

# Entrainment of the *Drosophila* clock by the visual system

Matthias Schlichting 

Howard Hughes Medical Institute, Brandeis University, Waltham, MA, USA.

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**ABSTRACT:** Circadian clocks evolved as an adaptation to the cyclic change of day and night. To precisely adapt to this environment, the endogenous period has to be adjusted every day to exactly 24 hours by a process called entrainment. Organisms can use several external cues, called zeitgebers, to adapt. These include changes in temperature, humidity, or light. The latter is the most powerful signal to synchronize the clock in animals. Research shows that a complex visual system and circadian photoreceptors work together to adjust animal physiology to the outside world. This review will focus on the importance of the visual system for clock synchronization in the fruit fly *Drosophila melanogaster*. It will cover behavioral and physiological evidence that supports the importance of the visual system in light entrainment.

**KEYWORDS:** *Drosophila melanogaster*, Circadian Clock, Entrainment, Light

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**CORRESPONDING AUTHOR:** Matthias Schlichting, Howard Hughes Medical Institute, Brandeis University, 415 South Street, Waltham, MA 02453-2728, USA. Email: mschlichting@brandeis.edu

## Introduction

The switch from day to night is one of the most ancient environmental cues every organism is confronted with. As an adaptation to this predictable change, circadian clocks have evolved in many organisms and allow them to predict dusk and dawn. Work over the last several decades has shown that the key mechanism underlying circadian rhythms is a transcriptional–translational feedback loop. In a simplified view, transcriptional activators (Clock [CLK] and Cycle [CYC]) activate the transcription of hundreds (if not thousands) of clock-controlled genes. These include other genes encoding important proteins for time-keeping (Period [PER] and Timeless [TIM]), which act as transcriptional inhibitors: once their protein concentrations peak toward the end of the night, they inhibit their own transcription and hence start a new cycle (reviewed by Hardin<sup>1</sup> and by Top and Young<sup>2</sup>).

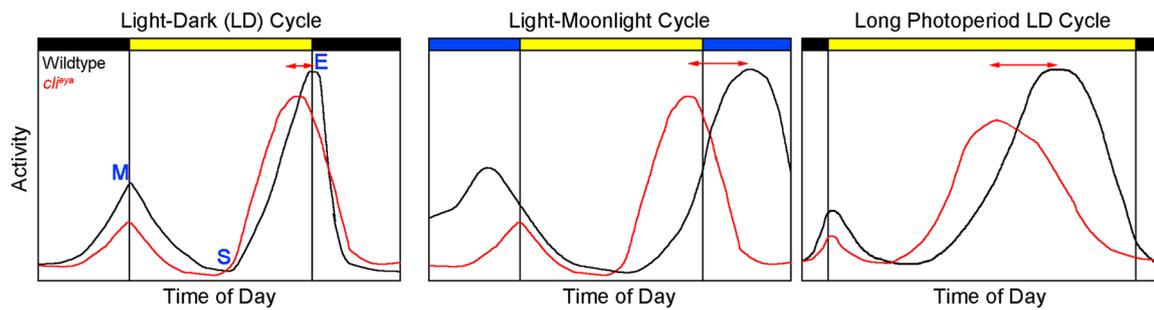
In the fruit fly, this molecular mechanism is expressed in a specialized area of the brain, the central clock, which comprises approximately 75 pairs of neurons.<sup>3</sup> These neurons orchestrate the behavioral output of the fly, which is characterized by a morning (M) peak, a siesta (S), and an evening (E) peak of activity (Figure 1A). Subpopulations of clock neurons are implicated in specific features of this behavioral output. The small ventral lateral neurons (sLN<sub>v</sub>s) are essential for the M peak,<sup>4,5</sup> whereas 3 of the 6 lateral dorsal neurons (LN<sub>d</sub>s) and the fifth sLN<sub>v</sub> are important for E activity.<sup>4,5</sup> The dorsal neurons 1 (DN<sub>1</sub>s) and the lateral posterior neurons (LPN<sub>s</sub>) are implicated in temperature entrainment<sup>6,7</sup> and were recently identified to regulate sleep and siesta,<sup>8–11</sup> whereas the DN<sub>2</sub> neurons are associated with temperature entrainment and temperature preference rhythms.<sup>12,13</sup> The DN<sub>3</sub> subgroup is not extensively studied yet; however, *nocturnin* expression in these neurons appears to contribute to light-evoked behaviors in the fly.<sup>14</sup> More recent work suggests, however, that complex interactions of these neurons regulate fly behavior.<sup>15–22</sup>

A key feature of circadian clocks is their ability to adjust to the switch from day to night by a process called entrainment. To do so, the central clock can use external stimuli, called zeitgebers, which will allow the animal to predict dawn or dusk (reviewed by Golombek and Rosenstein<sup>23</sup>). The most potent zeitgeber is light, which can be received via the visual system of the fly or via the circadian photoreceptor cryptochrome (CRY). The latter is expressed within the *Drosophila* clock neurons (most lateral and some dorsal neurons), as well as some other brain areas and the visual system,<sup>24,25</sup> and its function has been extensively studied. On illumination, CRY degrades TIM via the proteasome pathway and thereby “resets” the clock at the beginning of the day under regular light–dark (LD) conditions (reviewed by Foley and Emery<sup>26</sup>). However, not all clock cells express CRY and flies deficient for CRY are still able to entrain to LD cycles, demonstrating the importance of the visual system for clock synchronization.<sup>27–29</sup> This is further supported by the observation that both, CRY expression and the visual system, need to be ablated before a fly fails to entrain to LD cycles.<sup>30</sup> Over the past several years, a number of studies have deepened our understanding of how entrainment via the visual system impacts *Drosophila* at both behavioral and physiological levels. These studies are the focus of this review.

## Subpopulations of Photoreceptor Cells Contribute to Clock Synchronization

The visual system of *Drosophila* consists of 7 eye structures: 3 ocelli, 2 Hofbauer–Buchner (HB) eyelets, and 2 compound eyes.<sup>31</sup> The most prominent organs are the compound eyes, which are comprised of approximately 800 identical structural units, which are called ommatidia. Each ommatidium contains 8 photoreceptor cells (R). R1–6 are located in the periphery, span the whole length of the ommatidium, and send their axons into the lamina. R7 and R8 are located in the





**Figure 1.** Activity profiles of wild-type and *eyes absent* (*cliPya*) flies at different light conditions. (A) Wild-type flies show a bimodal activity pattern in LD 12:12 with a morning (M) peak at lights-on and an evening (E) peak at lights-off. Both peaks are clearly separated by a siesta (S) during which flies tend to sleep. *Eyes absent* flies also show a bimodal activity pattern with a reduced M peak amplitude and an advanced E peak. (B) Moonlight simulation phase-advances the M peak and phase-delays the E peak in wild-type flies, whereas the activity profile of *eyes absent* flies remains unchanged compared to A. (C) Behavior of wild-type and *eyes absent* flies in long day conditions. Under long days, flies adjust their behavior by delaying the timing of the E peak. The timing of the peaks is uncoupling from lights-off in both cases. Flies without eyes shift the activity peak about 1.5 hours less than wild-type flies. LD indicates light–dark.

center, with R7 in the distal and R8 in the proximal part of the eye. Both cells send their projections to the medulla (reviewed by Montell<sup>32</sup>). Each receptor cell has a microvillar structure called rhabdomere. It contains the light-absorbing molecules (rhodopsins, Rh) as well as the molecular machinery to convert light information into action potentials. In short, a photon activates the rhodopsin by converting cis-retinal into all-trans retinal. This change leads to the activation of the heterotrimeric G-protein phospholipase C (encoded by the *norpA* gene) and to the opening of Trp and Trp1 channels. When opened, these channels allow Na<sup>+</sup> and Ca<sup>2+</sup> to enter and depolarize the cell, which leads to the release of histamine at the synapse.<sup>32</sup> The compound eyes express 5 different rhodopsins in a well-defined manner: R1–6 express Rh1, whereas the central receptor cells express Rh3 to Rh6. In approximately 30% of the ommatidia (pale subtype), R7 express Rh3 and R8 express Rh5. In 70% of the ommatidia (yellow subtype), R7 express Rh4 and R8 express Rh6.<sup>33</sup> An exception to this rule is the dorsal rim area, in which both R7 and R8 express Rh3.<sup>34</sup>

The first evidence of the compound eyes being involved in light entrainment comes from photoreceptor mutants. Under equinox conditions, wild-type flies show a bimodal activity pattern with an M peak at lights-on and an E peak at lights-off (Figure 1A). Flies lacking compound eyes (*cli<sup>eya</sup>*) or flies deficient in *norpA* show an advanced timing of the E peak and a decreased M peak amplitude.<sup>35,36</sup> They further lack the ability to appropriately entrain to different photoperiods<sup>36–38</sup> (Figure 1C).

Over the past years, several experiments have addressed the importance of specific photoreceptors for clock synchronization. Key experiments included the investigation of nature-like light conditions: simulation of moonlit nights shifted the E peak of activity and caused an increase in the nocturnal activity levels.<sup>39</sup> The compound eyes are essential

for this behavioral transition. In particular, flies lacking Rh1 or Rh6 showed strongly reduced nocturnal activity in this condition<sup>39,40</sup> (Figure 1B). Similar results were obtained by investigating twilight conditions.<sup>41,42</sup> Even though these experiments demonstrated the contribution of specific rhodopsins to light synchronization, they did not address the question of whether photoreceptor subtypes are sufficient to entrain the circadian clock via the visual system. To answer this question, the Rouyer lab rescued *norpA* in specific photoreceptor subtypes in a *norpA<sup>P24</sup> cry<sup>02</sup>* mutant background.<sup>43</sup> These mutants failed to re-entrain to phase-shifted LD cycles. Rescue of *norpA* in all but the *rh5*-positive photoreceptors (and the ocelli) allowed the fly to re-synchronize to an altered light regime. Notably, the rescue appeared to be more efficient in phase advancing the clock than phase delaying it.<sup>43</sup> The key to these experiments was using low light intensities as flies lacking *norpA* and *cry* (*norpA<sup>P41</sup> cry<sup>b</sup>*, similar to the mutants above) are still able to entrain to LD cycles of higher intensities.<sup>28,44</sup> This suggested a *norpA*-independent pathway in the retina for clock synchronization. Most interestingly, this independent pathway was localized to the Rh5 and Rh6 expressing photoreceptors: quadruple mutants for *norpA*, *rh5*, *rh6*, and *cry* fail to re-entrain to phase-shifted LD cycles.<sup>44</sup> Recent work also demonstrated that the Rh5 and Rh6-positive receptors dominate the residual electroretinogram response of *norpA* mutants and suggested the importance of PLC21C, the second phospholipase C of the fly genome, as the target of these rhodopsins.<sup>45</sup>

Besides the *norpA*-independent pathway, Rh6-expressing cells appear to be key neurons that drive many circadian behaviors. Flies lacking Rh6 fail to appropriately adjust to high light intensities and fail to appropriately adjust to long photoperiods.<sup>37,46</sup> These data suggest that the Rh6-expressing photoreceptors might be similar to the mammalian retinal ganglion cells, as both are essential for high light

intensity entrainment. This notion is further supported by a recent study demonstrating that these cells not only act as photoreceptors but also as interneurons via the histamine receptor *HisCl*.<sup>47</sup> Flies lacking histamine receptors display a deficiency to re-entrain to shifted LD-cycles, which could be improved by rescuing *HisCl* in *rh6*-positive photoreceptors.

However, Rh6 is not only expressed in the compound eyes but also in the Hofbauer-Buchner eyelets. These photoreceptors are located underneath the retina and are comprised of 4 receptor cells. They are the remainders of the larval eyes, Bolwig organ, which undergoes complex reorganization during pupal development.<sup>48</sup> The eyelets appear to be histaminergic as well as cholinergic and contribute to light synchronization.<sup>49</sup> As mentioned previously, flies deficient of *norpA* and *cry* are able to re-synchronize to high light intensity; however, additional blocking of neurotransmitter output from *rh5*-GAL4 positive neurons (the driver is expressed in the eyelets) strongly reduced this ability.<sup>50</sup> Also, the inability to adjust to high light intensities appears to be due to the eyelets; flies lacking compound eyes (*cli<sup>eya</sup>*) but retaining eyelets can adapt to high light intensities, whereas the additional removal of Rh6 (*cli<sup>eya</sup>; rh6<sup>1</sup>*) renders the flies blind to high light intensities.<sup>46</sup>

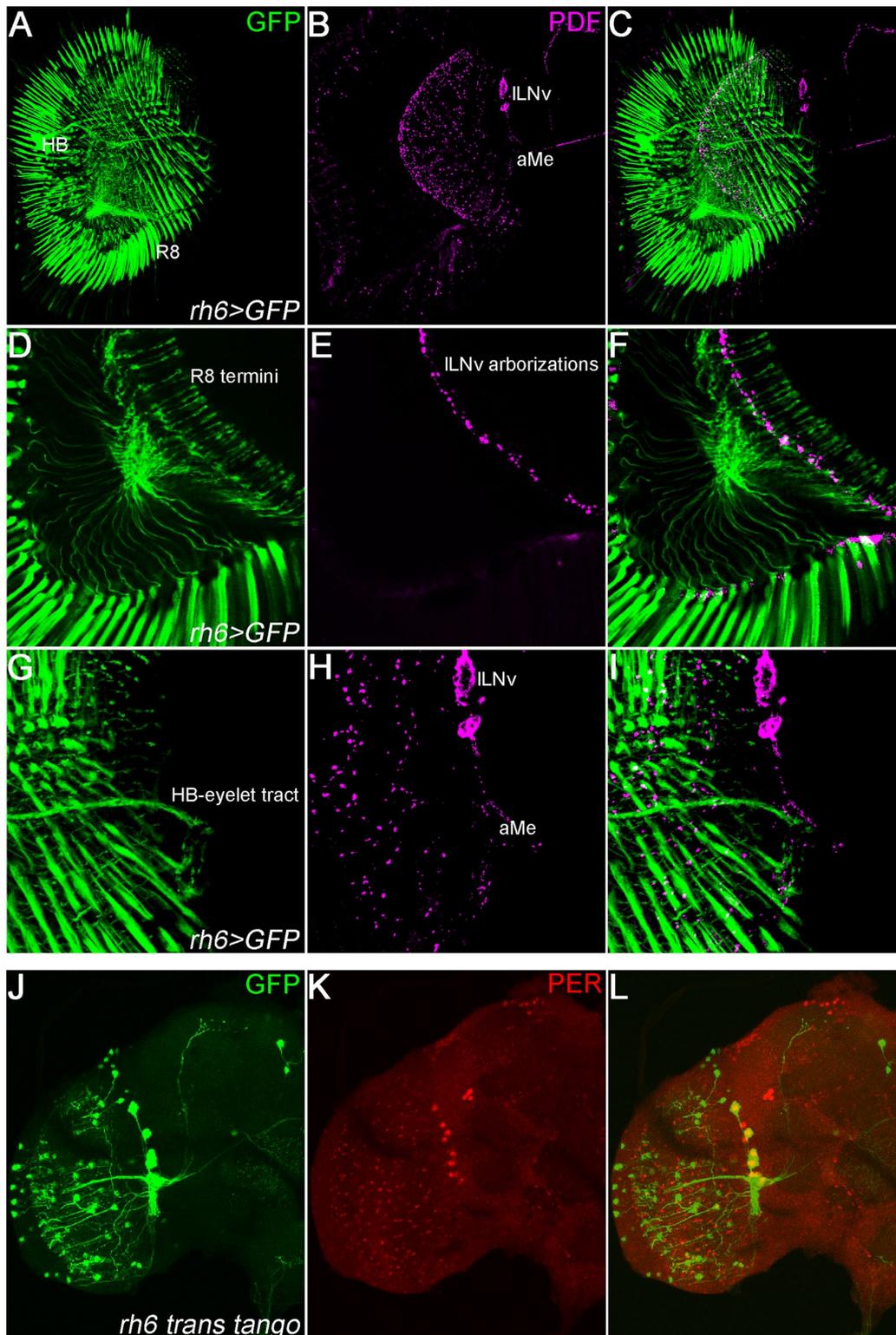
### The Photoreceptors Target Different Subsets of *Drosophila* Clock Neurons

The photoreceptors of the compound eyes terminate in different layers of the fly brain. R1-6 terminate in the lamina where they connect to interneurons which innervate the medulla. Both inner receptor cells directly innervate the medulla where they lie near the fibers of the pigment dispersing factor (PDF) neurons.<sup>32</sup> The HB-eyelets directly innervate the accessory medulla where they terminate close to the sLNvs<sup>48,51,52</sup> (Figure 2A-I). This anatomy suggested a direct connection of the visual system with the PDF neurons. This is indeed true for the HB-eyelet: GRASP (GFP Reconstitution Across Synaptic Partners) implicated that the HB-eyelets synaptically connect to both, the sLNvs and the ILNvs.<sup>53</sup> Exciting the HB eyelets lead to increases in Ca<sup>2+</sup> and cAMP in the sLNvs. The activation of the sLNvs likely leads to an increased release of PDF, which adjusts PER cycling in downstream target cells like the DN1 and LNd neurons.<sup>46</sup> In contrast, the ILNvs are receptive to bath-applied histamine,<sup>53</sup> suggesting that histamine released by the HB-eyelets could impact the ILNvs. This does not imply a direct connection, but trans-tango experiments suggest that the eyelet also directly connects to the ILNvs (Figure 2J-L). In contrast, the compound eyes do not appear to directly connect to the PDF neurons, and GRASP experiments did not show any GFP signal in the medulla suggesting interneurons being involved in signal transduction from the compound eyes to the clock neurons. However, electrophysiological evidence suggests a

connection between the compound eyes and the ILNvs. Recordings from these ILNv neurons with the retina attached to the brain significantly altered their firing pattern.<sup>55</sup> This change in firing pattern was mediated via cholinergic interneurons in the lamina. Similar results were obtained by a more recent study<sup>56</sup> that further extended our knowledge by recording from not only the ILNvs but also other clock neuron subgroups. The surprising finding was that most clock neuron clusters (sLNvs, ILNvs, fifth sLNv, CRY + LNds, DN1a, DN2, and DN3a) fired action potentials on light stimulation when the preparations included the compound eyes. Removing the compound eyes abolished the changes in physiology, suggesting that the eyes are able to activate an array of clock neurons. All of these neurons share projections into the accessory medulla, an important pacemaker center. Ablating this area lead to the loss of photosensitivity of the clock cells,<sup>56</sup> suggesting that visual information is integrated in this area.

Despite these advances in our understanding, it was still unclear which of these light responses drive the rhythmic behavior of the fly. Early evidence suggested the PDF-neurons as key players: *cry<sup>01</sup> pdf<sup>01</sup>* mutants lack the evening peak of activity which suggested that eye-mediated signaling is integrated into the system via PDF.<sup>57,58</sup> Similarly, Rh6-positive neurons (R8 from the compound eyes and/or the HB-eyelets) target the sLNvs in equinox conditions, where they interfere with the clock machinery and most likely PDF release into the dorsal brain.<sup>45,46</sup> In long day conditions, the compound eyes contact the ILNvs where they trigger PDF release in the accessory medulla, which signals directly to the E cells.<sup>37,53</sup> Moreover, silencing or ablating the PDF neurons reproduced the advanced E peak phenotype of flies lacking compound eyes, whereas changing the firing pattern of DN1s and/or LNds had different effects on behavior.<sup>9,36,37,59,60</sup> All of these experiments point to a prominent role of the PDF neurons in light synchronization via the visual system, which parallels what is known about mammalian mechanisms.<sup>61</sup> However, flies lacking CRY and PDF are still able to entrain to LD cycles, suggesting that other neurons can receive light information from the compound eyes. How these connections contribute to light synchronization in wild-type flies is still unknown.

In summary, recent research has provided a substantial body of evidence on the ways light information is integrated in the *Drosophila* clock neuron network. Key players include Rhodopsin 6 expressing photoreceptors as well as PDF neurons, as ablating these neurons largely reproduces the behavior of *eyes absent (cli<sup>ya</sup>)* mutants. However, a PDF-independent pathway allows the animal to synchronize to LD cycles in its absence. This pathway likely includes another rhodopsin (Rh7), which appears to be expressed within the clock neuron network and subpopulations of R8 of the compound eyes.<sup>62,63</sup>



**Figure 2.** (A-I) Termini of rhodopsin 6 expressing photoreceptors reside near PDF-neuron arborizations. (A-C) Brain and compound eyes of *rh6-GAL4 UAS-GFP* flies stained against GFP (green) and PDF (magenta). Rh6 is expressed in 70% of R8 which directly innervate the medulla. The HB-eyelet expresses Rh6 and sends its projections toward the accessory medulla (aMe). (D-F) Single layer from the posterior part of the brain. R8 termini (GFP, green) innervate the medulla, where they are near the fibers of the PDF neurons (magenta). (G-I) Z-stack of 4 layers in the anterior part of the brain. The HB-eyelet directly innervates the accessory medulla (aMe) (adapted from Schlichting et al<sup>46</sup>). (J-L) *Rh6-GAL4 trans-tango QUAS-GCamp6* flies were stained against GFP (green) and Period (red). Trans-tango was recently developed to investigate synaptic partners of neurons of choice (for details see Talay et al<sup>54</sup>). Neurons expressing *GCamp* (green, labeled with GFP antibody) lie downstream of *rh6*-expressing photoreceptors. As expected, downstream neurons include several medulla neurons likely involved in visual information transduction. Co-labeling with anti-PER reveals the sLNvs and also the ILNvs as direct targets, whereas other clock-neurons appear to be indirect targets of the photoreceptor cells. HB indicates Hofbauer-Buchner; PDF, pigment dispersing factor; PER, Period.

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## Author Contributions

Matthias Schlichting performed experiments, wrote and revised the manuscript.

## ORCID iD

Matthias Schlichting  <https://orcid.org/0000-0002-0822-0265>

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