

Photoreceptors of the compound eyes mediate E peak timing and nocturnal activity

To elucidate the contribution of the different compound eye rhodopsins to the adaptation of fly activity patterns to the four light regimes, we chose a light intensity of 100 lux, because the $WT_{CantonS}$ control flies strongly responded to twilight and moonlight at this light intensity.

We found that E peak timing and nocturnal activity levels depended significantly on the compound eyes under all four light regimes ($p<0.001$) (Fig. 2, 3): The more photoreceptor cells or rhodopsins were absent, the earlier the evening peak and the lower the nocturnal activity. This can be best seen in Figure 3, where we arranged the photoreceptor mutants according to their phenotype severity with the mutants lacking most photoreceptors to the right.

In the following we will not consider absolute differences in E peak timing and nocturnal activity between controls and mutants but concentrate on the responses to twilight and moonlight of each mutant, since this was the main aim of this study. Eyeless cli^{eya} mutants did neither respond to moonlight nor to twilight; their E peak always occurred at about 1.5h before lights-off ($p>0.472$; Fig. 3A, B) and they always spent about 10% of their daily activity in the night (Fig. 3C, D). $ninaE^{17}$ mutants that lack the outer photoreceptor cells (R1 to 6) responded to twilight by advancing their E peak in LMR as compared to LM ($p=0.002$) and showed a tendency to do so in LDR compared to LD ($p=0.062$) (Fig. 2A, B). In addition they slightly but significantly reduced nocturnal activity in response to twilight (LDR and LMR; $p\leq0.02$) (Fig. 2C, D). $rh5^{2};rh3^{1}rh4^{1}rh6^{1}$ quadruple mutants, in which both inner photoreceptor cells were impaired, always showed their E peak at the same time (~1h before lights-off; $p>0.386$) as did completely eyeless mutants. Nevertheless, these mutants clearly responded to moonlight and twilight with respect to nocturnal activity (Fig. 3C, D). This result indicates that different photoreceptors contribute to the phasing of the E peak and the control of nocturnal activity. The inner photoreceptor cells appear essential for phasing the E peak, while all photoreceptor cells seem involved in adjusting nocturnal activity levels.

Rhodopsin 6 (and rhodopsin 1) play a special role in sensing moonlight

Next, we aimed to unravel the role of the different rhodopsins in sensing moonlight and twilight. Clearly, all five rhodopsins of the compound eyes contributed to the increase of nocturnal activity in response to moonlight, since only completely eyeless flies failed to increase their activity in moonlit nights whereas all tested single or double mutants were able to do so (Fig. 3D). Similarly, all four rhodopsins of the inner photoreceptor cells contributed to the shift of the E peak into twilight, given only quadruple mutants ($rh5^{2};rh3^{1}rh4^{1}rh6^{1}$), that lack the four rhodopsins, failed to shift the E peak into dusk in

LDR and LMR. Flies lacking only rhodopsin 3 and 4 (*sev*^{LY3} and *rh3*^{1}*rh4*^{1} mutants) or rhodopsin 5 and/or 6 (*rh5*^{2}, *rh6*^{1}, *rh5*^{2}*;rh6*^{1} mutants) were still able to shift their E peak into dusk in LDR ($p<0.011$) and LMR ($p<0.004$) (Fig. 3A, B).

In spite of the obvious interplay of all rhodopsins in twilight and moonlight detection, rhodopsin 6 (and to some degree also rhodopsin 1) appear to have a special role in detecting moonlight in the presence of twilight. Mutants lacking rhodopsin 6 (*rh6*^{1}, *rh5*^{2}*;rh6*^{1} and *rh5*^{2}*;rh3*^{1}*rh4*^{1}*rh6*^{1} mutants) or rhodopsin 1 (*ninaE*^{17}) barely responded to moonlight when twilight was additionally present (Fig. 4A). *sev*^{LY3} mutants were exceptional, because they increased nocturnal activity in response to moonlight more than wild-type flies did ($p<0.001$). This may most likely be caused by their high rhodopsin 6 expression (Chou et al., 1999; Vasiliauskas et al., 2011). We found that virtually all R8 cells of our *sev*^{LY3} mutants expressed rhodopsin 6 (Fig. 4B, C). In contrast, *rh3*^{1} *rh4*^{1} double mutants showed a wild-type rhodopsin 6 distribution (Fig. 4B, C) and also responded to moonlight in a wild-type manner (Fig. 4A).

DISCUSSION

The main aim of the present study was to reveal the impact of combined twilight and moonlight on daily activity patterns of fruit flies. We tested the behavior of three wild-type strains to unravel the principal effects of the applied light regimes as well as the behavior of eight photoreceptor mutants to reveal the role of compound eyes, different photoreceptor cells and rhodopsins.

Twilight dominates over moonlight

The presence of twilight significantly reduced fly nocturnal activity in moonlit nights hence making their activity pattern look more similar to that observed under out-door conditions (Vanin et al., 2012). Under natural light- and temperature conditions flies were not nocturnal, neither were they in full-moon nights. Our results indicate that the lack of nocturnal activity in nature is not only caused by lower temperatures at night, but also by the presence of twilight that dominates over the moonlight effects even under lab conditions. Most interestingly, all tested wild-type strains behaved in a very similar way. Slight differences were evident in the shift of the E peak into the night and the increase of nocturnal activity under moonlight conditions with increasing daylight intensity. Here, the *s-tim* flies, such as $WT_{Lindelbach}$, responded most sensitively to an increase of daylight intensity, whereas the *ls-tim* flies, such as $WT_{CantonS}$, showed from the beginning onwards a rather late E peak and high nocturnal activity. E peak timing and nocturnal activity level were only moderately altered when daylight intensity increased. WT_{ALA} flies carrying a mixture of both *tim* alleles behaved intermediate to the two others. This is consistent with previous studies showing that the clock of *s-tim* flies is more light sensitive compared to *ls-tim* flies (Sandrelli et al., 2007; Tauber et al., 2007). It is very likely that also other still unknown gene polymorphisms contribute to fly responses to twilight and moonlight. Nevertheless, for the present study it is most important to note that the here reported principal responses are very similar in all three investigated wild-type strains. Thus, these seem rather independent of the genetic background.

Simultaneous simulation of twilight and moonlight combines masking and clock effects in behavior

Generally, light has two principal effects on fly activity: (1) direct effects (inhibiting or promoting activity) and (2) phase-shifting (entraining) effects on the circadian clock that controls fly activity rhythm. The direct light effects on activity are also known as “masking” because they may hide the clock-mediated effects. Most importantly, both light effects

have the adaptive value of confining animals to their temporal niche (Redlin, 2001). Masking often complements the circadian clock in fine-tuning activity patterns in response to environmental stimuli. In *D. melanogaster* clock neurons, cryptochrome can integrate photons over time and effectively phase-shift the clock (Tang et al., 2010; Kistenpfennig et al., 2012; Vinayak et al., 2013; Guo et al., 2014). In contrast, the compound eyes mediate mainly masking effects of light, although they seem to be also involved in entrainment, especially under long photoperiods (Rieger et al., 2003; Helfrich-Förster, 2014).

Moonlight provokes clear masking effects as it stimulates nocturnal activity. This stimulation depends on rhodopsin 1 and rhodopsin 6 in the compound eyes (Schlichting et al., 2014) and it is completely independent of a functional clock (Kempinger et al., 2009). On the other hand, moonlight affects the phase in PER cycling of certain clock neurons leading to an advance of the M peak and a delay of the E peak compared to LD conditions (Bachleitner et al., 2007). These phase changes are mediated by several rhodopsins in the outer and inner photoreceptor cells, but again rhodopsin 6 played a prominent role (Schlichting et al., 2014).

For twilight, the fraction of masking and clock-mediated effects on fly activity have not been evaluated, yet. Simulated twilight (LDR) clearly affects the clock because it facilitates entrainment to long photoperiods (Rieger et al., 2012). In addition, the gradual increase/ decrease of light may directly stimulate fly activity leading to the sharp activity peaks at dawn and dusk. The fact that these sharp peaks take place at an irradiance of ~5 lux, which coincides with the irradiance at which flies are preferrably active (Rieger et al., 2007; Kempinger et al., 2009), strongly suggest that the sharp peaks are masking effects. Here, we applied irradiances between 10 and 10000 lux and found that the timing of the E peak delays with increasing irradiance. This finding is consistent with the results of Rieger et al. (2007) and with the idea that the sharp E peak always takes place at the same dim light intensity, which occurs later during dusk at higher day-light intensities (since twilight duration was kept constant at 1.5h).

Furthermore, LMR advances the E peak into dusk compared to LM conditions, where the E peak occurs in the night. This effect cannot be regarded as pure masking of twilight, because the E peak occurrs significantly later under LMR than under LDR. Thus, the clock-dependent delay of moonlight on the E peak is also present.

The inner photoreceptor cells R7 and R8 are essential for the response to twilight, whereas all photoreceptor cells contribute to the responses to moonlight

Our results from the different photoreceptor mutants show that the inner photoreceptor cells R7 and R8 are essential for phase advancing the E peak in response to twilight. The phase delaying effect of moonlight in presence of twilight (LMR-LDR) was mainly

dependent on rhodopsin 6, which fits perfectly to the results obtained from LM alone (Schlichting et al., 2014). The nocturnal activity stimulating (masking) effect of moonlight in presence of twilight (LMR-LDR) was also mediated by several photoreceptors, but again rhodopsin 6 - and in this case also rhodopsin 1 - was important. Once more this result fits to the effects of pure moonlight on nocturnal activity (Schlichting et al., 2014). Most importantly, sev^{LY3} mutants with a higher amount of rhodopsin 6, showed significantly more nocturnal activity in LMR than wild-type flies, which again points to the prominent role of rhodopsin 6.

Concluding remarks

At present we are not able to clearly distinguish masking effects from entraining effects of twilight and moonlight as well as the role of the different photoreceptor cells and rhodopsins in masking and entrainment. Nevertheless, we demonstrate a prominent role of the compound eyes and their rhodopsins in fine-tuning fly activity pattern to twilight and moonlight with a special influence of rhodopsin 6. Future studies with clockless mutants will have to reveal whether the sharp activity peaks seen under simulated twilight are indeed pure masking effects of light. In addition, the universal phase delaying effect of photoreceptors in the compound eyes on the E peak observed under all light regimes should be further investigated.

ACKNOWLEDGEMENTS

We thank Nina Vogt, Claude Desplan, Steve Britt, Erich Buchner and Christopher Schnaitmann for providing fly lines and Steve Britt and Claude Desplan for providing antibodies. We are very grateful to Marta Beauchamp for language corrections. Further, we thank the Graduate School for Life Sciences Würzburg for supporting Rudi Grebler and Matthias Schlichting. Matthias Schlichting was further sponsored by a Hanns-Seidel-Foundation excellence grant funded by the BMBF (German Ministry for Education and Research). The study was funded by the Deutsche Forschungsgemeinschaft (DFG), collaborative research center SFB 1047 "Insect timing", Project A1 and A2.

NOTE

Supplementary material is available on the journal's website at http://jbr.sagepub.com/supplemental.

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FIGURE LEGENDS

Figure 1 Rhythmic behavior of $WT_{CantonS}$ flies under four different light regimes: light-dark cycles (LD, D indicated by black), light-dark cycles with simulated twilight (LDR, twilight (R) indicated by light gray), light-moonlight cycles (LM, M indicated by dark gray) and light-moonlight cycles with simulated twilight (LMR, twilight (R) indicated by light gray, M indicated by dark gray). **A** Average activity profiles under the four light regimes with daylight intensity increasing from 10 to 10000 lux (left to right). Black lines represent the mean of at least 17 flies, the grey lines represent the SEM. **B** Timing of the E peak under LD (upper panel) and LDR (lower panel). Dotted lines indicate lights-off (at Zeitgeber Time (ZT) 12). Timing of the E peak depended on daylight intensity (the higher the light intensity the later the E peak). Furthermore, the E peak occurred significantly earlier under LDR than under LD at all four daylight intensities ($p<0.001$) (as indicated by asterisks on top of B). **C** Timing of the E peak under LM (upper panel) and LMR (lower panel). Again the E peak occurred earlier in LMR compared to LM ($p<0.001$) indicating a dominant role of twilight for timing of the E peak. Labeling as in B. **D** Percentage of nocturnal activity (from whole day activity) in LD and LDR. Under LDR nocturnal activity was significantly lower than under LD ($p<0.001$) as indicated by the asterisks on top. **E** Percentage of nocturnal activity (from whole day activity) in LM and LMR. Moonlight generally increased nocturnal activity and this effect was significantly higher under LM than under LMR, at least between 100 and 10000 lux (see asterisks on top). n.s. not significant; ** $p<0.001$.

Figure 2 Average activity profiles of $WT_{CantonS}$ flies and all investigated photoreceptor mutants in LD, LDR, LM and LMR (from left to right) at a daylight intensity of 100 lux. As in Fig. 1A, the average values of at least 17 flies (±SEM) were depicted in each diagram. Activity in completely dark nights is represented in black, during moonlight in dark grey, during dawn and dusk in light grey and in times of maximal light intensity in white. All flies were able to entrain to the investigated light conditions with a prominent E peak and a smaller M peak. Nocturnal activity and the timing of the E peak were clearly influenced by the absence of certain photoreceptor cells or rhodopsins. The highest nocturnal activity was present in sev^{LY3} mutants, while nocturnal activity was wild-type like in $rh3^{1}rh4^{1}$ mutants and reduced in all other mutants. The E peak occurred earlier with increasing severity of the photoreceptor mutation being the earliest in complete eyeless flies (cli^{eya}). In addition, E peak timing and nocturnal activity depended on the light regime. For quantification of E peak timing and nocturnal activity levels see Figure 3.

Figure 3 Timing of E peak and levels of nocturnal activity in $WT_{CantonS}$ flies and all investigated photoreceptor mutants. Values are calculated from the data shown in Figure 2. E peak timing and nocturnal activity are compared between LD and LDR (**A, C** respectively) and between, LM and LMR (**B, D** respectively). Significant differences between the two conditions are indicated as asterisks on top. n.s.: not significant; *p<0.05 **p<0.001. Details see text.

Figure 4 Effect of the different rhodopsins on moonlight detection in the presence of twilight plus rhodopsin 5/6 distribution in the retina of selected mutants. **A:** Differences in nocturnal activity between LMR and LDR were calculated for $WT_{CantonS}$ flies and all investigated mutants (± SEM). Asterisks on top of the diagrams indicate significant effects of moonlight in presence of twilight. n.s. not significant; *p<0.05 **p<0.001. sev^{LY3} mutants strongly increased nocturnal activity in LMR compared to LDR, whereas $rh3^{1}rh4^{1}$ mutants behaved wild-type-like. **B**: Distribution of rhodopsin 5 and 6 in the retina of 18d old $WT_{CantonS}$ flies, $rh3^{1}rh4^{1}$ and sev^{LY3} mutants. sev^{LY3} mutants almost exclusively expressed rhodopsin 6 in photoreceptor cell R8, whereas $rh3^{1}rh4^{1}$ mutants were wild-type like. The scale bar applies to all images. **C**: Calculated percentage of rhodopsin 5 and rhodopsin 6 expression in R8. $WT_{CantonS}$ flies and $rh3^{1}rh4^{1}$ mutants showed the expected ratio of 30:70, whereas sev^{LY3} mutants expressed rhodopsin 6 in virtually 100% of R8 cells.

Figure 1

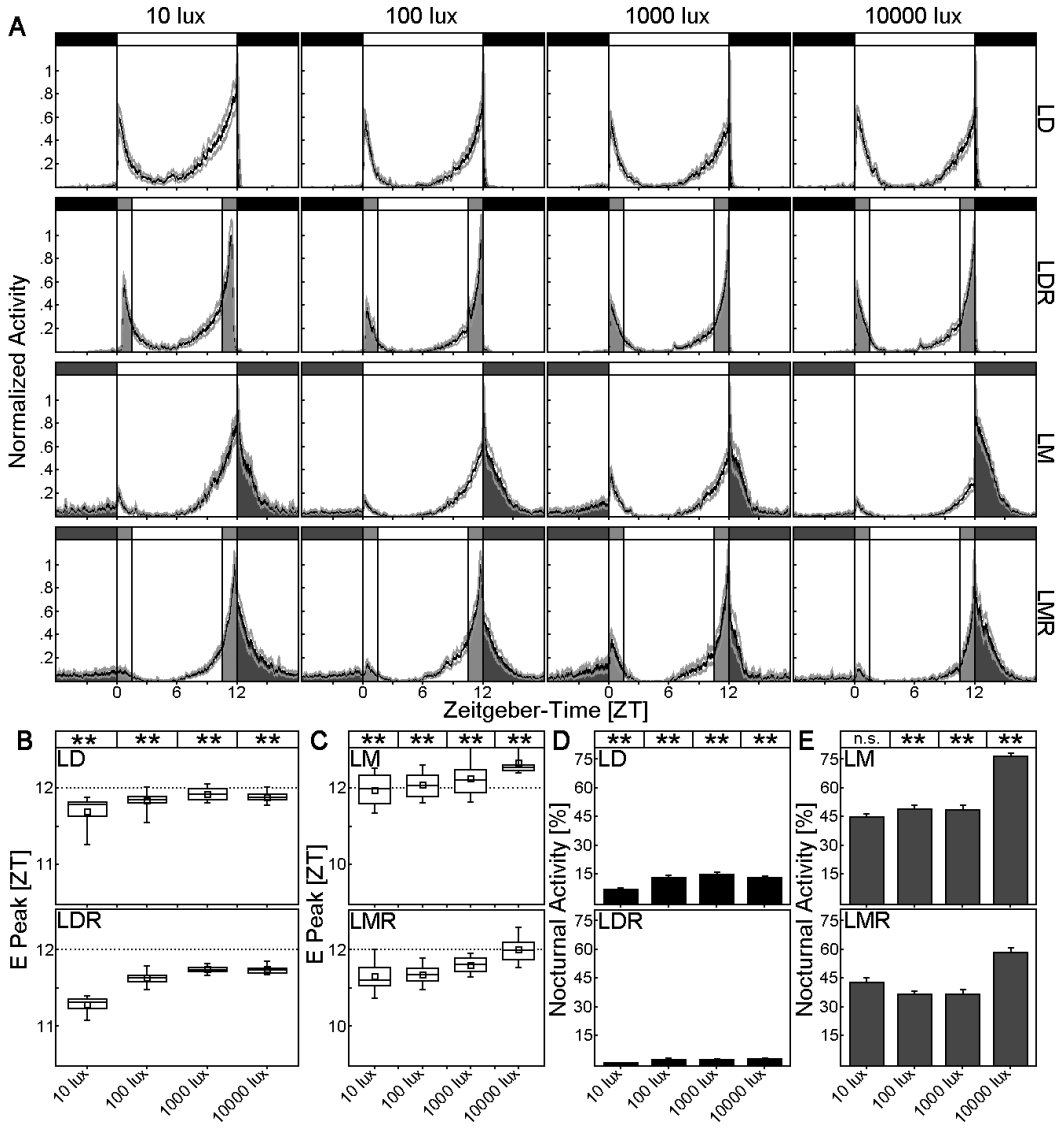
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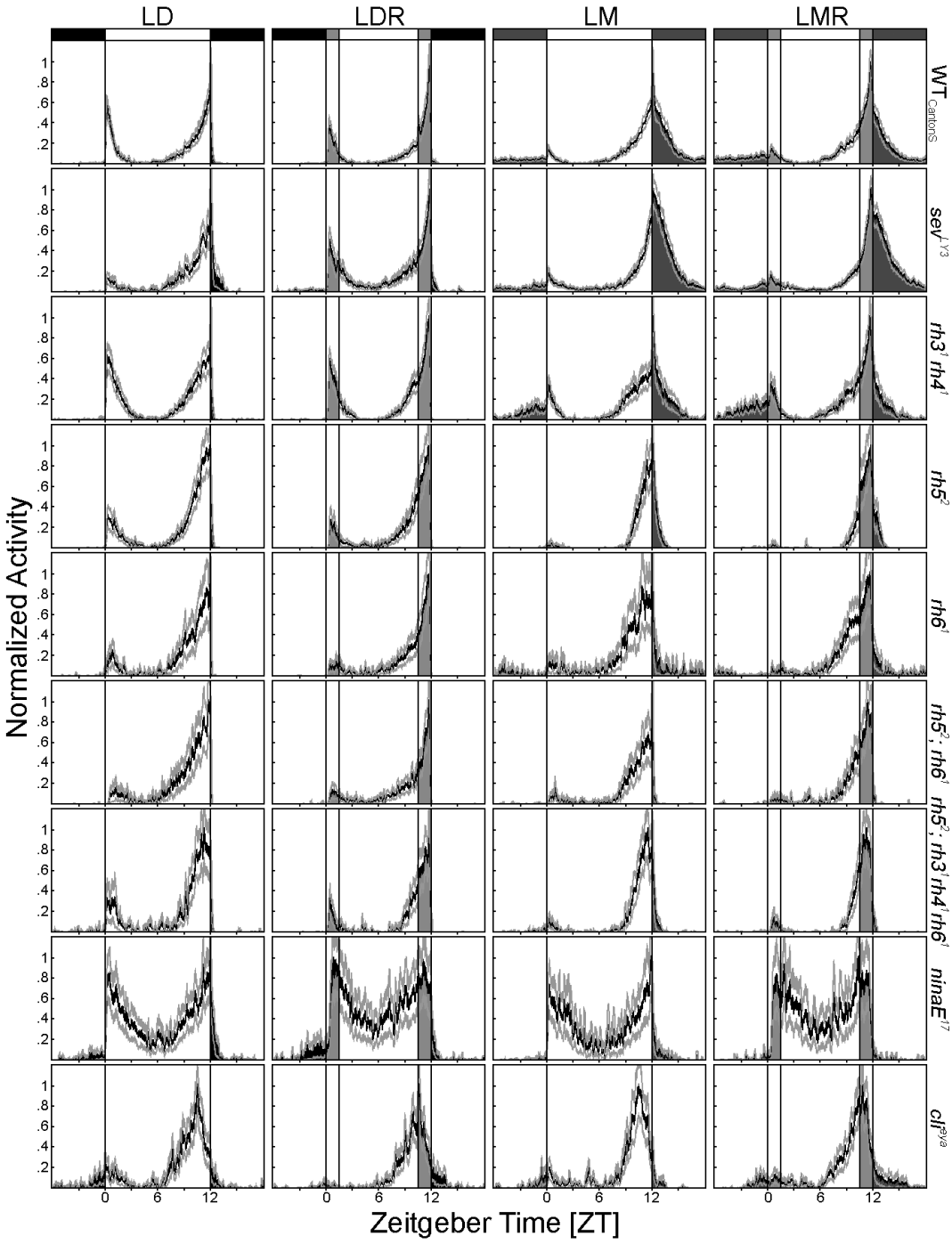
Figure 2


LD
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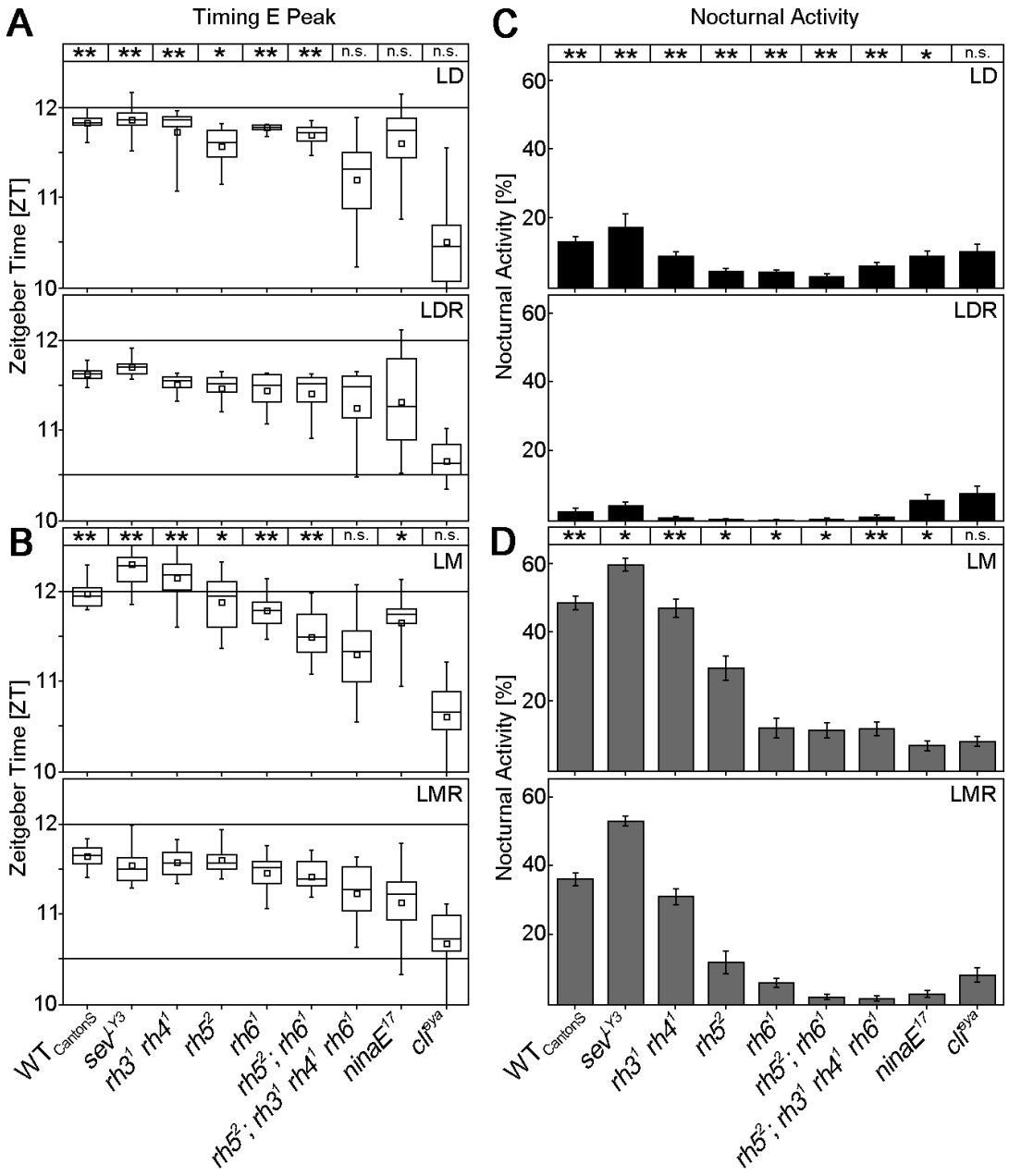
rh5^2; rh3^1rh4^1rh6^1
ninaE^17
clf^3va

Figure 3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rh6 1
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ninaE 17
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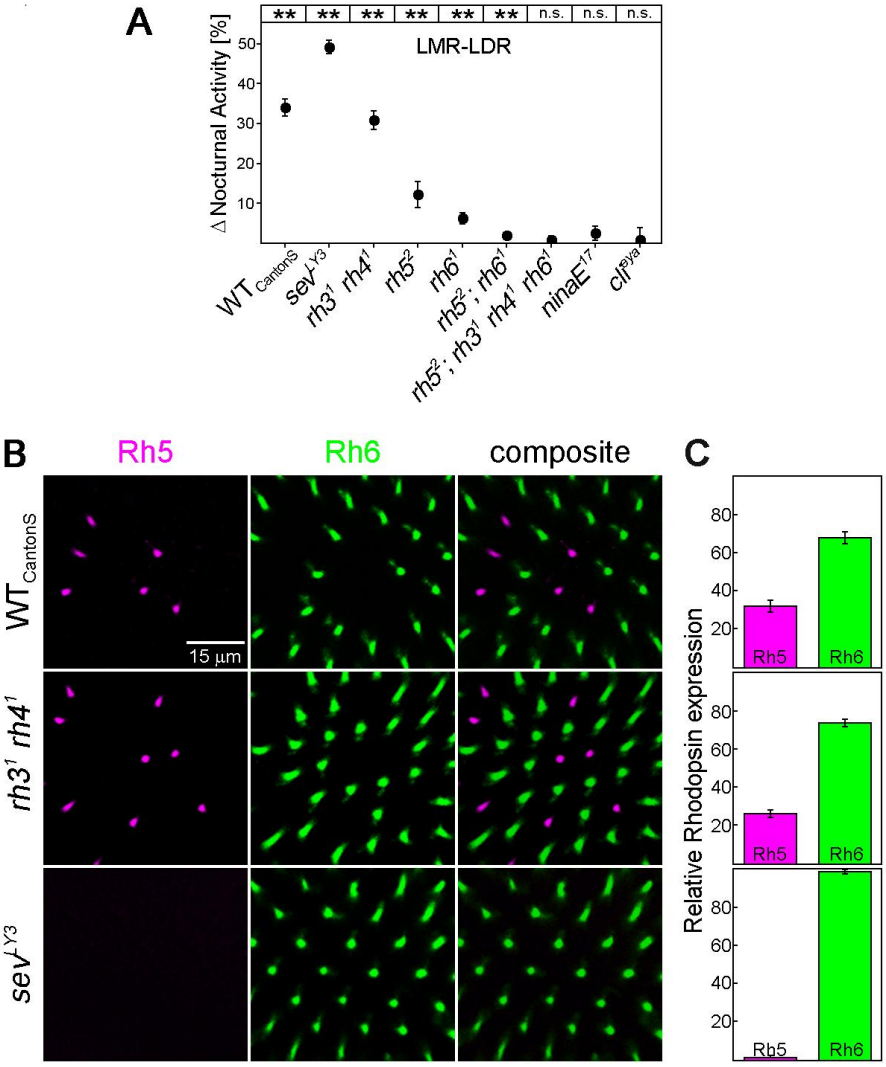
Figure 4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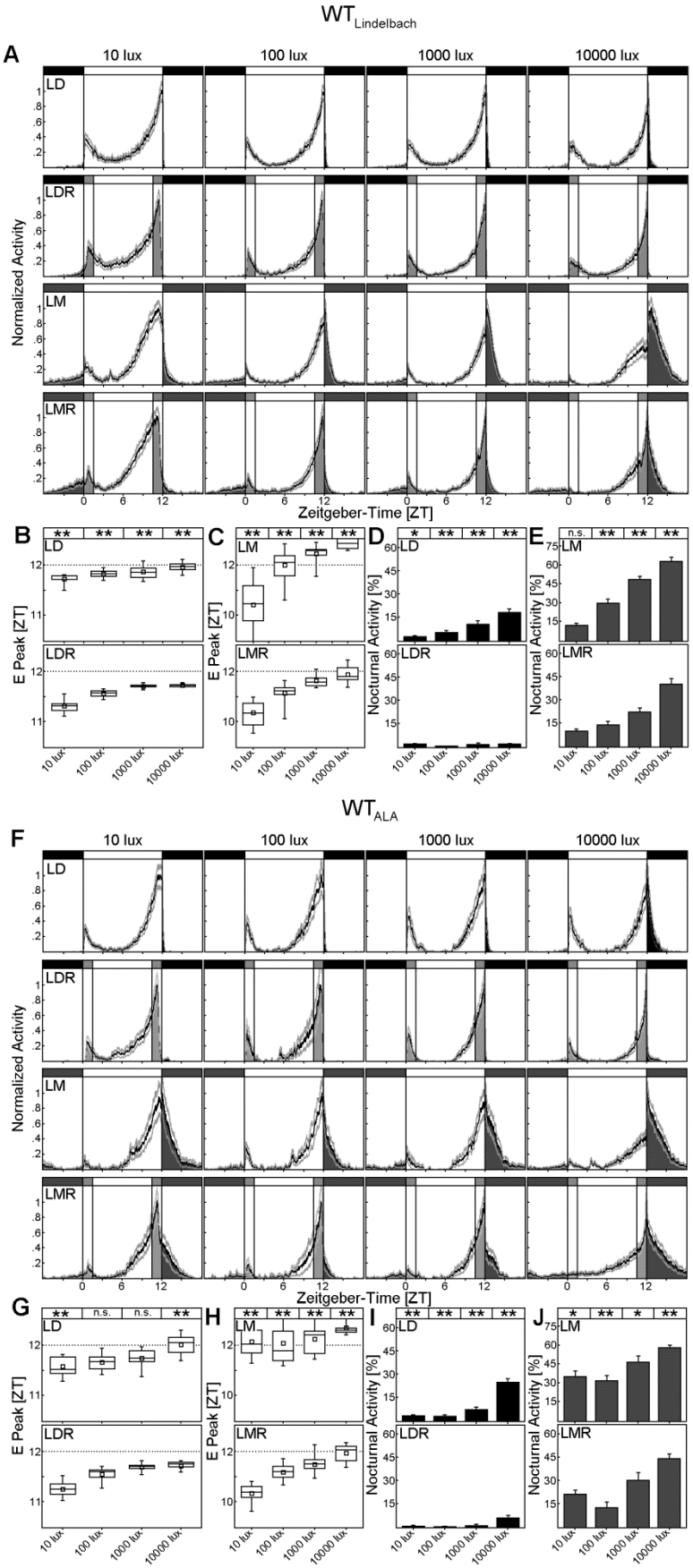
rh3^1 rh4^1
rh5^2
rh6^1
rh5^2; rh6^1
rh5^2; rh3^1 rh4^1 rh6^1
ninaE^17
ctr^9ya
B
Rh5
Rh6
composite
WT CantonS
rh3^1 rh4^1
sev^LY3
15 µm
C
Relative Rhodopsin expression
80
60
40
20
Rh5
Rh6

Supplement:

Figure S1 Rhythmic behavior of two different wild-type strains $WT_{Lindelbach}$ and WT_{ALA} under four different light regimes: light-dark cycles (LD, D indicated by black), light-dark cycles with simulated twilight (LDR, twilight (R) indicated by light gray), light-moonlight cycles (LM, M indicated by dark gray) and light-moonlight cycles with simulated twilight (LMR, twilight (R) indicated by light gray, M indicated by dark gray). **A, F:** Average activity profiles under the four light regimes with daylight intensity increasing from 10 to 10000 lux (left to right). Black lines represent the mean of at least 17 flies, the grey lines represent the SEM. **B, G:** Timing of the E peak under LD (upper panel) and LDR (lower panel). Dotted lines indicate lights-off (at Zeitgeber Time (ZT) 12). Timing of the E peak depended on daylight intensity (the higher the light intensity the later the E peak). Furthermore, the E peak occurred significantly earlier under LDR than under LD at all four daylight intensities ($p<0.001$) (as indicated by asterisks on top of B). **C, H:** Timing of the E peak under LM (upper panel) and LMR (lower panel). Again the E peak occurred earlier in LMR compared to LM ($p<0.001$) indicating a dominant role of twilight for timing the E peak. Labeling as in B, G. **D, I:** Percentage of nocturnal activity (from whole day activity) in LD and LDR. Under LDR nocturnal activity was significantly lower than under LD ($p<0.001$) as indicated by the asterisks on top. **E, F:** Percentage of nocturnal activity (from whole day activity) in LM and LMR. Moonlight generally increased nocturnal activity and this effect was significantly higher under LM than under LMR, at least between 100 and 10000 lux (see asterisks on top). n.s. not significant; ** $p<0.001$.

Figure S1:


WT Lindelbach
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10 lux
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Normalized Activity
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6.5. Fly Cryptochrome and the visual system

Fly cryptochrome and the visual system

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Edited by Jay C. Dunlap, Dartmouth Medical School, Hanover, NH, and approved February 25, 2013 (received for review July 18, 2012)

Cryptochromes are flavoproteins, structurally and evolutionarily related to photolyases, that are involved in the development, magnetoreception, and temporal organization of a variety of organisms. *Drosophila* CRYPTOCHROME (dCRY) is involved in light synchronization of the master circadian clock, and its C terminus plays an important role in modulating light sensitivity and activity of the protein. The activation of dCRY by light requires a conformational change, but it has been suggested that activation could be mediated also by specific "regulators" that bind the C terminus of the protein. This C-terminal region harbors several protein–protein interaction motifs, likely relevant for signal transduction regulation. Here, we show that some functional linear motifs are evolutionarily conserved in the C terminus of cryptochromes and that class III PDZ-binding sites are selectively maintained in animals. A coimmunoprecipitation assay followed by mass spectrometry analysis revealed that dCRY interacts with Retinal Degeneration A (RDGA) and with Neither Inactivation Nor Afterpotential C (NINAC) proteins. Both proteins belong to a multiprotein complex (the Signalplex) that includes visual-signaling molecules. Using bioinformatic and molecular approaches, dCRY was found to interact with Neither Inactivation Nor Afterpotential C through Inactivation No Afterpotential D (INAD) in a light-dependent manner and that the CRY–Inactivation No Afterpotential D interaction is mediated by specific domains of the two proteins and involves the CRY C terminus. Moreover, an impairment of the visual behavior was observed in fly mutants for dCRY, indicative of a role, direct or indirect, for this photoreceptor in fly vision.

Circadian clocks synchronize physiology and behavior of living organisms with 24-h environmental cycles. In *Drosophila*, the resetting of the clock depends mostly on light-mediated degradation of the clock protein TIMELESS (dTIM), which, in turn, affects the stability of its partner PERIOD (dPER). Light signals are received through the blue-light photoreceptor CRYPTOCHROME (dCRY), the expression of which is under clock control. dCRY associates with dTIM in a light-dependent manner and promotes its proteasome-mediated degradation (1). Cryptochromes are flavoproteins highly similar to photolyases, from which they have probably evolved, but across evolution they have lost or reduced the photolyase activity and gained roles in signaling (2). Cryptochromes consist of two protein domains: an N-terminal domain homologous to photolyases (Photolyase Related, or PHR), and a very divergent C-terminal tail (3). A class of cryptochromes, CRY-DASH (drosophila, arabidopsis, synechocystis, homo), with single-stranded DNA repair activity and without the C terminus tail, has been described in bacteria, plants, and animals (2). The role of cryptochromes in the circadian clock differs among the different species. Cryptochromes have merely a blue-light photoreceptor activity in plants whereas in mammals they are part of the central clock mechanism, and this function is not light dependent (4). In *Drosophila*, the unique CRY acts as a circadian photoreceptor in the master clock (5) whereas, in other insects, only the vertebrate-like CRYs play a role as transcriptional repressor (6). Moreover, dCRY has been shown to play a fundamental role in the fly's magnetosensitivity, i.e., the use of the Earth's magnetic field for orientation and navigation (7). dCRY is rhythmically expressed. Protein levels oscillate only under light–dark cycling conditions, with a peak in the late night; in constant darkness, they increase, reaching a plateau (8). dCRY resets the clock by interacting with dTIM in the presence of light: subsequent to this interaction, dTIM is phosphorylated and targeted for degradation through a ubiquitin-proteasome mechanism that involves JETLAG, an E3-ubiquitin ligase complex component (9). Upon light activation, dCRY also interacts with JETLAG and is degraded via proteasome (9). dCRY interacts also with the kinase shaggy/GSK3 (SGG), and the cryptochrome's stability in light is considerably increased by this interaction whereas the inactivation of the kinase leads to the degradation of dCRY in darkness (10). The molecular mechanism by which dCRY is activated by light is still not fully understood, but a regulatory role for the C terminus of the protein has been demonstrated by several studies (3, 5, 11–13). The activation of dCRY by light requires a conformational change (13), but the release of a putative repressor cannot be excluded (11). In fact, it has been hypothesized that the activation of dCRY by light is mediated also by specific "regulators" that bind its C terminus, known to regulate the light dependence of dCRY activity (13). This hypothesis was supported by the observation that the C terminus of dCRY is a hotspot for molecular interactions: by in silico analysis and experimental validation, we could identify several protein–protein interaction motifs in this small region and, among them, two class III PDZ-binding motifs (3). PDZ (postsynaptic density protein 95, *Drosophila* disk large tumor suppressor, and zonula occludens-1 protein) domains are modular domains that play a crucial role in the assembly of large protein complexes involved in signaling processes. These domains have a conserved fold consisting of five or six β-strands and two to three α-helices forming a β-stranded sandwich. PDZ domains typically recognize the extreme C terminus of target proteins (14). Distinct PDZ domains bind to optimal sequences, and the structural analysis of known binding sites of PDZ domains and their ligands has provided insight into the specificity of PDZ protein–protein interactions (15). The preference of each residue of a binding peptide is related to the physical-chemical characteristics of different relevant residues on specific secondary structural elements forming the PDZ-binding pocket (16). Three major classes of PDZ-binding motifs have been established (17).

Author contributions: G.M., S.M., R.C., and S.C.E.T. designed research; G.M., A.R., E.L., M.M., C.B., L.C., B.S., A.J.M.M., M.S., R.G., and S.C.E.T. performed research; G.M., C.B., and R.C. analyzed data; and G.M., C.B., C.H.-F., R.C., and S.C.E.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1212317110/-/DCSupplemental.

NEUROSCIENCE

Here, we show that some functional linear motifs are evolutionarily conserved in the C terminus of cryptochromes, with class III PDZ-binding sites selectively maintained in animals. We detected the presence of dCRY in a multiprotein complex (the Signalplex) involved in the visual-signaling pathway (18), and we found that the interaction with this comlex is mediated by Inactivation No Afterpotential D (INAD), a scaffold protein with five structural PDZ domains. Moreover, we detected a role for dCRY in fly vision.

Results

Functional Motifs Are Conserved in CRY Across Species. We searched for the evolutionary conservation of linear motifs in the C terminus of CRY throughout a broad range of organisms. Linear motifs are short sequences that mediate molecular interactions and very often reside in disordered or nonglobular regions of proteins. Unraveling the evolution of linear motifs is problematic, as these sites tend to be unstable over long evolutionary distances or to jump between different sequence positions inside nonglobular regions. dCRY is an excellent test case for this assumption, as it bears a highly variable C-terminal region that has undergone rapid evolution while maintaining overall similar roles in circadian rhythmicity. An unrooted neighbor-joining phylogenetic tree was constructed using amino acid sequences from various members of the CRY family from plants to humans (Fig. S1). Animal cryptochromes were clustered in four different groups: vertebrate, vertebrate-like (including invertebrate species), CRY4, and *Drosophila*-like. CRY sequences show many linear motifs that are not evenly distributed in all species investigated (Fig. S1). Among them, PDZ domains recognize short sequences at the C terminus of proteins and have an important role in mediating interactions for the assembly of large multiprotein complexes involved in signaling processes at specific subcellular locations. Interestingly, among the three major classes of PDZ-binding motifs, class III is evolutionarily conserved in the CRY C-terminal sequence across animal species (Fig. S1). We speculated that a protein partner of dCRY could be a PDZ domain-containing protein and searched the STRING database (19) for possible candidates. In this database, connections between proteins are based on several methods, including computational predictions. Fig. 1*A* shows the distribution of interactors for dCRY. The results showed a weak connection to No Receptor Potential A (NORPA), a protein belonging to the phototransduction complex (20).

dCRY Interacts with the Phototransduction Complex. In an attempt to identify new partners of dCRY, a coimmunoprecipitation assay, followed by mass spectrometry analysis, was performed on transgenic flies overexpressing a hemagglutinin (HA)-tagged form of dCRY (HACRY; 13) raised in 12:12 light:dark cycles and collected at Zeitgeber Time 24 (ZT24), before lights on, and after a 15-min light pulse. An ~115-kDa species was observed in the sample in the dark and an ~180-kDa species after the light pulse, which were not present in the respective negative controls (Fig. 1*B*). These protein bands were digested in-gel, and the peptide mixtures were analyzed by liquid chromatography–mass spectrometry (LC-MS)/MS using an ESI-QTOF mass spectrometer (21). Analysis of the MS/MS data using the MASCOT software yielded the identification of two proteins involved in the fly visual-signaling pathway: Retinal Degeneration A (RDGA) in the dark and Neither Inactivation Nor Afterpotential C (NINAC) after 15 min of light pulse (Fig. S2*A*) (18, 20). Although RDGA was identified on the basis of the MS/MS spectra of six different tryptic peptides, in the case of NINAC, the identification was based on the MS/MS spectrum of only one peptide displaying a significant score in MASCOT (Fig. S2*B*). The presence of NINACp174 in the complex with HA-tagged form of dCRY (HACRY) was also confirmed by Western blot with an antibody specifically raised against the p174 isoform of the protein that is localized in the rhabdomeres of photoreceptor cells in the fly's eye (22). By this procedure, NINACp174 was also detected in the dark, albeit at lower levels than under light conditions (Fig. 1*C*). The difference between NINACp174/HACRY ratios under light and dark conditions was significant ($P < 0.03$, Mann–Whitney U test) (Fig. S2*C*).

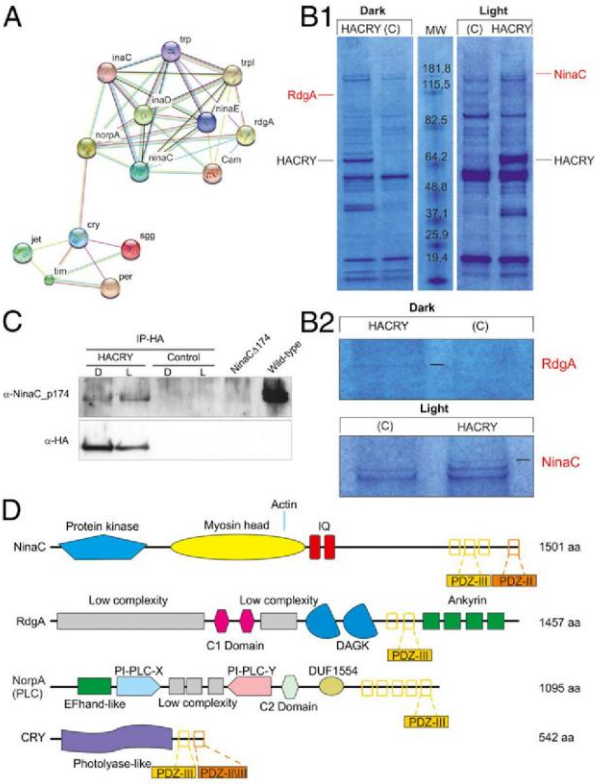

Fig. 1. Interaction of dCRY with the phototransduction complex. (*A*) Protein interaction network surrounding dCRY and INAD. The STRING interaction network is shown for dCRY, INAD, and their main interaction partners with edge colors representing different detection methods. Note that the edge between dCRY and NORPA is based on phenotypic enhancement assays and thus may not necessarily represent a true physical interaction. (*B1*) Coomassie blue-stained gel of heads of protein extracts coimmunoprecipitated with an anti-HA antibody. HACRY-overexpressing flies (HACRY, *yw;tim*-GAL4/+; UAS-*HAcry*/+) and relative controls (C, *yw;tim*-GAL4) were reared in 12:12 light: dark and collected in the dark (ZT24) and in the light (ZT24 + 15-min light pulse). Molecular masses of markers are indicated (BenchMark Pre-Stained Protein Ladder; Invitrogen). MW, molecular weight. Bands corresponding to HACRY are indicated in black, while stained proteins excised and characterized by mass spectrometry are indicated in red. (*B2*) Zoom of regions of the gel-bearing dRDGA and dNINAC bands. (*C*) Coimmunoprecipitation and Western blot confirming the interaction between HACRY and NINAC in HACRY-overexpressing flies (*yw;tim*-GAL4/+; UAS-*HAcry*/+). *tim*-GAL4 flies were used as control. Heads were collected as in *B1*. Membranes were probed with anti-NINACp174 and anti-HA antibodies. *NinaC*$^{\Delta 174}$ and w^{1118} flies, collected at ZT1, were used as negative and positive control, respectively. (*D*) Schematic domain distribution for known and putative INAD interacting proteins. Each protein is drawn proportional to its size, with solid shapes representing different protein domains and their name from the Pfam database. Note that low-complexity regions, shown as light-gray rectangles, are not a proper domain. PDZ-binding motifs are shown as white rectangles with yellow (class III), orange (type II), or peach (overlapping classes II/III) borders.

dCRY Interacts with the Phototransduction Complex Through INAD. Many of the elements of this visual cascade are assembled in a multiprotein-signaling complex (Signalplex) organized by

INAD, a scaffold protein with five structural PDZ domains, each of which binds to a specific partner (20). A schematic representation of the functional domains of NINAC, RDGA, NORPA, and dCRY is given in Fig. 1*D*. To test whether dCRY interacts with the phototransduction complex through INAD, we searched for INAD in the immunocomplex formed by dCRY. Indeed, a Western blot with an anti-INAD antibody (23), performed on head protein extracts from HACRY-overexpressing flies immunoprecipitated with an anti-HA antibody, revealed that INAD interacts in vivo with dCRY (Fig. 2*A*). The interaction is quite strong in the light, but traces of INAD are visible also in the dark. The difference between INAD/HACRY ratios under light and dark conditions was significant ($P < 0.02$, Mann–Whitney U test) (Fig. S2*D*).

The physical interaction between dCRY and INAD was further analyzed using a yeast two-hybrid system (24), in which a full-length dCRY, directly fused to LexA (bait), was initially challenged with full-length INAD as prey (Fig. 2*B* and Table S1). A strictly light-dependent interaction between the two proteins was observed (Fig. 2*B*, dCRY), which is completely abolished when part of the dCRY C terminus (aa 521–540) is removed. As this region contains the binding motifs for PDZ domains, the 22 C-terminal amino acids of dCRY were tested for the ability to interact with INAD. A light-independent interaction between INAD and the extreme C-terminal tail of dCRY was observed (Fig. 2*B*, dCRY). To examine which domains of INAD are responsible for the interaction with dCRY, prey fusions expressing individual PDZs or different combinations of them were generated and tested for the interaction with full-length dCRY as bait (Fig. 2*B*, INAD). Single PDZ domains did not interact with dCRY, although all of the fusion proteins were correctly expressed in yeast cells. In fact, before the β-galactosidase assay, the expression of all fusion proteins was analyzed by Western blot on yeast lysate with an anti-HA antibody (Fig. S3 and Table S2). For PDZ1, PDZ3, and PDZ4, in addition to the expected signal, a band of molecular weight compatible with a dimeric organization was observed (Fig. S3). However, dimerization of PDZ domains seems not to influence binding to their partners, as the sites involved in the two events are different (25). Because some PDZ domains need other PDZ domains connected in tandem to fold properly and interact with their partners (25), the interaction between dCRY and INAD may also require tandem PDZ domains. However, prey fusions expressing tandems of PDZ linked by their native spacer sequences were still not able to interact with dCRY (Fig. 2*B*, INAD). An in silico analysis performed with CSpritz (26) revealed the presence of an α-helical motif upstream from the PDZ2 domain, specifically the motif MAKI (aa 235–238), which could form a unique extension of the PDZ domain and is also part of the known calmodulin-binding motif. An "extended" version of the PDZ2-PDZ3 tandem prey fusion was generated to include the predicted sites, ranging from residues 207 to 448, and this sequence showed high affinity for dCRY (Fig. 2*B*, INAD). These data suggest that the interaction between INAD and dCRY is mediated by the PDZ2-PDZ3 tandem, but that the PDZ2 domain needs to be extended upstream, with respect to the canonical PDZ domain boundary. Longer fusion sequences were prepared by adding a third PDZ domain; three different portions of INAD, including PDZ1–3 (aa 17–448), PDZ2–4 (aa 249–577), and PDZ3–5 (aa 364–664), respectively, were tested. Only the fusion expressing the N-terminal PDZ1–PDZ3 domains showed affinity for dCRY (Fig. 2*B*, INAD), suggesting that PDZ4 and PDZ5 are not involved in the interaction between dCRY and INAD. The higher binding affinity for the extended PDZ2-PDZ3 tandem compared with larger INAD fragments may be explained by the PDZ2 domain having a non-canonical structure, conferring a higher binding affinity for the dCRY motif. This affinity is likely reduced when PDZ1 is present due to entropy losses caused by increased structural rigidity. The expression levels of all fusions, analyzed by Western blot on yeast lysate with an anti-HA antibody, were comparable (Fig. S3).

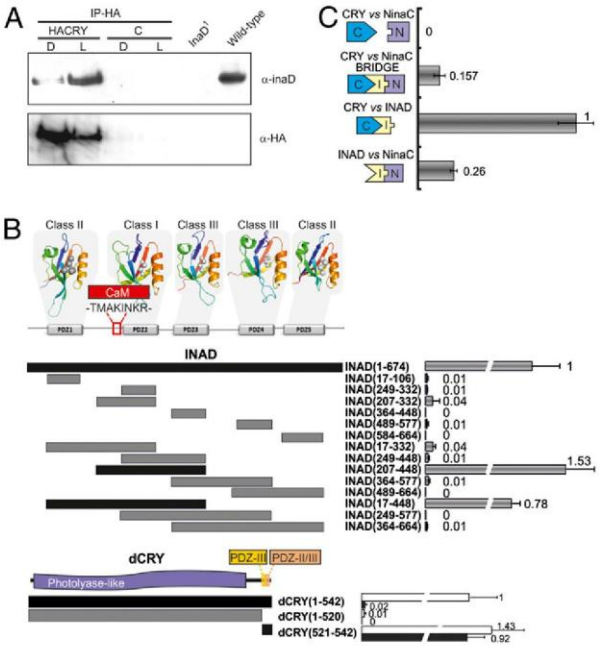

Fig. 2. dCRY interacts with INAD. (*A*) Coimmunoprecipitation and Western blot confirming the interaction between HACRY and INAD in flies overexpressing HACRY (*yw;tim*-GAL4/+; UAS-*HAcry*/+). *tim*-GAL4 flies were used as control ("C"). Heads were collected as in Fig. 1*B*. Membranes were probed with anti-INAD and anti-HA antibodies. *inaD*^{1} and *w*^{1118} flies, collected at ZT1, were used as negative and positive controls of the antibody, respectively. (*B*) Identification of the interaction domains of dCRY and INAD using the yeast two-hybrid system. The five INAD PDZ domains are shown where modeled and assigned to putative PDZ subtypes depending on the residue types at the peptide-binding site. Relevant sequence motifs are shown as empty rectangles in the INAD and CRY sequence diagrams. Different domains of INAD were tested for interaction with the full-length dCRY in the presence of light, and different domains of dCRY were tested for interaction with the full-length INAD under both light and dark conditions (open and filled bars, respectively). Interacting fusions are shown in black, and relative β-galactosidase activity (Miller units) is reported for each fusion. Mean ± SEM of at least seven independent clones for each fusion, analyzed in triplicates, is shown. An extended version of the PDZ2–3 tandem, INAD (207–448), exhibits a significantly stronger affinity for dCRY compared with the whole protein ($F_{14,87} = 67.81$, $P < 0.0001$). The interaction between dCRY and INAD occurred in a light-dependent fashion with the C terminus of dCRY being crucial. On the other hand, these last 22 amino acids of the protein showed a light-independent affinity for INAD with a significantly stronger interaction in the light compared with the dark ($t_{13} = 2.6$, $P = 0.02$). (*C*) Yeast two- and three-hybrid assays highlighting that the interaction between dCRY and NINAC is mediated by INAD. The schematic shows the different proteins used as bait or prey fusion: C, dCRY; N, NINAC; I, INAD. Relative β-galactosidase activity (Miller units) is reported for each fusion. Mean ± SEM of at least six independent clones for each fusion, analyzed in triplicates, is shown. The expression of dCRY and NINAC alone does not result in the activation of the reporter gene. The expression of INAD in the yeast nucleus, to generate a three-hybrid system, shows that INAD acts as a structural bridge (BRIDGE) between the two proteins ($F_{3,24} = 57.20$, $P < 0.0001$). The interactions of dCRY–INAD and INAD–NINAC are also shown.

The reported interaction between INAD and NINAC in the formation of the Signalplex (23), together with the interaction between INAD and dCRY that we observed, suggest that the interaction between dCRY and NINAC may be specifically mediated by INAD. To detect whether dCRY, INAD, and NINAC form a ternary protein complex, we devised a three-hybrid system,

in which dCRY was used as bait and NINAC as prey and a FLAG-tagged form of INAD was selectively expressed in the yeast nucleus. The expression of all fusions was tested by Western blot on yeast lysate with anti-HA antibody for NINAC and anti-FLAG antibody for the nuclear INAD (Fig. S3). When we expressed dCRY as bait and NINAC as prey alone, no direct interaction between the two proteins was observed, whereas expression of INAD in the nucleus resulted in the activation of the reporter gene, indicating that the formation of a three-component complex is necessary to restore the activity of the transcription factor (Fig. 2*C*).

dCRY Is Involved in Visual Behavior. The surprising presence of dCRY associated with the visual cascade complex could underline a role, direct or indirect, for this photoreceptor in fly vision, which has not been entertained as yet.

To investigate a possible involvement of dCRY in the fly eye-mediated light response, the electroretinogram (ERG) of flies in which dCRY was completely knocked out (cry^{01}) (27) was analyzed. Moreover, we studied the optomotor and phototactic behavior of cry^{01} flies or flies in which dCRY lacked the C terminus tail (cry^{M}) (5). Wild-type flies are known to show a diurnal rhythm in visual sensitivity determined by ERG recordings, with maximal sensitivity in the first half of the night (28). A comparable rhythm was found in control flies [Canton S (CS) × w^{1118}] with a pronounced sensitivity and a maximum in the middle of the night (Fig. 3*A*). In contrast, the visual sensitivity of cry^{01} mutants was not dependent on the time of day albeit their ERG profiles were normal (Fig. S4*D*). The same was true for the optomotor turning response of the flies. Although the optomotor response of wild-type flies depended significantly on the time of the day (as already observed in ref. 29), it did not in cry^{01} mutants (Fig. 3*B*). cry^{01} mutants responded less to visual stimuli throughout the day than control flies, but this impairment was most evident during the first half of the night, around the wild-type flies' maximum in optomotor turning response (Fig. 3*B*). The optomotor response was analyzed with two different setups (*SI Materials and Methods*) with similar results (Fig. 3 *B* and *C* and Fig. S4 *A* and *B*). Like cry^{01} mutants, cry^{M} mutants also displayed a similar impairment in their optomotor turning response (Fig. S4 *A* and *B*). In a phototaxis assay using counter-current distribution, in which wild-type flies orient and move toward a light source (30), cry^{01} and cry^{M} mutants showed a reduced performance index of 0.41, compared with 0.63 of the progeny of the CS × w^{1118} cross used as control (Fig. S4*C*). To test whether the impaired optomotor response depends on CRY function in the compound eyes, we selectively rescued CRY in the eyes with the help of the upstream activating sequences (UAS)-GAL4 system, driving GAL4 under control of the eye-specific *glass multiple reporter* (*gmrGAL4*) (31). *gmr-GAL4* is known to disturb the structure of the compound eyes in a dose- and temperature-dependent manner (32). As a consequence, $gmrGAL4;cry^{01}$ control flies showed a lower optomotor response than the other cry^{01} mutants (Fig. 3*C*). Nevertheless, the expression of the $HAcry^{7M}$ construct (Fig. S5) in the compound eyes restored the optomotor response of cry^{01} mutants to almost wild-type levels.

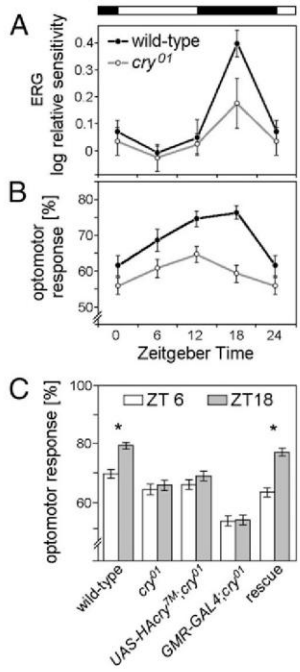

Fig. 3. Visual behavior of wild-type flies, cry^{01} mutants, and cry^{01} mutants with CRY rescue in the eyes. (*A*) Visual sensitivity of cry^{01} and wild-type controls (CS × w^{1118}) during the course of a day. Sensitivity was calculated as the reciprocal of the photon flux needed to evoke a criterion response of 6 mV in the ERG receptor potential. Within each genotype, sensitivity values were normalized to the average sensitivity at ZT6. Each point represents the average of values estimated for a minimum of 9 and a maximum of 13 flies. Mean values ± SEM are given. ANOVA revealed that sensitivity values were significantly dependent on the time of day for CS × w^{1118} ($F_{3,38} = 15.649$, $P < 0.001$) but not for cry^{01} ($F_{3,39} = 1.775$, $P = 0.168$). Note that the value at ZT4 is repeated at ZT0 to improve clarity. (*B*) Optomotor responses of cry^{01} and wild-type controls (CS × w^{1118}) during the course of a day. Each point represents the average of 32 flies. The nonparametric Kruskal–Wallis test revealed that optomotor response values were significantly dependent on the time of day for CS × w^{1118} ($P < 0.001$) but not for cry^{01} ($P = 0.181$). Furthermore, two-way ANOVA showed that optomotor response was highly dependent on the genotype ($F_{1,251} = 31.411$, $P < 0.001$), meaning that wild-type flies generally showed a higher optomotor response than cry^{01} mutants. Note that the value at ZT24 is repeated at ZT0 to improve clarity. (*C*) Optomotor responses at ZT6 and ZT18 for wild-type flies, cry^{01} mutants, and flies with CRY rescued in the compound eyes ($UAS\text{-}HAcry^{7M};cry^{01}$ × $gmrGAL4;cry^{01}$). A total of 100 flies per genotype were analyzed in each experimental condition. Only wild-type and CRY-rescued flies showed a significant difference in optomotor response between the ZT6 and ZT18 (wild type: $t_{198} = 5.23$, $P < 0.0001$; rescued flies: $t_{198} = 6.53$, $P < 0.0001$).

Discussion

The analysis of the linear motifs present in the C terminus of CRYs showed that they were not evenly distributed in all species investigated. The class III PDZ motif is present in all animal phyla, suggesting a functional constraint on the evolving sequence, as the motif is maintained although it is not being conserved in the same sequence stretch. Our results clearly indicate that the circadian blue-light photoreceptor dCRY interacts with the visual transduction complex (Signalplex) through the scaffold protein INAD. The interaction between the two proteins is mediated by a specific region of INAD, which includes the PDZ2-PDZ3 tandem, but is extended upstream with respect to the canonical PDZ domain boundary to include a stretch of amino acids known to be part of a calmodulin-binding motif. Interactions modulated by multiple INAD PDZ domains have already been described (33). It has also been reported for other PDZ-containing proteins that two or three PDZ domains connected in tandem may exhibit different specificity in their target-binding properties compared with isolated domains (34). We also established that the 22-amino acid C-terminal sequence of dCRY is involved in binding to INAD, in accordance with the presence of either class III or class II/III PDZ-binding motifs predicted by the eukaryotic linear motif (ELM) program in the C terminus of the protein and also with the notion that PDZ domains preferentially interact with the absolute carboxyl-terminal

ends of their target proteins (14). The interaction between dCRY and INAD is particularly effective in the light, and it is well recognized that the activity of both proteins is modulated by light. However, the light-independent interaction of the C-terminal fragment of dCRY with INAD suggests that the influence of light in the interaction of the full-length proteins is due to the PHR domain of dCRY. Supporting this hypothesis is the fact that the INAD PDZ4 and -5, known to be regulated by light-dependent conformational changes (33), are not involved in the interaction. The interaction between dCRY and NINAC observed in vivo represents quite an unexpected result. A connection between dCRY and a cardinal component of the fly visual cascade (23) was established, and the mediator role of INAD in the interaction was demonstrated. We also showed that this interaction has a functional importance for vision. In CRY-knockout flies, the diurnal cycling of photoreceptor sensitivity and motion vision typical of wild-type flies (28, 29) is abolished. Furthermore, the CRY-knockout flies are slightly but significantly impaired in motion vision. The diurnal rhythm in optomotor response was recovered when CRY was expressed in all photoreceptor cells of the compound eyes, showing that CRY in the photoreceptor cells is responsible for wild-type rhythms in motion vision. Motion detection depends mainly on intact vision in photoreceptors R1–6 with minor contribution from R7 and R8 (35, 36), whereas phototaxis is mediated by all eight photoreceptors in the compound eyes (37). dCRY is expressed in the entire cytoplasm of the photoreceptor cells and seems to have the highest density close to the rhabdomers, the place of the visual cascade (38). Therefore, dCRY may easily interact with INAD and eventually modulate the transient receptor potential (TRP) and TRP-like (TRPL) channel opening in interplay with the other PDZ proteins of the Signalplex. Interestingly, small amounts of CRY seem to be sufficient for this interaction as the optomotor response was highest at the end of the day until the middle of the night (ZT12–18) when CRY levels are low (9). Recently, dCRY was shown to be also involved in the membrane excitability (K^+ channel conductance) of the large ventral Lateral clock Neurons (l-LN_v) (39). These neurons fire action potential upon illumination with blue light, and this firing is dependent on dCRY. Although the way in which dCRY regulates the l-LN_v firing rate in relation to K^+ channel conductance remains unclear, our results further support an involvement of dCRY in membrane potential modulation. Here, we show that dCRY may be the link that couples the clock with the PDZ proteins of the Signalplex, in this way modulating vision in a circadian fashion. A functional circadian clock in the photoreceptor cells is obviously important to control visual coding efficiency in *Drosophila* and to optimize vision under different light intensity regimes (29). In fact, wild-type flies show circadian changes in the size of certain brain regions (e.g., optic lobes) and in photoreceptor cell terminals that control the sensitivity of photoreceptors to circadian variations in light levels (29). This structural plasticity is still maintained in period $(per)^{01}$ flies, which lack a key component of the circadian machinery, but it is exclusively light-driven as there is no longer "anticipation" of the light/dark transitions (29). In most invertebrates, the components of visual signaling are localized on the rhabdomeres (40), whereas a ciliary vision (rods and cones) is predominant in the vertebrate retina (41). An important difference between the two kinds of photoreceptors is the biochemical cascade used to transduce photic signals in electric signals. In fact, rods and cones use a cascade involving cyclic guanyl monophosphate as a second messenger whereas rhabdomeric photoreceptors use a phosphoinositide-signaling cascade involving the enzyme phospholipase C (PLC) (41). Retinal photoreception in mammals includes a subset of retinal ganglion cells that are able to respond to light even in the absence of synaptic inputs (42). These cells, called "intrinsically photosensitive retinal ganglion cells" (ipRGCs), use melanopsin as photopigment and send their axons directly to the suprachiasmatic nucleus, the site of the primary circadian pacemaker in mammals (18, 41). ipGRCs have been shown to use a rhabdomeric-like phosphoinositide cascade involving the effector enzyme PLC (18, 41). Very recently, it has been observed that these melanopsin-expressing ganglion cells extend their projections toward the thalamo-cortical neurons implicated in pattern vision, establishing melanopsin-based photoreception as a significant source of visual information to the thalamo-cortical pathway, independent of rods or cones (43). The ipGRCs and the fly phototransduction mechanisms also share other similarities: both require a member of the Gq/11 family of G proteins as a mediator of the phototransduction cascade, and, in both cases, the phototransduction cascade is tightly coupled to the plasma membrane and involves light-sensitive channels belonging to the TRP family (44). The similarity of the photoreception cascade between *Drosophila* and the mammalian ipRGCs, and also the expression of CRY in both photoreceptor cells (45), raises the question of whether mammalian CRYs could contribute to the circadian functions of ipRGCs by specifically binding to the phototransduction complex. Although a homologous complex of the fly Signalplex has not been described in ipRGCs, several components of this multiprotein complex seem to be conserved (18). Specifically, a protein homolog of dINAD, INAD-like (INADL), bearing seven PDZ domains, has been identified in humans (46). A search for a functional protein interaction network, performed with the STRING database (Fig. S6), showed that INADL can be a functional partner of Crumbs homolog 1 precursor, a factor involved in retinal photoreceptor organization (47). This renders INADL a good candidate for a scaffold protein that organizes and maintains the phototransduction complex in ipRGCs. Our results extend the role of dCRY to fly visual biology and provide a tantalizing glimpse of a phylogenetically conserved possible role for CRY that may have circadian implications in mammalian vision also.

Materials and Methods

Bioinformatic Analyses. The computational search for dCRY protein–protein interactions combined the results from the STRING database (19) of protein–protein interactions with the domain organization of proteins from Pfam (48). Relevant proteins were analyzed with CSpritz (26), which predicts intrinsic disorder in the sequence as well as linear motifs coding for common protein–peptide interactions taken from ELM (49). The X-ray structures of INAD PDZ domains were retrieved from the Protein Data Bank for domains 1 and 5 [Protein Data Bank (PDB) codes 1IHJ and 2QKT]. The three remaining domains were identified (50) and modeled based on PDB codes 2FNE (chain C) and 1Z87 (chain A) as templates for PDZ2-PDZ4 and PDZ3, respectively (Fig. S7).

Coimmunoprecipitation and Mass Spectrometry. Head extracts from HACRY-overexpressing flies were subjected to coimmunoprecipitation as previously described (3). After the separation of proteins by SDS/PAGE, Coomassie-stained protein bands were excised, in-gel digested (21), and analyzed by LC-MS/MS on a Micromass CapLC unit (Waters) interfaced to a Micromass Q-Tof Micro mass spectrometer (Waters). MS/MS data were analyzed by MASCOT software (Matrix Science; www.matrixscience.com/) against the *Drosophila* sequences of the Swiss-Prot database (release 2011_03).

Western Blots. Immunocomplexes were analyzed by Western blotting using the following antibodies: rabbit polyclonal anti-INAD (1:500) (25), rabbit polyclonal anti-NINACp174 (1:500) (22), and mouse anti-HA (Sigma; 1:5,000).

Yeast Two- and Three-Hybrid Tests. dCRY, either full-length or fragments, was fused to the LexA moiety in the bait vector (pEG202), and INAD (full length or fragments) was fused to the "acid-blob" portion of the prey vector (pJG4-5) (24). In the yeast three-hybrid assay, dCRY was used as bait and NINAC as prey, and a FLAG-tagged full-length INAD was expressed in the nucleus. Quantification of β-galactosidase activity was performed in liquid culture as in Ausbel et al. (51).

Visual Sensitivity Determined by ERG Recordings. Visual sensitivity was obtained from the irradiance response curves (IRC) recorded at four different ZTs. The ERG responses to light stimuli of different intensities were used to determine the IRCs. ERGs were recorded as in ref. 28.

Analysis of Optomotor Activity. The walking optomotor test was performed as in ref. 52 (setup 1 in *SI Materials and Methods*). Details of setups 1 and 2 are given in *SI Materials and Methods*.

Phototaxis. The experiments for phototaxis were performed as described in ref. 30. See details in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Michele Vidotto (University of Padova) for help with the initial linear motif analysis; Paola Cisotto (University of Padova) for technical support; Matteo Simonetti (University of Padova) for graphical support; Alberto Biscontin (University of Padova) for help with quantitative RT-PCR; Craig Montell (The Johns Hopkins University School of Medicine) for anti-INAD and anti-NINACp174 antibodies; Dr. Susan Tsunoda (Colorado State University) for $inaD^{1}$ flies; the Bloomington Stock Center for $NinaC^{\Delta 174}$ flies; and Dr. Taishi Yoshii (Okayama University) for $gmrGAL4;cry^{01}$ flies. We thank Reinhard Wolf (Virchow Center, University of Würzburg) for help with the optomotor response experiments and Reinhard Wolf and Bambos Kyriacou (University of Leicester) for helpful comments on the manuscript. This work was funded by grants from the European Community (Sixth Framework Project Entrainment of the Circadian Clock 018741) and Fondazione Cariparo (Progetti di Eccellenza 2011–2012) (R.C.); University of Padova Grant CPDA099390/09 (to G.M.); and University of Padova Grants CPDA098382 and CPDR097328 and Fondo Investimento Ricerca di Base (FIRB) Futuro in Ricerca Grant RBFR08ZSXY (to S.C.E.T.). R.C. was supported by the Italian Space Agency [Disturbo del Controllo Motorio e Cardiorespiratorio (DCMC) grant] and the Ministero dell'Università e della Ricerca. C.H.-F. was supported by the Deutsche Forschungsgemeinschaft (Fo207/10-3), R.G. by the Graduate School of Life Sciences (University of Würzburg), and M.S. by the Hanns Seidel Foundation.

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Supporting Information

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SI Text

We supposed that the interaction between *Drosophila* CRYPTOCHROME (dCRY) and Inactivation No Afterpotential D (INAD) occurs through the conserved class III PDZ-binding motif in the C-terminal tail of dCRY and one or more of the five PDZ domains of INAD. To identify the possible PDZ domain responsible for the interaction, we investigated the binding specificity of each domain. Because the binding preference of any PDZ domain is significantly determined by the chemical characteristic of the αB1 residue of the binding pocket and the p(−2) residue of the ligand motif, we collected structural and sequence information about PDZ domains and ligands of INAD. For two of the five PDZ domains, PDZ1 and PDZ5, a crystal structure had been previously solved (1, 2) whereas for the other three PDZ domains (PDZ2, PDZ3, and PDZ4) we built a model using a homology modeling approach. To identify relevant positions on the peptide-binding pocket of each domain, we built a structural alignment of the five domains. As described in Fig. S7, each domain has the highly conserved carboxylate-binding loop (X-Φ-G-Φ motif, where X is any amino acids and Φ is a hydrophobic residue) between βA and βB strands. Furthermore, the chemical characteristics of the distinct residues at position αB1 suggest a possible binding preference for each PDZ domain (3). On the basis of these speculations, PDZ1 could be classified in the IIB subclass where the negative or polar residue at αB1 has been associated with tyrosine (Tyr) or phenylalanine (Phe) at the p(−2) position in the ligand motif (3). However, PDZ1 was crystallized with the C terminus peptide of No Receptor Potential A (NORPA) phospholipase C (PLC)-β, revealing a unique mode of interaction that consists of a disulfide bond between cysteine 31 (Cys31) of PDZ1 and Cys(p-1) of the NORPA peptide (1). Furthermore, the C-terminal sequences of NORPA (PLC-β) and Neither Inactivation Nor Afterpotential C (NINAC) (-EFCA; -AVDI; respectively), another previously identified partner of INAD (4), matched well with the class II PDZ domain-binding motif Φ-X-Φ-COO-. The other domain of INAD that could be assigned to this class is the PDZ5 domain, which has an aromatic residue (Phe) at the αB1 position. PDZ5 was also found to interact with NORPA (PLC-β) (5), and the interaction seems to occur through the C-terminal class II PDZ-binding motif or through an internal region of NORPA (6). Different groups proposed that the INAD–NORPA interaction occurs either with both PDZ1 and PDZ5 domains (7) or with PDZ5 only (5). The lack of interaction with NORPA (PLC-β) reported by other groups (2, 8) was probably due to interference of the experimental conditions used (e.g., posttranslational modification) as PDZ5 is phosphorylated by PKC (8, 9) and undergoes a redox conformational switch that dramatically reorganizes the binding pocket (2, 10). The PDZ2 domain, with a His residue at the αB1 position, was assigned to class I PDZ interactions. These PDZ domains recognize ligands that contain either serine or threonine (Thr) at the −2 postion. The PDZ2 of INAD was found to recognize the unique class I PDZ-binding motifs predicted by the eukaryotic linear motif (ELM) program at the C-terminal sequence of eye-PKC (-ITII) (11). The class III PDZ-binding motifs instead could be preferential ligands for PDZ3 and PDZ4 domains, which have Tyr and Thr, respectively, at the αB1 position. The hydroxyl group of these residues can bind a negatively charged amino acid in the p(−2) position of the ligand motif. PDZ3 was identified as a target PDZ domain for the TRP calcium channel (5, 12) and PDZ4 as a target for eye-PKC (5). It has been demonstrated (8) that either PDZ3L (extra 28 residues COOH-terminal to PDZ3) or PDZ4 was sufficient to bind opsin, TRPL, and PKC. In this work, Xu et al. (8) demonstrated that the binding with PKC could occur through a binding site in the C terminus different from those at the extreme C terminus interacting with PDZ2. Furthermore, PDZ3 and PDZ4 domains, using a different interface, also mediate the INAD homo-multimerization (8). Little is known about the ligand motifs mediating interactions with domains PDZ3 and PDZ4, but all of the detected interacting proteins contain several class III PDZ-binding motifs predicted by the ELM program in the internal sequence of their C terminus (Fig. 1*D*).

SI Materials and Methods

In Silico Protein–Protein Interactions. The computational search for protein–protein interactions was started by using the annotated STRING database (13) of known and predicted physical and functional protein–protein interactions. Using STRING in protein mode, we obtained the protein interaction network of dCRY (Fig. 1*A*) and selected interactions with high confidence levels. To explore the domain organization of proteins (Fig. 1*D*), identified by SMART (14) and predicted or found to be related to CRY, we used the interactive view of the STRING network. The CRY C termini were analyzed using CSpritz (15), which predicts intrinsic disorder in the sequence as well as secondary structure preferences. Linear motifs coding for common protein–peptide interactions, taken from ELM (16), are also predicted in CSpritz.

Phylogenetic Tree Reconstruction. A multiple sequence alignment with 98 sequences from Photolyase/Cryptochrome families available in UniProt database (www.uniprot.org) was generated with ClustalW2 (17). Sequences are representative of either different cryptochrome families or different animal/plant phyla. Alignments were manually verified and a phylogenetic tree was generated using neighbor-joining methods (18) with a complete deletion mode (Fig. S1). A total of 1,196 sites (953 variable and 52 conserved sites), including gap sites, were used in the phylogenetic analysis. Bootstrap tests were performed with 1,000 replications. PAM matrix correction distance was adopted, and rates among sites were set as uniform. This analysis was performed in April 2011.

In Silico Analysis of INAD. Domain organization was defined by data retrieved from the Pfam resource (19). Secondary structure was predicted using a consensus method (20), and disordered regions were searched for with CSpritz (15). The crystal structure of INAD PDZ1/PDZ5 domains was retrieved from the Protein Data Bank (PDB) database (21) (PDB codes 1IHJ and 2QKT). Models for the other three INAD-PDZ domains (Fig. 2*B*) were constructed using the HOMER server (http://protein.bio.unipd.it/homer/). The automatic template search with MANIFOLD (22) indicated 2FNE (chain C) and 1Z87 (chain A) as templates for PDZ2/PDZ4 and PDZ3, respectively. The raw models generated from these templates were completed by modeling the divergent regions with LOBO, a fast divide-and-conquer method (23). The final models were subjected to a short steepest-descent energy minimization with GROMACS (24) and evaluated with QMEAN (25, 26). The structure was visualized using PyMOL (DeLano Scientific; http://pymol.sourceforge.net/). To predict potential functional motifs, protein sequences were analyzed using ELM (16).

Fly Strains. The following *Drosophila* strains were used: w^{1118}; *Oregon-R*; WT-ALA (27); the progeny of crosses *Canton S* × w^{1118} and WT-ALA × w^{1118} as wild-type controls; $inaD^{1}$ (28); $NinaC^{\Delta 174}$ (29); cry^{0} (30); cry^{M} (31); *yw; timGal4* (32); *w; UAS-HAcry* 16.1 (33), and *UAS-HAcry*^{7M};cry^{0}, *gmrGal4;*cry^{0}. All flies were reared on a standard yeast–glucose–agar medium and maintained at 23 °C, 70% relative humidity, on a 12-h light:12-h dark cycle.

Coimmunoprecipitation. Three- to five-day-old flies overexpressing HA-dCRY (*yw*; *tim-GAL4/+; UAS-HAcry/+*) were collected at Zeitbeger time 24 (ZT24) (ZT0 lights-on and ZT12 lights-off in a 12:12 light–dark cycle) and after a 15-min light pulse given at the same time point. Heads were homogenized in extraction buffer [20 mM Hepes, pH 7.5, 100 mM KCl, 2.5 mM EDTA, pH 8, 5% glycerol, 0.5% (vol/vol) Triton X-100, 1 mM DTT, complete protease inhibitors (Roche)] and centrifuged at maximum speed for 10 min, and the supernatant was precleared with protein-G agarose beads (Sigma) for 20 min. The extract was then incubated with anti-HA (1:1,000; Sigma) for 2 h at 4 °C before the addition of 30 μL of protein G agarose beads (1:1 slurry) for 1 h. The beads were precipitated by centrifugation at 2,000 × *g* and then washed three times with 1 mL of extraction buffer and once with 1 mL of 20 mM Hepes, pH 7.5.

For electrophoresis, proteins were detached from the beads by the addition of NuPAGE LDS sample buffer (Invitrogen) and heating at 70 °C for 10 min and analyzed by SDS/PAGE on 4–12% (wt/vol) NuPAGE Novex Bis-Tris Gels (Invitrogen).

Protein Identification by Mass Spectrometry. After the separation of proteins by SDS/PAGE, Coomassie-stained protein bands were excised and digested in-gel (34). Briefly, gel pieces were destained, and the proteins were reduced with DTT, alkylated with iodoacetamide, and digested with porcine trypsin (modified sequencing grade; Promega) overnight at 37 °C. The supernatants were then transferred to other tubes, and residual tryptic peptides were extracted upon incubation of gel spots with 25 mM NH_4HCO_3 at 37 °C for 15 min, followed by shrinking of gel pieces with acetonitrile and incubation with 5% (vol/vol) formic acid at 37 °C for 15 min, followed by shrinking with acetonitrile. The extracts were combined with the primary supernatant and dried in a SpeedVac centrifuge (Savant Instruments Inc.). Protein digests were then resuspended in 0.1% (vol/vol) trifluoroacetic acid and 5% (vol/vol) acetonitrile and analyzed by liquid chromatography–mass spectrometry (LC-MS)/MS. LC-MS/MS analyses were performed on a Micromass CapLC unit (Waters) interfaced to a Micromass Q-Tof Micro mass spectrometer (Waters) equipped with a nanospray source. Tryptic digests were loaded at a flow rate of 20 μL/min onto an Atlantis dC18 Trap Column. After valve switching, the sample was separated on a Symmetry C_{18} column (150 × 0.075 mm, 3.5-μm particle size) (Waters) at a flow rate of 3.8 μL/min using a gradient from 5 to 15% (vol/vol) B in 3 min and from 15 to 50% (vol/vol) B in 22 min [solvent A: 95% (vol/vol) H_2O, 5% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid; solvent B: 5% (vol/vol) H_2O, 95% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid]. Instrument control and data acquisition and processing were achieved with MassLynx V4.1 software (Waters). MS/MS data were analyzed by MASCOT software (Matrix Science; www.matrixscience.com/) against the *Drosophila* sequences of the Swiss-Prot database (release 2011_03). The following parameters were used in the MASCOT search: trypsin specificity; maximum number of missed cleavages—3; fixed modification—carbamidomethyl (Cys); variable modifications—oxidation (Met); peptide mass tolerance—± 0.5 Da; fragment mass tolerance—± 0.5 Da; protein mass—unrestricted; mass values— monoisotopic.

Western Blot. Following transfer onto nitrocellulose filters, proteins were analyzed by Western blotting using the following antibodies: rabbit polyclonal anti-INAD (4; 1:500), rabbit polyclonal anti-NINACp174 (29; 1:500), mouse anti-HA (Sigma; 1:5,000), and mouse anti-FLAG (1:5,000). For quantification of the immunodetected signals, each film was analyzed with Image J software (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health). Relative abundance of NINAC and INAD were defined as a ratio with HA-tagged form of dCRY (HACRY) (NINAC/HACRY and INAD/HACRY, respectively).

Yeast Two-Hybrid Assays. All of the experiments were performed in the EGY48 yeast strain (MATα, *ura3*, *trp1*, *his3*, 3*Lex*A-operator-*LEU*), and dCRY, either full length or in fragments, was fused to the LexA moiety in the bait vector (pEG202); INAD (full length or in fragments) was fused to the "acid-blob" portion of the prey vector (pJG4-5) (35). The full-length INAD-coding sequence was amplified from cDNA extracted from heads of w^{1118} flies with primers INAD-FL-F (Table S1), which add NdeI-EcoRI-AatII restriction sites, and INAD-FL-R (Table S1), which add XbaI, XhoI, and HinIII restriction sites, by using the Phusion High-Fidelity DNA Polymerase (New England Biolabs). The PCR product was digested with EcoRI and XhoI and directionally cloned in the pJG4-5 vector. All of the constructs with the different INAD fragments were obtained with the same strategy—by using pJG-INAD full length as template. The primers used are listed in Table S1; the reverse primers incorporate a TAG stop codon before the XbaI restriction site. All of the constructs were fully sequenced to assess the in-frame insertion of the cDNA and to control for unwanted mutations. The reliable expression of prey fusions in the EGY48 yeast strain (MATα, *ura3*, *trp1*, *his3*, 3*Lex*A-operator-*LEU*) transformed with the bait vector and *LacZ* reporter plasmid pSH18-34 was confirmed by immunoblot. Protein extracts were obtained as in ref. 36, subjected to SDS/PAGE (NuPAGE-Invitrogen), and probed with a specific anti-HA antibody (Sigma; 1:5,000). Expected molecular weights for the tested fusions are listed in Table S3. Quantification of β-galactosidase activity was performed in liquid culture as in ref. 36, and each experiment was repeated at least three times.

Yeast Three-Hybrid Assay. In this experiment, dCRY full-length was used as bait and NINACp174 as prey. The coding sequence of NINACp174 was amplified from cDNA extracted from heads of w^{1118} flies with primers NINAC-5F and NINAC-PBR (Table S1) that add a SalI site at both ends. The PCR product was digested with SalI and cloned in the pJG4-5 vector linearized with XhoI. Clones with the insert in the right orientation were fully sequenced to assess the in-frame insertion of the cDNA and to control for unwanted mutations. The expression of NINAC was assessed by Western blot with the anti-HA antibody. The expression of INAD in the yeast nucleus was achieved by cloning the full-length cDNA in pLEU, a modified version of the pDBLeu vector (Invitrogen), where the DNA-binding domain was removed by restriction with HindIII and SalI. The coding sequence of INAD was amplified with primers inaD-NLS-FLAG_F that add an HindIII site at the 5′ end, in-frame with sequences for a nuclear localization signal and a FLAG tag, and inaD-Xho-R that adds an XhoI site at the 3′ end. The PCR fragment was digested with HindIII and XhoI and directionally cloned in pLEU HindIII-SalI. Positive clones were sequenced to check for unwanted mutations. The expression of the nuclear form of INAD was assessed by Western blot on protein extracts with a specific anti-FLAG antibody (Sigma; 1:500). β-Galactosidase activity was quantified as previously described.

Visual Sensitivity (Electroretinograms). ***Preparation and Recording.*** Male flies at the age of 6–8 d, between ZT6 and ZT24, were slightly anesthetized with carbon dioxide and fixed with their ventral side to a small acrylic glass plate using dental wax (ESPE

Protemp II). Legs, wings, proboscis, and heads were also fixed to the plate without impairing the respiratory movement of the fly. The preparation was then transferred to the stage of a stereo microscope. A chloridized silver wire ($D = 0.38$ mm) that served as the reference electrode was inserted into the thorax of the fly. The recording electrode, a glass microelectrode pulled from borosilicate capillaries (i.d. = 0.58 mm, OD = 1.00 mm, $L = 80$ mm with filament) with a DMZ puller (Zeitz Instruments) and filled with Insect Ringer, was placed on the surface of the compound eye. The stereo microscope as well as the reference and recording electrode were placed in a Faraday cage to reduce the background noise. Voltage signals from the electrodes were preamplified with a Neuroprobe Amplifier Model 1600 (A-M Systems) and further amplified with a differential amplifier (custom-made). Both amplifiers were operated in DC mode with 10× and 50× gain, respectively. The amplified signals were displayed on an analog oscilloscope (HAMEG Instruments) via a DS1M12 Pocketscope (Meilhaus Electronic) on the PC using the data-logging software EasyLogger (Meilhaus Electronic). A halogen lamp (Spindler & Hoyer) was used for the generation of white light. The light beam passed through a KG heat filter (Schott), an electronic shutter (Melles Griot), and a plano-convex lens. The lens focused the light beam on a quartz glass fiber (LOT-Oriel) that transferred the light to the fly. Neutral density filters (Schott) were used to attenuate the light intensity. Light intensity was measured at the position of the fly with the QE6500 spectrometer (Ocean Optics). The maximum light intensity was 9.75×10^{14} photons·cm^{-2}·s^{-1}. Before the start of each experiment, flies were dark-adapted for 15 min. Light stimuli of 400-ms duration and different intensities were applied with an interstimulus interval of 20 s to keep the flies in a reasonably dark-adapted state. Experiments were run starting with the lowest light intensity to minimize adaptation effects.

Analysis. The receptor-potential amplitudes of the electroretinogram (ERG) responses to nine different intensities were plotted as a function of the related light intensity for ZT6, ZT12, ZT18, and ZT24 to yield the irradiance response curves. Each curve was obtained from $n = 9$–13 flies. Afterward, the photon flux needed to elicit a criterion response of 6 mV was determined for each ZT. Finally, the reciprocal of the photon flux was normalized to the according mean value at ZT6 and plotted as a function of the related ZT to yield the circadian fluctuations in ERG sensitivity.

Optomotor Activity Test. ***Setup 1.*** The walking optomotor test was performed as in ref. 37. Specifically, 3- to 8-d-old flies (entrained in a 12:12 light:dark cycle) were placed in a T-shaped tube with the longer arm painted black, located in the center of an arena inside a rotating drum, and tested between ZT1 and ZT4 (Fig. S4*A*) or ZT6 and ZT18 (Fig. 4*C*) . The internal walls of the drum were painted with alternating black and white stripes, and the apparatus was illuminated from above with a white light (2,000 lx). Attracted by the light, tested flies exited the darkened arm of the T tube and were then exposed to the black-and-white rotating drum. Normal flies tend to move in the same direction as the rotating environment. The test was repeated 10 times for each fly: 5 times with clockwise and 5 times with counterclockwise rotations randomly distributed. Each fly was thus scored for the number of correct turns taken in the 10 trials.

Setup 2. Five- to six-day-old flies were starved for 3 h before the experiments to increase the general activity level. Between ZT11 and ZT12 (Fig. S4*B*), when flies are usually active, or at ZT6 and ZT18 (Fig. 4*B*), single flies were put into a walking chamber (circular arena: Ø 3 cm; height: 0.15 cm) in the center of a transparent Plexiglas cylinder (Ø 3.4 cm; height: 1.5 cm), which was placed in the middle of an upright cylinder (Ø 8 cm; height: 4.5 cm). The walls of the outer cylinder were covered with six equally spaced vertical black stripes (width: 30°). Hence, the outer cylinder constitutes a striped drum with a pattern wavelength of $\lambda = 60°$, which was rotated around the arena with an angular velocity of $\omega = 60°/s$ (10 revolutions per minute). Accordingly, the effective optomotor stimulus was given by a contrast frequency of $\omega/\lambda = 1$ Hz. The illumination was provided by a ring of white light-emitting diodes (LEDs) surrounding the striped drum (Ø = 19 cm; $n = 15$ LEDs; light intensity in the center of the striped drum = 23 μW/cm^2). Before the experiment, the flies were dark-adapted for 10 min. For recording of the optomotor response (OR), the cylinder was rotated clockwise (cw) for 5 min and then counterclockwise (ccw) for another 5 min. Between cw and ccw rotation, a darkness period of 5-s duration was inserted. The optomotor response was calculated as $OR = (rev_{cw} + rev_{ccw})/(n_{cw}+n_{ccw}) \times 100\%$, where rev_{cw} indicates the observed number of cw revolutions of the fly during the first 5 min, rev_{ccw} the observed number of the fly's ccw revolutions during the second 5 min, n_{cw} and n_{ccw} are the numbers of revolutions of the striped drum during the cw and ccw periods, respectively. Please note that in this paradigm a result of "OR = 0%" would indicate "no optomotor response" (completely motion-blind flies), whereas, in contrast, a result of "0%" in setup 1 would indicate "100% wrong choice," which would be interpreted as a 100% negative optomotor response. For each genotype, 32 flies were tested at ZT 11–12.

Phototaxis. The light source for the experiment was a fluorescent lamp (intensity of the light at the apparatus: ~3,000 lx). The experiment consisted of five cycles, whereby the flies were able to run from one tube into another for 15 s. At the end, the flies were distributed within six tubes. By counting the number of flies in the different tubes, a performance index was calculated with 0 meaning "no fly showed phototaxis" and 1 meaning "all flies showed phototaxis five times." For each genotype, about 400 flies were tested.

Statistical Analysis. All of the results were expressed as means ± SEM. Data were tested for normal distribution using the Kolmogorov–Smirnov test and further compared by ANOVA, unpaired Student *t* test, or Mann–Whitney *U* test to determine significant differences (SYSTAT 11). P values <0.05 were considered statistically significant. Bonferroni's Multiple Comparison test was applied for post hoc comparison.

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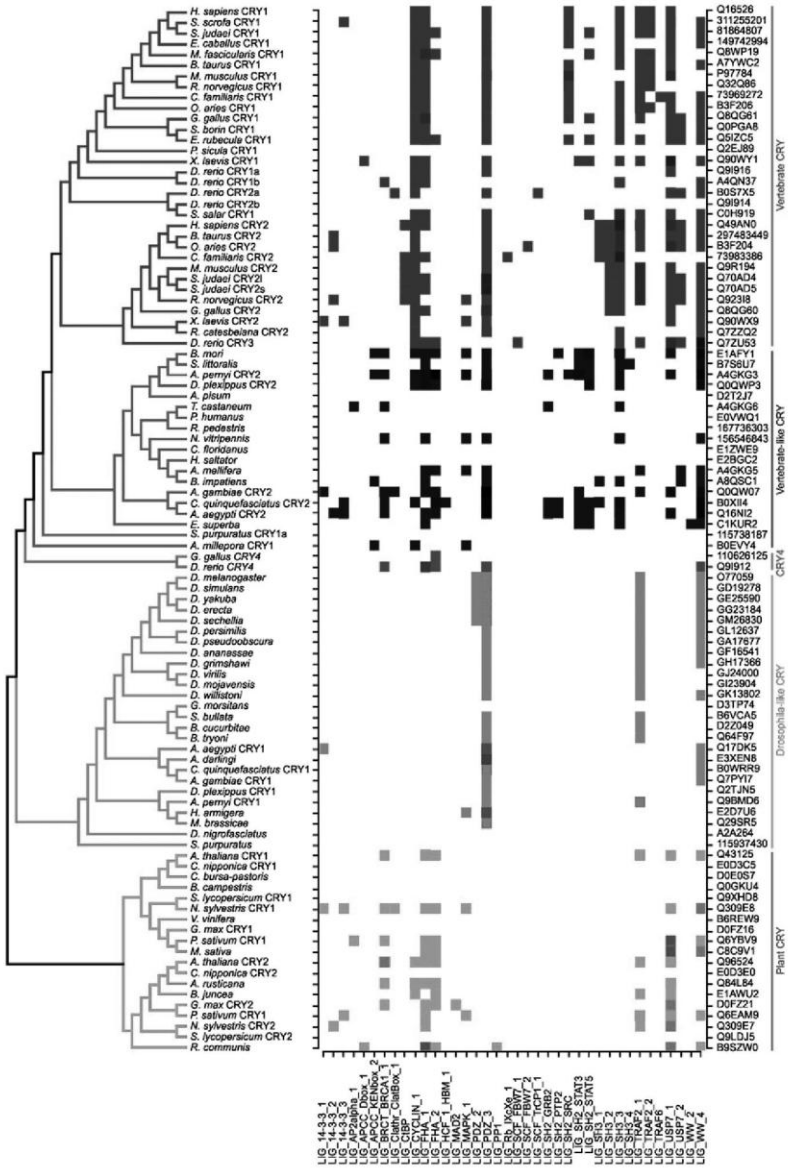

Fig. S1. Distribution of functional motifs in CRY across species. The neighbor-joining phylogenetic tree of 98 known CRY and CRY-like proteins is shown (*Left*) for the N-terminal photolyase-like domain and related to the presence of several functional motifs (identified in the highly variable C terminus) through colored squares (*Center*). The functional motifs are taken from ELM, limited to true binding motifs, and listed with their names on the bottom row. UniProt sequence accession numbers are shown on the *Right* with the high-level taxonomic grouping of the sequences. The latter is also used to color both the phylogenetic tree and the *Center* boxes. Note that darker box colors correspond to more motifs of the same type found in the sequence. The presence of long vertical stripes indicates the evolutionary conservation of a particular functional motif, with the class III PDZ-binding motif corresponding to the longest of such stripes. This is of particular relevance, given the potentially high error rate of single-motif instances.

A

Gel band	Acc. No.	MM (Da)	Protein name	Sequences of the tryptic peptides
RDGA (~ 115 kDa)[a]	Q09103-3	115,579 (Isoform B)[b]	Retinal DeGeneration A protein (RDGA)	GDVQVNPLEK FEESISYPK QVFDLTQGGPK ALGWGGGYTDEPIGK NANEIATYLESQER AFIVKPIPSPEVIPVIVFINPK
NINAC (~ 180 kDa)[a]	P10676	174,300 (Isoform B, long form) 131,836 (Isoform A, short form)	Neither Inactivation Nor Afterpotential protein C (NINAC)	ALGVLDTVIAR[c]

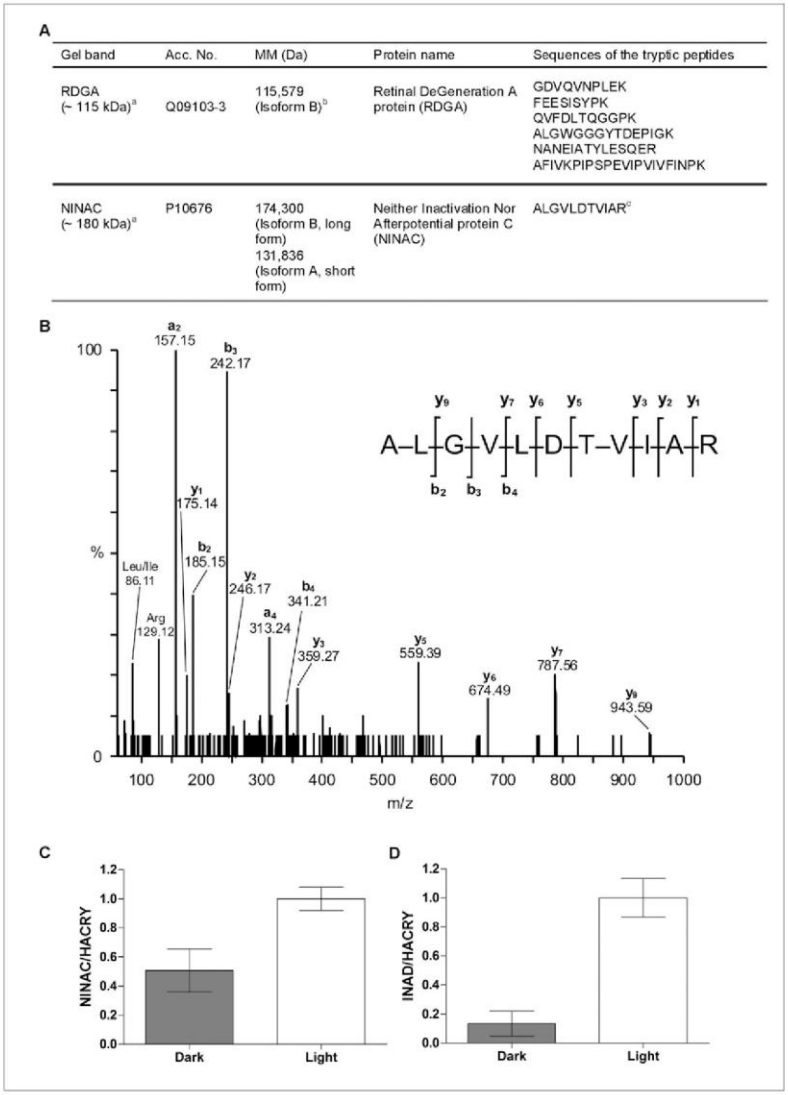

Fig. S2. Proteins identified by LC-MS/MS and quantification of dCRY in the phototransduction complex. (*A*) [a]For the electrophoretic bands corresponding to RDGA and NINAC (Fig. 1), the identified proteins with their accession numbers and molecular masses (MM) are listed. Protein identification was performed with the MASCOT software searching LC-MS/MS data against the sequences of *Drosophila* in the Swiss-Prot database. The fifth column contains a list of the peptides that were sequenced by MS/MS. [b]The peptide sequence NANEIATYLESQER is present only in isoform B of RDGA. [c]In the case of the band corresponding to NINAC, only one peptide sequenced by LC-MS/MS matched the protein with a significant score. The MS/MS spectrum of this peptide is reported in *B*. (*B*) MS/MS spectrum of the doubly charged ion at 564.39 *m/z*. A database search using MASCOT associated this MS/MS spectrum with a significant score to the tryptic peptide ALGVLDTVIAR of the protein NINAC (*Drosophila melanogaster*). In the mass spectrum, the **y**, **b**, and **a** ions are indicated. The corresponding localization of the product fragments **y** and **b** in the sequence of the peptide is also shown. (*C* and *D*) The interaction of NINAC (*C*) and INAD (*D*) for dCRY at ZT24 (dark) and after 15 min of light was quantified as the ratio NINAC/HACRY and INAD/HACRY, respectively. Mean levels normalized to values obtained after 15 min of light ± SEM of three replicates are shown. The differences between NINACp174/HACRY and INAD/HACRY ratios under light and dark conditions were significant ($P < 0.03$ and $P < 0.02$, respectively, Mann–Whitney *U* test).

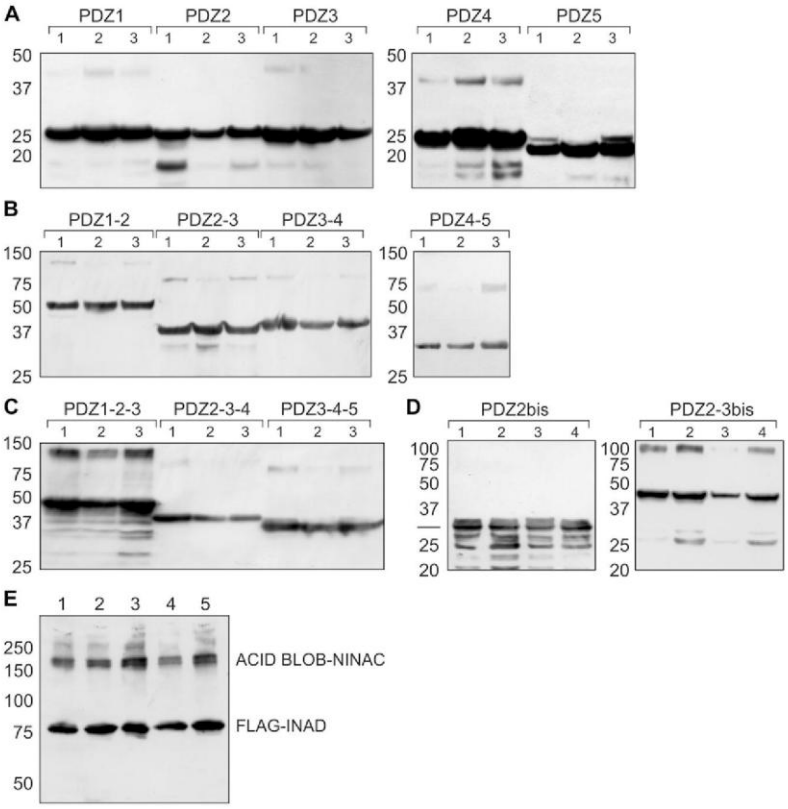

Fig. S3. Western blot analysis of independent yeast clones for prey fusions expressing different combinations of PDZ domains or NINAC as prey fusion and INAD in the nucleus. Three or four independent yeast clones for each prey fusion were probed with anti-HA antibody (Sigma; 1:5,000). The signals corresponding to the fusions are shown. See Table S3 for expected molecular masses. Images originating from different films have been reported in separate panels. (*A*) Single PDZs. The ~20- to 25-kDa signals indicate that all of the fusions are expressed in yeast cells and the absence of interaction cannot be explained by the absence of expression. A band of molecular mass compatible with a dimer is visible in the PDZ1, PDZ3, and PDZ4 lanes. (*B*) Tandem PDZs. (*C*) Three PDZs. All of the fusions are correctly expressed in yeast cells, and traces of dimerization are visible in all of the combinations. (*D*) Extended version of PDZ2 and PDZ2–3 tandem, including the CaM motif upstream from the canonical PDZ2 boundary. (*E*) Independent yeast clones expressing dCRY as bait, NINAC as prey (AcidBlob-NinaC) and a FLAG-tagged form of INAD specifically in the yeast nucleus were probed with anti-HA and anti-FLAG antibodies. The signals corresponding to the expressed fusions are shown.

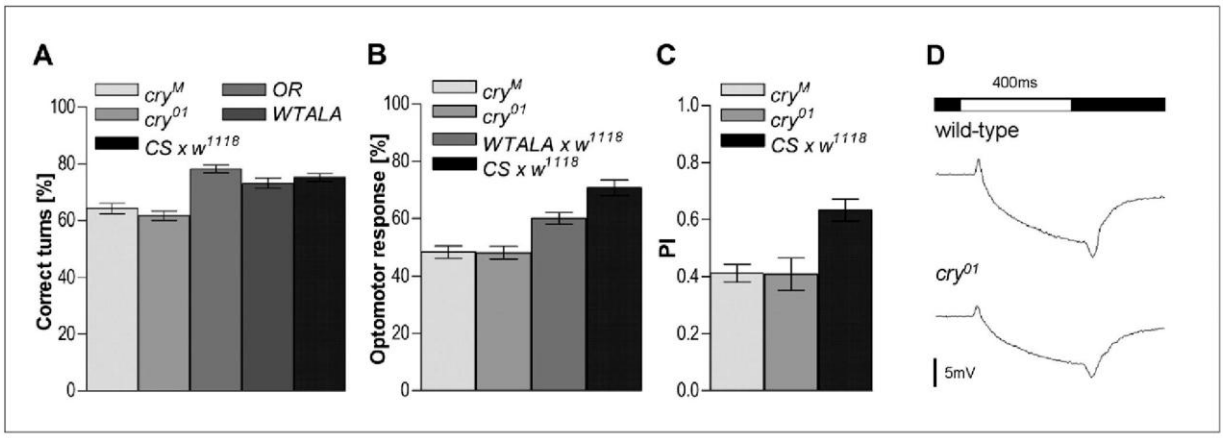

Fig. S4. Visual behavior of dCRY mutants. (*A*) Percentage of correct choices with respect to the direction of the stripe motion (optomotor stimulus) inside a T-shaped tube (setup 1, *SI Materials and Methods*). Male cry^{M}, cry^{01}, Oregon R, WT-Alto Adige (ALA), and CS × w^{1118} flies (100 for each genotype) were analyzed between ZT1 and ZT4. cry^{01} and cry^{M} exhibited 61.6 and 64.2% of correct turns, respectively, whereas wild-type controls (Oregon R, WT-ALA, and the progeny of a CS × w^{1118} cross) achieved 78.2, 73.2, and 75.2%, respectively. Mean values ± SEM are given. Both cry^{01} and cry^{M} displayed an impairment in their optomotor turning response with respect to controls ($F_{4,495}$ = 19.53, $P < 0.0001$). No difference was found between the two *cry* mutants ($P > 0.05$). (*B*) Optomotor response for cry^{M}, cry^{01}, WT-ALA × w^{1118}, and CS × w^{1118} males, with setup 2 (*SI Material and Methods*). The mean optomotor response of single flies placed in a Plexiglas arena is expressed as the percentage of fly revolutions with respect to the number of revolutions of the optomotor stimulus (striped drum). Both cry^{01} and cry^{M} mutants showed an optomotor response (OR) of about 48% whereas control flies (the progeny of CS × w^{1118} and WT-ALA × w^{1118} crosses) showed an OR of about 71 and 60%, respectively. Thirty-two flies for each genotype were analyzed between ZT11 and ZT12. Mean values ± SEM are given. As in setup 1, both *cry* mutant flies showed a reduced optomotor response with respect to the wild-type flies ($F_{3,124}$ = 22.35, $P < 0.0001$). Whereas the 50% level in *A* denotes random choice behavior (no optomotor response to the moving stripes), the same value of 50% in *B* would indicate that the fly completed 50% of the revolutions imposed by the rotating striped drum. Therefore, the mutants' OR levels in *B* correspond to about 68–80% of the WT OR. Both optomotor experiments reveal a significant OR reduction in cry^{01} and cry^{M}. (*C*) Phototaxis response for cry^{M}, cry^{01}, and CS × w^{1118}. The performance index (PI) is expressed as the number of times that flies show phototaxis in a five-cycle test, with 0 meaning "no fly showed phototaxis" and 1 meaning "all flies showed phototaxis five times." About 400 flies for each genotype were tested between ZT11 and ZT12. Both cry^{01} and cry^{M} flies showed a significant reduction in the phototactic response with respect to the wild-type flies ($F_{2,26}$ = 8.2, P = 0.002). (*D*) Electroretinograms of wild-type flies and cry^{01} mutants. cry^{01} mutants exhibit normal, wild-type-like ERG responses upon illumination with white light. The electroretinograms were recorded at ZT18 with white light pulses of 400-ms duration and an intensity (*I*) of 9.75 × 10^{14} photons·cm^{-2}·s^{-1}.

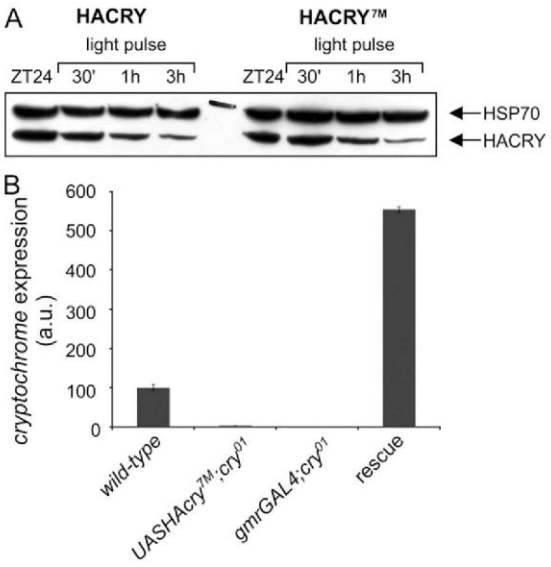

Fig. S5. Molecular characterization of $HAcry^{7M}$ strain. We had previously obtained a *UAS-HAcry*^{7M}, an HA-tagged variant of d*cry* in which a tyrosine (amino acid 497 in the chimeric protein) was substituted with alanine. (*A*) Western blot analysis, performed with anti-HA antibody, showing that the Y497A substitution does not influence the temporal light degradation profile of cryptochrome. *UAS-HAcry*^{7M} flies were crossed with flies carrying the *timGAL4* driver, and the progeny was entrained for 3 d under standard light–dark conditions. Individuals were then collected at ZT24 and after 30 min, 1 h, and 3 h of light exposure. As control, the progeny resulting from *UAS-HAcry* crossed with *timGAL4* were used. The housekeeping protein HSP70 was used as loading control. (*B*) Analysis of cryptochrome expression by quantitative RT-PCR. The expression levels of *cry* were analyzed in the following lines used in rescue experiments: wild type (w^{1118}), *UAS-HAcry*^{7M}*;cry01*, *gmrGAL4;cry01*, and rescue (progeny of the cross *UASHAcry*^{7M}*;cry01* × *gmrGAL4;cry01*).

Table S1. Nucleotide sequences and position of primers used

Primer	Length (bp)	Position	Direction	Sequence (5′→3′)
InaD_FL_F	36	1–18	F	CATATGGAATTCGACGTCATGGTTCAGTTCCTGGGC
InaD_FL_R	36	2007–2025	R	TCTAGACTCGAGAAGCTTCTAGGCCTTGGGTGCCTC
InaD_PDZ1_F	36	48–66	F	CATATGGAATTCGACGTCATGGTGACCCTGGACAAG
InaD_PDZ1_R	42	297–318	R	TCTAGACTCGAGAAGCTTCTAGTCGAAGGTCTGAATCTCCAG
InaD_PDZ2_F	36	744–762	F	CATATGGAATTCGACGTCAGGATCGAGGTCCAGAGG
InaD_PDZ2bis_F	42	559–582	F	CATATGGAATTCGACGTCGACGAGGACACCCGGGACATGACC
InaD_PDZ2_R	39	978–996	R	TCTAGACTCGAGAAGCTTCTAGCGTCGCGAGGTGATCAT
InaD_PDZ3_F	36	1080–1098	F	CATATGGAATTCGACGTCTTTCCCAAGGCGCGCACG
InaD_PDZ3_R	45	1311–1335	R	TCTAGACTCGAGAAGCTTCTACAATAGAATCATGGTCACTACGCC
InaD_PDZ4_F	42	1464–1488	F	CATATGGAATTCGACGTCCTCATTGAGTTGAAGGTGGAAAAG
InaD_PDZ4_R	39	1713–1731	R	TCTAGACTCGAGAAGCTTCTAAGGATCAGCGCGGAAGAC
InaD_PDZ5_F	41	1749–1772	F	CATATGGAATTCGACGTCAACGTTGACCTTATGAAAAAAGC
InaD_PDZ5_R	40	1973–1992	R	TCTAGACTCGAGAAGCTTCTACTTGGGTCGTGTCACTTCC
CRYdeltaF	29	1561–1581	F	CCGAATTCCCGCATTGCCGACCATCCAAC
CRYR	33	1604–1629	R	CCCTCGAGTCAAACCACCACGTCGGCCAGCCAG
InaDNLSFLAG_F	112	1–31	F	CCAAGCTTGAATTCATGGATTACAAGGATGACGACGATAAGG GTGCTCCTCCAAAAAAGAAGAGAAAGGTAGCTGGTATCAA TAAAGTTCAGTTCCTGGGCAAACAGGGCACCG
InaDXhoR	31	2006–2025	R	GGTCGACTCGAGCTAGGCCTTGGGTGCCTCC
Nina5F-Sal	32	1–24	F	CCGTCGACATGATGTATTTACCGTACGCGCAA
NinaPB3-Sal	31	4484–4506	R	GGGTCGACTTAGATATCGACGGCATAGCCTG

Position reflects nucleotide location in FlyBase: Fbgn0001263 (INAD), Fbpp0079064 (NINAC), and Fbpp0083150 (dCRY).

Table S2. Expected molecular masses of fusions used in yeast experiments

Fusion (amino acids)	Short name	MM (kDa)
A-inaD (17–106)	PDZ1	21.7
A-inaD (249–332)	PDZ2	20.8
A-inaD (364–448)	PDZ3	20.9
A-inaD (489–577)	PDZ4	21.5
A-inaD (584–664)	PDZ5	20.6
A-inaD (17–332)	PDZ1–2	46.9
A-inaD (249–448)	PDZ2–3	33.1
A-inaD (364–577)	PDZ3–4	35.6
A-inaD (489–664)	PDZ4–5	31.2
A-inaD (207–332)	PDZ2bis	28.2
A-inaD (207–448)	PDZ2-3bis	40.5
A-inaD (17–448)	PDZ1–3	59.2
A-inaD (249–577)	PDZ2–4	47.8
A-inaD (364–664)	PDZ3–5	45.1
A-inaD (1–674)	INAD	86.1
A-NinaC	NINAC	186.1
Nuclear FLAG-inaD (1–674)	NFLAG-INAD	82.1

A, acid blob (prey fusion); MM, molecular mass.

6.6. Cryptochrome plays different roles in the fruit fly´s compound eyes and clock neurons

Cryptochrome plays different roles in the fruit fly's compound eyes and clock neurons

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Key words: phototransduction, f-actin, moonlight, activity rhythms

Classification: BIOLOGICAL SCIENCES, Neuroscience

Author contributions: CHF and RC conceived and supervised the study. MS performed and analyzed the behavioral experiments, RG performed and analyzed the ERG experiments, RG and MS did the CRY-immunostaining in the compound eyes, AF determined histamine in the flies head by mass spectrometry, PM contributed to the behavioral experiments and immunohistochemistry, MM and GMM performed the co-immunoprecipitation assays with CRY followed by mass spectrometry as well as the yeast two-hybrid experiments with CRY and actin. CHF, GMM, MS, RG and RC wrote the manuscript. The authors declare no competing financial interests. Correspondence should be addressed to CHF.

Significance Statement

Cryptochromes (CRYs) are a class of flavoproteins which sense blue light. In animals, CRYs are expressed in the eyes and in the clock neurons that control sleep/wake cycles, and are implied in the generation and/or entrainment of circadian rhythmicity. Moreover, CRYs are sensing magnetic fields in insects as well as in humans. Here we show that in the fruit fly *Drosophila melanogaster*, CRY increases light sensitivity of the eyes enhancing the dim-light-driven shift of evening activity into moonlit nights. In contrast, CRY expression in neurons controlling evening activity (E clock neurons) advances locomotor activity. These opposite effects of CRY may be important for a balanced control of activity patterns, likely not only in flies.

Cryptochrome (CRY) is expressed in approximately half of the circadian clock neurons in fruit flies and plays a crucial role in entraining the clock to light-dark cycles. However, CRY is also present in the *Drosophila* compound eyes and has been recently shown to be involved in the visual transduction cascade and to influence fly vision. Besides their role in image formation, the compound eyes contribute to circadian clock entrainment and are especially important for sensing daylight intensity and low nocturnal light, as moonlight, thus modulating fly nocturnal behavior. Here we aimed to decipher the role of CRY in the compound eyes and in the circadian clock neurons in sensing daylight intensity and moonlight. We found that CRY in photoreceptor cells 1-6 of the compound eyes enhances fly sensitivity to daylight and moonlight, possibly by retaining the components of the phototransduction cascade in the rhabdomeres via interaction with F-actin and INAD scaffolding protein. In the evening clock neurons, CRY has opposite effects on the behavioral responses to moonlight: it reduces nocturnal activity by retaining evening activity in the day. These contrasting effects of CRY may be essential for activity pattern fine tuning.

Nearly all living organisms use daily patterns of day and night to entrain their endogenous circadian clocks. These responses utilize photic input from both visual photoreceptors and non-visual photopigments (reviewed in (1, 2)). Cryptochromes (from the Greek κρυπτό χρώμα*, hidden colour*) are a class of flavoproteins, non-visual photopigments present in plants and animals, which sense blue light. CRYs are involved in the generation and/or synchronization of circadian rhythms of plants and animals, in developmental processes in plants and in the sensing of magnetic fields in a number of species (3-5). The two principal types of CRYs are the light-sensitive plant/insect type 1 CRY and the mammalian type 2 CRY; the latter is a component of the molecular circadian clockwork and retains light responsiveness only under special conditions (4, 6).

The fruit fly *Drosophila melanogaster* possesses a single form of type 1 CRY, which appears to have different functions: in *Drosophila* circadian clock neurons, CRY acts as

circadian photopigment (7-9); upon light-activation, it interacts with the clock protein Timeless (TIM) and provokes its degradation via the proteasomal pathway, therefore resetting the molecular clock (10, 11). In peripheral tissues, including the compound eyes, CRY appears to be an integral component of the molecular clock (12, 13). In the compound eyes and in a clock neuron subgroup, CRY is additionally associated with the cytoplasmic membrane and appears to interfere with the phototransduction cascade (14) or with light-induced membrane depolarization (15).

The function of *Drosophila* CRY associated with the cytoplasmic membrane is so far not well understood. In its C terminus, CRY carries several protein-protein interaction motifs, including two class III PDZ-binding motifs that play a role in the assembly of large protein complexes involved in signaling processes (PDZ = **P**ostsynaptic density protein 95, **D**rosophila disk large tumor suppressor, **Z**onula occludens-1 protein) (14). In the photoreceptor cells of the compound eyes, CRY interacts with its PDZ domains in a light-dependent manner with the scaffolding protein INAD (**I**nactivation **N**o **A**fterpotential **D**) which seems, in turn, to enable interaction between CRY and other phototransduction components (14). INAD is important to fix the components of the phototransduction cascade to the membrane of the rhabdomers and it is further bound to F-actin filaments via myosin III (NINAC). Especially in the dark, INAD binds via its PDZ-domains 4/5 to TRP-channels and keeps them in the rhabdomeres – ready for activation, whereas after light-adaptation TRP channels move into the cell body (16). Most interestingly, CRY appears to enhance photosensitivity mainly during the night (14). Perhaps CRY enhances the interaction between INAD, NINAC and F-actin during the night hence enhancing the activation of TRP channels. However, this hypothesis limps, because the CRY-INAD interaction has only been found after light exposure and it has not yet been demonstrated that CRY is present in the rhabdomeres. Furthermore, if CRY is indeed involved in photoreception, one should also see differences in fly daily activity patterns when CRY is missing in the compound eyes. The compound eyes have been shown to fine-tune daily activity according to fluctuations in environmental light (17, 18). In particular, they seem responsible for setting the ratio of diurnal/nocturnal activity. Flies generally prefer being active at low light

intensities and consequently reduce diurnal activity with increasing daylight intensity (19). Similarly, under simulated moonlit nights in the lab flies shift activity into the night (20). Both responses are mediated by the compound eyes with a special importance of photoreceptor cells 1-6 (17, 18).

Here we show that CRY is present in the rhabdomeres of all photoreceptor cells, that it interacts with F-actin, in addition to INAD, and may therefore enhance the binding of the phototransduction cascade signaling components to the rhabdomere cytoskeleton. In contrast to the CRY/INAD interaction, the CRY/F-actin interaction is light-independent, possibly retaining the signaling components close to the membrane and ready for activation during day and night. Indeed, electroretinogram (ERG)-recordings show that CRY facilitates dark-adaptation in the photoreceptor cells after light-exposure. Furthermore, CRY in the rhabdomeres is not degraded by light, thus permitting the interaction with the signaling components even during long lasting light-exposure. Flies lacking CRY shift less activity from the day into the night in response to increasing day-light intensities, suggesting that the compound eyes of such flies are less light-sensitive. The wild-type behavior is fully rescued by expressing CRY in photoreceptor cells R1-6. CRY also plays a prominent role in fly behavioral responses to moonlight, but, unexpectedly, CRY in the compound eyes and in the E clock neurons have antagonistic effects on fly nocturnal activity, which may explain why the role of CRY for detecting moonlight passed unnoticed in our previous study (20). We propose a model for CRY action in the eyes and the clock neurons that has to be tested in the future.

Results and Discussion

CRY interacts with F-actin

F-actin, one of the major cytoskeletal components, is highly expressed in the rhabdomeric microvilli of fly photoreceptors and helps maintaining their structure (21). In addition, F-actin seems to be involved in subcellular localization and functional coupling of the phototransduction components, putatively via interaction with the myosin III protein NINAC (see Fig. 1E; (22)). NINAC also interacts with the scaffolding PDZ-domain protein INAD (see Fig. 1E) and, thus, could contribute to movements of phototransduction components into or out of the rhabdomeres and hence interfering with photosensitivity and light-adaptation (reviewed in (16)); however, no such role of NINAC was found so far. Its main function seems to lie in the inactivation of Metarhodopsin by accelerating the binding of Arrestin (23). Here, we asked whether CRY, which was also found to interact with INAD (14), could cover this function, since the screening of ~ 76.000 clones of an adult head cDNA library (see Methods) led to the identification of Actin57B as putative CRY partner.

The physical interaction between dCRY and Actin57B was confirmed using a yeast two-hybrid system, in which a full-length CRY, directly fused in frame with the DNA binding domain of Gal4 (aa1-147; GAL4-DB_bait), was directly challenged with the full-length Actin57B as prey. A light-independent interaction between the two proteins was observed (Fig. 1A). In addition, a co-immunoprecipitation assay, followed by 2D electrophoresis and mass spectrometry analysis, was performed on transgenic fly heads overexpressing a hemagglutinin (HA)-tagged form of CRY (HACRY) in all clock cells (24). Flies were raised in 12:12 light:dark cycles and collected before lights-on. Two spots of ~40 kDa (X1 and X2 in Fig. 1B) were observed in the sample but absent in the negative control (Fig. 1B). These protein bands were digested in-gel and the peptide mixtures were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (25). Analysis of the LC-MS/MS data using the MASCOT software yielded the identification of Actin-87E and Actin-5C in spot X1 and of Actin-57B in spot X2 (Table S1). *Drosophila melanogaster* has six actin encoding genes, localized on

different chromosomes. Nevertheless, the encoded proteins show only subtle sequence differences (Fig. S1). It is currently unknown which forms of F-actin are present in the rhabdomeres, but since we show that CRY can interact with at least three of the six its interaction with F-actin in the rhabdomeres is very likely.

CRY is expressed in the rhabdomeres of the photoreceptor cells, remains stable after photoactivation and enhances the sensitivity of the compound eyes to light

Our data suggest that CRY is bound to F-actin during light and darkness and could consequently stabilize INAD also after prolonged illumination. This hypothesis requires CRY to remain stably present under light, which is in contrast to previous observations showing a quick degradation of CRY after light onset in clock neurons and S2 cells (11, 26-28). To test the presence of CRY in the rhabdomeres, we immunostained retinas of flies kept in complete darkness from egg hatching onward as well as retinas of flies initially raised under the same conditions but then exposed for 2h to bright light (1000lux). CRY immunostaining was not visible in cry^{01} mutants but present in all rhabdomeres of wild-type (WT) flies (Fig. 1C). No sign of CRY degradation could be detected 2h after light exposure (Fig. 1D). This suggests that CRY is stably bound to rhabdomeric F-actin (Fig. 1E), which may prevent its degradation in the proteasome. It is unknown whether the light-activated E3 ligase complex, essential for light-mediated CRY degradation, is present in the rhabdomeres, but it is known that the same E3 ligase complex components that induce CRY ubiquitination, such as the BRWD3 protein Ramshackle and the Cullin4-RING Finger E3 Ligase are associated with chromatin and the nucleus (27, 29, 30).

To test whether the putative bond between F-actin, CRY and INAD affects light-sensitivity of the photoreceptor cells, we performed ERG recordings on completely dark-adapted flies (raised in darkness) and flies that were exposed to 2h of light. After complete dark-adaptation, we found no difference in the ERG-amplitude between WT flies and cry^{01} mutants (Fig. 1F). The same was true 1 min after the 2-h light exposure. As expected, both fly

strains showed a significantly reduced response, indicating that *cry*^{01} mutants exhibit a WT-like light-adaptation (Fig. 1F). However, when flies were allowed to re-adapt to darkness 15 min before the ERG recordings, WT flies almost returned to their normal dark-adapted response, whereas *cry*^{01} mutants still remained significantly less sensitive to light (Fig. 1F). Our results indicate that rhabdomeric CRY does not prevent light-adaptation per se, but that it speeds up the dark-adapting process, perhaps by hindering the light-induced migration of the phototransduction components out of rhabdomeres, which accompanies the normal light-adaptation process (16). Such a reduced migration caused by CRY-binding would make the phototransduction components more readily available for the next round of light-signaling and the eyes generally more light-sensitive.

CRY in the compound eyes contributes to measuring daylight intensity and adapting fly diurnal/nocturnal activity levels

After having shown that CRY interferes with light-signaling in the compound eyes we wanted to test whether this has any biological meaning for the flies, in addition to the already shown small visual impairments of *cry*^{01} mutants (14). The fly circadian clock is known to be very sensitive to light, and, therefore, it is an ideal system to study possible influences of CRY (31, 32). CRY is one of the major light-input pathways to the clock neurons , but its role in the compound eyes for circadian entrainment is so far not understood. The compound eyes seem not important for fast clock responses to light (8, 9, 33, 34), but they rather appear to fine-tune fly daily activity to different light-conditions (17) and to set the diurnal/nocturnal activity level ratio (19, 20). We could recently show that photoreceptors R1-6 are responsible for measuring daylight intensity (18). If CRY in R1-6 contributes to this, one would expect different relative nocturnal activity levels in *cry*^{01} mutants. In order to test this, we recorded fly activity rhythms under 12:12 light-dark (LD) cycles of different daylight intensities (10, 100, 1000 and 10000 lux) and determined the percentage of nocturnal activity from whole-day activity (Fig. 2; S2). We found that the increase in relative nocturnal activity with increasing daylight intensity was

significantly stronger in WT flies than in *cry*^{01} mutants. To ensure that CRY in the compound eyes is responsible for the observed differences, we expressed CRY under control of the rhodopsin1 promotor (*ninaE*) only in R1-6 in an otherwise *cry*^{01} background. We found that such flies behaved in a WT-like manner (Fig. 2; S2), indicating that CRY in R1-6 is indeed involved in measuring daylight intensity, probably by interfering with phototransduction in these photoreceptor cells.

CRY in the compound eyes and in the E clock neurons has antagonistic effects on fly responses to moonlight

Next, we aimed to unravel the contribution of CRY to the amount of fly nocturnal activity in response to moonlight. In the lab, moonlight has two different effects on fly activity: it phase delays the clock in the E clock neurons and in this way shifts E activity into the night and it strongly stimulates fly activity during the night (20). Both moonlight effects are mediated by the compound eyes and seem independent of CRY in the clock neurons. However, the contribution of CRY in the compound eyes to the moonlight effects have not been evaluated in detail. Therefore, we recorded flies without CRY and with CRY only in photoreceptor cells R1-6 under light-moonlight (LM) cycles with 0.01 lux light in the night and 100 lux during the day. We found that flies with CRY in the compound eyes were significantly more nocturnal than the relevant *cry*^{01} controls (Fig. S3), strongly suggesting that CRY in R1-6 does not only contribute to measuring daylight intensity (see above), but that it also enhances moonlight sensitivity of the flies.

Surprising results emerged when we compared activity patterns of Canton S WT flies and of our newly cantonized *cry*^{01} mutants under LM cycles. Instead of shifting less activity into the night, *cry*^{01} mutants shifted even more activity into the night than the WT controls (Fig. 3A, B). The high nocturnal activity of *cry*^{01} mutants was caused by a strong delay of the E activity (Fig. 3C), which was not observable in the *cry*^{01} controls shown in Fig. S3. To find the cause for the strongly delayed E activity in CRY-less flies, we down-regulated CRY in the compound

eyes and in the four CRY-positive E clock neurons (Fig. S4), respectively. In both cases, CRY-down-regulation was successful (see Fig. S4B for the CRY-positive E neurons). CRY down-regulation in the E clock neurons strongly phase-delayed the E activity whereas it had no effect in the compound eyes (Fig. 3C). This result indicates that CRY in the E clock neurons normally keeps E activity in the day or may even phase-advance it, an effect that is completely opposite to the phase-delaying effect of the compound eyes. The phase-advancing effect of CRY can be explained by its interaction with TIM. At the beginning of the day, CRY leads to TIM degradation, which is followed by a decline in PER (35-37). E activity usually starts after complete TIM and PER disappearance (36), hence CRY is one of the factors determining E activity onset and keeping E activity in the day. A recent study indicates that CRY in the clock neurons can integrate photons over a long time eventually leading to TIM degradation and that this is especially true for one of the four E neurons targeted in our study (the 5^{th} LN_v, Fig. 4, Fig. S4; (32)). In eyeless flies, CRY in the E neurons is the major factor setting E activity and, consequently, E activity occurs rather early with its maximum clearly before lights-off (Fig. 4; (17, 18, 38)). In the absence of CRY, though, TIM and PER remain more stable and E activity onset is mainly controlled by signals coming from the compound eyes. Light-input from the compound eyes decelerates molecular oscillations in the E neurons and consequently delays E activity (39). Thus, cry^{01} mutants have a late E activity, which is clearly visible under long photoperiods (33, 38). Under the here applied 12-h days, the delaying effects of the compound eyes on E activity are only evident under moonlit nights.

The compound eyes need histamine signaling to phase delay E activity

An important open question remains: why didn't we observe the late E activity in the cry^{01} mutants shown in Fig. S3? The only obvious difference between the two cry^{01} mutants is the genetic background: the cry^{01} mutants used in Fig. S3 are in a white-mutant (w^{1118}) background (40), whereas the here cry^{01} mutants used in Fig. 3 are in a WT (CantonS) background. White is an ABC-transporter necessary for pigment precursors (ommochromes and pteridins)

transport into the pigment granules of the compound eye pigment cells (41). Therefore, its absence results in white eyes. However, White also appears to transport histamine – the compound eye neurotransmitter - from the synaptic cleft into glia cells (42). Histamine recycling via glia cells is essential for normal vision (43-45); weakened recycling in w^{1118} reduces histamine levels (42) and consequently reduces signaling from the photoreceptor cells to downstream interneurons in the optic lobe. Thus, white-mutant flies bear impaired synaptic transmission and should shift less activity into moonlit nights, despite their eyes being more light-sensitive due to the loss of red-eye pigments (46). This was proven true (Fig. 3D) and seems to apply even to transgenic w^{1118} flies that carry the *mini-white*$^{+}$ gene and have subsequently red eyes (Fig. 3B, C). The original cry^{01} mutants also carry the *mini-white*$^{+}$ gene, because they were generated from w^{1118} flies by homologous recombination, in which the entire coding sequence of the *cry*$^{+}$ allele was replaced by *mini-white*$^{+}$ (40). Thus, mutant ($w^{1118};;cry^{01}$) and control flies (w^{1118}) had the same genetic background except for the *cry* and the *mini-white*$^{+}$ gene.

In order to test whether *mini-white*$^{+}$ can rescue histamine levels, we measured the histamine content in the head of WT (CantonS) and cantonized cry^{01} mutants with that of w^{1118} flies and $w^{1118};;cry^{01}$ mutants. We found no difference in histamine levels between *mini-white*$^{+}$ flies and white-eyed w^{1118} flies (Fig. 3G). Histamine was reduced almost 2-fold in w^{1118} flies and $w^{1118};;cry^{01}$ mutants as compared to WT (CantonS) and cantonized cry^{01} mutants (Fig. 3G). Thus, *mini-white*$^{+}$ can obviously only rescue the eye-color, but not photoreceptor signaling via histamine, at least not when it is controlled by the *cry*-promoter.

This means that the light-signals from the eyes are reduced in w^{1118} flies (with or without *mini-white*$^{+}$) and consequently the phase-delaying effect of the eyes on E activity is rather weak. We found that nocturnal activity levels still increased upon moonlight in comparison to dark nights in w^{1118} flies (Fig. 3H), but that CRY in the E neurons could completely counterbalance the moonlight-induced delay in E activity (Fig. 3I). In $w^{1118};;cry^{01}$ mutants, E activity significantly delays, but this delay is minor compared to the delay in cry^{01} mutants with

intact histamine signaling (Fig. 3I). Flies with normal histamine strongly delay E activity upon moonlight, even in the presence of CRY (Fig. 3A,C, I). CRY in the E neurons can only partly counteract this compound-eye-induced delay by advancing E activity. As soon as CRY is absent, the compound eyes dominate and flies dramatically phase-delay E activity (Fig. 3A, C, I).

We wondered whether we could rescue the E activity delay in $w^{1118};;cry^{01}$ mutants by exposing the flies to a higher light intensity, because this would increase histamine signaling. Indeed, we found that at 10000 lux $w^{1118};;cry^{01}$ mutants delayed E activity to the same extent as cantonized cry^{01} mutants did at 100 lux (Fig. 3F, I).

Concluding remarks

Here we propose different roles for CRY in the compound eyes and in the E clock neurons (summarized in Fig. 4). In the compound eyes, CRY interacts with the phototransduction cascade and by doing so it slightly increases light-sensitivity of the eyes. Consequently, WT flies sense day-light and moonlight as being brighter than cry^{01} mutants do and shift more activity into the night. In contrast, light-activated CRY in the E clock neurons keeps E activity in the day. The phase-advancing effects of CRY in the E neurons are clearly stronger than the phase-delaying effects of CRY in the compound eyes. The delaying effects of the compound eyes on E activity are more dependent on intact histamine signaling than on CRY and, therefore, become most evident under moonlit nights in cantonized cry^{01} mutants.

In summary, we show that CRY is a versatile molecule that can play multiple roles. Most likely, this is also true for mammals. Although the light-sensitivity of mammalian CRYs has only been shown in a cell-based assay (47), they are expressed in the retina, especially in the ganglion cells responsible for circadian entrainment and pupillary responses (48). Nowadays, it is clear that melanopsin - not CRYs - in the retinal ganglion cells is the major mammalian circadian photopigment (49, 50). Nevertheless, several reports suggest that CRYs affect circadian photoreception and pupillary responses (51, 52). Notably, here we propose a role of CRY in the fly retina that is rather independent of its function as photopigment. In our

study CRY seems to act as a stabilizing protein keeping the INAD signalplex linked to the F-actin and therefore to the rhabdomere internal membrane. Perhaps mammalian CRYs fulfill similar functions in the mammalian retina. This is conceivable because melanopsin ganglion cells have an insect-like (rhabdomeric) phototransduction cascade employing Gq/11-class G proteins and phospholipase C (53). Another fascinating analogy between flies and mice is that mammalian CRY1 and CRY2 have antagonistic effects on regulation of rhythmicity in the retina and in the circadian clock in the brain (54) as does *Drosophila* CRY in the retina and in the E neurons.

Methods

Fly stocks. *Cry*^{01} flies (40) were either compared to *w*^{1118} or to CantonS after back-crossing the flies to CantonS for 5 generations. Co-IP was performed with *yw; tim*-GAL4/+; UAS-*Hacry*/+ (24). For CRY knockdown in the eyes or the CRY-positive E clock neurons *ninaE*-GAL4 (BL: 30540) or *R78G02*-GAL4 (BL: 40010) (Fig. S3) were used in combination with UAS-*dcr2* (VDRC: 60012) and UAS-*cryRNAi* (BL: 25859,). Rescue experiments were conducted with *ninaE*-GAL4 and UAS-*cry* (7) crossed into the *cry*^{01} background. *R78G02-GAL4* expression pattern was analyzed using UAS-stinger2 (55).

Yeast-two-hybrid screening. The research of dCRY partners has been performed by means of the ProQUESTTM Two-Hybrid System (LIFE TECHNOLOGIES®), in the Mav203 strain, using *LacZ* (encoding β-galactosidase) as reporter gene. dCRY cloned in the bait vector pDBLeu was challenged to a cDNA expressing library from wild-type Drosophila heads (See Supplementary Information for details). The transformants were then tested for *LacZ* reporter gene expression the filter assay according to Breeden and Nasmyth (56). Positive clones were isolated from the yeast strain and re-transformed in the Mav-dCRY for further test. Quantitative CPRG assay was performed according to ProQUESTTM Two-Hybrid System manual (LIFE TECHNOLOGIES®).

Co Immunoprecipitation and 2D SDS PAGE. Coimmunoprecipitation was performed as in Mazzotta*, et al.* (14). The 2D electrophoresis has been performed according to Khoudoli, Porter, Blow and Swedlow (57), with some modifications. See Supporting Information for details.

Protein identification by mass spectrometry. After separation on the gel, Coomassie-stained protein spots were excised and in-gel digested, as previously described (14, 25). MALDI-TOF and LC-MS/MS data were analyzed by the online MASCOT software (Matrix

Science, http://www.matrixscience.com) against the *Drosophila* (fruit flies) sequences of the Swiss-Prot database (release 2012_04).

Determination of histamine in fly heads. Histamine was analysed with ultra performance liquid chromatography coupled to quadrupole/time -of-flight mass spectrometry using AccQ Tag Ultra Derivatisation Kit (all Waters) with modifications. See supplementary for details.

Electroretinogram recordings. Electroretinograms (ERGs) were recorded from male flies at the age of 6-9d using the preparation and the recording device described in (14). Flies were either raised in constant darkness or in constant darkness followed by 2h of white light emitting diode (LED) light exposure (10 lux) and dark adaptation before the recording. Irradiance response curves were obtained upon white light exposure from the amplitude of the ERG receptor potential. For further details see supplementary.

Immunostaining, microscopy and image analysis. Retinas were dissected from male flies at the age of 6-9d. After raising the flies either in constant darkness or in constant darkness followed by a 2h exposure to white LED light (1000lux) they were immediately fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (pH = 7.4) for 2.75h in darkness. Afterwards retinas were dissected in PBS with 0.1% Triton X-100 (PBST) (pH = 7.4). Blocking, washing and incubation with the primary and secondary antibody was performed analogous to Hsiao*, et al.* (58) with the modification of a 2d incubation in the primary antibody solution. Brains were dissected as described in (36). The primary antibody solutions contained 5% normal goat serum, PBST and antibodies against CRY (1:2000; (28)) and either Rh1 (1:30; 4C5, Developmental Studies Hybridoma Bank, Iowa City, IA) or PDF (1:1000, C7, Developmental Studies Hybridoma Bank, Iowa City, IA) and VRI (1:2000, (59)) for knockdown experiments. To investigate the *R78G02-GAL4* expression pattern antibodies against CRY, PDF and GFP (1:2000, abcam®) were used All secondary antibodies (Alexa Fluor) were

diluted 1:200. After mounting on glass slides with Vectashield (Vector Laboratories, Burlingame, CA) image stacks from retinas and brains were recorded using the laserscanning microscope (Leica TSC SPE with Leica DM 5500 Q microscope, Leica, Germany). Image size, brightness and contrast were adjusted with GIMP (2.8.6, Kimball and Mattis) and Powerpoint 2010 (Microsoft Office) or ImageJ (FIJI, available at http://fiji.sc/Downloads). Absolute retinal CRY staining intensity was measured with ImageJ in the rhabdomeres of R1-6 in grey-level values within a fixed area.

Recording of locomotor activity rhythms and data analysis. Locomotor activity was recorded under constant temperature (20°C) from 2-6d old male flies using the custom-made system. Flies were exposed to a one-week light-dark cycle of 12h light and 12h darkness at either 10, 100, 1000 or 10000 lux followed by a one-week light-moonlight cycle at 0.01 lux moonlight intensity. For details see supplement.

Statistical analysis. Statistical analysis was performed with Systat11 or Graphpad Prism v4. After checking for normal distribution data were compared by either a one- or two-way ANOVA followed by a pairwise comparison or Tukey's multiple comparisons test if normality was retained. If normality was rejected a Mann-Whitney-U or Wilcoxon-test were applied.

Acknowledgements

We thank Stephane Dissel, Patrick Emery and David Dolezel for providing fly lines, Stephen Goodwin (University of Oxford, UK) for providing the cDNA library, Paul Hardin for providing antibodies, Barbara Spolaore (Centro Ricerche Interdipartimentale Biotecnologie Innovative – CRIBI, Padova, Italy) for mass spectrometry analysis, Michel Mayr for excellent technical assistance, Wolfgang Engelmann, Christiane Hermann-Luibl, Nicolai Peschel, Taishi Yoshii and Günter Brönner for critical comments on the manuscript as well as Marta Beauchamp for editing the language. Stocks obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) for providing transgenic RNAi fly stocks used in this study. This work was funded by grants from the German Research Foundation (DFG; Fo207/10-3 and SFB1047, INST 93/784-1) (C.H.-F.), the European Community (6th Framework Project EUCLOCK no. 018741) (C.H.-F. and R.C.), the Fondazione Cariparo (Progetti di Eccellenza 2011–2012) (R.C.), and the Epigenomics Flagship Project 2012 – EPIGEN (Consiglio Nazionale delle Ricerche – CNR) (R.C.). M.S. was sponsored by a Hanns-Seidel-Foundation excellence grant funded by the BMBF (German Ministry for Education and Research) and R.G. by a grant of the German Excellence Initiative to the the Graduate School of Life Sciences, University of Würzburg.

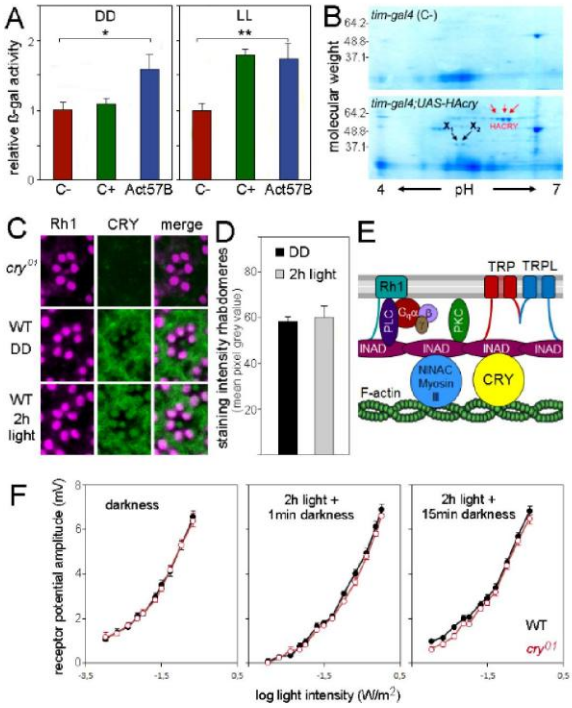

Figure 1

CRY interacts with F-Actin, is expressed in the rhabdomeres of the photoreceptor cells and speeds-up dark-re-adaptation after light-exposure. A: Yeast two-hybrid assays showing a light-independent interaction between CRY and Act57B (DD: constant darkness; LL: constant light). The negative control (C-) is represented by the empty prey vector, while the positive control (C+) is the fragment of PER (aa 233-685) known to interact with CRY in a light-dependent manner. Relative β-galactosidase activity (Miller units) is reported. Mean (+ SEM) of 10 independent clones, analyzed in triplicates, is shown (* $p< 0.05$; ** $p<0.001$). **B**: Coomassie blue-stained 2D gel of head protein extracts co-immunoprecipitated with an anti-HA antibody. HACRY overexpressing flies (*yw;tim-gal4/+;UAS-HAcry/+*) and relative control (*yw;tim-gal4* (C-)) have been reared in 12:12 LD and collected in the dark (ZT24). Protein complexes have been subjected to 2D separation (1st dimension: IPG STRIP pH 4-7; 2nd dimension NuPage ZOOM gel 4-12% Invitrogen). Red arrows indicate the spots relative to HACRY, while X1 and X2 are spots corresponding to putative HACRY partners. **C**: Cross sections of one ommatidium, respectively, stained with anti-Rh1 (magenta) and anti-CRY (green). No CRY staining is present in *cry*^{01} mutants, whereas in wild-type flies (WT) CRY is detected in all 8 photoreceptor cells including their rhabdomeres. After 2h illumination with 1000 lux, rhabdomeric CRY staining appears even stronger. **D**: Quantification of CRY staining intensity in the rhabdomeres of WT flies raised in constant darkness (DD) and after subsequent

2-h exposure to 1000lux. Means (+ SEM) of 10 independent retinas, respectively, are shown. CRY-staining was not reduced after light-exposure (p=0.771). Bars represent the mean of 35 ommatidia out of 7 retinas including SEM. **E**: Putative position of CRY in the phototransduction cascade of the fly rhabdomere. The cartoon is modified after Wang and Montell (16). INAD is a crucial PDZ-scaffold protein which interacts with many components of the cascade. It is connected to F-actin via the MyosinIII protein NINAC as well as via CRY (according to the present results). In addition, INAD interacts with rhodopsin 1 (Rh1), the transient-receptor-potential channels TRP and TRPL, Phospholipase C (PLC) and Phosphokinase C (PKC). **F**: Electroretinogram recordings (ERG) of receptor potentials evoked by 410 ms light-pulses of increasing intensity in WT (CantonS) flies and cantonized cry^{01} mutants. Flies were either raised in constant darkness (DD) before measuring the ERG (left diagram) or subsequently exposed to 2h of light followed by 1 min dark-adaptation (middle diagram) or to 2h of light followed by 15 min dark-adaptation (right diagram). No differences in the ERG responses were observed in DD flies (p=0.745) as well as in flies exposed to 2h of light followed by 1 min dark-adaptation (p=0.587). However, after 15 min dark-adaptation, cry^{01} mutants responded significantly less at lower light-intensities than wild-type flies (p=0.013). Each curve represents the average of at least 7 individuals including SEM.

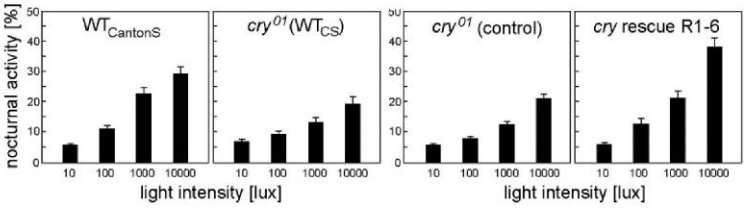

Figure 2

CRY enhances sensitivity to daylight. Mean percentage nocturnal activity of total daily activity calculated for WT flies ($WT_{CantonS}$), *cry*^{01} mutants (*cry*^{01}(WT_{CS}) and *cry*^{01} controls) and *cry*^{01} mutants with CRY rescued in photoreceptor cells 1 to 6 (R1-6) under light-dark cycles with different daylight intensities. A two-way ANOVA showed that relative nocturnal activity depended significantly on daylight intensity ($F_{(3,584)}$=142.066; p<0.001) and on the strain ($F_{(3,584)}$=35.831; p<0.001) and that there was a significant interaction between the two ($F_{(9,584)}$=10.584; p<0.001), indicating that nocturnal activity increased differently with increasing daylight intensity in the different strains. Post-hoc analysis revealed significant differences between *cry*^{01}(WT_{CS}) mutants and $WT_{CantonS}$ flies (p=0.022) as well as between *cry*^{01} controls and *cry*^{01} mutants with CRY rescued in photoreceptor cells R1-6 (p<0.001). No differences occurred between *cry*$^{01}_{CantonS}$ mutants and *cry*^{01} controls (p=0.782). Relative nocturnal activity levels of $WT_{CantonS}$ flies and flies with CRY rescued in R1-6 were also comparable with exception of 10000 lux daylight intensity, at which the flies with CRY only in R1-6 were more nocturnal (p=0.018).

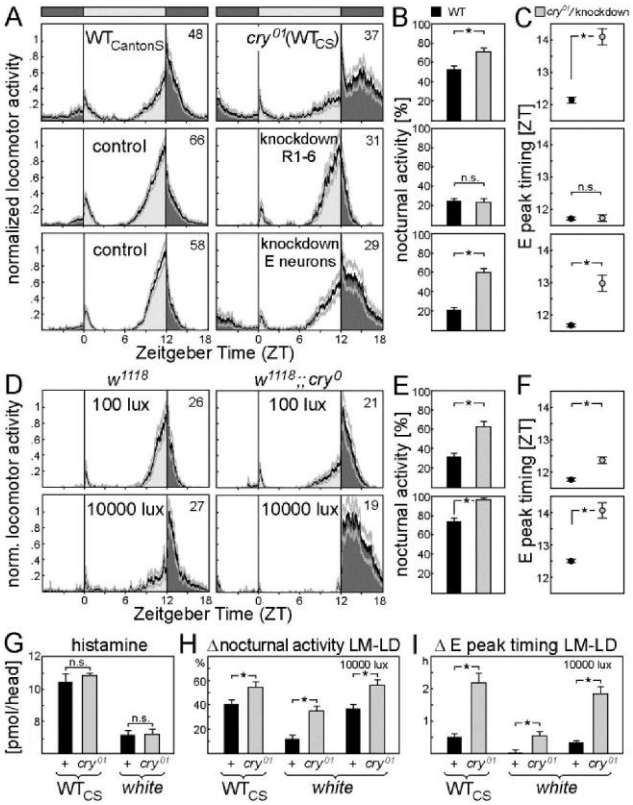

Figure 3

Fly response to moonlight strongly depends on CRY and on the genetic background. **A**: Average locomotor activity profiles of WT flies ($WT_{CantonS}$), cantonized *cry*^{01} mutants (*cry*^{01}(WT_{CS})) and flies with downregulated *cry* in photoreceptor cells R1-6 (knockdown R1-6) or in the four CRY-positive E clock neurons (knockdown E neurons) under light-moonlight (LM) cycles. Labeling as in Figure 2 with the exception that moonlit nights are labeled in dark grey opposed to the black of completely dark nights in Figure 2. **B**: Relative nocturnal activity of the three fly strains in percent of total daily activity. **C**: Timing of evening activity (E peak) in the three fly strains in Zeitgeber Time (ZT). ZT12 is lights-off. Values of wild-type flies ($WT_{CantonS}$ and controls) are shown in black and those of flies with impaired *cry* in light grey. **D**: Average locomotor activity profiles of flies with and without CRY in the *white* mutant background (*w*^{1118}). **E**: Relative nocturnal activity of these strains, and **F**: Timing of evening activity. At 100 lux, flies shifted significantly less activity into the night than their red-eyed siblings (compare to B and C). At 10000 lux their behavior was wild-type like. **G**: Histamine level in the head of flies in the $WT_{CantonS}$ and *white* background. **H**: Difference in nocturnal activity (Δ LM-LD) between light-moonlight and light-dark conditions. **I**: Difference in E peak timing in the same strains.

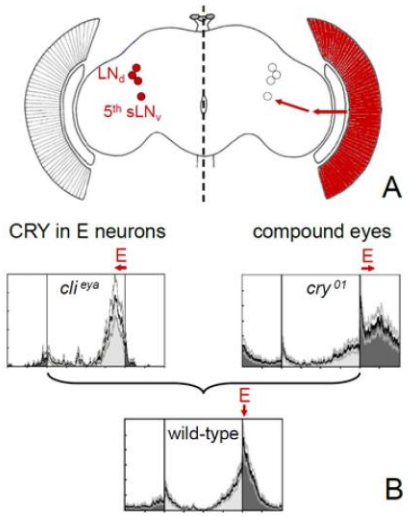

Figure 4

Model of CRY action in the compound eyes and the E clock neurons. A: schematic view of the fly brain with the four here manipulated CRY-positive E neurons (three LN_d and the 5^{th} sLN_v) highlighted in red in the left half and the compound eyes and their putative signaling pathway toward the E clock neurons highlighted in the right half. Signaling from the compound eyes toward the E clock neurons depends on histamine. Its mechanism is not yet completely understood and most probably indirect (red long arrows). **B**: Average activity profiles of eyeless flies (*cli*^{eya}), *cry*^{01} mutants and WT flies under light-moonlight cycles. The activity profile of *cli*^{eya} mutants is derived from (18), the other activity profiles are from the present paper. CRY in the E neurons advances E activity to a rather early phase (small red arrow directed to the left), which becomes evident in flies that lack the influence of the compound eyes (*cli*^{eya}). The compound eyes delay E activity into the night (small red arrow directed to the right), which becomes evident in flies that lack CRY (either completely or only in the E neurons). E activity timing in WT flies (small vertical red arrow) is intermediate between that of *cli*^{eya} and *cry*^{01} mutants and probably determined by the interaction of the two here shown light-input pathways. For further explanation see text.

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Cryptochrome plays different roles in the fruit fly's compound eyes and clock neurons

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Supplementary Information

METHODS

Yeast-Two Hybrid. Bait construction. The full length coding sequence of dCRY was cloned in the bait vector pDBLeu (derivative of pPC97_ Chevray and Nathans, 1992), in frame with the DNA binding domain of Gal4 (aa1-147; GAL4-DB), using standard molecular biology techniques. The pDB-CRY construct was transformed in Mav203 and checked for correct expression with a specific rabbit anti-dCRY antibody (1:1000_Neosystem Laboratoire, Strasburg, France, aa 76-90_ DGRGRLLVFEGEPAY).

Library screening. The expression library, kindly provided by Dr. S. Goodwin, University of Oxford, UK, was prepared from cDNA of wild-type Drosophila heads (Canton S strain) cloned in pPC86 vector (Life Technologies®). The Mav203 strain containing the bait construct (Mav-dCRY1) was transformed with the cDNA library according to the modified procedure of the lithium acetate method (Gietz et al., 1992). The transformants were then tested for *LacZ* reporter gene expression the filter assay according to Breeden and Nasmyth (1985). Positive clones were isolated from the yeast strain and re-transformed in the Mav-dCRY for further test. Clones that resulted positive at this further screening were sequenced with primers pPC86_F: 5'-TATAACGCGTTTGGAATCACT-3' and pPC86_R: 5'-GTAAATTTCTGACGAGGTAGAC-3', designed on the plasmid sequence. Quantitative CPRG assay was performed according to ProQUESTTM Two-Hybrid System manual (LIFE TECHNOLOGIES®). Statistic analysis was performed with Graphpad Prism v4 using one-way ANOVA followed by Tukey's multiple comparisons test.

Co Immunoprecipitation and 2D SDS PAGE. Three to five days old flies overexpressing HAdCRY (Dissel et al., 2004; yw; tim-GAL4/+; UAS-Hacry/+) were collected at ZT24 (ZT 0 lights-on and ZT 12 lights-off in a 12:12 light–dark cycle). Heads were homogenized in

extraction buffer [20 mM Hepes, pH 7.5, 100 mM KCl, 2.5 mM EDTA, pH 8, 5% glycerol, 0.5% Triton X-100, 1 mM DTT, complete protease inhibitors (Roche)], centrifuged at maximum speed for 10 min and the supernatant pre-cleared with protein-G agarose beads (Sigma) for 20 min. The extract was then incubated with anti-HA (1:1000, Sigma) for 2 h at 4°C before the addition of 30 µl of protein G agarose beads (1:1 slurry) for 1 h. The beads were precipitated by centrifugation at 2000g and then washed three times with 1 ml of extraction buffer and once with 1 ml of 20 mM Hepes, pH 7.5.

The 2D electrophoresis has been performed according to Khoudoli et al. (2004), with some modifications. Protein complexes were solubilized by heat treatment (5 min at 95°C) in presence of 100mM DTT and 0.2% SDS, precipitated in 80% acetone at -20°C and solubilized for 6 hours in resuspension buffer (30 mM Tris Base, 7 M Urea, 2 M Thiourea, 1.2% CHAPS, 0.14% ASB14, 0.25% Ampholytes, 43 mM DTT), with the addition of 60 mM Acrylamide after 3 hours, in order to alkylate the proteins (Mineki et al., 2002). Isoelectric focusing (IEF) was performed in 7 cm IPG strips of pH range 4–7 (ReadyStrip™_Bio-rad); strips have been passively rehydrated for 16 hours and then iso-electro focused by a two-phase protocol: 30 min at 250 V, 3 h and 30 min at 5500 V and 500 V until the complete focusing. After IEF, strips were equilibrated in Equilibration buffer (50 mM Bis-Tris pH 6.4, 6 M Urea, 30% (w/v) glycerol, 2% SDS) containing 50 mM DTT for 20 min and 360 mM Acrylamide for further 20 min. Strips were then placed on a 4-12 % pre-cast “ZOOM NuPAGE gel” (Invitrogen®) with the help of a 0.5% agarose matrix and and run at room temperature at 50 V.

Protein identification by mass spectrometry. After the separation of proteins on the gel, Coomassie-stained protein spots were excised and in-gel digested, as previously described (Wilm et al., 1996). Briefly, gel pieces were destained and the proteins digested with porcine trypsin (modified sequencing grade; Promega, Madison, WI, USA) overnight at 37 °C. The supernatants were then transferred to other tubes and residual tryptic peptides were

extracted upon incubation of gel spots with 25 mm NH_4HCO_3 at 37 °C for 15 min followed by shrinking of gel pieces with acetonitrile, and then upon incubation with 5% (v/v) formic acid at 37 °C for 15 min followed by shrinking with acetonitrile. The extracts were combined with the primary supernatant and dried in a SpeedVac centrifuge (Savant Instrument Inc., NY, USA). LC-MS/MS analyses were performed on protein digests dissolved in 0.1% trifluoroacetic acid, 5% acetonitrile and using a Micromass CapLC unit (Waters) interfaced to a Micromass Q-Tof Micro mass spectrometer (Waters) equipped with a nanospray source. Tryptic digests were loaded at a flow rate of 15 µl/min onto an Atlantis dC18 Trap Column. After valve switching, the sample was separated on a Symmetry C18 column (150 x 0.075 mm, 3.5 µm particle size) (Waters) at a flow rate of 3.5 µl/min using a gradient from 1% B to 40% B in 43 min and from 40% to 70% B% in 7 min (solvent A: 95% H2O, 5% acetonitrile, 0.1% formic acid; solvent B: 5% H2O, 95% acetonitrile, 0.1% formic acid). Instrument control, data acquisition and processing were achieved with MassLynx V4.1 software (Waters). MALDI mass spectrometry measurements were performed on a MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany) operating in the positive-ion reflectron mode. A saturated solution of α-cyano-4hydroxycinnamic acid in water, 0.1% TFA/acetonitrile (1/1 v/v ratio) was used as matrix and mixed at a v/v ratio of 1:1 with the digests dissolved in 0.5% TFA aqueous solution. MALDI-TOF and LC-MS/MS data were analyzed by the online MASCOT software (Matrix Science, http://www.matrixscience.com) against the Drosophila (fruit flies) sequences of the Swiss-Prot database (release 2012_04). The following parameters were used in the MASCOT search: trypsin specificity; maximum number of missed cleavages: 1; fixed modification: propionamide (Cys); variable modifications: oxidation (Met); peptide mass tolerance: ± 0.2 Da; (fragment mass tolerance: ± 0.5 Da for the MS/MS data); protein mass: unrestricted; mass values: monoisotopic.

Determination of histamine in fly heads. For determination of histamine in 6 day old male flies were collected 1 h after lights on, frozen at -80 °C, shaken to decapitate them and the

raw extract of heads were analyzed using ultra high pressure liquid chromatograph coupled to a time of flight mass spectrometer (Acquity UPLC - Synapt G2 HDMS, Waters) equipped with electrospray ion source operated at positive mode. Heads from 10 flies were homogenized in 250 µl of extraction solution using a ball mill (21 Hz, 5 min). The extraction solution was 50% methanol, 50% water containing 250 ng 1-methylhistamine (Sigma). After centrifugation (20800 g, 10 min), the supernatant was evaporated until dryness in a vacuum concentrator and derivatised using ACCQ-Tag Chemistry Kit (Waters) according to the suggested protocol. Chromatographic separation was carried out on a BEH C18 column (2.1x100 mm I.D., 1.7 µm, Waters) at a flow rate of 0.3 mL/min using linear binary solvent gradient of 0 to 10% eluent B over 10 min. Eluent A was 0.1% formic acid in water and eluent B was acetonitrile. The capillary voltage was set to 0.8 kV and nitrogen was used as desolvation gas (350° C, 800 L/h). Data was acquired over the mass range of 50−1200 Da. Instrument control, data acquisition and data preprocessing were achieved with MassLynx software (version 4.1; Waters). The retention time (rt) of histamine was 4.38 ± 0.02 min in the extracted ion chromatogram at m/z of 282.135 ± 0.03 Da. Internal standard approach using 1-methylhistamine (250 ng, rt of 4.55 min in the XIC of 296.150 ± 0.03 Da) as internal standard was used for the quantification of histamine from peak area using response factor of 0.96. The method was short validated by determining the response factor, repeatability (inter-day RSD < 4%), linearity (regression coefficient > 0.972 at concentration range of 10 and 5000 ng/sample) and sensitivity (limit of quantification 10 ng/sample).

Electroretinogram recordings. The illumination for ERG recordings was run with white light pulses of 410ms duration starting with the lowest light intensity. White light was generated by a halogen lamp (Spindler & Hoyer) with a KG heat filter (Schott) and attenuated using neutral density filters (Edmund Optics, Schott). The maximum intensity was measured with the QE6500 (Ocean Optics). The interval between the light pulses was 20s. The amplitude of the ERG receptor potential was measured relative to the baseline before lights on.

Recording of locomotor activity rhythms and data analysis. Both the custom made system used for recording the locomotor activity as well as the calculation of the average activity profile, the relative nocturnal activity level and the timing of the evening (E) peak are described in Schlichting and Helfrich-Förster (2015).

RESULTS

Table 1

List of the proteins identified by MALDI-TOF and LC-MS/MS analyses [a]

Spot	Protein name	Accession no. [b]	Theoretical MW (kDa)/p*I* [c]	MALDI-MS analysis: Matched peptides	MALDI-MS analysis: Coverage %	MALDI-MS analysis: Score	MS/MS peptides [d]
X1	**Actin-87E**	P10981	41.6/5.30	13	50	96	3[e]
	Actin-5C	P10987	41.6/5.30	11	47	75	
X2	**Actin-57B**	P53501	41.6/5.23	11	46	84	2

[a] For the spots X1 and X2, the identified proteins with their accession numbers and theoretical molecular weights/p*I* are listed. Protein identification was performed with the MASCOT software searching MS data against the sequences of Drosophila of the Swiss-Prot database. [b] Accession no. from the UniProtKB database. [c] Theoretical MW and p*I* were calculated using the Compute pI/MW tool available on the ExPASy website (www.expasy.org). [d] Peptides sequenced by LC-MS/MS that matched to the protein with a significant score ($p<0.05$). [e] The peptides sequenced by LC-MS/MS are common to both Actin-87E and Actin-5C.

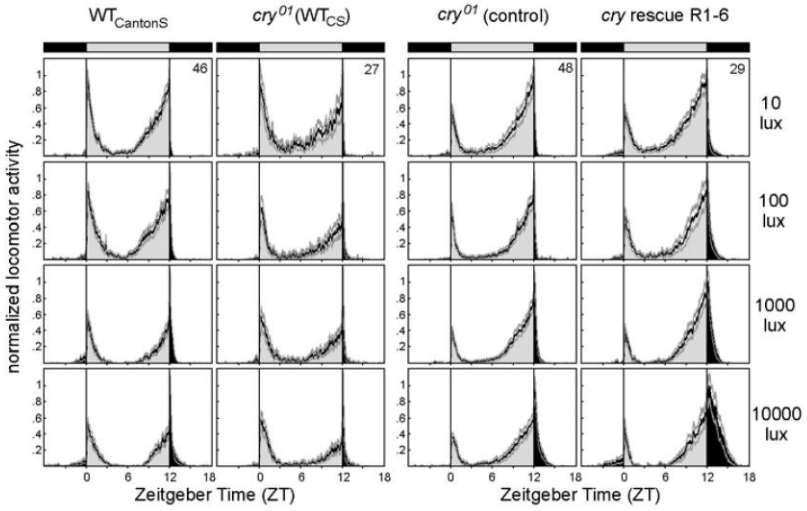
Figure S1

Clustal W alignment of proteins encoded by six actin genes in *Drosophila*.

* identical residues in all sequences, : highly conserved residues, . weakly conserved residues.

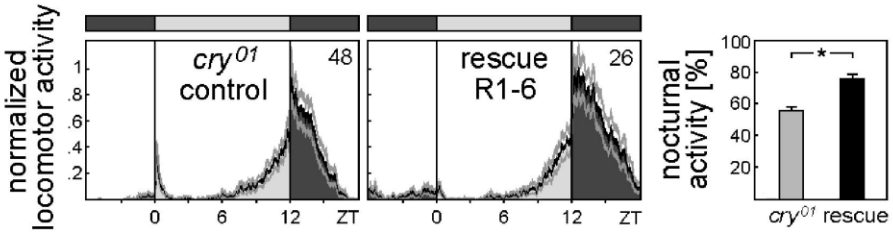
Actin 79B is encoded by gene CG7478 on the left arm of chromosome 3, Actin 88F by gene CG5178 on the right arm of chromosome 3, Actin 57B by gene CG10067 on the right arm of chromosome 2, Actin 87E by gene CG18290 on the right arm of chromosome 3, Actin 5C by gene CG4027 on the X-chromosome and Actin 42A by gene CG12051 on the right arm of chromosome 2.

Figure S2



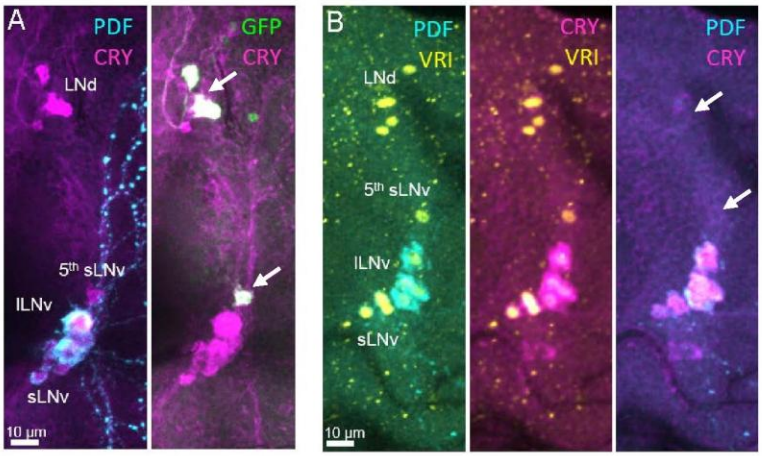
Average locomotor activity profiles of wild-type flies ($WT_{CantonS}$), *cry*^{01} mutants (*cry*^{01}(WT_{CS}) and *cry*^{01} controls) and *cry*^{01} mutants with CRY rescued in photoreceptor cells 1 to 6 (R1-6) under light-dark cycles with different daylight intensities. *cry*^{01} controls consist of ~half *w*^{1118}*;ninaE-gal4;cry*^{01} flies and half *w*^{1118}*;UAS-cry;cry*^{01} flies, respectively. We pooled the two controls, because they behaved similarly (p=0.176). Flies were recorded under light-dark cycles with 12 hours of light and 12 hours of darkness (LD 12:12) with daylight intensities of 10, 100, 1000 and 10000 lux, respectively. Light period (bar on top) and activity during the day are shown in light grey whereas the dark period (bar on top) and activity during the night are shown in black. Average activity profiles are normalized with maximal activity set to one. Faint grey lines above and below the average profiles represent standard errors of the mean (+SEM). Numbers of recorded animals are given in the right top corner of the upper diagram. In each fly strain, evening (E) activity decreased and nocturnal activity after lights-off increased with increasing daylight intensity.

Figure S3



CRY in the compound eyes enhances sensitivity to moonlight. A: Average locomotor activity profiles and relative nocturnal activity of *cry*^{01} control flies (~half *w*^{1118}*;ninaE-gal4;cry*^{01} and *w*^{1118}*;UAS-cry;cry*^{01} flies, respectively) and *cry*^{01} mutants with CRY rescued in photoreceptor cells 1 to 6 (rescue R1-6) under moonlight conditions. Light period (bar on top) and activity during the day are indicated in light grey whereas the moonlight period (bar on top) and activity during the night are shown in dark grey. The average activity profiles are normalized with maximal activity set to one. Faint grey lines above and below the average profiles represent standard errors of the mean (+SEM). Numbers of recorded animals are given in the right top corner of the diagrams. Flies with CRY in photoreceptor cells R1-6 shifted significantly more activity into the night than *cry*^{01} control flies, indicating that they are more sensitive to moonlight.

Figure S4



The GAL4 line R78G02 drives expression in the four CRY-positive E neurons and can reliably down-regulate CRY expression. A) GFP driven expression by the R78G02 within the clock neurons is restricted to four E neurons, the three CRY-positive LN_d and the 5th s-LN_v that expresses also CRY but is PDF negative (arrows). PDF and CRY immunostaining are shown in cyan and magenta, respectively. B) PDF (cyan), VRI (yellow) and CRY (magenta) immunopositive neurons in the brain of *UASdcr2;R78G02-GAL4;UAS-cryRNAi* flies. CRY expression is efficiently down-regulated in the R78G02 positive E neurons (arrows).

6.7. Rhodopsin five and six expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms of *D. melanogaster*.

Rhodopsin five and six expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms of *D. melanogaster*.

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Abstract

Circadian clocks were shown to be of high adaptive value for animals and plants. One of the key features of this clocks is that they can adjust their endogenous period of approximately 24h to exactly 24h if external stimuli are presented in a rhythmic fashion, which is called entrainment. Several studies demonstrated that light is the most important Zeitgeber entraining the clock. In *Drosophila* the blue-light photopigment Cryptochrome is expressed in about half of the clock neurons and resets the molecular clock mechanism to light-dark-cycles. However, also flies lacking CRY are able to adjust their endogenous period to 24h showing that the visual system of the fly is sufficient for entraining the clock in the fly. In this study we focus on the Hofbauer-Buchner-eyelet, a photoreceptor, which consists of only 4 receptor cells per hemisphere. Using new imaging approaches we are able to show a physiological connection between the Hofbauer-Buchner-eyelet and the lateral clock neurons for the first time. This connection appears to be mediated via acetylcholine and is able to phase-shift the activity-rest rhythm of the fly, suggesting a biological relevance of the investigated connection.

Introduction:

Circadian clocks create an endogenous sense of time that is used to produce daily rhythms in physiology and behavior (Aschoff, 1981a). A defining characteristic of a circadian clock is a modest deviation of its endogenous period from the 24.0-hour period of daily environmental changes (Aschoff, 1981b). For example, the average human clock has an endogenous period of 24 hours and 11 minutes (Czeisler et al., 1999), while the fruit fly *Drosophila melanogaster*'s clock has an average period of around 23-and-a-half hours (Dowse et al., 1987). Thus, in order to maintain a consistent phase relationship with the environment, the human clock must be sped-up by 11 minutes a day while the fly's clock must be slowed down by half an hour. A sensitivity of the circadian clock to environmental time cues (Zeitgebers) ensures that circadian clocks are adjusted daily to match the period of environmental change (Pittendrigh, 1981). This process, called entrainment, is fundamental to the proper daily timing of behavior and physiology (Roenneberg et al., 2003). For most organisms, daily light/dark cycles are the most salient Zeitgeber (Aschoff, 1981b).

Though most tissues express molecular circadian clocks in animals, small islands of neural tissue are responsible for producing sleep/activity rhythms and many other daily rhythms in physiology (Herzog, 2007). Within these islands a circadian clock neuron network (CCNN) functions as the master circadian clock (Nitabach and Taghert, 2008; Welsh et al., 2010). Subsets of neurons within the CCNN receive resetting signals from photoreceptors and connections between these neurons and their non-photoreceptive targets likely ensure light entrainment of the CCNN (Golombek and Rosenstein, 2009).

In both mammals and insects the CCNN receives light input from multiple photoreceptor types. In mammals these consist of rods, cones, and intrinsically photosensitive retinal ganglion cells, each of which is sufficient for the entrainment of sleep wake cycles (Guler et al., 2007). In *Drosophila* the CCNN is entrained by photoreceptors in the compound eye, the ocelli, the Haufbaur-Buchner (HB) eyelet, and by subsets of clock neurons that express the blue light photoreceptor *cryptochrome (cry)* (Helfrich-Förster, 2002). Understanding how multiple light input pathways modulate the CCNN to produce entrainment to the environmental light/dark cycle is critical for our understanding of the circadian system and its dysfunction in the face of the unnatural light regimes accompanying much of modern life (Münch and Bromundt, 2012). To what extent do these light input pathways converge on specific nodes within the CCNN? What is the

physiological basis for their effects? What are the effects of their excitation on sleep/activity rhythms?

Here we investigate the physiological basis and circadian role of a long-suspected circadian light input pathway in *Drosophila*: the HB-eyelets. These simple accessory eyes contain four photoreceptors located at the posterior edges of the compound eyes and project directly to the accessory medullae (Hofbauer and Buchner, 1989; Helfrich-Förster et al., 2002; Malpel et al., 2002), neuropils that support circadian timekeeping in insects (Helfrich-Förster, 1998). In *Drosophila* the AMe contain projections from ventral lateral neurons (LN_vs), important components of the CCNN that express the neuropeptide Pigment Dispersing Factor (PDF) (Helfrich-Förster, 1997; Helfrich-Förster, 1998), an output required for robust circadian rhythms in sleep and activity (Renn et al., 1999). The axon terminals of the HB-eyelet terminate near PDF positive projections (Helfrich-Förster et al., 2002; Malpel et al., 2002) and analysis of visual system and *cry* mutants supports a role for the HB eyelet in the entrainment of locomotor rhythms to LD cycles (Helfrich-Förster et al., 2001; Helfrich-Förster et al., 2002; Veleri et al., 2007), but how the eyelet influences the CCNN to support light entrainment is not well understood.

Here we examine the physiological nature of the connection between the HB-eyelet and the CCNN of *Drosophila* and present evidence that this circadian light input pathway specifically excites the small LN_vs among the PDF positive LN_vs and acts to phase-dependently advance free-running rhythms in sleep/activity. This work establishes for the first time the nature of a connection between the CCNN and an identified visual system pathway in the adult fly and indicates that input from external photoreceptors targets specific nodes within the fly CCNN.

Materials and Methods

Fly Rearing and Transgenic Strains

Flies were reared on cornmeal-yeast-sucrose media. Unless otherwise noted, flies were reared at 25˚C under a 12:12 light:dark cycle. All flies strains used in this study have been previously described. For GFP Reconstitution Across Synaptic Partners (GRASP) experiments we used the *LexAop-GFP-11* and *UAS-GFP1-10 elements* (Gordon and Scott, 2009) in combination with the *Rh6-GAL4* driver, which drives GAL4 expression in rhodopsin-six (Rh6) expressing photoreceptors (Sprecher and Desplan, 2008) and the *Pdf-LexA* element, which drives LexA in the PDF expressing LN_vs (Shang et al., 2008). To visualize the *Rh6-GAL4* expression pattern, we crossed this element to *UAS-cd8GFP* (Siegmund and Korge, 2001). For live imaging experiments, we used these same drivers along with the *Pdf(M)-Gal4* element (Renn et al., 1999) in conjunction with the *UAS-GCaMP3.0* (Tian et al., 2009), *UAS-P2X2* (Lima and Miesenböck, 2005), *UAS-Epac1-camps(50A)* (Shafer et al., 2008), *Lex- Aop-GCaMP3.0*, and *LexAop-Epac1-camps* (Yao et al., 2012) responder lines to drive the Ca^{2+} sensor GCaMP3.0 (Tian et al., 2009), the FRET based cAMP sensor Epac1-camps (Nikolaev et al., 2004), and the mammalian purinergic receptor P2X2 (Lima and Miesenböck, 2005) in rhodopsin-six (Rh6) expressing photoreceptors or LN_vs. For the TrpA1 mediated excitation of Rh6-expressing photoreceptors, the Rh6-GAL4 line above was combined with *UAS-TrpA1* (Hamada et al., 2008) in the hdc^{JK910}-mutant background. The hdc^{JK910}-mutant suffers a loss of function mutation in *histidine decarboxylase (hdc)* and therefore lacks histamine (Burg et al., 1993), the neurotransmitter of the compound eyes. Thus, in the hdc^{JK910}-mutant background the excitation of Rh6 expressing photoreceptors will not result in the release of histamine from Rh6 expressing photoreceptors in the compound eye, whereas the HB-eyelets will still produce and release acetylcholine upon excitation.

GRASP and Microscopy

To visualize the general projection pattern of the Rh6 expressing photoreceptors of the compound eyes (R8) and the HB-eyelet we crossed Rh6-GAL4 and UAS-cd8GFP flies and immuno-labeled F1 brains for anti-GFP and anti-PDF. To determine if the termini of Rh6 expressing photoreceptors and projections of the LN_vs were sufficiently close to allow for the reconstitution of GFP between these two cell types, we crossed flies containing complementary split GFP elements (*LexAop-GFP-11;UAS-GFP1-10*) with flies containing

both the *Rh6-GAL4* and the *Pdf-LexA* elements and processed FI brains only for anti-PDF, relying on basal GFP fluorescence to visualize reconstituted GFP. Both parental strains were independently crossed to w^{1118} flies and offspring served as negative GRASP controls.

For immunocytochemistry we submerged five to nine day old males of each genotype in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at room temperature. We washed these flies with four changes of PBS, removed the heads, and dissected brains from the cuticle and eye tissue. We blocked the brains in 5% normal goat serum (NGS) in PBST (0.5% TritonX) for three hours at room temperature and then stained the brains in primary antisera (anti-GFP and anti-PDF for mapping Rh6 expression, anti-PDF only for GRASP experiments). We used mouse anti-PDF (Developmental Studies Hybridoma Bank, The University of Iowa) at 1:1000 and chicken anti-GFP at 1:2000 (Abcam, Cambridge, MA) in 0,02% NaN_3 and 5% NGS in PBST. Brains were kept in primary antibody over night at room temperature (RT). Following exposure to primary sera, we rinsed the brains with five 10-minute rinses in PBST and transferred the brains into secondary antisera consisting of Alexa Fluor 635 conjugated goat anti-mouse (for GRASP experiments) or, in the case of rh6-GAL4/uas-cd8GFP brains, Alexa Fluor conjugated 488 goat anti-chicken, and Alexa Fluor 635 conjugated goat anti-mouse. We exposed brains to secondary sera for three hours at room temperature. All secondary sera were diluted 1:200 in 5% NGS in PBST and were purchased from Life Technologies (Grand Island, NY). We rinsed the secondary sera from brains with five 10-minute rinses in PBST and mounted brains on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

We imaged brains using a Leica TCS SPE scanning confocal microscope (Leica, Wetzlar, Germany). We used 488 and 635 nm laser diodes to excite GFP and the fluorophores of the secondary antibodies using confocal steps of 2 µm. The laser settings were kept constant within each experiment. All images were analyzed using the Fiji in ImageJ.

Live Imaging

We performed live imaging experiments as previously described (Lelito and Shafer, 2012; Yao et al., 2012) using an Olympus FV 1000 laser-scanning microscope (Olympus, Center Valley, PA) and a 60X 1.1N/A W, FUMFL N objective (Olympus, Center Valley, PA). We anesthetized flies over $C0_2$ and dissected brains under HL3 saline (Stewart et al., 1994). We mounted brains on the bottom 35-mm FALCON culture dishes

(Becton Dickenson Labware, Franklin Lakes, NJ) under a drop of HL3 saline in a Petri Dish Insert (PDI, Bioscience tools). We allowed brains to settle for 5-10 minutes before imaging. We established continuous perfusion of HL3 while the regions of interest (ROIs) over LN_v somata or the HB-eyelet nerve were selected using Olympus Fluoview software (Olympus, Center Valley, PA). We performed Ca^{2+} imaging using the sensor GCaMP3.0, scanning brains with a 488 nm laser at 1 Hz and collecting GFP emission. We performed cAMP imaging using the FRET sensor Epac-1cAMPS, scanning brains with a 440 nm laser at 1 Hz and collecting CFP and YFP emission. We processed GCaMP3.0 fluorescence and Epac1-camps inverse FRET (CFP/YFP) as previously described (Lelito and Shafer, 2012; Yao et al., 2012).

Each imaging experiment began with the acquisition of 30 seconds of baseline fluorescence. At 30s, we switched perfusion channel to a second channel, which contained either test compounds dissolved in HL3 or HL3 alone as a vehicle control, for 30s, after which we switched back to the first HL3 channel for the remainder of the 5-minute time-course. We purchased all chemicals from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Waltham, MA). We performed statistical tests on all live imaging data using Prism 5 (GraphPad, San Diego, CA) and compared maximum changes in GCaMP3.0 fluorescence or the Epac-1caMPs inverse FRET ratio between vehicle and test compounds. We used the Mann-Whitney U test for pairwise comparisons of maximum changes, and the Kruskal-Wallis one-way ANOVA with Dunn's post-test for multiple comparisons. All plots were generated in Prism 5.

Analysis of HB-Eyelet Induced Behavioral Phase Shifts

To address the effects of HB-eyelet excitation on free running locomotor rhythms, we expressed the heat activated cation channel TrpA1 (Hamada et al., 2008) in the Rh6 expressing photoreceptors of the compound eyes and HB-eyelets in a loss of function *hdc*^{JK910} mutant background, thereby removing the influence of the photoreceptors of the compound eyes. We performed an anchored phase-response curve (PRC) in these flies using pulses of high heat. As controls for our experimental line (w; *hdc*^{JK910};rh6-GAL4/UAS-TrpA1) we crossed the *w; hdc*^{JK910}*;UAS-TrpA1* and *w; hdc*^{JK910}*;rh6-GAL4* parental lines to *hdc*^{JK910} mutants resulting in the GAL4 (*w;hdc*^{JK910}*;rh6-GAL4/*+) and the UAS (*w;hdc*^{JK910}*;UAS-TrpA1/*+) controls. We used the Trikinetics *Drosophila* Activity Monitoring (DAM) system (Trikinetics; Waltham, MA) to record the number of beam crosses in one-minute intervals. We singly loaded two-to-five-day old male flies into capillary tubes containing a sucrose agar media and entrained the flies to a 12:12

light/dark (LD) cycle for 7 days at 20°C followed by constant darkness (DD). We delivered a two-hour heat pulse of 30°C at five different times during the final night of LD and during the first subjective day of DD and continued to record locomotor activity DD condition for 10 days at 20°C.

Raw data were plotted as actograms using ActogramJ (Schmid et al., 2011). To analyze phase shifts we used the open access program ChronoShop (Dr. Kamil Spoelstra, Netherlands) to determine the center of gravity (COG) for each fly for each day of the experiment, as this is the most reliable means to determine the phase of flies lacking input from the compound eyes. To determine the COG under entrained conditions, we averaged the COG of the last 2 days in LD. To determine the phase shift induced by the heat pulse, we calculated the difference between the COG on day 1 (COG1) after the HP and the COG in entrained conditions. The same was done for day 2 after the HP (COG2). To compensate for differences in the free-running period of different genotypes, we subtracted the shift caused by the free-running period at COG1 and COG2 for each single fly. We calculated the shift caused by the HP as the mean of ΔCOG1 and ΔCOG2. The free-running period was analyzed using chi^2 analysis. Only flies in which the free-running period as well as both COGs could be determined were used for analysis, leading to a sample size of between 18 and 26 flies for each experiment.

We statistically compared phase shifts using Systat11 and tested data for normal distributions using one-way Kolmogorov-Smirnoff tests. Normally distributed data were compared using one-way ANOVA, non-normally distributed data were compared using a Mann-Whitney-U-test. In both cases p-values were adjusted using a Bonferroni correction.

Results

The Rhodopsin-6 photoreceptor termini rest in close apposition to the LN_vs in the accessory medulla but not in the distal medulla.

In order to express transgenes in the HB-eyelet, we made use of the *Rh6-GAL4* driver, as the eyelet expresses Rhodopsin-6 strongly in the adult (Helfrich-Förster et al., 2002). The R8 photoreceptors of the compound eye also express Rhodpsin-6 and *Rh6-GAL4* (Yasuyama and Meinertzhagen, 1999) and we are not aware of a driver that drives strong expression exclusively in the HB-eyelet in the adult fly. R8 photoreceptors terminate in the distal medulla of the optic lobes whereas the eyelet projects to the accessory medulla where it terminates near the LN_vs (Helfrich-Förster et al., 2002; Malpel et al., 2002) and Fig 1A-C). If synaptic connections exist between the Rh6-expressing photoreceptors and the LN_vs, split GFP constructs driven independently in these cell types should result in the reconstitution of GFP and reveal fluorescence at synaptic sites (Feinberg et al., 2008). A lack of reconstitution would argue against the presence of direct connections between these photoreceptors and the LN_vs. Expression of split GFP in these cell types resulted in the reconstitution of GFP specifically in the accessory medulla in 16 of 16 brains imaged. In 13 of these brains we also observed GFP reconstitution along the previously described ventral elongation of the LN_v projections (Fig. 1E and G-I). No GFP reconstitution was detected in other regions of the brain, not even in the distal medulla where R8 termini reside near projections of the large LN_vs (l-LN_vs) (Fig. 1G-I). We detected no GFP fluorescence in control genotypes containing either the genetic drivers alone or the split GFP elements without drivers (Fig. 1D and F). These results support the hypothesis that the eyelets form direct connections on the LN_vs in the accessory medulla and suggest that the R8 photoreceptors do not form synapses on the l-LNvs within the distal medulla.

Histamine has no measurable effects on Ca^{2+} or cAMP in the LN_vs.

In the adult fly, the HB-eyelet is immunoreactive to antisera raised against both choline acetyl transferase (ChAT) and histamine (Pollack and Hofbauer, 1991; Yasuyama and Salvaterra, 1999) suggesting the presence of both acetylcholine and histamine in the eyelet nerve. It is not known if the eyelet employs both neurotransmitters in the adult or if anti-ChAT immunosignals simply represent a non-functional, waning pool of ChAT left over from the cholinergic Bolwig's nerve, which is remodeled to become the

eyelet in adults. It is not known if either neurotransmitter is used to relay light information from the eyelet to the clock neurons in the aMe, though previous work established that the LN_vs, both large and small, are receptive to acetylcholine (McCarthy et al., 2011; Lelito and Shafer, 2012). Mapping of the inhibitory histamine receptor HisCl indicated that the large but not the small LN_vs are receptive to histamine (Hong et al., 2006). We first asked if bath applied histamine had measurable effects on LN_v Ca^{2+} levels. We found no evidence that bath applied histamine caused significant changes in Ca^{2+} (Fig. 2A and B) or cAMP (Figure 2C and D) levels compared to vehicle controls in either the large or small LN_vs. Histamine acts through inhibitory receptors and inhibition is often difficult to detect with existing genetically encoded sensors. We previously established that GABA mediated inhibition could be detected in the LN_vs by co-applying GABA with the cholinergic agonist nicotine, in which case GABA significantly reduced the excitatory Ca^{2+} and cAMP response to nicotine. For both large and small LN_vs, the co-application of 10^{-2}M histamine did not significantly reduce the excitatory effects of 10^{-4}M nicotine (Fig. 2E-H), suggesting that the former neurotransmitter either does not inhibit either class of LN_vs or that such inhibition is not detectable with our sensors.

Excitation of Rhodopsin-six expressing photoreceptors causes Ca2+ and cAMP increases in the small but not the large LN_vs.

The lack of histamine effects on the adult LN_vs, along with previous work establishing that these neurons are receptive to acetylcholine, suggested that the HB-eyelet might act to excite the LN_vs rather than inhibiting them, as suggested by the anti-histamine immunosignals that appear eyelet nerve during metamorphosis. To determine if an excitatory connection exists between the HB-eyelet and the LN_vs, we rendered the HB-eyelet nerve excitable by ATP through the expression of the mammalian purinergic receptor P2X2 (Fig. 3A), via *Rh6-GAL4* mediated expression of *UAS-P2X2*. When we excited P2X2 expressing HB-eyelets with ATP application, no Ca^{2+} responses were detected in the large LN_vs (Fig. 3B). In contrast, the small LN_vs displayed significant Ca^{2+} increases in response to eyelet excitation (Fig. 3C). This excitatory response was not due to non-specific effects of ATP or to leaky P2X2 expression in the small LN_vs, as flies containing the *UAS-P2X2* responder element without an Rh6-GAL4 driver did not display Ca^{2+} increases in response to ATP application (Fig. 3D).

These results suggest that the HB-eyelets provide excitatory drive to the small but not the large LN_vs. The presence of anti-ChAT immunosignals in the eyelet nerve suggests that acetylcholine mediates this excitation. We previously showed that

cholinergic agonists increase cAMP levels in both the large and small LN_vs. Thus, if the eyelet specifically excites the small but not the large LN_vs, P2X2 mediated excitation of the eyelet should cause cAMP increases in the former but not the latter neuron class. Indeed, excitation of the eyelet produced no significant cAMP changes in the large LN_vs but caused clear increases in cAMP in the s-LN_vs (Fig. 4). These results suggest that among the LN_vs the HB-eyelet specifically excites the s-LN_vs, important clock neurons for the maintenance of strong, normally phased activity rhythms.

Thermogenetic excitation of Rhodopsin-six expressing photoreceptors causes phase dependent advances in free-running locomotor rhythms.

An excitatory connection between the HB-eyelets and the small LN_v clock neurons leads to the predication that HB-eyelet excitation would result in phase shifts in the fly's free-running locomotor rhythm, as the acute excitation of LN_vs results in phase dependent advances and delays in this behavioral rhythm. To determine if HB-eyelet activity is sufficient for phase shifting locomotor rhythms we expressed the heat gated cation channel TrpA1 by combining *UAS-TrpA1* with *Rh6-GAL4*, thereby rendering Rh6-expressing photoreceptors excitable by high temperature (30°C) pulses. In order remove the influence of histaminergic R8 photoreceptors, we conducted this experiment in a hdc^{JK910} mutant, which is unable to synthesize histamine. Thus, in hdc^{JK910}*;Rh6-GAL4/UAS-TrpA1* flies, TrpA1 mediated excitation of Rh6 expressing photoreceptors should result in acetylcholine release from the HB-eyelet in the absence of neurotransmitter release from the R8 photoreceptors in the compound eye.

At low temperature (20°C), the expression of TrpA1 in the Rh6 expressing photoreceptors of hdc^{JK910} mutants caused a significant decrease in the free-running period of locomotor rhythms relative to controls (Table 1). We excited the Rh6 expressing photoreceptors of experimental flies at different times within the circadian cycle with two-hour pulses of high temperature, comparing the phase responses of experimental flies to heat pulses to those of genetic controls that lacked either *Rh6-GAL4* or *UAS-TrpA1* elements. Heat pulses delivered between ZT14 and 16 and ZT21 and 23 on the last night of the LD cycle caused small (40-80min) but significant phase advances in the experimental flies, whereas a pulse delivered between these two time-points (ZT 18-20) caused no significant phase changes (Fig 5). During the subjective day the heat pulses caused advances in both experimental and control lines, with no obvious differences between experimental and control flies (Fig 5B). Thus, HB-eyelet output modestly but

significantly shifted the phase of free running locomotor rhythms in time dependent manner.

Discussion

The Nature of the eyelet to LNv connections

The HB-eyelets have long been implicated as a circadian light input pathway, based on anatomical and genetic evidence (Helfrich-Förster et al., 2001; Helfrich-Förster et al., 2002; Malpel et al., 2002; Veleri et al., 2007). However, the apparent presence of both excitatory (ACh) and inhibitory (histamine) neurotransmitters in the eyelet (Pollack and Hofbauer, 1991; Yasuyama and Salvaterra, 1999) and the inability to observe the physiological responses of clock neurons during eyelet excitation, have precluded a determination of how the eyelet relays information about environmental light to the circadian clock neuron network (CCNN). Our observation of GRASP signals between the eyelet and the LN_v clock neurons both within the heart of the accessory medulla (AMe) and along it's ventral elongation are consistent with connections between the eyelet and both the large and small LN_vs, as projections of both the large and small LN_vs reside in the AMe while the large LN_vs likely give rise to the ventral elongation (Helfrich-Förster et al., 2007).

The expression of histamine within the eyelet nerve (Pollack and Hofbauer, 1991) and the histamine receptor HisCl in the large LN_vs but not the small LN_vs (Hong et al., 2006) suggests that the HB-eyelet likely inhibits the large LN_vs. Unfortunately, we were unable to address this possibility physiologically because our genetically encoded sensors were unable to detect histamine responses in the large LN_vs. This is consistent with our previous experience indicating that Ca^{2+} and cAMP sensors are often unable to detect inhibitory responses within the clock neuron network (Lelito and Shafer, 2012). It was clear, however, that the HB-eyelet does not reliably excite the large LN_vs, despite the presence of anti-ChAT immunosignals in the eyelet (Yasuyama and Salvaterra, 1999) and nicotinic ACh receptors in the large LN_vs (McCarthy et al., 2011; Lelito and Shafer, 2012). Absent direct physiological evidence for the inhibition of the large LNvs, it is possible that there is no connection between the eyelet and the large LN_vs and that the GRASP signal along the ventral elongation is an artifact. Nevertheless, we suggest that our GRASP results along with previous work establishing that the large LN_vs express inhibitory histamine receptors (Hong et al., 2006) are most consistent with a model in which histaminergic subsets of the eyelet nerve inhibit the large LN_vs through inhibitory synapses along the ventral elongation of the AMe.

The HB-eyelet develops from the simple larval eye, called Bolwig's organ, during metamorphosis (Helfrich-Förster et al., 2002; Malpel et al., 2002). The axons leading from larval eyes project into the larval brain where they form excitatory cholinergic synapses on the PDF expressing larval LN_vs (Yuan et al., 2011; Yao et al., 2012), cells that persist to become small LN_vs in the adult (Helfrich-Förster, 1997). Thus, the precursor of the HB-eyelet acts to excite the precursor of the small LN_vs in the larval brain. The expression of histamine in the eyelet nerve (Pollack and Hofbauer, 1991) suggested that this photoreceptor to clock neuron connection might be transformed from an excitatory to an inhibitory connection during metamorphosis. If this were the case, given our inability to measure inhibitory histamine responses in the adult LN_vs, we would expect that the excitation of the HB-eyelet would have no measurable effects on the small LN_vs of the adult brain. In contrast, the excitation of the eyelet, along with the histaminergic R8 photoreceptors of the compound eye, resulted in the reliable excitation of the small LN_vs, suggesting that the anti-ChAT immunosignals detected in the eyelet (Yasuyama and Salvaterra, 1999) reflect the presence of functional cholinergic axons in the adult eyelet nerve. These results suggest that light falling on the HB-eyelet results in the excitation of the s-LNvs along with increases in cAMP.

The small LN_vs play a critical role in the maintenance of circadian rhythms in sleep and activity (Renn et al., 1999) and the exogenous excitation of these neurons is sufficient for phase resetting of these behavioral rhythms under free-running conditions (Guo et al., 2014). Furthermore, light input from external photoreceptors in the absence of the deep brain photoreceptor cryptochrome is sufficient for the light induced phase resetting of such behavioral rhythms (Kistenpfennig et al., 2012). We therefore predicted that exogenous excitation of cholinergic output in the eyelet would induce phase shifts in the free-running rhythm of sleep and activity. Indeed, such excitation produced phase dependent advances in sleep activity rhythms. It is not clear why such excitation did not mimic the effects of light pulses or the excitation of PDF neurons under free-running conditions, both of which can produce both advances and delays when delivered at night. We note that unlike light pulses or exogenous LN_v excitation, our eyelet excitation experiments were characterized by relatively low excitation that would have been specific only to the small-LN_vs. This suggests that the activity of the large LN_vs is likely important for the production of the phase delays.

Our results indicate that the specific excitation of the small LN_vs by the HB-eyelet is sufficient to advance free running behavioral rhythms. These advances are likely produced through both the resetting of the molecular clock within the small LN_vs and through the modulation of subsets of the remaining clock neuron network through the

release of sLN_v PDF (Renn et al., 1999; Shafer et al., 2008), a peptide that serves to synchronize some but not all PDF-receptive clock neurons within the network (Peng et al., 2003; Lin et al., 2004; Im and Taghert, 2010; Yao and Shafer, 2014). Taken together, our results provide the first physiological evidence in support of the longstanding hypothesis that the HB-eyelet physiologically modulates the circadian clock neuron network. They also provide strong evidence that light input through the HB-eyelet specifically excites the critical small LN_vs to produce small phase changes in the clock neuron network and the sleep activity rhythms they produce. These results represent an important first step in understanding the network properties and physiological basis of light entrainment of the fly's circadian clock neuron network.

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Figure Legends

Figure 1 *Rh6*-GAL4 expression pattern (**A-F**) and GRASP experiments (**G-L**). *Rh6*-GAL4 is expressed in 70% of R8 of the compound eyes and in the HB-eyelet. The axons of R8 terminate in the distal part of the medulla and are in close vicinity to the dendritic arborizations of the lLN_v (**A+D**). The axons of the HB-eyelets directly innervate the accessory medulla and form a bouton-like structure. At least one of the 4 axons per hemisphere also innervates the ventral elongation of the accessory medulla which is formed by the lLN_v (**A-C**, **E+F**). **G-L** GRASP experiments revealed reconstituted GFP in the accessory medulla and its ventral elongation. Whereas we found a strong signal by expressing the split-GFP constructs using *rh6*-GAL4 and *pdf*-lexA (**H**) no reconstituted GFP was found in both of the controls (**G+I**). Reconstituted GFP was found within the accessory medulla and its ventral elongation allowing communication between the HB-eyelet with both PDF-positive neuron clusters (sLN_v and lLN_v) (**J-L**).

Figure 2 Application of histamine (Hist) did neither increase cAMP nor Ca^{2+} levels in the PDF-positive lateral neurons. Calcium imaging from small and large LN_v did not show an increase of the signal after Hist application (**A+B**). The same was true for cAMP for both neuronal subgroups (**C+D**). Using the Ca^{2+} and cAMP sensors we were only able to determine excitatory responses, whereas histamine might have an inhibitory function. To test this possibility we co-applied nicotine (Nic) to excite the neurons and investigated, whether histamine would decrease the Nic mediated response (**E-H**). We found increases in cAMP in both neuron clusters (sLN_v and lLN_v) upon the application of nicotine (**E+F**). The co-application of histamine did not result in a reduction of the cAMP-response (for quantification see **G+H**) in both neuron clusters.

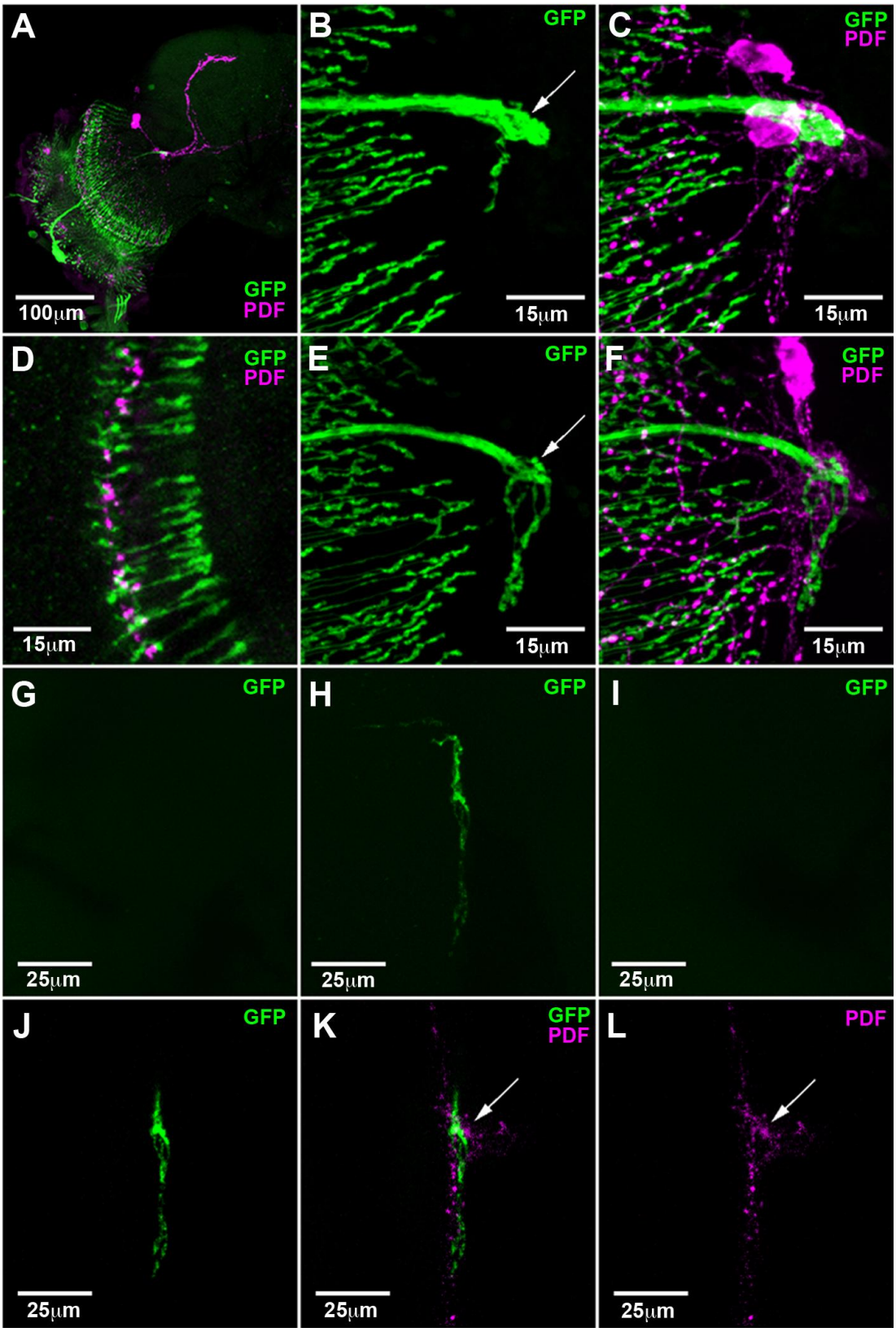
Figure 3 Calcium responses after activating the HB-eyelet using the P2X2 system. The application of ATP leads to the opening of the P2X2-channel and hence to the depolarization of the HB-eyelet. To test, whether we are able to activate the HB-eyelet we expressed the P2X2 channel as well as the Ca^{2+}-sensor in the eyelet and found a significant increase of Ca^{2+} upon ATP application in the eyelet (**A+B**). In a second set of experiments we again expressed the P2X2 channel in the HB-eyelet but imaged from the PDF-positive neurons. The application did have no effect on the Ca^{2+}-level in the lLN_v

(**C+D**) but significantly increased Ca^{2+}-levels in the sLN_v (**E+F**). In a control experiment lacking a driver line for the P2X2 no Ca^{2+}-increase was observed (**G+H**).

Figure 4 cAMP responses of the PDF-positive lateral neurons after activation of the HB-eyelet using the P2X2 system. The lLN_v did not show any response upon the application by ATP (**A+B**) whereas the sLN_v reacted with a significant increase of cAMP (**C-D**). This increase appeared again to be mediated by the activation of the HB-eyelet as the control experiments (lacking the driver for P2X2) showed only slight changes in cAMP levels upon ATP application in both neuronal subgroups (**E-H**).

Figure 5 Behavioral shifts after activating the HB-eyelet in the *hdc*^{JK910} mutant background. Flies were entrained in LD 12:12 at 20°C and in the first night after LD a heat pulse (2h 30°C) was applied during different time points of the day. **A** Single actograms of the experimental strain (*w*;*hdc*^{JK910};*rh6*-GAL4/UAS-*TrpA1*) and the UAS-control (*w*;*hdc*^{JK910};*UAS-TrpA1*/+). After entraining the flies in LD at 20°C a HP (red arrow) was applied from ZT 14-16 and flies were afterwards recorded for 10 days in constant darkness at 20°C. For each fly we determined the center of gravity (COG) for the entrained condition (green line) and the COGs following the HP (blue dots) as well as the free-running period (blue line). The experimental line significantly phase-advanced the COGs after the HP, whereas the control flies did not show any phase shift after applying the HP. **B** Quantification of the phase shift after activating the HB-eyelet. We applied a HP at 3 different time-points in the subjective night (upper panel) and 2 during the subjective day (lower panel). We observed a significant phase advance compared to both controls if a HP was applied between ZT14-16 and ZT 21-23, whereas we were not able to find differences if a HP was applied at the other time-points.

Figure 1: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G
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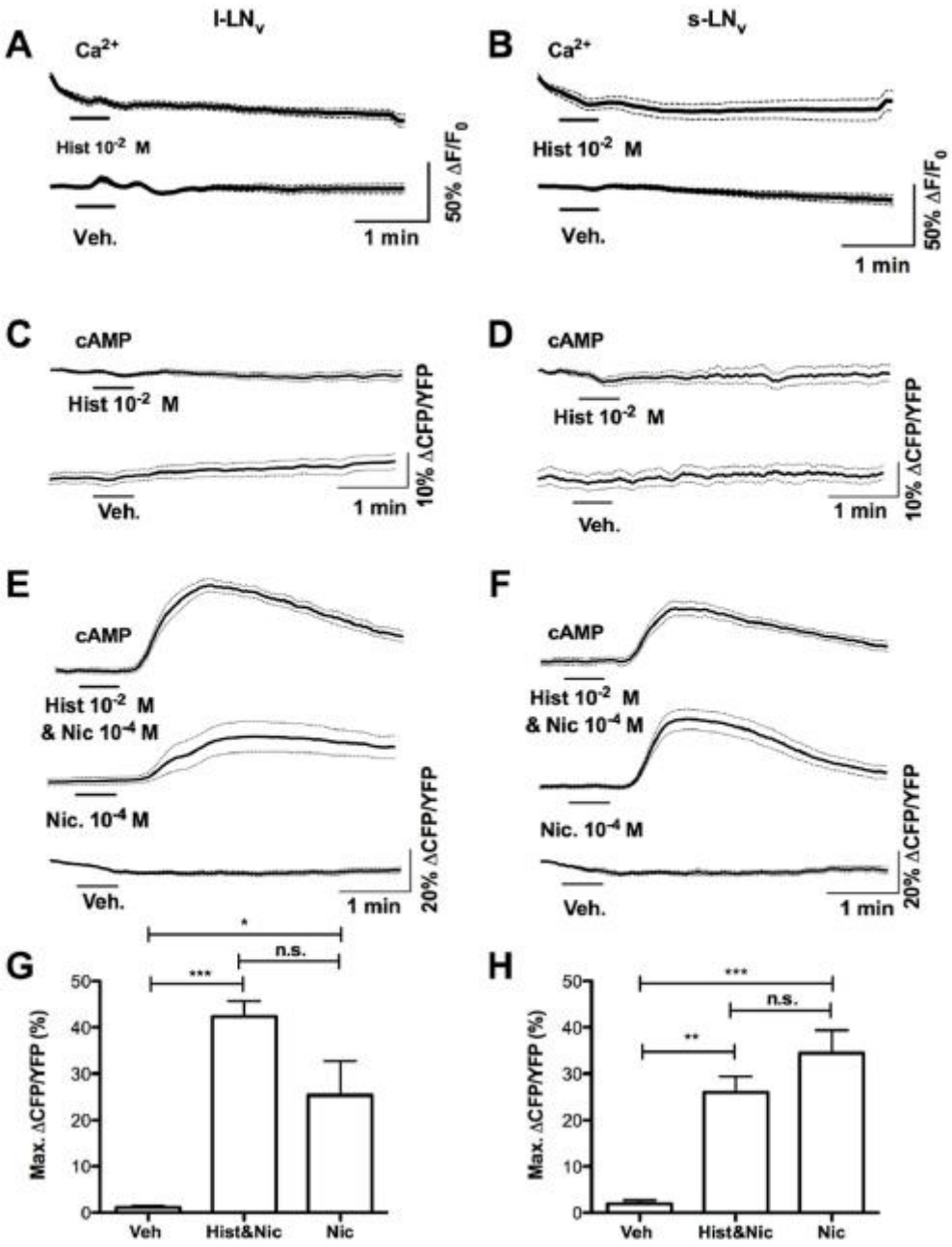
Figure 2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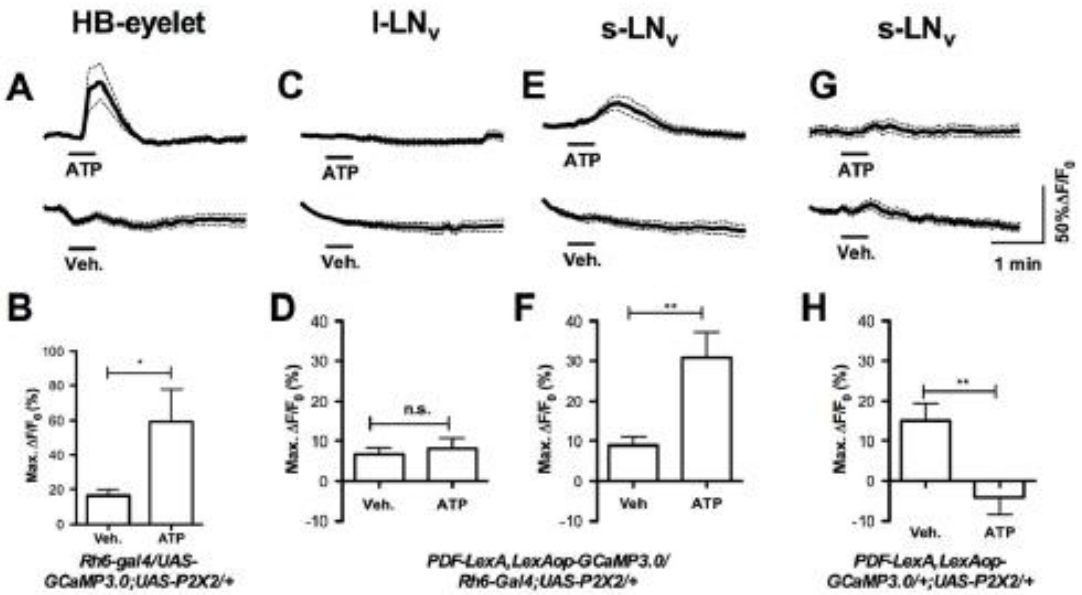
s-LN$_v$
A
B
Ca^{2+}
Hist 10^{-2} M
Veh.
1 min
50% ΔF/F$_0$
C
D
cAMP
10% ΔCFP/YFP
E
F
Hist 10^{-2} M & Nic 10^{-4} M
Nic. 10^{-4} M
20% ΔCFP/YFP
G
H
Max. ΔCFP/YFP (%)
Veh
Hist&Nic
Nic
n.s.
*
**
***

Figure 3


HB-eyelet
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s-LNv
s-LNv
A
C
E
G
ATP
Veh.
50%.ΔF/F0
1 min
B
D
F
H
Max. ΔF/F0 (%)
Veh.
ATP
n.s.
Rh6-gal4/UAS-GCaMP3.0;UAS-P2X2/+
PDF-LexA,LexAop-GCaMP3.0/Rh6-Gal4;UAS-P2X2/+
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Figure 4

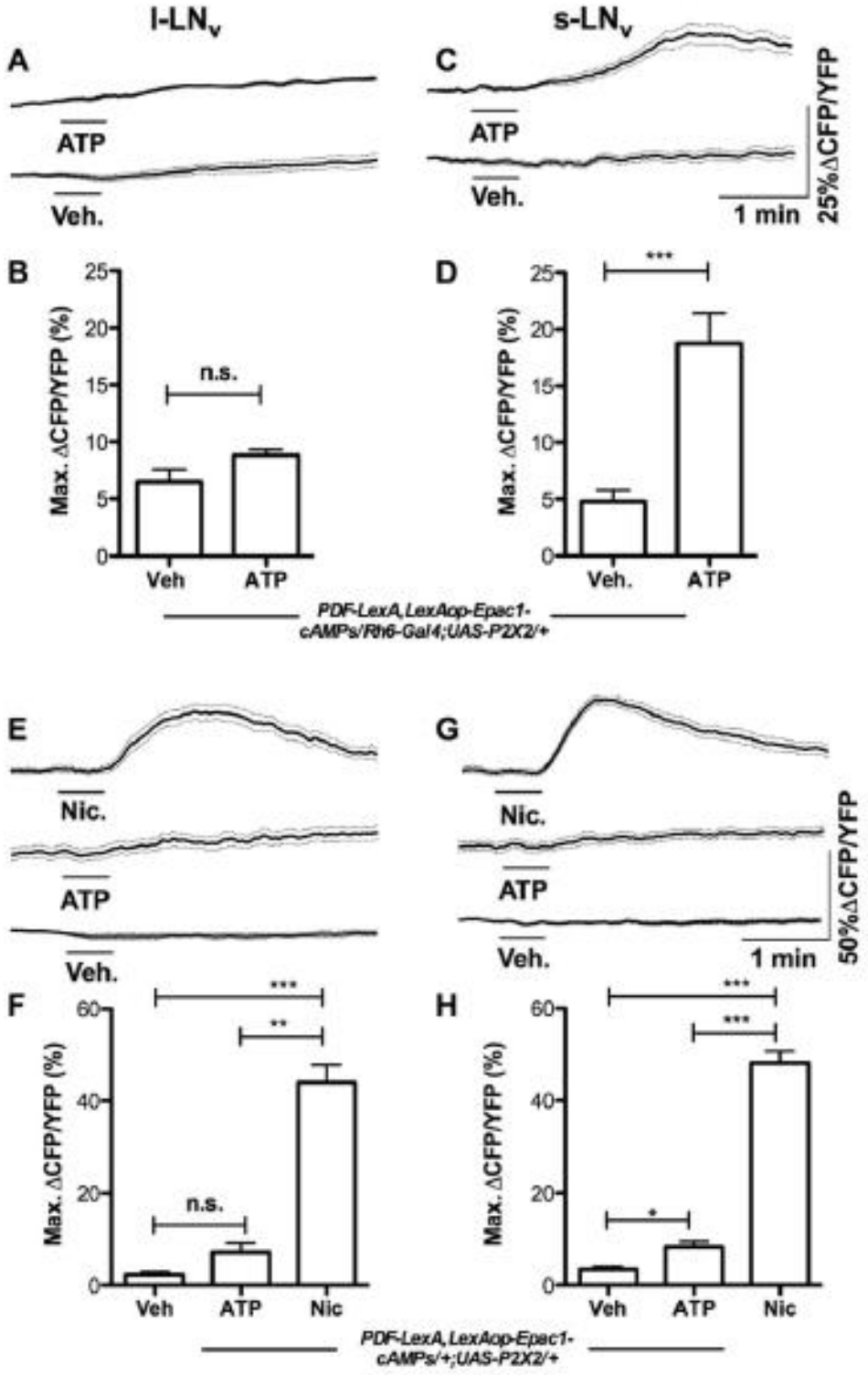
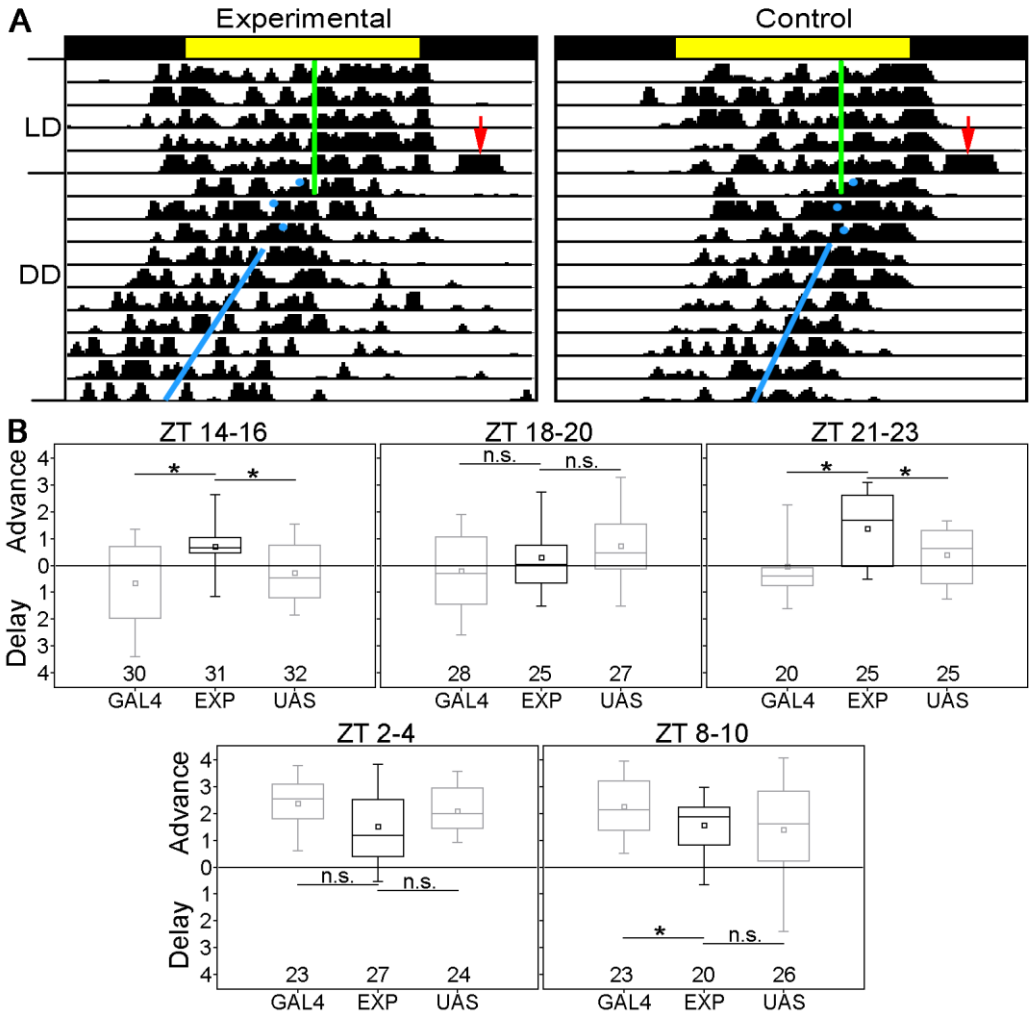

l-LN$_v$
s-LN$_v$
A
C
ATP
Veh.
1 min
25%ΔCFP/YFP
B
D
Max. ΔCFP/YFP (%)
n.s.
***
Veh
ATP
PDF-LexA,LexAop-Epac1-cAMPs/Rh6-Gal4;UAS-P2X2/+
E
G
Nic.
50%ΔCFP/YFP
F
H
**
*
Nic
PDF-LexA,LexAop-Epac1-cAMPs/+;UAS-P2X2/+

Figure 5


A
Experimental
Control
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DD
B
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ZT 18-20
ZT 21-23
Advance
Delay
n.s.
*
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31
32
28
25
27
20
25
25
GAL4
EXP
UAS
ZT 2-4
ZT 8-10
23
27
24
23
20
26

Acknowledgements

First of all I would like to thank Prof. Dr. Charlotte Förster for giving me the opportunity to work on this project under her supervision, thank you for your support and your infectious enthusiasm for science that made me go on in the field.

I am also very thankful to Prof. Dr. Simon Sprecher and Dr. Johannes Spaethe for being my Co-Supervisors for this project. Thank you for the fruitful discussions and supporting me whenever I had any question or problem.

Moreover I would like to thank Prof. Rodolfo Costa and Prof. Orie Shafer for the collaborations and the discussions which really helped a lot. Also a special thanks to all the co-authors of the papers included in this thesis. I further want to extend my gratitude to the members of the Department of Cellular and Developmental Biology at the University of Regensburg for "adopting" me while the rest of the Förster lab already moved to Würzburg.

Along this line I would like to thank all the members of AG Förster and AG Wegener for making such a great atmosphere at the University of Würzburg. A special thanks to Dr. Pamela Menegazzi, Dr. Christiane Luibl, Verena Dusik and Rudi Grebler for the help with experiments and the discussions. Furthermore I would like to thank Irina Wenzel, Petra Baron, Konrad Öchsner and Angelika Kühn for excellent technical assistence.

I am also very grateful to the Hanns-Seidel-Stiftung for funding the first years of my PhD. Due to the scholarship I got to know a lot of interesting people.

Last but not least I would like to thank my family for supporting me through all the years and encouraging me in every step I took.

Curriculum Vitae

Declarations

"Dissertation Based on Several Published Manuscripts"

Statement of individual author contributions and of legal second publication rights

Publication: Mazzotta G, Rossi A, Leonardi E, Mason M, Bertolucci C, Caccin L, Spolaore B, Martin AJ, Schlichting M, Grebler R, Helfrich-Förster C, Mammi S, Costa R, and Tosatto SC (2013) Fly cryptochrome and the visual system. PNAS 110:6163-6168.					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	G.M	S.M.	R.C.	S.C.E.T.	
Data Collection	G.M.	A.R.	E.L.	M.M.	M.S.
Data Analysis and Interpretation	G.M.	C.B.	R.C.		
Manuscript Writing	G.M.	C.B.	C.H.-F.	R.C.	S.C.E.T.

Publication: Schlichting M, Grebler R, Peschel N, Yoshii T, and Helfrich-Förster C (2014) Moonlight detection by *Drosophila*'s endogenous clock depends on multiple photopigments in the compound eyes. Journal of biological rhythms 29:75-86.					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	C.H.-F.	M.S.	T.Y.		
Data Collection	M.S.	R.G.	N.P.		
Data Analysis and Interpretation	M.S.	R.G.	C.H.-F.		
Manuscript Writing	M.S.	C.H.-F.			

Publication: Schlichting M, and Helfrich-Förster C (2015) Photic Entrainment in *Drosophila* Assessed by Locomotor Activity Recordings. In Methods in Enzymology,Sehgal B (Ed), Elsevier, pp105-123.					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	C.H.-F.	M.S.			
Data Collection	M.S.				
Data Analysis and Interpretation	M.S.	C.H.-F.			
Manuscript Writing	M.S.	C.H.-F.			

Publication: Schlichting M, Grebler R, Menegazzi P, Helfrich-Förster C Twilight dominates over moonlight in adjusting *Drosophila´*s activity pattern **In Press in the Journal of Biological Rhythms**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	C.H.-F.	M.S.			
Data Collection	M.S.	R.G.	P.M.		
Data Analysis and Interpretation	M.S.	C.H.-F.			
Manuscript Writing	C.H.-F.	M.S.			

Publication: Schlichting M, Menegazzi P, Helfrich-Förster C Normal vision can compensate for the loss of the circadian clock. **Submitted to BMC Biology**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	M.S.	C.H.-F.			
Data Collection	M.S.	P.M.			
Data Analysis and Interpretation	M.S.	P.M.	C.H.-F.		
Manuscript Writing	M.S.	C.H.-F.	P.M.		

Publication: Schlichting M#, Grebler R#, Mason M, Fekete A, Menegazzi P, Mazzotta G, Costa R, Helfrich-Förster C Cryptochrome plays different roles in the fruit fly´s compound eyes and clock neurons. **Submitted to PNAS**					
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Study Design	C.H.-F.	R.C.	M.S.	R.G.	
Data Collection	M.S.	R.G.	M.M.	A.F. P.M.	G.M.
Data Analysis and Interpretation	M.S.	R.G.	P.M.	G.M.	C.H.-F.
Manuscript Writing	C.H.-F.	R.C.	R.G.	M.S.	

Publication: Schlichting M#, Lelito KR#, Denike J, Helfrich-Förster C, Shafer OT Rhodopsin five and six expressing photoreceptors modulate the small ventral lateral neurons and shift the free-running circadian rhythms of *Drosophila melanogaster*. **Close to submission**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	O.T.S.	C.H.-F.			
Data Collection	M.S.	K.R.L	J.D.		
Data Analysis and Interpretation	M.S.	K.R.L	J.D.		
Manuscript Writing	O.T.S.	C.H.-F.	M.S.	K.R.L.	

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I also confirm my primary supervisor's acceptance.

Matthias Schlichting 02.04.2015 Würzburg

Doctoral Researcher's Name Date Place Signature

Affidavit

I hereby confirm that my thesis “Light entrainment of the circadian clock: the importance of the visual system for adjusting *Drosophila melanogaster´*s activity pattern” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Hiermit erkläre ich an Eides statt, die Dissertation „Lichtentrainment der Inneren Uhr: Die Bedeutung des visuellen Systems für die Anpassung des Aktivitätsmusters von *Drosophila melanogaster*.“ eigenhändig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
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Würzburg, 02.04.2015 _____
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