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MINI-REVIEW

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Circadian light-input pathways in *Drosophila*

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ABSTRACT

Light is the most important environmental cue to entrain the circadian clock in most animals. In the fruit fly *Drosophila melanogaster*, the light entrainment mechanisms of the clock have been well-studied. The *Drosophila* brain contains approximately 150 neurons that rhythmically express circadian clock genes. These neurons are called "clock neurons" and control behavioral activity rhythms. Many clock neurons express the Cryptochrome (CRY) protein, which is sensitive to UV and blue light, and thus enables clock neurons deep in the brain to directly perceive light. In addition to the CRY protein, external photoreceptors in the *Drosophila* eyes play an important role in circadian light-input pathways. Recent studies have provided new insights into the mechanisms that integrate these light inputs into the circadian network of the brain. In this review, we will summarize the current knowledge on the light entrainment pathways in the *Drosophila* circadian clock.

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Circadian pacemaker neurons in the *Drosophila* brain

Most animals possess circadian clocks that measure the time of day and allow the organism to adapt to daily environmental changes. The clock generates circadian rhythms of approximately 24 hours in many biological processes, such as behavior, metabolism, and physiology, enabling animals to anticipate and adapt to environmental changes. The oscillatory mechanism of the clock is self-sustained but must be synchronized to external time cues in order to allow adaption to changing environmental conditions. In most cases, light is the most critical entrainment cue and must be integrated into circadian clock circuits in the brain.

The brain of most animals contains a central clock. In the fruit fly *Drosophila melanogaster*, approximately 150 neurons in the brain have been identified as clock neurons based on the cyclic expression of the genes and proteins that play central roles in the circadian clock. These molecules are referred to as clock genes or proteins. Clock neurons are located in distinct clusters in the *Drosophila* central brain. Each cluster is named according to its location and the size of individual neurons, as shown in Figure 1. It remains unclear whether all clusters of clock neurons are important for controlling behavioral rhythms; however, several studies have suggested that they are not all of equal importance.[2]

The best-studied clock neurons are the large and small lateral ventral neurons (l-LN_vs and s-LN_vs). These neurons express Pigment-dispersing factor (PDF), a neuropeptide that acts as a circadian neuromodulator.[3] PDF plays an important role in the circadian network as an intercellular messenger, synchronizing daily rhythms between PDF neurons and other clock neurons.[4-8] *Pdf*-null flies display weak circadian activity rhythms with a shortened period of approximately 22 hours when kept in constant darkness (DD) and a phase-advanced evening activity peak in the presence of a 12-hour:12-hour light-dark cycle (LD).[9] Interestingly, flies lacking the l-LN_v and s-LN_v neuron clusters show identical behavioral phenotypes, suggesting that PDF is the principal output of these neurons. Thus, PDF-expressing clock neurons as well as PDF itself strongly influence activity rhythms.

The Gal4/UAS system is a genetic tool commonly used for tissue-specific manipulation of gene expression in *Drosophila*.[10] Several useful Gal4 lines have been available since the year 2000 that target subsets of clock neurons, allowing us to study their function. Pioneering studies using the Gal4/UAS system were performed by Grima et al. and Stoleru et al. in 2004.[11,12] These studies investigated different subsets of clock neurons by restoring clock

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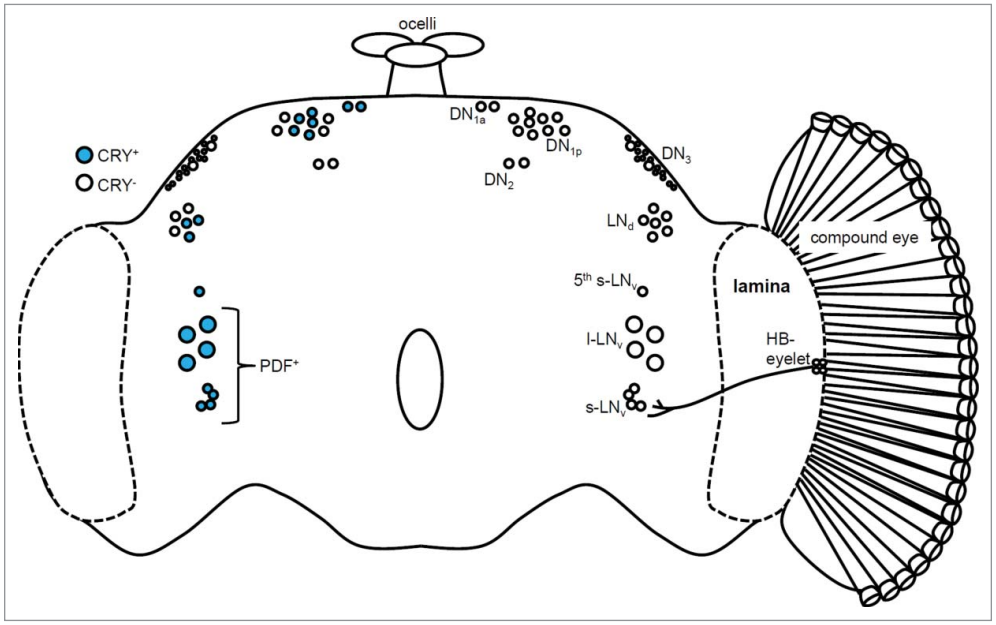

Figure 1. The circadian clock network in the *Drosophila* brain. The left hemisphere shows the distribution of the Cryptochrome (CRY)-positive (blue) and CRY-negative (white) clock neurons. The right hemisphere shows the compound eye and the Hofbauer-Buchner (HB) eyelet. The ocelli are located on top of the brain. The small and large lateral neuron (s-LN_v and l-LN_v) clusters express the Pigment-dispersing factor (PDF) protein and are regarded as the clock neurons controlling the morning (M) activity peak. The lateral dorsal neurons (LN_d) and the 5th s-LN_v are the evening (E) clock neurons. The dorsal neuron (DN) clusters are not well characterized. The HB eyelet is directly connected to the s-LN_v neurons through projections to the surrounding area. In contrast, the connections between the compound eyes and the clock neurons have not yet been elucidated.

function to specific neuron clusters in the clock-impaired *period* (*per*) mutant or by ablating clusters expressing the apoptosis-inducing *head involution defective* gene. The flies without functional PDF neurons lost anticipatory morning (M) activity, whereas the flies without functional lateral dorsal (LN_d) and dorsal neuron (DN) clusters lost anticipatory evening (E) activity under LD conditions. Thus, both studies concluded that the PDF-positive clock neurons are important for morning activity, and LN_d and DN clusters are important for evening activity. In 2006, the 5th s-LN_v was identified as a non-PDF-expressing clock neuron that was located in close proximity to the l-LN_v cluster and was classified as an E clock neuron.[13]

CRY function in clock neurons

The structure of the CRY protein is very similar to that of the bacterial 6–4 photolyase. The CRY protein was first identified in *Arabidopsis thaliana*,[14] suggesting that it is highly conserved. *Cry^b* mutant flies display failures in light entrainment.[15-17] Whereas wild-type flies are able to synchronize their circadian rhythms to a new LD condition within one day, *cry* mutant flies require approximately 7 d to adapt. The circadian clock responds to a light pulse during the night by advancing or delaying activity rhythms, depending on the time at which the light-pulse is given. These responses can be described by a phase-response curve. Compared to wild-type flies, *cry^0* mutants are significantly less sensitive to light pulses and display reduced phase responses.[18]

CRY is expressed in many clock neurons, including M and E neurons.[19,20] Upon light exposure, CRY binds to the Timeless (TIM) protein, an essential clock component, and leads to its ubiquitination by the Jetlag protein and subsequent degradation.[21,22] The light-induced degradation of TIM destabilizes the Period (PER) protein, another core clock component and a binding partner of TIM, and thus pauses the circadian oscillation of PER and TIM levels.

These interactions between the PER, TIM, and CRY proteins provide a simple and clear-cut explanation of how the *Drosophila* clock is reset by light. However, not all clock neurons express CRY, and levels of CRY expression differ among clock neurons.[20] Furthermore, not all clock neurons show similar responses to light-pulses given during the night.[23] For example, Tang et al. (2010) demonstrated that a light pulse early in the night induces degradation of TIM in E neurons, but not in M neurons, suggesting that the phase resetting of the clock by a light pulse is not the same in all clock neurons and depends on the time of day.[23] More interestingly, CRY-dependent light input to M neurons can cause CRY-independent TIM degradation in E neurons.[24] Thus, M neurons

can direct the light response of E neurons through neuronal communication.

A bright light pulse lasting for several minutes during the night is sufficient to induce TIM degradation in all clock neurons.[25,26] In response to a light pulse of reduced intensity and increased duration, the 5th s-LN$_v$ neuron shows the most efficient TIM degradation compared to other clock neurons,[27] suggesting that individual clock neurons differ in CRY-dependent light sensitivity. These studies used light pulses to examine light responses, revealing fine temporal properties of the clock in response to a short light-pulse. However, responses to light pulses differ from entrainment to LD conditions that consist of distinct periods of day and night.

When CRY is re-expressed in E neurons using the Gal4/UAS system in the *cry* mutant background, these flies show responses similar to those of wild-type flies to an 8-hour phase delay of the LD cycle, re-entraining nearly within one day.[26] In contrast, *cry^0* mutants require nearly a week to completely synchronize to the phase-delayed LD cycle. Interestingly, E neurons in *cry^0* mutants show rapid re-entrainment of molecular cycling of the clock protein Par Domain Protein (PDP1). However, re-entrainment of clock protein cycling in other clock neurons occurs slowly, similar to re-entrainment of behavioral rhythms.[26] Thus, E neurons are the first to reset in response to light input from the visual system, followed by other clock neurons.

Light-activated CRY also influences the neural activity of l-LN$_v$ neurons by increasing action potential firing.[28,29] This effect is independent of the CRY-TIM interaction and opsin-based photoreception. The role of these CRY-induced increases in neuronal activity is thus far unknown, but a possible involvement in light entrainment should be considered.

Light entrainment via the visual system

Drosophila have 3 different external photoreceptors: compound eyes, ocelli, and Hofbauer-Buchner eyelets (HB eyelets). The compound eyes are the largest photoreceptive structure and are thought to be the most important to light entrainment.[30] Studies using eye mutants have demonstrated that the compound eyes play roles in measuring day length and detecting moonlight.[31-33] Flies display 2 distinct activity peaks at approximately dawn and dusk in 12 hour:12 hour LD cycles, termed the M and E peaks, respectively.[34] These 2 activity peaks respond to changing photoperiod and to dim light during the night. Under long-day conditions or during nights with moonlight, the M and E peaks move away from one another, thus creating a larger phase angle between the peaks.[2] *Drosophila* mutants lacking compound eyes cannot adapt their M and E activity peaks to long-day conditions.[31] and do not respond to moonlight.[32,33] These findings suggest that the light input from the visual system affects the M and E neurons in opposite manners, namely phase-advances the M oscillator and phase-delays the E oscillator. Moonlight also has direct effects on activity, known as masking effects, in that it increases nighttime activity levels.[35] These masking effects are mediated by the compound eyes but are not influenced by CRY.[31,33] The compound eyes consist of approximately 800 ommatidia, each of which contains 8 photoreceptor cells (R1–8) expressing different rhodopsins (Rh1–6).[36] Mutant flies lacking Rh1 and Rh6, which are expressed in R1–6 and R8 cells, respectively, do not display masking effects on activity behavior in response to moonlight.[33]

Twilight, the gradual change in light intensity at dawn and dusk, also affects activity rhythms, though it is often neglected in laboratory experiments. Simulation of twilight conditions causes shifts of the M and E activity peaks into the dim light zones.[37] The effects of twilight on behavior exceed those of moonlight, and activity rhythms during twilight simulation more strongly resemble activity rhythms under natural conditions.[38-41] The effects of twilight on behavior are also mediated by the compound eyes, particularly by the 2 inner photoreceptor cells R7 and R8.[40] Thus, different light-sensing mechanisms have different roles in modulating and entraining activity rhythms.

The role of the ocelli in circadian light entrainment has not been well studied. Comparisons between mutants that lack all external photoreceptors or lack only the compound eyes showed significant contribution of the compound eyes to entrainment, compared to the minor contribution of the ocelli.[31] The same is true for the HB eyelets, which are remnants of the larval photoreceptors, the Bolwig's organs, and express the rhodopsin 5 and 6 (*rh5* and *rh6*) genes.[42,43] In both larval and adult brains, the projections of Bolwig's organs and HB eyelets directly contact the PDF-positive LN$_v$s.[30,44,45] The larval Bolwig's organs use acetylcholine as a neurotransmitter, whereas the adult HB eyelets express both acetylcholine and histamine.[43,46] Application of cholinergic agonists increases Ca^{2+} and cyclic AMP (cAMP) levels in both dissociated larval LN$_v$s and in adult LN$_v$ clusters in intact brains.[47,48] Bolwig's organs, together with CRY, are essential for light entrainment of the larval clock neurons.[49] However, it remains unclear how significantly HB eyelets contribute to light entrainment of the adult clock. Two studies have investigated the roles of the Rh5 and Rh6 proteins that are expressed in the HB eyelets and in photoreceptor cell R8 of the compound eyes.[50,51] Flies with

triple-mutation of *rh5, rh6*, and *cry* display slower light re-entrainment than *cry* single mutants in following a 6-hour phase shift of the LD cycle, suggesting that the HB eyelets contribute to light entrainment.

The compound eyes use histamine as a neurotransmitter. Flies with double-mutation of *cry* and the *hdc* gene encoding histidine decarboxylase are unable to synchronize to an LD cycle.[31] *Drosophila* express 2 histamine receptor genes: *ort* and *hisCl1*. Both receptors are expressed in interneurons located between the lamina and the medulla and may also be present in other brain regions.[52,53] Serotonin and dopamine may be involved in intermediate pathways.[54,55] However, it is not yet known how histamine receptor-positive cells transmit signals to clock neurons.

CRY is also expressed in the compound eyes. A recent study demonstrated that CRY interacts with the phototransduction complex through the Inactivation No Afterpotential D protein in the compound eyes.[56] *Cry* mutant flies show reduced nocturnal light sensitivity in electroretinograms and a weaker optomotor response compared to wild-type flies. However, CRY expressed in the eyes may have a minor contribution to light entrainment, since flies expressing *cry* only in the eyes do not show significant improvement in entrainment compared to *cry* mutants.[26,57]

PDF neurons and light entrainment

PDF is a circadian neuropeptide specifically expressed in the l-LN_v and s-LN_v clusters and serves as an intercellular communication signal between clock neurons.[3,6] The PDF receptor (PDFR) is expressed in many clock neurons including the s-LN_vs.[58] *Pdf* mutants or flies lacking PDF-expressing neurons show a phase-advanced E activity peak under LD conditions.[9] They are also incapable of shifting M and E peaks under long-day conditions.[7] These phenotypes are similar to those observed in eye mutants.[31] Furthermore, ablation of the l-LN_v cluster attenuates the response to a light-pulse late in the night.[59] These results suggest that PDF and the PDF neurons are important for light entrainment.

Flies with double-mutation of *Pdf* and *cry* show an intriguing phenotype, completely lacking the E peak under LD conditions.[60,61] This phenotype suggests a model in which CRY and the visual system entrain the M and E neurons by either direct or indirect mechanisms; furthermore, PDF-expressing neurons transduce light input from the visual system to E neurons, such that E neurons in *Pdf-cry* double mutants are unable to receive light input. Im and Taghert (2011) additionally demonstrates that the M peak in the double mutants is a masking effect but is not driven by the circadian clock, indicating that PDF and CRY are essential for light entrainment.[62]

Thermogenetics is a suitable genetic tool for the manipulation of clock neuron activity because the effect of temperature on the clock is relatively moderate compared to that of light, which is used for optogenetics. TrpA1 is a temperature-sensitive cation channel that induces neuronal depolarization in response to increasing temperature.[63] Interestingly, it appears that phase-response curves in response to temperature pulses applied to flies overexpressing *trpA1* in PDF neurons mimic phase-response curves in response to light pulses.[64] Thus, light inputs, likely from the visual system, may normally excite PDF neurons, consequently resetting downstream neurons such as the E neurons and leading to behavioral phase shifts. This pathway may be mediated by specific adenylate cyclases, cAMP, and the protein kinase A signaling cascade that promotes TIM degradation in E neurons.[64-67] The neuronal circuits linking the visual system to the clock neurons have not yet been described, with the exception of the direct connection between HB eyelets and PDF neurons described above. It has not yet been demonstrated that light inputs from all visual organs converge on the PDF neurons, not on the E neurons, which would support the hypothesis that PDF neurons provide the only pathway for light inputs to the clock that are not mediated by CRY. This has already been shown for the larval brain, which has simpler circadian circuits involving only 18 clock neurons and only 2 light-input pathways: Bolwig's organs and the CRY protein.[49]

Conclusions

Previous studies have revealed the astonishing complexity of light entrainment in the circadian system of the fly, which has otherwise been considered a rather simple organism. The compound eyes convey light signals to the PDF neurons via histaminergic signaling through interneurons, leading to the resetting of the molecular clock in the PDF neurons. Bolwig's organs and HB eyelets use both acetylcholine and histamine to signal to PDF neurons; this pathway seems to consist of a direct connection between the visual organs and clock neurons. Both pathways first reset PDF-expressing neurons, which in turn reset the PDF-negative clock neurons. CRY is expressed in the clock neurons and directly interacts with TIM to reset the molecular clock. Interestingly, light entrainment of the E neurons is especially important for behavioral rhythms. Although more detailed studies are required for an exhaustive understanding of the complete mechanism, including unknown light-input pathways,[68,69] the relentless efforts of many researchers in our field have been steadily revealing new details on how the *Drosophila* light-input systems entrain the neuronal clock network (Fig. 2).

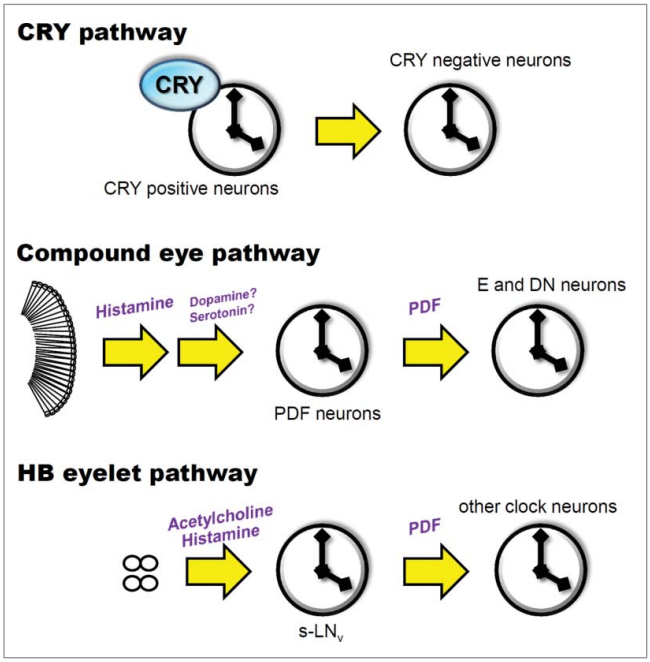

Figure 2. Three light-input pathways to the *Drosophila* clock. CRY is expressed in many clock neurons, which transmit light information to CRY-negative clock neurons. Histamine, dopamine, and serotonin have been suggested as neurotransmitters that convey light inputs from the compound eyes. The HB eyelets use acetylcholine and histamine as neurotransmitters and directly target the PDF neurons. PDF plays a role in intercellular communication between the PDF neurons and other clock neurons that express the PDF receptor.

Abbreviations

CRY	Cryptochrome
cyclic AMP	cAMP
DD	constant darkness
DN	dorsal neuron
E	evening
HB eyelets	Hofbauer-Buchner eyelets
LD	light-dark
LN	lateral neuron
M	morning
PDF	Pigment-dispersing factor
PDP1	Par Domain Protein 1
PER	Period
TIM	Timeless

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