Cellular/Molecular

Functional Analysis of Circadian Pacemaker Neurons in *Drosophila melanogaster*

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The molecular mechanisms of circadian rhythms are well known, but how multiple clocks within one organism generate a structured rhythmic output remains a mystery. Many animals show bimodal activity rhythms with morning (M) and evening (E) activity bouts. One long-standing model assumes that two mutually coupled oscillators underlie these bouts and show different sensitivities to light. Three groups of lateral neurons (LN) and three groups of dorsal neurons govern behavioral rhythmicity of *Drosophila*. Recent data suggest that two groups of the LN (the ventral subset of the small LN cells and the dorsal subset of LN cells) are plausible candidates for the M and E oscillator, respectively. We provide evidence that these neuronal groups respond differently to light and can be completely desynchronized from one another by constant light, leading to two activity components that free-run with different periods. As expected, a long-period component started from the E activity bout. However, a short-period component originated not exclusively from the morning peak but more prominently from the evening peak. This reveals an interesting deviation from the original Pittendrigh and Daan (1976) model and suggests that a subgroup of the ventral subset of the small LN acts as "main" oscillator controlling M and E activity bouts in *Drosophila*.

Key words: period; *timeless*; dual oscillators; internal desynchronization; constant light; cryptochrome

Introduction

Daily biological rhythms are governed by inherent timekeeping mechanisms, called circadian clocks. Such clocks reside in discrete sites of the brain and consist of multiple autonomous single-cell oscillators (Helfrich-Förster, 2004). Within each neuron, interacting transcriptional and translational molecular feedback loops as well as ionic signaling pathways constitute the oscillatory mechanism of the clock (Nitabach et al., 2002, 2005; Hardin, 2004). It is not understood how individual pacemaker neurons interact to drive behavioral rhythmicity. The long-standing model of Pittendrigh and Daan (1976) assumes that the clock consists of two groups of oscillators with different responsiveness to light, one governing the morning (M) and the other the evening (E) activity of the animal. Typical M and E activity bouts are present in animals ranging from insects to mammals and suggest that the two-oscillatory model is generally valid (Aschoff, 1966; Helfrich-Förster, 2001; Dunlap et al., 2003). Recently, Stoleru et al. (2004) and Grima et al. (2004) showed that M and E bouts could be eliminated or reinstated by manipulating different circadian pacemaker neurons in *Drosophila melanogaster*. This work suggested that the ventral (LN_v) and dorsal (LN_d) subsets of the lateral neurons (see Fig. 1) are the neuronal substrates for the M and E oscillators. It is not known whether these two oscillators respond differently to light (Schwartz, 2004).

The particular power of the two-oscillator model is that it explains observed adaptations to seasonal changes in day length. The model predicts that the M oscillator will shorten and the E oscillator will lengthen its period when exposed to extended constant light (LL) (Pittendrigh and Daan, 1976; Daan et al., 2001). As a consequence, the M activity occurs earlier and the E activity occurs later in long summer days, helping day-active animals avoid the midday heat (Majercak et al., 1999). The model also predicts that the M oscillator will free-run with short period and the E oscillator with long period when animals are placed in constant light. However, such internal desynchronization between oscillators does not occur, because high-intensity constant light usually results in arrhythmicity (Aschoff, 1979; Konopka et al., 1989). In *D. melanogaster*, the clock protein Timeless (TIM) is permanently degraded during light-induced interaction with Cryptochrome (CRY), leading finally to the arrest of the clock (Ceriani et al., 1999; Emery et al., 2000; Rosato et al., 2001; Busza et al., 2004). Without functional CRY, this does not happen. Indeed, internal desynchronization into two free-running components (one with a short period and the other with a long period) was described recently for cry^b mutants under constant-light conditions (Yoshii et al., 2004). In that study, behavioral rhythm dissociation was associated with a dissociation of Period (PER) expression between ventrally and dorsally located pacemaker neurons, but the authors could not distinguish between the LN_v and LN_d. The present study aims to analyze the molecular

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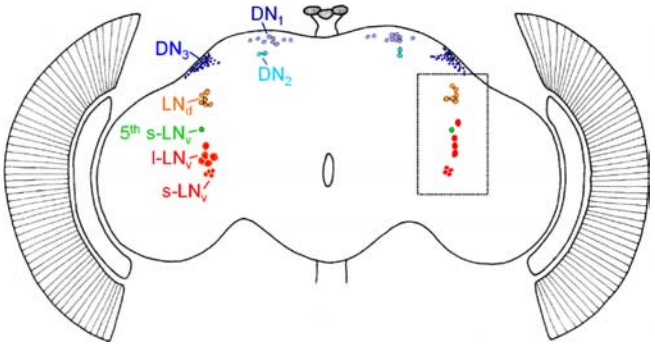

Figure 1. Circadian pacemaker neurons in the brain of the fruit fly. The three clusters of dorsal neurons (DN_1, DN_2, and DN_3 cells) are shown in blue colors, the three clusters of lateral neurons are shown in red (PDF-positive s-LN_v and l-LN_v cells) or orange (LN_d cells), and the PDF-negative 5th s-LN_v cell is depicted in green. The square in the right brain hemisphere indicates the area of the sections shown in Figures 4*A–D*, 6, and 7.

state of all clock gene-expressing neurons during behavioral rhythm dissociation to test the Pittendrigh–Daan model and refine the neuronal substrates of the E and M oscillators.

Materials and Methods

Fly strains and recording of locomotor activity. We used red-eyed cry^b mutants (+/+; $cry^{b\ rec9\ s}$) (Stanewsky et al., 1998), cli^{eya};cry^b double mutants (Rieger et al., 2003), and the wild-type strain Canton S for the present experiments. The main difference between cry^b mutants and cli^{eya};cry^b double mutants is the absence of the compound eyes in the cli^{eya};cry^b flies. Flies were raised on cornmeal medium at 25°C under a 12 h light/dark cycle (LD). At the age of 1–3 d, individual male flies were transferred into the recording chambers. Locomotor activity was recorded photoelectrically at 20°C as described previously (Helfrich-Förster, 1998). Halogen photooptic lamps (Xenophot; Osram) served as a light source, and intensity was adjusted to 500 $\mu W/cm^2$ with a dimmer. Activity was recorded for up to 7 d under LD, and then the flies were transferred to LL of the same intensity. To better see the rise of the M activity bout in cry^b mutants, which usually occurs before lights on but was essentially suppressed by darkness in these mutants, one group of cry^b flies was exposed to weak light (0.25 $\mu W/cm^2$ equaling full-moon light) during the dark phase of their LD cycle. Afterward, they were transferred to LL of the same light intensity (500 $\mu W/cm^2$) as the first group. Wild-type Canton S flies were recorded under LL conditions of moon light intensity (0.25 $\mu W/cm^2$) additionally to that of 500 $\mu W/cm^2$, because all flies became immediately arrhythmic at the high luminance.

Data analysis. The raw data were displayed as actograms (double plots) to judge the activity pattern, and the periods under LL were determined by the Sokolove-Bushell periodogram analysis (program El Temps; Diez-Noguera, Barcelona, Spain). To reveal the behavior of all cry^b flies on days 1, 5, and 11 in LL, average ± SE activity profiles were calculated for these single days as described previously (Helfrich-Förster, 2000).

Immunohistochemistry and quantification of staining intensity. Brains of wild-type and cry^b mutants flies were examined with respect to the position of the different circadian pacemaker groups (Fig. 1). For that purpose, 10 wild-type and 10 cry^b mutants were collected 1 h before lights on of LD because, at that time, TIM and PER levels are close to their peak levels.

To gauge the extent of neuronal desynchronization, 10 wild-type and 10 cry^b flies were collected at four different time points in LL (see Fig. 3, a, b on the first day in LL and c, d on the fifth day in LL). As additional controls, 10 eyeless cli^{eya};cry^b flies were collected at their activity minimum and maximum on the fifth day in LL. At the different collection times in LL, flies were quickly killed by submersion in 4% freshly prepared paraformaldehyde in phosphate buffer (PB) with 0.5% Triton X-100. After 2 h fixation, the flies were rinsed three times for 15 min in PB, and their brains were dissected as whole mounts. After blocking in 5% normal goat serum overnight, triple immunostainings were performed on the whole-mount brains with a rabbit anti-PER serum [diluted 1:1000 (Stanewsky et al., 1997)], a rat anti-TIM serum [diluted 1:1000 (Kaneko et al., 1997)], and the monoclonal mouse antibody nb33 (diluted 1:100). The latter recognizes the neuropeptide pigment-dispersing factor (PDF)-positive neurons (Veleri et al., 2003). Antibody labeling was performed for 48 h at 4°C. After rinsing five times in PB (0.5% Triton X-100), secondary fluorescence-conjugated antibodies were applied overnight: Alexa Fluor 488 (goat anti-rabbit), Alexa Fluor 568 (goat anti-rat), and Alexa Fluor 647 (goat anti-mouse), all diluted 1:200 (Invitrogen, Carlsbad, CA). Triple-labeling (green channel, PER; red channel, TIM; infrared channel converted to blue, PDF) was visualized by laser-scanning confocal microscopy (LSM 510 META; Zeiss, Oberkochen, Germany). To exclude bleed through, we used sequential scans of the three laser lines. Images presented in figures are overlays of several confocal stacks.

After discriminating between the different neurons on the overlays, scoring of staining intensities was performed on single optical sections that contained the nucleus for each neuron separately. For that purpose, the green (PER) and red (TIM) confocal channels were separately converted into grayscale. The grayscale pictures were imported into the program NIH Image J (version 1.33u; Wayne Rasband, freely available at http://rsb.info.nih.gov/ij/). For each neuron, mean pixel intensity of PER or TIM staining was measured for either the cytoplasm or the nucleus, depending on which compartment was more strongly stained. Only subset 3 of the dorsal neurons (DN_3) were scored as an entire group (using the magic wand in the plug-in Yawi 2D) without discriminating between nuclear and cytoplasmic PER and TIM expression, because these cells were too small and too numerous to be scored individually. Grayscale units ranged between 0 (black) and 255 (white). A mean staining intensity was calculated from all neurons within one group for each hemisphere separately. Within the LN_d, one neuron was consistently more strongly stained than the others. The staining intensity of this neuron was not averaged with the remaining cells of the group but was treated separately as the "extra LN_d." A background staining level was measured in the surrounding field of each neuronal group and subtracted from the mean pixel intensities measured for the cells. A staining index for each cell group was calculated by multiplying the staining intensity (minus background) with the number of stained cells divided by the maximum number of stained cells within this specific cluster. The maximum number of cells for the different cell clusters was as follows: PDF-positive s-LN_v, 4; PDF-negative small (s) LN_v, 1; large (l) LN_v, 5; extra LN_d, 1; remaining LN_d, 6; DN_1, 17; DN_2, 2; and DN_3, 40.

Statistics. Staining indices were tested for significant influences of strain and time point using two-way ANOVAs. A subsequent *post hoc* test with Bonferroni's adjustment was applied for pairwise comparisons of staining indices (Systat 10; SPSS, Chicago, IL). Values were regarded as significantly different at $p < 0.05$.

Results

Behavioral rhythms

The activity rhythm of 20 wild-type flies, 40 cry^b mutants, and 33 cli^{eya};cry^b double mutants was recorded for 7 d under LD and subsequently for 3 weeks under LL (light intensity, 500 $\mu W/cm^2$). Under LD, all flies showed the typical bimodal activity pattern consisting of M and E activity bouts (Figs. 2, 3). Consistent with previous reports, the M activity bout was weaker than the E activity bout. After transfer into LL, all wild-type flies became immediately arrhythmic (Fig. 2*A*), whereas the cli^{eya};cry^b flies remained rhythmic and free-ran with a period close to 24 h (Fig. 2*E*, Table 1) (see also below). In the majority of cry^b single mutants, the M activity bout became invisible immediately after transfer into LL (Fig. 3). Thus, the activity changed from bimodal to unimodal with one major activity bout corresponding to the E peak. The same behavior is typical for wild-type flies after transfer into constant darkness (DD), but this does not mean that the putative M oscillator lost its activity (see Fig. 9, Table 3) (cf.

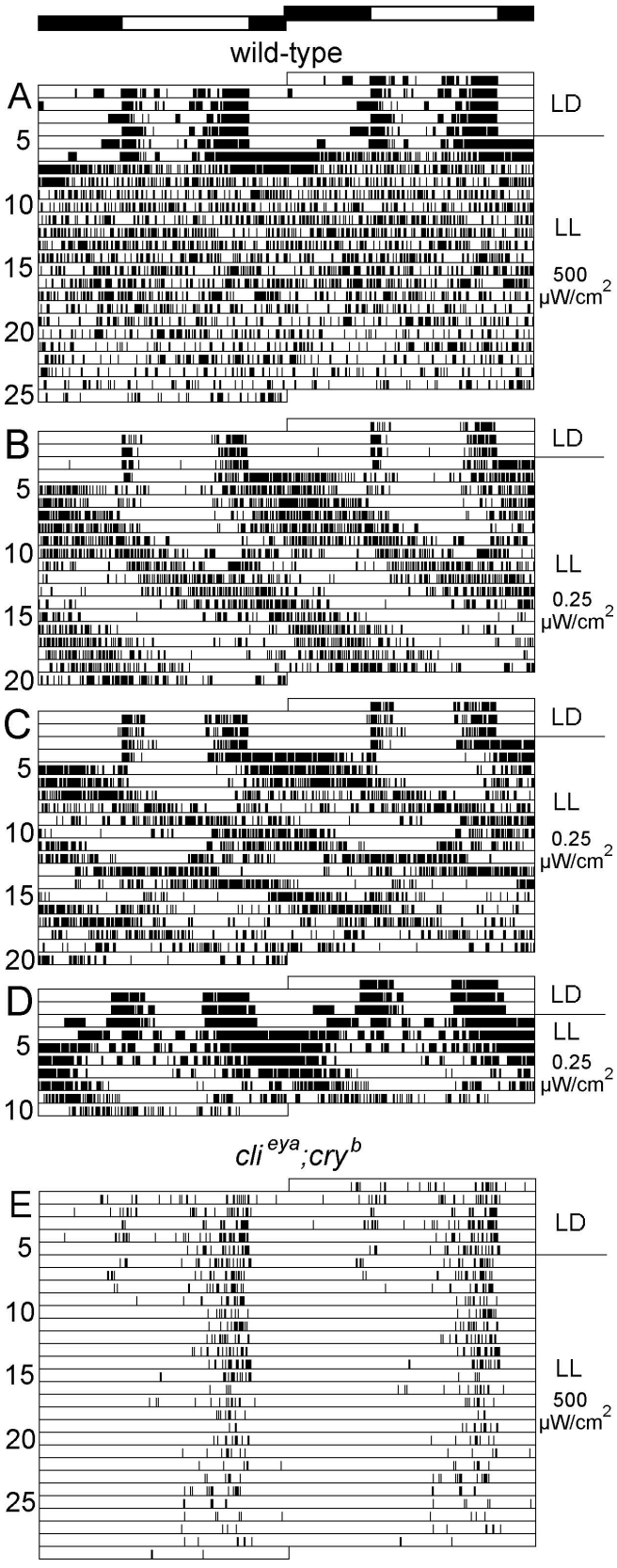

Figure 2. Constant dim light induces arrhythmicity and/or desynchronization in wild-type flies. Actograms of four wild-type flies (***A–D***) and $cli^{eya};cry^{b}$ mutants (***E***) are shown that were recorded under an LD cycle and subsequently under LL conditions. The light program is indicated as white and black bars on top. The actograms are shown as double plot to better see the activity pattern under LL. During LD, light intensity was 500 $\mu W/cm^2$ for all flies; during LL, light intensity was kept at 500 $\mu W/cm^2$ for the $cli^{eya};cry^{b}$ mutant (***E***) and the wild-type fly (***A***); for the other wild-type flies, light intensity was reduced to 0.25 $\mu W/cm^2$. Under 500 $\mu W/cm^2$ light, the wild-type fly became arrhythmic immediately after transfer into LL (***A***), whereas the $cli^{eya};cry^{b}$ mutant remained rhythmic (***E***). At 0.25 $\mu W/cm^2$, the wild-type flies showed internal desynchronization into two free-running components, one with a longer period and the other with a shorter period (see Table 1). Long and short components stayed together after the first cross-over in the fly shown in ***B***, whereas they showed full cross-over for two times in the fly shown in ***C***. In ***B*** and ***D***, the short-period component appears to originate from the morning peak and the long-period component from the evening peak.

Helfrich-Förster, 2001). For sake of clarity, we will now concentrate on the clearly visible E activity bout and refer to the M activity bout later. After a few days in LL, the E activity bout showed internal desynchronization into two activity components with short and long periods, respectively (Fig. 3). The frequency of internal desynchronization was found previously to be positively correlated with the light intensity, and the period of the short component became shorter with increasing light intensity and that of the long component became longer (Yoshii et al., 2004). Both findings indicate that internal desynchronization gradually increases with light intensity. In our experiment, all 40 flies showed internal desynchronization, with the short component having a period of 22.5 h and the long component a period of 25.2 h (Table 1). These coincide exactly with the periods observed by Yoshii et al. (2004) at the relevant light intensity. On the fifth day in LL, both components were 12 h out-of-phase with each other, and, after 11 d, they were in-phase again (Fig. 3). This behavior had a very similar time course in all flies, as can be seen in the average activity profile of all 40 flies on days 1, 5, and 11 (Fig. 3). After having met on day 11, both components separated again (full crossing over) in half of the flies; in the other half, the components remained coupled and kept an intermediate period (23.5 h) (Table 1). The intermediate period was clearly closer to the period of the previous short component than to that of the longer component.

The eyeless $cli^{eya};cry^{b}$ flies were tested to identify the photoreceptors responsible for the observed period lengthening and shortening under LL. The compound eyes are the most likely candidates because these are necessary for the adequate timing of M and E activity bouts in long summer days and short winter days (Rieger et al., 2003). As discussed above, this is a central task of M and E oscillators. Indeed, none of the 16 $cli^{eya};cry^{b}$ double mutants showed internal desynchronization, and all free-ran with a wild-type-like period (Fig. 2*E*, Table 1). This indicates that the compound eyes are the photoreceptors that transmit the light information to the putative M and E oscillators, provoking a period shortening in the former and a period lengthening in the latter.

To ensure that the observed internal desynchronization is not a peculiarity of cry^{b} mutants, we also studied the behavior of 23 wild-type flies under LL conditions of very-low-light intensity (0.25 $\mu W/cm^2$). Under these conditions, only one fly became arrhythmic, seven flies free-ran with a rather long period (~27 h), and 15 flies showed two free-running periods as revealed by periodogram analysis, one with a mean of 23.7 h and the second with 28.2 h (Fig. 2*B–D*, Table 1). The shorter period component was not as clear as in cry^{b} mutants, but it could be seen in each actogram for at least 3 d (Fig. 2*B*). We conclude that the ability to show internal desynchronization into two activity components during light is a general feature of the locomotor rhythm of *D. melanogaster*. As for cry^{b} mutants, half of the wild-type flies showed full crossing over of both activity components under low light (Fig. 2*C*), whereas in the other half, the components coupled

and continued with an intermediate period (Fig. 2*B*, Table 1). Note that one fly showed a very prominent M activity bout under LD (Fig. 2*D*). In this fly, the short-period component appeared to originate from the M activity bout. Unfortunately, this fly died on day 10, so we could not further observe its behavior.

Characterization of the different groups of clock neurons

D. melanogaster possesses six groups of circadian pacemaker neurons: three groups of lateral neurons (the s-LN_v, l-LN_v, and LN_d cells) and three groups of dorsal neurons (the DN_1, DN_2, and DN_3 cells) (Fig. 1). The s-LN_v cluster can be further divided into four cells that express the PDF and in one cell that is PDF negative and known as 5th s-LN_v (Kaneko et al., 1997).

The first aim was to distinguish these neurons unequivocally from each other as well as to discriminate cytoplasmic from nuclear PER and TIM staining. The latter point seems trivial, but we found these proteins frequently located close to the nuclear membrane (Fig. 4) (and below), making it difficult to decide between nuclear and cytoplasmic staining, especially in small neurons. Generally, we defined a protein location as nuclear if substantial staining was also found in more central parts of the nucleus and as cytoplasmic if no staining was visible in these places. To identify the individual clock neurons, we performed triple immunostainings with antibodies against the clock proteins PER and TIM and against the neuropeptide PDF at the time at which PER and TIM were close to peak levels (1 h before lights on in LD). To ensure that the secondary antibodies from rat and mouse do not show any cross-reaction, we applied both secondary antisera also to brains that were either single labeled with only mouse anti-nb33 or only rat anti-TIM. We found no cross-reaction of the goat anti-mouse antibody with the rat anti-TIM, but we detected a weak cross-reaction of the anti-rat secondary antibody with mouse anti-nb33. This cross-reaction was restricted to the cytoplasm of the l-LN_v, which contains a very large amount of PDF and was consequently very strongly labeled by the mouse anti-nb33. All other PDF-positive structures involving the strongly labeled arborizations arising from the l-LN_v cells, as well as the s-LN_v with their arborizations were not double labeled by the anti-rat secondary antibody. We conclude that the secondary antibodies are sufficiently specific for our needs, but we excluded the observed TIM staining in the cytoplasm of the l-LN_v from additional analysis.

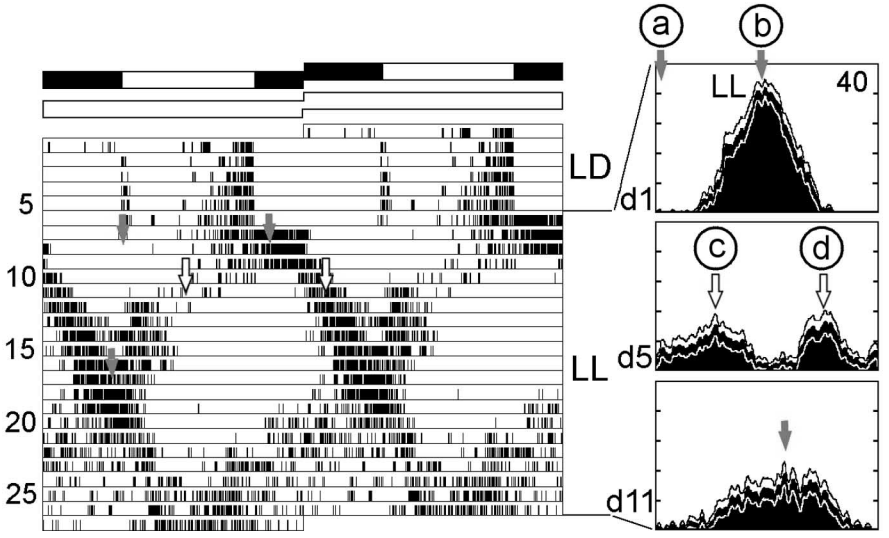

Figure 3. Internal desynchronization in *cry*^{b} mutants. Actogram of a typical *cry*^{b} fly recorded for 7 d under an LD and for an additional 20 d under LL. The light program is indicated as white and black bars on top. Light intensity was 500 μW/cm^2. The actogram is shown as double plot and reveals two free-running rhythm components, one with a short period (τ of ~22 h) and the other with a long period (τ of ~25 h). On day 1 in LL (d1), both rhythms were in-phase and the average ± SE activity profile calculated from all 40 flies showed one single activity bout (top right). On day 5 in LL (d5), the long and short components were 12 h out-of-phase, with two activity bouts visible in the average activity profile (middle right). On day 11, the two components crossed and therefore appear as a single peak in the average activity profile (bottom right). The flies were fixed on day 1 and day 5 at the indicated times (time points a and b, gray arrows on day 1; time points c and d, open arrows on day 5).

The anti-PDF labeling was necessary to distinguish the PDF-positive s-LN_v and l-LN_v cells (Helfrich-Förster, 1995; Renn et al., 1999) from the LN_d and the 5th PDF-negative s-LN_v cell (Fig. 1). The 5th s-LN_v cell has only been described in larvae, because it was easily discerned from the few other larval clock neurons (Kaneko et al., 1997). During metamorphosis, glia cells start to express the clock proteins, and, because many PER-positive glia cells are found in close vicinity to the LN cells, it was hard to discern the 5th s-LN_v among them in adults. Interestingly, we found only weak PER and TIM labeling in glia cells, and, as a consequence, we could unequivocally detect one PER–TIM-positive, but PDF-negative, neuron among the LN_v cells. We regarded this cell as the 5th PDF-negative s-LN_v cell. This neuron was not located among the four PDF-positive s-LN_v cells but rather was among the more anteriorly located four to five PDF-positive l-LN_v cells (Fig. 4*A–C*). Depending on the position of the l-LN_v cells in the dorsoventral axis, the 5th s-LN_v was located among them (Fig. 4*H–K*), dorsally to them, or slightly ventral to them (Table 2).

The l-LN_v were the cells with the most variable position. In

Table 1. Rhythmic activity patterns under LL

Genotype/condition	*n*	Arrhythmicity	1 rhythmic component	τ (h)	2 rhythmic components	τ_1 (h)	τ_2 (h)	Full crossover	τ (h) if no crossover
WT/LL (500 μW/cm^2)	16	16 (100%)							
WT/LL (0.25 μW/cm^2)	23	1 (4%)	7 (30%)	27.3 ± 0.18	15 (65%)	23.7 ± 0.13	28.2 ± 0.31	7 (47 %)	27.5 ± 0.35
cry^b/LL (500 μW/cm^2)	40	0 (0%)			40 (100%)	22.5 ± 0.04	25.2 ± 0.05	21 (53 %)	23.5 ± 0.03
cli^{eya};*cry*^{b}/LL (500 μW/cm^2)	33	0 (0%)	33 (100%)	23.8 ± 0.08					

WT, Wild-type.

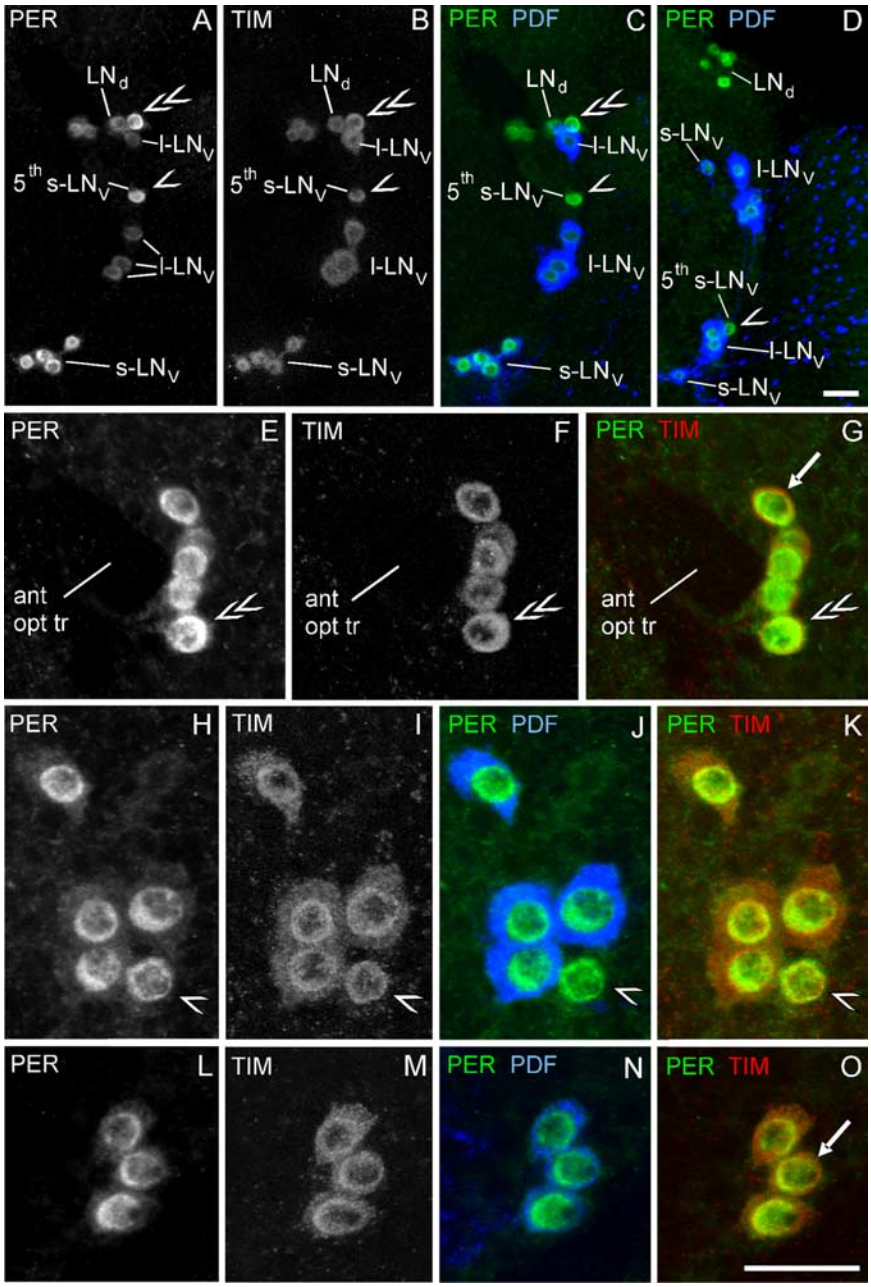

Figure 4. PER, TIM, and PDF expression in the lateral neurons of wild-type flies. For clarity, single labeling is shown in gray, and double labeling is shown in color. ***A–C***, ***E–G***, ***H–K***, and ***L–O*** show the same optical sections, respectively. Brains were stained 1 h before lights on in an LD cycle. At this time point, PER and TIM were predominantly nuclear in all lateral neurons: the LN$_d$ (***E–G***), the l-LN$_v$ (***H–K***), the 5th s-LN$_v$ (arrowhead in ***A–D***, ***H–K***), and the s-LN$_v$ (***L–O***). The strongest PER and TIM labeling was found close to the nuclear membrane, whereby TIM was always located slightly lateral of PER (see arrows in ***G*** and ***O***). PDF was exclusively found in the cytoplasm of the l-LN$_v$ (***J***) and s-LN$_v$ (***N***), as well as in their arborizations (e.g., the network on the surface of the medulla in ***D***). Note that the nuclei of all groups of lateral neurons were more or less of similar size, but the cytoplasmic area was larger in the l-LN$_v$. The 5th PDF-negative s-LN$_v$ (arrowhead) could be distinguished from the other cells because it lacked PDF. It was located among the l-LN$_v$ (***A–D***, ***H–K***). The LN$_d$ were usually grouped around the anterior optic tract (ant opt tr; ***E–G***) at the place in which it enters the central brain. One cell of the LN$_d$ was usually more prominently stained than the others and appeared slightly larger (double arrowhead, ***A–C***, ***E–G***). In the brain shown in ***D***, the LN$_d$ were displaced dorsally toward the DN$_3$ (not in the focal plane). Furthermore, two l-LN$_v$ and three s-LN$_v$ cells (2 of the latter are below the displaced l-LN$_v$ and are hard to see) were displaced toward the original location of the LN$_d$. In ***A–C***, one l-LN$_v$ was displaced toward the LN$_d$. Note that the extra LN$_d$ (double arrowhead), the 5th s-LN$_v$ (arrowhead), and the PDF-positive s-LN$_v$ are the cells that are most prominently stained by anti-PER in ***A***. In ***A–D***, 10 confocal sections of 5 μm were combined; ***E–O*** consist of five confocal sections of 1 μm. ***A–D*** and ***E–O*** are of the same magnification, respectively. Scale bars, 20 μm.

almost half of the brain hemispheres, at least one l-LN$_v$ cell was dorsally dislocated and found among the LN$_d$ (Fig. 4*A–C*, Table 2). In two wild-type brain hemispheres (of different brains), three of four stained l-LN$_v$ were not only displaced dorsally but additionally posteriorly; these were found on the posterior surface of the brain instead of the anterior surface. In one hemisphere of a *cry^b* brain, all four l-LN$_v$ were ventrally displaced. This was the only case in which we found a ventral displacement of the l-LN$_v$.

The position of the LN$_d$ was the next most variable. In 20–30% of the brain hemispheres, they were situated dorsally among the DN$_3$ (Fig. 4*D*, Table 2). The s-LN$_v$ held the most constant position, but we still found one to three s-LN$_v$ cells situated in dorsal position in 5–10% of the brain hemispheres (Fig. 4*D*, Table 2). χ^2 analysis revealed no significant difference in dislocalized cell groups between wild-type and *cry^b* flies (Table 2).

In addition to their variable position, we found the LN$_d$ stained to different degrees by PER and TIM antibodies. In 65% of the brains, one LN$_d$ was more strongly stained than the others. This single neuron was characterized by a slightly larger nucleus (Fig. 4*E–G*) and cytoplasmic area (see Fig. 6) compared with the majority of LN$_d$ cells. This neuron will be referred to as extra LN$_d$ cell.

Among the DN cell groups, the two DN$_2$ cells held the most constant position and were easy to identify, because they were always associated with the terminals of the PDF-positive s-LN$_v$ cells (Fig. 5). The DN$_1$ were more difficult to characterize. These consisted of up to 17 cells that were quite variable in their position; in half of the cases, these were rather clustered, and, in the other half, they were dispersed in the dorsal brain. The staining intensity within the DN$_1$ cells was quite variable. Approximately half of the DN$_1$ cells was strongly stained by anti-PER and anti-TIM, whereas the other half was only weakly labeled (Fig. 5*B*, *C*). Comprising as many as 40 cells, the DN$_3$ cluster was the largest group. The size of the great majority of these neurons was rather small, but, in approximately one-third of the cases, we found up to five larger neurons in a more lateral position (Fig. 5*A*). These cells were clearly different from dorsally dislocated LN$_d$ cells.

In summary, we conclude that the LN$_d$, the DN$_1$, and the DN$_3$ may not represent homogeneous groups of neurons and that this fact has to be taken into account in this study. Similar observations were made in a contemporary study by O. T. Shafer and P. H. Taghert (unpublished observation).

Intracellular location of PER and TIM

As expected, we found PER and TIM predominantly in the nucleus 1 h before lights on in LD. These proteins were not uni-

formly distributed in the nucleus but were restricted to certain compartments, whereby the unmarked regions within the nucleus might correspond to regions of the nucleolus (Fig. 4*E–O*). The strongest PER immunoreactivity was found at the border of the nucleus close to the nuclear membrane. TIM showed a similar distribution, but the most prominent TIM immunoreactivity was found slightly centrifugal (lateral) of the PER immunoreactivity, suggesting that TIM forms a ring around the nuclear membrane, remaining partly on the cytoplasmic site, whereas PER is mainly located inside the nucleus. This was true for all clock neurons, and it is shown in detail for the three groups of lateral neurons (Fig. 4*E–O*). PDF remained entirely cytoplasmic and therefore served as good marker for the cytoplasm in the l-LN_v (Fig. 4*H–K*) and s-LN_v (Fig. 4*L–O*). The l-LN_v showed a large cytoplasmic area (Fig. 4*J*), whereas the s-LN_v contained only small amounts of cytoplasm around the nucleus (Fig. 4*N*). The cytoplasmic area of the PDF-negative 5th s-LN_v, which was always found in vicinity to the l-LN_v, was more difficult to judge, but it seemed similar to that of the s-LN_v (Fig. 4*H–K*).

Table 2. Location of the 5th s-LNv and displacement of the other lateral neurons (l-LN_v, LN_d, and s-LN_v)

		Location of the 5ths-LNv		
Genotype	*N*	Among l-LN_v	Slightly dorsal of l-LN_v	Slightly ventral of l-LN_v
WT	20	10 (50 %)	7 (35 %)	3 (15 %)
cry^b	20	9 (45 %)	9 (45 %)	2 (10 %)
	$\chi^2 = 0.50; p = 0.78$*			
		Hemispheres with displacement of		
		l-LN_v	LN_d	s-LN_v
WT	20	9 (45 %)	6 (30 %)	1 (5 %)
cry^b	20	8 (40 %)	4 (20 %)	2 (10 %)
	$\chi^2 = 0.16; p = 0.92$*			

*No significant difference was found between wild-type (WT) flies and *cry*^{b} mutants concerning the location of the lateral neurons.

Clock protein rhythms in LL

After ensuring that we could unequivocally identify the different groups of clock neurons, we determined whether the molecular cycling in individual pacemaker neurons becomes internally desynchronized after some days under LL in a manner parallel to the activity rhythm. In contrast to their bimodal activity pattern under LD, wild-type flies have been shown to exhibit a unimodal rhythm in clock protein accumulation. PER and TIM levels are highest ~3 h before lights on and lowest 12 h later, as revealed by Western blots on head extracts (Marrus et al., 1996). This means that PER and TIM are at their peak levels at the time of the M activity bout and at trough level at the time of the E activity bout. In the clock neurons, the cycling of PER and/or TIM has been revealed immunohistochemically by several investigators (Zerr et al., 1990; Shafer et al., 2002). These studies revealed no evident phase differences between the diverse neuronal groups. All neurons appear to cycle more or less in-phase and to have the lowest clock protein levels during the flies' main activity peak (the E activity bout). This appears also true for the *cry*^{b} mutants, but only the amplitude of the cycling was reduced in the LN_d, l-LN_v, and DN_2 cells (Helfrich-Förster et al., 2001; Yoshii et al., 2004). Therefore, we expected all clock neurons to cycle in-phase on the first day in LL, whereas some of them might be out-of-phase several days later under the state of internal desynchronization. This was most easily investigated in *cry*^{b} mutants because, in all *cry*^{b} flies, the two activity components were in-phase on day 1 and 12 h out-of-phase on day 5 of LL (Fig. 3).

To determine the molecular cycling profiles in the different pacemaker neurons, we performed triple labeling with anti-PER, anti-TIM, and anti-PDF at four critical time points: (1) at the peak and trough of the main activity bout on day 1 when both components were in-phase and (2) at the peak points of both activity components on day 5 when these were 180° out-of-phase (Fig. 3). In wild-type control flies, PER and TIM levels in the clock neurons were extremely low already on the first day of LL, and no staining at all was found on day 5 (data not shown). This result correlates well with the arrhythmic behavior of the flies under LL conditions at 500 $\mu W/cm^2$. In *cry*^{b} mutants, PER and TIM continued to cycle under LL in the s-LN_v and the LN_d (Yoshii et al., 2004 and this study). On day 1, we found strong immunostaining at the trough point of the E activity bout and weak immunostaining at its peak point, coinciding nicely with previous results (Yoshii et al., 2004). Highly significant cycling of clock proteins was found in the 5th s-LN_v cell, the PDF-positive s-LN_v cells, and the extra LN_d cell (Fig. 6). The extra LN_d cell was now discernible as a very prominently stained cell in 95% of the brains. The clock proteins were nuclear at the trough point of the E activity bout and cytoplasmic at its peak point, showing that they undergo not only a cycling in abundance but also in subcellular location, as shown previously for wild-type flies under constant-dark conditions (Shafer et al., 2002). Some discrepancy occurred in PER and TIM staining levels in the s-LN_v cells. Although the difference in PER staining at the two time points was highly significant ($p < 0.001$), TIM staining was not significantly different in the s-LN_v cells. Nevertheless, TIM was nuclear at the time point of high PER staining and cytoplasmic at the time point of low PER staining. Thus, TIM clearly cycled in subcellular location in the PDF-positive s-LN_v cells. The same was true for the remaining LN_d and for the DN_2. There was a cycling in subcellular location of PER and TIM, but the observed staining differences turned out to be not significant in the *post hoc* test with Bonferroni's adaptation.

The l-LN_v, DN_1, and DN_3 cells showed similar overall staining intensities at both time points. In the l-LN_v, PER was found in the nucleus at both time points, whereas TIM appeared to remain in the cytoplasm (see also below). This suggests that there is no clock protein cycling in the l-LN_v as was found in wild-type flies under DD conditions (Kaneko et al., 2000; Yang and Sehgal, 2001; Veleri et al., 2003). It should, however, be noted that Peng et al. (2003) found the rhythm in *tim* mRNA cycling to come back in these neurons after 9 d in DD. In the DN_1 and the DN_3, both clock proteins were found in the nucleus in some cells and in the cytoplasm in other cells (Fig. 5*C*), suggesting that these groups may consist of at least two subclusters of cells that may cycle out-of-phase with each other. Because we had no markers for the different subclusters, it was impossible to unequivocally distinguish the subgroups described above (the different DN_1 cells and the laterally located DN_3 cells with larger cell bodies).

On day 5 in LL, no cycling of PER and TIM expression were observed for the l-LN_v, DN_1, and DN_3. No evident difference in level and location of the clock proteins occurred between the two time points (Fig. 7). The other groups of neurons appeared to maintain a cycling in PER and TIM, which, for the LN_d and DN_2 cells, was again not statistically significant (Fig. 7). Most notably, the phase of the four PDF-positive s-LN_v cells was now reversed in respect to the other cycling pacemaker groups. The PDF-positive s-LN_v cells were strongly stained at the activity peak point of the long-period component (the activity trough of the

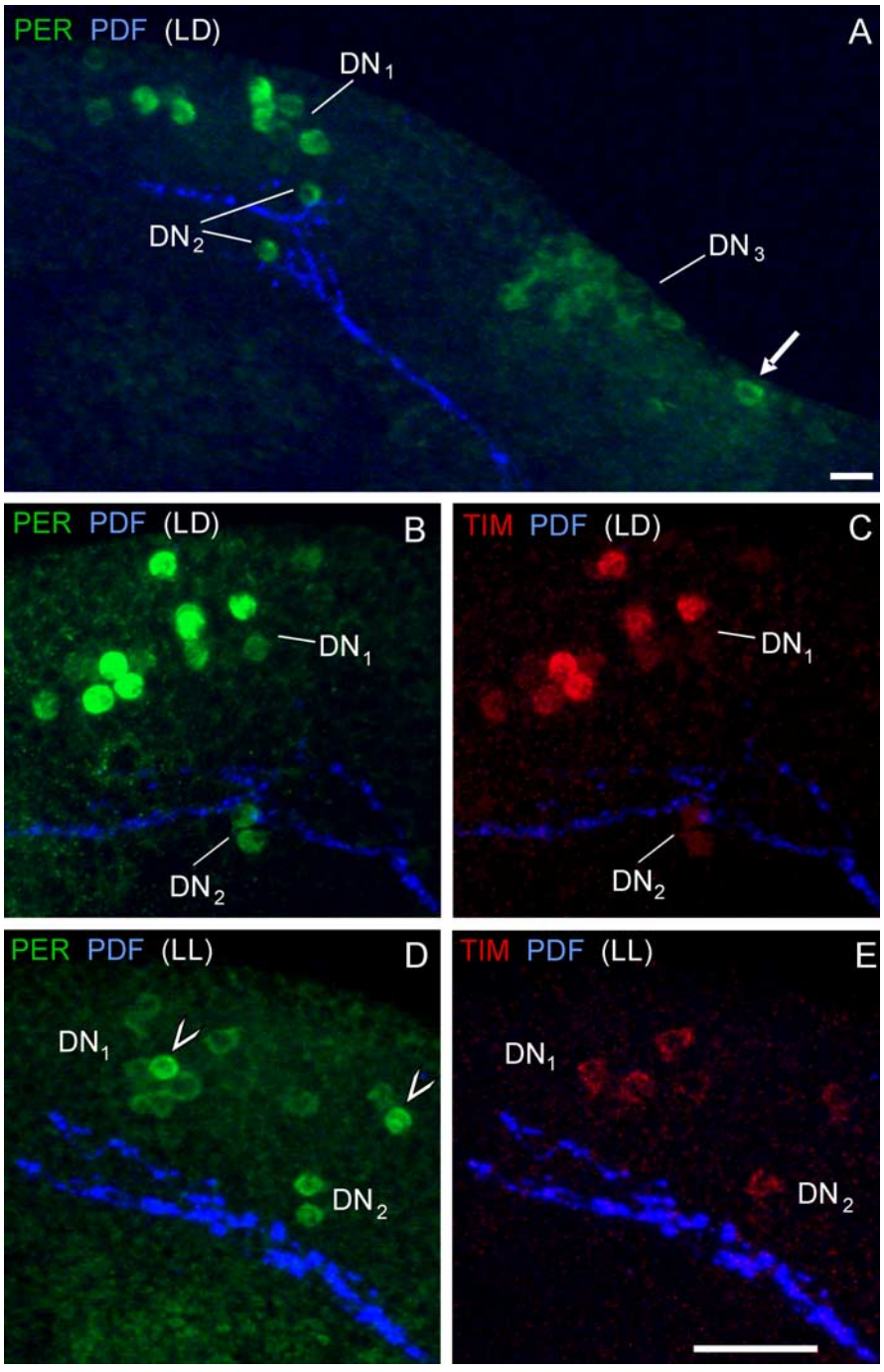

Figure 5. The dorsal neurons of *cry*^{b} flies stained with anti-PER (green), anti-TIM (red), and anti-PDF (blue) 1 h before lights on in an LD cycle (***A–C***) and on the fifth day of LL at time point c (***D***, ***E***). One hour before lights on (***A–C***), PER and TIM were nuclear in most dorsal neurons (the DN_1, DN_2, and DN_3 cells) except for a few DN_3 cells (arrow in ***A***). The DN_1 consisted of up to 17 cells that showed a different intensity of PER and TIM labeling; half of the cells were strongly labeled, and the other half were only weakly labeled (***B***, ***C***). The two DN_2 cells were always close to the terminals of the s-LN_v (blue), and they showed rather weak TIM labeling. On the fifth day of LL, TIM labeling was very weak in all dorsal neurons and was restricted to the cytoplasm (***D***, ***E***). PER labeling was nuclear in some DN_1 cells and cytoplasmic in others. Note that nuclear PER labeling was found in two DN_1 cells that showed no TIM labeling at all (arrowheads in ***D***). ***A*** consists of 12 confocal sections (5 μm). ***B*** and ***C*** are composed of five confocal sections (2 μm) and are of the same magnification. Scale bars, 20 μm.

short-period component), whereas the other neuronal groups (especially the 5th PDF-negative s-LN_v cell and extra LN_d cell) were maximally stained at the peak point of the short-period component (the activity trough of the long-period component) (Fig. 7). This suggests that the four PDF-positive s-LN_v cells control the short-period component, whereas the 5th PDF-negative s-LN_v cell and extra LN_d cell control the long-period component. Obviously, internal desynchronization has occurred between the PDF-positive s-LN_v cells and the 5th s-LN_v and extra LN_d. Thus, input from the compound eyes seems to shorten the period of the PDF-positive s-LN_v cells and to lengthen the period of the 5th PDF-negative s-LN_v and the extra LN_d.

To ensure that the observed internal desynchronization between PDF-positive s-LN_v and the other two neurons does indeed correlate with the behavioral splitting of *cry*^{b} flies and does not generally occur after several days under LL conditions, we stained *cli*^{eya};*cry*^{b} flies at their E activity peak and trough on day 5. These flies do not show any behavioral desynchronization on day 5 under LL (Fig. 2*E*), and, consistent with this behavior, we observed no internal desynchronization between the neuronal groups (Fig. 8). All cell groups were found to be in-phase with each other. Actually, the staining pattern was very similar to *cry*^{b} flies on the first day in LL (Fig. 6), with the exception that TIM levels were lower and thus similar to those of *cry*^{b} flies on the fifth day in LL (Fig. 7).

To exclude the possibility that the observed internal desynchronization between the neuronal groups is a kind of stationary state in *cry*^{b} flies, we also stained flies at two additional time points, just between the activity peaks of both components on day 5 in LL. As expected, we found intermediate staining intensities in all neurons (data not shown), indicating that PER and TIM do indeed cycle in the different neurons. This is consistent with the results of Yoshii et al. (2004), who stained flies every 3 h throughout day 1 and day 4–5 in LL and additionally on day 9, at which both activity components including both groups of neurons were in-phase again.

Although our study is of correlative nature, the results of these experiments further support the model in which behavioral desynchronization is caused by the internal desynchronization between the PDF-positive s-LN_v cells and the 5th s-LN_v and the extra LN_d.

Differences in PER and TIM labeling

The cycling of both clock proteins appeared to occur principally in parallel, but we observed certain differences in PER and TIM labeling under LL. TIM staining was generally weaker than PER staining (except for the l-LN_v, which showed high cytoplasmic TIM labeling at all time points investigated, but this might be partly attributable to the slight cross-reaction we found for the secondary anti-mouse and anti-rat antibodies and will not be considered further). The low TIM levels are especially evident

in the dorsal neurons after 5 d exposure to constant light. TIM was rather weak and mainly cytoplasmic in all three DN clusters (Figs. 5*D*,*E*, 7). This suggests that the dorsal neurons possess an additional photopigment that is different from CRY but that may likewise lead to a light-dependent degradation of TIM. The existence of such a still unknown photopigment was predicted by previous studies (Rieger et al., 2003; Veleri et al., 2003). Interestingly, the virtual absence of TIM had little effect on the PER levels in these cells. PER did even continue to move into the nucleus (Fig. 5*C*). These results corroborate previous studies by Shafer et al. (2002, 2004) showing that PER and TIM do not necessarily have to enter the nucleus together within pacemaker neurons. In support of this contention, we revealed putative phase differences between PER and TIM in the PDF-positive s-LN_v.

Do the PDF-positive s-LN_v cells compose the M oscillator and the LN_d the E oscillator in the Pittendrigh–Daan model?

Previous studies suggested that the PDF-positive s-LN_v control the M activity bout, whereas the LN_d govern the E activity bout (Grima et al., 2004; Stoleru et al., 2004). According to the Pittendrigh–Daan model, the PDF-positive s-LN_v cells underlie the M oscillator and should shorten their period under constant-light conditions, whereas the LN_d underlie the E oscillator, which lengthens its period under light. For period shortening and lengthening, this is essentially what we observed. However, we found that only one LN_d (the so-called extra LN_d) showed a significant cycling in PER and TIM staining intensity under LL conditions and thus putatively represents an E oscillator. By this same criterion, the 5th s-LN_v cell might be an essential component of the E oscillator. Despite these deviations, our results principally support the hypothesis of Grima et al. (2004) and Stoleru et al. (2004) that the s-LN_v and the LN_d are neuronal substrates of putative M and E oscillators in *D. melanogaster*.

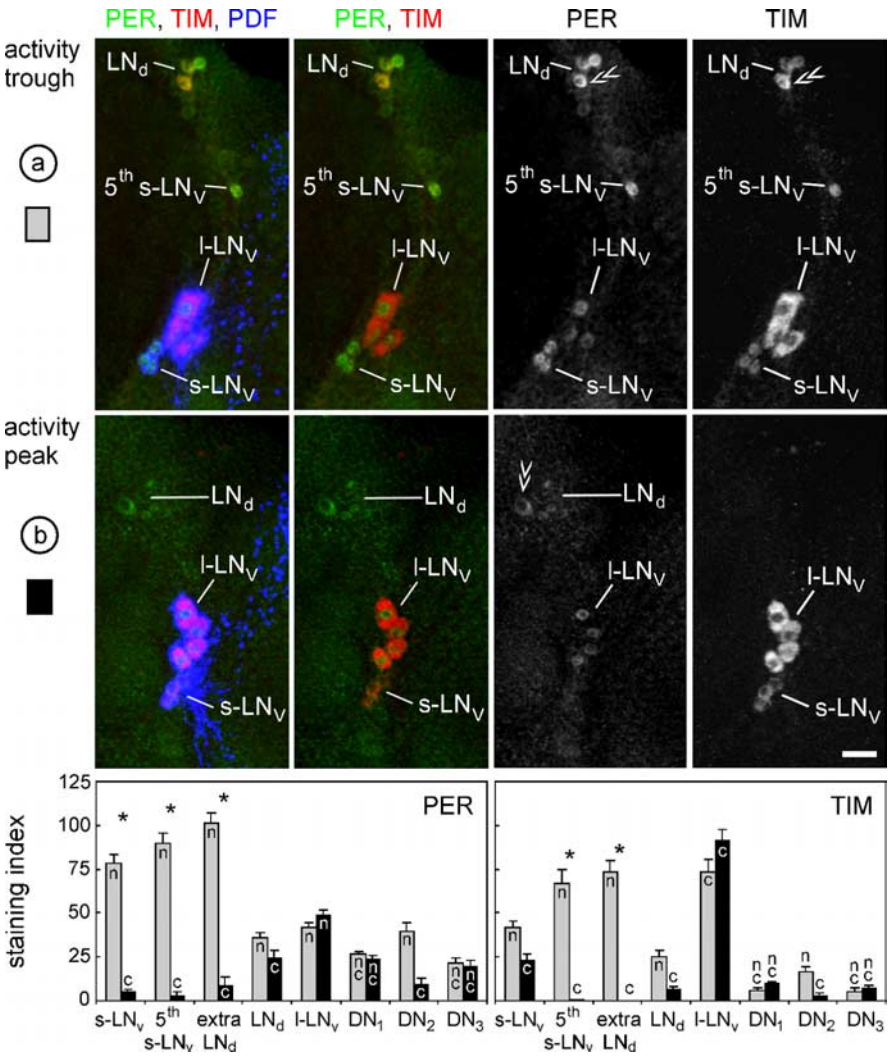

Figure 6. Lateral neurons of internally synchronized *cry*^{b} flies stained on day 1 of LL with anti-PER, anti-TIM, and anti-PDF at the time points of low and high locomotor activity. Single labeling is shown in gray, and double labeling is shown in color. Top, PDF (blue) is used as marker for the four s-LN_v and all l-LN_v cells plus their neurites. At time point a (activity trough, see Fig. 3), strong PER and TIM staining was found in the nuclei of all neurons, except the l-LN_v cells, in which TIM is predominantly cytoplasmic. At time point b (activity peak, see Fig. 3), PER and TIM staining was weaker in most cells, and both proteins were in the cytoplasm. In the l-LN_v cells, PER remained nuclear and TIM was found in the cytoplasm, as was the case in time point a. Double arrowheads point to the extra LN_d that showed the highest staining index among the LN_d at time point a and appears to have a larger cytoplasmic area than the other LN_d cells, as visible at time point b. Scale bar, 20 μm. Bottom, Quantification of the staining intensity for PER and TIM in all neurons at the time points a (gray columns) and b (black columns). Localization of proteins: n, nuclear; c, cytoplasmic; n + c, nuclear in some cells and cytoplasmic in other cells. Error bars represent SEs, and asterisks indicate significant differences in staining index between the two time points ($p < 0.001$).

However, unlike Grima et al. (2004) and Stoleru et al. (2004), our behavioral data suggest that the s-LN_v and the LN_d cells (the putative E and M oscillators) both underlie the evening peak of activity. In the actogram shown in Figure 3, both components started from the E activity bout, whereas the M activity bout disappeared immediately after transfer into LL. This was typical for most flies. Nevertheless, in 55% of the flies, we saw an additional weak short-period component running in parallel to the first one (Table 3). This component usually appeared after 1–2 weeks in LL, and it could always be extrapolated back to the M activity bout (Fig. 9*A*). In rare cases (7.5%), this short-period component originated directly from the M activity bout (Table 3). To better judge the behavior of the M activity bout, we conducted an additional experiment, in which we offered 34 flies a weak night light. Light intensity during the night was adjusted to 0.25 μW/cm^2 (approximate moon light) and was kept at 500 μW/cm^2 during the light phase and the following LL, as before. Under these conditions, we could not only see the M activity bout more clearly but we observed the M component of the majority of flies starting to free-run with short period already before transfer into LL (Fig. 9*B–D*, Table 3). We never found a long-period component originating from the M activity bout, indicating that the M oscillator is indeed the one that shortens its period under LL conditions, as predicted by the Pittendrigh–Daan model. However, the putative M oscillator (the PDF-positive s-LN_v) of *Drosophila* seems to control some aspects of the E activity bout, because a second, and even more pronounced, short-period component detached always from the E activity bout as soon as

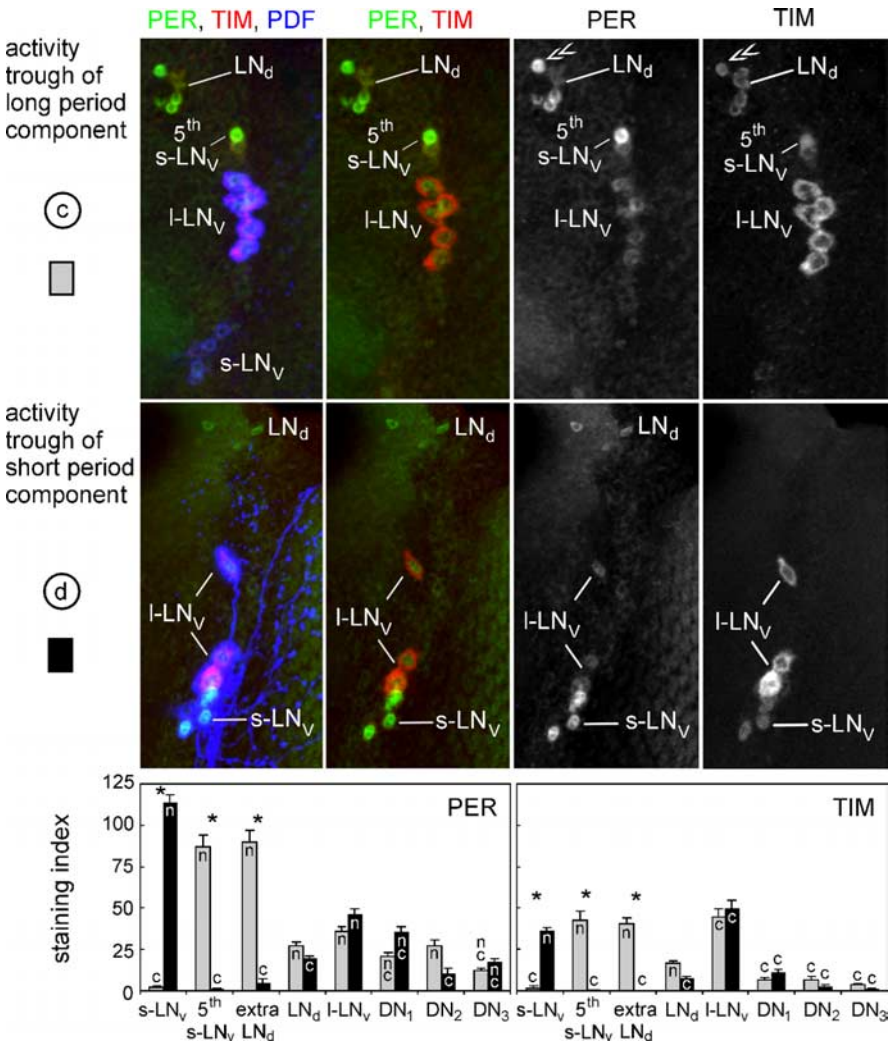

Figure 7. Lateral neurons of internally desynchronized *cry*^{b} flies stained on day 5 of LL with anti-PER, anti-TIM, and anti-PDF at the activity trough points of the long-period (time point c) and the short-period (time point d) components (labeling as in Fig. 6). Top, At time point c, strong PER and TIM staining was found in the nuclei of the LN$_d$ cells (especially the extra LN$_d$ cell; double arrowhead) and the PDF-negative 5th s-LN$_v$ cell. Note that the LN$_d$ cells are differently stained for PER and TIM (details in Results). At time point d, strong PER and TIM staining was found in the nuclei of the PDF-positive s-LN$_v$ cells. The l-LN$_v$ cells showed nuclear PER and cytoplasmic TIM at both time points (compare with Fig. 6). Bottom, Quantification of the staining intensity for PER and TIM at the time points c and d. Note that staining indices for TIM are rather low, and the protein is mainly cytoplasmic in all three DN groups. Error bars indicate SE.

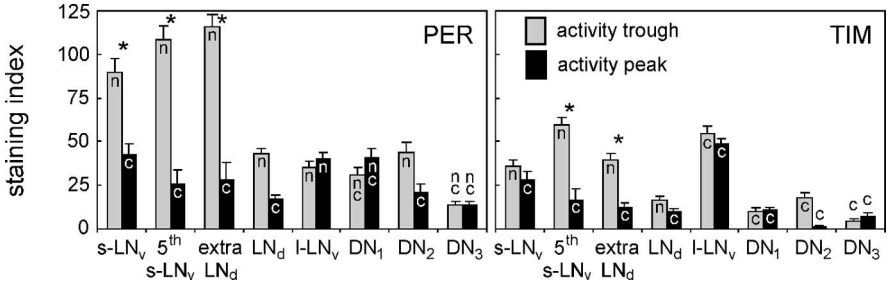

Figure 8. Quantification of the staining intensity for PER and TIM in eyeless *cli*^{eya};*cry*^{b} flies on the fifth day in LL at the times of high and low activity (labeling as in Fig. 6). Note that all neurons that show significant differences (asterisks) in staining intensity appear to cycle in-phase. Error bars indicate SE.

the M activity bout started to free-run (Fig. 9*B–D*). Thus, the E activity bout might be controlled by both oscillators (the assumed M and E cells) and therefore shortens and lengthens its period simultaneously.

If true, the PDF-positive s-LN$_v$ cells would control M and E activity bouts, and the extra LN$_d$ and the 5th s-LN$_v$ cell solely control the evening activity bout (see Discussion). This would introduce an interesting deviation from the original Pittendrigh–Daan model, because we have now no pure M oscillator but rather an "M–E" oscillator that shortens its period during illumination and controls aspects of both the morning and evening bouts of activity. This situation might be special for *Drosophila* and may account for the very prominent E peak of this species. Despite this deviation, the principle of the Pittendrigh–Daan model still appears valid. One oscillator (the combined M–E oscillator) is shortened by light, and the other (the E oscillator) is lengthened by light. As a consequence, the activity band would become broader under prolonged light conditions, thus adapting the activity to long summer days.

Discussion

Role of the PDF-positive s-LN$_v$ and LN$_d$ cells in the dual-oscillator system

In this study, we support the notion that the activity rhythm of *Drosophila* is controlled by at least two sets of neuronal oscillators. Furthermore, we refine the definition of these neuronal substrates of both oscillators more precisely than done before. As proposed by Pittendrigh and Daan (1976), the two oscillators show different responses to light: one is accelerated and the other decelerated by constant light. However, we observed a deviation from the original model. In contrast to previous observations (Grima et al., 2004; Stoleru et al., 2004), our results suggest that the PDF-positive s-LN$_v$ cells control not only the M but also the E activity bout. Therefore, we should perhaps not talk of a "morning" oscillator but rather of an M–E or "main" oscillator (to keep the "M"), for the following reasons. The PDF-positive s-LN$_v$ cells are essential for maintaining activity rhythms after several days under constant conditions (Renn et al., 1999; Blanchardon et al., 2001; Helfrich-Förster et al., 1998; Grima et al., 2004; Stoleru et al., 2004), and electrical silencing of the LN$_v$ cells severely impairs free-running rhythms (Nitabach et al., 2002). In the present study, the PDF-positive s-LN$_v$ cells appear to dominate the rhythms in those flies that did couple E and M components after the first crossing-over on day 11 in LL, because such flies free-ran with short period (Fig. 9*A*).

Our hypothesis that the PDF-positive LN$_v$ cells control not only the M activity but also partly the E activity can also explain

Table 3. Properties of the M component in *cry*^{b} mutants under LD, light/moonlight (LM), and LL conditions

Condition	*n*	No M activity bout	M activity bout entrained	M activity bout free-running with short period
LD	40	2 (5 %)	38 (95 %)	0
LM	34	1 (3 %)	14 (41 %)	19 (56 %)
		No evident short period component originating from M peak	Short period component can be extrapolated back to the M peak	Short period component continuing directly from M peak
LL (after LD)	40	15 (37.5 %)	22 (55 %)	3 (7.5 %)
LL (after LM)	34	6 (18 %)	11 (32 %)	18 (53 %)

other findings. The E activity bout is always the most prominent peak, which persists under constant-dark conditions, whereas the M activity bout is much reduced under such conditions and may even disappear (Wheeler et al., 1993; Helfrich-Förster, 2000). Thus, mainly the E component constitutes the free-running rhythm, and it seems implausible that the neurons responsible for rhythmicity under these conditions should have no impact on the E component. Indeed, Veleri et al. (2003) found that the s-LN$_v$ showed the most robust cycling after extended time under constant conditions. Furthermore, during the preparation of this manuscript, a new study by Stoleru et al. (2005) appeared that emphasizes the importance of the s-LN$_v$ cells for the timing of activity peaks under constant conditions.

Despite their dominance, the PDF-positive s-LN$_v$ cells depend on functional LN$_d$ and DN cells to provoke a normal E activity bout under light-dark conditions (Grima et al., 2004; Stoleru et al., 2004). Flies with the clock gene PER present only in PDF-positive LN$_v$ cells have a prominent M activity bout but lack the E activity bout (Grima et al., 2004). It is unclear whether this was attributable to the E activity fusing with the M activity or whether the E activity is suppressed, but these findings show that the output from the PDF cells requires PER in the LN$_d$ and DN cells to manifest wild-type activity patterns.

Role of the 5th s-LNv and the DN

We found that the PDF-negative 5th s-LN$_v$ cell cycles in-phase with the LN$_d$ cells under LL and thus may contribute to the E component. Notably, the PDF-negative 5th s-LN$_v$ cell shows high-amplitude cycling (Figs. 6, 7). Although this is not proof of the involvement of this cell, it suggests that it is an important circadian pacemaker neuron. Little is known about this cell because it could not be distinguished from the other lateral neurons in the former studies in which single-labeled clock protein staining was performed (Grima et al., 2004; Stoleru et al., 2004), but the PDF-negative 5th s-LN$_v$ cell is the only clock cell beside the PDF-positive s-LN$_v$ cells that appears to work from the first larval instar onward (Kaneko et al., 1997). Thus, it might have the same strong impact on the activity rhythm that was revealed for the PDF-positive s-LN$_v$ cells (Renn et al., 1999; Blanchardon et al., 2001) (for review, see Helfrich-Förster, 2005). More work is necessary to reveal the role of the PDF-negative s-LN$_v$ cell in more detail.

Additional studies are also necessary to fully reveal the function of the DN cells. Our results suggest that the DN$_1$ and the DN$_3$ cells may contain different subclusters. Indeed, the DN$_1$ cells develop at different times and appear to have distinct projection patterns (Kaneko et al., 1997; Kaneko and Hall, 2000) (Shafer and Taghert, unpublished observation). It is very likely that some DN$_1$ cells contribute to the M oscillator whereas others supply the E oscillator. Again, there are data that support this hypothesis: if the lateral neurons (s-LN$_v$, l-LN$_v$, and LN$_d$) are absent as a result of mutation or genetic ablation but the dorsal neurons (DN$_1$, DN$_2$, and DN$_3$) are left intact, morning and evening activity bouts are still present under LD conditions, although with reduced amplitude and changed phase (Hardin et al., 1992; Helfrich-Förster, 1998; Veleri et al., 2003). The DN$_2$ cells might play a special role for bimodal activity patterns because, in wild-type flies, they cycle 12 h out-of-phase with the s-LN$_v$ and LN$_d$ cells under DD conditions (Veleri et al., 2003). The present study indicates that this is not the case in *cry*^{b} flies under LL conditions, because the DN$_2$ cells were in-phase with all other neurons on the first day in LL. The same applies for wild-type flies under LD conditions (Kaneko et al., 1997). In their recent study, Stoleru et al. (2005) showed that the DN$_2$ are indeed pacemaker neurons that cycle independently of the s-LN$_v$ cells. However, despite their autonomous function, the DN$_2$ cells did not visibly contribute to the activity patterns of the flies under constant darkness. This suggests a minor role of the DN$_2$ cells in the control of the activity rhythm, but we cannot exclude that the DN$_2$, together with the other DN groups, may contribute to morning and evening activity bouts under certain conditions.

Photoreceptors responsible for period shortening and lengthening

The blue-light photopigment cryptochrome is regarded as the main photoreceptor of the fruit flies' circadian clock (Emery et al., 2000). We show here that the compound eyes are responsible for period shortening and period lengthening of the molecular oscillations in different subsets of pacemaker neurons (the M and E oscillators) under LL. Their special role may lie in the adaptation of the clock to seasonal changes. This is in line with previous findings showing that the compound eyes are necessary for the adequate timing of M and E activity bouts in long summer days and short winter days (Rieger et al., 2003). Cryptochrome, conversely, appears to lengthen the period in all clock neurons as can be deduced from the periods of the wild-type flies that showed internal desynchronization under "moonlight LL." In such flies, the periods of both components were clearly longer than those of internally desynchronized *cry*^{b} flies (Table 1).

Dual-oscillator systems

The internal desynchronization of activity into long- and short-period components described here is reminiscent of previous results for *Drosophila* mutants with primarily reduced optic lobes (Helfrich, 1986) or ectopic expression of PDF (Helfrich-Förster, 2000). Both of these fly strains have ectopic PDF-containing nerve fibers in the dorsal brain that might lead to elevated and/or nonrhythmic secretion of PDF in this brain area and may disturb normal communication between the pacemaker cells. It is unknown whether such a perturbed communication results in internal desynchronization between the s-LN$_v$ and the 5th s-LN$_v$ and extra LN$_d$ as observed in the present study. Dual-oscillator

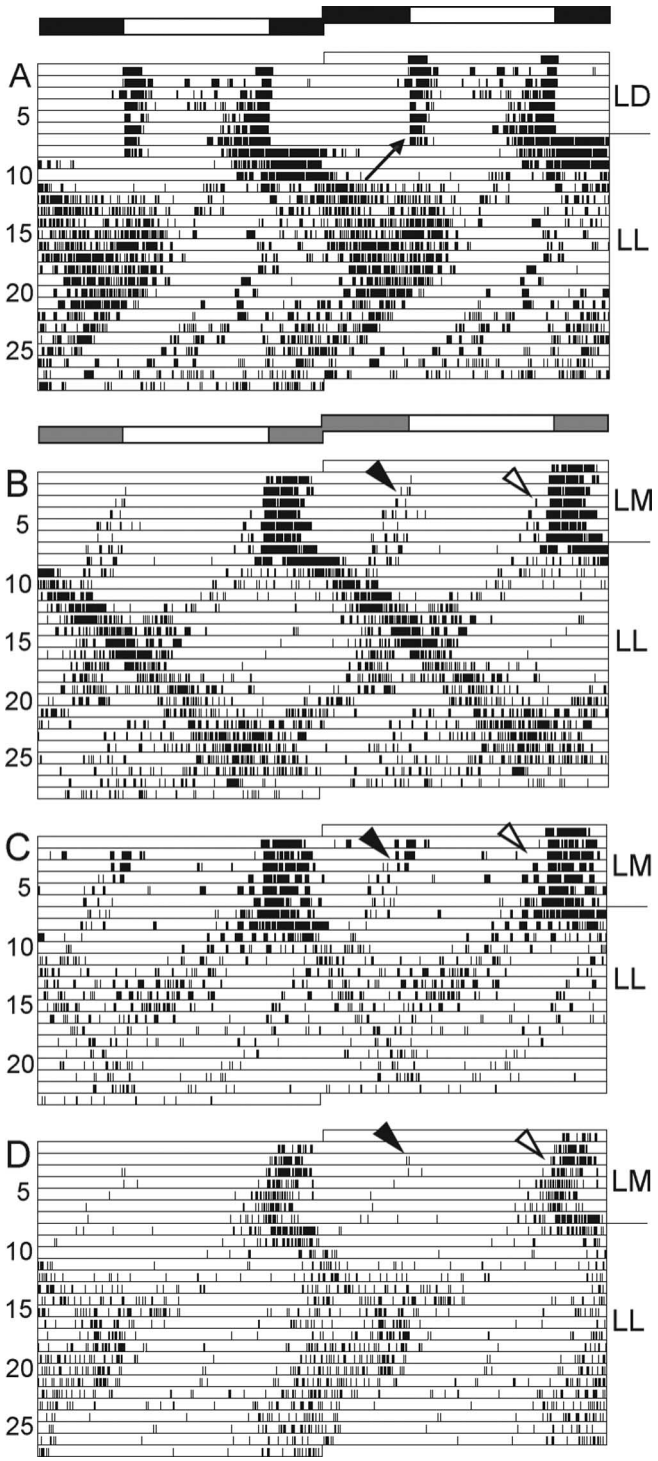

Figure 9. Actograms of *cry*^{b} flies showing a short-period component originating from the M activity bout. The fly shown in ***A*** was kept in the usual LD cycle for the first 7 d and then transferred to LL; the other three flies (***B–D***) received dim light during the night and thus had a light/moonlight cycle (LM) (night, gray bars). ***A***, A second short-period rhythm appears on day 8 in LL that could be extrapolated back to the morning activity bout (arrow). From day 10 onward in LL, the fly free-ran with the short period. ***B–D***, The morning activity bout started to free-run with a short period already during the LM cycle (filled arrowhead). At the same time, a short-period component also detached from the E activity bout (open arrowhead). In LL, the short-period components originating from the M and E activity bouts continued to free-run, indicating that the neurons free-running with short period under LL control M and E activity bouts simultaneously.

systems have been also described for mammals, but in no case they could be traced to the level of single neurons (Shinohara et al., 1995; Jagota et al., 2000; de la Iglesia et al., 2004). Like the circadian pacemaker center of flies, the mammalian pacemaker center, the suprachiasmatic nucleus (SCN), contains a heterogeneous neuronal population (Lee et al., 2003). A recent study has shown that internal desynchronization of motor activity into short and long periods similar to the one shown here can be provoked in rats by special light schedules (de la Iglesia et al., 2004). Like in *Drosophila*, both components reflect the separate activities of two oscillators in anatomically defined subdivisions of the SCN. Furthermore, there is some evidence to suggest that the SCN is composed of two oscillating M and E components (Jagota et al., 2000). These results underline the universality of dual-oscillator systems.

The work of Grima et al. (2004) and Stoleru et al. (2004) strongly implicated the PDF-expressing LN_v and the LN_d cells as the respective neuronal loci for the morning and evening activity bouts. Despite the near 12 h phase difference between the morning and evening locomotor peaks under LD, no obvious molecular phase differences between these pacemakers have been observed that would explain them (Grima et al., 2004). Work in mammals suggests that the relationship between molecular phase and locomotion is complex. For example, nocturnal and diurnal rodents show the same phases of PER oscillations (Smale et al., 2003). Furthermore, different rat strains that displayed unimodal or multimodal activity patterns, respectively, all exhibited the same unimodal rhythm in melatonin synthesis (Klante et al., 1999). Individual Nile grass rats changed their activity patterns from unimodal– diurnal to bimodal–nocturnal after introducing a running wheel (Blanchong et al., 1999). Despite this dramatic effect on the activity patterns, the wheel had little effect on the circadian pacemaker, and the spatial and temporal patterns of c-Fos expression in the SCN remained similar (Smale et al., 2003). All of these data indicate that the relationship between molecular and behavioral phase is not straightforward. Clearly, a multitude of phase relationships between the molecular rhythm and behavior are possible. Brain regions outside the pacemaker center may be responsible for these different phases as was shown recently for mammals (Nixon and Smale, 2004; Schwartz et al., 2004; Saper et al., 2005; Schwartz and Smale, 2005). It appears that the same is true within the circadian system of the fly. The present data as well as previous data (Yoshii et al., 2004) show that, during the internally synchronized state, the trough in PER level of all neurons correlates with the main activity bout (the E peak). No second trough appears to correlate with the M peak. However, a second small peak can be seen at closer inspection of the PDF immunoreactivity in the terminals of the s-LN_v (Park et al., 2000, their Fig. 4). This suggests that the unimodal rhythm in clock protein cycling might be converted into a bimodal output already within the neurons. We admit, however, that the bimodality in PDF cycling was not stated in this paper and that additional experiments are necessary to confirm it.

During the state of behavioral desynchronization under LL conditions, we observed simultaneously an internal desynchronization in PER oscillations among subsets of pacemaker neurons. One interpretation of our data is that constant light causes internal desynchronization between these pacemaker neurons that then in turn drive the behavioral outputs. However, it must be acknowledged that this is only a correlation, and, although we favor the hypothesis that the split molecular rhythms are driving the split locomotor rhythms, it is possible that they are merely tracking or entraining to a split rhythm driven by other pacemak-

ers. For example, the split rhythms might be driven by subsets of dorsal neurons. We prefer the hypothesis that the split behavioral rhythms were driven by the desynchronized PDF-positive LN_v and the 5th s-LN_v/extra LN_d cells for two reasons. First, accumulating evidence points to the lateral neurons (LN_v and LN_d cells) as major pacemaker cells, whereas the dorsal neurons (the DN_1, DN_2, and DN_3 cells) are not sufficient for locomotor rhythms under constant darkness (for review, see Hall, 2005; Helfrich-Förster, 2005). Second, in rodents, a similar behavioral desynchronization was correlated with a dissociation of clock gene expression between ventrolateral and dorsomedial subdivisions of the SCN (de la Iglesia et al., 2004). The established role of this brain center as the circadian clock led to the uncontroversial conclusion that the split molecular oscillations were driving the split behavioral oscillations. We suggest that the same phenomenon is occurring in main (i.e., small LN_v cells) and evening (i.e., 5th s-LN_v and extra LN_d cells) neuronal oscillators in *Drosophila*.

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