The Lateral and Dorsal Neurons of *Drosophila melanogaster*: New Insights about Their Morphology and Function

C. HELFRICH-FÖRSTER,* T. YOSHII,* C. WÜLBECK,* E. GRIESHABER,* D. RIEGER,*
W. BACHLEITNER,* P. CUSUMANO,[†] AND F. ROUYER[†]

*University of Regensburg, Institute of Zoology, 93040 Regensburg, Germany;

[†]Institut de Neurobiologie Alfred Fessard, CNRS UPR2216, 91198 Gif-sur-Yvette, France

This chapter summarizes our present knowledge about the master clock of the fruit fly at the neuronal level. The clock is organized in distinct groups of interconnected pacemaker neurons with different functions. All of these neurons appear to communicate with one another in order to produce the species-specific activity rhythm, which is organized in morning (M) and evening (E) activity bouts. These two activity components are differentially influenced by distinct groups of pacemaker neurons reminiscent of the Pittendrigh–Daan dual oscillator model. In the original work (Grima et al. 2004; Stoleru et al. 2004), the ventrolateral (LN_v) and dorsolateral (LN_d) plus some dorsal groups (DN) of clock neurons have been defined as M and E cells, respectively. We further specify that the clock neurons belong to the M and E oscillators and define a more complex picture of the *Drosophila* brain clock.

INTRODUCTION

During the past years, the fruit fly *Drosophila melanogaster* has been very helpful in understanding the neuronal organization and function of the circadian clock in the brain. As is the situation in the mammalian clock, the fly clock consists of a network of morphologically different neurons that have putatively different functions and outputs (Hall 2005; Helfrich-Förster 2006; Taghert and Shafer 2006). Fortunately, the *Drosophila* clock neurons are few in number (~150), and they can be easily genetically manipulated. Nevertheless, the activity rhythm of *Drosophila* is similarly as complex as that of mammals. It consists of two main activity bouts, one in the morning (M) and the other in the evening (E), that are timed in a sophisticated manner to dawn and dusk. Most significantly, two groups of neurons in the lateral brain—the ventral and dorsal lateral neurons—have been implicated in controlling M and E activity, respectively (Grima et al. 2004; Stoleru et al. 2004). The lateral neurons are regarded as the main pacemaker cells, because they were shown to be necessary and sufficient to drive behavioral rhythmicity in the absence of external time cues (Ewer et al. 1992; Frisch et al. 1994).

This chapter reviews our current knowledge about the morphology and function of these main clock neurons and other players. It includes morphological details that were not reported in previous papers (Shafer et al. 2006; Helfrich-Förster et al. 2007), and it shows that the functional division in the M and E oscillators is more complex than initially thought.

CLOCK NEURONS OF *DROSOPHILA* *MELANOGASTER*

The overall anatomical organization of *Drosophila*'s pacemaker system was the topic of two recent papers and one recent review (Shafer et al. 2006; Taghert and Shafer

2006; Helfrich-Förster et al. 2007) and is only briefly summarized here (Fig. 1). According to their location in the brain, the clock neurons are traditionally divided into six groups: three dorsal ones (DN_{1–3}) in the superior brain and three lateral ones (LN_d, l-LN_v, and s-LN_v) in the anterior brain; but there is an additional group of lateral neurons in the posterior brain called the LPN (Kaneko et al. 1997; Kaneko and Hall 2000; Yoshii et al. 2005; Shafer et al. 2006). The projections of the LPN are still unknown, but they seem to overlap completely with those of the other neurons (O. Shafer and C. Helfrich-Förster, unpubl.).

The clock neurons have two main projection targets: the accessory medulla that appears to be the principal pacemaker center of insects and the dorsal brain that houses the hormonal center (pars intercerebralis and lateralis) of insects and also has connections to most brain areas (Homberg et al. 2003; Shiga 2003; for discussion, see Jaramillo et al. 2004; Lear et al. 2005a). The clock neurons form a well-defined fiber network in these brain areas, putatively allowing considerable cross-talk between them (Fig. 1).

Recent studies indicate that the classical division of the clock neurons is insufficient, because several of these groups are rather heterogeneous (Lee et al. 2006; Shafer et al. 2006; Helfrich-Förster et al. 2007). A further subdivision is particularly appropriate for s-LN_v, LN_d, DN₁, and DN₃. Of the s-LN_v, four cells express the neuropeptide pigment-dispersing factor (PDF) and one is PDF-negative (Kaneko et al. 1997). The projection of the PDF-negative fifth s-LN_v appears to be similar to that of the PDF-positive s-LN_v, but its neurotransmitter still remains elusive (Helfrich-Förster et al. 2007).

The six LN_d cells consist of three neurons that are strongly cryptochrome (CRY)-positive, whereas the remaining three cells appear to express less or no CRY (Klarsfeld et al. 2004; Picot et al. 2007; T. Yoshii, unpubl.). The CRY-positive LN_d cells are also revealed by the *Mai179-gal4* enhancer-trap line (see Fig. 4) (Siegmund and Korge 2001; Grima et al. 2004; Picot et al.

Figure 1. Arborization patterns of the lateral neurons (LN_{ds} , $I-LN_s$, and $s-LN_s$) and dorsal neurons (DN_1 , DN_2 , DN_3). Their fibers overlap in the dorsal brain and the accessory medulla (aMe). (Reprinted, with permission, from Helfrich-Förster et al. 2007 [© Wiley].)

2007), but the nature of the gene hosting the *Mai179* insertion is still unknown. Furthermore, it has been reported that in male flies, three LN_d cells express the neuropeptide F (NPF), a homolog of the mammalian neuropeptide Y (NPY) (Lee et al. 2006). It is unclear whether the three NPF-positive cells are identical to the CRY- and *Mai*-positive LN_d cells. The LN_d cells seem not only heterogeneous in their neurohistochemistry, but also heterogeneous in their arborizations (Helfrich-Förster et al. 2007). The three CRY- and *Mai*-positive cells appear to project ipsilaterally and contralaterally, whereas the axons of the remaining LN_d cells run only to the contralateral side of the brain (T. Yoshii et al., unpubl.).

DN_1 encompasses a large and obviously rather heterogeneous group of clock neurons. They consist of approximately 17 neurons that are spread in the dorsal brain. Two of the DN_1 are conspicuous because (1) they are already present in larvae, (2) they are the only DN_1 cells that lack the transcription factor *glass* and are present in *gl^{60J}* mutants, and (3) they are clearly located in an anterior position in the adult brain and thus separated from the others (Klarsfeld et al. 2004; Shafer et al. 2006). These cells were accordingly named $DN_{1anterior}$ (DN_{1a}). These two neurons express the neuropeptide IPN-amide (Shafer et al. 2006). Furthermore, they show anti-CRY immunoreactivity (Klarsfeld et al. 2004) and are strongly labeled by the *cry-gal4* line (#13) (Shafer et al. 2006; Helfrich-Förster et al. 2007). Similarly, CRY-positive cells are two further DN_1 cells that lie among the other DN_1 cells in the posterior brain and were named DN_{1p} . The DN_{1a} and the two DN_{1p} cells appear to show the same arborization pattern; they project ventrally to the ipsilateral accessory medulla (Fig. 1) (Shafer et al. 2006; Helfrich-Förster et al. 2007). Of the remaining DN_1 cells, some may run also to the accessory medulla, others may cross to the contralateral brain in a dorsal commissure, and others even may do both.

With about 40 neurons, the DN_3 cluster covers the largest number of clock neurons. Again, these neurons are heterogeneous in size and projection pattern (Shafer et al. 2006). Two DN_3 neurons with larger soma are labeled by the *cry-gal4* line (#13), and these neurons project ventrally to the ipsilateral accessory medulla, whereby their neurites overlap with those of the CRY-positive LN_d (Helfrich-Förster et al. 2007).

FUNCTIONAL DISSECTION OF THE FLY'S PACEMAKER SYSTEM

The Lateral Neurons as Master Clocks

As mentioned above, the current view is that the LN clusters (specifically, the $s-LN_s$ and LN_{ds}^1) are *Drosophila*'s main clock neurons, whereas the DN groups seem to be less important. This conclusion is based on experiments that restricted PER expression to certain subsets of clock neurons: Flies that expressed PER in the LN clusters but not in the DN clusters showed rhythmic locomotor activity under constant dark (DD) conditions (Frisch et al. 1994), whereas PER expression in brain cells other than the LN clusters was not sufficient to produce robust activity rhythms (Ewer et al. 1992). Furthermore, PER expression restricted to the PDF-expressing LN_v cells is sufficient to drive robust rhythms in DD (Fig. 2, top) (Grima et al. 2004), whereas rhythmic LN_d cells are sufficient to drive robust rhythmicity in LL (Fig. 2, bottom) (Picot et al. 2007). Conversely, the *disconnected* (*disco*) mutation that causes degeneration of many optic lobe neurons including the LN clusters during development, but that leaves the DN clusters intact, resulted in arrhythmic behavior under DD conditions (Fig. 3) (Steller et al. 1987; Dushay et al. 1989). Similarly, transgenic flies, in which the LN_v cells were ablated specifically by activation of cell death or apoptosis genes, became arrhythmic under DD conditions (Renn et al. 1999; Blanchardon et al. 2001). The same happened after electrically silencing the LN_v cells (Nitabach et al. 2002). Altogether, this points to the LN clusters as main pacemakers. But there is one major caveat. Nobody has so far shown that ablating all DN and leaving the LN intact will preserve behavioral rhythmicity. It is only known that elimination of the *glass*-positive DN_1 (in *gl^{60J}* mutants) does not lead to arrhythmicity (Helfrich-Förster et al. 2001; Klarsfeld et al. 2004). Although PER expression in the DN is neither required nor sufficient for sustained

¹The role of the $I-LN_s$ cells is still unknown. These cells do not show apparent oscillations in clock proteins under DD conditions and are therefore not regarded as master clock neurons (Stanewsky et al. 1998; Yang and Sehgal 2001; Shafer et al. 2002; Lin et al. 2004).

Figure 2. Averaged group actograms ($n = 16$) of flies, in which PER was restricted to the PDF-positive s-LN_v cells (only the M oscillator is functional; *top*) or to the fifth s-LN_v, the CRY-positive LN_d, and perhaps the DN_{1a} cells (predominantly the E oscillator is functional; *bottom*). (*Top*) The flies with a functional clock only in the M oscillator show only the M peak plus a lights-off peak under LD, and the free-run under subsequent DD conditions started from the M peak. (*Bottom*) The flies with a functional clock in the E oscillator also carry the *cry^b* mutation. They remain rhythmic under LL conditions, and activity clearly starts from the E-activity peak.

behavioral rhythms in DD, the function of DN_{1a}, DN₂, and DN₃ in the brain clock remains unknown.

The Dorsal Neurons as Poorly Characterized Players in the *Drosophila* Circadian System

Several studies have shown that the DN cells are involved in the control of behavioral rhythmicity. Most *disco* flies missing the LN clusters showed rather organized activity patterns under LD conditions with clearly recognizable M and E peaks (Fig. 2) (Hardin et al. 1992; Helfrich-Förster 1998). Some of them even showed residual rhythmicity for the first few days under constant conditions, indicating that they still possess a dampened circadian clock (Fig. 2). Indeed, PER levels were found to vary cyclically in the DN and in the glial cells of *disco* mutants (Zerr et al. 1990), and this cycling persisted even in the absence of the LNs for at least 2 days under constant conditions (Blanchardon et al. 2001). With the help of a luciferase-based *per* reporter system, a stable *per* cycling in the DN₂ and DN₃ clusters was revealed for many days under DD conditions (Veleri et al. 2003). This cycling continued even in absence of PER in the LN cells and was just self-sustained. The same may apply to the DN₁. Most recent studies showed that under certain circumstances, a subset of the DN₁ cells is clearly cycling and that these cells may even control rhythmic behavior under constant light (LL) conditions (Murad et al. 2007; Stoleru et al. 2007; see below for further discussion).

Mysterious LPN with Unidentified Function

LPNs were originally described as cells that expressed the clock protein Timeless (TIM) but not PER (Kaneko and

Figure 3. Activity patterns of one phenotypically wild-type fly (*pdf⁰¹* mutant, in which PDF expression was rescued in the PDF neurons), two *pdf⁰¹* mutants, and one *disco⁰¹* mutant. The first *pdf⁰¹* mutant appears to lack the morning activity under LD conditions (except for a lights-on peak) and free-runs with short period under DD conditions before rhythmicity slowly vanishes. Most importantly, free-run started from the middle of the night. The second *pdf⁰¹* fly showed activity in the second half in the night under LD conditions and the free-running activity in DD started indeed from this nocturnal activity. Thus, the midnight activity might relate to a strongly advanced M peak. The *disco* mutant, which lacked all LN_v and most probably also LN_d cells still showed M and E peaks under LD conditions, but became quickly arrhythmic after transfer into DD (for details, see Helfrich-Förster 1998).

Hall 2000), and more recently, LPNs were confirmed as bona fide clock neurons expressing PER as well and specifically implicated in temperature entrainment (Yoshii et al. 2005; Shafer et al. 2006; Miyasako et al. 2007).

The Neuropeptide PDF as Internal Synchronizer

In sum, there is evidence that all clock neurons work together in controlling behavioral rhythmicity. They form a sophisticated interactive network, in which each neuronal cluster might have its specific role. This does not exclude a certain hierarchy in the network. The LN clusters appear to have a dominant role in the network, and this role becomes most evident in the absence of external time cues (e.g., under DD conditions). Most interestingly, the neuropeptide PDF, which is produced in most of the LN_v cells, has a prominent role in coordinating the oscillations of all clock neurons (Peng et al. 2003; Klarsfeld et al. 2004; Lin et al. 2004; for review, see Taghert and Shafer 2006). *pdf⁰¹* mutants entrain to LD cycles showing rather normal activity patterns, except for an advanced E peak and a barely visible if not absent M peak (Fig. 3) (Renn et al. 1999; however, see below). When transferred to DD, these flies maintain a rhythm with a short period for at least 1 week under DD (Fig. 3). Then, the oscillations in the clock neurons become asynchronous and the flies become arrhythmic (Peng et al. 2003; Lin et al. 2004). These results indicate that the main role of PDF is to coordinate and synchronize the oscillations of the other clock neurons in the absence of external time cues. A similar role of PDF was also suggested for larger insects (Petri and Stengl 2001; Singaravel et al. 2003; Schneider and Stengl 2005).

PDF has no homolog in mammals, but most interestingly, the PDF receptor (Hyun et al. 2005; Lear et al. 2005b; Mertens et al. 2005) shows similarity to the mammalian VPAC2 receptor that binds the vasoactive intestinal polypeptide (VIP). VIP and VPAC2 are essential to produce a coherent oscillating output of the mammalian pacemaker center, the SCN (Maywood et al. 2006). Similar to the situation in *pdf⁰¹* mutant flies, VIP and VPAC2 mutant mice become arrhythmic under constant conditions.

THE DUAL OSCILLATOR MODEL

Diurnal animals are not just active when light turns on and inactive when light turns off, but they entrain their clock in a sophisticated manner to the environmental conditions and they are well capable of adapting to seasonal changes in day length and temperature (Majercak et al. 1999; Rieger et al. 2003). Most diurnal species show a broader activity band under long summer days as compared to short winter days. If M and E activity components are present, the M component couples to dawn and the E oscillator couples to dusk, and a pronounced midday trough becomes visible in long summer days. This means that M and E activity components change their phase relationship to adapt to seasonal changes in day length. These adjustments can be best explained by assuming two oscillators with different properties: one accelerating and the other decelerating with increasing light (Pittendrigh and Daan 1976). In this two-oscillator model, the M oscillator would lock to dawn and the E oscillator would lock to dusk. The anatomical substrates of M and E oscillators remained unknown until Grima et al. (2004) and Stoleru et al. (2004) found that the LN_v and the LN_d cells are good candidates for M and E oscillators, respectively. Stoleru et al. (2004) genetically eliminated either the PDF-positive

LN_v or the LN_d cells (plus the fifth s-LN_v and some DN cells) and found in the first case that the M peak disappeared and in the second case that the E peak vanished. Grima et al. (2004) limited *per* expression to the PDF-positive LN_vs and found that the flies showed a normal M peak but lacked the E peak (Fig. 2, top), whereas adding PER expression to some LN_d cells induced an evening peak. Both studies suggest that PDF-positive LN_v and the LN_d cells (plus at least the fifth s-LN_v) actually are the M and E oscillators proposed by Pittendrigh and Daan (1976). It was shown recently that the M and E cells are inhibited and activated, respectively, by light at the output level, suggesting a switch between M and E oscillators under LD conditions (Picot et al. 2007). Finally, Stoleru et al. (2007) proposed a clever model showing how M and E oscillators adapt to seasonal changes (the E cells being DN₁ neurons in this study). In this model, the M cells are “dark oscillators” that dominate under short photoperiods, whereas the E cells are “light oscillators” that dominate under long photoperiods. Our findings are principally in concert with the M/E oscillator model, but we have indications that the separation in M and E cells is far too simple. In the following, we describe our current view based on published and still unpublished findings.

The Nature of the M Cells

There are indications that the PDF-positive s-LN_v are not the only cells that generate the M peak under LD conditions but that some DN cells contribute. Stoleru et al. (2004) found that flies without a functional clock in the PDF-positive s-LN_v cells still showed M and E activity bouts. The same happened after restricting *per* expression to the DN cells and a few LN_d using a promoter-less *per* construct (Veleri et al. 2003). These data suggest that the DN cells contribute not only to the E peak, but also to the M peak. This is in agreement with the findings for *disco* mutants that lack all LNs but still show some bimodality under LD conditions (Fig. 3). Thus, the DNs alone can mediate aspects of normal activity under LD conditions, and a combination of DN and LN_d cells is sufficient to cause almost wild-type-like activity patterns under LD (Veleri et al. 2003; Stoleru et al. 2004). This applies also to a certain degree if PDF is absent. A closer look at the *pdf⁰¹* mutants that should lack the M oscillator (Fig. 3) shows that these flies do not exhibit a clear M peak; however, several of them show elevated activity in the second half of the night as if they had an extremely advanced M peak. In agreement with such an advanced phase, the free-running activity of all *pdf⁰¹* flies starts from this point when the flies are released into constant conditions (Fig. 3). Thus, a weakly coordinated M peak with advanced phase could be present in these “M-oscillator-less” flies. This advanced M peak could stem from the DN cells that contribute to the M oscillator.

To complicate the story, there are also indications that some of the LN_d cells may contribute to the M oscillator. Flies are very light-sensitive, and they phase-advance and phase-delay their M and E peaks not only under long days, but also when exposed to dim light during the night (Fig. 4A) (Bachleitner et al. 2007). This phase-advance and phase-delay was not only observed for behavior, but also

for the molecular clocks in the LN. As expected, the s-LN_v cells phase-advanced and thus behaved as an M oscillator. But unexpectedly, the majority of LN_d cells also advanced (Fig. 4A). Only the fifth s-LN_v cell behaved as an E oscillator and phase-delayed. Thus, the LN_d cells as an entity did not behave as a bona fide E oscillator under these conditions. However, it is still possible that some LN_d cells acted as an E oscillator and phase-delayed. The peak in PER/TIM immunoreactivity in the LN_d cells did considerably broaden when the flies were exposed to the nocturnal dim-light conditions. This is consistent with the idea that the LN_d cells are a heterogeneous cell group (see above) consisting of M and E oscillators.

To test this idea, we recorded flies that express PER only in the CRY- and Mai-positive LN_d cells and in the fifth s-LN_v cell (*per⁰;Mai179-gal4/pdf-gal80;uas-per¹⁶/+* flies)

under the LD-LM (light moonlight) paradigm. The CRY- and Mai179-positive LN_d cells are those that appear to act as E oscillators under LD and LL conditions (Fig. 2, bottom) (Grima et al. 2004). Indeed, these flies showed a normal E peak and no clearly visible M peak under LD conditions, although some activity before lights on was present (Fig. 4B). However, when the flies were exposed to nocturnal moonlight, a prominent advanced M peak became visible and after transfer into constant moonlight, free-running activity started from the M and E peak (Fig. 4B). This behavior is hard to reconcile with the existence of only the E oscillator. Either some of the three LN_d cells must behave as an M oscillator or additional clock neurons must express PER and have this role. Picot et al. (2007) found weak cycling PER expression in the DN_{1a} cells of a few brains under LD conditions, raising the possibility that

Figure 4. Under nocturnal dim light, flies shift M and E peaks into the night. (A) Histology and behavior of the wild type under light-dark (LD), light-moonlight (LM), constant moonlight (MM), and constant darkness (DD) (for details, see Bachleitner et al. 2007). (B) Flies with PER restricted to the CRY- and Mai179-positive LN_d and to the fifth s-LN_v still show M and E peaks under LM conditions and rhythmicity under MM but not under DD.

the DN_{1a} cells contribute to the M peak in LM. Future studies will have to determine whether these cells start to cycle robustly and with advanced phase under LM conditions or whether some of the LN_d cells behave as an M oscillator under LM conditions. The comparison between LD and LM conditions indicates that the early M activity peak is promoted by light, which was suggested to be a feature of the E cells (Murad et al. 2007; Stoleru et al. 2007; Picot et al. 2007).

There is a further study that is in favor of our idea that the LN_d cells are a mixture of M and E cells (Rieger et al. 2006). The M and E oscillator model predicts that the M oscillator will free-run with a short period and the E oscillator will free-run with a long period when animals are placed in constant light. Indeed, internal desynchronization into two free-running components (one with short period and the other with long period) could be observed in wild-type flies under dim-light conditions (Rieger et al. 2006). In *cry^b* mutants in which the immediate degradation of TIM by light is prevented, this internal desynchronization could even be observed at higher light intensities and it occurred simultaneously in all flies (Fig. 5) (Yoshii et al. 2004). This allowed testing whether PER/TIM cycling in the clock neurons is similarly desynchronized. Indeed it was. In agreement with the M and E model, the PDF-positive s-LN_v cells appeared to control the short-period component, whereas the fifth s-LN_v appeared to control the long-period component. Of the LN_d cells, one cell clearly behaved as an E oscillator showing the same phase as the fifth s-LN_v, whereas the others could not be unequivocally attributed to M or E oscillators (Rieger et al. 2006). In retrospect, we looked again at the stainings and found that three of the six LN_d cells were always stained together with the s-LN_v and three to four LN_d cells (including the one with larger soma that could be unequivocally assessed from the beginning) were stained together with the fifth s-LN_v (Fig. 5). Thus, three LN_d cells may act as an M oscillator and three LN_d cells may act as an E oscillator. In the future, we will determine which of these different LN_d groups are CRY- and Mai-positive and which express the neuropeptide NPF. Nonetheless, there are already indications that the three CRY- and Mai⁷⁹-positive LN_d cells may act as E oscillators in LL. These cells showed a robust cycling in *cry^b* mutants under LL conditions that was in-phase with the fifth s-LN_v cell (Picot et al. 2007). In this experiment, the M cells (PDF-positive s-LN_v) had only a slightly earlier phase than the E cells, probably due to the fact the flies did not show evident internal desynchronization into short and long free-running components at that time (the recordings lasted only for 6 days and the cycling was determined on days 2 to 3; in the previous study, both oscillators were 180° out of phase not before days 4 to 5). Furthermore, flies carrying the *white* eye color mutation were used in the Picot et al. (2007) study, whereas red-eyed flies, which are much more prone to splitting, were used in the Rieger et al. (2006) study.

When the M cells (PDF-positive s-LN_v) were made arrhythmic by expressing CRY in an otherwise *cry^b* background, the flies showed a long period under LL conditions (Murad et al. 2007; M. Picot et al., in prep.). The same situation occurs in a wild-type background after

down-regulating CRY in all clock cells except for s-LN_v. These results are consistent with the previous findings where the E cells free-run with a long period (Rieger et al. 2006). However, the opposite prediction that flies showing oscillations only in the PDF-positive s-LN_v cells (with all other clock cells made arrhythmic by the above-mentioned methods) should show a short period under LL conditions was not fulfilled. Unexpectedly, these flies were behaviorally arrhythmic (Stoleru et al. 2007; Picot et al. 2007). The ultimate explanation of this result is that the PDF-positive s-LN_v cells are not capable of driving rhythmicity under LL conditions.

But how do these results fit with the simultaneous occurrence of short and long periods in *cry^b* mutants under LL conditions? One cannot exclude that the behavior of the multioscillator clock in LL is more complex than predicted by the E/M model (MO accelerates and EO slows down). One possibility is that the short-period component has nothing to do with the M oscillator, but stems from the E oscillator that splits into two components. In favor of this hypothesis is the finding that the short-period component usually appears to start from the evening peak (Fig. 5). But then, why do the M cells (PDF-positive s-LN_v) cycle in-phase with the short-period component? And why does a second short-period component often start from the M peak (Helfrich-Förster 2006; Rieger et al. 2006)?

We propose another hypothesis. There is one fundamental difference in the different experimental designs. Whereas Rieger et al. (2006) tested flies in which all clock neurons carried the *cry^b* phenotype and thus were all rhythmic in LL, Picot et al. (2007) and Stoleru et al. (2007) tested flies in which only the PDF-positive s-LN_v carried this phenotype. As outlined earlier, the PDF-positive s-LN_v cells are most likely not the only M cells. Some LN_d and some DN₁ cells seem also to belong to the M cells, and these cells may be crucial for a normal function of the M oscillator under LL conditions.

Interestingly, the DN_{1a} and the DN_{1p} cells are likely to be among the cells that are responsible for rhythmic behavior under LL conditions after overexpression of PER² (Murad et al. 2007). Our flies with limited PER expression in the three Mai-positive LN_d, the fifth s-LN_v (and possibly the DN_{1a} under certain conditions), were similarly rhythmic under constant moonlight but became arrhythmic after a transfer into DD (Fig. 4B). This raises the possibility that the DN_{1a} cells are important for rhythmicity in low light, in addition to being candidates for the

²This is not stated in the paper, but can be concluded from the strains the authors have used. They report that strains which overexpress PER in all clock cells (under control of the *tim-gal4* driver) are rhythmic under LL conditions. Flies that overexpress PER in all clock cells except the PDF-positive LN_v (*tim-gal4;pdf-gal80;uas-per*) are still rhythmic under LL conditions, indicating that the PDF-positive cells are not responsible for this rhythmicity. When PER is overexpressed in all clock cells except for those that express CRY (*tim-gal4;cry-gal80;uas-per*) the flies are arrhythmic in LL as are wild-type flies. This means that cells that are *cry-gal80*-positive, but *pdf*-negative, are responsible for LL rhythmicity. These are the LN_d, two DN₃, and the DN_{1a} and two DN_{1p}. Because Murad et al. (2007) found molecular oscillations under LL only in some DN₁, the LN_d, and DN₃ can be excluded as source for LL rhythmicity. Thus, the DN_{1a} and DN_{1p} are the best candidates.

Figure 5. Internal desynchronization of a *cry^b* mutant in constant light (LL of 500 $\mu\text{W}/\text{cm}^2$) and the corresponding desynchronization in the lateral neurons on days 4–5 in LL. Flies were immunostained at the two indicated time points, and representative examples for PER and PDF labeling of the lateral neurons are shown below. Because PER staining is at its trough when activity is high, the cells that are strongly stained at the activity peak of the long component (red point) control the short-period component and those that are stained at the activity peak of the short component (blue point) control the long period (for details, see Rieger et al. 2006). Accordingly, the PDF-positive s-LN_v and three LN_d cells appear to govern the short-period component, whereas, the fifth s-LN_v cell, a strongly stained LN_d cell (arrow) and two to three other LN_d cells appear to govern the long-period component.

M oscillator (see above). Most significantly, the DN_{1a} cells have arborizations in close vicinity to the s-LN_v terminals in the dorsal brain and to the dendrites of the s-LN_v in the accessory medulla (see Fig. 1) (Shafer et al. 2006). Thus, the DN_{1a} may mutually interact with the s-LN_v. It is quite likely that the normal function of the M oscillator depends on the DN_{1a} and DN_{1p} under LL conditions, whereas it does depend on the s-LN_v under DD conditions. Both cell types are anatomically well suited to get light input from the compound eyes and/or the H-B eyelet

via the accessory medulla. This light input might interfere with the interaction between the two cell types and might trigger the switch in dominance between both, as it has been proposed between the morning PDF-positive and evening PDF-negative LN (Picot et al. 2007). These ideas about a switch in pacemaker dominance between LL and DD are in line with those of Stoleru et al. (2007) that some clock neurons are the dominant pacemaker cells under short days and others, under long days. According to our results, it is, however, more likely that the dominant pace-

makers under LL are not exclusively E cells but that some M cells (e.g., the DN_{1a}) are included in the “LL clock.” In contrast, some E cells may contribute to rhythmicity under DD conditions.

The Nature of the E Cells

The E cells are less well defined than the M cells. In the Stoleru et al. (2004) study, E cells were ablated by using *cry-gal4;pdf-gal80;uas-hid* flies. In these flies, all CRY-positive cells except the PDF-positive LN_v are ablated. Thus, such flies lack all LN_d,³ the fifth s-LN_v, the DN_{1a}, DN_{1p}, and two DN₃ cells. Among these cells, the three CRY- and Mai179-positive LN_d and the fifth s-LN_v are bona fide E oscillators, whereas the role of the two DN₃ cells is unknown, and the CRY- and Mai179-negative LN_d cells as well as the DN_{1a}, DN_{1p} cells could be more likely M oscillators (see above). This means that not only E cells were ablated in these flies, but also certain M cells. Furthermore, most probably not all E cells were removed, because other DN₁ (not the DN_{1a}, DN_{1p}) and some DN₂ and DN₃ cells seem to act as E oscillators. In later work, the E cells were manipulated using a *tim-gal4;pdf-gal80* driver (Stoleru et al. 2005, 2007). This driver indeed hits all E cells, but additionally also some M cells. How can we reinterpret the published findings taking this knowledge into account? First of all, one would expect that the still present E cells in *cry-gal4;pdf-gal80;uas-hid* flies would influence the activity rhythm. This is indeed true and has been discussed previously (Helfrich-Förster 2006). These flies still showed signs of bimodality in their activity pattern under LD. Besides the normal M peak, a strongly delayed E peak is visible. This peak is somehow comparable with the advanced M peak visible in flies without functioning PDF-positive s-LN_v (*pdf⁰¹* mutants; see Fig. 3). Under DD conditions, the E peak is apparent as shoulder following the putative M peak (see Fig. 2d in Stoleru et al. 2004). This remnant E peak was previously discussed as consequence of the dominant PDF-positive s-LN_v cells that also control aspects of the E peak (see also Rieger et al. 2006; Taghert and Shafer 2006; Miyasako et al. 2007). After our present state of knowledge, we see this remnant E peak as output from the DN cells that act as an E oscillator.

CONCLUSIONS

We have depicted a rather complex picture in which several groups of clock neurons interact to produce *Drosophila*'s typical M- and E-activity bouts. In this picture, the M and E cells are not confined to certain regional clusters of clock neurons, but instead are distributed among several different clusters. Figure 6 summarizes our current view. Certainly, this picture is still partly hypothetical. It relies on several preliminary results that must be confirmed in future studies. Nevertheless, we are approaching an adaptable layered system with emerging properties at multiple levels of organization. Without any doubt, working on the *Drosophila*'s clock remains exciting.

³The used *cry-gal4* (#13) is expressed in all six LN_d. Therefore, all LN_d cells are absent in these flies, including the three cells that show no CRY immunoreactivity and are Mai179-negative.

Figure 6. Semischematic representation of putative M and E cells in the *Drosophila* brain. (Red) M cells; (blue) E cells. Note that the division of the LN_d and the DN₁ in M and E cells is still hypothetical. We know that these cell groups are composed of M and E cells, but we do not know yet which individual cell behaves as M and which as E oscillator. (Gray) All cells for which the engagement in M or E oscillators is not at all clear. For better clarity, the LPN as well as the arborizations of the DN₂ and DN₃ cells are omitted. Only some of the DN₁ (~17 cells) are shown.

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