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REVIEW

Unique Self-Sustaining Circadian Oscillators Within the Brain of *Drosophila melanogaster*

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ABSTRACT

In *Drosophila* circadian rhythms persist in constant darkness (DD). The small ventral Lateral Neurons (s-LN_v) mainly control the behavioral circadian rhythm in consortium with the large ventral Lateral Neurons (l-LN_v) and dorsal Lateral Neurons (LN_d). It is believed that the molecular oscillations of clock genes are the source of this persistent behavior. Indeed the s-LN_v, LN_d, Dorsal Neurons (DN)-DN_2 and DN_3 displayed self-sustained molecular oscillations in DD both at RNA and protein levels, except the DN_2 oscillates in anti-phase. In contrast, the l-LN_v and DN_1 displayed self-sustained oscillations at the RNA level, but protein oscillations quickly dampened. Having self-sustained and dampened molecular oscillators together in the DN groups suggested that they play different roles. However, all DN groups seemed to contribute together to the light–dark (LD) behavioral rhythm. The LD entrainment of LN oscillators is achieved through Rhodopsin (RH) and Cryptochrome (CRY). CRY's expression in all DN groups implicates also its role in LD entrainment of DN, like in DN_1. However, mutations in *cry* and *glass* that did not inflict LD synchronization of the DN_2, DN_3 oscillator implicate the existence of a novel photoreceptor at least in DN_3.

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1. INTRODUCTION

Circadian rhythms are oscillations in the biochemical, physiological, and behavioral function of organisms with a periodicity of ~24 h. True circadian rhythms free-run in constant darkness (DD) and are entrainable to the day–night changes in the environment. Circadian rhythms are present in almost all eukaryotes examined so far (Edery, 2000). *Drosophila melanogaster* has been used as a model system to study the circadian rhythmicity (reviews: Edery, 2000; Stanewsky, 2002; 2003): mainly the locomotor rest-activity rhythm (LRAR) and eclosion rhythm (ER) were widely explored in this organism (reviews: Hall, 2000; Helfrich-Förster, 2003; Helfrich-Förster and Engelmann, 2002; Jackson et al., 2001). A behavioral screen focused on both ER and LRAR has paved the way to the discovery of the first clock gene *period* (*per*) involved in the control of circadian rhythm (Konopka and Benzer, 1971). Thereafter, an underlying molecular mechanism for the circadian rhythm has been envisaged (Hardin et al., 1990; review: Dunlap, 1990). The clock gene per has been shown to be expressed in certain clock cells in the central nervous system, as well as in several peripheral tissues (Hall, 1995; Kaneko, 1998; Kaneko and Hall, 2000; Krishnan et al., 1999; Liu et al., 1988; Plautz et al., 1997; Reppert, 1998). The clock gene expression is rhythmic in these clock cells in light–dark cycles (LD) (Kaneko and Hall, 2000).

The LRAR and ER free-run in DD in *Drosophila*. It is believed that the cycling levels of clock gene expression maintain the free-running LRAR in DD. However, this was not proved beyond skepticism because the molecular cycling had been dampening quickly in DD (e.g., Stanewsky et al., 1997a). Nevertheless, in a subset of clock cells located in the lateral brain clock gene cycling in early DD was reported (Blanchardon et al., 2001; Klarsfeld et al., 2004; Shafer et al., 2002; Yang and Sehgal, 2001). This finding was supported by demonstrating *per* cycling in DD in vivo for an extended time period, at the molecular level (Veleri et al., 2003). This was attained by employing a real-time luciferase reporter system (Stanewsky et al., 1997a), adopted for restricted spatial expression. In conjunction with immunostainings this reporter system has identified a novel molecular circadian oscillator in the dorsal brain of *Drosophila melanogaster* besides the oscillator in the lateral brain (Veleri et al., 2003). Nevertheless, not all clock gene-expressing neurons showed circadian rhythms in DD. One group of clock neurons in the dorsal brain became arrhythmic after transfer into DD (Klarsfeld et al., 2004), this was also true for one group of clock neurons in the lateral brain (Shafer et al., 2002; Veleri et al., 2003; Yang and Sehgal, 2001). This suggests that only some clock cell groups behave as a real pacemaker in the brain of *Drosophila*. Only these real pacemaker cells may be capable to maintain sustained molecular oscillation in DD.

This article briefly summarizes our present knowledge about the so-far identified behavioral circadian pacemaker neurons of the adult *Drosophila* and further discusses the self-sustained molecular oscillators found in the brain.

2. COMPONENTS OF THE MOLECULAR OSCILLATOR

In 1971, Konopka and Benzer made the first discovery connecting the circadian rhythm to a gene by showing that the *per* mutants exhibited varied period lengths in pupal eclosion and adult activity rhythms. Two decades later a second crucial clock gene, *tim* was discovered (Sehgal et al., 1994). More clock genes like *Clock* (*Clk*), *cycle* (*cyc*), *double-time* (*dbt*), *vrille* and *shaggy* (Allada et al., 1998; Blau and Young, 1999; Martinek et al., 2001; Price et al., 1998; Rutila et al., 1998) were added to the repertoire of the clock-controlled genes in successive years.

All these clock genes and their products are orchestrated, by virtue of two interlinked autoregulatory positive–negative feedback loops, to achieve the overt circadian rhythm. In the positive limb of the loop, the hetero-dimeric transcription activating factor CLOCK (CLK)/CYCLE (CYC) binds to the E-box present in the promoter region of both *per* and *tim*, to switch on their transcription. In the negative limb, the hetero-dimer PERIOD (PER)/TIMELESS (TIM) binds back onto the CLOCK/CYCLE hetero-dimer and thus inhibits transcription of *per* and *tim* (Stanewsky, 2002).

The molecular oscillations of PER and TIM could be visualized in the clock neurons (Helfrich-Förster et al., 2001; Kaneko, 1998; Yang and Sehgal, 2001). To gain an in-depth understanding of the circadian clock the spatio-temporal expression patterns of clock genes in these neurons were widely sought out in *Drosophila* (Kaneko, 1998; Kaneko and Hall, 2000). Therefore, the expression profiles of both *per* and *tim* genes (Stanewsky et al., 2002) and their gene product (Kaneko, 1998), may serve as a tool to identify and characterize novel circadian oscillators.

3. CLOCK NEURONS

The molecular-genetic studies enabled the identification of numerous putative clock cells throughout the fly's body (reviewed by Hall, 1995). Out of all of these clock cells, the brain clock neurons were the main focus of many studies (Ewer et al., 1992; Helfrich-Förster, 1995; Kaneko, 1998; Kaneko and Hall, 2000; Zerr et al., 1990), after Konopka et al. (1983) had already located the pacemaker neurons controlling rhythmic behavior into the brain. These brain clock neurons are classified into two main groups based on their anatomical positions. Laterally positioned groups of neurons called Lateral Neurons (LN) and dorsally positioned groups of neurons called Dorsal Neurons (DN) (Fig. 1). Both the LN and DN are divided into three subgroups: small Lateral Neuron ventral (s-LN_v), large Lateral Neuron ventral (l-LN_v), and Lateral Neuron dorsal (LN_d); DN_1, DN_2, and DN_3. All these neuronal groups are placed symmetrically in both brain hemispheres. The s-LN_v consist normally of 4–5 cells, the l-LN_v are of 4–6 cells, and the LN_d are 5–6 cells (Kaneko, 1998). The DN_1 are located in the dorsal-most cortex of the brain and consist of 8–17 cells (Kaneko, 1998). The DN_2 are situated at the roof of calyces of mushroom body and typically consist of two cells, positioned either in a vertical or horizontal line to each other (Fig. 1). Due to the similarity in their larval and adult positions it was suggested that the origin of the DN_2 is the same (Kaneko 1997). The DN_3 are located in the lateral superior protocerebrum and are $\sim$40 neurons with

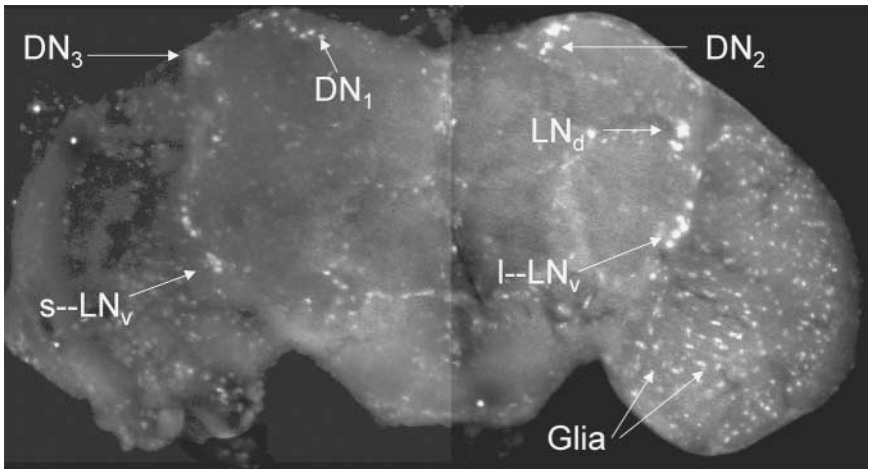

Figure 1. Depicts the *Drosophila melanogaster* brain stained with anti-PER antibody. The various groups of clock neurons were marked. The DN_1, DN_2, and DN_3 represent the dorsal neurons 1, 2, and 3, respectively. The s-LN_v, l-Nv represent small and large lateral ventral neurons. The LN_d represents the dorsal lateral neurons.

small somata. All these LN and DN express PER and TIM rhythmically in LD (Kaneko, 1998; Yang and Sehgal, 2001).

Besides the LN and DN numerous glia cells also express PER (Ewer et al., 1992; Kaneko, 1998; Zerr et al., 1990) and TIM rhythmically (unpublished observation, Veleri). Furthermore, the analysis of a *per-β-galactosidase* fusion gene expression pattern revealed that *per* was expressed rhythmically in peripheral tissues like cells in antennae, proboscis, eyes, in the thoracic ganglion, gut, Malpighian tubules, ovarian follicles, and testes (Giebultowicz et al., 2000; Liu et al., 1988). It was also suggested that many of these peripheral tissues contain intrinsic oscillators (Liu et al., 1988). Later, by using a luciferase reporter system the sensory bristles in legs and wings have been demonstrated to host independent light entrainable clocks (Plautz et al., 1997).

Presently it is believed that the s-LN_v harbor the central behavioral circadian pacemaker, arguing in these cells at least PER continued to cycle in DD for one or two days (Shafer et al., 2002; Yang and Sehgal, 2001; reviewed by Helfrich-Förster, 2003). Besides controlling the circadian output the s-LN_v together with the l-LN_v may play a role in coupling single pacemaker cell groups and thus accomplish the synchronization among all these cells (Helfrich-Förster, 1998; Peng et al., 2003).

A role for the LN_d in LRA is inferred from the studies of Blanchardon et al. (2001) and Renn et al. (1999). A functional knock-out of the s-LN_v and l-LN_v, either by cell ablation or by mutation (pdf^{01}), had a much milder effect on the LRA than the knock-out of all LN (s-LN_v, l-LN_v, and LN_d) by the *disco* mutant (which have disconnected optic lobes). The pdf^{01} flies for example displayed a rhythm with short periods for at least one week in DD (Helfrich-Förster, 2002), which may be generated from the LN_d (Helfrich-Förster, 2002).

Compared to the LN for which at least a putative role has been postulated in the control of behavioral oscillator, almost nothing is known about the function of the DN, despite the fact that their projections have been studied intensely (Helfrich-Förster, 2003; Kaneko and Hall, 2000). Recently, a role for DN_1 toward the light sensitivity of the clock controlling LRAR has been implicated (Klarsfeld et al., 2004). So far, no function has been assigned to the other DN.

4. THE SELF-SUSTAINED MOLECULAR CIRCADIAN OSCILLATORS

The LRAR sustains for weeks in *Drosophila* in DD (Dowse et al., 1987; Helfrich, 1986). It appears paradoxical, however, that the molecular oscillations studied in living flies, with the help of the luciferase reporter system dampened quickly after transfer into DD (Giebultowicz et al., 2000; Plautz et al., 1997; Stanewsky et al., 1997a). This is in contrast to the apparent circadian cycling of PER in the s-LN_v (Shafer et al., 2002; Yang and Sehgal, 2001). Therefore, it is believed that the genuine molecular circadian oscillation, which persists in DD, is a "*uniqueness*" of a few groups of neurons in the brain which drive circadian behavior (Frisch et al., 1994; Helfrich-Förster, 1998). A possible explanation for this discrepancy may be derived from an exquisite qualitative difference between the real pacemaker oscillator (e.g., oscillator that drives the LRAR) and the peripheral oscillator (e.g., oscillator in the eye). Perhaps the genuine pacemaker oscillators are the only ones qualified to maintain sustained molecular oscillation under DD (Veleri et al., 2003). Looking at such a group of clock neurons individually might help to identify self-sustained molecular oscillators.

In this respect, it was necessary to demonstrate in a single fly the self-sustained molecular oscillations for more than just one or two days in DD. It is possible that clock gene expression oscillates for one or two days but becomes arrhythmic later, or that clock gene expression is initially arrhythmic and becomes rhythmic after a few days in DD as reported for cycling in the l-LN_v (Peng et al., 2003). To demonstrate the self-sustained molecular oscillations for an extended time period, a real-time reporter system like the luciferase reporter system is best suited (Stanewsky et al., 1997a) because it allows us to measure the gene expression repeatedly in real time in individual organisms. However, to realize two aims in one experimental setup i.e., to look at an individual group of clock neurons and simultaneously report in real time, a design was required. In this context, one transgenic line carrying a 7.2 kb promoterless *per* DNA subsegment was demonstrated to express *per* in a restricted subset of clock neurons. This construct was also capable of rescuing LRA in per^{01} flies in DD, albeit with a 2 h longer period as compared to the wild-type flies (Frisch et al., 1994). Therefore, combing a similar *per* DNA subsegment with luciferase reporter gene could possibly achieve the goal to demonstrate the self-sustained molecular circadian oscillations in DD.

Indeed a 8.0-*luc*:9 transgenic line that carried a 8.0 kb promoterless *per* genomic DNA fused to luciferase cDNA displayed nondampening molecular circadian bioluminescence rhythms in DD in real-time (Veleri et al., 2003) (Fig. 2). Since the rhythm of 8.0-*luc*:9 transgenics was nondampening, it was suggestive that it might

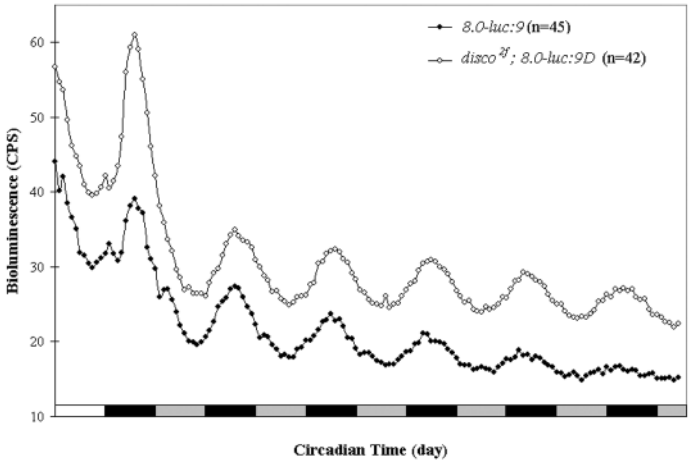

Figure 2. Bioluminescence rhythm records from 8.0-*luc*:9 transgenic files in wild-type and *disco* genetic backgrounds. The 8.0-*luc*:9 transgenic files showed a nondampening bioluminescence record both in wild-type and *disco* backgrounds (*disco* had an improved performance than the wild-type). The bioluminescence record was averaged for many animals (indicated number in the legend), which qualified statistically "rhythmic." The white and black bars in the bottom indicate the light on (12 h) and light off (12 h) in LD, respectively. The gray bars indicate subjective day in DD (12 h).

be emanating from some of the pacemaker clock cell groups. The per^{01} flies are arrhythmic in DD (Konopka and Benzer, 1971). Bringing the 8.0-*luc* transgene into the per^{01} background should rescue the LRAR in DD as demonstrated for the 7.2-kb *per* subsegment (Frisch et al., 1994), if the transgene is expressed in the LN. Surprisingly, the 8.0-*luc*:9 transgenic line could not rescue the wild-type LRAR in DD in per^{01} flies. Nevertheless, it restored the typical wild-type LRAR in LD (Veleri et al., 2003).

The next step was to check whether the observed rhythm was independent of the LN. Analysis of the 8.0-*luc*:9 transgenic flies in a *disco* background, which largely eliminated LN (Helfrich-Förster, 1998), conclusively proved that the 8.0-*luc* driven bioluminescence oscillations (Fig. 2) did not originate from the LN, but rather stemmed from a different oscillator independent from the LN. Finally, immunocytochemical studies on 8.0-*luc*:9 transgenic flies traced the expression of the transgene mainly to the DN (Veleri et al., 2003).

4.1. Molecularly Self-Sustained Oscillators

The next question was whether all DN show a self-sustained cycling of PER in DD. In order to address this problem, anti-PER immunocytochemistry has to be performed on wild-type flies kept several days in DD. On the fifth day of DD, anti-PER staining revealed cycling in the s-LN_v, LN_d, DN_2, and DN_3 but no cycling in l-LN_v and DN_1 (Veleri et al., 2003). A lack of PER protein cycling after 1 or 2 days

in DD in the l-LN_v was shown independently by Shafer et al. (2002) and Yang and Sehgal (2001); but a more recent study reported that PER cycles robustly in the l-LN_v for at least 2.5 days in DD (Klarsfeld et al., 2004). This apparent discrepancy of PER expression in the l-LN_v may be due to the differences in the measured amplitude of the PER expression in the studies mentioned. Whatsoever, the PER cycling in s-LN_v in DD is consistently reported for multiple days (Shafer et al., 2002; Veleri et al., 2003; Yang and Sehgal, 2001), indicating that at least these cells harbor a self-sustained molecular oscillator.

Notably, in the DN_2, PER was cycling in antiphase with the s-LN_v and DN_3 in DD (Veleri et al., 2003). Antiphase cycling of PER as well as of TIM is a feature that has already been reported for the larval DN_2 under LD and free-running conditions (Kaneko et al., 1997; Klarsfeld et al., 2004). In contrast to the larval data PER has been shown to cycle in DN_2 of adult flies in synchrony with the other clock neurons under LD conditions as well as for the first day in DD (Blanchardon et al., 2001). The return to antiphase cycling after a longer time in DD suggests that the antiphase cycling of the DN_2 is a default state of the system in absence of entraining LD cycles. One explanation for the larval DN_2 antiphase cycling is that the DN_2 might not get any light input. Indeed, the blue-light photopigment *cryptochrome* (*cry*) is not expressed in the larval DN_2 (Klarsfeld et al., 2004). As soon as *cry* is artificially expressed in the larval DN_2, the phase of PER rhythm reverted and oscillated in synchrony with the s-LN_v and DN_3 (Klarsfeld et al., 2004). One may argue that the DN_2 in larvae was secluded from light inputs until a certain stage of the metamorphosis was and that this is the reason that the PER oscillation remained in an antiphase default state (Kaneko et al., 1997). Like in larvae, in adult flies the DN_2 also resumed the default state in absence of any photic signal (Veleri et al., 2003).

4.2. Dampened Molecular Oscillators in Free-running Conditions

The l-LN_v and DN_1 behaved more as dampened molecular oscillators as shown by PER cycling profiles in DD (Klarsfeld et al., 2004; Shafer et al., 2002; Veleri et al., 2003; Yang and Sehgal, 2001). In the presence of *Zeitgeber* signals, they were running in synchrony with the s-LN_v like peripheral oscillators. According to Helfrich-Förster (1998) the l-LN_v might be involved in coupling the s-LN_v oscillators between both brain hemispheres. The l-LN_v may even play a role in geotaxis. It has been reported that pdf^{01} mutants that lack the neuropeptide Pigment Dispersing Factor (PDF) in the l-LN_v and s-LN_v showed that male pdf^{01} mutants exhibited negative geotaxis similar to the male *Hi*5 mutant (Toma et al., 2002 and references therein).

The DN probably may contribute to the LD behavioral rhythm or to the overall robustness of behavioral rhythm because even when the LN were absent the LD behavioral rhythm remained intact in *disco* flies at least for a few days (Hardin et al., 1992; Renn et al., 1999). Klarsfeld et al. (2004) postulated that the PER oscillations rapidly dampened out in DN_1 in DD because of absence of the PDF signaling from the LN_v. Similarly, in *disco* flies (which have no LN, thus no PDF) PER was not cycling in the DN_1 on the fifth day in DD, whereas in wild-type flies PER was cycling

in the DN_1 with low amplitude, though it seemed insignificant (Veleri et al., 2003). Obviously, PER cycling dampens faster in the *disco* DN_1 than in wild-type DN_1.

4.3. Unresolved Problems—Transcriptional vs. Posttranscriptional Regulation of Oscillatory Status

In contrast to the dampened PER rhythmicity described so far for the l-LN_v and DN_1, in DD, *tim* mRNA was reported to cycle still in these cells on the eighth day of DD (Peng et al., 2003). These profound differences in expression pattern of PER and *tim* mRNA are problems yet to be reconciled by further studies. Peng et al. (2003) also claimed that mRNA oscillation of the l-LN_v adapt to constant conditions by becoming rhythmic once again after about 2 days in DD in contrast to the dampened protein oscillations in these cells. Conceivably there may be differential gene regulation at various levels of gene expression such as transcription (mRNA) and translation (protein) (Stanewsky et al., 1997b, 2002). The meaning of this divergence for the regulation mRNA and protein rhythms in various types of circadian oscillators is yet to be understood.

5. A NOVEL PHOTORECEPTION PATHWAY IN THE DN_3 OSCILLATOR INVOLVED IN LD ENTRAINMENT

The circadian oscillators are entrainable to LD cycles. For the LD entrainment of the circadian oscillators in the brain light signals are received through *rhodopsin* and *cryptochrome* (*cry*) encoded photoreceptor proteins (Helfrich-Förster et al., 2001). In *Drosophila* there are many *rhodopsin*-expressing cells such as the R1-R8 photoreceptors in the compound eyes, the ocelli and a photoreceptive structure called H-B eyelet (Hofbauer and Buchner, 1989), as well as perhaps the DN_1 themselves (Klarsfeld et al., 2004). The H-B eyelet sends axons to the s-LN_v (Helfrich-Förster, 2002; Malpel et al., 2002). A mutation in the zinc-finger transcription factor *glass* (*gl*) eliminates almost all the *rhodopsin*-based photoreceptors in *Drosophila* (Moses et al., 1989), and it also removes the H-B eyelet and most of the DN_1 cells except two (Helfrich-Förster et al., 2001). The photoreceptor *cry* is necessary for mediating the blue light-based photoreception to the circadian clock, which is blocked in cry^b mutant flies (Stanewsky et al., 1998). *Cry* is strongly expressed in the LN (Emery et al., 2000) and apparently also in the majority of DN (Klarsfeld et al., 2004). Double mutations for both *glass* (gl^{60j}) and *cry* (cry^b) render the circadian clock blind (Helfrich-Förster et al., 2001).

The DN appear to be substantially involved in the entrainment of the flies to LD cycles, since *per* expression in the DN alone (in per^{01}; 8.0-*luc*:9) could rescue normal entrainment (Veleri et al., 2003). Similarly, *disco* flies can entrain to LD cycles, although the normal connection between compound eyes and brain is disturbed, and these mutants additionally lack all LN (Helfrich-Förster, 2002; Veleri et al., 2003). Therefore, neither the compound eyes nor the LN are able to entrain the *disco* flies. This suggests that photopigments in the DN themselves mediate the LD entrainment. Indeed, the DN_1 co-expressed *cry* and *gl* (Klarsfeld et al., 2004),

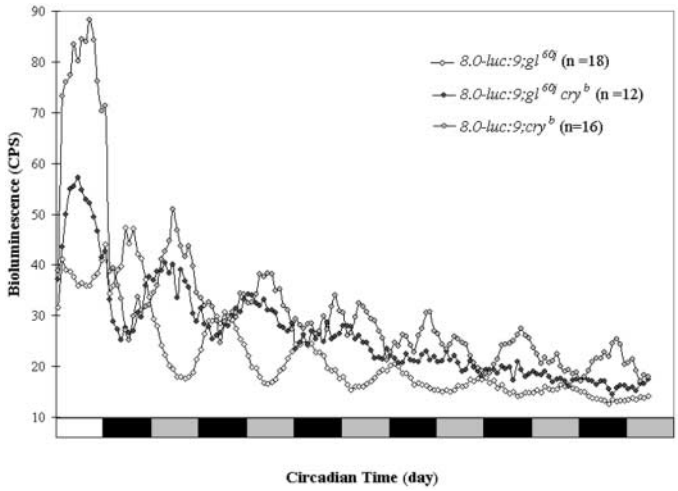

Figure 3. Bioluminescence rhythms recorded from 8.0-*luc*:9 transgenic flies in various photoreceptor mutant backgrounds. 8.0-*luc*:9 transgenic flies carrying the rhodopsin (gl^{60j}) and/or cryptochrome (cry^{b}) deficient mutations were analyzed in DD after initially being entrained to at least 3 days in LD (12 h:12 h) at 25°C. All genotypes showed synchronized rhythms. The genotypes with cry^{b} depict a phase of peak delayed with respect to the gl^{60j}. The white, black, and gray bars are as seen in Fig. 2.

suggesting that Cryptochrome (CRY) and a Rhodopsin (RH) might be the photopigments in question. Klarsfeld et al. (2004) found that the DN_1 contribute significantly to the light sensitivity of the circadian system, and that this contribution depends entirely on CRY and not on rhodopsin. Similarly, rhodopsins were not necessary to entrain the DN_3: 8.0-*luc*:9 transgenic flies brought into gl^{60j} genetic background still nicely entrained to LD cycles and retained the synchronization for more than 6 days in DD (Veleri et al., 2003) although they missed all rhodopsin-based photoreceptors (Fig. 3). When, the 8.0-*luc*:9; cry^{b} was brought into the cry^{b} mutant background the DN_3 were still capable of synchronizing to the LD cycles (Fig. 3), but the maximum of the oscillations was delayed by several hours as compared to the wild-type. This was also true in the doubly mutant background, 8.0-*luc*:9; $gl^{60j}cry^{b}$ (Veleri et al., 2003). This observation ruled out the possibility that *cry* is essential for circadian photoreception in DN_3 but suggested that *cry* sets the phase of the rhythm. Indeed it was reported that c*ry* plays a role in maintaining the robustness and phase of molecular cycles in isolated peripheral tissues (Levine et al., 2002). In conclusion, a novel photoreceptor may be involved in the photic entrainment of DN_3.

6. CONCLUSIONS

In DD circadian behavioral rhythms persist possibly owing to the molecular oscillations of clock genes. The s-LN_v, which is thought to be the main behavioral

pacemaker, have been shown to host self-sustained molecular circadian oscillator in DD both at transcriptional and translational levels. The LN_d, DN_2, and DN_3 were also displaying persistent molecular rhythms at all levels like the s-LN_v. The DN_2 and DN_3 oscillators can function in the absence of the LN oscillators. In in vivo recordings, the DN_3 oscillator showed self-sustained cycling in DD for more than 6 days independent of the LN. Surprisingly, the DN_2 cycled in antiphase to all other neurons. The l-LN_v and DN_1 behaved differently: while protein cycling quickly dampens in DD, self-sustained oscillations appear to persist at the RNA level. The implication of this difference in the role of gene expression levels is yet to be addressed, but from the present point of view the protein oscillations are more relevant for overt circadian rhythm than the RNA oscillations. The present understanding of the various circadian oscillators in the *Drosophila* brain are depicted in Fig. 4.

Though the precise functions of the neurons with or without self-sustained circadian oscillators are unknown, it appears clear that they have different roles. The s-LN_v, perhaps together with the LN_d, seem to control the LRAR in DD, whereas the DN_3 oscillator together with the dampened DN_1 oscillator apparently contribute to LRAR only in LD. It is unclear why the DN_3 show such robust self-sustained oscillations in DD, although they only contribute to LRAR in LD, but it is possible that the DN_3 are involved in the control of other rhythm outputs, perhaps in olfactory sensitivity rhythms. The DN_2 may participate in the LD entrainment,

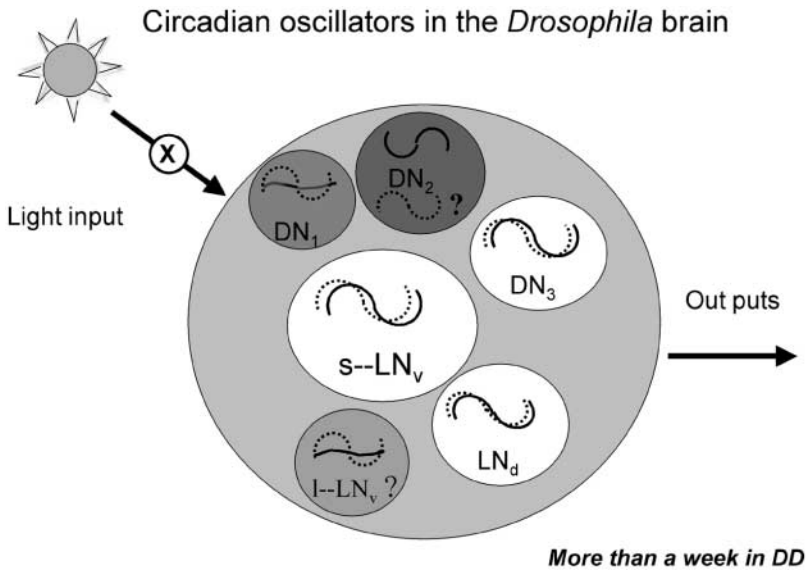

Figure 4. Schematic model depicts the present knowledge about the circadian oscillators in the *Drosophila* brain: nondampening and dampening oscillators in DD. The s-LN_v, l-LN_v, LN_d and DN_1, DN_2, DN_3 are as seen in Fig. 1. The oscillating waves inside the circles represent nondampened circadian oscillations (solid and dotted lines indicate protein and RNA oscillations, respectively). The flat solid lines inside the circles indicate dampened protein oscillations. Note that in DN_2 the protein oscillation is in antiphase with other oscillators. The question marks indicate that either it is not clear or there is some discrepancy.

whereas the l-LN_v may play a role in the coupling of clock cell groups and thus to fine-tune the main circadian oscillator.

Another unresolved question is the precise role of the various photoreceptors in the entrainment of different circadian oscillators in the brain. The LN oscillators are mainly entrained by the rhodopsins in the photoreceptors of compound eyes and H-B eyelets, as well as by *cry*. The DN can be entrained independent of the LN. A role for the DN_1 in LRAR in LD is implicated, which is *cry* dependent (in the entrainment of the DN_1 oscillator the rhodopsin may also be involved). In the DN_3 oscillator CRY and rhodopsins were dispensable for LD synchronization but CRY was necessary to set the phase of the circadian oscillations.

In conclusion, the various *per* and *tim* expressing clock neurons play different roles in the circadian organization of *Drosophila*. Though more and more is known about the function of LN_v as a behavioral oscillator, the precise function of DN remains a challenging question. Future research directed specifically to unravel the unknown circadian outputs may be able to decipher the function(s) of the dorsal neurons.

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